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ORIGINAL PAPER

Nitroaniline Isomers Interaction with Bovine Serum Albumin and Toxicological Implications

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Abstract The interactions of 2-nitroaniline (2-NA), 3-nitroaniline (3-NA) and 4-nitroaniline (4-NA) with bovine serum albumin (BSA) have been investigated by means of fluorescence spectrometry, synchronous fluorescence spectrometry and UV absorption spectrometry under the simulative physiological conditions. Association constants (K_A) were estimated by the remarkable static quenching effect of 2-NA, 3-NA and 4-NA to the intrinsic fluorescence of BSA, and thermodynamic parameters such as enthalpy change (ΔH) and entropy change (ΔS) were calculated according to van't Hoff equation. The results show that hydrophobic force plays a main role in the interaction of nitroanilines to BSA, nitroanilines have high affinity to BSA and the affinity order is as follows: 4-NA>2-NA>3-NA. On the basis of this study, it is found that percents of the binding of nitroanilines to BSA are almost no relative to the concentrations of nitroanilines, and correlation between K_A and $\log K_{ow}$ is disclosed. In the meantime, relationships between the combination of nitroanilines with BSA and toxicological implications were also discussed. In addition, synchronous fluorescence method was used to study the interaction mechanisms between nitroanilines and BSA, and energy transfer distances from BSA to nitroanilines were estimated based on the Förster's non-radiation energy transfer theory. The results suggest that the binding site for nitroanilines on BSA is close to the sub-domain IIA where Trp 214 is located.

Keywords Bovine serum albumin · Nitroanilines · Interaction · Toxicological implications

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Introduction

Nitroanilines are nitro-aromatic compounds which are largely used as intermediates in the synthesis of dyes, pharmaceuticals, pesticides, and herbicides [1–3]. These compounds exist in environment widely, and are harmful to the environment potentially. Acute or chronic exposure to nitroanilines can produce symptoms of headache, dizziness, nausea [4]. The acute toxicity mechanism of these compounds is that both nitro and amino groups on aromatic cycle can be reduced or oxidized to reactive nitroso and hydroxylamine groups which can oxidize the ferrous iron (Fe^{2+}) in hemoglobin to ferric iron (Fe^{3+}) and form methemoglobin. The formation of methemoglobin which is incapable of transporting oxygen and results in impaired oxygen delivery.

Albumin represents 52–60% of the total plasmatic protein content and plays an important role in transport endogenous ligands and xenobiotics mostly through the formation of non-covalent complexes at specific binding sites, actuating in the regulation of their plasmatic concentrations [5, 6]. These compounds include metal ions, fatty acids, amino acids, diverse drugs and toxicants. The binding of toxicants to serum albumin has toxicological importance, since it controls their free, active concentrations and affects duration and intensity of their effects [7]. The free concentration available for the toxic action can be effectively reduced for toxicants with high binding to proteins [8].

It is well-known facts that tryptophan and tyrosine residues in proteins show high fluorescence intensity, in other words, the intrinsic fluorescence of albumins results from tryptophan and tyrosine residues, so they always function as natural probes. The two aromatic amino acid residues have distinct excitation and emission wavelengths,

and differ greatly in their quantum yields. While excited at a wavelength of 285 nm, the fluorescence of bovine serum albumin (BSA) is mainly due to tryptophan residues. So tryptophan residue is used as a natural probe most frequently. Fluorescence quenching technique is an important method to study the interactions of several substances with protein, which can reveal the accessibility of quenchers to albumin's fluorophore groups, help us to understand the albumin's binding mechanisms to these substances and provide clues to the nature of the binding phenomenon [9–11]. In the past years, many researches had been concentrated on the binding of drugs to albumin. Nowadays, some researches on the binding of organophosphorous pesticides to BSA or HSA have been carried out [5]. However, study on the interaction of nitroaniline isomers and serum albumin has not been reported.

In the present work, we studied in vitro interaction of 2-nitroaniline (2-NA), 3-nitroaniline (3-NA) and 4-nitroaniline (4-NA) with BSA in the simulative physiological conditions by using the fluorescence quenching method, UV absorption spectrometry and synchronous fluorescence techniques. Association constants, thermodynamic parameters such as enthalpy change (ΔH) and entropy change (ΔS), and energy transfer distance between nitroanilines and BSA were estimated. On the basis of this study, correlation between association constants (K_A) and $\log K_{ow}$ was disclosed, the interaction mechanisms and relationships between the combination of nitroanilines with BSA and toxicological implications were also discussed.

Experimental section

Apparatus

The fluorescence spectra and intensities were measured on a model F-2500 spectrofluorimeter (Hitachi, Japan) with a quartz cell (1×1 cm^2 cross section) equipped with a xenon lamp and a dual monochromator, both entrance and exit slits for all fluorescence measurements were maintained at 5 nm. All absorption spectra were measured on a UV-2401PC spectrophotometer (Shimadzu, Japan).

Reagents

2-NA, 3-NA, 4-NA and BSA were obtained from Shanghai Chemical Reagent Co. Ltd., China. Stock solutions of 2-NA, 3-NA and 4-NA (2.0×10^{-3} mol L^{-1}) were prepared by dissolving the corresponding chemicals in ethanol, all the stocking solutions were kept in the dark. A stock solution of BSA (2.0×10^{-5} mol L^{-1}) was prepared by dissolving the corresponding BSA in water, and stored in a

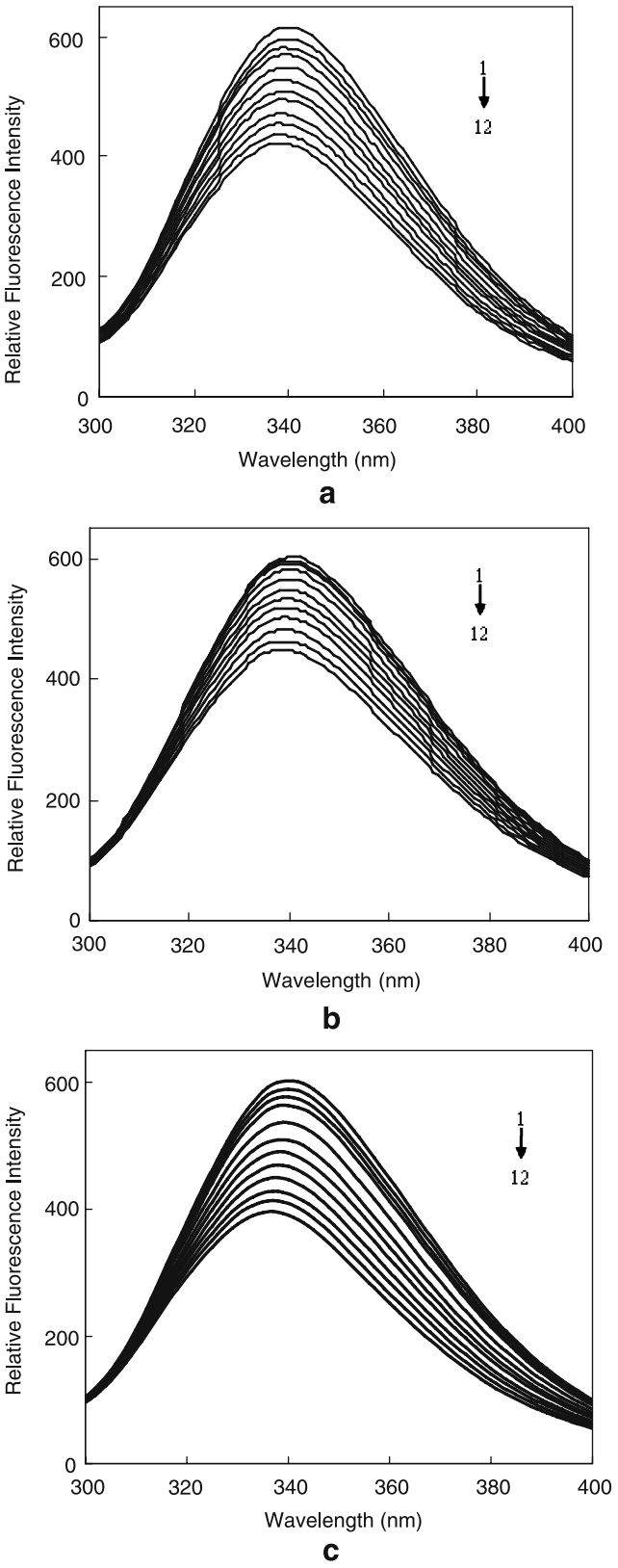

Fig. 1 Effect of 2-NA, 3-NA and 4-NA on the fluorescence intensity of BSA. [BSA]=2.0×10^{-6} mol L^{-1}; λ_{ex}=285 nm; T=298 K. **a** 2-NA, **b** 3-NA, **c** 4-NA. From 1 to 12: 0, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, and 3.6 ($\times10^{-5}$ mol L^{-1})

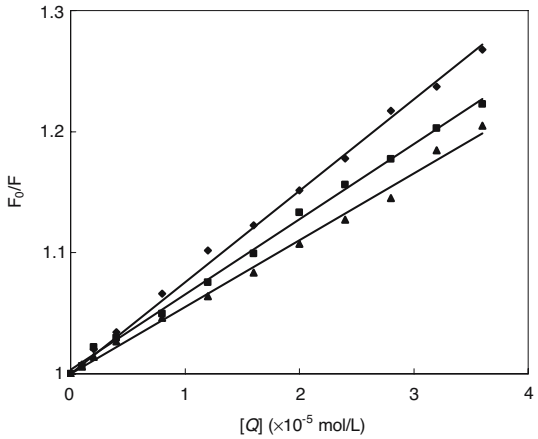

Fig. 2 The Stern–Volmer curves of nitroaniline concentrations versus the fluorescence intensity of BSA. C_{BSA}=2.0×10^{-6} mol L^{-1}; T=298 K; pH=7.40; 2-NA (*filled square*); 3-NA (*filled triangle*); 4-NA (*open square*)

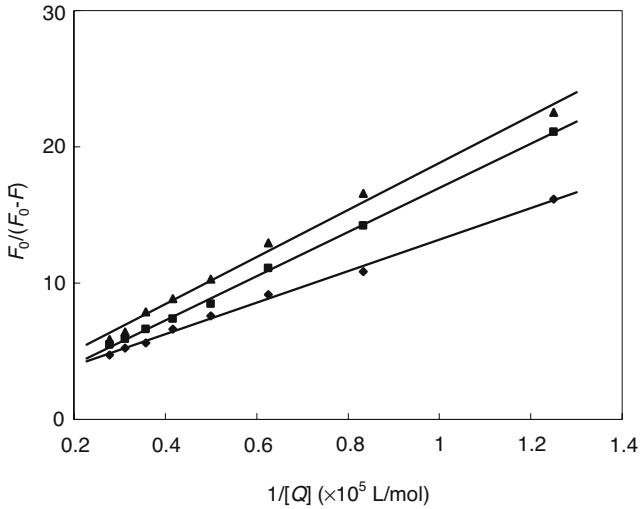

Fig. 3 The Lineweaver–Burk double-reciprocal curves of nitroaniline concentrations versus the fluorescence intensity of BSA. C_{BSA}=2.0×10^{-6} mol L^{-1}; T=298 K; pH=7.40; 2-NA (*filled square*); 3-NA (*filled triangle*); 4-NA (*open square*)

refrigerator at 4°C until used. A 0.2 mol L^{-1} Tris buffer (pH=7.40) was prepared by dissolving the corresponding Tris in water and adjusting pH with hydrochloric acid to give a final total volume of 500 mL. A 1.0 mol L^{-1} NaCl solution was prepared in order to maintain ion strength of the mixture solution. Other chemicals used were analytical grade reagents and double distilled water was used throughout the experiment.

Procedures

Fluorometric titration experiments were taken as follows: to a 10 mL test-tube, 1.0 mL of 2.0×10^{-5} mol L^{-1} BSA, 1.0 mL of 0.2 mol L^{-1} Tris buffer solution (pH=7.4) and 1.0 mL of 1.0 mol L^{-1} sodium chloride were added, diluted to 10.0 ml with water, and then shaken gently to uniformity. The test tube containing 10.0 mL mixture solution of 2.0×10^{-6} mol L^{-1} BSA, 0.02 mol L^{-1} Tris buffer and 0.1 mol L^{-1} sodium chloride was allowed to stand for 30 min at the constant temperature (detailed temperature depends on the experiments demand). The mixture solution was titrated by successive additions of 2.0×10^{-3} mol L^{-1} stock solutions of 2-NA, 3-NA and 4-NA, volume of each addition is 5, 5, 10, 20, 20, 20, 20, 20, 20, 20 and 20 μL, respectively. All the final concentrations of 2-NA, 3-NA and 4-NA were ranging from 1 to 36 μmol L^{-1}. For every addition, the mixture solution must be shaken and allowed to stand for 5 min at the corresponding temperature, and then the fluorescence intensities were measured with an excitation wavelength of 285 nm, the emission spectra was read at 300–450 nm. In the meantime, the synchronous fluorescence intensity of the mixture solution was measured

Table 1 Quenching rate constants at different temperatures

Temperature (K)		Quenching rate constant K_q (L (mol s)$^{-1}$)	Dynamic quenching constant K_{sv} (L mol^{-1})	Correlation coefficient r
2-NA	293	7.03×10^{11}	7.03×10^3	0.999
	298	6.29×10^{11}	6.29×10^3	0.998
	304	5.94×10^{11}	5.94×10^3	0.999
	310	5.44×10^{11}	5.44×10^3	0.993
3-NA	293	6.11×10^{11}	6.11×10^3	0.997
	298	5.68×10^{11}	5.68×10^3	0.998
	304	5.54×10^{11}	5.54×10^3	0.997
	310	5.27×10^{11}	5.27×10^3	0.999
4-NA	293	7.35×10^{11}	7.35×10^3	0.994
	298	6.83×10^{11}	6.83×10^3	0.997
	304	6.37×10^{11}	6.37×10^3	0.996
	310	6.31×10^{11}	6.31×10^3	0.999

Table 2 Association constants between nitroanilines and BSA

Temperature (K)		Dissociation constant K_D (mol L^{-1})	Association constants K_A (L mol^{-1})	Correlation coefficient r
2-NA	293	1.48×10^{-4}	6.76×10^{3}	0.997
	298	1.62×10^{-4}	6.17×10^{3}	0.998
	304	1.81×10^{-4}	5.52×10^{3}	0.992
	310	1.96×10^{-4}	5.11×10^{3}	0.992
3-NA	293	1.55×10^{-4}	6.45×10^{3}	0.999
	298	1.71×10^{-4}	5.85×10^{3}	0.998
	304	1.85×10^{-4}	5.41×10^{3}	0.994
	310	2.02×10^{-4}	4.95×10^{3}	0.994
4-NA	293	1.42×10^{-4}	7.02×10^{3}	0.989
	298	1.55×10^{-4}	6.45×10^{3}	0.994
	304	1.73×10^{-4}	5.78×10^{3}	0.998
	310	1.85×10^{-4}	5.41×10^{3}	0.994

at λ_{ex}=285 nm, $\Delta\lambda$=20 nm and $\Delta\lambda$=60 nm, respectively. After every determination, the residue solution in the 1-cm quartz cell must be returned to the test tube. In the course of successive additions, concentrated stock solutions of nitroanilines were chosen so that volume increment (the total increment volume is 0.18 mL) was negligible compared with the 10.0 mL mixture solution. Titrations were done manually by using trace syringe. The temperature of samples was kept by recycle water throughout experiments.

To evaluate existing inner filter effects of protein and ligand, absorbance measurements were performed at excitation and emission wavelengths of albumin. Observed F values were corrected using the equation [9]:

$$F_{cor} = F_{obsd} e^{\frac{A_{285}+A_{341}}{2}} \quad (1)$$

where A_{285} and A_{341} are the sum of the absorbances of protein and ligands at excitation and emission wavelengths, respectively.

Table 3 Number of the binding sites of nitroanilines with BSA

Temperature (K)		Number of the binding sites n	Correlation coefficient r
2-NA	293	1.01	0.997
	298	0.99	0.998
	304	0.97	0.999
	310	0.98	0.984
3-NA	293	0.97	0.995
	298	0.95	0.997
	304	0.94	0.989
	310	0.92	0.998
4-NA	293	1.03	0.983
	298	1.00	0.994
	304	0.99	0.997
	310	0.97	0.995

Results and discussion

Fluorescence quenching mechanism

Fluorescence spectroscopy is an appropriate method to study the interactions between small molecule ligands and biomacromolecule. From measurements of emission peaks, transfer efficiency of energy, lifetime and so on, a vast amount of information about the interaction will be given.

The effects of 2-NA, 3-NA and 4-NA on the fluorescence intensity of BSA were shown in Fig. 1. It can be seen from Fig. 1 that the fluorescence intensity of BSA decreases regularly with concentration increase of nitroanilines, which is called the fluorescence quenching effect. Moreover, the maximum emission wavelengths of BSA are changed by 2-NA, 3-NA and 4-NA from 342 to 339, 338, and 336 nm, respectively, which indicates that BSA conformations are changed. The fluorescence quenching effect may result from a variety of processes such as excited state reactions, ground-state complex formations and collisional processes.

The static quenching is due to the formation of ground-state complex between fluorophores and quencher. However, the collisional quenching or dynamic quenching results from the collisions between fluorophores and quencher, and can be mathematically expressed by the Stern–Volmer equation [11]:

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities of protein in the absence and presence of quencher, respectively. K_q is quenching rate constant of biomolecule, K_{sv} is dynamic quenching constant, τ_0 is average lifetime of molecule without quencher and $[Q]$ is concentration of quencher.

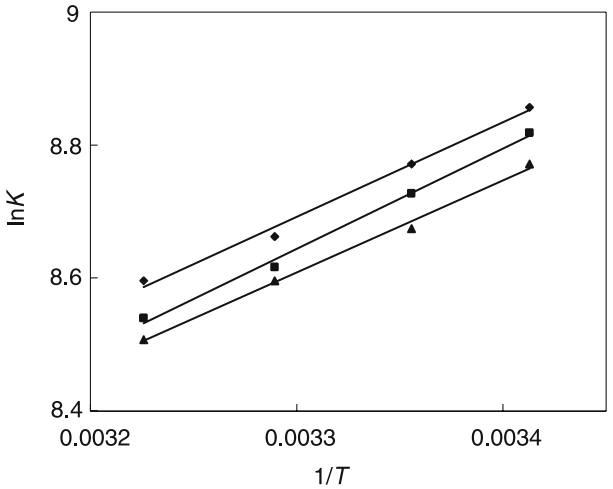

Fig. 4 The Van't Hoff curves. C_{BSA}=2.0×10^{-6} mol L^{-1}; pH=7.40; 2-NA (*filled square*); 3-NA (*filled triangle*); 4-NA (*open square*)

The graphs plotted according to the Stern–Volmer equation were shown in Fig. 2. Dynamic quenching constants K_{sv} were obtained by the slope of regression curves in the linear range, and quenching rate constants K_q were calculated based on the fluorescence lifetime of biopolymer about 10^{-8} s [12]. The results were shown in Table 1. Generally speaking, for the dynamic quenching mechanism, quenching rate constants of the fluorescent complexes will increase with a rise in temperature, and the maximum scatter collision quenching constant of biomacromolecule by all kinds of quenchers is 2.0×10^{10} L mol^{-1} s^{-1}. On the contrary, for the static quenching mechanism, quenching rate constants of the fluorescent complexes will decrease with a rise in temperature. It can be seen from Table 1 that quenching rate constants of BSA decrease with a rise in temperature, and it is far greater than the maximum scatter collision quenching constant of biomacromolecule (2.0×10^{10} L mol^{-1} s^{-1}) [13]. Thus, the fluorescence quenching effects of 2-NA, 3-NA and 4-NA are not initiated by the dynamic collision, and caused by the static quenching of compounds formation.

Association constants and number of binding sites

On the basis of the above conclusion, it is postulated that the fluorescence quenching of BSA is a static quenching process, then the static quenching can be mathematically expressed by Lineweaver–Burk formula [14]:

$$\frac{1}{F_0 - F} = \frac{1}{F_0} + \frac{K_D}{F_0[Q]} \tag{3}$$

where K_D is dissociation constant, $[Q]$ is concentration of quencher.

The Lineweaver–Burk double-reciprocal plots were constructed based on the relationship of $(F_0-F)^{-1}$ versus various concentrations of nitroanilines (see Fig. 3). From the regression equation of curves, association constants (K_A=1/K_D) between nitroanilines and BSA were obtained (see Table 2). It can be seen from Table 2 that association constant values are great, which indicates that nitroanilines had high affinity to BSA. The affinity order is as follows: 4-NA>2-NA>3-NA.

The Scatchard equation can be used to estimate the number of the binding sites between organic micromolecule and biological macromolecule based on the above conclusion that the fluorescence quenching is caused by the static quenching of compounds formation, it is described as follows [15]:

$$\lg\frac{F_0 - F}{F} = \lg K_A + n\lg[Q] \tag{4}$$

where K_A represents the static association constants, n is the number of the binding sites, $[Q]$, F_0 and F represent the same meanings as the above equations.

The plots were constructed based on the relationships of lg[(F_0-F)/F] versus lg[Q]. From the regression equation of

Table 4 Thermodynamic parameters of the interaction between nitroanilines and BSA

Temperature (K)		Association constants K_A (L mol^{-1})	Free energy change ΔG (kJ mol^{-1})	Enthalpy change ΔH (kJ mol^{-1})	Entropy change ΔS (J (mol K)$^{-1}$)
2-NA	293	6.76×10^3	−21.45	−12.53	30.38
	298	6.17×10^3	−21.59		
	304	5.52×10^3	−21.74		
	310	5.11×10^3	−21.97		
3-NA	293	6.45×10^3	−21.33	−11.51	33.46
	298	5.85×10^3	−21.45		
	304	5.41×10^3	−21.69		
	310	4.95×10^3	−21.89		
4-NA	293	7.02×10^3	−21.54	−11.78	33.27
	298	6.45×10^3	−21.70		
	304	5.78×10^3	−21.86		
	310	5.41×10^3	−22.12		

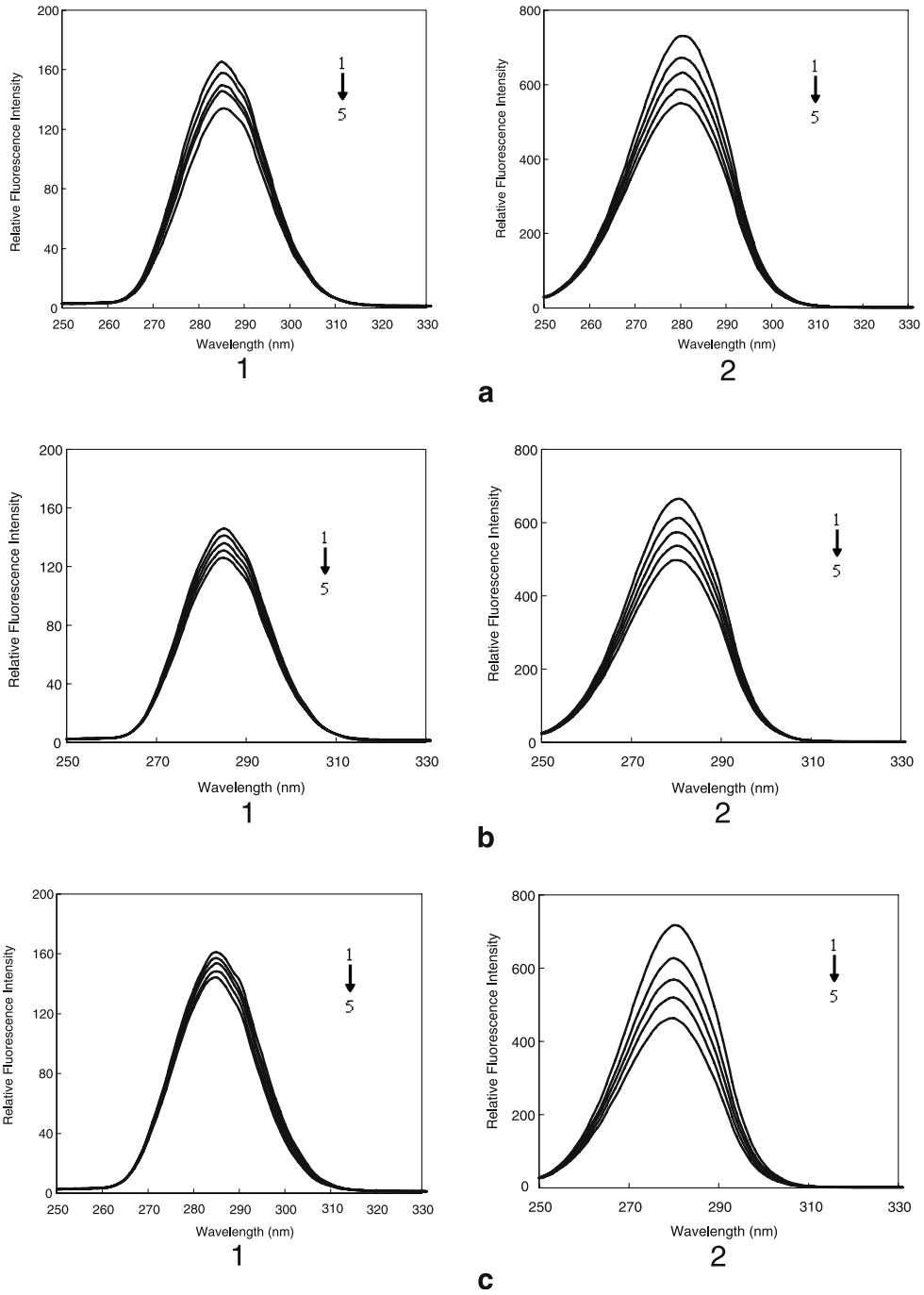

Fig. 5 Effect of nitroaniline on synchronous fluorescence spectra of BSA. C_{BSA}=2.0×10^{-6} mol L^{-1}; T=298 K; pH=7.40; (1) Δλ=20 nm; (2) Δλ=60 nm; from *curves* 1 to 5, C=0, 0.8, 1.6, 2.4, and 3.2 (×10^{-5} mol L^{-1}). **a** 2-NA system, **b** 3-NA system, **c** 4-NA system

curves, association constants (K_A) and number (n) of the binding sites were obtained. The results show that association constants estimated by this method are basically in accordance with these obtained by the above Lineweaver–Burk equation. The number of the binding sites at different temperatures was shown in Table 3. Table 3 shows that number of the binding sites of nitroanilines with BSA is 1, which indicates that a nitroaniline molecule bound to one BSA molecule.

Thermodynamic parameters and binding modes

The interaction forces between small molecule ligands and biomacromolecule may include hydrophobic force, electrostatic interaction, van der Waals interaction, hydrogen bonds, and so forth. Energy change of the quenching process can be calculated by the slope of a plot of bimolecular quenching constant versus 1/T (T, absolute temperature). If enthalpy change (ΔH) does not vary significantly over the

temperature range studied, then its value and entropy change (ΔS) can be estimated by the van't Hoff equation:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{5}$$

where K is association constant at the corresponding temperature and R is gas constant. T is absolute temperature. Enthalpy change (ΔH) and entropy change (ΔS) are calculated based on the slope of the van't Hoff relationship (Fig. 4). Free energy change (ΔG) is estimated according to the following relationship:

$$\Delta G = \Delta H - T\Delta S \tag{6}$$

The values of ΔH, ΔS, and ΔG were shown in Table 4. According to the accepted viewpoints [16], when $\Delta S > 0$, the possible interaction forces are hydrophobic force and electrostatic interaction; when $\Delta H \approx 0$ and $\Delta S > 0$, the possible interaction force is electrostatic interaction; when $\Delta H < 0$, the main interaction force is electrostatic interaction. It can be seen from Table 4 that all the values of ΔG and ΔH are negative, while the value of ΔS is positive. The negative value of free energy (ΔG) shows that the interaction process is spontaneous. The positive values of enthalpy (ΔH) and entropy (ΔS) indicate that the binding of nitroanilines and BSA is mainly entropy-driven, and the enthalpy is unfavorable for it. Thus, it was concluded that the hydrophobic force played a major role in the interaction, but it did not mean that the electrostatic interaction was excluded [16].

Binding sites of the interaction of nitroanilines with BSA

The synchronous fluorescence spectra can give some information about the molecular environment in a vicinity of chromophoric molecules. In the synchronous spectra, the sensitivity associated with fluorescence is maintained, while offers several advantages: spectral simplification, spectral bandwidth reduction and avoiding different disturbing effects. As known to all, the intrinsic fluorescence of albumins results from the tryptophan and tyrosine residues, and the normal emission spectra of tryptophan and tyrosine residues are overlap. When the wavelength difference ($\Delta\lambda$) of excitation and emission wavelengths was fixed at 20 and 60 nm, the synchronous fluorescence would give the only characteristic information of tyrosine and tryptophan residues, respectively [17]. The effects of nitroanilines on the synchronous fluorescence spectra of BSA were shown in Fig. 5. It can be seen from Fig. 5 that a stronger fluorescence quenching effect of tryptophan residues compared with the tyrosine residues is observed

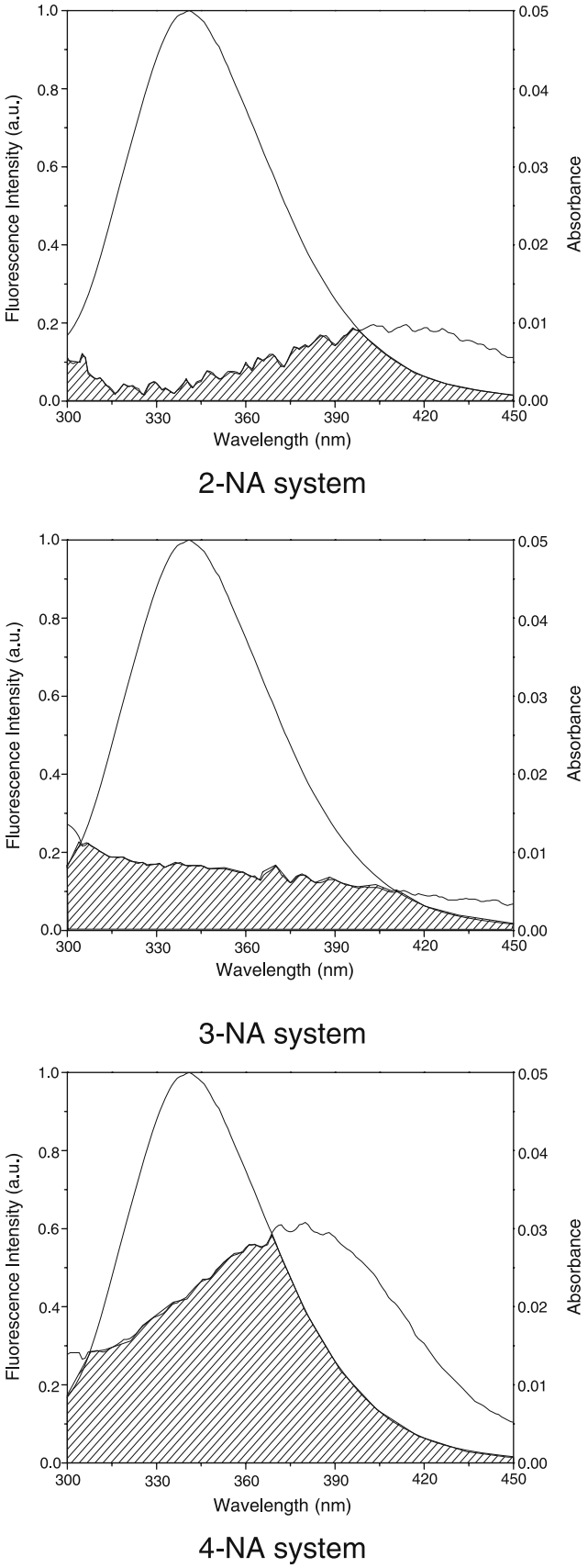

Fig. 6 Spectral overlap of nitroanilines absorption with BSA fluorescence. $C_{BSA}=C_{NA}=2.0\times10^{-6}$ mol L^{-1}; pH=7.40

after nitroanilines are added. This difference indicates that the binding site of nitroanilines is nearer to tryptophan than that of the tyrosine residues.

BSA consists of 582 amino acid residues forming a single polypeptide with well-known sequence, which contains three homologous α-helices domains (I–III). Each domain is divided into two sub-domains (A and B). The adherence of two sub-domains with their grooves towards each other forms a domain, and three of such domains make up an albumin molecule [7, 18]. BSA has two tryptophan residues (Trp 135 and Trp 214) located in sub-domains IA and IIA, respectively [7]. A large hydrophobic cavity is present in the IIA sub-domain, and a wide variety of arrangements can take place in this sub-domain [19]. Trp 214 is deeply buried in sub-domain IIA where the hydrophobic molecules of 2-NA, 3-NA and 4-NA can better penetrate, whereas Trp 135 is located in sub-domain IA which is more exposed to a hydrophilic environment. Therefore, it can be inferred that the primary binding target of nitroanilines is sub-domain IIA of BSA where Trp 214 is located. This conclusion is in accordance with the reported results on the interaction of drugs with BSA [20].

Energy transfer distance between nitroanilines and BSA

According to the Förster's non-radiation energy transfer theory [21], energy transfer can occur only when the fluorescence emission spectra of the donor and the absorption spectra of the acceptor have enough overlap and the distance between the donor and the acceptor is not longer than 7 nm [22, 23] (Fig. 6). According to this theory, the distance r of energy transfer between nitroanilines and BSA can be described by the following Eqs. 7, 8, and 9:

$$E = \frac{R_0^6}{R_0^6 + r^6} \tag{7}$$

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \Phi J \tag{8}$$

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \mathrm{d}\lambda}{\int_0^\infty F(\lambda)\mathrm{d}\lambda} \tag{9}$$

where E is the efficiency of transfer between the donor and the acceptor, R_0 is the critical distance when the efficiency of transfer is 50%, r is the distance between the donor and the acceptor, K^2 is the space factor of orientation, n is the refracted index of medium, Φ is the fluorescence quantum yield of the donor, J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range λ to λ+Δλ, and ε(λ) is the extinction coefficient of the acceptor at λ. The efficiency of transfer (E) can be obtained by the equation:

$$E = 1 - \frac{F}{F_0} \tag{10}$$

In the present case, K^2=2/3 [24], N=1.336, and Φ=0.15 [25]. According to the Eqs. 7, 8, 9, and 10, the overlap integral J, R_0, E, and r can be evaluated. The results were shown in Table 5. It can be seen from Table 5 that all the distances r are less than 7 nm, which indicates that energy transfer from BSA to nitroaniline occurs with high probability. At the same time, it is proved again that the fluorescence quenching of BSA is caused by the static quenching of compounds formation, and the conclusion that the primary binding site for nitroaniline on albumin is close to tryptophan residues 214 of BSA is auxiliarily proved.

Correlation between association constants (K_A) and K_{ow}

Since the main force between nitroanilines and BSA is hydrophobic force, maybe octanol/water partition coefficient (K_{ow}) as an important index of hydrophobicity has some correlation with association constants (K_A) of nitroanilines with BSA. A plot of association constants (K_A) versus the corresponding logK_{ow} values of 2-NA, 3-NA and 4-NA was constructed in order to explore the correlation between association constants (K_A) and K_{ow}. logK_{ow} values of 2-NA, 3-NA and 4-NA are 1.44, 1.37 and 2.66, respectively, they are from the International Chemical Safety Cards. The logK_{ow} values' order for 2-NA, 3-NA and 4-NA are in accordance with the affinity order of nitroanilines to BSA, which indicates that the combination of toxicants with BSA is direct correlation with K_{ow} of toxicants. At the room temperature (298 K), the correlation coefficient (r) between association constants (K_A) and the corresponding logK_{ow} is 0.871. The correlation coefficient is not very

Table 5 Energy transfer parameters between nitroanilines and BSA

Parameters	Overlap integral J (cm^3 L mol^{-1})	Efficiency of transfer E	Critical distance R_0 (nm)	Energy transfer distance r (nm)
2-NA	3.51×10^{-15}	0.0298	2.12	3.78
3-NA	5.84×10^{-15}	0.0236	2.31	4.29
4-NA	1.83×10^{-14}	0.0354	2.79	4.84

good, perhaps the localization of $-NO_2$ group influences the dimension of the whole molecule, which can produce different steric hindrance when it interacts with BSA, consequently, influencing the interaction with BSA.

Combination of nitroanilines with BSA and toxicological implications

The binding of toxicants to serum albumin has toxicological importance, since it controls their free, active concentrations and affects duration and intensity of their effects [7]. Thus, it is necessary that the percents of the binding of nitroanilines to BSA were estimated. On the basis of the Scatchard equation and association constants (K_A) at 37°C, the concentration of BSA is fixed at 5.8×10^{-4} mol L^{-1} (approximate to the physiological concentration of albumin), when the concentrations of nitroanilines change from 1.0×10^{-5} to 1.0×10^{-6} mol L^{-1}, the percents of the binding of nitroanilines to BSA change from 68.0 to 68.2% for 2-NA, 55.8 to 55.9% for 3-NA and 73.6 to 73.8% for 4-NA. From the above results, it indicates that the percents of the binding of nitroanilines to BSA are almost no relative to the concentrations of nitroanilines. Additionally, many albumins such as HSA are similar to BSA, some of our conclusions on the interaction of nitroanilines to BSA can also be applied to understand other albumins, so some toxicological phenomena can be easily explained based on the results. For example, for nitroaniline isomers, which the localization of $-NO_2$ group influences the dimension of the whole molecule and results in influencing the interaction with BSA, generally speaking, the better of the molecular symmetry, the more serious of its toxicity, the toxic order of the three isomers should be p−>m−>o− position. However, from the toxicological data of acute exposure (rat intake by mouth), LD_{50} values of 2(o)-NA, 3(m)-NA and 4(p)-NA are 1,600, 536 and 750 mg g^{-1}, respectively. From the above toxicological data of acute exposure, it seems that the toxicity of 3-NA is the most serious, which disobeys the general rule. Actually not, it is easily explained for the binding percent of 4-NA is obviously greater than 3-NA, more amount of 4-NA is bound to albumin than that of 3-NA and its toxic effects are postponed, so LD_{50} of 4-NA is greater than 3-NA. Therefore, the combinations of nitroanilines to albumin have significantly toxicological importance, when nitroanilines are invaded into the biological organism, more than 50% of the toxicants are bound to albumin, and the free concentration available for the toxic action can be effectively reduced for toxicants with high binding to proteins.

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