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Spectral Studies on the Interaction of Toluidine Blue O with Bovine Serum Albumin

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Abstract — Interaction between toluidine blue O (TBO) and bovine serum albumin (BSA) was investigated by UV-visible absorption and emission spectroscopy. It is proposed that the fluorescence quenching of BSA by TBO was mainly a result of the formation eof TBO–BSA complex and electrostatic interactions played an important role to stabilize the complex. The Stern–Volmer quenching constant K_{SV} and corresponding thermodynamic parameters ΔH^0, ΔG^0, and ΔS^0 were evaluated. Results suggest the interaction process to be comparable with the reversible biological processes. Effect of TBO on the conformation of BSA has been analyzed by means of synchronous fluorescence spectroscopy. IR spectra also proved that the interaction of TBO with BSA had changed the conformation of TBO.

Keywords : *Toluidine blue O; Stern-Volmer constant; BSA; fluorescence quenching.*

INTRODUCTION

Toluidine blue O (TBO) [3-Amino-7(dimethyl amino)-2-methyl phenothiazin-5-ium chloride], a cationic fluorescent dye which belongs to a thiazine class shows a pronounced ability to permeate through the cellular membrane and to perform a photo-bacterial activity [1–2]. More traditionally, it is widely employed in the quantitative estimation of important anticoagulant molecules, *viz.*, heparin [3–4]. The TBO dye is also used as stain. The most evident effect shown by TBO, among many other dyes examples is so called metachromasia, *i.e.*, the pronounced blue shift in the visible spectrum due to the aggregation phenomenon and the electrostatic interaction with the charged synthetic and biological polymers [5–6]. Recently, TBO molecules

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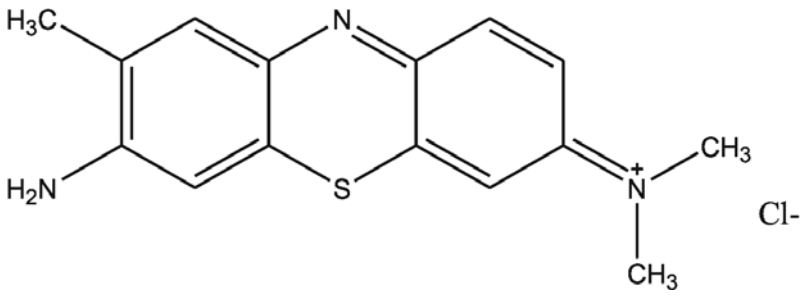

Scheme 1. Structure of Toluidine Blue O.

combined with gold chloride used as a stain to investigate chick embryo neural tissue and poly (toluidine blue) film electrode are reported as nitrite amperometric sensors [7–8]. Phenothiazine dye derivatives are most extensively studied and widely used in solar energy conversion. However, these dyes form photoinactive dimers in concentrated aqueous solution [9–10].

Serum albumin (SA) is the most abundant protein constituent of the blood plasma, and facilitates the disposition and transport of various exogenous and endogenous ligands to particular biotargets [11–13]. The specific delivery of ligands by bovine serum albumin (BSA) originates from the presence of two major and structurally selective sites, namely, site I and site II, which are located in three homologous domains that form a roughly heart-shaped protein [14–16]. The binding affinity offered by site I is mainly through hydrophobic interactions, whereas site II involves a combination of hydrophobic, hydrogen bonding and electrostatic interactions [17–18]. Globular proteins play an important role to catalyze the enzyme reactions, adsorb to surface and to form molecular aggregates [19]. BSA has 583 amino acids residues in single polypeptide chain. The protein contains 17 disulfide bridges, one free –SH group, which can cause it to form a covalent linkage. BSA is commonly used for research purposes due to its stability, water solubility, and versatile binding capacity. It can bind to a variety of ligands such as bilirubin, fatty acids, dye, surfactants, hematin and drugs, *etc*. [20–25].

Kolekar *et al*. [26] has studied the effect of temperature and pH on the interaction between BSA and CPB. Spectroscopic studies on the interaction of drug and BSA have been studied by many authors [27–29]. Ling *et al*. [30] and Jing *et al*. [31] have studied the interaction of BSA with Tween-20 and fluorinated surfactants. It is important to understand and predict ligand/drug displacement interactions for a variety of endogenous and exogenous ligands/drugs. However, detailed investigations

on the interaction of BSA with TBO have yet to be conducted.

Fluorescence quenching is an outcome of decrease in the quantum yield of a fluorophore induced by a variety of molecular interactions with quencher molecule. Application of the spectral methods can reveal the reactivity of chemical and biological systems in low concentration under physiological conditions, and there have been several studies on fluorescence quenching of albumin induced by drugs or other bioactive small molecules [32–34].

In this paper the interaction of TBO with BSA was investigated by absorption and emission spectroscopic methods in order to determine the binding nature between dye and protein. No such work has been done with this protein albumin. The thermodynamic parameters such as changes in standard free energy (ΔG^0), enthalpy (ΔH^0) and entropy (ΔS^0) have been determined at different temperatures. The state of the dye-polymer aggregates were also investigated by way FTIR studies. The present set of studies are expected to shed light in the domain of dye-polymer interaction which could eventually be extended to the drug-biomolecule interactions and subsequent drug efficacy studies. Moreover, the present set of studies has another novelty in the sense that usually dye-polymer interactions studies are performed keeping a fixed concentration of dye. In the present case, as BSA itself shows characteristic absorption and emission spectra, hence its interaction with TBO was investigated keeping the concentration of BSA fixed (10μM) and varying the concentration of dye TBO (0 to 35 μM).

EXPERIMENTAL

Materials :

Toluidine blue O (TBO) and BSA were purchased from Sigma-Aldrich (USA). The buffer Tris had a purity of no less than 99.5% and NaCl, HCl, etc., were all of analytical grade from Shanghai Chemical Reagent Plant, China. The samples were dissolved in Tris–HCl buffer solution (0.05 mol L^{-1} Tris, 0.10 mol L^{-1} NaCl, pH 7.4±0.1).The solvents used were HPLC grade products from E. Merck, Germany. All solutions were prepared with doubly distilled water.

Methods :

Instrumentation — Absorption spectra were recorded using a spectrophotometer Lambda 25 (Perkin-Elmer, USA) while the fluorescence spectra were recorded using spectrofluorimeter RF-5000 (Shimadzu, Japan) using a glass cuvette of 1.0 cm optical path length. Experimental temperature was controlled by TB-85 circulating water bath (Shimadzu, Japan). The FTIR spectra were obtained on Perkin-Elmer Spectrum 100.

The solid BSA and TBO were used directly to obtain FTIR spectra.

Spectral measurements — In order to study the interaction between TBO and BSA the concentration of BSA was kept fixed at 10 μM and the TBO concentration was varied from 0 to 35 μM. The excitation wavelength was set at 280 nm. Fluorescence measurements were carried out at different temperatures (298, 303, 308 and 313 K) in the range 300–450 nm.

Evaluation of the excited state binding constant between BSA and TBO :

In the present set of studies, it was observed that the fluorescence of BSA got quenched in the presence of varying amount of TBO. The fluorescence quenching follow the Stern-Volmer formalism :

$$\frac{F_0}{F} = 1 + K_{SV}\,[Q] \qquad (1)$$

where, F_0 and F denote the steady-state fluorescence intensities in the absence and in the presence of quencher (TBO), respectively. K_{SV} is the Stern-Volmer quenching constant and [Q] is the concentration of the quencher. Hence, Eq. (1) was applied to determine the excited state binding constant (K_{SV}) between BSA and TBO by linear regression of a plot of F_0/F against [Q]. In many instances, the fluorophore can be quenched both by collision and by complex formation with the same quencher. In the present case, the Stern-Volmer plot exhibited an upward curvature, concave towards the Y-axis at high [Q], and F_0/F is related to [Q] by the following form of the modified Stern-Volmer equation [35–36] :

$$\frac{F_0}{F} = (1 + K_D\,[Q])(1 + K_s\,[Q]) \qquad (2)$$

where, K_D and K_S are the dynamic and static quenching constants, respectively. The first factor on the right-hand side in eq.(2) describes the "dynamic" quenching, resulting from encounters of quencher and fluorophore during the excited state and the second factor describes the "static" quenching, that is quenching by formation of a complex between quencher and fluorophore predating the excitation. It will be noticed that if the quenching effect of either of these two kinds is much greater than the other, the quenching follows the Stern–Volmer linear dependence of F_0/F upon [Q] (Eq. (1)). If there is a departure from linearity, due to the term in $[Q]^2$ being appreciable, it is certain that both ground-state complexes and excited-state interactions contribute to the quenching, which accounts for the upward curvature observed at high [Q] when both static and dynamic quenching occur for the same fluorophore.

RESULTS AND DISCUSSION

Effect of TBO on emission spectra of BSA :

A variety of molecular interactions can result in fluorescence quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. The different mechanisms of quenching are usually classified as dynamic quenching, static quenching or static and dynamic quenching can participate simultaneously. Dynamic and static quenching can be distinguished by their differing dependence on temperature and viscosity. Dynamic quenching depends upon diffusion. Since higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature. In contrast, increased temperature is likely to result in decreased stability of complexes, and thus lower values of the static quenching constants.

The effect of TBO on BSA fluorescence intensity is shown in Fig. 1. It was observed that the addition of increasing concentrations of TBO caused a progressive reduction of the fluorescence intensity. Fig. 2 displays the Stern-Volmer plots for the quenching of BSA tryptophan residue fluorescence by TBO at different temperatures. Table 1 summarizes the calculated K_{SV} at each temperature; the results show that the Stern-Volmer quenching constant K_{SV} is inversely correlated with temperature, which indicate that the probable quenching mechanism of TBO-BSA binding reaction is initiated by the formation of excited state complex rather than by dynamic collision [35]. In other words, the fluorescence quenching of BSA results from complex formation is predominant, while from dynamic collision could be negligible. For reconfirming the probable quenching mechanism of fluorescence of BSA by TBO is mainly initiated by ground-state complex formation, we used the difference absorption spectroscopy to obtain spectra. The UV-vis absorption spectrua of BSA and the difference absorption spectra between BSA-TBO and TBO at the same concentration could not be superimposed within experimental error (figure not shown). The results re-confirm that the probable quenching mechanism of fluorescence of BSA by TBO is mainly a static quenching procedure [36].

Determinations of the binding constant and the number of binding sites :

For the static quenching process, it has been assumed that there are ‘n’ equivalent and independent binding sites in the protein. The binding constant (K_a) and number of binding sites (n) were calculated using the following equation [37] :

$$\log = \frac{F_0 - F}{F} = \log K_a + n\log [Q] \qquad (2)$$

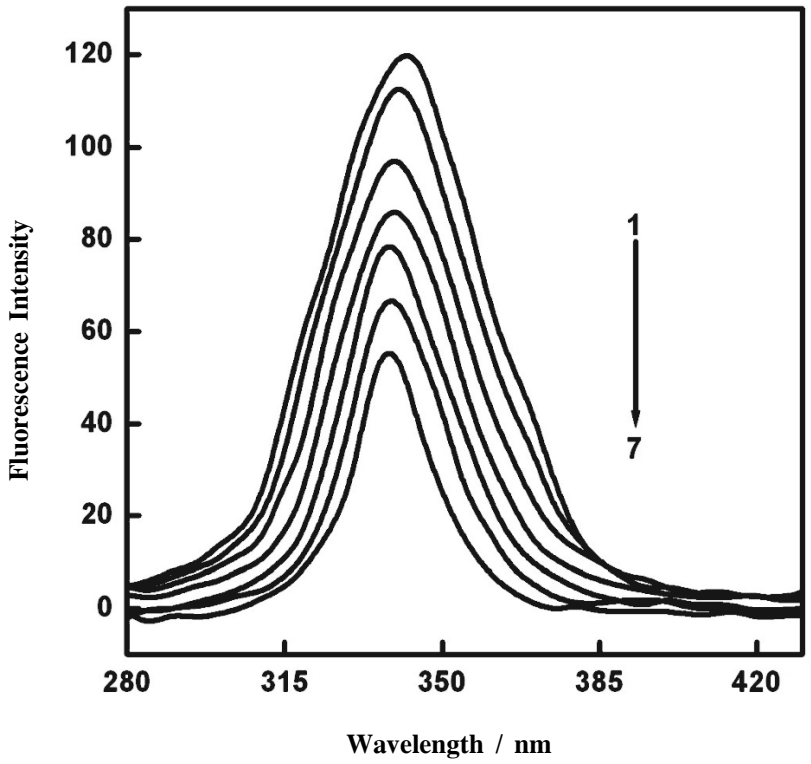

Fig. 1. Effect of TBO on fluorescence of 10 μM BSA at 298K. Concentration of TBO/μM : 1–7 : 0, 5, 10, 15, 20, 25 and 35.

where, F_0 and F are the fluorescence intensities of tryptophan residue of BSA in the absence and presence of the quencher TBO, respectively. [Q] is the quencher concentration. Plots of log (F_o-F/F) vs log [Q] for the BSA-TBO system at different temperatures are shown in Fig. 3. The plots exhibit good linearity and the values of number of binding sites and binding constant for the BSA-TBO system are presented in Table 2. The results show that the binding constants increased with temperatures, which manifests the endothermicity of the binding process [38] whereby a stable BSA-TBO complex is formed. The values of 'n' are approximately equal to unity, indicating the existence of just a single site in BSA for TBO.

Thermodynamic parameters and nature of binding forces :

The forces of interaction between the dye and protein molecules include hydrogen

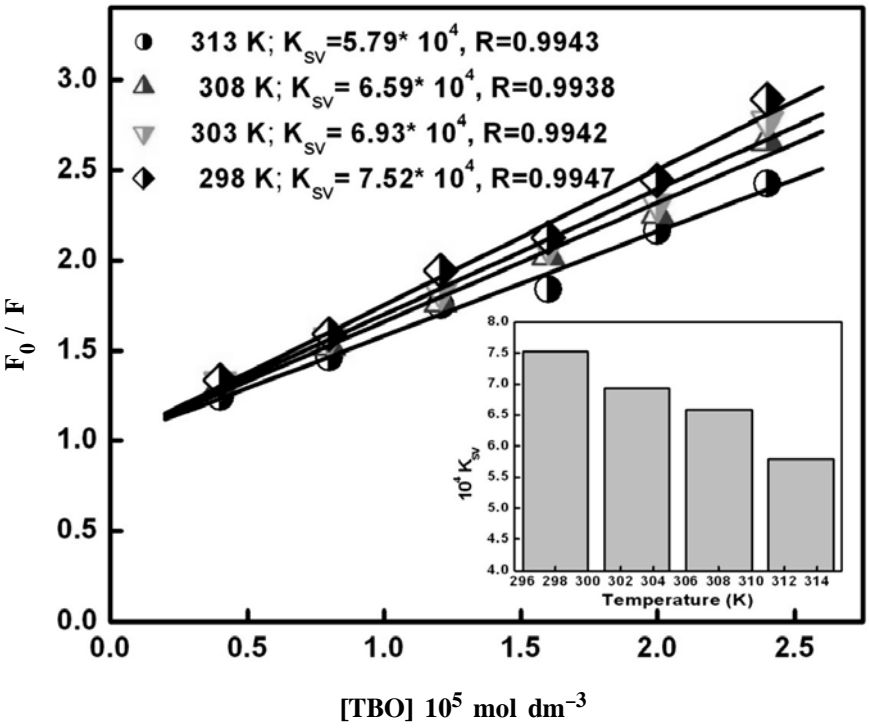

Fig. 2. Stern-Volmer plots for the quenching of BSA by TBO at different temperatures. The inset shows the relationship of the Stern-Volmer quenching constants K_{SV} and temperature T.

TABLE 1.

Stern-Volmer quenching constants of the system of TBO–BSA at different temperatures

Temperature (K)	K_{SV} (10^4) M^{-1}	$R^{2,\ a}$
298	7.52	0.9947
303	6.93	0.9942
308	6.59	0.9938
313	5.79	0.9943

[a]R = correlation co-efficient

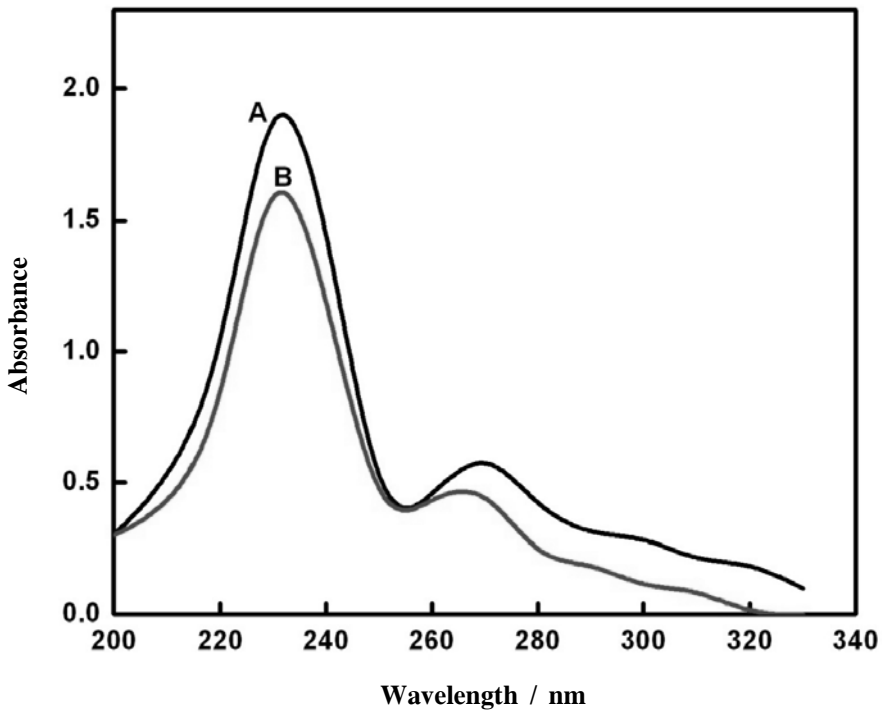

Fig. 3. UV-vis spectra of BSA in the presence of TBO. (A) The absorption spectrum of BSA only; (B) the difference absorption spectrum between TBO–BSA and TBO at the same concentration; [BSA] = [TBO] = 10 μM.

TABLE 2

Binding constant (K_a) and binding site (n) for TBO-BSA system at different temperatures

pH	Temperature (K)	K_a (10^4) M^{-1}	n	$R^{2,a}$
7.40	298	1.15	0.919	0.9998
	303	1.42	0.926	0.9985
	308	1.69	0.948	0.9978
	313	2.31	0.970	0.9961

[a]R = correlation co-efficient

bonds, van der Waals forces, as well as electrostatic and hydrophobic interactions. The thermodynamic parameters were calculated from the van't Hoff plots for the study of TBO-BSA interaction. If the enthalpy change (ΔH^o) does not vary significantly overthe temperature range studied, then its value and that of entropychange (ΔS^o) can be determined from the van't Hoff equation :

$$\ln K = - \frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

where, associative binding constants K is analogous to K_{SV} and R is the gas constant. The temperatures used were 298, 303, 308 and 313 K. The changes in standard enthalpy (ΔH^o) is calculated from the slope of the van't Hoff relationship. The free energy change (ΔG^o) is estimated from the following relationship :

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

The van't Hoff plot of *ln* K against 1000/T based on Eq. (4) gives the values ΔH^o and ΔS^o, as presented in Table 3, were obtained from the slope and intercept, respectively as shown in Fig. 4. From Table 3, it can be seen that the negative sign for freeenergy (ΔG^o)means that the binding process is spontaneous. The negative enthalpy (ΔH^o) and positive entropy (ΔS^o) values of the interaction of TBO and BSA indicate that the specific electrostatic interactions played major role in the reaction [39]. BSA is characterized by two tryptophan residues : Trp-134 is thought to be located on the surface (sub-domain IA) and Trp-212 is considered to be located within a hydrophobic pocket [40], which is in a well-characterized binding cavity (sub-domain IIA) for smallcharged aromaticmolecules.

Effect of TBO on BSA conformation :

The synchronous fluorescence spectra (SFS) can provide the information on the molecular micro-environment, particularly in the vicinity of the fluorophore functional groups [41]. To investigate the structural change of BSA by the addition of TBO, SFS for BSA with various concentrations of TBO were recorded. According to Miller *et al.* [42], the difference between excitation and emission wavelength ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) reflects the spectra of a different nature of chromophores, with large $\Delta\lambda$ values such as 60 nm, the synchronous fluorescence of BSA is characteristic of tryptophan residue and the small $\Delta\lambda$ values such as 15 nm is a characteristic of tyrosine. The SFS of BSA with various concentrations of TBO was recorded at $\Delta\lambda$ of 60 nm as shown in Fig. 5. With the addition of TBO the intensity of fluorescence is quenched along with a blue shift. The blue-shift effect implies the change in the conformation of BSA. It is also indicated that the polarity around the tryptophan residues was

TABLE 3

Thermodynamic parameters for TBO-BSA interactionsat differenttemperatures

Temp. (K)	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol/K)	$R^{2,a}$
298	–27.83			
303	–28.09	–12.50	51.45	0.9927
308	–28.35			
313	–28.60			

[a]R = correlation co-efficient

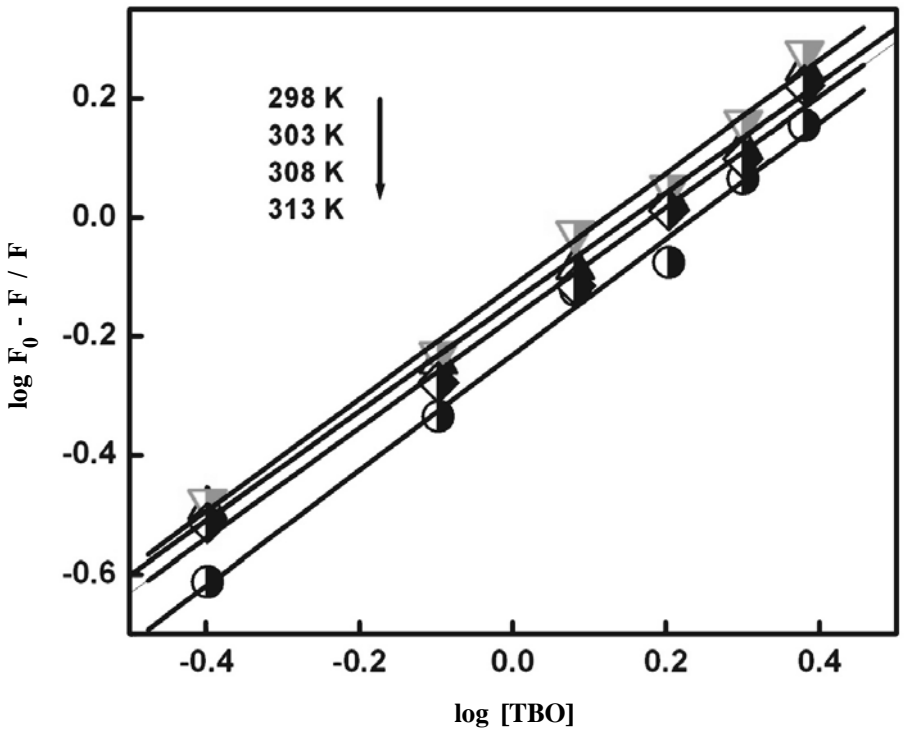

Fig. 4. Plot of *log* (F_o-F/F) vs *log* [TBO] for the BSA-TBO system at different temperatures. Temperatures (in K) are mentioned inside the figure.

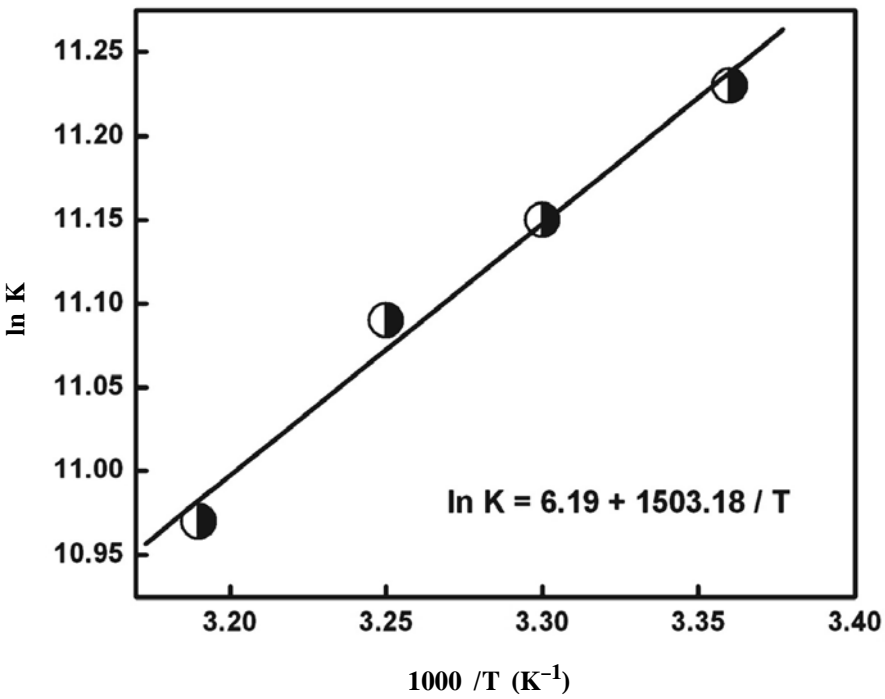

Fig. 5. van't Hoff plot for BSA-TBO aggregates at pH = 7.40. [BSA] was fixed at 10 μM while the TBO concentration was varied in the range of 5 – 35 μM.

decreased and the hydrophobicity was increased [25].

FTIR studies on the BSA-TBO complex :

FTIR studies are considered to provide useful information on the nature and binding of the of the ligand (herein TBO) around a host molecule (herein BSA). Fig. 6 demonstrates the FTIR spectra of BSA, TBO and TBO-BSA complexes. For BSA, the characteristic bands appeared at 1653, 2136 and 3363 cm^{-1} and the bands were shifted to 1647, 2112 and 3306 cm^{-1}, respectively, for TBO-BSA complex which confirmed the interaction between TBO and BSA.

For TBO, weak peaks locating at 3395, 3122, 2924, 2853 and 2699 cm^{-1} were observed in the infrared spectrum. However, these characteristic bands in the FTIR spectrum of TBO-BSA complex shifted from 3122 and 2853 to 3131 and 2868 cm^{-1}, respectively, proving the interaction between TBO and BSA. The disappearance of

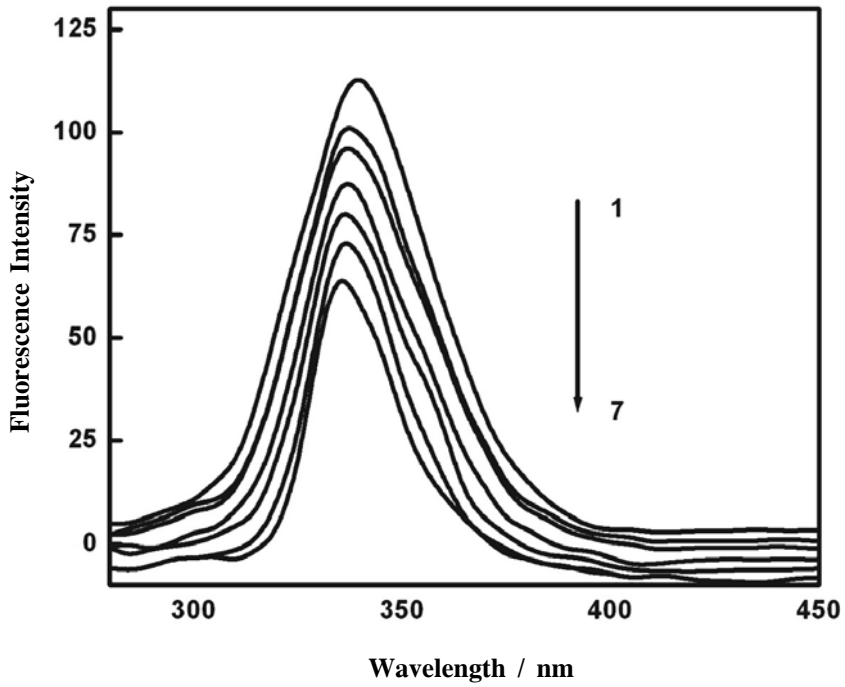

Fig. 6. Synchronous fluorescence spectra of 10 μM BSA at 298K. [TBO]/μM : 1–7 : 0, 5, 10, 15, 20, 25 and 35.

the absorption band at 3395, 2924 and 2699 and strong peak at 1705 cm^{-1} in TBO-BSA complex are the evidence that the interaction have altered the conformation of TBO [31].

CONCLUSION

It is of great importance to study the binding of dye to protein in drug delivery, cosmetic preparation and often detergent action. In this paper, the interaction of TBO with BSA was studied by spectroscopic methods including fluorescence spectroscopy and UV-vis absorption spectroscopy. Fluorescence quenching indicates that the probable quenching mechanism of TBO-BSA binding reaction is initiated by formation of excited state complex rather than by dynamic collision. In other words, the fluorescence quenching of BSA results from complex formation is predominant, while from dynamic collision could be negligible. The binding reaction was spontaneous

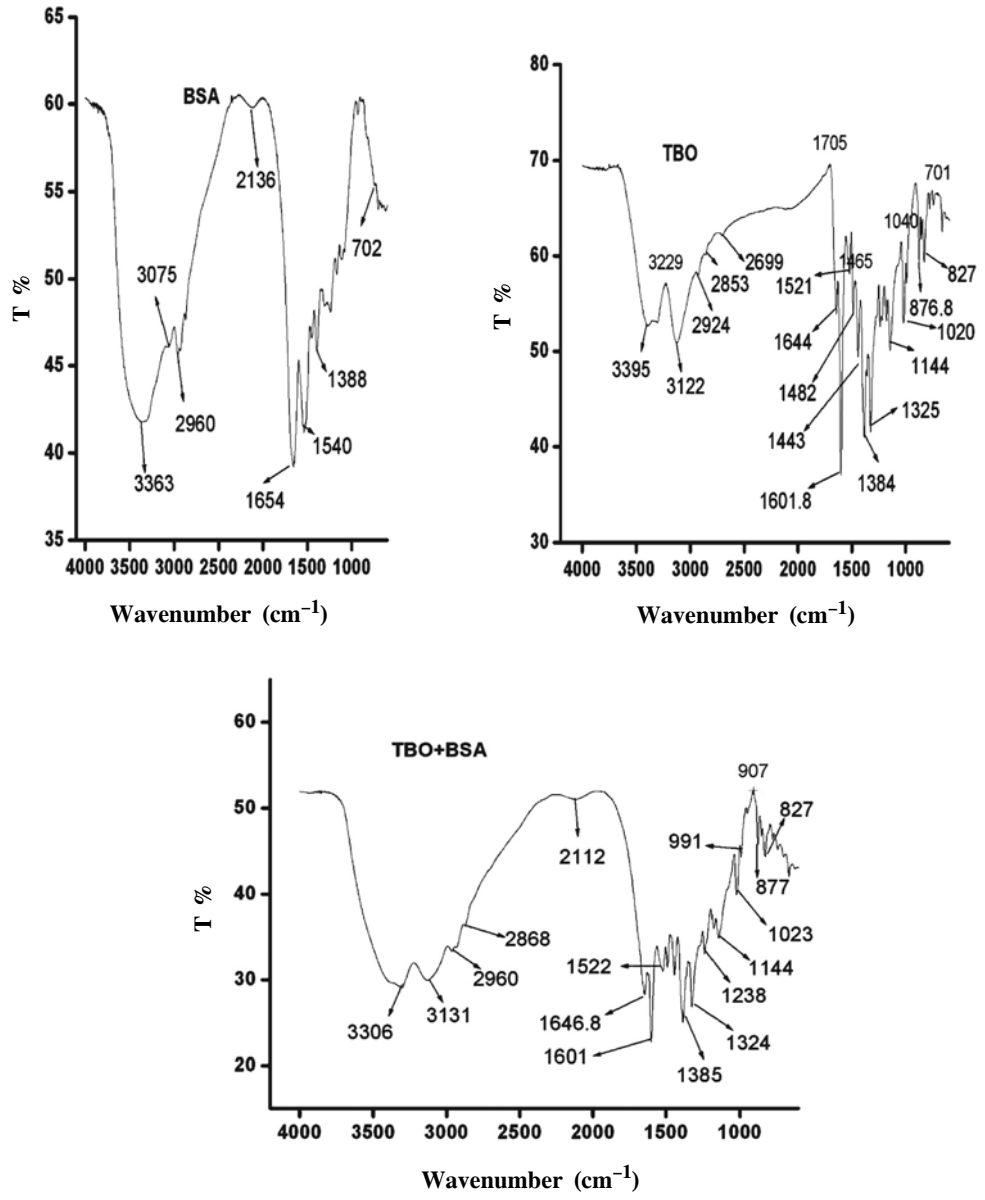

Fig. 7. FTIR-spectra of TBO-BSA complex. Systems : a, 10 μM TBO; b, 10 μM TBO and 10 μM BSA; c, 10 μM BSA.

and endothermic. The negative sign for freeenergy (ΔG^o) indicate that the binding process is spontaneous. Thenegative enthalpy (ΔH^o) and positive entropy (ΔS^o) values of the interaction of TBO and BSA also indicate that the specific electrostatic interactions played major role in the reaction. The results of synchronous fluorescence spectra indicate that the secondary structure of BSA molecules is changed dramatically in the presence of TBO. From IR spectra it was proved that interaction between TBO-BSA system has occurred and conformation of TBO has been altered.

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