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# Investigation on the toxic interaction of toluidine blue with calf thymus DNA

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ABSTRACT

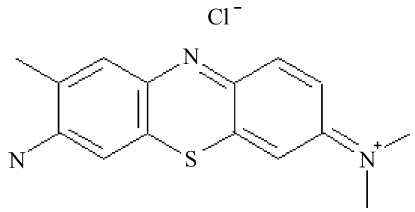
The gene toxic interactions of toluidine blue (TB) with calf thymus DNA (ct-DNA) were examined in vitro with UV–visible absorption spectroscopy, fluorescence spectroscopy, fluorescence polarization and circular dichroism techniques. The experimental results showed that TB interacted with ct-DNA by two binding modes. At low TB concentrations, TB intercalated into the DNA base pairs. At higher TB concentrations, TB was attached to the negatively charged phosphate groups. For the intercalation binding mode and electrostatic binding mode, the binding constants were $1.76 \times 10^6$ L mol$^{-1}$ and $6.18 \times 10^5$ L mol$^{-1}$ and the number of binding sites was 0.48 and 0.79, respectively. Circular dichroism results showed that the two binding modes had different effects on the ct-DNA conformation. At high dye concentrations, Z-form DNA appears, while at low TB concentrations, a B to A form transition is observed.



## 1. Introduction

The study on the interaction between DNA and DNA targeting molecules has been an active field [1]. It is of great significance for determining the interaction mechanisms of anticancer/antiviral drugs with DNA, in vitro selection of drugs and the mechanisms of DNA damage by carcinogens [1].

Using spectroscopic methods, we have studied the gene toxicity interaction of nanoAg-cetylpyridine bromide combination with ct-DNA in vitro from the viewpoint of molecular toxicology [2]. In this article, we discuss the gene toxic interaction of toluidine blue (TB) with ct-DNA. The structure of TB is shown as follows:




Toluidine blue, a positively charged phenothiazine type dye, is highly soluble in water (3.82 g/100 mL) [3,4]. It has been widely used for different purposes in several fields such as medicine, textiles and biotechnology [5]. There have been many reports on the toxicity of TB. Using the Ames test, Dunipace et al. [6] found that TB has a mutagenic effect. Van Duijn [7] found that TB can significantly decrease the mean half-life of motile bull spermatozoa which indicates an intrinsic toxicity of TB. Krüger et al. [8] found that TB can induce structural changes of rat mast cells. Popa and Bosch [9] reported the interaction of ribonucleic acid and toluidine blue by gel electrophoresis and spectrophotometry and found that TB forms two types of complexes with rRNA depending on the molar ratios of dye and rRNA nucleotides.

TB has toxic in the level of organism [7], cell [8] and has toxic interaction with RNA [9]. TB may also interact with DNA to show toxic, but research on the interactions of TB and DNA has not been reported. By using spectroscopic methods (including UV–visible absorption, fluorescence, fluorescence polarization and circular dichroism techniques), we investigated the interactions between TB and ct-DNA and the conformational changes of ct-DNA induced by exposure to TB. This work provides evidence for a possible genotoxic mechanism of TB in vivo.

## 2. Experimental

### 2.1. Apparatus

Fluorescence measurements were made with a HITACHI Model 850 fluoro-spectrophotometer. Fluorescence polarization measurements were made using a LS-55 spectrofluorimeter (PE). UV–visible absorption spectra were measured on a UV-2450 spectrophotometer (SHIMADZU, Kyoto, Japan). All CD spectra were measured on a J-810 circular dichroism spectrometer (JASCO). All

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pH measurements were made with a pHs-3C acidity meter (Pengshun, Shanghai, China).

### 2.2. Reagents

Stock solutions of nucleic acids were prepared by dissolving commercial calf thymus DNA (ct-DNA, Sigma) in 50 mL calibrated flask. This solution was stored at 0–4 °C and shaken gently as needed. The nucleotide concentration was $2.26 \times 10^{-4}$ mol $L^{-1}$, determined by the absorbance at 260 nm ($\varepsilon_{260}$ = 6600 L $mol^{-1}$ $cm^{-1}$).

A TB stock solution ($1.0 \times 10^{-3}$ mol $L^{-1}$) was prepared by dissolving 0.0306 g of TB (Sinopharm Chemical Reagent Co., Ltd.) in 100 mL of water. This solution was further diluted as required. When preparing the reaction systems in 10-mL standard flask, 1.0 mL of Britton–Robinson buffer (BR) solution was used to control pH which consisted of a mixture of 0.04 mol $L^{-1}$ $H_3BO_3$, 0.04 mol $L^{-1}$ $H_3PO_4$ and 0.04 mol $L^{-1}$ $CH_3COOH$ that had been titrated to the desired pH with 0.2 mol $L^{-1}$ NaOH.

All the chemicals used were analytical-reagent grade and ultrapure water was used throughout.

### 2.3. Procedures

After prepared, the reaction systems were placed for incubation of 20 min, then, the spectra were measured.

#### 2.3.1. Fluorescence spectra

The fluorescence intensities of the TB solution ($F_0$) and the mixed TB/ct-DNA solution ($F$) were measured on the Hitachi 850 fluorospectrophotometer. The excitation wavelength ($\lambda_{ex}$) was 633 nm. The excitation and emission slit widths were set at 5.0 nm. The fluorescence quenching [taken as the value of ($F_0/F$)] was thus obtained.

#### 2.3.2. Fluorescence polarization experiments

The fluorescence polarization measurements were carried out on the PE LS-55 spectrofluorimeter. The excitation and emission wavelength was 627 nm and 645 nm, respectively. The excitation and emission slit widths were 10.0 nm.

## 3. Results and discussion

### 3.1. The interaction of TB with DNA determined by UV–visible spectrophotometry

To investigate the interaction of TB with ct-DNA, the absorption spectra of several concentrations of TB in the presence of different concentrations of ct-DNA were measured (Fig. 1).

As reported in other dye-DNA systems [10–12], a hypochromic effect and blue shift of the absorption spectra are signs of external binding, while a red shift of the spectra is a sign of intercalation. It can be seen from Fig. 1 that TB had an absorption peak at 632 nm. When $\gamma$ ($\gamma$ = [DNA]/[TB]) was low ($\leq$2.26; curves a–g), the absorption peak decreased with increasing ct-DNA concentration, which demonstrates that TB was attached to the helix exterior. When $\gamma$ was higher ($\geq$4.52; curves h–k), as ct-DNA increased, the absorption peak increased and red shifted (from about 632 nm to about 645 nm), which demonstrates that TB intercalated into DNA base pairs. A red shift has also been detected in the spectra of some three-dimensional dyes which bind to the groove in DNA [13,14]. However, TB has a planar structure, so we believe that TB interacts with DNA by intercalation.

### 3.2. Fluorescence spectra

The effect of DNA concentration on the fluorescence of TB was studied (Fig. 2). Free TB displayed an excitation maximum at 633 nm and an emission maximum at 658 nm. It can be seen from Fig. 2(A) that the fluorescence of TB was efficiently quenched by ct-DNA. The quenching extent increased with the increasing of ct-DNA concentration. The position of the emission maximum remained unchanged. The phenomena are in agreement with the experimental results reported by Guo et al. [15] who used this effect to measure the concentration of nucleic acids.

Fluorescence quenching includes dynamic quenching and static quenching. Dynamic quenching only affects the excited state of the fluorescence molecules, so the absorption spectra of the fluorescence molecules do not change. In contrast, static quenching often causes changes of the absorption spectra of the fluorescent molecules [16]. The change of the absorption spectra in Fig. 1 indicates that the fluorescence quenching process of TB by DNA is electrostatic binding. Utilizing the formula $F_0/F = 1 + KsvC$, where $C$ is the concentration of DNA, we got the quenching constant ($Ksv$) under the electrostatic binding mode (Fig. 2(B)) which was $1.58 \times 10^3$ L $mol^{-1}$. At higher DNA concentrations, under the intercalative binding mode, the quenching curve deviate from the original straight line toward abscissa (Fig. 5). The phenomena indicate that DNA has a stronger quenching ability under the electrostatic binding mode than intercalative binding mode.

#### 3.2.1. Effect of TB concentration on fluorescence quenching

We investigated the effect of TB concentration on the fluorescence quenching of TB by ct-DNA (Fig. 3). The quenching extent ($F_0/F$) increased and then decreased with increasing TB. The quenching extent reached its peak at a TB concentration of $4.0 \times 10^{-6}$ mol $L^{-1}$. We selected $4.0 \times 10^{-6}$ mol $L^{-1}$ for further study.

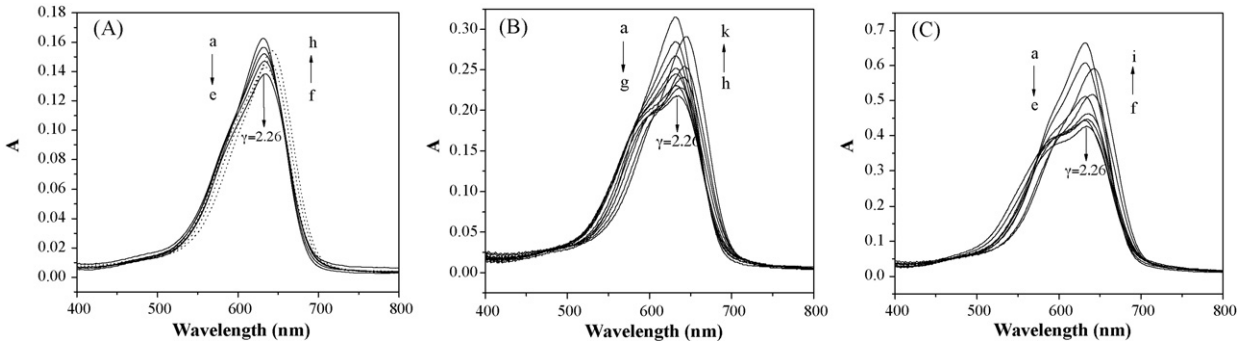


**Fig. 1.** The UV–visible absorption spectra of TB in the presence of different concentrations of ct-DNA at room temperature (blank vs. the same concentration of buffer). Conditions—(A) TB: $5.0 \times 10^{-6}$ mol $L^{-1}$; ct-DNA/($10^{-6}$ mol $L^{-1}$, nucleotide concentration): a, 0; b, 3.05; c, 6.09; d, 9.14; e, 11.30; f, 18.27; g, 30.45; h, 60.9. (B) TB: $1.0 \times 10^{-5}$ mol $L^{-1}$; ct-DNA/($10^{-6}$ mol $L^{-1}$): a, 0; b, 2.26; c, 4.52; d, 6.78; e, 9.05; f, 13.57; g, 22.62; h, 45.24; i, 67.85; j, 113.08; k, 180.92. (C) TB: $2.0 \times 10^{-5}$ mol $L^{-1}$; ct-DNA/($10^{-5}$ mol $L^{-1}$): a, 0; b, 1.10; c, 2.86; d, 4.17; e, 4.52; f, 4.872; g, 6.09; h, 12.18; i, 18.27; pH 7.5.

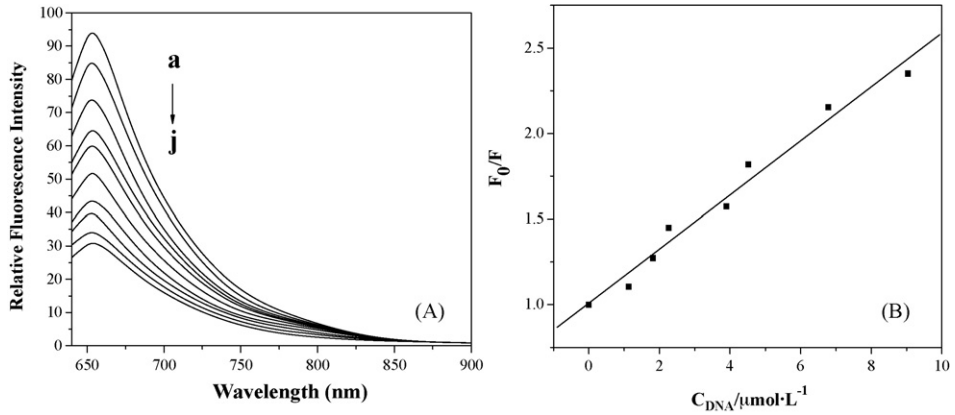


**Fig. 2.** (A) The fluorescence spectra of TB in the presence of ct-DNA. (B) The Stern–Volmer plot for the quenching of TB by ct-DNA under the electrostatic binding mode at room temperature. conditions: TB: $4.0 \times 10^{-6}$ mol L$^{-1}$; ct-DNA/($10^{-6}$ mol L$^{-1}$, nucleotide concentration): a, 0; b, 1.13; c, 1.81; d, 2.26; e, 3.9; f, 4.52; g, 6.78; h, 9.04; i, 11.3; j, 13.56; pH 7.5.

### 3.2.2. Effect of pH on fluorescence quenching

The effects of pH on the fluorescence quenching of TB by ct-DNA are shown in Fig. 4. The quenching extent was quite stable in the pH range 6.5–8.5. We selected the physiologically relevant pH 7.5 for further study.

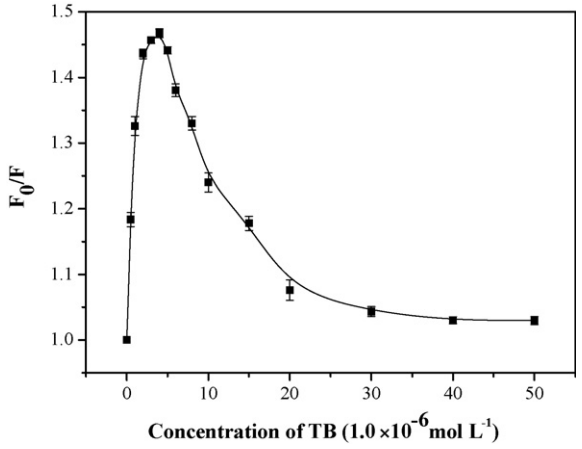


**Fig. 3.** Effect of TB concentration on fluorescence quenching at room temperature. Conditions: ct-DNA: $2.26 \times 10^{-6}$ mol L$^{-1}$ (nucleotide concentration); pH 7.5.

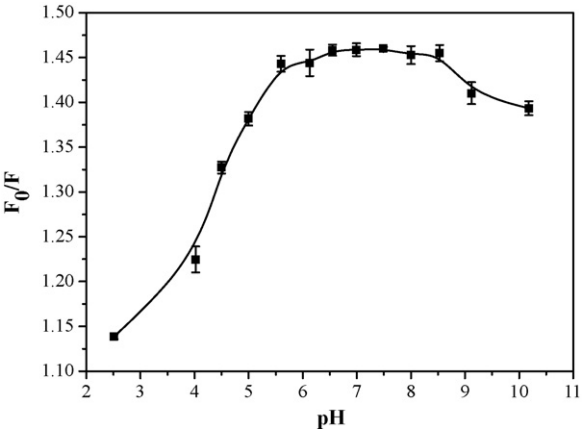


**Fig. 4.** Effect of pH on fluorescence quenching at room temperature. Conditions: TB: $4.0 \times 10^{-6}$ mol L$^{-1}$; ct-DNA: $2.26 \times 10^{-6}$ mol L$^{-1}$ (nucleotide concentration).

### 3.2.3. Effect of native and denatured DNA on fluorescence quenching

The effects of the native and denatured DNA on the fluorescence of TB ($F_0/F$) were compared (Fig. 5). Double strand DNA (dsDNA) was converted into single strand DNA (ssDNA) by incubating at 100 °C for 10 min, then quenching in ice-water. When small molecules bind to the phosphate backbone of DNA, the effects of dsDNA and ssDNA on the quenching of their fluorescence should be uniform. If small molecules are intercalated into the helix stack, the fluorescence quenching by ssDNA would be smaller than that by dsDNA [17].

When $\gamma$ was low ($\leq$2.26), there was little difference between the effects of dsDNA and ssDNA on the quenching of TB fluorescence (Fig. 5), which indicates that TB bound to the phosphate backbone of ct-DNA. At higher $\gamma$ ($\geq$4.52), the quenching of TB fluorescence by ssDNA was smaller than that by dsDNA, which indicates that TB intercalated into the ct-DNA helix. The ssDNA can still quench the fluorescence of TB because of the interaction between TB and ssDNA bases.

### 3.3. Fluorescence polarization measurements

The fluorescence polarization of small molecules is small due to their rapid tumbling motion. If small molecules intercalate into the helix of DNA, their rotational motion should be restricted, hence

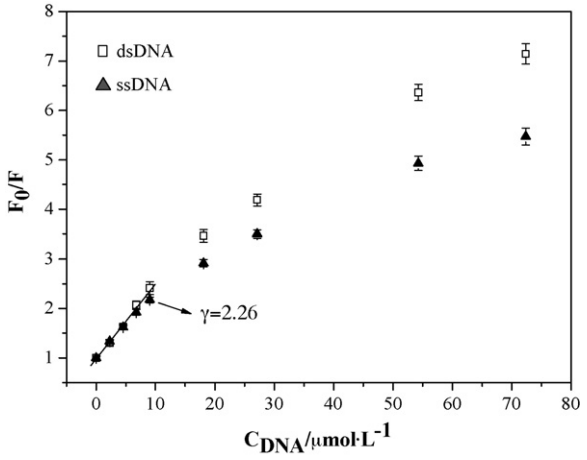


**Fig. 5.** Comparison between the effects of dsDNA and ssDNA on the quenching of TB fluorescence. Conditions: TB: $4.0 \times 10^{-6}$ mol L$^{-1}$; pH 7.5.

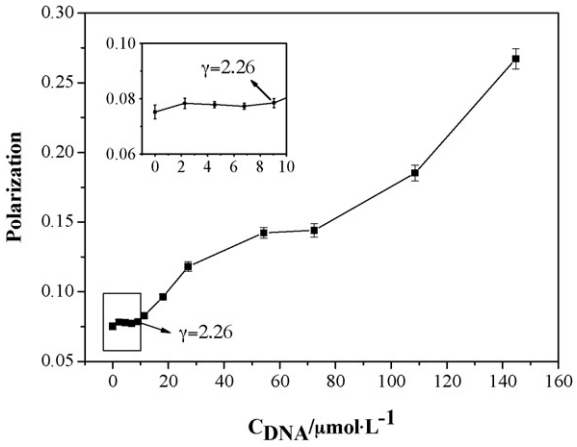


**Fig. 6.** Effect of ct-DNA on the fluorescence polarization of TB. Conditions: TB: $4.0 \times 10^{-6}$ mol L$^{-1}$; pH 7.5.

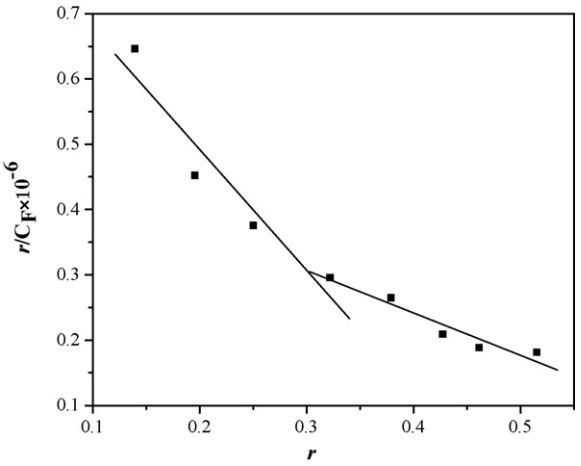


**Fig. 7.** Fluorescence Scatchard plot for the binding of TB to ct-DNA at room temperature. Conditions: TB: $4.0 \times 10^{-6}$ mol L$^{-1}$; pH 7.5.

the fluorescence polarization increases. Mere binding to the phosphate backbone or to the DNA grooves does not result in enhanced fluorescence polarization [18].

As shown in Fig. 6, when $\gamma$ was low ($\leq$2.26), the fluorescence polarization of ct-DNA–TB system was small and there was no obvious change of the polarization, which indicates that the binding mode is a non-intercalative mode. When $\gamma$ was higher ($\geq$4.52), the fluorescence polarization increased with ct-DNA concentration. The large increase of fluorescence polarization upon TB binding to ct-DNA supported the intercalation of TB into the DNA helix.

The conclusion of the fluorescence polarization results is in agreement with that of the UV–visible spectra and the fluorescence spectra. Popa and coworkers [9,19] studied ribonucleic acid–TB complexes by gel electrophoresis and spectrophotometry and obtained a similar conclusion. They found that at low dye concentrations, natural RNA and TB form the so-called Complex I, in which intercalation of TB between Watson and Crick type base pairs is assumed to occur. At higher dye/nucleotide ratios TB is attached to the negatively charged phosphate groups of rRNA nucleotides to form Complex II.

### 3.4. Determination of binding constants from a Scatchard plot

The binding constant between ct-DNA and TB can be evaluated by the Scatchard equation [20]:

$$\frac{r}{C_F} = kn - kr \tag{1}$$

where $k$ is the binding constant, $n$ is the number of binding sites on the DNA molecule, $r$ is the moles of bound TB per mole DNA ($r = C_B/[\text{DNA}]$) and $C_F$ and $C_B$ are the concentrations of free and bound TB, respectively.

$C_F$ and $C_B$ were evaluated by the following equations [21,22]:

$$C_F = \frac{C_T(I/I_0 - q)}{(1 - q)} \tag{2}$$

$$C_B = C_T - C_F \tag{3}$$

where $C_T$ is the total concentration of TB, $I$ and $I_0$ are the fluorescence intensity of TB with and without DNA, respectively, $q$ is the ratio of the observed quantum yield of fluorescence of the totally bound TB to that of the free TB. By plotting $I/I_0$ vs. $1/[\text{DNA}]$, a straight line can be obtained where $q$ is the $y$-axis intercept of the line.

Based on the calculation of the fluorescent data of TB under different DNA concentrations utilizing the equations mentioned above, a plot of $r/C_F$ vs. $r$ at room temperature is shown in Fig. 7. The Scatchard plot is non-linear which indicates that there is more than one binding mode between TB and ct-DNA. The experimental binding constant $k$ for electrostatic binding and intercalation binding are $6.18 \times 10^5$ L mol$^{-1}$ and $1.76 \times 10^6$ L mol$^{-1}$, respectively. The number of binding sites on the DNA molecule is 0.79 and 0.48, respectively.

### 3.5. Interaction between TB and ct-DNA measured by circular dichroism

Circular dichroism (CD) can show if TB binding can alter the ct-DNA conformation.

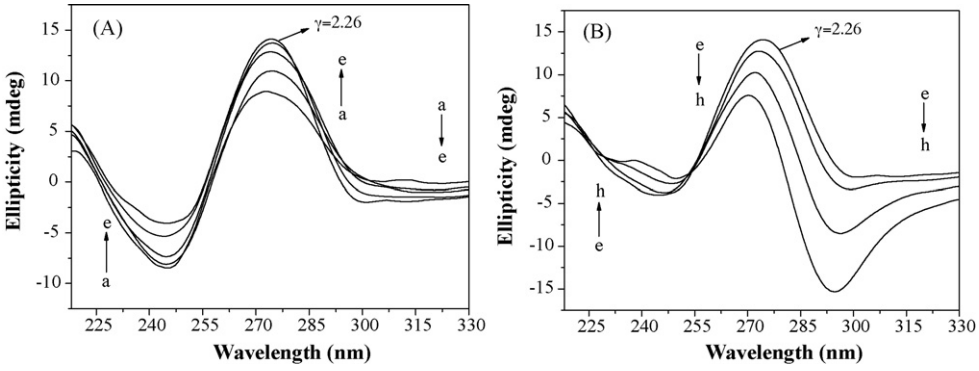


**Fig. 8.** Circular dichroism spectra of ct-DNA at different TB concentrations. Conditions: ct-DNA: $1.13 \times 10^{-4}$ mol L$^{-1}$ (nucleotide concentration); TB/($10^{-5}$ mol L$^{-1}$): a, 0; b, 1; c, 2; d, 4; e, 5; f, 6; g, 8; h, 10; pH 7.5.

It can be seen from Fig. 8(A) that the CD spectrum of free ct-DNA consists of a negative band at 245 nm and a positive one at 273 nm, characteristic of the B-form DNA [23]. When $\gamma$ was high ($\geq$2.26) (Fig. 8(A)), corresponding to the intercalation binding mode, the negative band at 245 nm decreased and the positive band at 273 nm increased as TB increased, which are characteristic of a B $\rightarrow$ A conformational transition of DNA [23,24]. When $\gamma$ was low ($\leq$2.26) (Fig. 8(B)), corresponding to the electrostatic binding model, with increasing TB, the negative band at 245 nm decreased accompanied by a red shift and the positive band at 273 nm decreased accompanied by a blue shift. A new negative peak also appeared at about 295 nm which is characteristic of Z-form DNA [25].

TB can change the conformation of ct-DNA. At high TB concentrations, Z-form DNA appears, while at low concentrations, a B to A form transition is observed.

## 4. Conclusions

We explored the mechanisms of interaction of TB with ct-DNA. The results of UV-absorption, fluorescence spectra and fluorescence polarization agree with each other. At low dye concentrations ($\gamma \geq 4.52$), TB intercalates into the DNA base pairs. At higher dye concentrations ($\gamma \leq 2.26$), TB is attached to the negatively charged phosphate groups. The CD results show that the two binding modes have different effects on the DNA conformation. At high dye concentrations, Z-form DNA appears, while at low TB concentrations, a B to A form transition is observed.

The binding constant of the intercalation binding mode was $1.76 \times 10^6$ L mol$^{-1}$ and the number of binding sites on the DNA molecule was 0.48. For the electrostatic binding mode, the binding constant was $6.18 \times 10^5$ L mol$^{-1}$ and 0.79 binding sites are calculated per DNA molecule.

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