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Study on the interaction between methylene violet and calf thymus DNA by molecular spectroscopy

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Abstract

The fluorescence and solid surface room temperature phosphorescence (SS-RTP) spectral properties of methylene violet (MV) were investigated. And the factors influencing the phosphorescence emission were discussed. The interaction of MV and ctDNA was studied by molecular spectroscopy. Deduced from the fluorescence, SS-RTP and resonance light-scattering spectral data, there were two different interaction mechanism involved in the whole interaction process depending on the *R*-values(*R*: the molar ratio of MV to ctDNA) at pH = 7.56. The value $R = 1.9$ is the turning point; when $R < 1.9$, enhanced resonance light-scattering (RLS) was observed, which indicated long range assembly of MV on the ctDNA molecular surface. In this process long-range assembly is the main binding mode. The second process occurs when $R > 1.9$, where MV intercalated into the ctDNA helix, the intercalative binding is predominant, supported by fluorescence polarization experiment and potassium iodide quenching study. Based on the fluorescence quenched experiment of MV by ct DNA, the binding constant of MV binding to ctDNA was 1×106 L/mol, the binding site was 1.0.

Keywords: Methylene violet; ctDNA; Interaction; Fluorescence; SS-RTP; RLS

1. Introduction

The binding of small molecules to DNA has been of great interest due to the importance in understanding the drug–DNA interactions and the consequent design of new efficient drugs targeted to DNA [1–4]. There are three modes about binding of small molecules to DNA double helix: electrostatics binding, groove binding and intercalative [5,6]. Electrostatic binding are the interactions between cationic species and the negatively charged DNA phosphate backbone, which are along the external DNA double helix and do not possess selectivity [5]. Groove binding interacted with two grooves of DNA double helix generally involves direct hydrogen bonding or van der Waals' interactions with the nucleic acid bases in the deep major groove or the wide shallow minor groove of the DNA helix. Stacking interactions between nucleobases and aromatic ligands are important in defining the intercalative binding, which is defined when a planar, hetero-aromatic moiety slides between the DNA base pairs and binds perpendicular to the helix. Therefore, it is apparent that the intercalative binding and groove binding are related to the grooves in the DNA double helix but the electrostatic binding can take place out of the groove. Understanding the modes of the binding of small molecules to DNA and the factors that can affect the binding is of fundamental importance in understanding DNA binding in general. Among the three modes, the most effective mode of the drugs targeted to DNA isintercalative binding [7]. Thus the research of the interactive model can provide a start for the design of the structure of new and efficient drug molecules. In general, planarity was suggested to be one of the important features needed for efficient intercalators [8]. A non-negative charge on the small molecule is considered another important feature, otherwise electrostatic repulsion between anionic DNA polymer and small negative molecules inhibit interaction efficiency.

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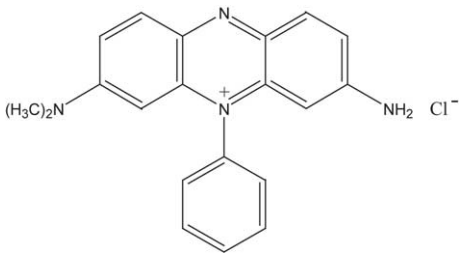

Scheme 1. Structure of methylene violet (MV).

The planar dye molecules, such as phenazine dye, which can interact with DNA by intercalation, had been testified with several methods [9]. Methylene violet (MV) (Scheme 1), a nontoxic water-soluble dye of class, with sharp absorption peak in visible region, is an ideal substrate for photometric monitoring. It was used as a photosensitizing dye for pathogen inactivation has been studied in both red cell and platelet blood components [10]. MV can destroy bacteria and viruses when irradiated with visible light and it is believed that this is connected with photosensitizing DNA damage [11]. MV features a planar phenazine ring, which is expected to facilitate intercalation of MV into the relatively nonpolar interior DNA helix. In addition, cationic charge on the MV molecule is also expected to improve the DNA binding affinity due to the increased electrostatic attraction between the MV molecule and the DNA phosphates. What we reported here on the interaction between MV and DNA may be relevant to our understanding of the mechanism of bioactivity of phenazine derivatives on the bio-molecular level and the consequent design of new efficient drugs targeted to DNA.

Spectral analysis methods have been employed to investigate DNA binding and DNA determination including absorption, fluorescence and resonance light scattering (RLS) spectrum [12–14]. Phosphorescence is another spectral technique expected used in this field for its longer emission wavelength and larger Stokes' shifts, which makes it possible to avoid the interference from the background in the biological and clinic sample. Solid surface substrate room temperature phosphorescence (SS-RTP) is an analytical technique combining microtechnique with trace analysis, and it had been applied widely in the fields of environmental research, biochemistry, clinical chemistry and pharmaceutical analysis [15,16] for its simplicity, high sensitivity and selectivity. The employment of SS-RTP in the study of DNA provided more useful messages and favorable evidence for the DNA binding. There are a few papers [17,18] to examine the DNA binding by SS-RTP and they all focused on the phyrin macromolecules, while the study of small molecule dyes binding to DNA by SS-RTP have not been reported. In this paper, molecular spectroscopy including fluorescence and SS-RTP was introduced to investigate the interaction with DNA. The essence of the different interactive model depending on the R (C_{MV}:C_{DNA}) was discussed.

2. Experimental

2.1. Apparatus

In this paper, the fluorescence, RLS and SS-RTP measurements were made with a F-4500 fluorescence spectrophotometer (HITACHI, Japan), which was equipped with a 150 W xenon arc lamp, a recorder, dual monochromators and a quartz glass (1 cm × 1 cm). A sample holder (made by Institute of Opto-Electronics, Shanxi University) was used for solid substrate and a phosphorescence attachment. A drying box, equipped with a 250 W IR lamp, was used to dry the samples, and temperature was controlled automatically (±1 °C). Filter paper purchased from Hangzhou Xinhua Paper Factory was used as a substrate. An amount of 5 μL syringes (Shanghai Medicine Laser Instruments Factory) were used for delivery of samples and heavy atom solution. The pH of solution was measured on Model pHS-29 A pH-meter (Shanghai Second Analysis Instrumental Factory, China).

2.2. Regents and materials

Calf thymus DNA (ctDNA) was commercially purchased from Sigma and used without further purification. It was dissolved in NaCl (0.1 mol/L) at a final concentration of 1 mg/ml and stored at 0–4 °C, the concentration was determinated by the absorption of ctDNA at 260 nm after establishing the absorbance ratio of A_{260}/A_{280} in the range of 1.8–1.9, and molarities of ctDNA was calculated based on $\varepsilon_{260} = 6600\ \text{L mol}^{-1}\ \text{cm}^{-1}$. Methylene violet was of analytical reagent grade (the third reagent factory, Shanghai, China). The stock solution of 1×10^{-3} mol/L was prepared by directly dissolving the compound in doubly distilled water, and the working solution was prepared by appropriate dilution before use. Briton-Robinson buffer controlled the pH of the working solution. Water was doubly distilled water in a sub-boiling still. All other reagents and inorganic salts were analytical reagent grade.

2.3. Procedure

2.3.1. Fluorescence

A 1.0 ml of buffer solution (pH = 7.56) and certain volume of MV solution were transferred to a 10 ml volumetric flask, and an appropriate amount volume of ctDNA standard solution was added. The mixed solution was diluted to the final volume with doubly distilled water and shaken thoroughly, then equilibrated for 5 min. Record the fluorescence spectra of the mixed solution and the reagent blank on a F-4500 fluorescence spectrophotometer. Measure the fluorescence intensities of the mixed solution and the reagent blank with the following settings of the F-4500 fluorescence spectrophotometer: excitation wavelength, 538 nm; emission wavelength, 605 nm; excitation slit, 10 nm; emission slit, 5 nm.

2.3.2. SS-RTP

The filter paper was cut into 17 mm × 14 mm strips on which one line with an interval of 0.4 cm was engraved with a graver at the position of the excitation light spot in order to limit the extent of sample solution. A heavy atom solution of 5 μL was spotted onto the surface of the filter paper strip by using a 5 μL microsyringe. The paper strip was pre-dried at 88–90 °C for 40 s, then a sample solution of 5 μL was deposited on the same position. The paper was then dried for 2 min. The filter paper strip was fixed to a solid substrate holder and covered with a quartz glass piece to avoid humidity. Then the sample holder was placed in the sample compartment immediately for measuring the SS-RTP intensity or recording the spectra.

Measurements of the SS-RTP intensity of MV were executed at the corresponding maximal of excitation and emission wavelength, and the widths of $slit_{ex}$ and $slit_{em}$ were set at 10/20 nm.

2.3.3. RLS

By scanning both the excitation and emission monochromators of a common fluorescence spectrophotometer with $\Delta\lambda = 0$ nm, resonance light-scanning (RLS) spectra had been developed [18] and proved to be able to investigate the aggregation of small molecules and the long-range assembly of organic dyes on biological template [20].

3. Results and discussion

3.1. Spectral properties of MV

The SS-RTP of MV was observed using filter paper as solid substrate and $Pb(Ac)_2$ as heavy atom perturbers, and room temperature fluorescence (RTF) was studied in aqueous solution. Figs. 1 and 2 showed the spectral properties of MV. The excitation and emission wavelengths were 538/605 nm for fluorescence and 538/715 nm for SS-RTP, respectively. It is obvious that longer emission wavelength and larger Stokes' shift are the advantages of SS-RTP over the fluorescence.

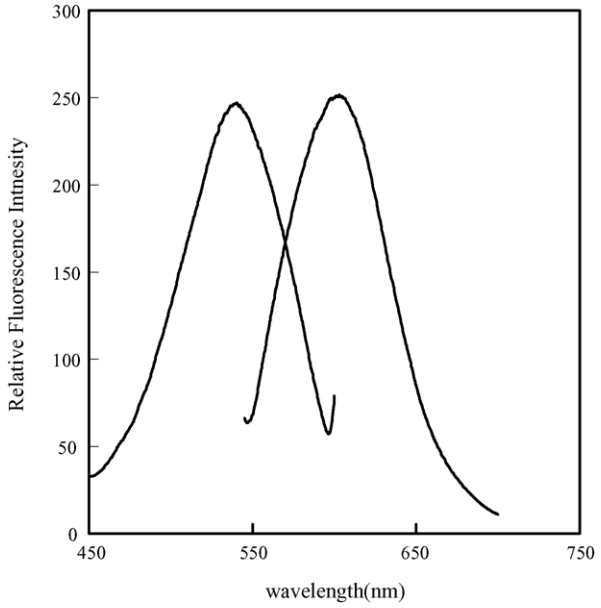

Fig. 1. The fluorescence spectra of MV (2×10^{-5} mol/L, pH = 7.56).

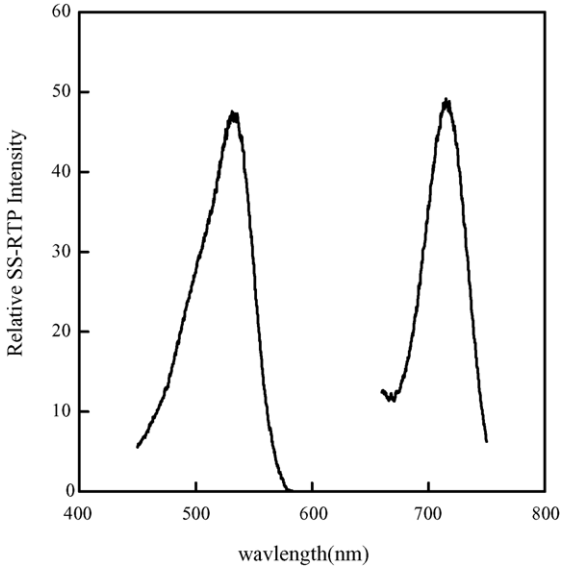

Fig. 2. The phosphorescence spectra of MV (1×10^{-3} mol/L, pH = 7.56).

3.2. Optimized measurement conditions of SS-RTP

3.2.1. Effect of filter paper substrate

China-made filter paper had been widely used as substrate in SS-RTP because of its availability of wide selection [21]. The SS-RTP spectra properties of MV adsorbed on several filter papers were examined and the slow speed quantitative filter paper was selected as the optimum substrate for inducing SS-RTP emission for its lower background and higher intensity. When different filter papers were applied there is no significant changes in the shape and position of the maximum of the excitation and emission spectra.

3.2.2. Effect of the heavy atom

The heavy-atom is necessary in inducing the SS-RTP [22]. In the presence of heavy atom, the mixing of the singlet state and triplet state takes place as a result of spin–orbit coupling. This condition favors a high probability of intersystem crossing from the lowest excited singlet state to triplet state in the molecule, which will induce and enhance the emission of phosphorescence. Different heavy atom has different enhancement action on phosphorescence. The effect of 30 inorganic salts as heavy-atom perturber on the SS-RTP intensities of MV adsorbed on filter paper was investigated. Among all the salts investigated, only the lead(II) acetate could induce the SS-RTP emission of MV. Considering the

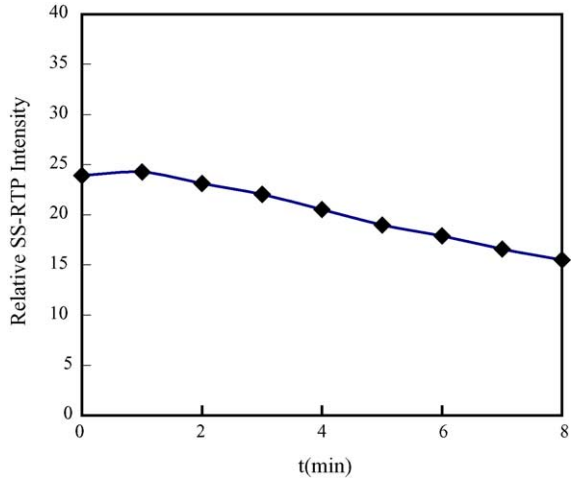

Fig. 3. Effect of laying time on SS-RTP intensity of MV (1×10^{-3} mol/L, pH = 7.56).

solubility of lead(II) acetate, the optimum $Pb(Ac)_2$ concentration 1.0 mol/L containing 2.0 mol/L acetate was chosen for the further work.

3.2.3. *Effect of drying temperature, drying time and stability*

The sample drying conditions, such as drying temperature and drying time, were examined in detail and the following results could be obtained. (1) The drying temperature is in the range of 88–90 °C. (2) The pre-drying time was the time to dry the wet substrate after spotting the heavy-atom solution before adding the sample solution. The appropriate pre-drying time was 40 s. (3) The RTP intensities varied with the drying time and the highest and the most stable RTP could be obtained after drying for 2 min.

The stability of SS-RTP of MV was examined and the SS-RTP intensities of MV changed slightly in the first 4 min and altered distinctly during 8 min (Fig. 3). However, the determination of one sample only needed 1 min. Consequently, the stability time of MV could meet the needs of the determination.

3.3. *The SS-RTP lifetime of MV*

In the study of quenching, energy transfer and photochemical reaction, the luminescence lifetime is an important parameter. The phosphorescence lifetime indicates the probability of intersystem crossing ($T_1 \rightarrow S_0$) and the triplet deactivation, and it is a measure by which the interaction mechanism between luminophor and substrate can be discussed. The phosphorescence lifetime can offer more information about the interaction of analyte and environmental factors there are some reports [23,24] involving the studies of phosphorescence lifetime

The measurement of lifetime follows:

$$\ln I_t = \ln I_0 - \frac{t}{\tau} \quad (1)$$

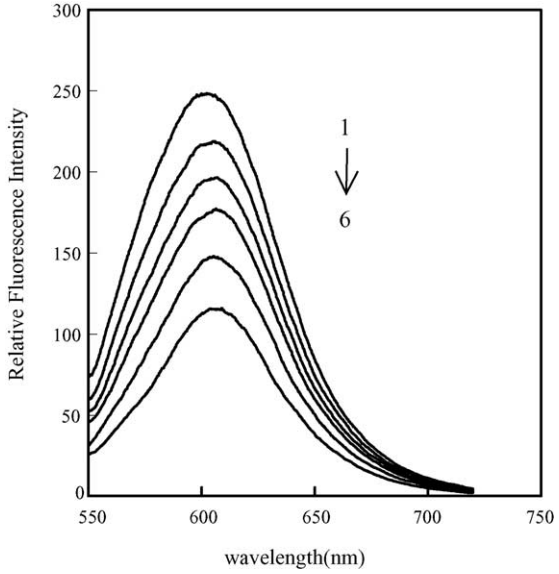

Fig. 4. The fluorescence spectra of MV (2×10^{-5} mol/L, pH = 7.56) in the different concentration of DNA. [ctDNA] = 1: 0, 2: 2.42×10^{-6}, 3: 4.84×10^{-6}, 4: 6.30×10^{-6}, 5: 7.75×10^{-6}, 6: 9.21×10^{-6} mol/L (from 1 to 6).

where τ is the lifetime; t the decay time; I_t the luminescence intensity at decay time t after excitation; I_0 is defined as pre-exponential factor of the decay curve, was the prompt transient luminescence intensity at zero decay time. I_t and I_0 could be written as F_0 and F_t in fluorimetry or P_0 and P_t in phosphorimetry, respectively. Therefore a calibration plot would be constructed directly from I_0. The value of τ can be obtained by Eq. (1).

Measurement of the SS-RTP lifetime of MV was taken at the corresponding maximal of excitation and emission wavelength and the concentration of working solution was 5×10^{-4} mol/L. The phosphorescence lifetime of MV was 165.9 ms in the absence of DNA, which showed that the phosphorescence of MV belongs to long lifetime luminescence. It was 305.8 ms in the presence of DNA of $6 \times 10-6$ mol/L. This result might occurred because the formation of MV with DNA leads to stronger rigidity and, therefore, to a longer lifetime.

3.4. *Possibilities of MV as probes for DNA*

Upon binding to DNA, the fluorescence and SS-RTP of MV were efficiently quenched by the DNA bases, with no shifts in the maximal emission. Figs. 4 and 5 displayed the fluorescence and SS-RTP features by fixing MV concentration and varying DNA concentration. As shown in Fig. 4, the fluorescence intensities of MV decreased clearly with the increase of DNA concentration. When the concentration of DNA increased to 9.21×10^{-6} mol/L, the change of fluorescence intensity of MV tended to be constant. The similar phenomenon is in the case of SS-RTP. The obvious quench of fluorescence and SS-RTP emission of MV after addition of

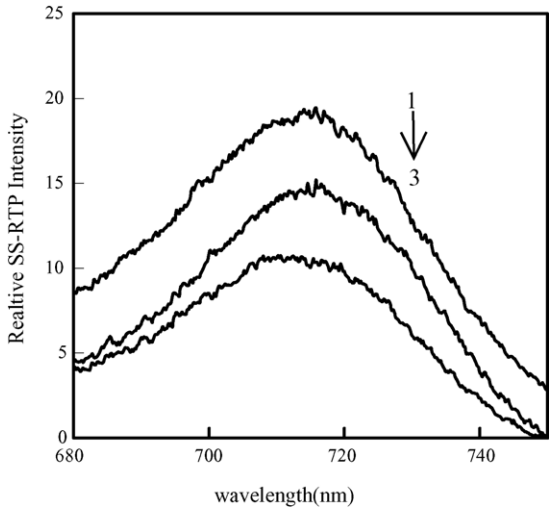

Fig. 5. The phosphorescence spectra of MV (1×10^{-3} mol/L, pH = 7.56) in the different concentration of ctDNA. [ctDNA] = 1: 0, 2: 2.42×10^{-6}, 3: 4.84×10^{-6} mol/L (from 1 to 3).

ctDNA showed that MV intercalated into the DNA bases and indicated that the presence of ctDNA resulted in the change of microenvironment of MV. That is to say, MV can be used as the probe of ctDNA potentially.

3.5. Investigation of interaction of MV with DNA

3.5.1. Effect of pH on the phosphorescence

Because MV has four nitrogens, it is required to study the effect of pH on the SS-RTP of MV in the absence and presence of DNA. Fig. 6 showed that the pH change influences the luminescent intensity distinctly. The quench is the largest when the pH of sample solution is in the range of 6.0–9.0, and the neutral condition of pH = 7.56 were selected for further work.

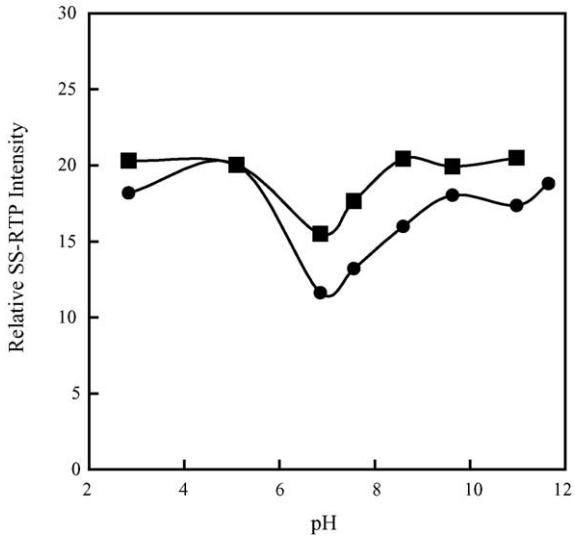

Fig. 6. Effect of pH on SS-RTP intensity of MV ([MV] = 1×10^{-3} mol/L, pH = 7.56) in the absence (■) and presence of DNA(●) ([ctDNA] = 3 mg/L).

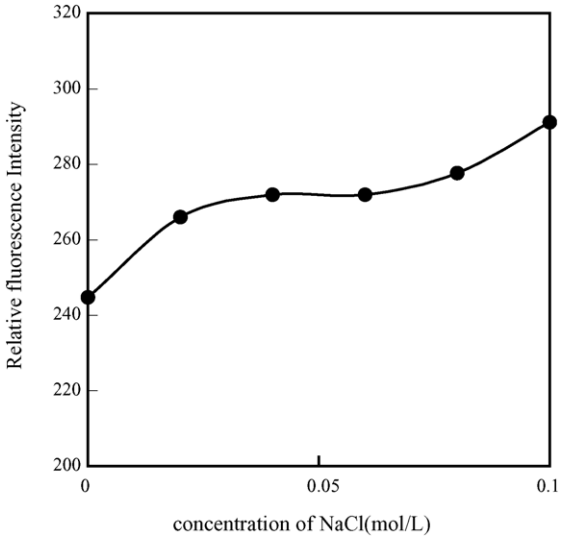

Fig. 7. Effect of ion strength on the fluorescent intensity of MV ([MV] = 1×10^{-5} mol/L, pH = 7.56) in the presence of DNA ([ctDNA] = 3mg/L).

3.5.2. Effect of ionic strength

As a controller of ionic strength of solution, Na^+ act as counter ions to decrease the unwinding tendency for electrostatic repulsion between the negatively charged phosphate groups on adjacent nucleotides [25]. That is to say, the ionic strength controller also leads to a change of the state of nucleic acids. In the other hand, electrostatic attraction between small molecule and DNA surface weakened by the addition of Na^+. The effect of the ionic strength on the MV fluorescence was tested by the addition of a strong electrolyte—NaCl (Fig. 7). In the presence of DNA, the fluorescence intensity of MV was enhanced by the addition of NaCl. This is due to the free of bound MV from the helix and the weakening of electrostatic attraction of MV and ctDNA surface.

3.5.3. Fluorescence quenching study

The fluorescence quenching experiment was performed with potassium iodide and the experimental data were plotted according to the Stern–Volmer equation:

$$\frac{I_0}{I} = 1 + K_{SV}(Q) \quad (2)$$

where I_0 and I are the fluorescence intensities in the absence and in the presence of potassium iodide (Q), and K_{sv} is the Stern–Volmer quenching constant.

Intercalation protected the bound molecules from the anionic quencher, owing to the base above and below the intercalator [26]. Groove binding exposes the bound molecules to the solvent surrounding the helix [27]. Compared with intercalative binding, grooving binding provides much less protection for the chromophore. If small molecule is intercalated into the helix stack, the magnitude of K_{SV} of the bound small molecule should be lower than that of the free

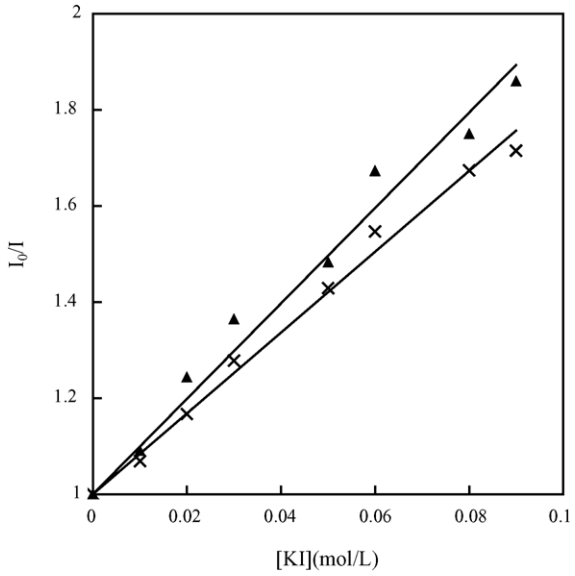

Fig. 8. Quench curve of MV (1×10^{-5} mol/L, pH = 7.56) in the absence (▲) and presence of DNA (×, [ctDNA] = 3 mg/L).

small molecule [28], in contrast, if small molecule binds to DNA in the groove, the magnitude of K_{SV} of the bound small molecule should be higher than that of the free small molecule [29]. In aqueous solutions, potassium iodide quenched the fluorescence of MV efficiently. Therefore, the potassium iodide quenching study was chosen to determine the accessibility of MV to aqueous anionic quenchers in the presence of ctDNA. Addition of potassium iodide to a mixture of MV and ctDNA resulted in decreased quenching of the fluorescence intensity (Fig. 8). K_{SV} values for the free MV and the bound MV with ctDNA at 3 mg/L were $K_{SV} = 9.924$ and 8.405 L/mol, respectively. The results showed that the K_{SV} of the bound MV was lower than that of the free MV, which indicated the MV intercalated into the DNA bases.

3.5.4. Comparison between the effects of double strand DNA and single strand DNA on the quenching of MV fluorescence

Double strand DNA (dsDNA) was converted into single strand DNA (ssDNA) with the open of its double helix by incubated at 100 °C for 10 min and cooled in ice water immediately. If MV intercalated into the helix stack, the quenching of the fluorescence from MV by ssDNA would be smaller than that by dsDNA [30]. The results of comparison of experiments, given in Table 1, showed that the quenching of fluorescence from MV by ssDNA was smaller than that by dsDNA, which also supported the intercalation of MV into the helix.

3.5.5. Binding constant and binding sites

A Scatchard plot is commonly used in the discussion of interaction of small molecules with macromolecules [31,32]

Table 1
Effects of dsDNA and ssDNA on the quenching of MV fluorescence quenching

Concentration of DNA (mg/L)	Kinds of DNA	Relative fluorescence intensity
1.0	dsDNA	240.9
	ssDNA	250.0
3.0	dsDNA	198.1
	ssDNA	213.3
5.0	dsDNA	185.4
	ssDNA	210.9

from,

$$C_F = C_T \left(\frac{I}{I_0} - P \right) (1 - P) \quad (3)$$

$$C_B = C_T - C_F \quad (4)$$

where C_F is the concentration of free luminophor, C_B the concentration of bound luminophor, C_T the total concentration of all luminophor, I the fluorescent intensity of MV at different concentration of DNA, I_0 the fluorescent intensity of MV in the absence of DNA and P the ratio of fluorescence quantum yield of free and bound luminophor. The plotting of I/I_0 versus 1/(DNA) yields the line, and the intercept is P.

According to the Eqs. (3) and (4):

$$\frac{r}{C_F} = -K r + K n \quad (5)$$

where r stands for (C_B/(DNA)), the plotting of r/C_F versus r yields the line and the slope is K, the intercept is Kn, and then we can calculate the binding constant and binding number. The Scatchard plot of MV bound to DNA is $r/C_F = 1.0 \times 10^6 r + 1.0 \times 10^6$ and the binding constant is 1.0×10^6 L/mol and the binding sites is 1.0. The large binding constant for MV indicated that the phenazine ring has high affinity for DNA base pairs. This observation of binding site gives evidence for an intercalation of MV into DNA according to the neighbor-exclusion model, since groove binding and electrostatics binding usually results in significantly higher n values.

3.5.6. Fluorescence polarization measurement

The measurement of polarization follows [33]

$$P = \frac{I_{VV} - G I_{HV}}{I_{VV} + G I_{HV}} \quad (6)$$

Here I_{VV} is the vertical polarization intensity vertical with excitation; I_{VH} is the horizontal polarization intensity vertical with excitation. G is the corrected factor. $G = I_{HV}/I_{HH}$. I_{HV} is the vertical polarization intensity parallel with excitation; I_{HH} is the horizontal polarization intensity parallel with excitation.

Small molecule weakly polarized due to the rapid tumbling motion in aqueous media. However, when small molecule chromophore intercalates into the helix, its rotational motion should be restricted and therefore the

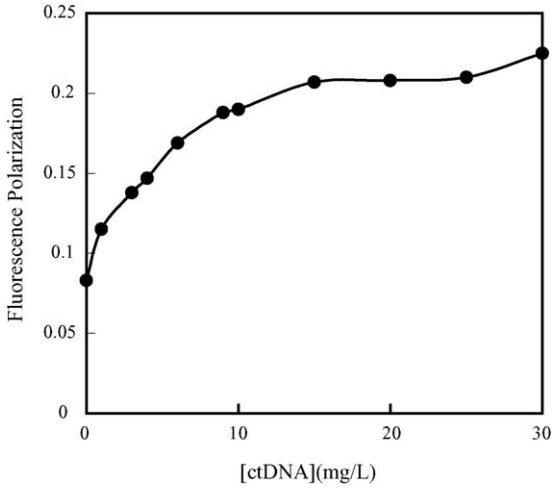

Fig. 9. Influence of ctDNA concentration on fluorescence polarization ([MV] = 2×10^{-5} mol/L, pH = 7.56).

fluorescence polarization of the bound chromophore should be increased. Mere binding to the phosphate backbone or to the DNA grooves does not result in enhanced fluorescence polarization [34]. In the absence of ctDNA, the fluorescence of MV was weakly polarized, and the fluorescence polarization of MV increased efficiently with the addition of ctDNA (Fig. 9), which is evidence that MV intercalated into the helix.

3.5.7. Resonance light-scattering spectrum

The binding of MV to DNA was further probed by RLS by fixing MV concentration and varying DNA concentration. The RLS intensity of MV increases dramatically by the DNA addition when $R < 1.9$, when $R > 1.9$ the RLS intensity of MV

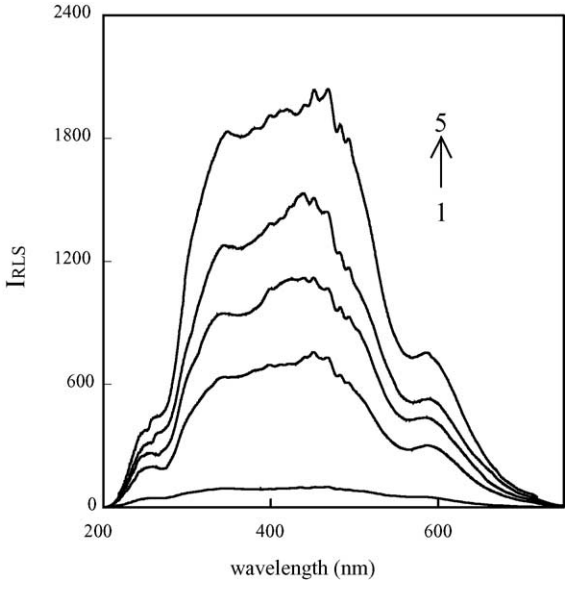

Fig. 10. The resonance light scattering spectra of MV ([MV] = 2×10^{-5} mol/L) in different concentration of ctDNA. Concentration of ctDNA (from 1 to 5): 1: 0, 2: 4.84×10^{-6}, 3:9.69×10^{-6}, 4: 1.45×10^{-5}, 5: 1.94×10^{-5} mol/L.

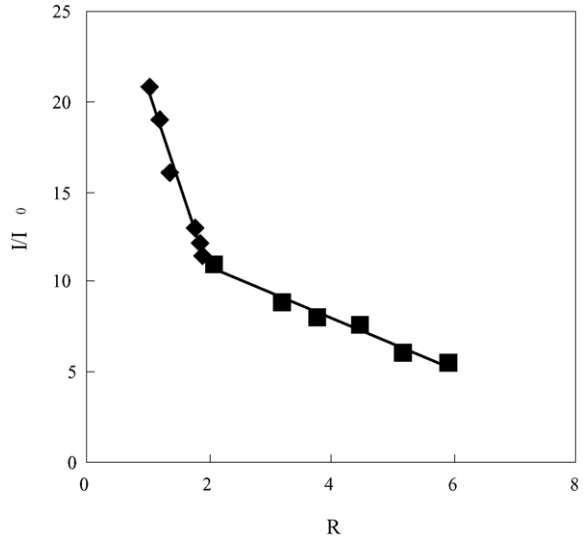

Fig. 11. The relationship between the R-value and the I/I_0 of RLS.

would not change with further increase of DNA concentration (Fig. 10). It is assumed that the enhancement of MV result from the long range assembly of MV on the molecular surface of nucleic acids when $R < 1.9$, in this process, nucleic acids act as a template and MV organized on the molecular surface of nucleic acids, which does not involve in groove or intercalative binding [35]. That is to say, the enhanced RLS is due to the long-range assembly of MV on the DNA surface. When $R > 1.9$, there is no obvious change in RLS, and MV intercalated into DNA helix. It is evident that there is two modes existed in the whole binding process of MV to DNA. The two modes changed into each other depending on the R-value. Relation between the R-value and RLS has been shown in Fig. 11. It is be obvious that the $R = 1.9$ is turning point. If $R > 1.9$, the intercalative binding was predominant, the case of $R < 1.9$, the long range assembly was driving.

4. Conclusion

The SS-RTP and fluorescence spectra of MV were located in the visible region. The longer emission wavelengths make it possible as probe in bio-analysis. Based on the measurement of fluorescence, SS-RTP, and RLS, the interactive model of MV with DNA was characterized. After binding to DNA, the fluorescence and SS-RTP of MV was quenched efficiently and RLS enhanced dramatically. The fluorescence polarization experiment, potassium iodide quenching study and investigation of effect on dsDNA and ssDNA indicate that MV intercalated into DNA helix. The enhancement of RLS of MV in the presence of ctDNA results from the long-range assembly of MV on the molecular surface of nucleic acids. In brief, the binding displayed two interactive process in the whole interaction process depending on R-values, when $R < 1.9$ long-range assembly is main, when $R > 1.9$, the interaction is predominant. Because intercalative model is the

most important interactive model in drug design MV would be potentially useful in pharmaceutics. DNA damage intercalated by MV can have positive applications in technical and therapeutic fields. For example, photosensitizing dye such as MV can be used to destroy tumour cells in treatment. Furthermore, if damage to DNA can be precisely controlled, to the extent that cleavage of the backbone can be limited to a single site, then this opens the door for design of sequence-specific DNA (artificial restriction nucleases).

Acknowledgement

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