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ORIGINAL PAPER

Interaction Between Methylene Blue and Calf Thymus Deoxyribonucleic Acid by Spectroscopic Technologies

Changlun Tong · Zhou Hu · Jianmin Wu

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Abstract Characterization of the interaction between methylene blue (MB) and calf thymus deoxyribonucleic acid (ctDNA) was investigated by UV absorption spectra, fluorescence spectra, fluorescence polarization and fluorescence quenching experiments by ferrocyanide. The above results indicated that the binding modes of MB to ctDNA were relative to the molar ratio γ (γ=[DNA]/[MB]). At low γ ratios (γ<4), remarkable hypochromic effect with no shift of λ_{max} in the absorption spectra of MB was observed in the presence of increasing amounts of ctDNA, the fluorescence of MB was efficiently quenched by the ctDNA bases and the fluorescence polarization of MB was slightly increased, which indicated that MB cations bound to phosphate groups of ctDNA by electrostatic interaction and then stacked on the surface of ctDNA helix. While at high γ ratios (γ>6), besides the fluorescence of MB was quenched efficiently by the ctDNA bases, a red shift (about 3 nm) in the absorption spectra of MB was observed and the fluorescence polarization of MB was obviously increased, which indicated the intercalation binding that MB molecules were intercalated into the space of two neighbouring DNA base pairs was the preferred mode. Effects of $K_4Fe(CN)_6$ on the fluorescence quenching of the MB-ctDNA system at low and high γ ratios were also performed. The results showed that at γ=1.7, the quenching effect by ferrocyanide was higher than that of pure MB, while at γ=13.6 a decreased quenching of the fluorescence intensity was observed as compared with that of pure MB, which further proved the above conclusion. In addition, the mechanisms of the hypochromic effect and the fluorescence quenching were also discussed in detail.

Keywords Calf thymus DNA · Methylene blue · Binding modes · Interaction · Spectrometry

C. Tong (✉) · Z. Hu
Institute of Environmental Science, Zhejiang University,
Hangzhou 310029, China
e-mail: cltong@zju.edu.cn

J. Wu (✉)
Department of Chemistry, Zhejiang University,
Hangzhou 310027, China
e-mail: wjm-st1@zju.edu.cn

Introduction

The binding of small molecules to DNA has been of great interest for a long time due to the importance in understanding the drug-DNA interactions in order to the consequent design of new efficient drugs targeted to DNA [1, 2]. There are three modes on the binding of small molecules to DNA double helix: electrostatic binding, groove binding and intercalative binding [3]. Electrostatic binding between cationic species and the negatively charged phosphate backbone of DNA is nonspecific and usually occurs along the external DNA double helix. Groove binding 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, heteroaromatic 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. However, the electrostatic binding can take place out of the groove. Understanding the binding modes of small molecules to DNA and the factors that can affect the binding

is of fundamental importance in understanding the DNA binding in general.

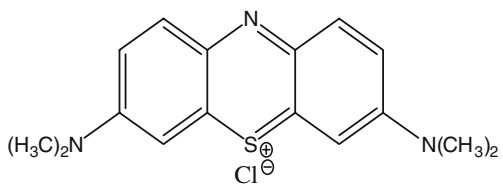
Methylene blue (MB, Scheme 1) is a phenothiazinyl dye, which is a kind of photosensitizer drug molecules showing promising applications in the photodynamic therapy (PDT) for anticancer treatment [4, 5]. Due to its planar structure, which is similar to acridine dyes, MB are known to interact with DNA and has been attracting the interest of many researchers [6–11]. Time-resolved fluorescence, fluorescence polarization anisotropy and transient photobleaching methods were employed to investigate the MB-DNA complexes over a range of salt concentration and a limited range of temperature and base composition [6]. It is generally thought that the binding mode of MB with DNA is the intercalative binding, which is also in agreement with theoretical modeling studies [7]. However, the evidence for this type of binding is rather indirect. Spectroscopic and electrochemical measurements of β-cyclodextrin inclusion complexes of MB with DNA revealed that the binding mode of confined MB to DNA is the electrostatic mode [8, 9]. In addition, some researchers showed that at low ionic strength buffer, MB must intercalate into DNA, while with the salt concentration increasing, MB may be both intercalated and externally bound to DNA [10, 11]. At present, the binding mode of MB with DNA remains an interesting matter of discussion.

MB as a near infrared (NIR) dye, which has a strong absorption band in the long wavelength region, it emits fluorescence in the NIR region from 600 to 1,000 nm where most biomolecules have no absorption. Based on its luminescence characteristic, we have reported it as a luminescence probe for DNA [12]. Meanwhile, an interesting phenomenon that the binding modes of MB with DNA are relative to molar ratio γ (γ=[DNA]/[MB]) was discovered. In the present work, characterization of the interaction between methylene blue (MB) and calf thymus deoxyribonucleic acid (ctDNA) has been investigated in detail. The interaction mechanism of MB with ctDNA was also discussed.

Experimental

Apparatus

The fluorescence spectra and intensities were acquired on a model F-2500 spectrofluorimeter (Hitachi, Japan) with a quartz cell (1 cm×1 cm cross-section) equipped with a xenon lamp (150 w) and dual monochromators. The fluorescence polarization measurements were carried out on a LS-55 luminescence spectrometer (Perkin-Elmer, USA). All absorption spectra were measured on a UV-2401PC spectrophotometer (Shimadzu, Japan). pH was measured on a MP220 pH meter (Mettler Toledo, China).



Scheme 1 The molecular structure of MB

Reagents

All chemicals were of analytical reagent grade, and distilled or deionized water was used. Commercially prepared calf thymus DNA was purchased from Sigma and used without further purification. It was suspended directly in 0.05 mol L^{-1} sodium chloride solution at a final concentration of 100 μg mL^{-1} as stock solution. After establishing the absorbance ratio A_{260}/A_{280} in the range of 1.80–1.90 for DNA, the concentration of DNA was determined according to the absorbance at 260 nm by using $\varepsilon_{DNA(P)}$= 6,600 L mol^{-1} cm^{-1}, the calculated concentration of DNA stock solution is 2.72×10^{-4} mol L^{-1}. Single strand DNA (ssDNA) was acquired according to the following procedures, native forms of ctDNA were thermally denatured by incubating them at 100 °C boiling water for 10 min, then followed by cooling in an ice–water bath. All of the stock solutions and their diluted solutions were stored in a refrigerator at 4 °C until used. All diluted solutions of DNA were used within 24 h. The standard stock solution (1.0×10^{-3} mol L^{-1}) of MB was prepared by dissolving the corresponding MB in water. Working solutions were prepared by appropriate dilution with water before use, and the concentration of MB solution was determined according to the absorbance at 664 nm by using ε_{MB}=76,000 L mol^{-1} cm^{-1}. A standard stock solution (0.01 mol L^{-1}) of $K_4Fe(CN)_6$ was prepared by dissolving the corresponding $K_4Fe(CN)_6$ in water and stored in dark. A 0.2 mol L^{-1} tris(hydroxyl–methyl)aminomethane (Tris) buffer solution was prepared by dissolving the corresponding Tris in water and adjusted the pH to 7.3 with hydrochloric acid to give a final total volume of 500 mL.

Methods

Absorption spectra

1.0 mL of 0.2 mol L^{-1} Tris buffer solution, 1.0 mL of 4.0×10^{-5} mol L^{-1} MB solution, and a known volume of ctDNA solution with appropriate concentration were transferred to a 10 mL test-tube. The mixture was diluted to 10.0 mL with water, shaken thoroughly and allowed to stand for 15 min. Pure MB solution was prepared according to the above procedure without ctDNA. The absorption spectra of the mixture solution and pure MB solution were measured on a

UV-2401PC spectrophotometer. All the absorption spectra have subtracted the background absorption from all the reagents by using a corresponding solution without MB as a reference solution.

Fluorescence spectra

Solutions used in the measurements of fluorescence spectra were prepared the same as the above measurements of the absorption spectra. The fluorescence spectra were recorded in the range of 650–750 nm at excitation wavelength of 630 nm. The entrance and exit slits for all fluorescence measurements were both maintained at 10 nm.

Fluorescence polarization

Fluorescence polarization was measured on a Perkin-Elmer LS-55 Fluorescence Spectrophotometer. Solutions used in the measurements of fluorescence polarization were prepared the same as the above measurements of absorption spectra. The polarization was calculated as follows:

$$P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH}) \quad (1)$$

where I_{VV} is the intensity with both excitation and emission polarizers vertical; I_{VH} is the intensity with excitation polarizer vertical and emission polarizer horizontal. $G=I_{HV}/I_{HH}$, I_{HV} is the intensity with excitation polarizer horizontal and emission polarizer vertical; I_{HH} is the intensity with both excitation and emission polarizers horizontal.

Ferrocyanide quenching measurements

The fluorescence quenching experiments by ferrocyanide were also performed as the following procedures. 1.0 mL of 0.2 mol L^{-1} Tris buffer solution, 1.0 mL of 4.0×10^{-5} mol L^{-1} MB solution, and a known volume of ctDNA stock solution were transferred to a 10 mL test tube. The mixed solution was diluted to 10.0 mL with water, shaken thoroughly and allowed to stand for 15 min. Fluorescence quenching titration profiles were measured by incrementally adding aliquots of ferrocyanide stock solution to the above mixture. In the meantime, the fluorescence quenching titration experiments without ctDNA were done according to the same procedures.

Results and discussion

Fluorescence spectra

The interaction of MB with ctDNA was investigated by the fluorescence spectra. Figure 1 showed that the characteristic fluorescence of MB was observed at 680 nm when excitation wavelength was fixed at 630 nm. Upon addition of ctDNA, the fluorescence of MB was efficiently quenched by ctDNA with no shifts in the emission wavelength. The fluorescence-quenching phenomenon suggested the changes of the excited-state electronic structure caused by the electronic interactions in the MB-DNA complexes [13].

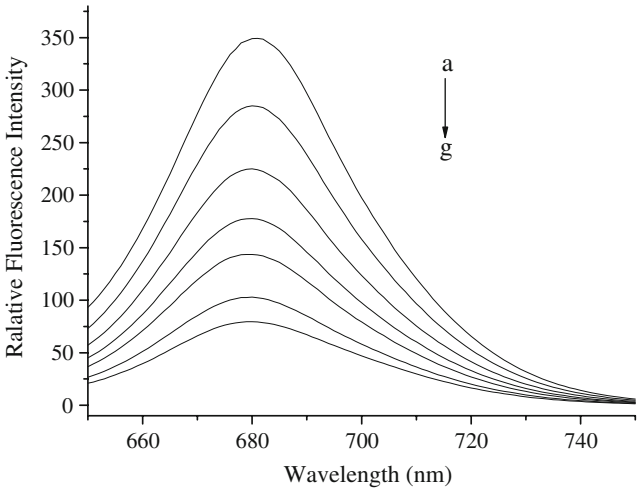


Fig. 1 The fluorescence spectra of pure MB and MB-ctDNA complexes in aqueous solution at room temperature. *Line a* represents the pure MB aqueous solution; *Lines b–g* are the above MB aqueous solutions in the presence of ctDNA with the concentrations of 2.78, 13.9, 20.85, 27.8, 41.7 and 55.6 μmol L^{-1}, respectively. MB: 4.0 μmol L^{-1}; pH=7.3; λex=630 nm

To ascertain the binding modes of MB with ctDNA, the comparison between the effects of double strand DNA (dsDNA) and single strand DNA (ssDNA) on the quenching of MB fluorescence was performed. The experimental data of the MB fluorescence quenched by dsDNA and ssDNA were plotted according to the Stern-Volmer equation [14, 15]:

$$F_0/F = 1 + K_{sv}[DNA] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher DNA, and K_{sv} is the Stern-Volmer quenching constant. Figure 2 showed that F_0/F varied linearly with the increasing amounts of DNA concentration. The Stern-Volmer quenching constant K_{SV} was evaluated by linear fitting of the data in terms of the above equation. K_{SV} values of 4.22×10^4 L mol^{-1} for dsDNA and 2.64×10^4 L mol^{-1} for ssDNA were acquired. The above results showed that the fluorescence quenching of MB by ssDNA was smaller than that by dsDNA, which indicated that the possible binding mode of MB with ctDNA was intercalation binding. This presumption consisted with the reported study [16].

The Scatchard equation could be used to estimate the binding constants and the number of the binding sites that

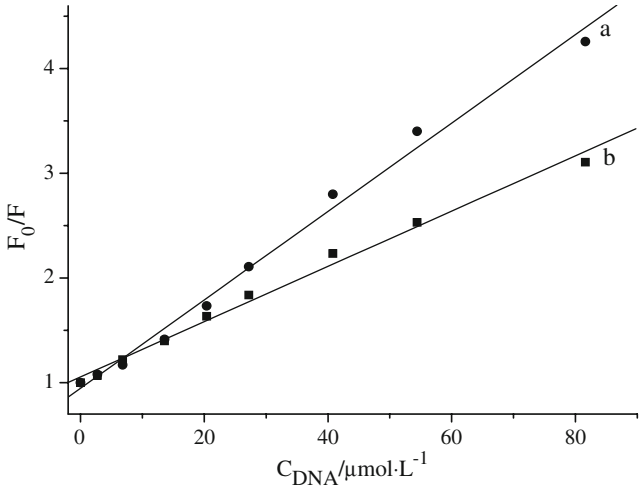


Fig. 2 The Stern-Volmer quenching plots of the MB fluorescence quenched by DNA. (a) dsDNA; (b) ssDNA; MB: 4.0 μmol L^{-1}; pH=7.3

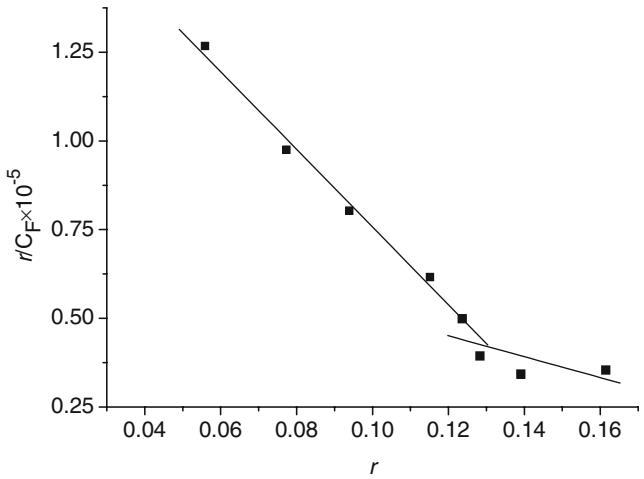


Fig. 3 Scatchard plots for the binding of MB to ctDNA. MB: 4.0 μmol L^{-1}; pH=7.3

small molecules bound to biomacromolecule (eg, DNA and protein), it was described as follows [17]:

$$C_F = C_T\left(\frac{I}{I_0} - q\right)/(1-q) \tag{3}$$

$$C_B = C_T - C_F \tag{4}$$

$$\frac{r}{C_F} = Kn - Kr \tag{5}$$

where C_F is the amount of free MB, C_T is the known added amount of MB, C_B is the amount of bound MB, q is the ratio of the observed quantum yield of fluorescence of the totally bound MB to that of the free MB, I_0 is the fluorescence of MB in the absence of DNA and I is the fluorescence of MB in the presence of DNA, r is the number of moles of bound MB per mole of DNA base pair, $r=C_B/[DNA]$, K is the intrinsic binding constant and n is the exclusion parameter in base pairs. q was determined by adding DNA to a known quantity of MB until no further change in fluorescence emission intensity was observed. q could be obtained by the intercept of a plot of I/I_0 versus 1/[DNA]. A Scatchard plot of r/C_F versus r was obtained as shown in Fig. 3. From Fig. 3, it could be seen that the Scatchard curve of MB-ctDNA was non-linear, which indicated more than one kind of binding modes existed in the MB-ctDNA system. Two kinds of binding constants (*K*), namely electrostatic binding and intercalation binding, were obtained according to the Scatchard equation of MB-ctDNA [18], its binding constants were 2.94×10^5 L $mol^{-1}$and 1.13×10^6 L mol^{-1}, respectively.

Absorption spectra

As could be seen in Fig. 4, the absorption spectra of MB in the visible region had a λ_{max} of 664 nm with a shoulder at 616 nm (curve a), which was consistent with the literature reported [19]. In the presence of increasing amounts of ctDNA, remarkable decreases of the absorbance of MB (hypochromic effect) and a small red-shift of the chromophore were observed in the absorption spectra, while in this region ctDNA did not have the absorption. Thus, it was suggested to be due to the strong interaction between the electronic states of the intercalated chromophore and DNA bases [13].

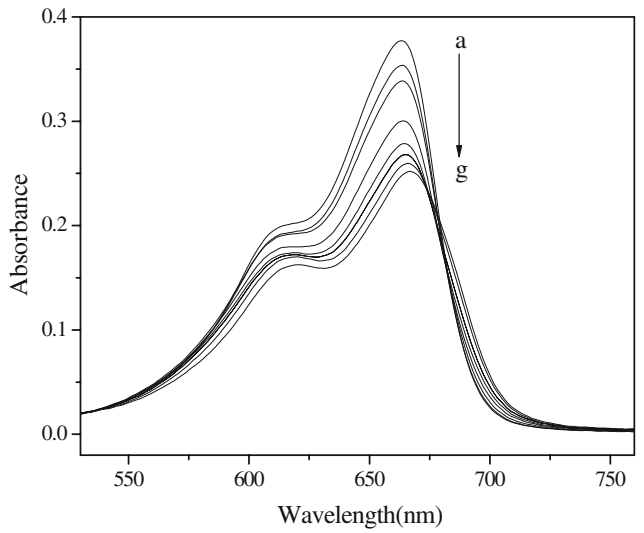


Fig. 4 The absorption spectra of pure MB and MB-ctDNA complexes in aqueous solution at room temperature. *Line a* represents the pure MB aqueous solution; *Lines b–h* are the above MB aqueous solutions in the presence of ctDNA with the concentrations of 2.78, 6.95,13.9, 20.85, 27.8, 41.7 and 55.6 μmol L^{-1}, respectively. MB: 4.0 μmol L^{-1}; pH=7.3

Figure 5 showed that the changes of the absorbance and λ_{max} of MB were relative to γ values (γ represents molar ratio of [DNA]/[MB]). At low γ value (γ<4), the interaction of MB with ctDNA presented a remarkable hypochromic effect and no obvious shift of λ_{max} of MB. While at high γ value (γ>6), the absorbance at λ_{max} decreased unceasingly with a red shift (about 3 nm) from 664 to 667 nm. The above results indicated that the binding mode of MB with ctDNA was likely to depend on the molar ratio of [DNA]/[MB], the subsequent study also showed it.

Fluorescence polarization

Effect of γ on the fluorescence polarization of MB was shown in Fig. 6. In the absence of DNA, the fluorescence of MB was weakly polarized due to the rapid tumbling motion in aqueous media. However, if the chromophore of MB molecule intercalated into the DNA helix, its rotational motion should be restricted and the fluorescence polarization of MB should be increased. While mere binding to the phosphate backbone or to the DNA grooves did not result in the enhancement of fluorescence polarization [3]. As could be seen in Fig. 6, when at low γ ratio (γ<4) the fluorescence polarization of MB was slightly increased, which might be due to electrostatic binding of MB to the external DNA helix. However, when at high γ ratio (γ>6) the fluorescence polarization of MB was greatly increased. It indicated that MB molecule possibly intercalated into the DNA helix.

Ferrocyanide quenching measurement

To further establish the binding properties of MB to ctDNA, the ferrocyanide quenching experiments were performed, for ferrocyanide was a rather efficient quencher of the MB fluorescence in aqueous solutions. It was a generally accepted fact that when the binding mode of small molecules with DNA was the groove binding, the quenching effect of the bound small molecules by ferrocyanide should be more efficient as compared with the pure solution of small molecules quenched by ferrocyanide [20]. Contrarily, if small molecules were intercalated into the helix stack, its quenching effect by ferrocyanide should be less efficient than that of pure solution of small molecules [21]. Maybe in the groove binding the bound molecules were exposed to the solvent surrounded by the DNA helix much more than that of the intercalation binding [22].

The quenching titration experiments were performed at different γ with increasing amounts of $K_4Fe(CN)_6$. As could be seen in Fig. 7, in aqueous solution ferrocyanide quenched the fluorescence of pure MB very efficiently. However, at γ=13.6, when addition of ferrocyanide to the

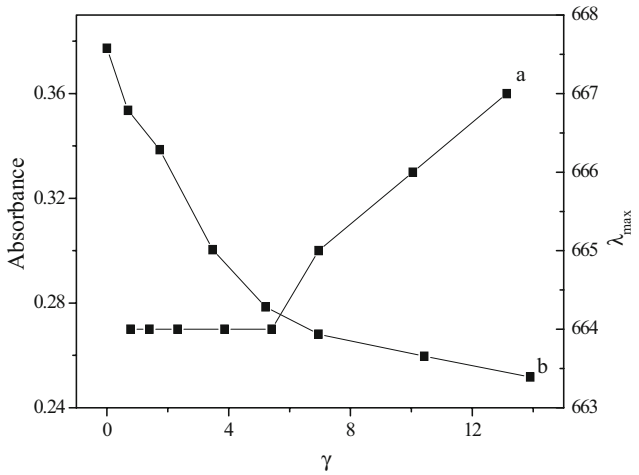


Fig. 5 Effect of γ on the absorption (b) and λ_{max} (a) of MB solution. γ represents [DNA]/[MB] molar ratio; MB: 4.0 μmol L^{-1}; pH=7.3

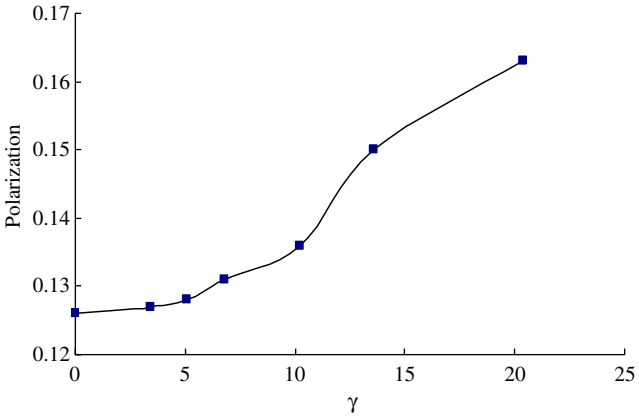


Fig. 6 Effect of γ on the fluorescence polarization of MB in aqueous solution at room temperature. γ represents [DNA]/[MB] molar ratios; MB: 4.0 μmol L^{-1}; pH=7.3

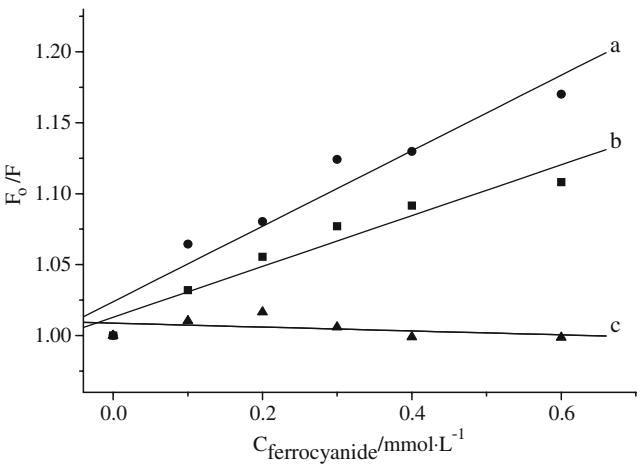


Fig. 7 Ferrocyanide induced emission quenching of pure MB and MB-ctDNA complexes in aqueous solution at room temperature. (a) γ=1.7; (b) γ=0 (pure MB); (c) γ=13.6; MB: 4.0 μmol L^{-1}; F_0 and F are the emission intensities without ferrocyanide and with ferrocyanide, respectively; MB: 4.0 μmol L^{-1}; pH=7.3

MB-DNA system, a weak quenching effect was observed as compared with the pure MB, which could presume that the main binding mode between MB and DNA was intercalation binding according to the above accepted fact. While at γ=1.7, the quenching effect by ferrocyanide was stronger than that of pure MB, which indicated that non-intercalative binding of MB to DNA occurred in this condition. The conclusion consisted with the above results obtained by the fluorescence polarization.

Mechanisms of the interaction between MB and DNA

MB is a cationic molecule and the electrostatic interaction seems to be important for its interaction with DNA [8]. However, experimental results indicated that maybe other interaction modes such as intercalation or groove binding existed in the MB-DNA system besides the nonspecific electrostatic binding. Generally speaking, red-shift and hypochromic effect were usually observed in the absorption spectra of small molecules if they intercalated into DNA [23]. In many dye-nucleic acid systems, blue shift and (or) hypochromic effect of the dye spectra were taken for the spectral signals of external association, and red-shift with or without hypochromic effect were taken for the spectral signals of intercalation [16, 24, 25]. As could be seen in Fig. 5, the interaction of MB with increasing concentration of ctDNA at low γ ratios (γ<4) produced the remarkable hypochromic effect but without any obvious shift of λ_{max} of MB, which suggested external electrostatic binding at these γ ratios. The remarkable hypochromic effect suggested that π electrons of the chromophore were perturbed considerably upon binding to DNA [26]. At high γ ratios (γ>6), a red shift about 3 nm was observed in the absorption spectra of the MB-ctDNA system, which indicated that the possible binding between MB and ctDNA was in the mode of intercalation binding in terms of the above theory. It should be noted that for many groove binding in their complexes with DNA, a red-shift also existed in the absorption spectra [27, 28], which made difficult to distinguish groove binding from intercalation only by absorption studies. In the present study, the chromophore of dye was a planar molecule, unlike the groove binders, which interrelated with bent structures and also had functional groups that could give additional stability to the binding in the groove. Therefore, the above changes in the absorption spectra suggested the possibility of intercalation of the dye between the base pairs in the double helical regions. When ligand molecules are intercalated into the space of two neighbouring DNA base pairs, π-electron stacking between the intercalated-ligands and DNA base pairs will take place, the overlap between π^* empty orbitals of the intercalated-ligands and π-electron orbitals of the base pairs will also take place and makes energy levels descending, and results in a decrease of π–π^* transition energy gap [29]. Accordingly, red-shift effect was observed in the absorption spectra of the MB-DNA system when MB molecules were intercalated into DNA. In addition, the hypochromic effect was also observed at high γ ratios, the possible causes were that some chromophores of MB were shielded after MB was intercalated into DNA [23], which caused free MB molecules decreasing. So the hypochromic effect was observed in the absorption spectra, for the absorption spectra were the sum spectra of free MB and intercalated MB.

Similarly, fluorescence quenching of small molecules was also found to be relative to the binding modes with DNA. It was reported that dimerization/aggregation of the dye molecules was associated with a decrease in the fluorescence intensity [30], and MB was known to readily dimeric and aggregate. As could be seen in Fig. 1, at the initial additions of ctDNA, a great decrease in the fluorescence intensity was observed, which indicated that MB bound to ctDNA as dimers or higher aggregates. When at high γ ratios (γ>6) MB molecules bound to ctDNA, the phenomena of fluorescence quenching were also observed. Moreover, the fluorescence polarization of MB (see Fig. 6) was greatly increased, which indicated that the MB molecule intercalated into the ctDNA helix according to the above conclusion in the fluorescence polarization section. The fluorescence quenching of MB could be due to the electron/H atom transfer from the nucleic acid bases to the intercalated dye molecules [31, 32].

Another strong evidence to support the above conclusion is the quenching tests of the MB-ctDNA system by ferrocyanide. As could be seen in Fig. 7, after addition of ferrocyanide to the MB-ctDNA system at γ=13.6, a decreased quenching of the fluorescence intensity was observed as compared with that of pure MB. In the presence of ctDNA, the intercalated MB was almost completely protected, for a negatively charged $[Fe(CN)_6]^{4-}$ would be repelled by the negatively charged phosphate backbone of ctDNA, and hindered the fluorescence quenching of the intercalated MB. In other words, a decreased quenching of the fluorescence intensity at γ=13.6 was due to the less accessibility of intercalated MB to the quenchers. While at γ=1.7, the fluorescence quenching of the bound MB by ferrocyanide was stronger than that of pure MB. The possible causes were that MB might stack on the surface of ctDNA helix through electrostatic attraction between MB cations and the negatively charged phosphate moieties on ctDNA, which resulted in the more accessibility of quenchers to bound MB as compared with the pure MB.

In addition, previous studies showed in the binding of aminoacridines, which were structurally similar to MB, with various ribo- and deoxy-ribonucleic acids, at low γ ratios (γ<4) the binding mode was the electrostatic interaction between the cationic dye and the negatively charged phosphates assisted by the stacking tendency of the dye molecules. While at high γ ratios (γ>10), the intercalation binding that

dye molecules were intercalated into the nucleic acid bases was the preferred mode [24, 25]. The above conclusion was in accordance with the previous studies.

Conclusions

Characterization of the interaction between MB and ctDNA has been investigated by absorption spectra, fluorescence spectra, fluorescence polarization and ferrocyanide quenching experiments. The above results indicated that the binding modes of MB to ctDNA were relative to molar ratio γ. At low γ ratios (γ=[DNA]/[MB], γ<4), maybe MB cations bound to phosphate groups of ctDNA by electrostatic interaction and then stacked on the surface of ctDNA helix, while at high γ ratios (γ>6) the intercalation binding that MB molecules were intercalated into the space of two neighbouring ctDNA base pairs was the preferred mode.

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