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Spectroscopic studies on the binding of methylene blue with DNA by means of cyclodextrin supramolecular systems

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Abstract

The interaction of methylene blue (MB) with calf thymus DNA (ctDNA) was comparatively investigated in detail by spectrometric methods in different kinds of cyclodextrin (CD) systems. Absorption spectra, fluorescence spectra and fluorescence polarization measurements indicated that the interaction mechanism of MB with DNA depended on the ratio (R) of the concentration of MB versus the concentration of ctDNA: intercalation into ctDNA base pairs at lower R value ($R < 0.125$); while electrostatic interaction at higher R values ($R > 0.125$). It was shown that the binding of MB with DNA at higher R value was inhibited by the anion CD derivatives, e.g., carboxymethyl β-CD (CMCD) and sulfated β-CD (SCD), and whereas this inhibition effect from the neutral CD was negligible. The inhibition effect from anion CDs on the MB/DNA binding is accounted for the competition between the negatively charged group of CMCD or SCD and the phosphate backbone (also negatively charged) of the DNA molecules, when these CDs and DNA molecules coexist with MB molecules. As a contrast, at lower R values, neutral as well as anion CDs were found to have similar effects on the absorption and fluorescence spectra of MB–ctDNA system, implying that different binding mechanism will be applied at low concentration ratio of MB/ctDNA. The corresponding intrinsic binding constants for these complexes were obtained by UV–vis and fluorescence spectroscopic methods, respectively.

Keywords: Methylene blue; Binding; Cyclodextrin; Spectroscopic; Fluorescence polarization

1. Introduction

Investigations of the interaction of small molecules with DNA have captivated scientists over the past years because it is fundamental for many intracellular processes and therefore of biotechnological relevance. The results of various interactive modes studies have been used in designing new and efficient drug molecules and studying the structure of DNA and even protein-nucleic acid recognition [1–3]. For the agents which bind to DNA, intercalation, groove binding and electrostatic binding are the three likely binding modes [4].

As a planar dye molecule, methylene blue (MB) has long been used for biological staining and diagnosis of disease including carcinoma [5–7]. The interaction of methylene blue with DNA has been studied with various methods [8–12]. Most studies indicated that at low ionic strength buffer and low concentration of DNA the major binding mode of MB with DNA was through intercalation and otherwise MB interacts with DNA by non-intercalative binding [10,13,14]. However, interpretations are not so much satisfactory and more studies are needed to elucidate the exact interactive mode of MB with DNA. In recent years, cyclodextrins were employed as a highly organized host media to investigate the interaction between organic dyes and DNA [15–17]. Cyclodextrin is found to be of great importance because of its amphiphilicity of combining the hydrophobic cavity with hydrophilic periphery. The hydrophobic cavity can serve as a selective container for organic molecules of proper size, and the hydrophilicity is really very convenient for carrying out experiments in aqueous solution. It has been confirmed that MB could enter the cavity of β-CD to form the inclusion complex. The interactive mode of MB with DNA has therefore been investigated by comparing the absorption and fluorescence spectra difference between the interaction of β-CD-MB with DNA and that of MB with DNA [17]. However, the evidence for the binding mode was still not sufficient, particularly when changing the concentration ratio of MB to DNA. In this

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paper, we report about absorption, emission and fluorescence polarization measurements by means of different cyclodextrin supramolecular systems to further elucidate the binding modes of MB with ctDNA. A reliable evidence for the binding of MB to DNA is hopefully provided. Besides neutral β-CD, we chose here sulfated β-CD and carboxymethyl β-CD as hosts considering the basic structure of DNA. In addition to the normal hydrophobic inclusion interaction, these CDs will have a further charge attraction to the charged group of MB which is similar with the electrostatic interaction between the intercalator with the phosphate group of DNA. This charge attraction combining with the hydrophobic inclusion may influence binding affinity of MB to DNA. More information of the interactive mode can be provided through the spectroscopic studies of these systems.

2. Experimental

2.1. Materials

Calf thymus DNA (ctDNA), type XV, was purchased from Sigma Chemical Co. (USA) and used as received. Purity of DNA was checked by monitoring the ratio of absorbance at 260–280 nm. The ratio was 1.89, indicating the DNA was free from protein [18]. DNA was dissolved in aqueous phosphate buffers (5 mM Na_2HPO_4 and 5 mM NaH_2PO_4; pH 6.98) at a concentration of 100 μM. These DNA solutions were stored at 4 °C more than 24 h with gentle shaking occasionally to get homogeneity and used within 5 days. The concentration of ctDNA stock solution was determined according to the absorbance at 260 nm by using the extinction coefficients of 6600 mol^{-1} cm^{-1} l [18]. Methylene blue was obtained from Sigma Chemical Co. (USA) and used without further purification. MB stock solution (100 μM) was prepared by dissolving the solid MB in deionized water and the solution was stored in the dark at 4 °C. β-CD and sulfated sodium salt β-CD (degree of substitution D.S. = 13, average MW = 2403) were both purchased from Sigma Chemical Co. (USA). Sodium salt of carboxymethyl β-CD (D.S. = 3, average MW = 1375) was purchased from CTD Inc. (FL, USA). The sterilized and deionized water was used in all solutions.

2.2. Methods

2.2.1. Sample preparation

Samples for absorption and emission experiments were prepared by mixing certain amounts of stock solutions of MB, ctDNA and or CD and diluting the mixture with 5 mM phosphate buffer (pH 6.98) to the appropriate concentrations. The concentration of MB is 4 μM.

2.2.2. Absorption studies

Absorbance spectra were recorded on a thermo Labsystems Multiscan Spectrum Microplate Spectrophotometer (Thermo Electron Co., USA) using a 1.0 cm path length cell. The intrinsic binding constant $K_{i,UV}$ of MB with ctDNA was determined by Eq. (1) [19]

$$\frac{[DNA]}{\Delta\varepsilon_a} = \frac{[DNA]}{\Delta\varepsilon} + \frac{1}{\Delta\varepsilon\, K_{i,UV}} \quad (1)$$

where [DNA] is the concentration of DNA, $\Delta\varepsilon_a = [\varepsilon_a - \varepsilon_f]$, $\Delta\varepsilon = [\varepsilon_b - \varepsilon_f]$, ε_a, ε_b and ε_f correspond to the apparent extinction coefficient of MB, the extinction coefficients of binding form of MB and free MB, respectively. The $K_{i,UV}$ value can be determined by the plot of [DNA]/$\Delta\varepsilon_a$ versus [DNA].

2.2.3. Fluorescence studies

The fluorescence spectra were measured on a FluroMax-3 Spectrofluorometer (Jobin Yvon, NJ) equipped with a 150 W xenon lamp for excitation.

The excitation and emission wavelength were 640 and 684 nm, respectively. Excitation and emission bandpass were both set at 5 nm. In fluorescence quenching experiments, the data were plotted according to the Stern–Volmer Eq. (2) [19a].

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

where I_0 and I are fluorescence intensities in the absence and in the presence of DNA, respectively. [DNA] is the concentration of DNA and K_{SV} is the Stern–Volmer quenching constant.

The intrinsic binding constant ($K_{i,FL}$) was also determined by fluorescence titrations. The value of $K_{i,FL}$ was obtained from the modified Scatchard Eq. (3) given by McGhee and von Hippel [20]

$$\frac{r}{C_F} = K_{i,FL}(1 - nr)\left[\frac{1 - nr}{1 - (n-1)r}\right]^{n-1} \quad (3)$$

In Eq. (3), n is the binding site size in base pairs, r is equal to C_B/[DNA]. The amount of bound probe (MB dye) (C_B) is equal to $C_T - C_F$ and C_T is the concentration of the probe added. C_F, the concentration of the free probe, was determined by Eq. (4) [21].

$$C_F = \frac{C_T(I/I_0 - P)}{1 - P} \quad (4)$$

where P is the ratio of the observed fluorescence quantum yield of the bound probe to that of the free probe. The value of P was given by the y-intercept of a plot of I/I_0 versus 1/[DNA].

2.2.4. Fluorescence polarization measurement

Fluorescence polarization measurements were performed on steady state MB–CD and MB–DNA solutions. The polarization ratio (Pr) was determined from the following equation [22]:

$$Pr = \frac{I_{VV} - G I_{VH}}{I_{VV} + G I_{VH}} \quad (5)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}.

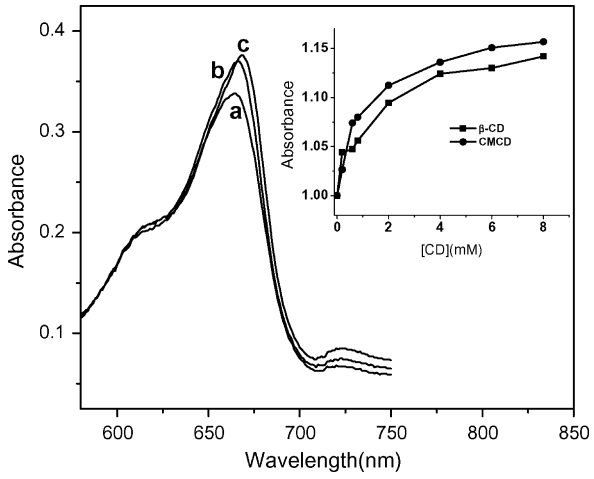


Fig. 1. Absorption spectra of MB in different media. C(MB) = 4×10^{-6} M; C(CD):4×10^{-3} M; (a) aqueous buffer, (b) β-CD and (c) CMCD. Inset: absorbance of MB in different concentration of β-CD and CMCD.

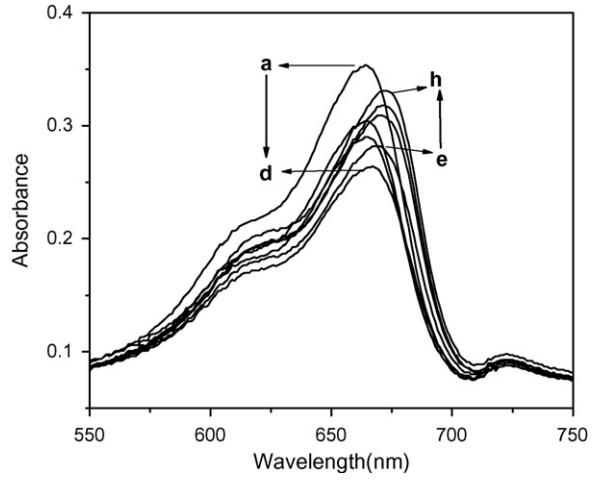


Fig. 2. Absorption spectra of MB in different concentrations of SCD. C(MB) = 4×10^{-6} M; C(SCD)/(10^{-5} M): (a–d) 0, 0.08, 0.6, 6; (e–h) 20, 40, 60, 80.

3. Results and discussion

3.1. Study on the inclusion complex of MB with CD

3.1.1. Absorption studies

Absorption spectra of MB upon adding of β-CD, CMCD and SCD were shown in Figs. 1 and 2, respectively. For β-CD and CMCD (Fig. 1), the absorbance of MB increased with the increase of CD concentration and the absorption peak red-shifted by 3 nm. This change became slow and tended to reach a plateau when the concentration of CD was more than 4 mM, indicating that MB was included by CD completely. SCD, however, has different results. As can be seen from Fig. 2, the absorbance decreased at lower SCD concentration (<6×10^{-5} M) with no noticeable peak shift, and then the absorption peak shifted to long wavelength by 8 nm and the intensity of the red shifted peak increased with further increase of SCD. These changes suggested that the binding process for MB to SCD was not a single mode. It can be inferred that the sulfate group linking at the edge of CD cavity interacted with the positive group of MB through electrostatic interaction, and induced the hypochromism at low SCD concentrations. The red shift of the absorption peak with further increase of SCD indicated the including of MB into SCD cavity (shown in Scheme 1).

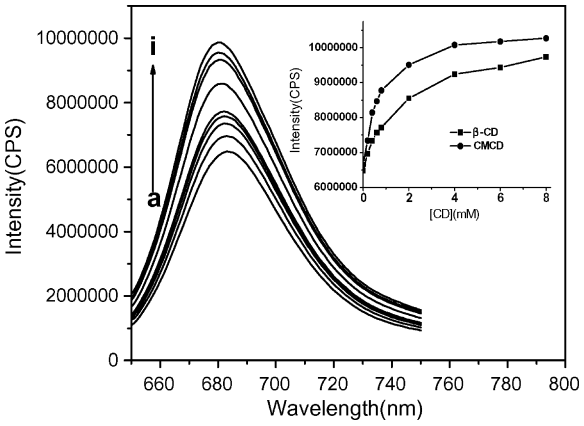
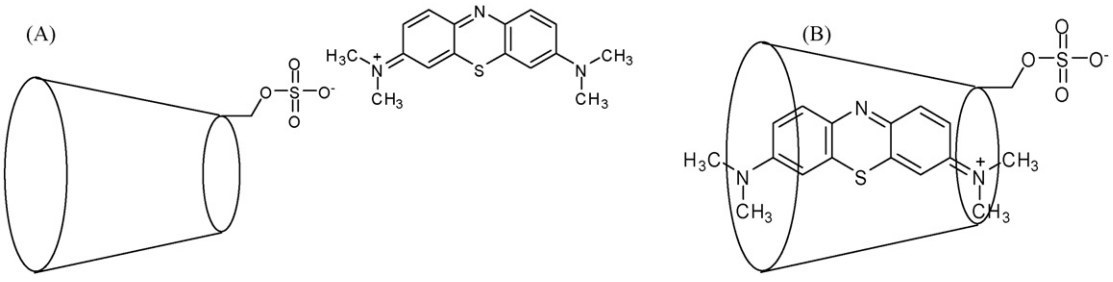


Fig. 3. Fluorescence spectra of MB in different concentrations of β-CD. C(MB) = 4×10^{-6} M; C(β-CD)/(10^{-3} M): (a–i) 0, 0.2, 0.4, 0.6, 0.8, 2, 4, 6, 8. Inset: fluorescence intensities of MB in different concentrations of β-CD and CMCD.

3.1.2. Fluorescence studies

3.1.2.1. Fluorescence spectra analysis. As shown in Fig. 3, the fluorescence intensities of MB increased with the increase of the concentrations of β-CD and CMCD, indicating the formation of MB–CD inclusion. However, as shown in Fig. 4, the addition of SCD quenched the fluorescence of MB in the first stage, and then the emission wavelength red-shifted by 3 nm and the fluorescence intensity increased when SCD concentration was more than 6×10^{-5} M. The decrease of fluorescence showed



Scheme 1. The binding models of MB and SCD at different SCD concentrations: (A) lower SCD concentration and (B) higher SCD concentration.

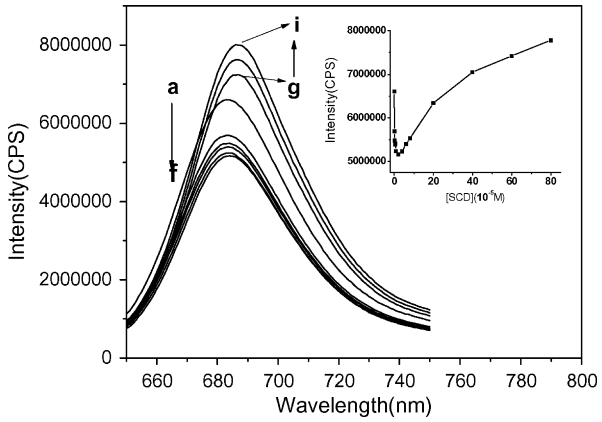


Fig. 4. Fluorescence spectra of MB in different concentrations of SCD. C(MB) = 4 × 10^{-6} M; C(SCD)/(10^{-5} M): (a–f) 0, 0.08, 0.2, 0.4, 0.8, 2; (g–i) 40, 60, 80. Inset: fluorescence intensities of MB in different concentration of SCD.

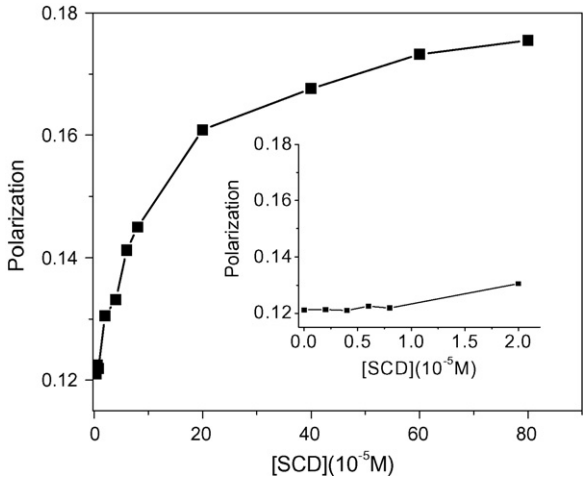


Fig. 6. Fluorescence polarization of MB in different concentration of SCD. Inset: polarization of MB at lower concentration of SCD. C(MB) = 4 × 10^{-6} M.

that MB can interact with SO_4^{2-}. The increase of fluorescence indicated the inclusion of MB into SCD cavity. These various spectra changes suggested different interactive modes of MB may be applied with different concentrations of SCD, which are consistent with the observed absorption results.

3.1.2.2. Fluorescence polarization measurement. Steady-state fluorescence polarization measurements monitor the rotational diffusion of fluorophores [22]. Substantial changes in its polarization occur when a fluorescent small ligand binds to a macromolecule, providing a convenient means of detecting and quantifying the association reaction between fluorescent small molecules and macromolecules. Polarization titrations of MB by increasing amounts of CD were shown in Figs. 5 and 6. As shown in Fig. 5, an obvious enhancement of polarization were observed with increase of the CD concentration in the β-CD-MB and CMCD-MB systems, supporting the inclusion of MB into the cavity of CD. The addition of SCD aroused no polarization enhancement at lower concentrations while the further addition of SCD increased the polarization. Since mere binding to the anion group does not cause enhanced fluorescence polarization, the changes in polarization are accounted for the electrostatic interaction between the sulfate groups of SCD and the positive group of MB at lower concentration level. With further increase of SCD, MB entered the SCD cavity with the result of enhanced polarization.

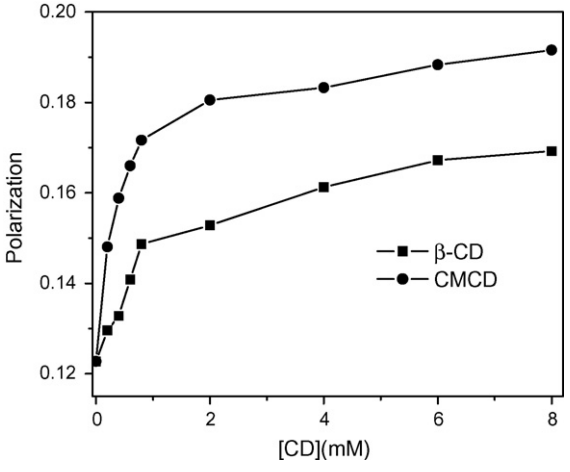


Fig. 5. Fluorescence polarization of MB in different concentration of β-CD and CMCD. C(MB) = 4 × 10^{-6} M.

3.2. Interaction of MB with DNA

3.2.1. Absorption studies

Hypochromism and red shifts of the absorption peak were used to characterize the binding of small molecules to DNA [23]. The absorption spectra in the presence of DNA were shown in Fig. 7. It can be seen that the absorption peak firstly decreased without wavelength shift and then increased with obvious red shift. The change of absorption peak was very similar to that of MB–SCD complex (Fig. 2). So it is reasonable to infer that the interaction modes at high and low concentrations of DNA

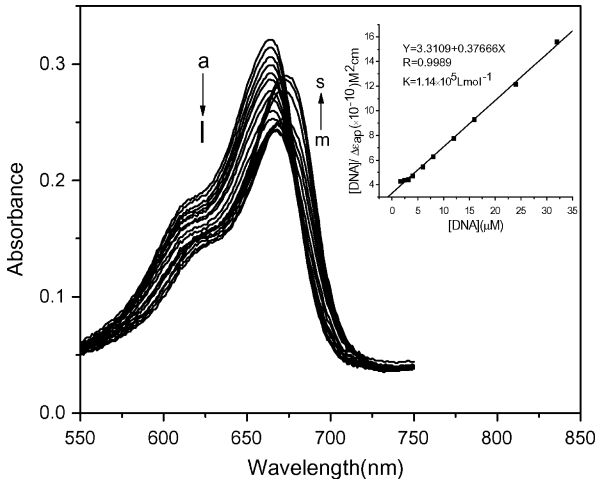


Fig. 7. Absorption spectra of MB in different concentration of ctDNA. C(MB) = 4 × 10^{-6} M; C(DNA)/(10^{-6} M); (a–l) 0, 0.8, 1.6, 2.4, 3.2, 4, 6, 8, 12, 16, 24, 32; (m–s) 40, 60, 80, 120, 160, 240, 320. Inset: half-reciprocal plot of MB binding with ctDNA.

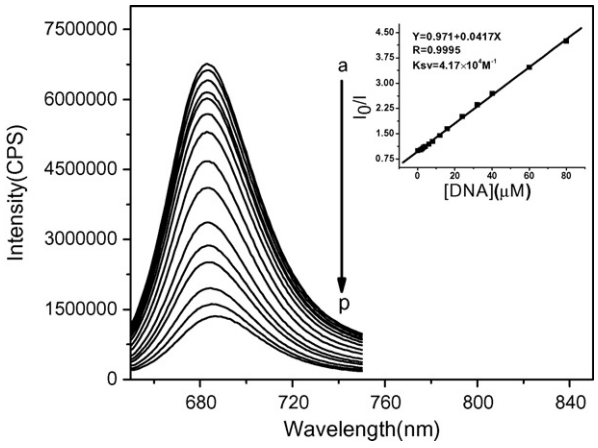


Fig. 8. Fluorescence spectra of MB in different concentration of ctDNA. C(MB) = 4 × 10^{-6} M; C(DNA)/(10^{-6} M); (a–p) 0, 0.8, 1.6, 2.4, 3.2, 4, 6, 8, 12, 16, 24, 32, 40, 60, 80, 120. Inset: Stern–Volmer quenching plot of MB with increasing concentration of ctDNA.

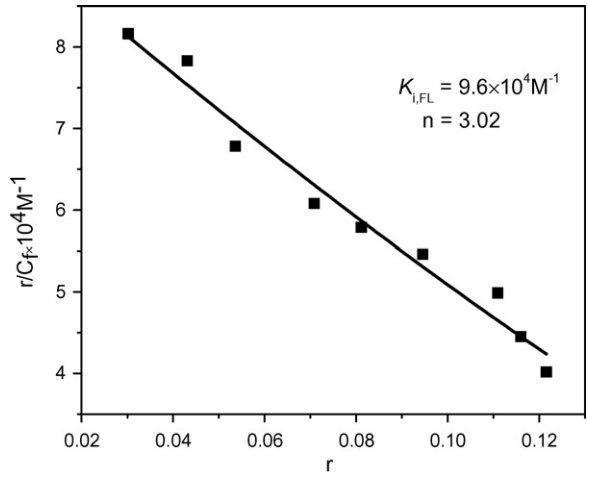


Fig. 9. Scatchard plot of the fluorescence titration data, with excitation at 640 nm and emission at 683 nm. C(MB) = 4 × 10^{-6} M.

are different. The further analysis of spectra indicated that the binding mode of MB with DNA was dependent on the ratio (R) of the MB to ctDNA. MB cationic group is attracted to the phosphate back bone of DNA through electrostatic interaction at higher R value. According to the absorption spectra, the absorption of MB–DNA complex decreased with increasing of DNA until the mole ratio of MB concentration to DNA concentration reached 0.125 (shown in Fig. 7). When $R < 0.125$, the absorption of MB–DNA complex increased and red shifted. This transition point indicated the change of binding mode with the change of R. The eventual red shift of absorption peak by 9 nm and the increase of absorbance at lower R value strongly supported the intercalation mode [24]. The data were fitted to Eq. (1), the plot of [DNA]/$\Delta\varepsilon_a$ versus [DNA] exhibiting a linear line. $K_{i,UV}$ was obtained as 1.14 × 10^5 l mol^{-1} from the ratio of the slope to the y-intercept.

3.2.2. Fluorescence studies

3.2.2.1. Fluorescence spectra analysis. Fig. 8 shows the fluorescence spectra of MB with ctDNA. It can be seen that the fluorescence of MB was efficiently quenched by ctDNA and the maximum emission wavelength at 683 nm for the free MB shifted on further addition of ctDNA to a maximum at 687 nm, indicating the MB binding to DNA changes the excited state electronic structure of MB fluorophore [24]. The fluorescence quenching constant, K_{SV}, evaluated using the Stern–Volmer Eq. (2) was 4.17 × 10^4 M^{-1}. It is clear that the fluorescence of MB was not completely quenched by ctDNA, indicating that only a fraction of the binding sites quenches the fluorescence. The binding constant $K_{i,FL}$, and the binding site size in base pairs, n, can be obtained from Eq. (3). Fitting fluorescence titration data to Eq. (3) (Fig. 9) gave $n = 3.02$ and a binding constant $K_{i,FL}$ = 9.6 × 10^4 M^{-1}, which is close to $K_{i,UV}$ = 1.14 × 10^4 M^{-1} obtained from absorption titration method (in Fig. 7) and is consistent with the reported value [11].

3.2.2.2. Fluorescence polarization measurement. When the chromophore intercalates into the double helix DNA, its rotational motion should be restricted and its fluorescence therefore should be polarized. Mere binding to the phosphate backbone or to the DNA grooves, however, will not affect the fluorescence polarization [25]. Fig. 10 shows the fluorescence polarization changes of MB upon the addition of ctDNA. It can be seen that the change of polarization is dependent on the ratio of MB to DNA and very similar to that of MB–SCD complexes. The polarization values stayed almost constant at higher R values while the large increase in the polarization at lower R values was observed (shown in Fig. 10), demonstrating the binding mode changed from electrostatic interaction to intercalation mode with the change of R value (shown in Scheme 2).

3.3. Study on the binding mode of MB with DNA in CD supramolecular systems

3.3.1. Absorption studies

The plots of absorption titration data of MB–CD–DNA systems with different concentrations of DNA were shown in Fig. 11. A_0, A are absorbance of MB in the absence and presence

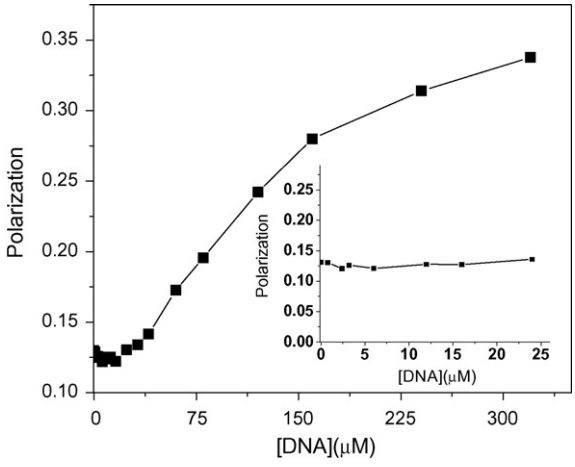
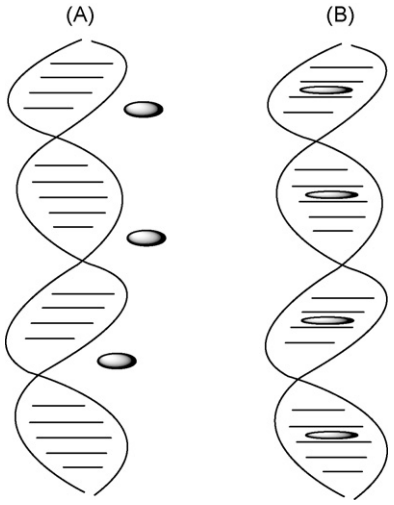


Fig. 10. Fluorescence polarization of MB in different concentration of ctDNA. C(MB) = 4 × 10^{-6} M.



Scheme 2. The binding models of MB with DNA: (A) electrostatic binding and (B) intercalative binding.

of DNA, respectively. The absorbance changes with increasing of DNA in MB–β-CD were found to be very similar to that of MB alone (the curve labeled with 'no CD'), suggesting that the existence of β-CD did not significantly affect the interaction of MB with DNA. However, the absorbance that was saturated by DNA in the presence of β-CD was slightly more than that in the absence of β-CD, indicating that MB was still included in the cavity of β-CD when it interacted with DNA. From Fig. 11 one can also see the absorbance of CMCD–MB and SCD–MB had no significant change with increasing concentrations of DNA and, however, only showed small decrease in the high concentrations of DNA. This result suggested that the presence of CMCD and SCD, especially SCD, dramatically influenced the interaction of MB with DNA. The anions of CMCD and SCD have a strong attraction to the cationic groups and intensively compete with phosphate groups in the backbone of DNA, inhibiting the binding of MB to DNA and causing the great spectra change of MB–DNA system.

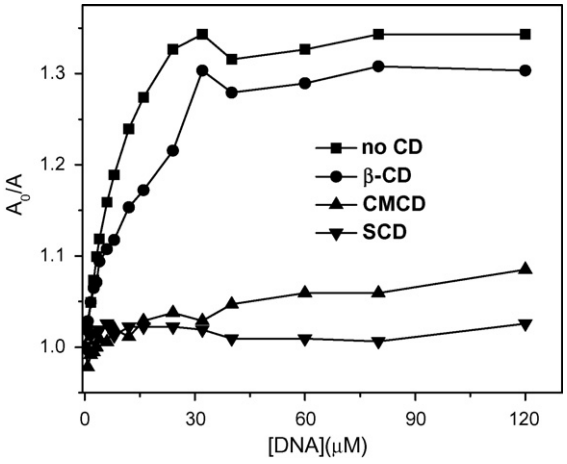


Fig. 11. Plots of the absorption titration data at the absorption peak of MB at different concentrations of ctDNA in different CDs. [MB] = 4×10^{-6} M, [CD] = 4×10^{-3} M.

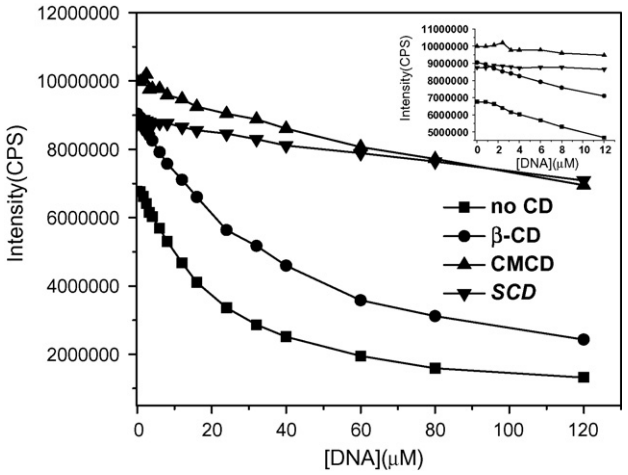


Fig. 12. Fluorescence intensity of MB at different concentration of ctDNA in different CDs. [MB] = 4×10^{-6} M, [CD] = 4×10^{-3} M.

Table 1
Stern–Volmer quenching constants (K_{SV}) of MB-ctDNA in different CDs

Media	No CD	β-CD	CMCD	SCD
K_{SV} (M^{-1})	4.17×10^4	3.36×10^4	3.93×10^3	1.97×10^3
Regression coefficient	0.9995	0.9963	0.9970	0.9958

3.3.2. Fluorescence studies

As can be seen from Fig. 12, the fluorescence intensity of β-CD–MB is quenched in the presence of DNA, quite similar to the results obtained for MB alone. The fluorescence intensities of SCD–MB and CMCD–MB in different concentration of DNA, however, stay constant at low concentration of DNA (see inset) and then decreased when the R value is lower than 0.3 (corresponding to 12 μM [DNA]). The quenching still conforms to Stern–Volmer equation. As shown in Table 1, the quenching constants, K_{SV}, in different media follow this magnitude order no CD > β-CD ≫ CMCD > SCD. These results indicated the binding of MB with DNA is inhibited by anionic CD derivative CD whereas neutral CD has little influence on the binding, further supporting that the electrostatic interaction is a one of binding modes between MB and DNA. SCD has a stronger attraction to the cationic groups and intensively compete with phosphate groups in the backbone of DNA, causing the great spectra change of MB–DNA system.

4. Conclusions

The interactive mode of MB with DNA has been studied by CD–MB supramolecular system and a reliable evidence for the binding of MB to DNA has been provided. UV–vis absorption, fluorescence quenching in combination with fluorescence polarization measurements indicated that the interaction of MB with DNA depended on the ratio of concentration of MB to the concentration of ctDNA (R). MB binds with DNA through electrostatic interaction at higher ratio (R) of MB to DNA ($R > 0.125$) and changes to intercalation interaction at lower R values ($R < 0.125$).

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