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ORIGINAL PAPER

Elucidation of the Binding Properties of A Photosensitizer to Salmon Sperm DNA and Its Photobleaching Processes by Spectroscopic Methods

Lei Zhang · Guo-Qing Tang

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Abstract Methylene blue (MB) is a tricyclic heteroaromatic photosensitizer with a promising application in the photodynamic therapy (PDT) for anticancer treatment. The binding properties of MB to salmon sperm DNA have been investigated by the measurements of absorption spectra, quenching experiments and the photobleaching processes. Remarkable hypochromic and bathochromic effects of MB in the presence of increasing amounts of DNA have been observed in the absorption spectra. The quenching of MB by the DNA bases obeys the Stern-Volmer equation and ferrocyanide quenching of MB in the absence and presence of DNA is also measured as extended experiments. Results from the above spectral measurements are all consistent with the intercalative binding mode of MB to DNA with the K_b value of $5.6\times10^3\,M^{-1}$. The photobleaching processes of MB and its DNA complex have also been studied, which indicate that the photobleaching of MB and its DNA complex proceed with different mechanisms and the reactive oxygen species are responsible for the self-sensitized photooxidation of MB.

Keywords Photosensitizer · DNA · Fluorescence · Photobleaching

Introduction

In recent years, there has been a growing interest in the study of various small organic molecules-DNA interactions because of its importance in understanding the drug-DNA interactions and the consequent design of new efficient drugs targeted to DNA [1–5]. It is generally accepted that three models about binding of small molecules to the DNA double helix have been identified: intercalative binding, groove (or surface) binding and electrostatic binding [6, 7]. Electrostatic binding interactions between cationic species and the negatively charged DNA phosphate backbone usually occur along the exterior of the 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 third type of binding mode known as the intercalative binding, which is defined when a planar, heteroaromatic moiety slides between the DNA base pairs and binds perpendicular to the helix axis. 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.

The research of the interactive model can provide a start for the design of the structure of new and efficient drug molecules [8]. Planar dye molecules, such as acridine dye that can interact with DNA by intercalation, have been tested with several methods [9–16]. Methylene blue (MB, Fig. 1) belongs to the phenothiazinium dye, which is a kind of photosensitizer drug molecules showing promising applications in the photodynamic therapy (PDT) for anticancer treatment [17–19]. Since MB has a planar structure that is similar to acridine dye, MB is known to interact with DNA and has been attracting the interest of many researchers [20–28]. Induced by visible light-excited MB, protective effects of rosemary [20] and vanillin [21] against oxidative DNA damage as a consequence of scavenging of singlet

L. Zhang (✉) · G.-Q. Tang
Institute of Modern Optics, Nankai University, Tianjin 300071, People's Republic of China
e-mail: leiz@sas.upenn.edu

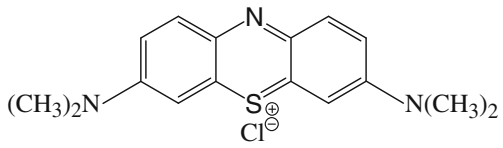

Fig. 1 The molecular structure diagram of methylene blue (MB)

oxygen ($^1O_2$) were reported quite recently. Time-resolved fluorescence, fluorescence polarization anisotropy, and transient photobleaching methods were also employed to investigate MB-DNA complexes over a range of salt concentrations and a limited range of temperature and base composition [22]. It is worthy of note that the interactive model of MB with DNA remains an interesting matter of discussion. Although it is generally thought that the interactive model of MB with DNA is intercalative binding, which is also in agreement with theoretical modeling studies [23], the evidence for this type of binding was rather indirect. In addition, some researchers showed that at low ionic strength buffer, MB must intercalate into DNA but with increasing salt concentration, MB may be both intercalated and externally bound to DNA [24–26]. Moreover, spectroscopic and electrochemical measurements of MB loaded β-cyclodextrin with DNA reveal that the binding model of confined MB to DNA is the electrostatic mode [27, 28]. The binding behavior of MB to DNA has also attracted our research interest. In this contribution, we report the investigation of the binding mode of MB to salmon sperm DNA, together with their photobleaching processes by spectroscopic methods. The reason to choose salmon sperm DNA as a model for investigation is two-fold. The first reason is that salmon sperm DNA is less studied comparing to those that have received most attention, such as calf thymus DNA; the second one is due to its minimal cross-reactivity as well as the availability.

Experimental

Materials

All the chemicals used in this work were of analytical reagent and used without further purification, unless otherwise stated. Double distilled water was used for solution preparation. Commercially prepared salmon sperm DNA was obtained from Sigma Chemical Co. and stored at 4 °C. To prepare stock solution, it was directly dissolved in water at a DNA concentration of 0.5 mmol/L in nucleotide phosphate, the concentration of which had been determined by absorption spectroscopy as described in the literature methods [29, 30]. The stock solutions of the photosensitizer MB and the quencher $K_4Fe(CN)_6$ were both 0.1 mmol/L in concentration, and stored in dark. The buffer solution of pH7.1 contains 0.5 mol/L NaCl and 0.05 mol/L Tris. All above solutions were further diluted as required. It should be noted that all the following spectroscopic measurements are performed in the aerated environment. We have also tested the influence of molecular oxygen on the absorption and emission spectra and found no variation of the above spectral features.

Absorption Spectra

UV-Visible absorption spectra in aqueous solution by using a quartz cell having 1.0 cm pathway were all recorded on a computer-controlled JASCO V-570 spectrophotometer. 1.0 ml of buffer solution (pH7.1) and 0.2 ml of MB solution were transferred to a 10 ml standard flask. A known volume (0.2, 0.4, 0.6, 0.8 ml) of DNA stock solution was also added. The above solutions were then mixed, diluted to the volume with water, and incubated for 5 min. Pure MB solution was prepared in a similar manner without DNA and buffer solution. The final concentration ratio of the dye to DNA (in nucleotide phosphate) ranges from 1:5 to 1:20 in this study.

Emission Spectra

All the emission spectra were measured on Acton Research SpectroPro-300i spectrometer with spectral CCD operating at −15 °C. Xenon arc lamp is used as the excitation light source in the measurements of emission spectra. Solution used in the measurement of emission spectra was prepared the same as the above measurement of absorption spectra. The excitation wavelength was set at 630 nm.

Ferrocyanide Quenching Measurements

The fluorescence quenching experiments with ferrocyanide ($K_4Fe(CN)_6$) were also performed. 1.0 ml of buffer solution (pH7.1), a known volume (0.2 and 0.8 ml) of DNA stock solution, 0.2 ml of MB solution and 1.0 ml of ferrocyanide solution were transferred to a 10 ml standard flask. The above solution was mixed, diluted to the volume with water, and incubated for 5 min. Pure MB solution with the quencher ferrocyanide was prepared in a similar manner without DNA and buffer solution.

Photobleaching Experiments

The photobleaching of the photosensitizer MB and its DNA complex as a result of the laser light irradiation was carried out in this work. Sealed in a tube, 50 μl of the MB solution (2 μmol/L) in the absence and presence of DNA (10 and 40 μmol/L) was irradiated with the instrument for PDT developed in our laboratory (670 nm semiconductor laser output, 35.0 mW laser

power at the focal point). Every 5 min, the emission spectra of MB solution were measured by xenon light excitation (λ_{ex}=630 nm), while the laser irradiation was turned off at that moment.

Results and Discussion

Absorption and fluorescence properties of the photosensitizer MB change dramatically as a result of binding to DNA, and these changes have been quantified to estimate binding constants and sequence specificities and to evaluate the DNA binding mode.

Absorption Spectra

Classically, the binding of dye molecules to DNA has been characterized through absorption titrations, following the hypochromism and bathochromism associated with the binding of colored dye to the helix [31]. The UV-Visible absorption spectra of the MB-DNA complexes in aqueous solution have been recorded with the MB concentration of 2 μmol/L and the DNA concentration in the range of 10–40 μmol/L. Strong absorption of these complexes in the UV region, which is mainly contributed by DNA, have been observed and increase with increasing amounts of DNA concentration. Both pure MB and its DNA complexes possess intense optical absorption in the visible region owing to the aromatic chromophores of MB. Figure 2a. displays a well-behaved titration of MB with salmon sperm DNA. Isosbestic points are observed at *ca.* 683 and 711 nm. Remarkable decrease of the peak intensity of MB (hypochromic effect) in the presence of increasing amounts of DNA is observed, while DNA do not absorb light in this region. This hypochromic phenomenon is suggested to be due to the strong interaction between the electronic states of the intercalated chromophore and DNA bases [32, 33]. Since the strength of this electronic interaction is expected to decrease as the cube of the distance separated between the chromophore and the DNA bases, the marked hypochromism indicates a close proximity of the chromophore to the DNA bases [33]. For example, intercalation of the aromatic chromophore into the helix and strong overlap of the π-π* states of MB with the electronic states of the DNA bases are consistent with the observed spectral changes. In addition to the decrease in absorption peak intensity, a small bathochromic shift of the chromophore is also observable in the spectra. These spectral features are immediately indicative of the intercalation of MB within the DNA helix, which results in an ordered stacking of MB between the aromatic heterocyclic base pairs and the intercalating surface is sandwiched tightly between the base pairs and stabilized electronically in the helix by π-π stacking and dipole-dipole interactions [32].

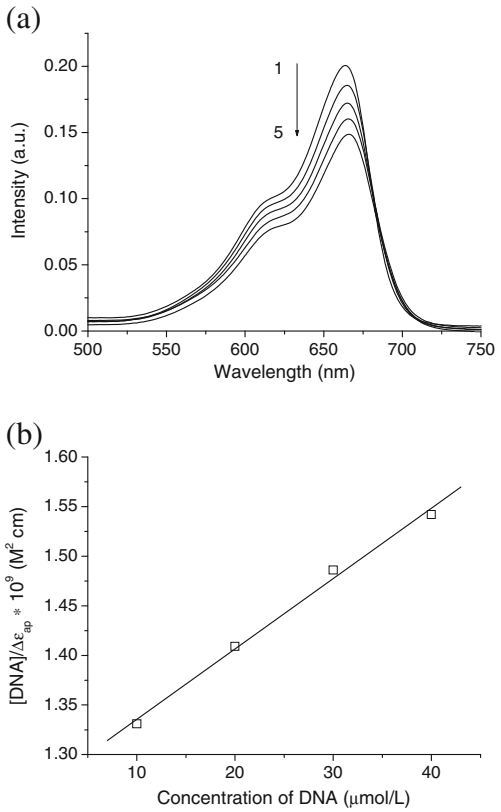

Fig. 2 **a** The absorption spectra of pure MB and MB-DNA complexes in aqueous solution at room temperature. Line 1 represents the pure MB aqueous solution (2 μmol/L); Lines 2–4 are the above MB aqueous solutions in the presence of salmon sperm DNA with the concentrations of 10, 20, 30 and 40 μmol/L, respectively. **b** The half-reciprocal plot of [DNA]/$\Delta\varepsilon_{ap}$ as a function of DNA concentration as determined from the absorption titration data. The solid line represents the best linear fit of the data

It is commonly known that the magnitudes of hypochromism are correlated with the strength of the intercalative binding interaction. Equilibrium constants were determined according to the previously reported procedures. When the ratio of bound MB to DNA base pairs is relatively low, the intrinsic binding constant, K_b, based upon these absorption titrations may be determined from a double-reciprocal plot of the changes in the apparent extinction coefficient of MB versus DNA concentration. The following equation represents this relationship [34, 35]:

$$\frac{1}{\Delta\varepsilon_{ap}} = \frac{1}{\Delta\varepsilon K_b[DNA]} + \frac{1}{\Delta\varepsilon} \quad (1)$$

where $\Delta\varepsilon_{ap} = |\varepsilon_a - \varepsilon_f|$, $\Delta\varepsilon = |\varepsilon_b - \varepsilon_f|$. The apparent extinction coefficient, ε_a, is obtained by calculating A_{obs}/[MB]. ε_f and ε_b correspond to the extinction coefficient for the free MB, and the extinction coefficient for MB in the fully bound

form, respectively. Multiplying by [DNA] puts the equation in the half-reciprocal form:

$$\frac{[DNA]}{\Delta\varepsilon_{ap}} = \frac{1}{\Delta\varepsilon}[DNA] + \frac{1}{\Delta\varepsilon K_b} \quad (2)$$

A plot of [DNA]/$\Delta\varepsilon_{ap}$ versus [DNA] will have a slope of $1/\Delta\varepsilon$ and a y-intercept equal to $1/(\Delta\varepsilon K_b)$. K_b is then given by the ratio of the slope to intercept. Since a double-reciprocal plot gives excessive weight to data points obtained at low [DNA], the half-reciprocal plot should generally be more accurate. The change in the absorbance of MB with increasing DNA concentration was used to construct the half-reciprocal plot as shown in Fig. 2b. The half-reciprocal plot of the absorption titration data according to eq. (2) gave a linear plot and resulted in an intrinsic binding constant of $5.6\times10^3 M^{-1}$ in DNA base pairs, which is consistent with the literature reports.

Emission Spectra

Binding of MB to salmon sperm DNA helix was found to quench the probe fluorescence very strongly. Excited by 630 nm light, the binding of the MB-DNA complex is also investigated by fluorescence spectroscopy. As shown in Fig. 3a, upon binding to DNA the fluorescence from MB is efficiently quenched by the DNA bases with no apparent shifts in the emission maximum. This emission-quenching phenomenon also reflects the changes in the excited-state electronic structure as a consequence of the electronic interactions in the MB-DNA complexes. The experimental data of the MB fluorescence quenched by DNA are plotted according to the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[\mathrm{DNA}] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher DNA. It was found that F_0/F varies linearly with the increasing amounts of DNA concentration (Fig. 3b). The Stern-Volmer quenching constant, K_{SV}, was evaluated by linear fitting of the data in terms of the above equation. K_{SV} value for the bound MB with DNA under this experimental condition was 2.51×10^4 M^{-1}. Considering the emission quenching phenomenon and the hypochromic and bathochromic effects in the absorption spectra, the above spectroscopic measurements fitted the intercalative mode of MB to DNA.

It should be noted that similar spectroscopic properties as mentioned above are occasionally observed for minor groove binders. Therefore, the binding mode of MB to salmon sperm DNA may be further questioned considering this point. We agree that the minor groove-binding mode may be probable for some organic molecules, but it should be unlikely for MB in this case. In support of our conclusions, a recent theoretical

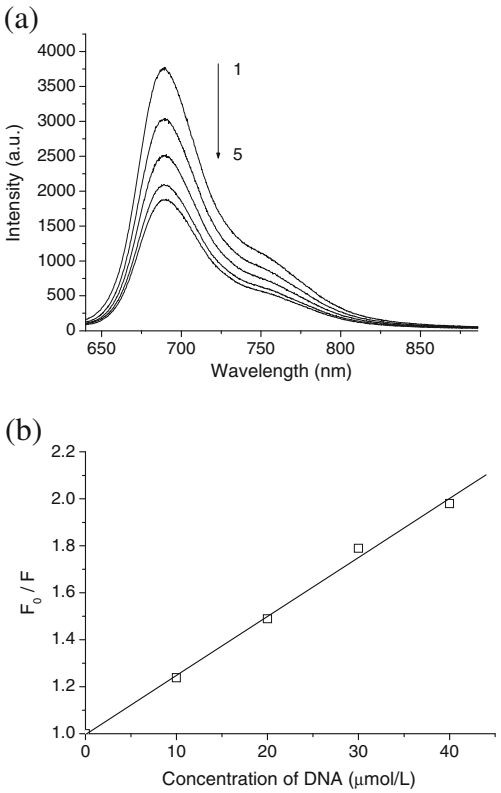

Fig. 3 **a** The emission spectra of pure MB and MB-DNA complexes in aqueous solution at room temperature. λ_{ex}=630 nm. Line 1 represents the pure MB aqueous solution (2 μmol/L); Lines 2–4 are the above MB aqueous solutions in the presence of salmon sperm DNA with the concentrations of 10, 20, 30 and 40 μmol/L, respectively. **b** The Stern-Volmer plot of the MB fluorescence quenched by DNA. The solid line represents the best linear fit of the data

modeling study reveals a preference for symmetric intercalation of MB to DNA with an alternating GC base sequence, while asymmetric intercalation and minor and major groove binding appear to be less favorable [23]. This result also matches with the published circular dichroism data [24]. It is also worthy of note a few recent publications by Zhao et al., in which spectroscopic and electrochemical measurements of MB loaded β-cyclodextrin with DNA reveal that the binding model of confined MB to DNA is the electrostatic mode [27, 28]. Considering diverse systems used (MB loaded β-cyclodextrin with DNA versus MB directly complexed with DNA), it is easy to understand why different binding modes are finally concluded.

Ferrocyanide Quenching Measurements

To further establish the DNA binding affinity of MB, the ferrocyanide quenching experiments were chosen because ferrocyanide is a rather efficient quencher of the MB fluorescence in aqueous solutions. A possible explanation for this phenomenon is that the negative charge on the CN^-

group would seem to add electrostatic interference in the quenching studies of MB bound to DNA. It is generally accepted that the groove binding exposes the bound molecules to the solvent surrounding the DNA helix much more than does the intercalation [36]. Therefore, if MB is intercalated into the helix stack, the ferrocyanide quenching of the bound MB should be less efficient than that of the free MB; in contrast, if MB binds to DNA in the groove, the quenching efficiency of the bound MB should be higher than that of the free MB. Excited by 630 nm light, the quenching experiments were performed with MB (2 μmol/L) and $K_4Fe(CN)_6$ (10 μmol/L) in the absence and presence of DNA solution (10 and 40 μmol/L). The emission spectra of pure MB quenched by $K_4Fe(CN)_6$ are shown in Fig. 4a, in which a marked decrease of the fluorescence intensity is observed. Seeing from Fig. 4b and c, the addition of $K_4Fe(CN)_6$ to the

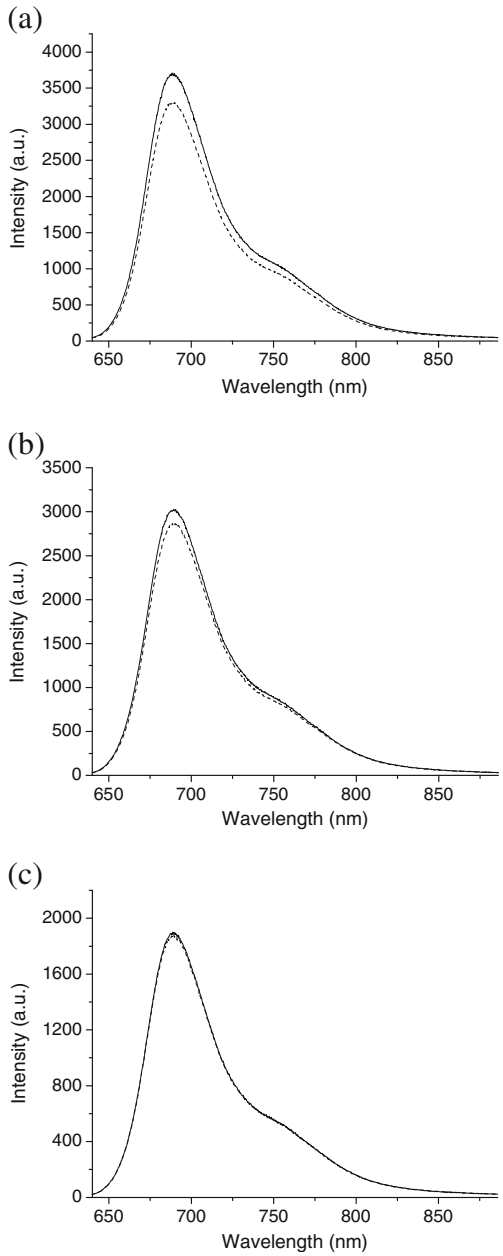

Fig. 4 **a** The emission spectra of pure MB in the absence (*solid line*) and presence (*dashed line*) of $K_4Fe(CN)_6$ in aqueous solution. **b** The emission spectra of MB-DNA (10 μmol/L) complex in the absence (*solid line*) and presence (*dashed line*) of $K_4Fe(CN)_6$ in aqueous solution. **c** The emission spectra of MB-DNA (40 μmol/L) complex in the absence (*solid line*) and presence (*dashed line*) of $K_4Fe(CN)_6$ in aqueous solution. λ_{ex}=630 nm

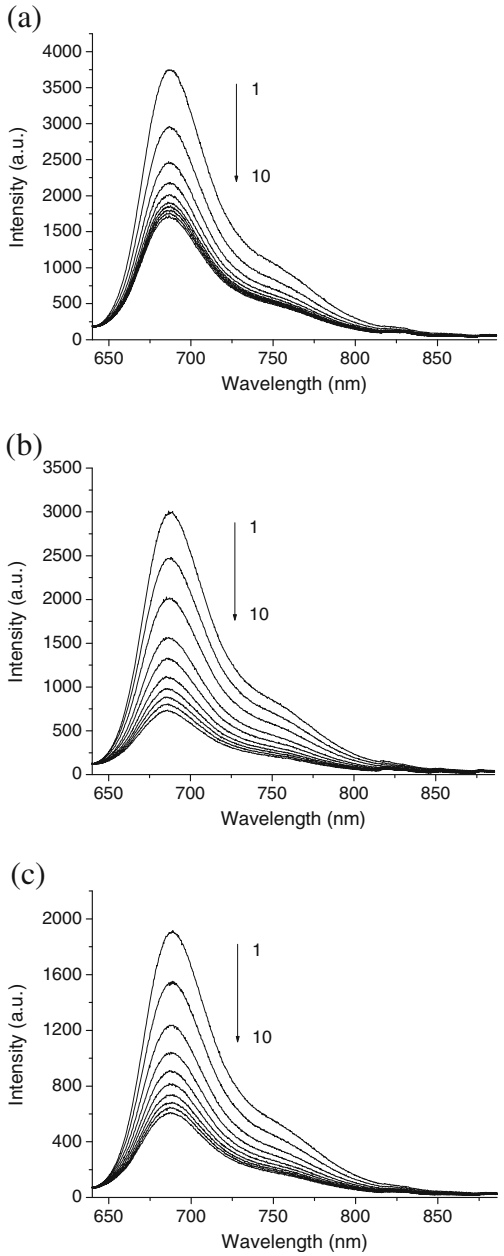

Fig. 5 **a** The emission spectra of pure MB irradiated with 670 nm laser light. **b** The emission spectra of the MB-DNA (10 μmol/L) complex irradiated with 670 nm laser light. **c** The emission spectra of the MB-DNA (40 μmol/L) complex irradiated with 670 nm laser light. Line 1 in these figures represents the emission spectrum recorded before laser irradiation and the following lines are the spectra hereafter recorded every 5 min. λ_{ex}=630 nm

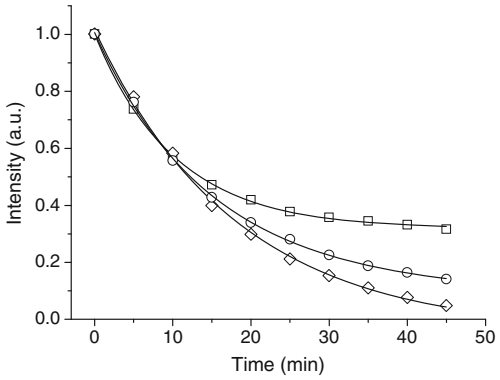

Fig. 6 The decay curves of the fluorescence intensities of pure MB (*white square*) and MB-DNA complexes with DNA concentrations of 10 μmol/L (*white diamond*) and 40 μmol/L (*white circle*) in aqueous solution at room temperature. The experimental data are fitted to the single- and bi-exponential decay functions (see the equations in the text) and the solid lines in this figure represent the best fitting curves. λ_{ex}=630 nm

MB-DNA complexes resulted in a decreased quenching of the fluorescence intensity caused by ferrocyanide. The higher the DNA concentrations, the less the variations of the emission spectra. These measurements well support the above intercalation mechanism of MB into the DNA bases. Extended spectroscopic experiments with different MB-to-$K_4Fe(CN)_6$ and MB-to-DNA ratios were also performed and essentially the same spectral variation features was found as above.

Photobleaching Experiments

To delineate the photobleaching process of MB and its DNA complex and gain a better understanding of the MB-DNA interactions, the photobleaching experiments were performed according to the procedure mentioned in the second section. Sealed in a tube and irradiated with the laser light (670 nm, 34.5 mW), the changes of the emission spectra of pure MB and its DNA (10 and 40 μmol/L) complexes are shown in Fig. 5a–c, respectively. In these measurements, the spectra were recorded every 5 min and exhibited a dramatic decrease of the MB fluorescence intensity. Figure 6 shows the fluorescence decay curves in which the experimental data correspond to the maxima emission values as revealed in Fig. 5. It is evident from Fig. 6 that the decay curves of pure MB fluorescence in aqueous solution fitted reasonably well to the single-exponential function $I(t)=I_0+A\exp(-t/\tau)$ with the τ value of 10.2 min. When MB was bound to DNA, the fluorescence decay profile should best be fitted as the bi-exponential function $I(t)=I_0+A_1\exp(-t/\tau_1)+A_2\exp(-t/\tau_2)$ giving the τ_1 and τ_2 values of 17.9 and 165.4 min (for 10 μmol/L DNA concentration) and of 14.3 and 120.9 min (for 40 μmol/L DNA concentration). Relevant control experiments indicated that the sensitizer, oxygen and light were all essential for this process. It is suggested that the photobleaching of MB and its DNA complex in aqueous solution were photodynamic processes. MB is a potent sensitizer for photodynamic therapy and a good generator of active oxygen species, including singlet oxygen $^1O_2$ and superoxide anion $O_2^{\bullet-}$ [17, 37]. It is generally agreed that the active oxygen species generated during MB photosensitization can attack the sensitizer itself, leading to the photochemical reactions of the sensitizer, which is the so-called self-sensitized photooxidation. Finally, the photooxidized products – endoperoxide and dioxirane are formed respectively, according to different types of photodynamic processes [38].

It should be noted that the singlet oxygen mechanism (Type II) is ordinarily regarded as the predominant one during the self-sensitized photooxidation of sensitizers, which could proceed without the involvement of DNA. However, the photobleaching processes of MB in the absence and presence of DNA in aqueous solution obey different decay functions. It had been previously demonstrated by spectrophotometric and EPR methods that the photoinduced electron transfer from DNA to long-lived excited triplet photosensitizers led to the generation of sensitizer radicals, which was unstable and rapidly recovered to the sensitizers [39]. Therefore, the bi-exponential decay nature of MB in its DNA complex indicated that a free radical mechanism (Type I), i.e. the superoxide anion radical mechanism, was also involved in the photobleaching processes as well as the singlet oxygen mechanism. From the above fitting data of fluorescence decay kinetic measurements, it apparently reveals that the Type I and II photoprocesses correspond to the slow (τ_2) and fast (τ_1) decay components, respectively. The τ_1 value obtained from pure MB fluorescence decay kinetics is slightly enlarged when the measurements are performed in the presence of DNA, which is probably due to the existence of two reaction channels in the latter case. Accompanying with the increase of DNA concentrations from 10 μmol/L to 40 μmol/L, a noticeable shortening of the slow decay component τ_2 from 165.4 min

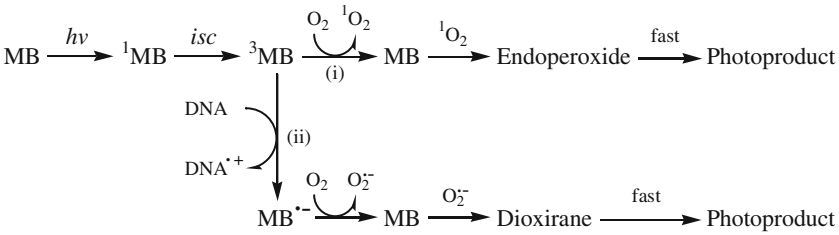

Fig. 7 The proposed pathways for photobleaching of MB and its DNA complex. Two pathways are denoted by process (*i*) and process (*ii*), respectively

to 120.9 min is observed. Considering a higher DNA-to-MB ratio evidently facilitates the Type I photoprocess, the variation of the τ_2 values well supports the forementioned assignment of decay components to the photoprocess types. By this time, it can be concluded that the photobleaching of MB and its DNA complex proceed with different mechanisms, which could be best outlined in Fig. 7.

Conclusion

In summary, the binding properties of photosensitizer MB to salmon sperm DNA have been studied by spectroscopic methods in this work. MB binds to double helical DNA with a high affinity. Remarkable hypochromic and bathochromic effects of MB in the presence of increasing amounts of DNA have been observed in the absorption spectra. The quenching of MB by the DNA bases obeys the Stern-Volmer equation and as further experiments, ferrocyanide quenching of MB in the absence and presence of DNA is also measured by fluorescence spectroscopy. Results from the above spectral measurements are all consistent with the intercalative binding mode of MB to DNA with the intrinsic binding constant (K_b) of $5.6\times10^3\,M^{-1}$. The photobleaching processes of MB and its DNA complex have also been investigated. On one hand, singlet oxygen is formed via energy transfer from triplet MB to molecular oxygen (process (i) in Fig. 7). On the other hand, MB withdraws one electron from DNA upon irradiation, which results in the formation of reduced MB radical that is the precursor of superoxide radical (process (ii) in Fig. 7). These reactive oxygen species are both contribute to the self-sensitized photooxidation of MB. Elucidation of the above photobleaching processes of MB helps us to obtain a better understanding of the MB-DNA interactions in vivo and may facilitate our future investigation of the photodynamic activities of MB in biological organisms.

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