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In Vitro Study on the Interactions between Native Herring Sperm DNA and Melamine in the Presence of Ca^{2+} by Spectroscopic and Voltammetric Techniques

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In vitro studies on the interactions between native Herring Sperm DNA (HS-DNA) and melamine (MM) in the presence of Ca^{2+}, have been investigated by spectrophotometric, spectrofluorometric, melting temperature (T_m), viscosimetric and cyclic voltammetric techniques. Comparative studies were carried out with those of the denaturalized DNA and absence of Ca^{2+}. Spectrophotometric and spectrofluorometric results showed a slight bathochromic shift (~ 2 nm) of the absorption band at the 258 nm, with a significant hypochromic effect (the absorbency at 258 nm decreased ~0.12, ~17 %). A small increase in melting temperature (2 ^{o}C) and an increase in the fluorescence of methylene blue (MB)-DNA solutions were observed. The cyclic voltammetric study results indicated that some electrochemical-active complexes (MM-Ca^{2+} and DNA-MM-Ca^{2+}) might be formed with an adsorption controlled quasireversible electrochemical process. For the MM-DNA system in the presence of Ca^{2+} (1.0 g L^{-1}, pH = 7.0), the charge transfer coefficients (α) were 0.27 and 0.25, with the velocity constant (k_s) at 0.32 and 0.36 s^{-1} before and after DNA added, respectively. The interaction constants of the MM-DNA-Ca^{2+} systems were found to be ~ 10^5 L mol^{-1}. The thermodynamic studies suggested that the interaction processes of those in the presence of Ca^{2+} were exothermic favored ($\triangle H$<0) and entropy favored ($\triangle S$>0), which indicated that the MM might interact with HS-DNA by a mixture mode of non-covalent groove binding *via* hydrogen bonds and electrostatic interaction with the negatively charged nucleic acid sugar-phosphate structure.

Keywords: Melamine (MM), Herring Sperm DNA (HS-DNA), Interaction, Ca^{2+}, Hydrogen bonds, Electrostatic interaction, Mixture mode of binding

1. INTRODUCTION

Melamine (MM), with a chemical formula of $C_3H_6N_6$, is an organic base and a trimer of cyanamide, with a 1,3,5-triazine skeleton. It is used primarily in the synthesis of melamine

formaldehyde resins with several industrial uses. If mixed with resins, it has fire retardant properties due to its release of nitrogen gas when burned or charred. Melamine is also one of the major components in Pigment Yellow 150, a colorant in inks and plastics [1]. Since it contains 66.6% of nitrogen by mass, MM is sometimes illegally added to food products in order to increase the apparent protein content. Standard tests, such as the Kjeldahl and Dumas tests, estimate protein levels by measuring the nitrogen content, so they can be misled by adding nitrogen-rich compounds such as melamine [2]. 2007's pet food incident and the following year's milk powder scandal were notorious typical examples. Although, MM is reported to have an oral LD_{50} of 3248 mg kg^{-1} based on rat data, and the clinic symptoms of the sufferers with melamine poisoning are related to kidney stones or bladder urolithiasis, other potential harmful effects are still uncertain [3-5]. A study performed by FDA described the risk to human health associated with eating products from animals that have been fed with MM [3]. The United Nations' food standards body, Codex Alimentarius Commission, has set the maximum amount of melamine allowed in powdered infant formula to 1 mg kg^{-1} and the amount of the chemical allowed in other foods and animal feed to 2.5 mg kg^{-1} [6]. Recent research showed that ingestion of melamine at levels above the safety limit may cause kidney failure and even death, particularly for vulnerable individuals such as infants and young children. Melamine is not approved by the US Food and Drug Administration (FDA) and Chinese government as a food additive in human food or animal feeds. High dosage of MM has shown to be carcinogenic for male rats [7]. Although at the "Post-Melamine Time", great attention has been paid to MM, however, mainly about the establishment of the detecting methods of it, studies on the evaluation of the safety performance of MM are still limited and of great challenges.

DNA is known to be a major target for drugs and some harmful chemicals to be attacked. It has been proved to be an efficient route to evaluate the safety performance of chemicals *via* the investigation of the interaction between DNA and target agent. Small molecules normally interact with DNA *via* non covalent interaction modes, *e. g.*, (i) intercalating between stacked base pairs, (ii) non-covalent groove binding, or (iii) electrostatic interaction with the negatively charged nucleic acid sugar-phosphate structure [8]. Studies on the binding mechanism of some small molecules with DNA have been identified as one of the key topics during the past few decades [9-15]. It is of great help to understand the structural properties of DNA, the mutation of genes, the origin of some diseases, the action mechanism of some anti-tumor and antivirus drugs. Recently, researches on the interaction between DNA and some harmful chemicals, such as environmental pollutants, pesticides, *etc*, gradually become a hot topic as a main way for the investigation of DNA damage, as well as understanding of toxic mechanism [9, 12, 15]. Some techniques, including gel electrophoresis [16], foot-printing technique, X-ray crystallography, Mass spectrum [17], NMR [18], fluorescence [19], UV/vis spectroscopy [20], electrochemical [21] *etc*., have been used to investigate this interaction. Among them, spectroscopic, voltammetric and viscosimetric techniques have been testified to be of high sensitivity, relatively low cost, direct monitoring and simplicity [19-26].

Our previous work has established a series method for the investigation of the interactions of native Herring Sperm DNA with small molecules [23-26]. As we all know, some important metallic elements present in food as well as in human bodies. The effect of these metallic elements on the interactions of native Herring Sperm DNA with melamine (MM) can not be ignored. In this work, Ca^{2+}

was selected as a model for the investigation of the interactions of native Herring Sperm DNA with melamine (MM) in the presence of metallic ions. As the normal concentration of Ca^{2+} in milk and milk powder is at around 1.0 g L^{-1} or 1.0 g kg^{-1} [27], the concentration of Ca^{2+} at 1.0 g L^{-1} in the solution was chosen for study. Spectrophotometric, spectrofluorometric, melting temperature (T_m), viscosimetric, and cyclic voltammetry techniques were applied for this study. Some valuable results were obtained. We hope this work will provide some useful information for the evaluation of the safety performance of MM through understanding their interaction with DNA in the presence of Ca^{2+}.

2. EXPERIMENTAL

2.1. Chemicals and reagents

Melamine (MM) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) with purity of 99%. Herring Sperm DNA (HS-DNA) was purchased from Shanghai Bio Life Science and Technology Co., Ltd (Shanghai, China). The stock solution of DNA was prepared by dissolving DNA in 0.01 mol L^{-1} of Tris buffer at pH 7.2 (0.01 mol L^{-1} of tris(hydroxymethyl)aminomethane (Tris) with NaCl concentration at 0.01 mol L^{-1}) and dialyzing exhaustively against the same buffer for 24 h, and used within 5 days. A solution of DNA gave a ratio of UV absorbance at 260 and 280 nm more than 1.8, indicating that DNA was sufficiently free from protein. The DNA concentration of the stock solution was determined by UV spectrophotometry, in properly diluted samples, using the molar absorption coefficient 6600 L mol^{-1} cm^{-1} at 260 nm; the stock solution was stored at 4 ^oC. A stock solution of MM (1×10^{-4} mol L^{-1}) or Ca^{2+} (100 g L^{-1}) was prepared by dissolving an appropriate amount of MM or $CaCl_2$ in Tris buffer. A fresh working solution was prepared daily by diluting the stock solution with Tris buffer and used for different studies. Other used chemicals were of analytical reagent grade.

2.2. Apparatus

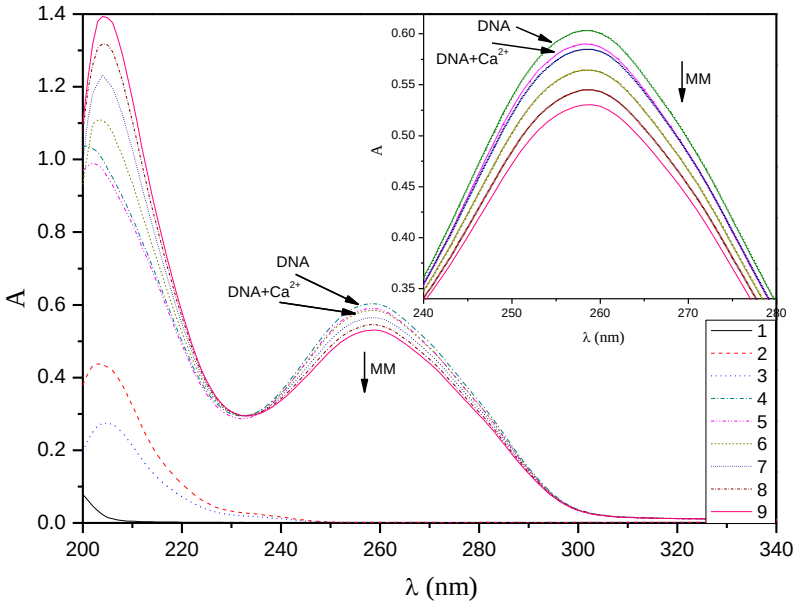
The UV-vis spectra for MM–DNA interactions were obtained by using a PE λ25 spectrophotometer. For DNA melting studies, the temperature of the cell holder was changed as 30 ^oC, 37 ^oC, 40 ^oC, 45 ^oC , 50 ^oC , 55 ^oC , 60 ^oC , 65 ^oC , 70 ^oC , 75 ^oC , 80 ^oC, 85 ^oC, and 90 ^oC. The fluorescence studies were carried out by using an F4500 fluorescence spectrophotometer with methylene blue (MB) as a fluorescent probe. Spectra were recorded at room temperature using a standard quartz cell of 1.0 cm path length, where 3.0 mL of Herring Sperm DNA solution with a concentration of 9.0×10^{-5} mol L^{-1} was placed. Once the first absorption spectrum was achieved, 30 μL of Ca^{2+} (c = 100 g L^{-1}) solution was added, which resulted in a new spectral acquisition. Then 10.0 μL of MM solution was added to obtain the 3rd absorption spectrum. This procedure allowed us to increase the MM after each addition without any appreciable change in the total volume, thus preserving DNA concentration. Therefore, obtained features for absorption bands over the whole series of spectra were comparable. For viscosity measurements, a Ubbelohde viscosimeter (0.6-0.7 type) was used, which

was thermostated at 25 ^{o}C by a constant temperature bath. Flow time was measured with a digital stopwatch; the mean values of three replicated measurements were used to evaluate the viscosity (η) of the samples (η was calculated by the equation $\eta = (t-t_0)/t_0$, where, t_0 is the flow time of the blank solution of Ca^{2+} (1.0 g L^{-1}); t is the flow time of the DNA solutions with the concentration ratios of [DNA]/[MM] from 0.05 to 1.20). The data were reported as $(\eta/\eta_0)^{1/3}$ *vs* the [DNA]/[MM] ratio, where η_0 is the viscosity of the DNA solution in the presence of Ca^{2+} (1.0 g L^{-1}). The cyclic voltammetry studies were carried out by using LK-2006 electrochemical system (Lanlike Co. Ltd, Tianjing, China). Electrochemical cell consisted of a glass container with a cap having holes for introducing electrodes and nitrogen. The cell was then maintained oxygen free by passing nitrogen over the solution. The reference electrode used was saturated calomel electrode (SCE), while the auxillary and working electrodes were platinum foil and glassy carbon electrode (GCE), respectively. In a typical cyclic voltammetric experiment of the reaction mixture consisted of MM and DNA in the presence of Ca^{2+} (1.0 g L^{-1}) in Tris buffer, a stream of nitrogen was passed over them and the reaction mixture was thermostated. The three electrodes were connected to a computer controlled potentiostat and required potential scan rate, current sensitivity, initial potential and final potential were given and the resulting current was measured as a function of applied potential.

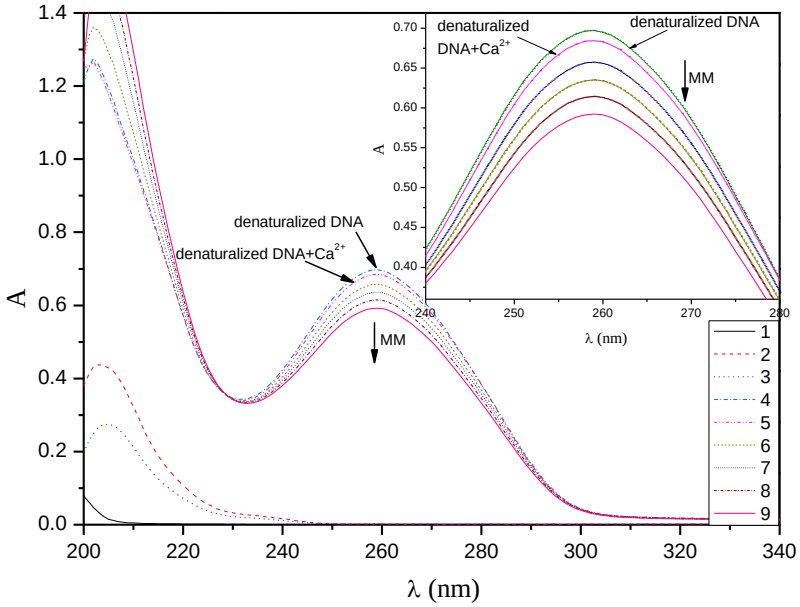
3. RESULTS AND DISCUSSION

3.1. Electronic spectra

The electronic spectra obtained *via* titration of DNA with MM solutions in presence of Ca^{2+} were shown in Figure 1 (a).



a



b

Figure 1. Electronic spectra of HS-DNA (a) and denaturalized HS-DNA (b) with MM in presence of Ca^{2+} in 0.01 mol L^{-1} of Tris buffer at pH 7.2(1) 0.01 mol L^{-1} of Tris buffer at pH 7.2, (2) Ca^{2+} ($c(Ca^{2+})$ =1.0 g L^{-1}), (3) MM (c(MM) =1.0 ×10^{-5} mol L^{-1}), (4) DNA (c(DNA)=9.0×10^{-5} mol L^{-1}), (5) DNA in presence of Ca^{2+} ($c(Ca^{2+})$=1.0 g L^{-1}), (6-9) MM-DNA, in presence of Ca^{2+}, (c(MM): (6) 3.3×10^{-6} mol L^{-1}, (7) 6.6×10^{-6} mol L^{-1}, (8) 1.0 ×10^{-5} mol L^{-1}, (9) 1.3 ×10^{-5} mol L^{-1})

As shown in Figure 1 (a), the spectrum of HS-DNA recorded in the presence of Ca^{2+} (1.0 g L^{-1}) represented a slight bathochromic shift (~2 nm) centered at the 258 nm with a slight hypochromic effect (the absorbency at 258 nm decreased ~0.02, ~3 %). After MM was added gradually, the spectra of DNA represented a significant hypochromic effect (the absorbency at 258 nm decreased ~0.12, ~17 %) with the increasing amounts of MM, which was almost 7% higher than that absence of Ca^{2+} in our previous finding (the absorbency at 258 nm decreased ~10 %) [24, 25].

Same experiments were carried out *via* titration of denaturalized DNA with the MM solution in the presence of Ca^{2+} (1.0 g L^{-1}), where the denaturalized DNA was obtained by heating the same HS-DNA solution in boiling water for 1 hr followed by cooling down in ice-water bath immediately. The results were shown in Figure 1 (b). As shown in Figure 1 (b), similar spectra of the denaturalized DNA were obtained. A slight bathochromic shift (~2 nm) centered at the 258 nm with a slight hypochromic effect (the absorbency at 258 nm decreased ~0.01, ~2 %) in the presence of Ca^{2+} (1.0 g L^{-1}). After MM was added gradually, the spectra of DNA represented a significant hypochromic effect (the absorbency at 258 nm decreased ~0.09, ~13 %) with the increasing amounts of MM, which was almost 4% less than that observed in nature DNA as described above (the absorbency at 258 nm decreased ~17 %).

The band at ~ 260 nm of DNA arises because of the π-π* transition of DNA bases. Changes in absorbance and wavelength shifts of this characteristic band reflect the corresponding structural changes of the DNA, including changes of stacking pattern, disruption of the hydrogen bonds between complementary strands, covalent binding of DNA bases, intercalation between aromatic rings of

molecules, *etc* [28-30]. The present finding of the slight hypochromic effect investigated in the spectrum of DNA in the presence of Ca^{2+} indicated that the conformation of DNA double-helix structure was changed after Ca^{2+} being added. This was consistent with the results in literature [31, 32]. Dickerson et al., has revealed the interaction mode between Ca^{2+} ions and DNA by using 1 Å resolution X-ray crystal structures of Ca^{2+} salts of the B-DNA that Ca^{2+} interact with DNA by binding to the major and minor grooves of DNA, as well as non-specific binding to backbone phosphate oxygen atoms [32]. Our previous work indicated that MM might interact with HS-DNA by a groove mode of binding *via* hydrogen bonds [24, 25]. After Ca^{2+} being added, on one hand, Ca^{2+} ions might bind to the phosphate *via* electrostatic interaction, as well as compete the binding sites with MM for groove bonding; on the other hand, the Ca^{2+}-MM complexes might be formed which could interact with DNA by binding to the grooves of DNA *via* hydrogen bonds as well as binding to backbone phosphate oxygen atoms *via* electrostatic interaction. Thus, the overall conformation changes of DNA structure may be a combination of a groove mode of binding through hydrogen bonds and electrostatic interaction [8, 28].

For further investigation the intensity of the interaction between the MM and DNA in the presence of Ca^{2+}, the interaction constants were evaluated by double reciprocal equation as listed in eq. 1 [8, 12].

$$1/(A_0\text{-}A) = 1/A_0 + 1/[K \times A_0 \times c(\text{MM})] \qquad (1)$$

Where A_0 and A are the absorbency at 258 of DNA in the presence of Ca^{2+} (1.0 g L^{-1}) before and after adding any of MM, where c(MM, mol L^{-1}) is the concentration of MM added and K (L mol^{-1}) is the interaction constant of MM-DNA, respectively.

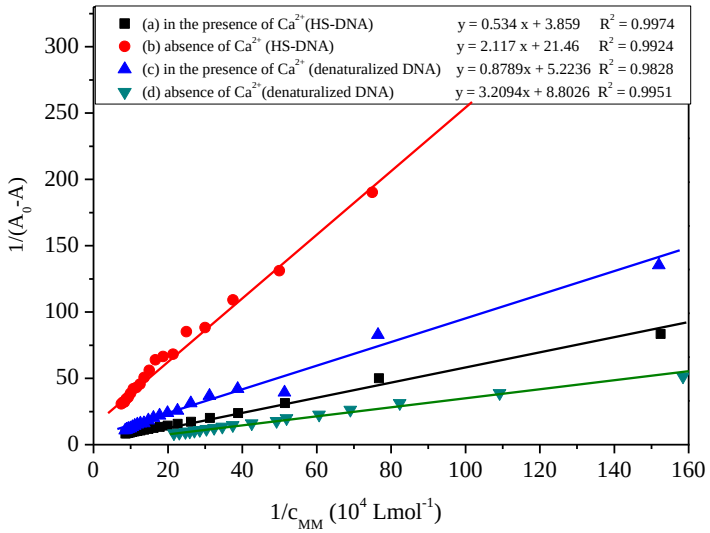


Figure 2. The interaction constant (37 ^{o}C) of the interaction between MM and HS-DNA (a) in the presence of Ca^{2+} (1.0 g L^{-1}), (b) absence of Ca^{2+}; and denaturalized DNA(c) in the presence of Ca^{2+} (1.0 g L^{-1}), (d) absence of Ca^{2+} (c(DNA) = 9.0×10^{-5} mol L^{-1}), c(MM) = 6.6×10^{-7} ~ 1.3×10^{-5} mol L^{-1})

By plotting of $1/(A_0-A)$ *vs* $1/c$(MM), K can be obtained from the slope and intercept of resulted curves, shown in Figure 2. The values of K for the MM-DNA complexes in the presence, or absence of Ca^{2+} (1.0 g L^{-1}) were 7.22×10^4 and 1.01×10^5 L mol^{-1}, respectively. Those for the denaturalized DNA were 5.94×10^4 and 2.74×10^4 L mol^{-1}, respectively.

To have a better understanding of thermodynamics of the interactions between MM and DNA in the presence of Ca^{2+}, it is useful to determine the contributions of enthalpy and entropy of the interactions. The evaluation of K for the MM-DNA complexes at different temperatures (320~350 K) allows to determine the thermodynamic parameters such as enthalpy (ΔH) and entropy (ΔS) of MM-DNA formation by Van't Hoff equation (as listed in eq. 2.) by plotting ln$K$ versus $1/T$. The results were summarized in Table 1.

$$\ln K = -\triangle H/RT + \triangle S/R \qquad (2)$$

Table 1. The thermodynamic parameters of MM-DNA interactions

system	ΔH (kJ mol^{-1})	ΔS (J mol-1 K^{-1})	K (L ×mol^{-1})	ΔG (kJ mol^{-1})
MM-DNA	-40.23	-31.03	1.02□105	-28.59
MM-DNA-Ca2+	-8.24	65.34	7.22□104	-27.72
MM denaturalized DNA	-29.23	-14.03	2.74□104	-25.33
MM denaturalized DNA-Ca^{2+}	-9.68	59.25	5.94□104	-27.25

As listed in Table 1, the $\triangle H$ and $\triangle S$ values of the MM-DNA complexes were -40.23 kJ mol^{-1} and -31.03 J mol^{-1} K^{-1} for MM-DNA absence of Ca^{2+}, -8.24 kJ mol^{-1} and 65.34 J mol^{-1} K^{-1} for that in the presence of Ca^{2+}, -29.23 kJ mol^{-1} and -14.03 J mol^{-1} K^{-1} for MM-denaturalized DNA absence of Ca^{2+}, -9.68 kJ mol^{-1} and 59.25 J mol^{-1} K^{-1} for that in the presence of Ca^{2+}, respectively. As proposed by P. D. Ross [33], when $\triangle H<0$ or $\triangle H\approx0$, $\triangle S>0$, the mainly acting force is electrostatic; when $\triangle H<0$, $\triangle S<0$, the mainly acting force is van der Waals or hydrogen bond and when $\triangle H>0$, $\triangle S>0$, the mainly force is hydrophobic. For the binding systems of the MM with DNA absence of Ca^{2+}, the $\triangle H$ and $\triangle S$ values were negative, indicated that hydrogen bonds might be the main acting force in the binding of the MM and DNA. However, in the presence of Ca^{2+}, although the $\triangle H$ values kept negative, $\triangle S$ values changed to be positive. These results indicated that in the presence of Ca^{2+}, hydrogen bonds might not be the only main acting force in the binding of the MM and DNA, while the contribution of the electrostatic interaction force might not be ignored. Overall, the values of K were at ~10^5, which was at least 100 times smaller than reported examples of traditional intercalating mode, such as daunomycin [34], cryptolepine [35], and chlorobenzylidine [36]. These results illuminated that the interactions between DNA and MM whether or not in the presence of Ca^{2+} did not follow the traditional intercalating mode, while the conformation changes of DNA structure may be realized *via* non-covalent groove binding and electrostatic interaction.

3.2. Fluorescence studies

The fluorescence studies of the interactions between MM and DNA were carried out in the presence of Ca^{2+} by using methylene blue (MB) as a fluorescent probe. Traditionally, the standard method for fluorescence enhancement of DNA is based on ethidium bromide (EB) usage. Recently, due to carcinogenic properties of EB, the methylene blue (MB) replaced EB and has become a safe reagent in nucleic acid chemistry. MB is a phenothiazinium dye that can interact with DNA not only by intercalation, but also by non-intercalation mode depending on the concentration of MB used. These results have been testified by several spectroscopic methods [9]. The fluorescence spectra of MB, MB-DNA complex before and after addition Ca^{2+} and MM were recorded and shown in Figure 3.

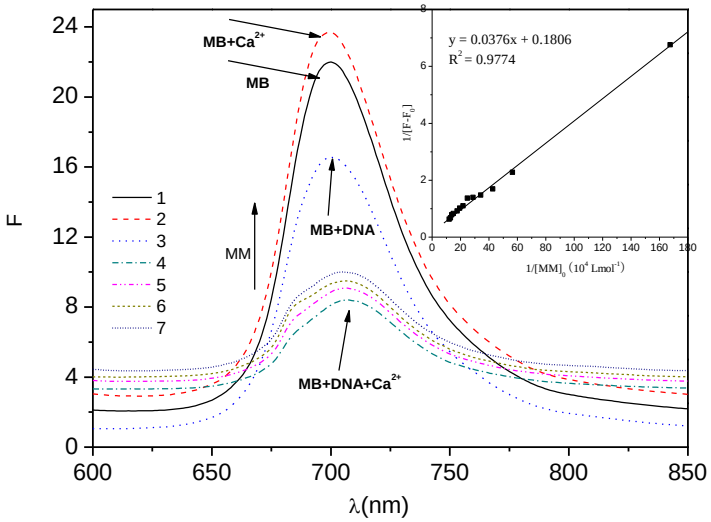


Figure 3. Fluorescence spectra of the MB-DNA-Ca^{2+} system (c(MB): 1.0×10^{-5}, c(DNA): 9.0×10^{-5} mol L^{-1}, c(Ca^{2+}): 1.0 g L^{-1} in the presence of the increasing MM concentrations ((1) MB, (2) MB+Ca^{2+}, (3) MB+DNA, (4) MB+DNA+Ca^{2+}, (5-7) MB+DNA+Ca^{2+}+MM (c(MM): (5) 1.0×10^{-6} mol L^{-1}, (6) 5.0×10^{-6} mol L^{-1}, (7) 1.0×10^{-5} mol L^{-1}) in 0.01 mol L^{-1} of Tris buffer at pH 7.2 at room temperature

As shown in Figure 3, by addition of Ca^{2+} (1.0 g L^{-1}), the fluorescence of MB was enhanced in some extent (Line 2). By addition of DNA, the fluorescence of MB was quenched significantly (Line 4) and the extent much lower than that absence of Ca^{2+} (Line 3). This emission-quenching phenomenon was due to the changes in the excited-state electronic structure in consequence of electronic interactions of MB-DNA-Ca^{2+} complex [8]. It should be noted that the effect of the MM on pure MB spectrum has been carefully checked, and no variation of the absorption and emission of spectrum was detected. By adding MM to the DNA-MB-Ca^{2+} solution, the fluorescence of MB was increased (Figure 3). The increase of the fluorescence intensity should be due to the fact that MB was released after the addition of MM. The formation of MM-DNA complex prevents MB binding to DNA. By using this phenomenon, the formation constant of MM to DNA was measured based on the recorded fluorescence data using the modified Benesi-Hildebrand equation, as described in eq. 3 [27]:

$$\frac{1}{F\text{-}F_0} = \frac{1}{\{KLQ[MB\text{-}DNA\text{-}Ca^{2+}]_0\}[MM]_0} + \frac{1}{(LQ[MB\text{-}DNA\text{-}Ca^{2+}]_0)} \quad (3)$$

where F_0 and F represent the fluorescence signals of MB-DNA-Ca^{2+} in the absence and presence of MM; $[MB\text{-}DNA\text{-}Ca^{2+}]_0$ and $[MM]_0$ represent the initial concentration of MB-DNA-Ca^{2+} complex and MM, L is the instrumented constant, K is formation constant of the MM-DNA complexes, and Q is the quantum yield for the MM-DNA-Ca^{2+} complex. By plotting of $1/(F\text{-}F_0)$ *vs* $1/[MM]_0$, the K can be obtained from the slope and intercept of the resulted curves, shown in Figure 3 (insert). The results showed that the value of K was 4.8×10^4 L mol^{-1}, which was a little bit less than that absence of Ca^{2+} (9.5×10^4 L mol^{-1}) [24]. The results were consistent with those obtained *via* electronic spectra method.

3.3. Viscosity measurements

Spectrophotometric and spectrofluorometric methods can provide necessary but not sufficient clues to support binding modes, whereas hydrodynamic measurements which are sensitive to the length change are regarded as the most critical tests of a binding model in solution [28].

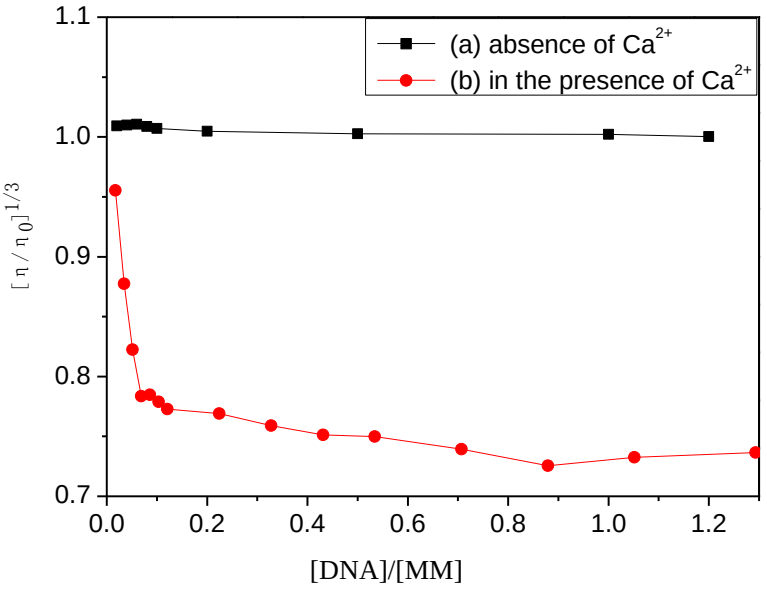


Figure 4. Effect of increasing amounts of MM on the viscosity of DNA (9.0×10^{-5} mol L^{-1}) (a) absence of Ca^{2+}, (b) in the presence of Ca^{2+} (1.0 g L^{-1}) in 0.01 mol L^{-1} Tris buffer (pH 7.2)

Thus, to further clarify the interaction between MM and DNA in the presence of Ca^{2+}, we carried out viscosity measurements. A classical intercalation mode is known to cause a significant increase in the viscosity of a DNA solution due to an increase in lengthening the DNA helix, while a non-classical intercalation or a groove mode would reduce the DNA viscosity. The viscosity

measurements were taken by varying the concentration ratio of DNA and MM in the presence or absence of Ca^{2+}. The values of relative specific viscosity $(\eta/\eta_0)^{1/3}$ *vs* [DNA]/[MM] were plotted in Tris buffers (Figure 4). As it was observed from Figure 4, the relative specific viscosity of DNA exhibited a dependence on the concentration of MM, which decreased with the value of [DNA]/[MM], and notablely, the decrease extent of the relative specific viscosity of DNA in the presence of Ca^{2+} was much large than that absence of Ca^{2+}. These results indicated that greater effect on the interaction between DNA and MM might exist when Ca^{2+} was presented, which might be the result of the mixture acting forces of hydrogen bonds and electrostatic interaction.

3.4. DNA denaturation temperature

Additional evidence for the binding mode between the MM and DNA in the presence of Ca^{2+} was obtained from DNA melting (T_m) studies by investigating the UV-vis spectra of MM-DNA at different temperatures (30-90 ^oC).

The changes in the absorbance at 260 nm as a function of temperature (30-90 ^oC) for HS-DNA in the presence or absence of MM were measured. f_{ss} was calculated as eq. 4. [37]:

$$f_{ss}=(A-A_0)/(A_f-A_0) \qquad (4)$$

where, A_f and A_0 were the maximum (double strand DNA fully separated to be single strand DNA) and minimum (double strand DNA) absorbances at 260 nm, respectively. A was the absorbances at 260 nm at any temperature, f_{ss} was the value of the hyperchromic effect of DNA. The DNA denaturation temperature (T_m) was defined as the temperature when f_{ss} was 0.5. By plotting of f_{ss} *vs* temperature (shown in Figure 5), the T_m can be obtained.

It has been reported that the intercalation of small molecules into the double helix is known to increase T_m significantly, while the interactions realized *via* non-traditional intercalation, or groove binding or electrostatic interaction mode are known to have little effect on T_m [38, 39].

As shown in Figure 5, T_m was increased to be 71.5 ^oC and 73.4 ^oC when Ca^{2+} was in absent or presented, with the ratios of the MM to DNA at 1.0, respectively. These results showed that either absence or in the presence of Ca^{2+}, T_m of the system with the MM added did not increase as much as that of previously observed for daunomycin [34], cryptolepine [35], and chlorobenzylidine [36], which were proved their interaction with DNA to be intercalative mode. However, an interesting phenomenon need to be paid attention to, *i. e.*, when Ca^{2+} existed, an extra ~ 2 ^oC was increased for the T_m of DNA, comparing to that absence of Ca^{2+}. The small increase of T_m might be due to the interaction of the Ca^{2+}-MM complex with DNA *via* electrostatic as well as hydrogen bonds, which subsequently lead the conformation of DNA being changed in some degree and the stabilization of the DNA-MM systems being increased. The results furtherly supported that that the MM might interact with HS-DNA by a mixture mode of non-covalent groove binding *via* hydrogen bonds and electrostatic interaction with the negatively charged nucleic acid sugar-phosphate structure.

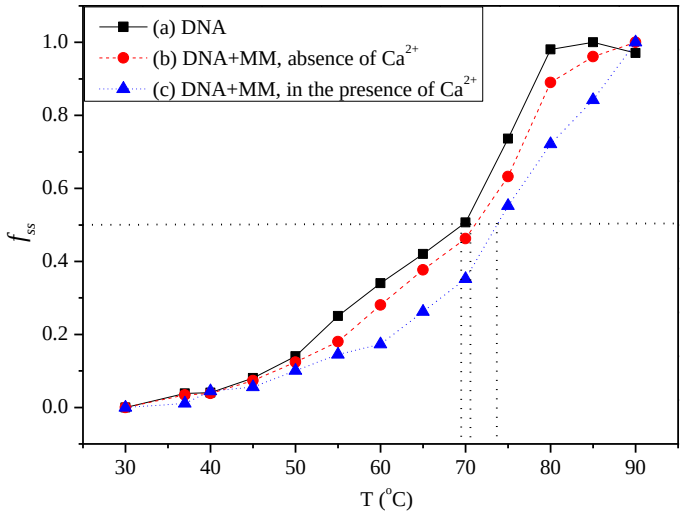


Figure 5. The relationship between f_{ss} of DNA and T before (a) and after (b and c) adding MM to the DNA solution in 0.01 mol L^{-1} Tris buffer (pH 7.2). (a) DNA (9.0×10^{-5} mol L^{-1}); (b) absence of Ca^{2+}, (c) in the presence of Ca^{2+} (1.0 g L^{-1})

3.5. Cyclic voltammetric studies

For thermodynamic studies, typical cyclic voltammetric curves of the MM with concentration at 1.0×10^{-5} mol L^{-1}, in the presence or absence of HS-DNA (9.0×10^{-5} mol L^{-1}) or Ca^{2+} (1.0 g L^{-1}) in 0.05 mol L^{-1} Tris (pH 7.0) with a scan rate of 100 mV s^{-1} were shown in Figure 6.

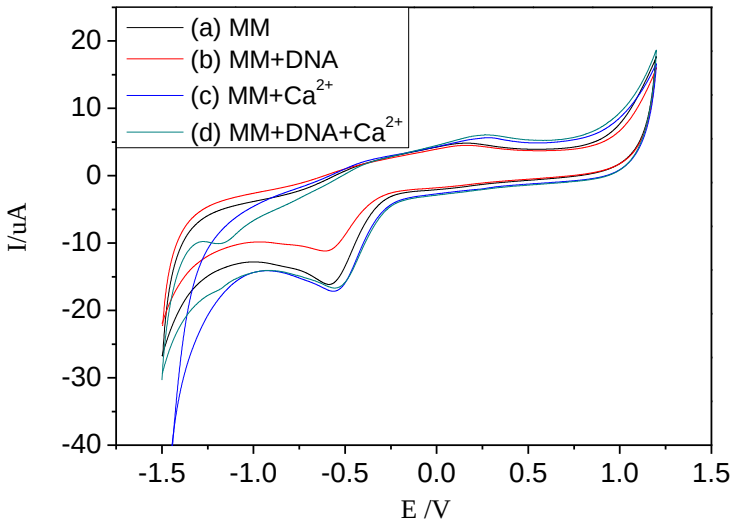


Figure 6. Cyclic voltametry (CV) of (a) MM, (b) MM-DNA, (c) MM-Ca^{2+} and (d) MM-Ca^{2+}-DNA system in 0.05 mol·L^{-1} Tris (pH 7.0), with a scan rate of 100 mV ·s^{-1}

As shown in Figure 6, in the case of free from Ca^{2+}, a pair of redox peaks for MM absence of HS-DNA appeared using a bare GCE in the range of -1.5 to +1.5 V (vs. SCE). The cathodic peak

potential (E_{pc}) was at -0.59 V for MM with a scan rate of 100 mV s^{-1}, while the anodic peaks for MM was not obvious. The reduction peak current of MM was obviously much higher than the oxidation peak current. This result indicated that the electrochemical process of the MM at a bare GCE was quasireversible. When HS-DNA was added into the MM solution, increase in reduction peak currents of MM (from -16.44 μA to -11.17 μA) with a slight negative shift in peak potential value (from -0.59 V to -0.60 V) was observed. While, in the case of Ca^{2+} presented, before HS-DNA was added, a decrease in reduction peak currents of MM (from -16.44 μA to -17.15 μA) with a slight positive shift in peak potential value (from -0.59 V to -0.57 V) was observed. When HS-DNA was added into the MM-Ca^{2+} solution, similar phenomenon, *i, e.,* increase in reduction peak currents of MM (from -17.15 μA to -16.68 μA) with a slight positive shift in peak potential value (from -0.57 V to -0.56 V) was observed. Moreover, relative obvious anodic peaks were observed at the peak potential value of +0.28 V with the peak currents of 5.66 μA and 6.05 μA for MM-Ca^{2+} and MM-Ca^{2+}-DNA systems, respectively. These results indicated that some electrochemical-active complexes might form between MM and Ca^{2+}, which might be the formation of MM-Ca^{2+} complex, and furtherly some electrochemical-active complexes might form between DNA and MM-Ca^{2+}.

For further investigation the intensity of the interaction between the MM and DNA in the presence of Ca^{2+} (1.0 g L^{-1}), cyclic voltametric experiments of MM with the varied concentrations in the presence and absence of HS-DNA 9.0×10^{-5} mol L^{-1}) were carried out. The results showed that the difference of the reduction peak current in absence and presence of DNA ($\triangle I_p$) gradually increased with the concentration of the MM and finally reached to a flatform (shown in Figure 7 (insert), which was the typical phenomenon for an adsorption controlled quasireversible electrochemical process.

Thus, the interaction constants were evaluated by double reciprocal equation as listed in eq. 5 [34].

$$\frac{1}{\Delta I_p} = \frac{1}{\Delta I_{pmax}} + \frac{1}{K\Delta I_{pmax}} \times \frac{1}{[MM]^s} \qquad (5)$$

Where $\triangle I_p$ and $\triangle I_{pmax}$ are the difference and the maximum difference of the reduction peak current absence and in the presence of DNA, respectively, while c(MM, mol L^{-1}) is the concentration of MM added, and K (L mol^{-1}) is the interaction constant, s is the binding number. Taken different values of s (s = 1, 2 .... n), by plotting of $1/\triangle I_p$ *vs* $[1/c(MM)]^s$, K can be obtained from the slope and intercept of resulted curves. In the present work, when s = 1, a good linear relationship between $1/\triangle I_p$ *vs* [1/c(MM)] can be obtained, as shown in Figure 7. The K values were 2.5×10^5 and 1.4×10^5 L mol^{-1}, for the systems in the presence of or absence of Ca^{2+} (1.0 g L^{-1}), respectively.

For kinetic investigation, cyclic voltammetric experiments of the MM absence and in the presence of Ca^{2+} and DNA in 0.05 mol·L^{-1} Tris (pH 7.0) with varied scan rate were recorded. The curves in the presence of HS-DNA were taken as examples shown in Figure 8. It was observed that the reduction peak current (I_{pc}) of MM, either in the presence or absence of Ca^{2+}, varied linearly with scan rate (v) rather than $v^{1/2}$. The results were summarized in Table 2, which indicated that the electrode processes were controlled by adsorption step [35].

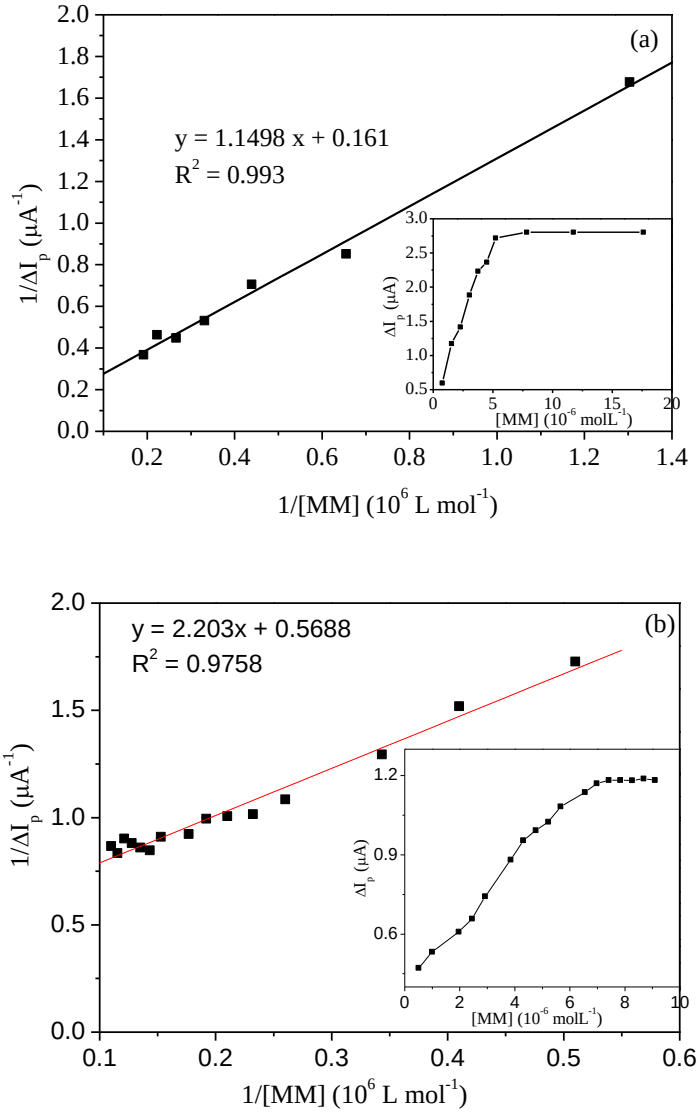
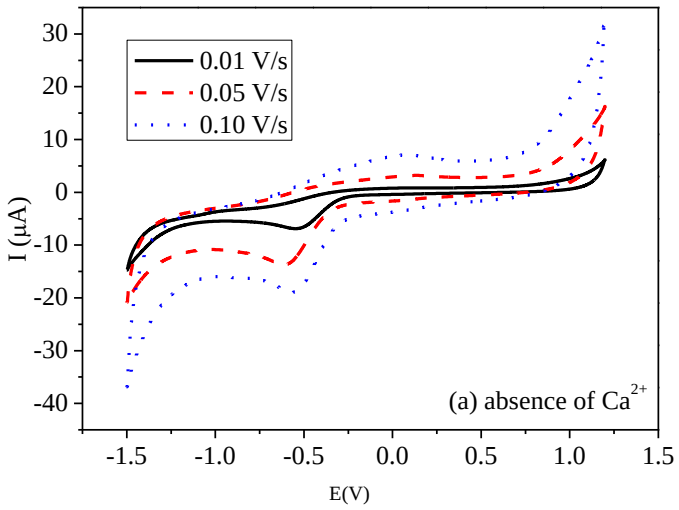


Figure 7. Relationship curves between ΔI_p^{-1} and $[MM]^{-1}$ (insert: Relationship curves between ΔI_p and [MM]; $\triangle I_p$: the difference of peak currents before and after addition of DNA; [MM] the equilibrium concentration of (a) absence of Ca^{2+}, (b) in the presence of Ca^{2+} (1.0 g L^{-1})



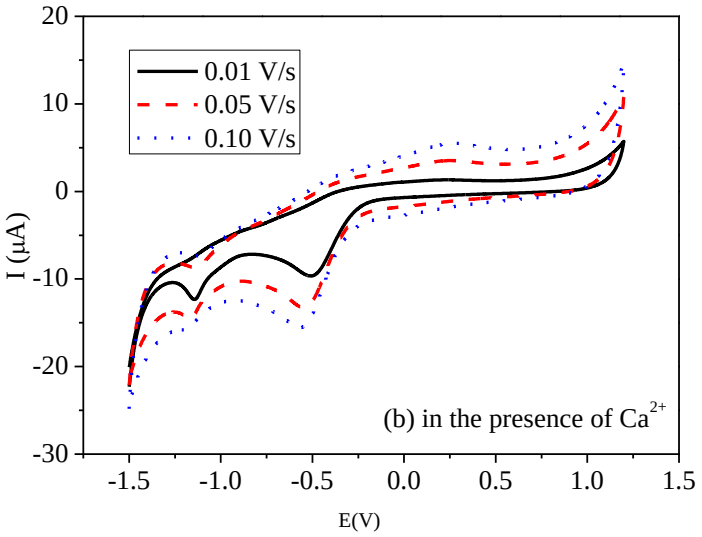


Figure 8. Cyclic voltametry (CV) of MM in presence of HS-DNA (c(DNA)= 9.0×10^{-5} mol L^{-1}) at different scan rate ((a) absence of Ca^{2+}, (b) in the presence of Ca^{2+} (1.0 g L^{-1})

Table 2. The relationship between I_p and v, the number of electrons transferred per molecule (n) of MM absence of Ca^{2+} and in the presence of Ca^{2+} (1.0 g L^{-1})

System	v($V\cdot s^{-1}$)	I_p(μA)	$I_p \sim v$	Q (μC)	n	n_{av}
MM	0.01	-9.63	I_p = -129.17 v - 8.8893 R^2 = 0.9626	-49.72	1.99	2.05
	0.03	-12.35		-20.63	2.05	
	0.05	-16.44		-15.94	2.12	
	0.07	-18.73		-14.32	1.92	
	0.10	-20.88		-9.84	2.18	
MM in the presence of Ca^{2+}	0.02	-9.65	Ip = -72.7 v - 8.594 R^2 = 0.9798	-26.37	1.88	2.04
	0.04	-11.80		-14.30	2.12	
	0.06	-13.26		-11.59	1.96	
	0.08	-14.50		-8.43	2.21	
	0.10	-15.57		-7.92	2.02	

For an adsorption controlled quasireversible electrochemical process, the relationship between the peak current (I_p) and the coulomb of adsorption (Q_a) obeys the following formula [36]:

$$I_p = n^2F^2A\Gamma v/4RT \qquad (6)$$

$$\text{Since, } Q_a = nFA\Gamma \qquad (7)$$

$$\text{Then, } I_p = nQ_aFv/4RT \qquad (8)$$

where R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the Kelvin temperature (T), F is the Faraday constant (96487 C mol^{-1}), n is the number of electrons transferred in reaction, A is the surface area of the working electrode (cm^2), v is the scan rate (V s^{-1}), Γ is the surface concentration of adsorption (mol cm^{-2}), Q_a is the coulomb in the process of adsorption (C), and I_p is the peak current (A).

By integrating the peak areas of the reduction peaks, Q can be obtained, therefore, the number of electrons transferred per molecule (n) were calculated as listed in Table 2 The number of electrons transferred per molecule (n) was found to be ~2.

As discussed above, the reduction peak current (I_{pc}) of MM varied linearly with scan rate (v) rather than $v^{1/2}$, indicating that the electrode process was controlled by an adsorption step. According to Laviron theory [35], for an adsorption controlled process, the relationship between E_p and lg$v$ should obey the following formula.

$$E_p = E_p^{0'} + 2.3RT/\alpha nF[\lg(RT/nF)\, k_s] - 2.3RT/\alpha nF \lg \upsilon \qquad (9)$$

where E_p is the peak potential (V), $E_p^{0'}$ is the formal potential (V), which can be obtained from the intercept of the resulted lines by plotting of $E_p \sim v$ [34,35], R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the Kelvin temperature (T), F is the Faraday constant (96487 C mol^{-1}), n is the number of electrons transferred in reaction, k_s is the standard rate constant (s^{-1}), α is the charge transfer coefficient, v is the scan rate (V s^{-1}).

By plotting of $E_p \sim \lg v$, the charge transfer coefficient α and the standard rate constant k_s before and after adding HS-DNA/Ca^{2+} can be obtained. The results were listed in Table 3. The results showed that the values of α and k_s before and after adding HS-DNA/Ca^{2+} were not much changed, which is consisted with the findings that the electrode process was controlled by an adsorption step [24, 26].

Table 3. The main electrochemical kinetic parameters of the MM and MM-DNA systems

System	$E_p \sim v$	$E_p^{0'}$(V)	$E_p \sim \lg v$	α	$k_s(s^{-1})$
MM	E_p=-1.0662v-0.5237 R^2 = 0.9504	-0.52	E_p=0.1032lgv-0.7222 R^2 = 0.986	0.28	0.26
MM-DNA	E_p=-0.8309v-0.6627 R^2 = 0.9793	-0.66	E_p=-0.1244lgv-0.8668 R^2 = 0.9641	0.24	0.28
MM in the presence of Ca^{2+}	E_p=-0.4988v-0.5254 R^2= 0.9812	-0.53	E_p=-0.1089lgv-0.6991 R^2 = 0.9643	0.27	0.32
MM-DNA in the presence of Ca^{2+}	E_p=-0.3306v-0.555 R^2 = 0.982	-0.56	E_p=-0.1174lgv-0.7262 R^2 = 0.9643	0.25	0.36

4. PRESUMED INTERACTION MECHANISM

As discussed above, it can be inferred that the MM interacted with HS-DNA in the presence of Ca^{2+}, presumably by a mixture mode *via* hydrogen bonds and electrostatic interaction. Recently, Zhong, et al, has systematically investigated the structure, spectroscopy and reactivity properties of melamine and its metallic complexes, $M(MM)_2(OH)_2$, by using density functional theory (DFT), conceptual DFT and time-dependent (TD) DFT, which proved that MM might be able to form Ca^{2+} complexes, $Ca(MM)_2(OH)_2$ [40]. Dickerson et al., has revealed the interaction between Ca^{2+} ions and DNA might be realized by binding to the major and minor grooves of DNA, as well as non-specific binding to backbone phosphate oxygen atom [32]. Base on literature reports [41, 42] and our previous findings [23-25], the hydrogen bonds might be the main acting force in the binding of the MM and DNA. We presumed the possible interaction mechanism might involve, (1) the formation of the Ca^{2+}-MM complex, (2) MM or Ca^{2+}-MM complex interacted with DNA by a groove mode of binding *via* hyrogen bonds, (3) Ca^{2+} or Ca^{2+}-MM complex interacted with backbone phosphate of DNA *via* electrostatic interaction. The presumed mechanism was presented in Figure 9. Adenine and Thymine (A/T) were taken as examples for depiction.

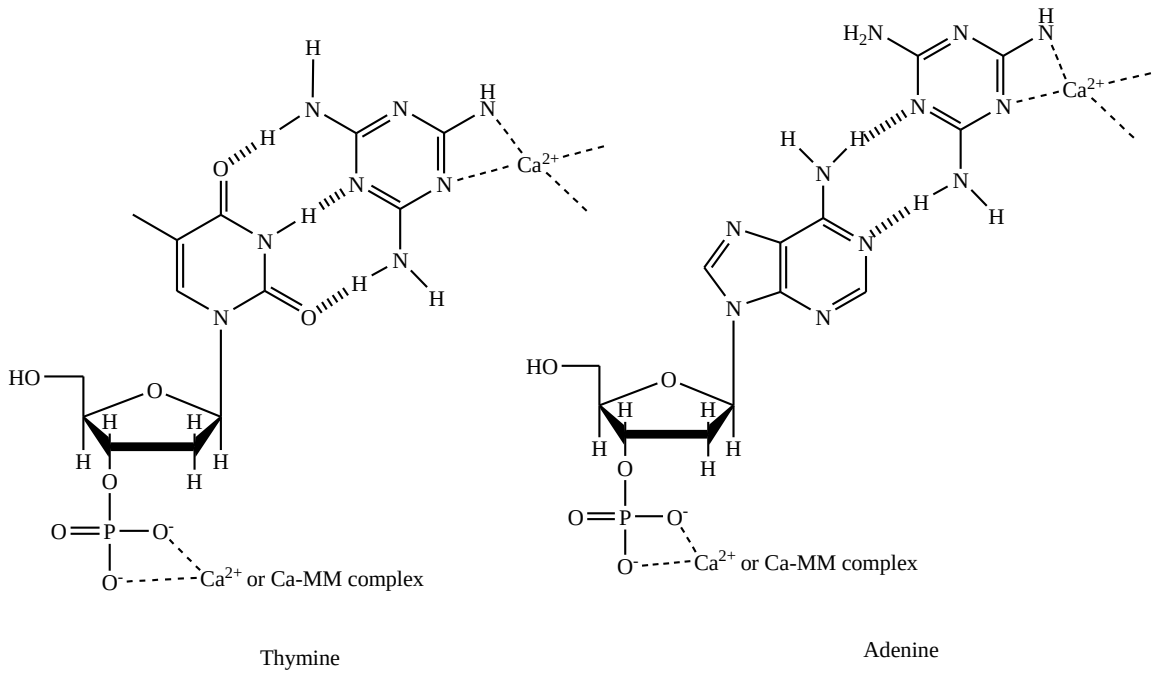


Figure 9. The presumed interaction mechanism of DNA and MM in the presence of Ca^{2+} (Thymine or Adenine taken as examples)

5. CONCLUSIONS

In summary, spectrophotometric, spectrofluorometric, melting temperature (T_m), viscosimetric and electrochemical studies showed that MM might interact with Herring Sperm DNA (HS-DNA) in the presence of Ca^{2+} (1.0 g L^{-1}) by a mixture mode *via* hydrogen bonds and electrostatic interaction.

This was supported by the following findings: (i) the UV slight bathochromic shift (~ 2 nm) of the absorption band at the 258 nm, with a significant hypochromic effect (the absorbency at 258 nm decreased ~0.12, ~17 %), in the presence of increasing amounts of MM; (ii) little increase of the DNA melting temperature of ~2 ^{o}C when molar ratio of [MM]/[DNA] is 1; (iii) a decrease in the relative specific viscosity of DNA for the DNA-MM-Ca^{2+} system; (iv) the recovery of MB fluorescence in some extent; (v) values of α and k_s before and after adding HS-DNA/Ca^{2+} were not much changed and the electrode process was controlled by an adsorption step, (vi) the interaction systems of MM with DNA were exergonic ($\triangle H$<0) and entropy favored ($\triangle S$>0), and (vii) the interaction constants at ~10^5 L mol^{-1} determined by spectrophotometric, spectrofluorometric methods and cyclic voltammetric studies.

Although the present results indicated that the MM might interact with native DNA in the presence of Ca^{2+} (1.0 g L^{-1}), presumably by a mixture mode *via* hydrogen bonds and electrostatic interaction, intensive investigation and mechanism studies need to be raised for deep attention.

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