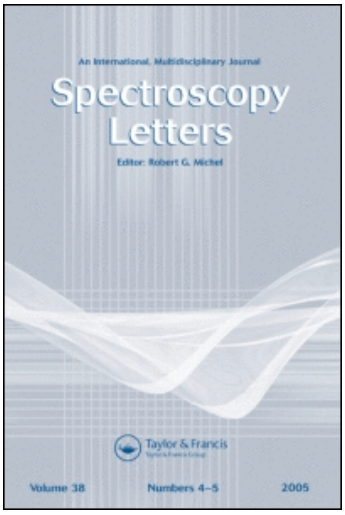

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Yunhua Wu[a]; Guangzhong Yang[b]
[a] College of Life Science, South Central University for Nationalities, Wuhan, P. R. China [b] College of Pharmacy, South Central University for Nationalities, Wuhan, P. R. China

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Interaction Between Garcigenrin and DNA by Spectrophotometry and Fluorescence Spectroscopy

Yunhua Wu[1] and Guangzhong Yang[2]

[1]College of Life Science, South Central University for Nationalities, Wuhan, P. R. China
[2]College of Pharmacy, South Central University for Nationalities, Wuhan, P. R. China

ABSTRACT The interaction of 12b-hydroxy-des-D-Garcigerin (GA) with DNA was studied by using acridine orange (AO) as a probe. The results indicated that there was a complex of GA and DNA, which was confirmed by absorption and fluorescence spectra. The influence of salt and temperature on the interaction of GA with DNA was studied. The results suggested that the intercalation and electrostatic binding should be the 2 major modes for interaction between GA and DNA.

KEYWORDS 12b-hydroxy-des-d-garcigerin, acridine orange, action mode, DNA

INTRODUCTION

Deoxyribonucleic acid (DNA) is an important genetic substance in each organism. The areas of DNA involved in vital processes such as gene expression, gene transcription, mutagenesis, carcinogenesis, and cell death are of particular interest as targets for a wide range of anticancer and antibiotic drugs.[1–7] So, the study of the interaction of drug and DNA plays a key role in pharmacology, and it is of great significance for designing and synthesizing the new drugs targeted to DNA. Their effectiveness depends on the mode and affinity of the binding. The interaction of DNA with drugs has been studied by various techniques including fluorescence,[8] UV method,[9] luminescence,[10] electrophoresis,[11] NMR,[12] quartz crystal microgravimetry,[13] and electroanalytical methods.[14]

The fluorescence quantum yield of DNA is about 10^{-4}–10^{-5} at room temperature,[15] and the intrinsic fluorescence from DNA is of little practical usefulness. The utility of fluorescence probes is that they can obtain the information of the structure and quantity of DNA. Many organic dyes—such as ethidium bromide (EB),[16] acridine orange (AO),[17] oxazide yellow homodimers,[18] Nile blue,[19] diphenylamine blue,[20] and neutral red[21]—have already proven to be sensitive probes of DNA. In this study, AO (Scheme 1a) was selected as a probe to investigate the interactions of xanthones with DNA by spectrometric methods.

In principle, there are three modes for reversible binding of molecules with double-helical DNA: (1) electrostatic attractions with the anionic

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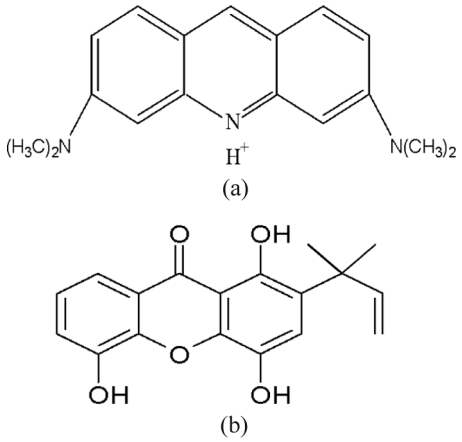
Address correspondence to Yunhua Wu, College of Life Science, South Central University for Nationalities, Wuhan 430074, P. R. China. E-mail: yunhuawu@yahoo.com.cn



SCHEME 1 **The structure of (a) acridine orange and (b) 12b-hydroxy-des-D-Garcigerin.**

sugar-phosphate backbone of DNA, (2) interactions with the DNA groove, (3) intercalation between base pairs via the DNA groove. Depending on structural features of both the molecule and DNA, many molecules show more than a single interaction mode with DNA.[22]

The natural xanthones from high plants have been obtained mainly from about 150 plants associated with four families—*Guttiferae, Gentianaceae, Moraceae*, and *Polygalaceae*[23]—having many pharmacological effects such as MAO inhibition, antitumor activity, cytotoxicity, antibacterial activity, antifungal activity, antiinflammatory properties, antioxidant activity, and tuberculoatatic activity.[24] Due to their strong bioactivities, some herbal medicines in *Polygalaceae* family containing xanthones have been used as antiinflammatory, antibacterial, and antirheumatism agents in clinical trials.[25] In the present work, 12b-hydroxy-des-D-Garcigerin (Scheme 1b) was selected as a probe to investigate the interactions of xanthones with DNA by spectrometric methods. Studies of the effect of ionic strength, the measurements of viscosity, melting temperatures, and the like were carried out to probe the binding mechanism.

MATERIALS AND METHODS

Apparatus

All fluorescence measurements were made with a Hitachi F-2500 spectrofluorimeter (Hitachi; Tokyo, Japan) equipped with a 1-cm quartz cell and a thermostat bath. A UV-757CRT visible ultraviolet spectrophotometer (Shanghai Precision & Scientific Instrument Co.; China) equipped with 1.0-cm quartz cells was used for scanning the UV spectrum. All pH measurements were made with a pHs-3 digital pH-meter (Shanghai Lei Ci Device Works; Shanghai, China) with a combined glass electrode. An electronic thermostat water bath (Tianjin Taisite Instrument Co.; Tianjin, China) was used for controlling the temperature. The viscosity determination was carried out using NDJ-79 viscosity meter (Yinhuan Flowmeter Co.; Zhejiang, China).

Reagents

All starting materials had analytical reagent grade, and double distilled water was used for all the measurements. Calf Thymus dsDNA (Sigma) was directly dissolved in water to prepare stock solutions and then stored at 4°C, and its concentration was determined to be 2.45×10^{-4} mol L^{-1} by absorption spectrometry, using the absorptivity $\varepsilon_{260} = 6600\,mol^{-1}\,cm^{-1}$. Purity of DNA was checked by monitoring the ratio of the absorbance at 260–280 nm. The solution gave a ratio of $A_{260}/A_{280} > 1.8$, indicating that DNA was sufficiently free from protein.[26] AO was purchased from Chemical Regents Co. (Shanghai, China). A stock solution of AO (5.00×10^{-3} mol L^{-1}) was prepared by dissolving an appropriate amount of AO in water and was stored in a cool and dark place. 12B-hydroxy-des-D-Garcigerin was isolated from *Garcinia xanthochymus*, and its spectroscopic data was in good agreement with literature values.[27] 1.22×10^{-3} Mol L^{-1} stock solution of GA was directly prepared in methanol. A tris-HCl buffer (pH 7.4) was used to control the pH of the reaction system. NaCl was used to adjust the ionic strength of the solution.

Procedures

UV-VIS Spectrometry

A 3.0-mL solution in 1.0-cm quartz cells, containing appropriate concentration of GA, was titrated by successive additions of a $2.45 \times 10^{-4}\,mol\,L^{-1}$ stock solution of DNA; and a 3.0-mL solution in 1.0 cm quartz cells, containing appropriate concentration of DNA or GA-DNA, was titrated by successive additions of a $5.00 \times 10^{-3}\,mol\,L^{-1}$ stock solution of AO. Titration was done manually by using microinjector. The aliquot of each injection was 10 μL to avoid the change of the volume. Appropriate blanks corresponding to the buffer were

used as the reference. The UV-VIS spectra and the absorption were measured after shaking for 5 min.

Fluorescence Spectrometry

A 3.0-mL solution in 1.0-cm quartz cells, containing appropriate concentration of AO-DNA, was titrated by successive additions of a 1.22×10^{-3} mol L^{-1} stock solution of GA. Titration was done manually by using microinjector. The aliquot of each injection was 10 μL to avoid the change of the volume. The widths of both the excitation slit and the emission slit were set to 5.0 nm with a nominal resolution of 0.5 nm. The excite wavelength was set at 514 nm. Appropriate blanks corresponding to the buffer were subtracted to correct background of fluorescence. After 5 min, the fluorescence spectra were measured.

Melting temperature of DNA, DNA-AO, or DNA-GA was determined by monitoring the maximum fluorescence of the system as a function of temperature ranging from 25 to 100°C at intervals of 5°C.

In the study of the binding of GA with DNA in the presence of AO, the fluorescence measurements were carried out in 3-ml pH-7.4 tris-HCl buffers by adding an appropriate amount of DNA and GA to get four series of solutions with various concentrations of GA and a constant concentration of DNA. Then each series of solutions was titrated by five successive additions of a stock solution of AO at 5.00×10^{-3} mol L^{-1}. Before measurements, the tubes were shaken up and placed into a thermostat water bath for 5 min. Then the assay solutions were transferred into a quartz cell, and fluorescence measurements were performed. At aforementioned fluorescence measurements, two groups with or without addition of NaCl were done as contrast to study the salt on the interaction of GA with DNA.

Viscosity Measurements

A series of solutions containing a constant amount of DNA and various concentrations of GA or AO were made, and the viscosity measurement was performed at 25°C.

RESULTS AND DISCUSSION

UV-VIS Absorption Spectra

Binding Properties of GA with DNA

UV-vis absorption spectra were obtained by titration of a GA solution at 1.62×10^{-5} mol L^{-1} with increasing concentration of DNA (Fig. 1). In the absence of DNA, the spectrum of the GA (Curve 2) was characterized by the peaks at 317 nm and 412 nm. With the addition of DNA, the intensity of both 317-nm and 412-nm peak absorbances increased slightly, and the maximum absorption peak was not changed (Curves 2–6). Generally, bathochromic and hypochromic effects are observed in the absorption spectra of small molecules if they intercalate with DNA.[28] The experimental results show that GA intercalates into the DNA bases and forms a GA-DNA complex.

Binding Properties of DNA in Presence of AO

Figure 2 shows the absorption spectra of DNA in presence of AO. It can seen that the absorption peaks of DNA are at 260 nm (Curve a), and the absorption peaks of AO are at 269 and 492 nm. There is also a small absorption peak at about 287 nm for AO (Curve b). With the addition of AO to the fixed DNA, the peak of DNA at 260 nm disappeared, and a new peak at 265 nm appeared. It indicated that a new complex between DNA and AO formed. Meanwhile, the peak at 492 nm of AO shifted to 495 nm (Curves c and d). The change of the absorbance of both AO and DNA confirms that AO intercalates into the base pairs in a helix of DNA and forms a DNA-GA complex. The π electrons of AO dye combine with the π electrons of DNA's bases, and the empty π^*

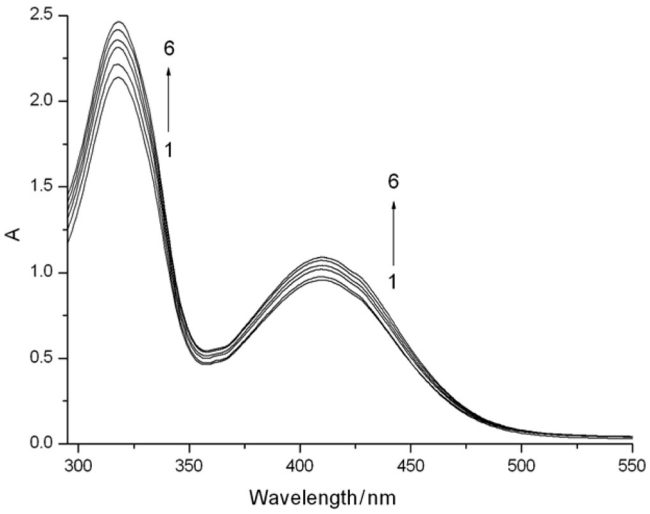


FIGURE 1 The absorption spectra of the DNA to GA in a tris-HCl buffer (pH = 7.4). $C_{GA} = 1.62 \times 10^{-5}$ mol/L; $C_{DNA} = 2.45 \times 10^{-4}$ mol/L (10 μL per scan), 1 ~ 6: 0 ~ 60 μL.

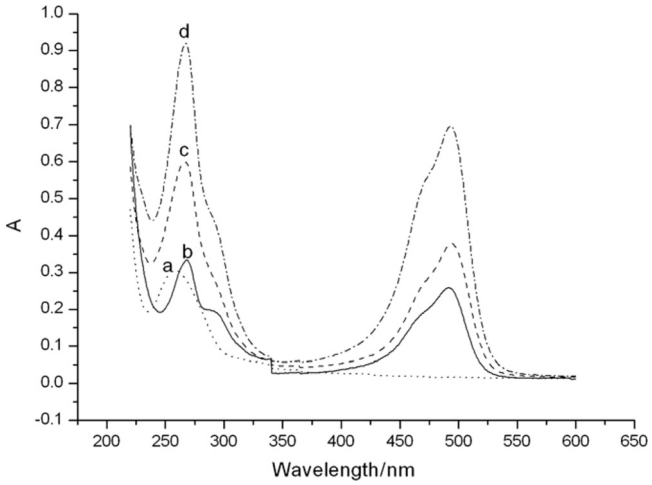


FIGURE 2 The absorption spectra of (a) DNA ($C_{DNA} = 6.00 \times 10^{-6}$ mol/L), (b) AO ($C_{AO} = 1.50 \times 10^{-6}$ mol/L), (c) AO-DNA ($C_{DNA} = 6.00 \times 10^{-6}$ mol/L; $C_{AO} = 2.10 \times 10^{-6}$ mol/L), and (d) AO-DNA ($C_{DNA} = 6.00 \times 10^{-6}$ mol/L; $C_{AO} = 4.20 \times 10^{-6}$ mol/L) in a tris-HCl buffer (pH = 7.4).

orbital of the AO dye couple with the π orbital of the bases. This facilitates a decrease in the energy of the π–π^* electron transition, which is reflected in the observed red shift.

Binding Properties of GA with DNA in Presence of AO

Figure 3 shows the absorption spectra of GA-DNA in presence of AO. It can seen that the absorption peaks of GA-DNA are at 264 nm (λ_{max} (DNA)), 317 nm (λ_{max} (GA)), and 412 nm (λ_{max} (GA)) (Curve b), and the absorption peaks of AO are at 269 nm, 287 nm, and 492 nm (Curve a). With increasing AO concentration, peak intensity gradually increases, and a slight red shift occurs from 264 nm (λ_{max} (DNA)) to 266 nm. Meanwhile, the peak at 492 nm of AO shifts to 495 nm (Curves c and d). The change of the absorption spectra of GA-DNA in presence of AO is similar to that of the absorption spectra of DNA in presence of AO, and the similarity indicated that GA intercalates into the DNA bases as AO.

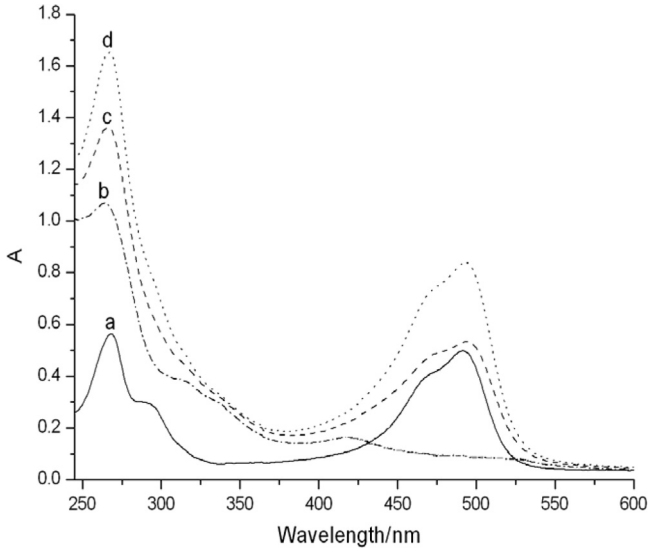


FIGURE 3 The absorption spectra of (a) AO ($C_{AO} = 2.10 \times 10^{-6}$ mol/L), (b) GA-DNA ($C_{GA} = 1.40 \times 10^{-5}$ mol/L, $C_{DNA} = 6.00 \times 10^{-6}$ mol/L), (c) GA-DNA-AO ($C_{GA} = 1.40 \times 10^{-5}$ mol/L, $C_{DNA} = 6.00 \times 10^{-6}$ mol/L, $C_{AO} = 2.10 \times 10^{-6}$ mol/L) and (d) GA-DNA-AO ($C_{GA} = 1.40 \times 10^{-5}$ mol/L, $C_{DNA} = 6.00 \times 10^{-6}$ mol/L, $C_{AO} = 4.20 \times 10^{-6}$ mol/L) in a tris-HCl buffer (pH = 7.4).

Fluorescence Spectrometry

Fluorescence Quenching

When GA is added into the solution of DNA-AO complex, the fluorescence intensity of DNA-AO complex decreases with the increasing concentration of GA (Fig. 4). Quenching can occur by different mechanisms, which usually are classified as dynamic quenching and static quenching. In order to confirm this point, the procedure was assumed to be dynamic quenching. The quenching equation is presented by

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{SV}[Q], \qquad (1)$$

where F and F_0 are the fluorescence intensities with and without quencher, K_q is the quenching rate constant of the biomolecule, K_{sv} is the Stern–Volmer

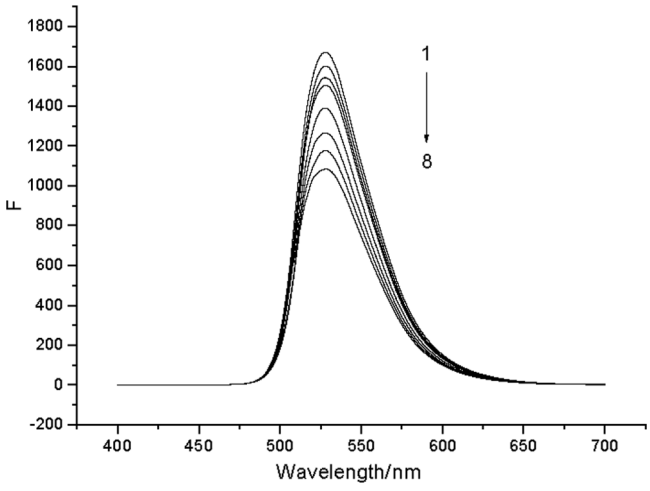


FIGURE 4 Effect of GA on fluorescence spectra of DNA-AO. C_{GA} (mol L^{-1}):(1)0; (2) 4×10^{-5}; (3) 8×10^{-5}; (4) 1.2×10^{-4}; (5) 1.6×10^{-4}; (6) 2×10^{-4}; (7) 2.4×10^{-4}; (8) 2.8×10^{-4}. ($C_{DNA} = 5.00 \times 10^{-6}$ mol/L; $C_{AO} = 2.50 \times 10^{-5}$ mol/L).

quenching constant, τ_0 is the average lifetime of the biomolecule without quencher, and [Q] is the concentration of quencher. Obviously,

$$K_q = \frac{K_{SV}}{\tau_0}. \quad (2)$$

The possible quenching mechanism can be interpreted by the fluorescence-quenching spectra of DNA-AO and the $F_0/F \sim C$ (Stern–Volmer) curves of DNA-AO with GA at 303 K as shown in Fig. 5. The result of linear regressions of Fig. 5 is $F_0/F = 0.93619 + 1952$ [GA]. Because the fluorescence lifetime of the biopolymer τ_0 is $10^{-8}\,s^{-1}$,[29] the corresponding Stern–Volmer quenching constant, K_q, was obtained as 1.952×10^{11} at 303 K. However, the maximum scatter collision quenching constant Kq of various quenchers with the biopolymer is $2 \times 10^{10}\,L\,mol^{-1}\,s^{-1}$.[30] Obviously, the rate constant of DNA-AO quenching procedure initiated by GA is greater than Kq of the scatter procedure. These results indicate that the probable quenching mechanism of fluorescence of DNA-AO by GA is not a dynamic quenching procedure but a static quenching procedure, and that confirms again that GA interacts with DNA and forms a GA-DNA complex.

Scatchard's Procedure

The binding mode between small molecules with DNA can be determined using Scatchard's procedure.[31] This method is based on the general equation

$$\frac{r}{c} = Kn - rc, \quad (3)$$

where r is in moles of AO bound per mole of DNA, c is the molar concentration of free AO, n is binding site multiplicity per class of binding sites, and K is the association-binding constant of AO with DNA. If GA interacts with DNA by intercalation mode, the value of n is constant and that of K changes in Scatchard plot. If GA interacts with DNA by intercalation mode or electrostatic interactions, both values of n and K change in Scatchard plot.[32] In order to investigate the effects of ionic strength on the interaction between GA and DNA, two groups of buffers with or without addition of NaCl were used. For NaCl, which is not an anionic quencher of DNA,[33] its influence on GA-AO-DNA comes only from the ionic strength. Collection of the fluorescence value of a fixed concentration of GA-DNA after interaction with different concentrations of AO r can be calculated where $r = \Delta F/F_0$, F_0 is the fluorescence value at a fixed concentration of GA-DNA, F is the fluorescence value of a fixed concentration of GA-DNA after interaction with different concentrations of AO, and ΔF is the difference between F_0 and F. As shown in Fig. 6, we prepared a calibration curve that related the value of r/c to the value of r. From the Scatchard plot, values of K and n were obtained. The results are shown in Table 1. From Fig. 6a, it can be seen that both values of n and K change with the different concentrations of AO. The result indicates that GA interacts with DNA by intercalation or electrostatic interactions. Comparing Fig. 6b with Fig. 6a, the value of n became smaller with added NaCl, indicating that GA interacts with DNA by some electrostatic interactions.

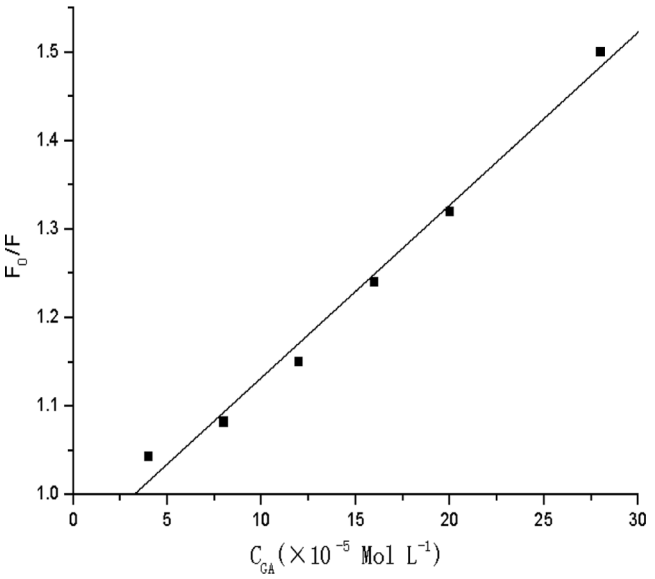


FIGURE 5 **The Stern–Volmer quenching curve of GA to AO-DNA (pH = 7.2, 25°C). Others are the same as those in Figure 4.**

Melting Studies

Heat and alkali can destroy the double helix structure of DNA and change it into a single helix at the melting temperature (T_m). Interaction of small molecules with the dsDNA can influence T_m. Intercalation binding can stabilize the double helix structure, and Tm increases by about 5–8°C, but the nonintercalation binding causes no obvious increase in T_m.[34] The values of T_m for DNA, AO-DNA, and GA-AO-DNA were determined, by monitoring the

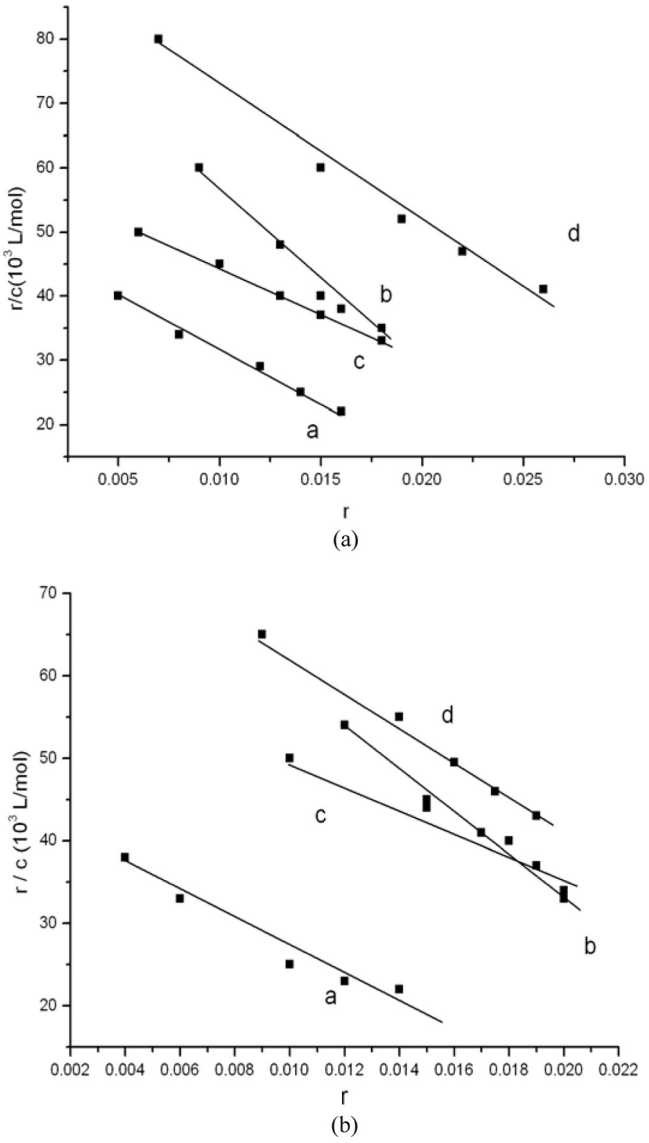


FIGURE 6 Influence of GA on the Scatchard plot of DNA-AO system (a) with NaCl and (b) without NaCl. $C_{GN} = 6.75 \times 10^{-5}$ mol/L; $C_{DNA} = 5.00 \times 10^{-6}$ mol/L; $C_{AO} = 4.00 \times 10^{-4}$ mol/L (10 μL per scan); $R_t = C_{GN}/C_{DNA}$; a, $R_t = 0.00$; b. $R_t = 0.20$; c. $R_t = 0.40$; d. $R_t = 0.60$.

maximum fluorescence of the systems as a function of temperature that ranged from 25 to 100°C. For each monitored transition, the T_m of the assay solution was determined at the transition midpoint of the melting curve. The melting curves are shown in Fig. 7. The value of T_m for DNA is 75°C under the experimental conditions. The observed melting temperatures of DNA-AO in the absence of and in presence of GA are 85°C and 90°C, respectively. The changes in T_m of DNA-AO after the addition of GA reveal that the binding modes of GA with DNA are intercalated.

TABLE 1 Binding Parameters for the Binding of GA to AO-DNA at PH 7.4 Measured by Scatchard Procedure

Curve	C_{GN}/C_{DNA}	NaCl (%)	Scatchard equation	K ($\times 10^5$ L/mol)	n
a	0	5.00	$1.49 \times 10^4 - 1.65 \times 10^5$r	1.65	0.09
		0	$1.92 \times 10^4 - 1.60 \times 10^5$r	1.60	0.12
b	0.20	5.00	$5.75 \times 10^4 - 2.50 \times 10^5$r	2.50	0.23
		0	$7.86 \times 10^4 - 2.91 \times 10^5$r	2.91	0.27
c	0.40	5.00	$5.13 \times 10^4 - 1.51 \times 10^5$r	1.51	0.34
		0	$4.18 \times 10^4 - 1.44 \times 10^5$r	1.44	0.29
d	0.60	5.00	$8.69 \times 10^4 - 2.23 \times 10^5$r	2.23	0.39
		0	$7.45 \times 10^4 - 2.07 \times 10^5$r	2.07	0.36

Note. CGN = 6.75×10^{-5} mol/L; CDNA = 5.00×10^{-6} mol/L; CAO = 4.00×10^{-4} mol/L (10 μL per scan); Rt = CGN/CDNA; a, Rt = 0.00; b. Rt = 0.20; c. Rt = 0.40; d. Rt = 0.60.

Viscosity Measurements

The binding mode of GA with DNA was further investigated by viscosity measurements. A classical intercalation model results in lengthening the DNA helix, as base pairs are separated to accommodate the binding ligand, leading to the increase of DNA viscosity. However, a partial and/or nonclassical intercalation of ligand may bend (or kink) DNA

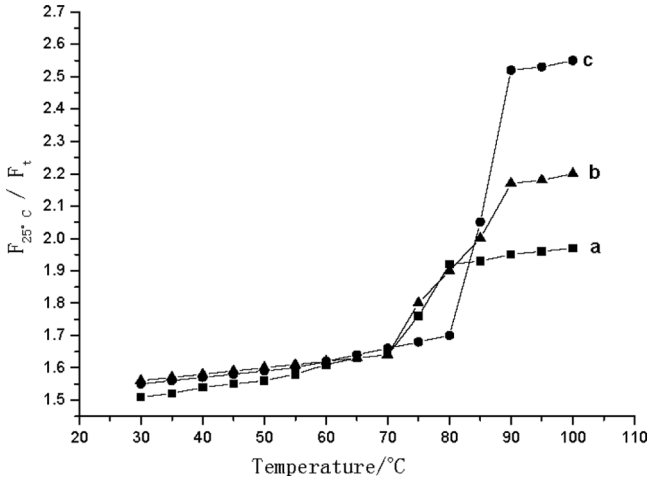


FIGURE 7 Melting curves of DNA (a), DNA-AO (b), and DNA-AO-GA (c). $C_{GA} = 1.40 \times 10^{-5}$ mol/L, $C_{DNA} = 6.00 \times 10^{-6}$ mol/L, $C_{AO} = 2.10 \times 10^{-6}$ mol/L.

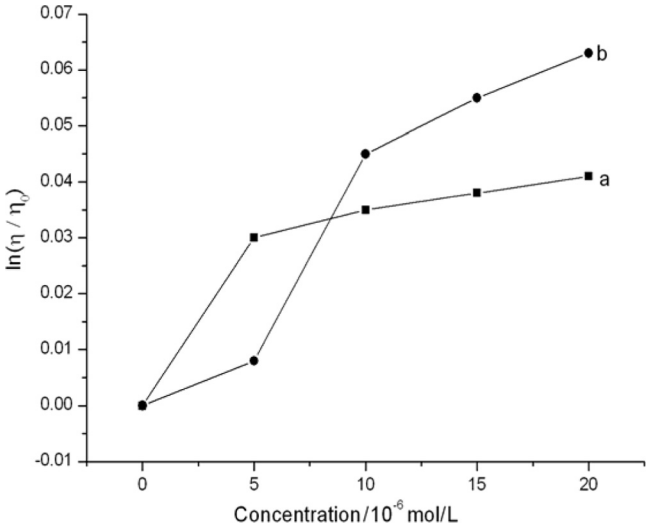


FIGURE 8 **Effect of increasing amounts of AO (a) and GA (b) on the relative viscosities of DNA at 25°C. $C_{DNA} = 2.45 \times 10^{-4}$ mol/L.**

helix, resulting in the decrease of its effective length and, concomitantly, its viscosity.[35] The effects of GA and AO on the viscosity of DNA are shown in Fig. 8. For GA or AO, as its concentration increased, the viscosity of DNA increased steadily, and their trend lines were similar. The experimental results suggested that GA and AO could bind DNA in electrostatic and intercalation modes.

CONCLUSIONS

The binding of 12b-hydroxy-des-D-Garcigerin to Calf Thymus dsDNA in aqueous solution was studied by fluorescence and UV-Vis spectroscopic methods. By using AO as a DNA probe, the fluorescence quenching was observed in the system of GA-DNA-AO, and the results show that GA has a strong ability to quench the DNA-AO fluorescence mainly through static quenching. The binding constants of GA with DNA in the presence of AO were calculated. By our taking account of the absorption spectra, ionic strength effects, the melting temperature determinations, and the viscosity measurements, the results revealed that intercalation and electrostatic binding are likely the two major modes for interaction between GA and DNA. The binding mode and binding constant of GA and DNA will provide necessary information on the mechanism of antitumor drugs' binding with DNA, and they will benefit the designing of new drugs.

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