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A Negative Deviation from Stern–Volmer Equation in Fluorescence Quenching

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A negative deviation from the normal Stern–Volmer equation shown in the fluorescence quenching of doxorubicin by adenosine 5′ monophosphate is interpreted in terms of doxorubicin exists in two different conformers in the ground state. An estimate of the Stern–Volmer constant for the excited-state quenching is about 218 M^{-1}. The fluorescence decay of free doxorubicin is a bi-exponential in polar protic and polar aprotic solvents. In the presence of adenosine 5′ monophosphate, doxorubicin shows a tri-exponential decay in water.

KEY WORDS: Fluorescence quenching; doxorubicin; adenosine 5′ monophosphate; solvent effect.

INTRODUCTION

The exponential decay of the fluorophore in the presence of the quencher obeys the well-known Stern–Volmer equation. However, many photochemical reactions have shown significant deviation from the Stern–Volmer equation [1]. A positive deviation is mainly due to the formation of a non-fluorescent complex between the fluorophore and quencher in the ground state. The two obvious phenomena that cause the negative deviation are (i) the presence of two fluorophores with different accessibility to the quencher and (ii) the occurrence of the reverse reaction in the photochemical system [2].

Doxorubicin, shown in Fig. 1, is a substituted derivative of a potent anti-leukemic drug daunorubicin. A considerable attention has been paid to the biochemical and spectroscopic properties of daunorubicin and doxorubicin during the last few years in order to correlate with their biological and pharmacological activities [3–5]. Quinizarin (1,4-dihydroxy anthraquinone) is the basic fluorophore of these two drugs and a great deal of spectroscopic studies has been extensively done on it in the past [6–8]. It is also widely known that the fluorescence intensity of many anthraquinones is quenched upon interaction with DNA through a charge transfer mechanism. However, the photophysical processes dealing with the basic fluorescence of these two drugs have been rarely found in the literature. The present paper is devoted to the study of steady-state fluorescence quenching of doxorubicin by adenosine 5′ monophosphate (AMP). AMP is a nucleotide that is formed from a phosphoric acid molecule, a five carbon sugar and a nitrogen-containing organic base. The fluorescence data suggests the possibility of doxorubicin exists in two conformers in the ground state.

Materials and methods

Doxorubicin and AMP were obtained commercially from Sigma Chemical Company and used as received. Methanol, isopropyl alcohol, chloroform, acetonitrile and acetone were obtained from Merck. Deionized water from VWR Scientific was used for sample preparations. The concentration of doxorubicin in all solutions was kept 5 μM to avoid the self-association.

Absorption spectra of doxorubicin with or without AMP were recorded with a Cary 500 scan UV-VIS-NIR spectrophotometer from Varian. Fluorescence emission and excitation spectra were measured with a FluoroMax-2 spectrofluorometer. Recorded fluorescence spectra were

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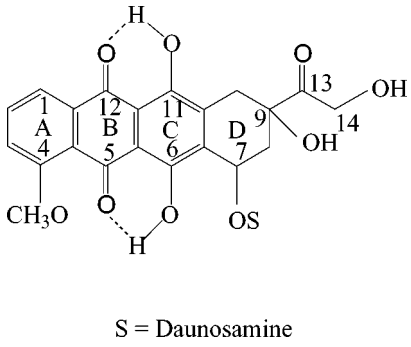


Fig. 1. A structure of doxorubicin.

corrected for the response characteristics from the spectrofluorometer components.

Time-resolved fluorescence spectra were measured by using a FluoTime 200 Time-resolved Spectrometer. A pulsed picosecond diode laser with the repetition frequency of 40 MHz between pulses was used as an excitation source. A minimum pulse width was about 54 ps and the instrument respond function was about 35 ps. And average peak power was about 1 mW. The excitation wavelength was set at 400 nm. The fluorescence signals were monitored at 590 nm and collected by a Time-Correlated Single Photon Counting. Fluorescence lifetimes were extracted from the fluorescence decay curve by use of Fluofit deconvolution software program.

RESULTS AND DISCUSSION

Absorption

An absorption spectrum of the aqueous solution of doxorubicin displays absorption maxima in the visible region at about 473 and 494 nm respectively (Fig. 2). The addition of AMP into the aqueous solution of doxorubicin caused no changes in the absorption spectrum. Therefore, doxorubicin does not form a complex with AMP in the ground state.

Steady-State Fluorescence

A fluorescence spectrum of doxorubicin obtained by a 400 nm excitation shows an emission maximum at about 590 nm in water. The excitation spectra of doxorubicin, measured at the fixed emission wavelengths of 590 and 550 nm, reveal the vibration structures with the excitation maxima at about 474 and 496 nm. Both absorption and emission spectra of doxorubicin in the aqueous solution agree very well with those previously reported in the literature [3,4].

As depicted in Fig. 3, AMP quenches the fluorescence intensity of doxorubicin. When I_0/I at 590 nm is

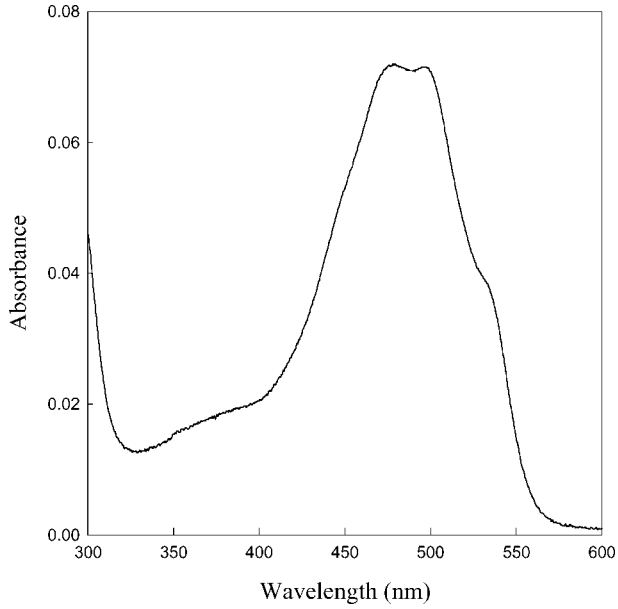


Fig. 2. An absorption spectrum of doxorubicin in water.

plotted against the concentration of AMP, the plot follows the Stern–Volmer linearity at lower concentrations of AMP while a significant downward deviation from the linearity is displayed at higher concentrations of AMP (Fig. 4). This could either be due to the presence of two fluorophores with different accessibility to AMP or the occurrence of a reverse reaction in the photochemical process. As no new fluorescence emission band was emerged upon the addition of AMP into the aqueous solution of doxorubicin, the negative deviation in Fig. 4 would most likely to be the presence of doxorubicin in two different conformers. As the plot in Fig. 4 can be put into a linear form by [9],

$$\frac{I_0}{I_0 - I} = \frac{1}{f} + \frac{1}{f\,K_{sv}[\mathrm{AMP}]} \qquad (1)$$

and the fraction of accessible fluorophore (f) is found to be unity (Fig. 5), it is concluded that only one conformer is accessible to AMP for which an estimate of Stern–Volmer constant (K_{sv}) is 218 M^{-1} implying that 5 mM of AMP concentration is required to quench one half of the fluorescence intensity of doxorubicin.

In full agreement with the absorption, the negative deviation in the fluorescence quenching of doxorubicin clearly rules out the formation of a non-fluorescent complex between doxorubicin and AMP in the ground state. Even if the static quenching arises due to the presence of AMP molecules in the vicinity of doxorubicin at the moment of excitation, it is expected to be very weak because the ground-state association constant (K_s) as evaluated

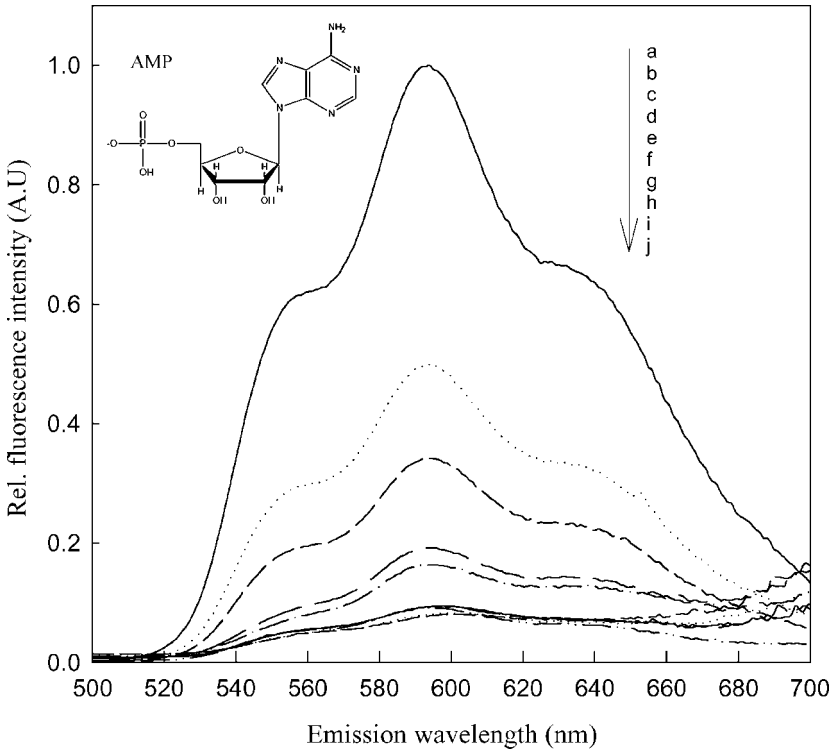


Fig. 3. Normalized emission spectra of doxorubicin in water with (a) no AMP (b) 5 mM (c) 10 mM (d) 20 mM (e) 30 mM (f) 40 mM (g) 60 mM (h) 80 mM (i) 100 mM (j) 120 mM of AMP.

from the fit of Fig. 4 is about 4.9 M^{-1}. It suggests that AMP concentration of about 0.20 M is needed to quench one half of the fluorescence intensity by the static process. This ensures that the static quenching does not take place within the used concentrations of AMP in this experiment.

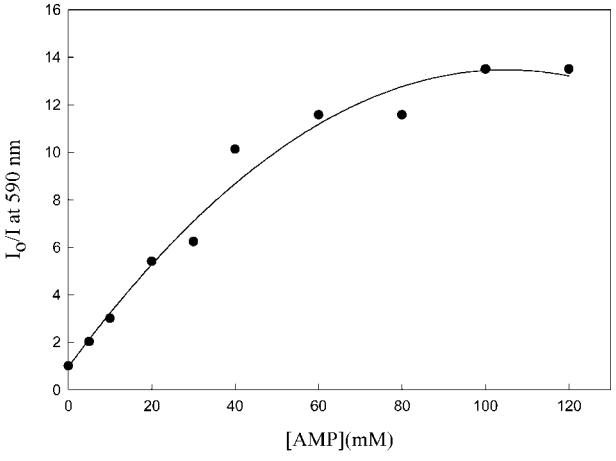


Fig. 4. A plot of I_0/I versus [AMP].

Time-Resolved Fluorescence Decays

Time-resolved fluorescence decays of doxorubicin were measured in the polar protic solvents (i.e. water,

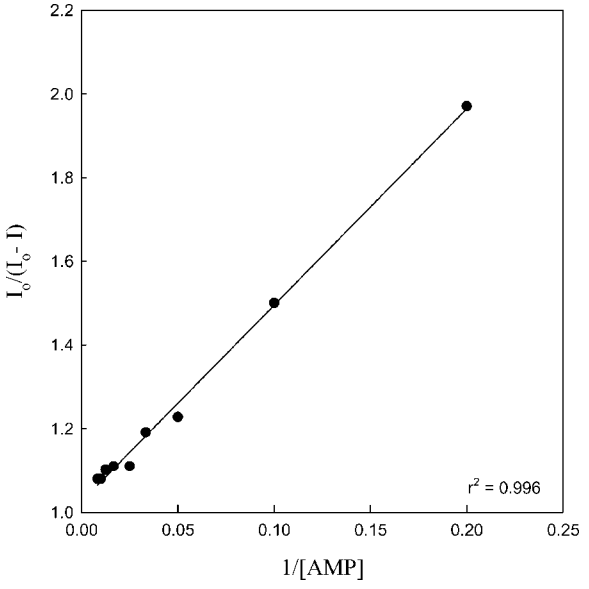


Fig. 5. A linear plot of $I_0/(I_0 - I)$ versus 1/[AMP].

Table I. Fluorescence Decays of Doxorubicin in Some Solvents

Solvent	A_1 (%)	τ_1 (ns)	A_2 (%)	τ_2 (ns)	χ^2
Water	23	0.63	77	1.1	1.1
Methanol	20	0.75	80	1.4	1.1
Isopropyl alcohol	10	0.52	90	1.5	1.1
Chloroform	9	0.50	91	1.5	1.1
Acetonitrile	16	0.60	84	1.5	1.1
Acetone	12	0.67	88	1.5	1.1

Table II. Fluorescence Decays of Doxorubicin at Some Temperatures

Temp (°C)	A_1 (%)	τ_1 (ns)	A_2 (%)	τ_2 (ns)
5	17	0.51	83	1.1
15	26	0.62	74	1.1
23	23	0.62	77	1.1
35	24	0.60	76	1.0
55	26	0.64	74	1.0

methanol, isopropyl alcohol and chloroform) and polar aprotic solvents (i.e. acetonitrile and acetone) at room temperature. As reported in Table I, the fluorescence decays in all solvents have shown bi-exponentials with the time constant τ_1 scattered around 600 ps with no appreciable change over the polarity of the used solvents while the other time constant τ_2 decreases with increasing polarity of the solvents (Fig. 6). In addition, the τ_1 and τ_2 in D_2O solvent are about 500 ps and 4.3 ns showing the change only in the τ_2 by a factor of four times longer in D_2O than in water.

The most stable structure of chromophore in doxorubicin is expected to be the one that forms in plane quasi-aromatic six-membered ring via intra-molecular hydrogen bondings between hydrogen atoms of hydroxyl groups that interact with the quinonoid oxygens [8]. In this context, it is worth to mention that the first absorption band of doxorubicin, appeared at 473 nm, was red-shifted by 134 cm^{-1} in chloroform compared to that in water. Therefore, the break up of at least one intramolecular hydrogen bond between the quinonoid oxygen and hydroxyl hydrogen in the ground state is expected in water, which is a better hydrogen donor than chloroform. Tables II and III

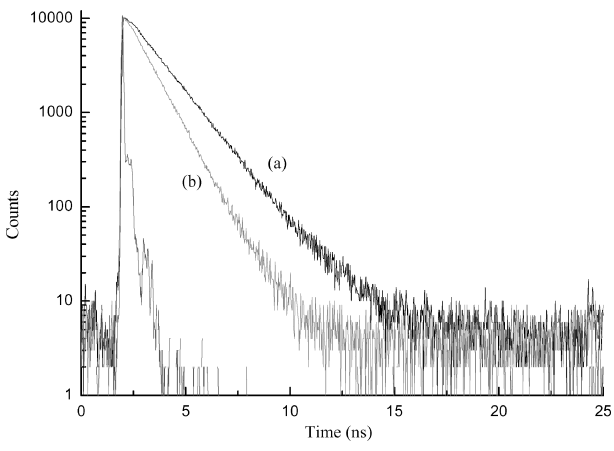


Fig. 6. Fluorescence decays of doxorubicin in (a) water and (b) chloroform at room temperature. ($\lambda_{exc} = 400$ nm, $\lambda_{em} = 590$ nm).

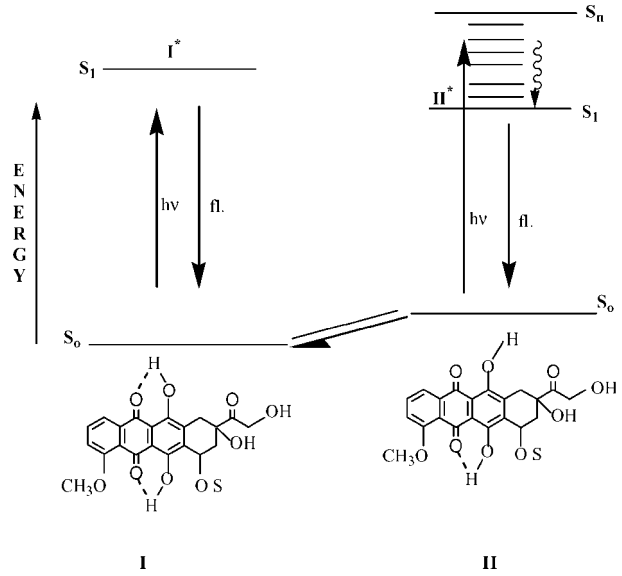
also show the independent nature of the fluorescence decays of doxorubicin over the temperature and acidity of the medium.

Quinizarin in its most stable configuration exhibits two intramolecular hydrogen bonds. Photochemical hole burning of quinizarin in a variety of host matrices at low temperatures with narrow band laser radiation has shown the proton rearrangement in quinizarin during which an intramolecular hydrogen bond between quinonoid oxygen and hydroxyl hydrogen is broken and a new intermolecular hydrogen bond is subsequently formed with a nearby host matrix [8]. Since quinizarin is a basic fluorophore of doxorubicin, it is likely that doxororubicin may exist in two different conformers in the ground state differing in their hydrogen bonding in a manner analogous to quinizarin.

It is, therefore, proposed that doxorubicin exists initially as two different conformers in the ground state where one conformer (I) may have two intramolecular hydrogen bonds while the other conformer (II) may be allowed to form at least one intermolecular hydrogen bond with a nearby water molecule from the medium as presented in Scheme 1. Subsequently, τ_1 and τ_2 may be taken as the lifetimes of the conformers II and I of doxorubicin. The pre-exponentials factors of τ_1 and τ_2 are also independent of the emission wavelengths across the steady-state emission spectrum of doxorubicin. Thus, it is believed that no dynamics establishes between the two conformers in the excited state. As the solvent is changed from chloroform to water, the competition of solvent with the hydroxyl hydrogen of the conformer I for forming a hydrogen bond with the quinonoid oxygen become apparent and may possibly lead to the rupture of at least one intramolecular hydrogen bond causing the τ_2 shorter in the

Table III. Fluorescence Decays of Doxorubicin at Selected pH

pH	A_1 (%)	τ_1 (ns)	A_2 (%)	τ_2 (ns)
1	30	0.63	70	1.1
3	15	0.41	85	1.0
5	20	0.60	80	1.0
7	26	0.62	74	1.1



Scheme 1

water. Since the intramolecular bond between C-11 hydroxyl and C-12 oxygen is weaker than the one between the C-6 hydroxyl and C-5 oxygen [10], the former may be the position where the solvent–solute interaction is taking place in the water.

In the absence of AMP, the fluorescence decay of doxorubicin in the water is a bi-exponential with $\tau_1 = 630$ ps (23%) and $\tau_2 = 1.1$ ns (77%). As discussed earlier, AMP quenches the fluorescence intensity of doxorubicin and the extent of quenching would primarily depend on the nature of AMP and the geometry of chromophore in doxorubicin. As summarized in Table IV, the fluorescence decay of doxorubicin in the presence of AMP shows a tri-exponential with the time constants τ_1', τ_2' and τ_3' whose values, on the average, are about 700 ps (17%), 2.5 ns (3%) and 70 ps (80%). τ_3' is the shortest time component and τ_2' is the longest time component. Presumably, τ_1' may be attributed to the decay component of the conformer II unbound to AMP since A_1' falls within the range of A_1 of the conformer II. AMP consists of a nitrogen-containing base and a back-bone with sugar-phosphate ester. The shortest time component τ_3' may be attributed to the decay component that experiences the most effective quenching through an electron transfer from the base of AMP to conformer I. As the concentration of AMP increases in the water, the competition of solvent with the hydroxyl hydrogen of the conformer I for forming a hydrogen bond with the quinonoid oxygen become less apparent leaving the conformer I with the intramolecular hydrogen bond that renders a better stability. Therefore, the longest time component τ_2' may be assigned to the decay component of the conformer I unbound to AMP. However, as can be seen from Table IV, only 2 to 4% of the conformer I stays unbound to AMP in water.

CONCLUSIONS

The fluorescence quenching of doxorubicin by AMP has been studied by the combination of steady-state fluorescence and time-correlated single-photon counting techniques. The quenching shows a negative deviation from the normal Stern–Volmer relationship indicating the existence of doxorubicin in two ground-state conformers in the aqueous solution. The Stern–Volmer constant for the excited-state quenching of doxorubicin by AMP is 218 M^{-1}. The fluorescence lifetime measurements in the presence of AMP indicate the occurrence of effective quenching through a charge transfer when doxorubicin bound to AMP.

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Table IV. Fluorescence Decays of Doxorubicin in the Presence of AMP in Water

Doxorubicin (M)	AMP (mM)	A_1' (%)	A_2' (%)	A_3' (%)	τ_1' (ns)	τ_2' (ns)	τ_3' (ns)	χ^2
5.0×10^{-6}	5	15	28	57	0.62	1.1	0.030	1.2
	10	19	17	64	0.66	1.2	0.044	1.3
	20	23	3	74	0.84	2.1	0.071	1.3
	30	18	4	78	0.82	2.7	0.070	1.4
	40	15	4	81	0.72	2.5	0.070	1.3
	60	12	2	85	0.72	2.1	0.068	1.7
	80	13	2	86	0.72	2.1	0.068	1.7
	100	10	2	88	0.48	2.0	0.078	1.3
	120	10	2	88	0.45	2.1	0.067	1.6

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REFERENCES

1. J. R. Lakowicz (1999). *Principles of Fluorescence Spectroscopy*, 2nd ed., Kluwer Academic/Plenum Publishers, New York.
2. T. Htun (2003). Excited-state proton transfer in nonaqueous solvent, *J. Fluoresc.* **13**(4), 323–329.
3. E. J. Gabby, D. Grier, R. E. Fingerle, R. Reimer, R. Levy, S. W. Pearce, and W. D. Wilson (1976). Interaction specificity of the anthracyclines with deoxyribonucleic acid. *Biochemistry* **15**, 2062–2070.
4. A. Andreoni, A. Colasanti, and G. Roberti (1992). Excited singlet state properties of anthracenedione photosensitizers. *J. Photochem. Photobiol., B: Biol.* **14**, 319–328.
5. V. Malatesta and A. Andreoni (1988). Dynamics of anthracyclines/DNA interaction: A laser time-resolved fluorescence study. *Photochem. Photobiol.* **48**, 409–415.
6. R. N. Capps and M. Vala (1981). Luminescence studies of quinizarin and daunorubicin. *Photochem. Photobiol*. **33**, 673–682.
7. J. D. Petke, P. Butler, and G. M. Maggiora (1985). Ab initio quantum-mechanical characterization of the electronic states of anthraquinone, quinizarin, and 1,4-diamino anthraquinone. *Int. J. Quant. Chem.* **27**, 71–87.
8. F. Drissier, F. Graf, and D. Haarer (1980). Light induced proton transfer in dihydroxyanthraquinone as studied by photochemical hole burning, *J. Chem. Phys.* **72**, 4996–5001.
9. S. E. Webber (1997). The role of time-dependent measurements in elucidating static versus dynamic quenching processes. *Photochem. Photobiol.* **65**(1), 33–38.
10. V. Malatesta, G. Ranghino, and F. Morazzoni (1990). Aminoanthracyclines: Physicochemical properties as predicted by ab-initio molecular orbital calculations. *J. Mol. Struct. (Theochem.)* **205**, 169–175.