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ORIGINAL PAPER

Wavelength Dependant Quenching of 2,5-Diphenyloxazole Fluorescence by Nucleotides

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Abstract The quenching of 2,5-diphenyloxazole (PPO) fluorescence by nucleotides has been investigated by electronic absorption and steady state fluorescence spectra. Five purine nucleotides AMP, ADP, ATP, GMP and dGMP, one pyrimidine nucleotide UMP and one dinucleotide NAD have been employed in the present study. Electronic absorption studies indicate that there is no ground state complexation between the nucleotides and PPO. The quenching of PPO fluorescence was investigated at two different wavelengths. When excited at 304 nm, the λ_{max} of PPO, the fluorescence spectra of PPO is quenched following Stern–Volmer kinetics. The quenching ability of nucleotides are in the order NAD>AMP>ADP>GMP> dGMP>UMP. The K_{SV} and k_q values obtained indicate that AMP is a better quencher of PPO fluorescence than GMP, which is contrary to commonly observed pattern. The quenching is found to be dynamic in nature. However, when excited at 260 nm, the absorption maximum of the nucleotides, the fluorescence intensity of PPO is reduced with increase in the concentration of the nucleotide. This is attributed to the primary inner filter effect arising due to the absorption of the incident radiation by the nucleotides. Thus the inner filter effect phenomenon can be employed to assay the non-fluorescent molecules by fluorimetry.

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Introduction

2,5-Diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)benzene (POPOP) are commonly used in liquid scintillation cocktails as primary and secondary fluors. PPO is used in fluorography techniques for the detection of tritium [1], tritiated glycopeptides [2], ^{3}H labeled thymidine, DNA [3], ^{35}S labeled proteins [4], for measuring methane and carbon dioxide in the gaseous phase present in the radioactive form [5], in scintillation proximity assays for the determination of protein function [6] and in elucidating potassium signaling [7]. The genotoxic and radioprotective properties of 2,5-diphenyloxazole and its derivatives on mice DNA structure in vivo have been studied [8].

The concentration and temperature dependent fluorescence spectra of PPO were investigated in benzene. The emission maximum shifted to longer wavelengths on increasing the concentration of PPO, while an increase in the temperature favored the emission from the monomer and a decrease in the temperature favored the emission from the PPO excimer [9]. The mechanism of quenching of PPO fluorescence by exogenous compounds has been thoroughly investigated [10, 11]. The interactions between ground and excited states of PPO and CCl_4 [12] were investigated employing cyclohexane and p-xylene as solvents. The quenching of PPO fluorescence by metal ions was reported by Hariharan et al. [13]. Photoluminescence and radioluminescence of PPO in 1,4-dioxane and water were reported [14]. The quenching of PPO fluorescence by 9,10-dibromoanthracene in glycerol and cyclo-

hexane was investigated and compared to the quenching of fluorescein by rhodamine B. Based on the shell model of a luminescent center and on the Forster theory of intermolecular energy transfer, an expression for the decrease in sensitizer quantum yield with increasing acceptor concentration has been derived [15].

Nucleotides and nucleosides are reported to be efficient quenchers of fluorescence. The interaction of nucleotides and DNA with fluorophores and chromophores has been widely studied [16–22]. In contrast to the efficient absorption of light, nucleotides, nucleosides and their bases exhibit poor fluorescence quantum yields (~10^{-5}) due to efficient non-radiative deactivation processes, thus making fluorescence, a less reliable technique for their detection [23]. The nucleotide specific quenching of fluorescent dyes has been investigated by electrochemical and spectroscopic methods [18]. The selective quenching of 2,3-diazabicyclo [2.2.2]oct-2-ene by nucleotides was studied and it was found that guanosine has the highest quenching efficiency [24]. Fluorescence quenching by electron transfer between various fluorescent dyes and nucleobases was thoroughly evaluated by Torimura et al. [25]. The quenching of benzo [a]pyrene tetraol metabolite model compound by 2′-deoxynucleosides was also reported [26]. Fluorescence quenching of ε-AMP [27], coumarin [28], tetracycline and oxytetracycline [23, 29, 30] and acridines [31–33] by purines and pyrimidines has been investigated. It was observed that quenching is strongly enhanced by polarity of the solvents.

Owing to the efficient fluorescence of PPO with a quantum yield of 0.85 and quenching ability of the biologically important nucleotides, it is considered worthwhile to investigate the interactions in the ground as well as in the excited states between PPO and a few nucleotides. In the present work, the effect of nucleotides namely AMP, ADP, ATP, GMP, dGMP, UMP and NAD on the steady state fluorescence of PPO in methanol has been investigated. Further the nucleotides displaying strong absorption at 260 nm causing a filtering effect on the incident irradiation, a method for their assay based on the additive characteristics of the spectral data has been presented. The effect of solvent on the emission intensity of [ATP–PPO] and [AMP–PPO] systems in methanol-dioxane, DCM, acetonitrile were studied.

Experimental

Scintillation grade PPO was obtained from Sigma chemicals and was recrystallized from 9:1, petroleum ether: methanol mixture. ATP, ADP, AMP, GMP, dGMP, UMP and NAD were obtained from Sigma, USA and were used without further purification. All nucleotides were stored at −80°C. The solvents methanol, DCM, acetonitrile, dioxane and chloroform were of spectroscopic grade and were used without further purification.

Absorption spectra were recorded on a Helios α spectrophotometer, Unicam, UK. Fluorescence measurements were carried out using a Hitachi F-4500 spectrofluorimeter. The entrance and exit slit widths were maintained at 5 nm in all the experiments. The stock solutions of nucleotides were freshly prepared in methanol on the day of the experiment and kept at 4°C, while the PPO solutions were freshly prepared on the day of the experiment in the respective solvents.

The fluorescence studies were carried out by measuring the emission intensity of PPO alone and in presence of nucleotides in methanol. The concentration of PPO was kept constant and that of the nucleotide was increased. The steady state fluorescence intensity of PPO at 374 nm was monitored with the excitation wavelength set at 260 and 304 nm for the calculation of Stern–Volmer constants. Ten to twelve sets were prepared and the fluorescence spectra recorded at each concentration.

Results and discussion

Absorption studies of PPO and nucleotides

The absorption spectra of PPO, AMP and PPO–AMP system in 40% methanol are shown in Fig. 1. From the figure it can be observed that PPO shows an absorption band at 304 nm, AMP at 261 nm while that of AMP–PPO system exhibits absorption peaks at both the wavelengths. It

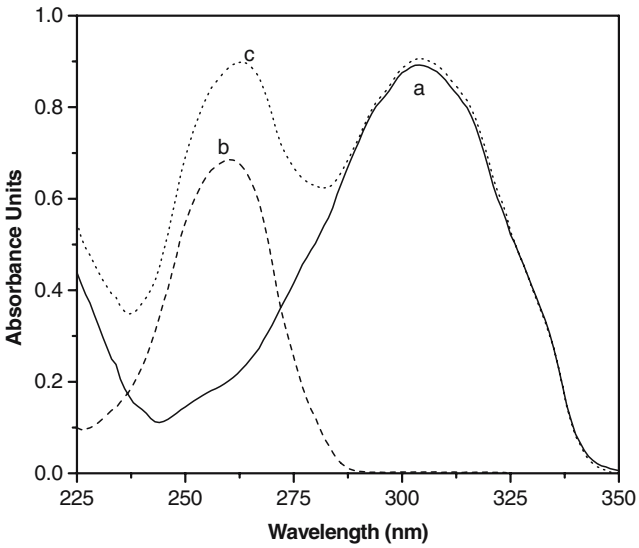

Fig. 1 Absorption spectra of (*a*) PPO (8.0×10^{-5} mol dm^{-3}), (*b*) AMP (8.0×10^{-5} mol dm^{-3}) and (*c*) PPO–AMP (8.0×10^{-5} mol dm^{-3} each) in 40% methanol

is observed that the nucleotides investigated do not show any effect on the absorption spectrum of PPO. PPO does not show any new absorption band or shift in the absorption maximum, indicating that there is no ground state interaction between PPO and the nucleotides. However, when the binary mixture of PPO and the nucleotides were taken the absorption maximum at 260 nm of the nucleotides underwent a hyperchromism. The increased intensity at this wavelength is due to the additional contribution of the PPO absorption. Similar results were obtained with other nucleotide-PPO systems.

Fluorescence studies of PPO and nucleotides

PPO displays an emission maximum at 374 nm, on excitation at its λ_{max} (304 nm) as well as at 260 nm. The emission spectra of nucleotides were recorded after excitation at two wavelengths, at 260 nm, their λ_{max}, and the other at 304 nm. When excited at 260 nm, a weak emission maximum was observed at 384 nm. However, when excited at 304 nm, an emission maximum with very low intensity appeared at 330 nm and practically no emission at 374 nm was noticed. Therefore, when PPO was excited in presence of nucleotides the latter do not contribute to the fluorescence intensity of former at 374 nm. Furthermore, on increasing the nucleotide concentration, formation of excimers causes further reduction in the emission of nucleotides.

The quenching effect of nucleotides on PPO was studied, by varying the concentration of nucleotides between 5.0×10^{-6} and 5.0×10^{-5} mol dm^{-3}, while that of PPO was kept constant (1.25×10^{-5} mol dm^{-3}) and in Fig. 2 the fluorescence spectra of PPO in presence of AMP are shown. From the figure it can be noticed that the emission intensity of PPO at 374 nm decreases gradually as the concentration of nucleotide increases. Though the spectra did not undergo any wavelength shifts, they exhibit isoemissive points at 330 nm and 450 nm. The emission spectra of PPO in presence of other nucleotides showed similar results. The dependence of the fluorescence intensity of PPO on nucleotide concentration was analyzed following the Stern–Volmer relationship,

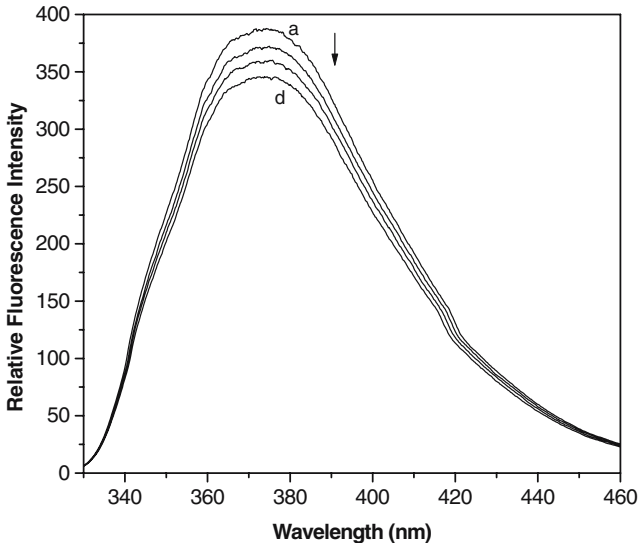

Fig. 2 Fluorescence spectra of PPO (1.25×10^{-6} mol dm^{-3}) in presence of AMP (*a*) 0, (*b*) 5.0×10^{-6}, (*c*) 10.0×10^{-6} and (*d*) 2.5×10^{-5} mol dm^{-3} in 40% methanol. λ_{ex} at 304 nm

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities measured in the absence and presence of the nucleotide respectively, [Q] is concentration of nucleotide and K_{SV} is Stern–Volmer quenching constant. Typical Stern–Volmer plots of PPO–nucleotide systems are shown in Fig. 3 and the data obtained are presented in Table 1. The bimolecular collisional quenching constants k_q were calculated using the Eq. 2.

$$K_{SV} = k_q \tau_0 \quad (2)$$

where k_q is bimolecular collisional quenching constant and τ_0 is the intrinsic fluorescence lifetime of PPO. The value of τ_0 (1.4 ns) was obtained from literature [34]. The k_q values obtained are shown in Table 1.

The quenching of PPO fluorescence by the addition of a nucleotide displays a linear Stern–Volmer relationship. The presence of isoemissive points at 330 nm and 450 nm indicate the formation of an exciplex between the excited state PPO and nucleotide which probably involves the quenching of the fluorescence of PPO. The Stern–Volmer quenching constants vary between 5.70×10^3 lit mol^{-1} for AMP and 1.11×10^2 lit mol^{-1} for UMP. The quenching ability of nucleotides are in the order AMP>ADP>GMP>dGMP>UMP. This is contrary to the observed quenching behavior of nucleobases. It is commonly reported that guanine is a greater quencher of fluorescence than adenosine for many organic fluorophores. The fluorescence quenching by nucleotides and nucleobases is explained in these cases by a photoinduced electron transfer mechanism. As a result, the quenching is dynamic in nature. In case of guanine both static and dynamic mechanisms have been reported to be responsible for fluorescence quenching [24]. It has also been observed that the nucleobase is responsible for the quenching process and hence the phosphate ester and the sugar ring have no contribution to the quenching process. However, our results have shown that AMP is a better quencher than GMP. This may be due to the fact that if only dynamic quenching is involved, AMP has greater ability than GMP or in the absence of any static quenching adenine base is a better quencher than guanine. From

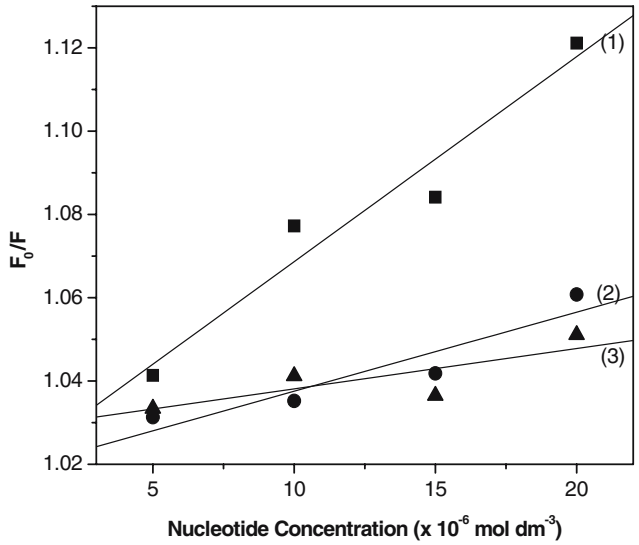

Fig. 3 Stern–Volmer plots of (*1*, *filled squares*) AMP, (*2*, *filled circles*) ADP and (*3*, *filled triangles*) GMP quenching of PPO fluorescence in 40% methanol. λ_{ex} at 304 nm and λ_{em} at 374 nm

Table 1 it is observed that the Stern–Volmer quenching constants vary in the order AMP>ADP>ATP. Similarly GMP>UMP and GMP>dGMP. From the above trends it can be inferred that the quenching ability of purine nucleotides is greater than pyrimidine nucleotides. Similarly, the quenching efficiency of nucleoside monophosphates is greater than the corresponding triphosphates, which in turn are more efficient quenchers than the deoxyribose nucleotides. A similar trend was reported by Dunn et al. [22]. The triphosphates however do not show any relationship in their quenching pattern. Linear Stern–Volmer plots indicate that only one type of quenching occurs and it is dynamic in nature. Further no ground state complexation was observed and the magnitudes of bimolecular collisional quenching constants further indicate the absence of static quenching.

Inner filter effects

From Fig. 1 it was noticed that the intensity of 260 nm band of PPO–AMP system is greater than the intensity of individual systems. Spectral properties are additive in nature and it is reasonable to predict that the enhanced intensity at 260 nm in AMP–PPO system is due to the combined absorption of AMP and PPO, with bulk of the intensity being contributed by the nucleotide. When PPO is excited at 260 nm in presence of nucleotides, the latter efficiently absorbs the incident light and makes PPO less fluorescent. The fluorescence emission of PPO monitored by excitation at 260 nm in presence of various concentrations of GTP is shown in Fig. 4. From the figure it is observed that the emission intensity of PPO at 374 nm decreases steadily with increase in GTP concentration. The quenching of PPO occurred without any change in either the peak shape or position which confirms the trivial absorption of radiation by nucleotides without formation of any ground or excited state complexes with the added nucleotide. If the quenching of PPO fluorescence is due to the competitive absorption of light by nucleotides i.e., due to inner filter effect [35], the former should be proportional to both the nucleotide concentration and its molar extinction coefficient. It is observed that in case of NAD, the quenching is much more pronounced than the remaining nucleotides as it contains two adenine moieties and its molar extinction coefficient is approximately about 1.5 times the values of the mono nucleotides. Hence the quenching is also expected in the same order of magnitude.

A plot of log of fluorescence intensity at 374 nm against the concentration of NAD is given in Fig. 5. The plot exhibited a linear relationship. Higher the concentration of nucleotide greater is the absorption of the incident radiation at 260 nm, by the nucleotide and larger is the fluorescence

Table 1 Stern–Volmer quenching constants, K_{SV} and bimolecular quenching constants k_q of PPO–nucleotide systems in methanol

Nucleotide	K_{SV} ($\times 10^2$)	k_q ($\times 10^{11}$)
AMP	57.0	40.7
ADP	26.4	18.8
GMP	5.39	3.85
dGMP	3.52	2.51
ATP	2.47	1.76
UMP	1.11	0.79

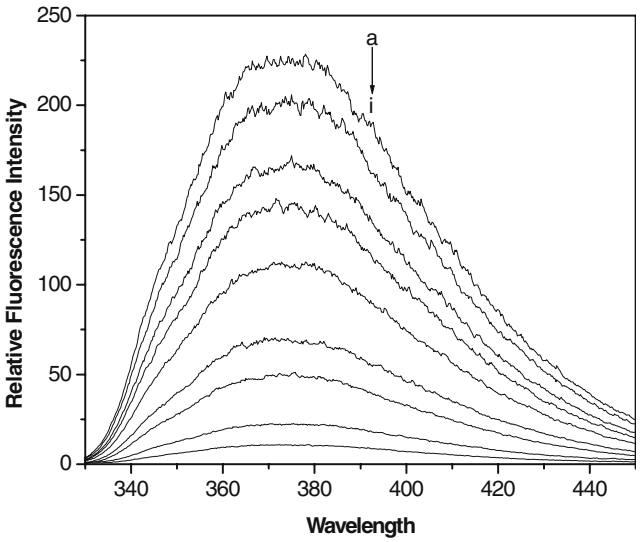

Fig. 4 Fluorescence emission spectrum of PPO (25×10^{-6} mol dm^{-3}) in presence of varying concentrations of GTP (*a–i*); (0, 10, 20, 40, 50, 80, 100, 150 and 200×10^{-6} mol dm^{-3}) in 40% methanol. λ_{ex} at 260 nm

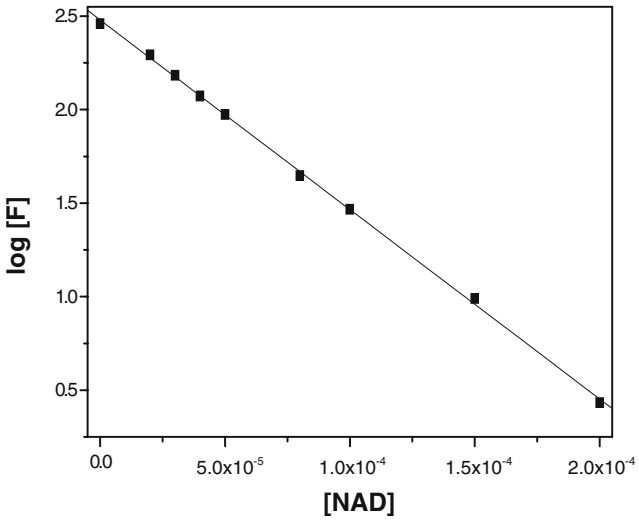

Fig. 5 Fluorescence emission intensity of PPO (25×10^{-6} mol dm^{-3}) in presence of varying concentrations of NAD (a–i); (0, 20, 30, 40, 50, 80, 100, 150 and 200×10^{-6} mol dm^{-3}) in 40% methanol. λ_{ex} at 260 nm and λ_{ex} at 374 nm

quenching of PPO. Similar relationship is also observed with other nucleotides Greater is the absorption of the incident light by the nucleotide higher is the slope, *K*, of the plot. All the *K* values derived from such plots are tabulated in Table 2. If the quenching of PPO fluorescence in presence of nucleotides, when monitored by excitation at 260 nm, obeys the Beer–Lambert law, the slopes obtained must directly correlate with the respective molar extinction coefficient (ε) of the nucleotides. Hence, in Fig. 6 a plot between the ε values of the nucleotides and the slopes obtained is given. From the figure it is obvious that the relationship is linear and therefore can be concluded that the quenching of PPO fluorescence by nucleotides when excited at their respective absorption maximum is due to inner filter effect. Thus, the quenching constant K depends on the molar extinction coefficient of the quencher and hence the quenching of PPO by compounds can be used as a sensitive fluorimetric method for their determination, though the compounds themselves are not fluorescent.

Table 2 Fluorescence quenching constants *K* and molar extinction coefficients (ε) for various nucleotides

Nucleotide	*K* (lit mol^{-1})	ε (lit mol^{-1} cm^{-1})
ADP	7,651	14,862
ATP	8,138	15,400
CTP	3,958	9,000
GMP	5,749	11,700
GTP	6,883	13,700
NAD	10,134	18,000
UTP	4,900	10,000

λ_{ex} at 260 nm and λ_{em} at 374 nm

Inner filter effect has been used as an analytical tool for the detection of non-fluorescent molecules by using fluorophores [36].

Conclusion

The effect of nucleotide on the fluorescence PPO was studied at two different excitation wavelengths. When PPO was excited at its absorption maximum i.e., 304 nm, the fluorescence quenching follows the Stern–Volmer model. From the K_{SV} and k_q values obtained it was observed that AMP shows greater quenching than GMP. This is contrary to the commonly observed quenching patterns for nucleotides. The quenching is higher for monophosphates than for the triphosphates. Similarly, the ribose nucleotides quench PPO fluorescence more efficiently than the deoxynucleotides. The nucleoside triphosphates do not show any definite trend in their quenching behavior. The quenching of PPO fluorescence by nucleotides probably arises due to an exciplex formation and is dynamic in nature. This is borne out of k_q values and also due to the fact that absorption spectra of PPO were not affected in presence of nucleotides indicating no interaction in the ground state which rules out static quenching. However, the quenching of PPO fluorescence by the nucleotides when excited at 260 nm arises due to the inner filter effect, due to the competitive absorption of incident radiation by the nucleotides. As the excitation wavelength is not the maximum absorption wavelength of PPO but the absorption maxima of nucleotides no exciplexes involving the excited state PPO are formed to quench its emission. The quenching is linearly dependant on the concentration and the molar

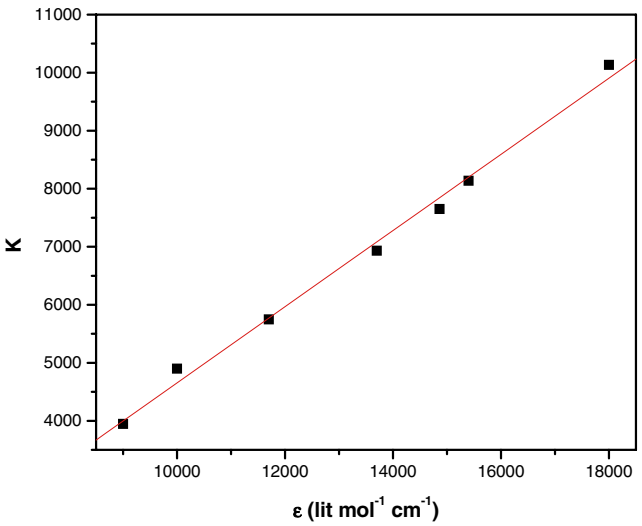

Fig. 6 Plot of fluorescence quenching constant *K* (the slope of Fig. 5) against molar extinction coefficient (ε) of nucleotides

extinction coefficient of the nucleotides. Therefore the inner filter effect can be used for the fluorimetric assay of nucleotides.

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