Spectroscopic Study on the Reaction of Singlet-Excited Nile Blue with Certain Antioxidants

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Abstract

The photoinduced interaction of nile blue (NB) with various antioxidant molecules was investigated by fluorescence quenching technique and lifetime measurements. The various substituted catecholic compounds are employed as quenchers to evaluate their antioxidant activity. The formations of ground state complex between NB and quencher molecules observed from the UV-Visible absorption spectroscopy. The bimolecular quenching rate constants ($k_q$) values depend on presence of substituent and its electronic properties of quencher molecules. Fluorescence quenching experiments have been performed at three different temperatures to assess the thermodynamic parameters. Time resolved fluorescence measurements suggest that the fluorescence quenching of NB with antioxidant molecules undergoes static quenching mechanism. The bond dissociation enthalpy (BDE) values reveal the discharge of $H_T$ from the antioxidant molecules. The electronic properties play an important role in the antioxidant activity of quencher molecules. The mechanism of fluorescence quenching between NB and quencher molecules are analysed based on the fluorescence quenching experiments, cyclic voltammetry experiments and BDE calculations.

1. Introduction

Nile blue (NB) is an attractive molecule (Structure 1) in the class of cationic oxazine dye. Nile blue derivatives have been shown to be potentially effective photosensitizers for photodynamic therapy of malignant tumours due to its absorption of light in the red region of spectrum. NB efficiently react with tumour cells than normal tissues and retard the tumour growth [1, 2]. NB and its derivatives are favorable materials for optical and photonic devices [3–5]. NB employed as a reagent to assess the antioxidant activity of commercial wine and fruit juice samples [6]. NB have strong binding affinity with DNA and show extensive biological applications [7, 8].

Catechols and catecholamines are potentive antioxidants and scavenge reactive oxygen species [9]. The antioxidant activity found to have direct relationship with their biological actions [10–11]. The antioxidant effect depends on the chemical composition, solubility in lipids, ability to scavenge free radicals, donation of hydrogen atoms and free radicals related with chain reactions. It has been observed that presence of two hydroxyl groups in catechol molecule is responsible for the antioxidant activity. The protective effects of antioxidants vary depending on the structure of molecules [12]. The presence of catechol compounds in olive oil is liable for higher antioxidant activity in biological environment [13]. The neurological disorder, Parkinson disease arises due to the non-production of dopamine treated by administering Levodopa [14].

Our prime intention is to investigate the interaction of antioxidant molecules with appropriate biological targets and in this perspective study on the fluorescence quenching of NB with catechol and phenol molecules is of utmost important in pharmaceutical point of view. We are interested in understanding the release of $H^+$ from antioxidant molecules as it may possibly solve biological related problems [15]. Besides, presence of substituent in catechol molecule influences the release of $H^+$ from aromatic hydroxyl
groups (-OH). Fluorescence quenching of 9-aminoacridine and acriflavine by various antioxidants such as estrogens, flavonoids, phenol and its derivatives, catechols and uracils were thoroughly investigated and found to undergo pronounced charge transfer process [16–21]. Recently, NB employed as a fluorescent probe to evaluate the antioxidants of uracil molecules by abstracting the release of $H^+$ from antioxidants via ground state complex formation [22]. Although reports on the antioxidant activity of catechol was available, but comparison on the antioxidant activity of quencher molecules with different substitution based on steady state measurements and density functional studies are not reported so far. Thus, the present work focus on the photo induced interaction of NB with various quencher molecules using steady state and lifetime measurements. Bond dissociation enthalpy calculations reveal the efficiency of antioxidants with respect to position of the substituent. The structure of the catechol derivatives chosen for this study is depicted in structure 2.

2. Experimental And Methods

2.1. Materials

Nile blue, catechol, pyrogallol, 4-t-butyl catechol, dopamine, levodopa and 4-aminophenol were purchased from Sigma-Aldrich and used without further purification. All the stock and aliquot solutions were prepared using double distilled water.

2.2. Methods

UV-Visible absorption and fluorescence spectra were recorded by UV-Visible absorption spectrophotometer (JASCO V-630) and Fluorescence spectrophotometer (JASCO FP-6500) respectively. The emission spectra were measured by exciting the NB at 636 nm and emission maximum of NB is observed at 669 nm in phosphate buffered at pH 7.4. In order to avoid the quenching by singlet oxygen, samples were degassed with pure nitrogen gas for 15 min. Fluorescence lifetime measurements were carried out in a time correlated single photon counting (TCSPC) spectrometer. The data were analysed through software provided by IBH (DAS – 6). The kinetic trace examined by non-linear square fitting of mono exponential method.

2.3. Cyclic voltammetry measurements

The reduction potential and oxidation potential of NB and quencher molecules were measured with potassium chloride (0.1 M) as the supporting electrolyte. The reduction potential for NB observed at -3.88 V versus SCE [22]. The experimental setup consist a platinum working electrode, a glassy carbon-counter electrode and a silver reference electrode. Irreversible peak potential of quencher molecules measured at different scan rates (0.05 V/s). All samples bubbled in presence of nitrogen gas for 5 min at room temperature for deaeration.

2.4. BDE calculation
All the organic quencher molecules were optimized by B3LYP [23–26] method with 6-31G** [27–29] basis set. Harmonic vibrational frequency calculations were carried out on these optimized geometries to confirm that they are saddle point with all positive frequencies. RB3LYP method applied for quencher molecules and UB3LYP method applied for radicals. All these computational methods have been used as employed in the Gaussian 16 software package [30].

The bond dissociation energy (BDE) of O–H bond was calculated by

\[ E_{\text{BDE}} = E_{\text{RO}^\bullet} + E_{\text{H}^\bullet} - E_{\text{RO}-\text{H}} \]

Were, \( E_{\text{RO}^\bullet} \), \( E_{\text{H}^\bullet} \) and \( E_{\text{RO}-\text{H}} \) represent the energies of \( \text{RO}^\bullet \), \( \text{H}^\bullet \) and \( \text{RO}-\text{H} \), respectively.

### 3. Result And Discussion

#### 3.1 UV-Visible absorption spectra

The absorption of NB is characterized by a strong band at 636 nm. UV Visible spectral studies have been performed to reveal the presence of ground state interaction between NB and quencher molecules. All the quencher molecules show no absorption bands in the range of 600–700 nm. Interestingly, addition of quencher molecules decreases the absorbance of NB followed with an observable red shift (longer wavelength). This shows the existence of ground state complex formation between NB and quencher molecules [31]. Figure 1 indicates UV-Visible absorption study of NB with increasing concentration of dopamine in phosphate buffered media at pH 7.4. It is worthy to note that similar behaviour noticed for other quencher molecules.

#### 3.2 Effect of quenchers in emission spectra of NB

The emission spectra of NB were measured in absence and presence of quencher molecules by exciting at 636 nm. It has been observed that on increasing the concentration of quencher molecules, the emission intensity of NB decreases. Figure 2 depicts the fluorescence quenching of NB in absence and presence of catechol. The Stern –Volmer rate constant (\( K_{sv} \)) calculated from the following Stern – Volmer equation as follows,

\[ \frac{I_0}{I} = 1 + K_{sv} [Q] = 1 + k_q \cdot \tau_0 \]

The Stern - Volmer (S-V) plot has been obtained from the plot of \( \frac{I_0}{I} \) versus quencher concentration. It yields a straight line as shown in Fig. 3. The bimolecular quenching rate constant (\( k_q \)) was calculated and compiled in table 1. The extent of quenching efficiency are found in the order as follows.

Dopamine > L-DOPA > pyrogallol > 4-aminophenol > 4-t-butyl Catechol > Catechol

Dopamine show higher \( k_q \) value than other quencher molecules. Dopamine consists of two hydroxyl groups at adjacent position with ethylamine as a side chain. The presence of electron releasing group
proliferate the electron density inside the ring and improves the antioxidant activity [32]. Thus, it leads to superior release of H’ . L-DOPA shows lesser $k_q$ than dopamine. The observed behaviour attributed to the effect of electron withdrawing nature of COOH group. The presence of electron withdrawing group diminishes the effect of electron releasing group (-NH$_2$) in the quencher molecule. Pyrogallol shows less $k_q$ value than dopamine and L-DOPA. Pyrogallol consists of three hydroxyl groups at adjacent positions but the absence of electron releasing species in the molecule might be plausible reason for lower $k_q$ value. 4-Aminophenol shows higher $k_q$ value than 4-t-butylcatechol. The presence of electron releasing amine group enhances the electron density and favour the possibility of releasing the H’ from the molecule. Unsubstituted catechol shows very less $k_q$ value among the quencher molecules due to the absence of electron releasing substituent in the molecule. The presence of electron releasing species in the antioxidants greatly influences the fluorescence quenching of NB. Similar type of observations documented in the literature [20–22].

The fluorescence quenching experiments was executed at different temperatures and observed the presence of significant change in the excited state of NB in existence and non-existence of quencher molecules. The quenching titrations were conceded at various temperatures ranging from 15 to 35$^\circ$C. The bimolecular quenching rate constant ($k_q$) falls with increasing the range of temperatures (shown in Table 1). The observed results specify the existence of static quenching between NB and quencher molecules.

### 3.3 Lifetime measurements

The fluorescence quenching of NB with quencher molecules were carried for understanding the decay mechanism through excited state lifetime measurements. The fluorescence quenching shall be either dynamic or static [33]. The lifetime of NB was recorded with different concentrations of quencher molecules. The decay curve properly fit well with single exponential decay. The excited state lifetime of NB was observed and found to be 1.74 ns [34]. The lifetime of NB in presence and absence of quencher molecules was noted. Interestingly, the lifetime of the NB molecule remains unaffected. The decay process was plotted and looks like single decay curve. The excited state lifetime measurement of NB in absence and presence of catechol was shown in Fig. 4 and indicate the existence of static quenching between NB and quencher molecules. The presence of static quenching embraces the possibility of ground state complex formation. Similar behaviour observed for NB in presence of other quencher molecules. Hence, the quenching pursues static mechanism.

### 3.4 Mechanism of fluorescence quenching

The fluorescence quenching of NB with quencher molecules can be rationalised by various mechanisms. The possibility of energy transfer mechanism can be eliminated as the absorption spectrum of catechol and phenol molecules unsuccessfully overlay with the fluorescence spectrum of NB. The prospect of either electron transfer or proton transfer mechanism, were evaluated by employing Rehm – Weller expression, shown as follows,
\[ \Delta G_{et} = E_{ox} (D) - E_{red} (A) - E^* + C \]

The \( \Delta G_{et} \) values were positive and imply the probability of proton transfer mechanism [35]. The obtained values are displayed in table 1.

The forces acting between NB and quencher molecules are favored with weak interactions forces such as hydrogen bond formation, electrostatic interaction, hydrophobic interaction and Vander Waals forces [36]. The binding mode is authenticated by using thermodynamic parameters, enthalpy change (\( \Delta H \)) and entropy change (\( \Delta S \)) of binding reaction. As based on thermodynamic point of view, \( \Delta H > 0 \) and \( \Delta S > 0 \) indicate a hydrophobic interaction; \( \Delta H < 0 \) and \( \Delta S < 0 \) implies the Vander Waals forces or hydrogen bond formation and \( \Delta H \sim 0 \) and \( \Delta S > 0 \) suggest an electrostatic force exist between fluorophore and quencher molecules [37].

The thermodynamic parameters were calculated using the following equation and the values are displayed in Table 2.

\[ \Delta G = -RT \ln K \]

\[ \ln K = \frac{\Delta H}{RT} + \frac{\Delta S}{R} \]

The \( \Delta G \) value is negative and signifies the interaction process is spontaneous. The \( \Delta H \) and \( \Delta S \) value point out the non-bonded (Van der Waals) interactions and hydrogen bond formation [37]. Thus, quencher molecules are destined to NB due to Van der Waals interaction and hydrogen bond formation. The \( \Delta H \) and \( \Delta S \) values predict the possibility of charge transfer and hydrogen bonding interaction. NB possesses high reduction potential and quencher molecules own oxidation potential. The charge transfer occurs between NB and quencher molecules. The charge transfer process might be one of the promising evidence for the quenching mechanism of the non-radiative processes.

### 3.5 Bond Dissociation Enthalpy calculation

Density functional theory (DFT) calculations were carried out to understand the radical scavenging performance of chosen phenolic quencher molecules, as shown in structure 2. Bond dissociation enthalpy (BDE) found to be as a common descriptor for radical scavenging activity of quencher molecules. The radical scavenging of quencher molecules was defined as

\[ Q-H + \text{ROO}^* \rightarrow Q^* + \text{ROOH} \]

where \( Q-H \) and \( \text{ROO}^* \) correspond to the quencher molecule and peroxyl radical, respectively.

The BDE of O-H bond in the quencher molecule may act as parameters to envisage the pathway of scavenging free radicals by quencher molecule. The lowest BDE, indicates the most preferred mechanism of scavenging the \( \text{ROO}^* \) radical. The lower the BDE value, weaker the O–H bond strength and greater the
free radical scavenging ability of organic quencher molecules. The weakest O–H bond are recognized for the quencher molecules and compared with corresponding \( k_q \) values (shown in Fig. 5).

The calculated BDE values are shown in Fig. 6. The quencher molecules with smallest BDE value, exhibit highest \( k_q \) values and correlate well between them. Intriguingly, analogous trend observed in the steady state measurements.

Among the quenchers, dopamine shows lower BDE (70.63 kcal/mol) value and found that outmost high antioxidant activity. This is owing to the electron rich olefin, electron releasing (-NH\(_2\)) group and especially, intramolecular hydrogen bond between two -OH group in dopamine [38]. On comparing L-DOPA and pyrogallol, the former show less BDE value due to presence of electron releasing -NH\(_2\) functional group and intramolecular hydrogen bond. Pyrogallol consists of three hydroxyl groups and one of the hydroxyl group in pyrogallol molecule show less BDE value and it account for high antioxidant activity than 4-aminophenol (70.36 kcal/mol). The trend indicates the presence of electron releasing species at C-4th position has great impact in radical scavenging potential of quencher molecules. Thus 4-t-butyl catechol show less BDE than catechol molecule. The observed behaviour is owing to the presence of electron releasing methyl group at the 4th position and intramolecular hydrogen bond. The existence of substituent at C-4th position plays an important role in assessing the BDE of quencher molecules. The interpretations disclose the prominence of H in determining the antioxidant activity. The present research reveals the activity of radical scavenging confides on the position and electron releasing property of substituent in quencher molecules.

4. Conclusion

The fluorescence quenching of NB in presence of antioxidant molecules investigated by using steady state, lifetime measurements and BDE calculations. The formation of ground state complexes between NB and quencher molecules were confirmed using UV-Visible spectroscopy and lifetime measurements. The calculated bimolecular quenching rate constant (\( k_q \)) depend on the substituent in quencher molecules. The effect on fluorescence spectra of NB in existence of quencher molecules were carried out at diverse temperatures on the way to estimate the thermodynamic parameters. The BDE value was deliberated to examine the discharge of H in the quencher molecules. Based on the fluorescence quenching experiments and BDE calculations, it suggests that the hydrogen atom transfer between the NB and quencher molecules as one of the possible quenching mechanism. The present study demonstrates a new way in designing novel molecules with great antioxidant activity.

5. Declarations

Conflicts of interest/Competing interests: Authors have no conflict of interest.

Ethics approval: Not applicable
Consent to Participate: Not applicable

Consent for Publications: Not applicable

Data availability: All data generated or analysed during this study are included in this published article.

Code Availability: Not applicable

Authors’ contributions: CM and SB has carried out the experimental parts and prepared the draft of the manuscript. VA has contributed in the revision of the manuscript.

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7. Tables

**Table 1** Fluorescence quenching rate constant and electrochemical data of NB with quencher molecules

| S. No | Quencher      | $k_q \times 10^{11} \text{M}^{-1}\text{s}^{-1}$ | $E_{\text{ox}}$ vs. SCE (V) | $\Delta G_{\text{et}}$ (eV) |
|-------|---------------|-----------------------------------------------|----------------------------|-----------------------------|
|       |               | $15 \degree \text{C}$ | $25 \degree \text{C}$ | $35 \degree \text{C}$ | | | | |
| 1     | Dopamine      | 11.32                                        | 9.83                       | 8.05                        | 0.67                        | 4.55                        |
| 2     | levodopa      | 9.65                                         | 7.73                       | 5.41                        | 0.58                        | 4.46                        |
| 3     | Pyrogallol    | 8.72                                         | 6.45                       | 4.54                        | 0.78                        | 4.66                        |
| 4     | 4-aminophenol | 7.72                                         | 5.72                       | 4.11                        | 0.39                        | 4.27                        |
| 5     | 4-t-butyl catechol | 6.68 | 5.35 | 3.85 | 0.6 | 4.48 |
| 6     | Catechol      | 5.34                                         | 4.16                       | 2.89                        | 0.8                         | 4.68                        |

$^a$ determined by steady state fluorescence quenching in phosphate buffer media ($\tau_o=1.74 \text{ ns}$)

$^b$ Oxidation potential of quencher molecules in V vs SCE

$^c$ Calculated by Rehm-Weller equation $DG_{\text{et}} = E_{\text{ox}} (D) - E_{\text{red}} (A) - E^* + C$, the reduction potential of NB is $-3.88 \text{ V vs. SCE}$, $E^* =1.93 \text{ eV}$.

Error $\pm 3 \%$

**Table 2** Thermodynamic parameters of NB with quencher molecules
| S. No | Quencher          | ΔG (kcal mol⁻¹) | ΔH (kcal mol⁻¹) | ΔS (cal K⁻¹ mol⁻¹) |
|-------|-------------------|-----------------|-----------------|-------------------|
| 1     | Dopamine          | -16.31          | -13.17          | -42.11            |
| 2     | Levodopa          | -16.93          | -21.13          | -52.43            |
| 3     | Pyrogallol        | -17.4           | -22.14          | -59.86            |
| 4     | 4-aminophenol     | -17.7           | -22.89          | -60.57            |
| 5     | 4-t-butyl catechol| -18.25          | -24.52          | -65.99            |
| 6     | Catechol          | -19.35          | -25.67          | -68.42            |

**Figures**

![Absorption Spectra](image_url)

**Figure 1**

Absorption spectra of NB (5 X 10⁻⁶ M) in the presence of various concentrations of dopamine (2,4,6,10 X 10⁻⁴ M) in phosphate buffered media at pH 7.4. (The arrows indicate decrease in absorbance followed with red shift)
Figure 2

Emission spectra of NB (5x10^-6 M) in the presence of Catechol (0 - 8 X 10^-5 M) in phosphate buffered media at pH 7.4. (The down arrow indicates decrease in emission intensity of NB).
Figure 3

Comparison of Stern - Volmer Plot of NB (5x10^-6 M) a) in the presence of quencher molecules at various concentrations (0 - 8 X 10^-5 M).
Figure 4

Fluorescence lifetime decay curve of NB (5x10^{-6} M) in absence and presence of pyrogallol (8 X 10^{-5} M) in phosphate buffered media at pH 7.4.
Figure 5

Comparison of bimolecular quenching rate constant with BDE value

Figure 6

Structure of quencher molecules with BDEs at B3LYP/6-31G**/LANL2DZ level of theory (energies are given in kcal/mol)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- structure1.png
- structure2.png