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ORIGINAL PAPER

Study and Application of the Interaction Between Asymmetrical Porphyrin and Ascorbic Acid

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Abstract The interaction between novel asymmetrical porphyrin 5,10,15-triphenyl-20-pyridyl porphyrin and ascorbic acid has been studied by fluorophotometry. It has been found experimentally that in the presence of CTMAB microemulsion, the absorption and fluorescence intensity of the porphyrin was greatly enhanced. The addition of ascorbic acid caused the decrease of the intensity, and there was a linear relationship between the fluorescence quenching value and the concentration of ascorbic acid in certain extent. Based on this phenomenon, a new fluorescence quenching method for the determination of ascorbic acid was established, and utilized in the determination of commercial tablets with satisfactory results. The quenching mechanism between porphyrin and ascorbic acid was preliminary studied, and the quenching was belonging to static quenching.

Keywords 5,10,15-triphenyl-20-pyridyl porphyrin · Ascorbic acid · Fluorescence quenching · Static quenching

Introduction

Porphyrin is a kind of special macrocycle aromatic compound with great conjugate π bond system. Owing to its special structure, superior physico-chemical properties, and its optical performance, porphyrin has broad application prospects in the fields of bionics [1, 2], material chemistry, photophysical chemistry [3], medicinal chemistry [4], analytical chemistry [5–7], and new arisen chemical biology. There are various natural porphyrin and its metal complexes in nature including human body, and they all play important roles in life courses. So porphyrin and its complex itself have rather high medical value. In clinical practice, porphyrin can be utilized in photodynamic therapy of cancer [8], and the therapy of porphyria, hematologic diseases and jaundice. For example, due to its tendency of aggregation around cancer cell, various anticancer drugs were modified on the porphyrin ring. Finally the targeting drug delivery was realized, and the pharmacological effects were correspondingly increased [9]. It can be seen that as an indispensable part of living body, there has been practical significance in the study between porphyrin and drug molecules.

When the special structure peripheral substituents were connected on the porphyrin, it could recognize many organic and biotic molecules as a host molecule. The recognition actions between porphyrin and biomacromolecule [10, 11] or small organic molecules such as amino acid [12] were reported at present, but there is little research reports study on the interaction between porphyrin and drug molecule. Herein, the interaction between novel asymmetrical porphyrin 5,10,15-triphenyl-20-pyridyl porphyrin(TPPyP) and ascorbic acid(AA) was studied using fluorescence quenching method. And based on the regular fluorescence quenching action of TPPyP by AA, a new determination method of AA was established.

AA is an important nutrient substance and drug. It has active effect on maintaining the normal physiological function and increasing body's defenses, and as a kind of drug, it can also prevent the production of carcinogen such as nitrosamine, and applied to the prevention of some diseases. Because the content of AA in practical samples is low and AA can be easily oxidized into dehydroascorbic acid, the establishment of a rapid and sensitive determination method has important significance. Up to now, there are several methods for the determination of AA, such as high-performance liquid chromatography [13], kinetic method

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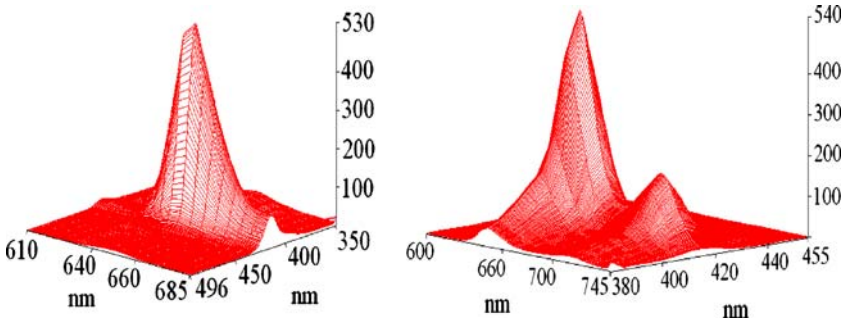

Fig. 1 The three-dimensional fluorescence spectrum. Conditions: $c_{TPPyP}=2.5\times10^{-7}$ mol·L^{-1}; pH=4.52; $V_{CTMAB}=0.5$ mL

[14], electrochemistry [15, 16], and chemiluminescence method [17, 18]. But the application of porphyrin as a spectral probe has not been reported. The method established in this paper has low detection limit and good selectivity when compared with other methods, and a satisfactory result was obtained when the phenomenon was applied to the determination of AA in practical samples.

The phenomenon of fluorescence quenching refers to any effects that cause the decrease of fluorescence quantum yield, and the quenching includes dynamic quenching and static quenching processes. Fluorescence quenching could be utilized in the investigation of reaction mechanism and the quantitative determination of various organic or inorganic samples [19]. The assignment of quenching process could be determined from multiple aspects, such as the relationship between quenching phenomenon and fluorescence lifetime, temperature or viscosity; and the changes of the absorption spectrum. In the last part of the paper, the quenching mechanism between TPPyP and AA was studied by the method.

Experimental

Apparatus and reagents

LS 55 Fluorescence Spectrometer (PerkinElmer, USA), Lambda 35 UV/VIS Spectrometer (PerkinElmer, USA) and PHS-3B pH meter (Shanghai) were used.

Solution of ascorbic acid (AA, 1 g·L^{-1}) was prepared by dissolving 0.1000 g AA with doubly distilled water and brought to 100 mL volumetric flask, then it was diluted to 10 μg·mL^{-1} when needed; Solution of 5,10,15-triphenyl-20-pyridyl porphyrin (TPPyP, 1.0×10^{-4} mol·L^{-1}) was prepared by dissolving 0.0031 g TPPyP with N,N-dimethylformamide (DMF) and brought to 50 mL volumetric flask, then it was diluted to 1.0×10^{-5} mol·L^{-1} when needed. Cetyltrimethylammonium bromide (CTMAB) micro-emulsion was prepared by mixing CTMAB: n-butanol: n-heptane: water = 1.0: 0.90: 0.10: 98(mass ratio). Britton-Robinson (B-R) buffer solution was prepared by mixing 0.2 mol·L^{-1} NaOH and 0.04 mol·L^{-1} CH_3COOH, H_3PO_4 and H_3BO_3 in different ratio and adjusting to appropriate values.

Unless otherwise mentioned, all chemicals were of analytical reagent grade and doubly distilled water was used throughout the experiment.

Procedure

In a 10 mL colorimetric tube, 0.25 mL of TPPyP (1.0×10^{-5} mol·L^{-1}), certain amount of AA standard solution (10 μg·mL^{-1}), 0.50 mL of CTMAB micro-emulsion and 1.50 mL of pH4.52 B-R buffer solution were added in order, and the solution was diluted to the mark with water and mixed thoroughly. After laying aside for 5 min at room temperature, the fluorescence spectra were monitored at 600~750 nm with fixed excitation wavelength at 414 nm, and excitation and emission slit widths were set at 7.5 nm/7.5 nm. Fluorescence intensities of sample (F) and the blank (F_0) (prepared in a similar manner without AA) were measured with $\lambda_{em}=648$ nm. Therefore the fluorescence intensity difference ($\Delta F=F_0-F$) was used to determine AA.

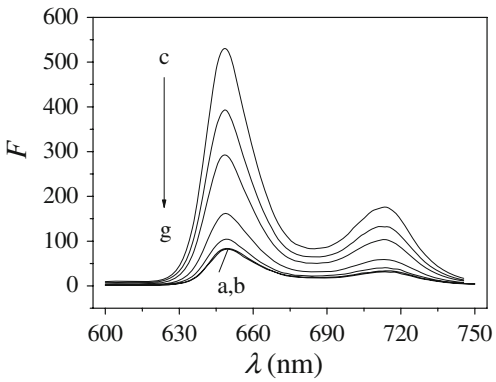

Fig. 2 Change of fluorescence Spectrum. **a** TPPyP + BR buffer solution; **b** TPPyP + AA + BR buffer solution; **c** TPPyP + CTMAB + BR buffer solution; d~g. TPPyP + AA(ρ_{AA} is 0.20 μg·mL^{-1}, 0.40 μg·mL^{-1}, 0.60 μg·mL^{-1}, 0.80 μg·mL^{-1} in sequence) + CTMAB + BR buffer solution. Conditions: $c_{TPPyP}=2.5\times10^{-7}$ mol·L^{-1}; $\rho_{AA}=0.40$ μg·mL^{-1}; pH=4.52; $V_{CTMAB}=0.5$ mL

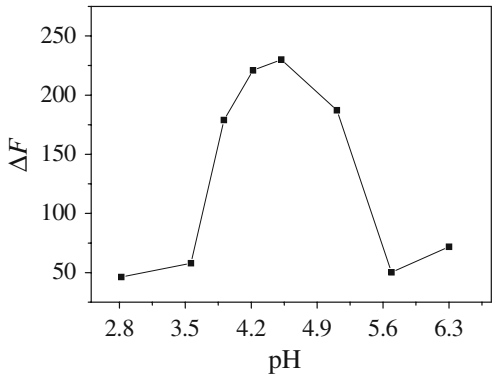

Fig. 3 The effect of acidity on the intensity. Conditions: $c_{TPPyP}=2.5\times10^{-7}$mol·L^{-1}; $\rho_{AA}=0.40$μg·mL^{-1}; $V_{CTMAB}=0.5$ mL

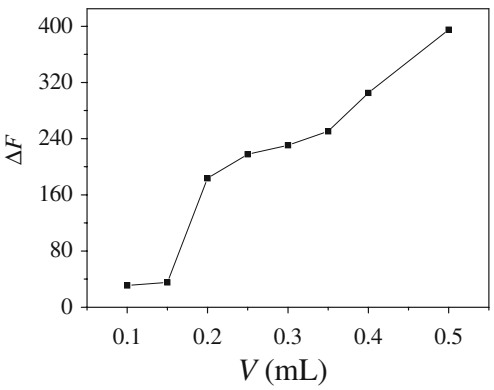

Fig. 5 The effect of the amount of TPPyP (1.0×10^{-5}mol·L^{-1}). Conditions: $\rho_{AA}=0.40$μg·mL^{-1}; pH=4.52; $V_{CTMAB}=0.5$ mL

Results and discussion

Fluorescence spectrum

According to the above experiment method, the three-dimensional fluorescence spectrum of the system was measured (Fig. 1), and it can be seen from the spectrum that the maximum excitation wavelength was 414 nm, and two emission peaks located at 648 nm and 715 nm, thus with this fixed maximum excitation wavelength of 414 nm, the emission spectrum was scanned between 600 nm and 750 nm.

It can be seen from Fig. 2 that, the fluorescence intensity of TPPyP was low without micro-emulsion, and there was no change on the intensity when AA was added; However, the fluorescence intensity of the porphyrin was greatly enhanced while the CTMAB micro-emulsion was added in the system, and the intensity especially the fluorescence intensity was enormously decreased when AA was added in the system.

Therefore, in the presence of CTMAB micro-emulsion and pH=4.52 B-R buffer solution, fixed the amount of porphyrin, the intrinsic fluorescence emission intensity reduced regularly when the concentration of AA increased, but the position and shape of fluorescence peak was unchanged. That is, the variation of fluorescence intensity reduction value with the concentration of AA was linear in porper range. And we proved that AA doesn't have any fluorescence emission at the excitation wavelength. Based on this, a new determination method of AA based on porphyrin as a fluorescence probe is established.

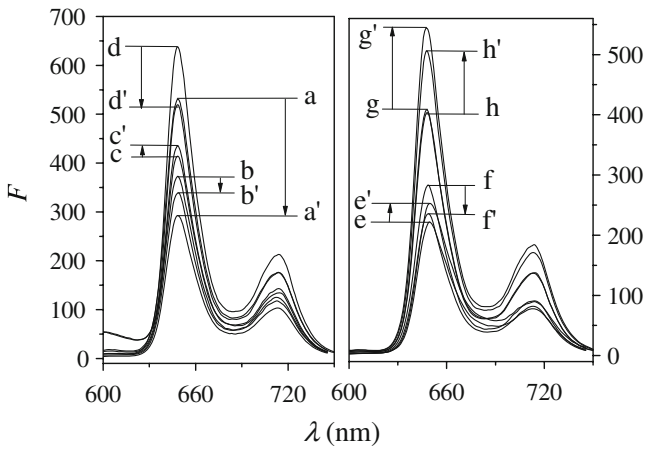

Fig. 4 Influence of different medium. a–a′: CTMAB micro-emulsion; b–b′: CTMAB micelle; c–c′: Tw-20 micro-emulsion; d–d′: Tw-80 micro-emulsion; e–e′: AOT micro-emulsion; f–f′: CPB micro-emulsion; e–e′: TX-100 micelle; g–g′: TX-10 micelle. a,b,c,d,e,f,g, h and a′,b′,c′,d′,e′,f′,g′ refer to the fluorescence spectra of TPPyP the absence and in the presence of AA respectively. Arrows indicate the directions of spectral changes. Conditions: $c_{TPPyP}=2.5\times10^{-7}$mol·L^{-1}; $\rho_{AA}=0.40$μg·mL^{-1}; pH=4.52

The selection of acidity

According to the experimental method, the influences of HAc-NaAc, KH_2PO_4-$Na_2B_4O_7$ and B-R buffer solution were investigated; it can be found that B-R buffer solution has the best effect. The influence of acidity on ΔF was investigated by changing the acidity of B-R buffer solution. The results were shown in Fig. 3.

Different kinds of buffer solution and different acidities all have great influence on the sensitivity of the system. It

Table 1 The effect of addition order

Addition order	ΔF
TPPyP + AA + CTMAB + pH4.52 B-R buffer solution	231.18
TPPyP + CTMAB + AA + pH4.52 B-R buffer solution	48.23
TPPyP + pH4.52 B-R buffer solution + AA + CTMAB	2.52
AA + pH4.52 B-R buffer solution + TPPyP + CTMAB	+0.29
AA + CTMAB + pH4.52 B-R buffer solution + TPPyP	44.65
CTMAB + TPPyP + AA + pH4.52 B-R buffer solution	+3.13
pH4.52 B-R buffer solution + TPPyP + AA + CTMAB	10.52

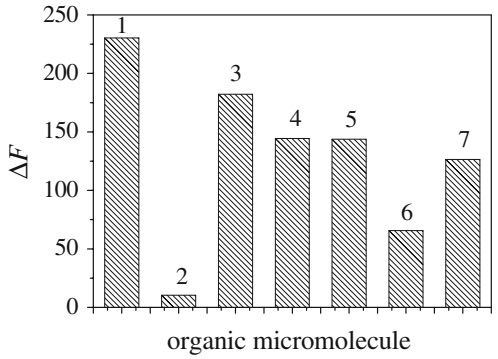

Fig. 6 The effect of different solvents. (1) water; (2) ethanol; (3) glycol; (4) propanol; (5) dimethyl sulfoxide; (6) acetone; (7) DMF

can be seen from Fig. 3 that, relative fluorescence intensity of the system was low in strong acidic medium. It was because porphyrin would protonate in strong acidic medium; the protonation caused the symmetry of porphyrin ring and electron density of the conjugated system all changed, and then ΔF decreased correspondingly. ΔF also decreased rapidly in alkaline condition, it was because OH^- reacted with acidic drug molecule, and the inner structure of the drug molecule was destroyed.

We can see that choosing appropriate acidity was important to the system. ΔF remained maximum and constant in the range of pH=4.20~5.10. Thus, the optimum pH to run the assay was pH4.52, and a final 1.50 mL of buffer solution was suitable.

The selection of reaction medium

Previous studies of our studying team [20, 21] proved that introducing surfactant into fluorescence system could significantly improve determination sensitivity and stability. This is because introduction of surfactant could decrease interfacial surface tension and shorten distance between two molecules, and shorter distance is beneficial to the improving of energy transfer efficiency. Fluorescence properties of fluorescent agent were different in various medium, so according to the experimental methods, the species of surfactant were changed, and the fluorescence intensity of TPPyP in the absence and in the presence of AA depended much on the nature of the surfactant (Fig. 4).

It can be seen from Fig. 4 that CTMAB micro-emulsion has the best sensitizing effect. The mechanism could be thought as the existence of CTMAB micro-emulsion not only changed the microstructure of the system, but also influence the rate of various physical processes. CTMAB micro-emulsion provided a protective environment to fluorescence particles, so the fluidity of the particles reduced. The environment decreased the particles' own concentration quenching and the quenching effect caused by external quencher. Therefore the lifetime of fluorescence molecular was prolonged, and the fluorescence quantum yield increased simultaneously.

So CTMAB micro-emulsion was chosen as the sensitizing agent and optimum dosage was 0.50 mL.

Effect of the concentration of TPPyP

Fixing the dosage of AA, and adding different amount of porphyrin, investigating the influence of porphyrin dosage, and the results were shown in Fig. 5.

As shown in Fig. 5, ΔF increased as the dosage of porphyrin increased and the trend was approximately linear. Based on comprehensive consideration of sensitivity and linear range, 0.25 mL of porphyrin was chosen by optimization selection.

Effect of different addition order of the reagents

The adding sequence of reagents has great influence in fluorophotometric analysis. It can be seen from Table 1 that, porphyrin and drug added first was beneficial to the binding reaction between them. The addition of micro-emulsion after the formation of the complex is helpful for the reaction. So the optimum addition order was the following: TPPyP, AA, CTMAB micro-emulsion and pH 4.52 B-R buffer solution.

Table 2 The effect of coexistent ions on the intensity (0.40 μg·mL^{-1} AA)

Substance	Concentration coexisting (μg·mL^{-1})	Relative error (%)	Substance	Concentration coexisting (μg·mL^{-1})	Relative error (%)
starch	20.0	5.7	*L*-phenylalanine	80.0	2.4
glucose	200.0	8.0	*DL*-serine	60.0	3.4
aminoacetic acid	10.0	3.5	Fe^{3+}	20.0	6.1
thiourea	50.0	5.0	Al^{3+}	50.0	5.2
BSA	60.0	3.7	Ca^{2+}	100.0	6.0
HSA	100.0	4.9	K^+	12.5	3.6
L-histidine	20.0	7.1	Pb^{2+}	15.0	8.3
L-tryptophan	50.0	3.9	Ni^{2+}	10.0	2.8

Table 3 The results for the determination of Vitamine C

Sample	Found (mg/g)	Conversion values (mg/Tablet)	Mean	RSD (%)	Recovery (%)
Saled Vitamin C tablet	571.3	93.7	93.4	0.58	98.7
	562.8	92.3			94.1
	575.6	94.4			102.5
	567.7	93.1			95.4
	569.5	93.4			97.7

Effect of organic micromolecule

The addition of organic micromolecule may have the effect of increasing the sensitivity. So the influence was tested by adding 0.50 mL of different kinds of micromolecule in the solution, and all the results were displayed in Fig. 6. As can be seen from Fig. 6, all the tested organic micromolecules caused the decrease of ΔF. So no organic micromolecule was added in the system.

Stability

TPPyP and AA could combine quickly at room temperature, and achieved stability after 5 min. The complex could remain stable for about 30 min. As time goes on, ΔF decreased slowly, and relative fluorescence intensity decreased by 20% after 3 h. The system has good stability, and 5 min was recommended in this study.

Influence of coexisting substances

Coexistence interference ion could influence determination results in actual sample determination. Under the optimum conditions, various coexisting substances including familiar amino acids, glucose and metal ions were examined for interference. The results were summarized in Table 2.

As shown in Table 2, most of examined coexisting substances have no marked interference on the assay. Therefore, the sample analysis could be performed without any special preparation.

The calibration graph and determination limit

Under the conditions given above, a linear relationship is obtained between the fluorescence intensity difference and the concentrations of AA. The linear regression equation is $\Delta F=54.57+487.6\rho$ ($\mu g{\cdot}mL^{-1}$) and the correlation coefficient is 0.9906. The linear range is from $0.10\,\mu g{\cdot}mL^{-1}$ to $0.80\,\mu g{\cdot}mL^{-1}$ with a detection limit of $0.026\,\mu g{\cdot}mL^{-1}$.

Analytical applications

Determination of AA in commercial vitamin C tablets

Take six commercial Vitamine C tablets (A certain company in Shandong), and grind the tablets in agate mortar. The sample solution was prepared by dissolving 20 mg sample with water and brought to 100 mL. Filtering with dry filter paper, the initial filtrate was discarded; and taking 2.00 mL continued filtrate, transferred to 100 mL volumetric flask. According to the experimental method,

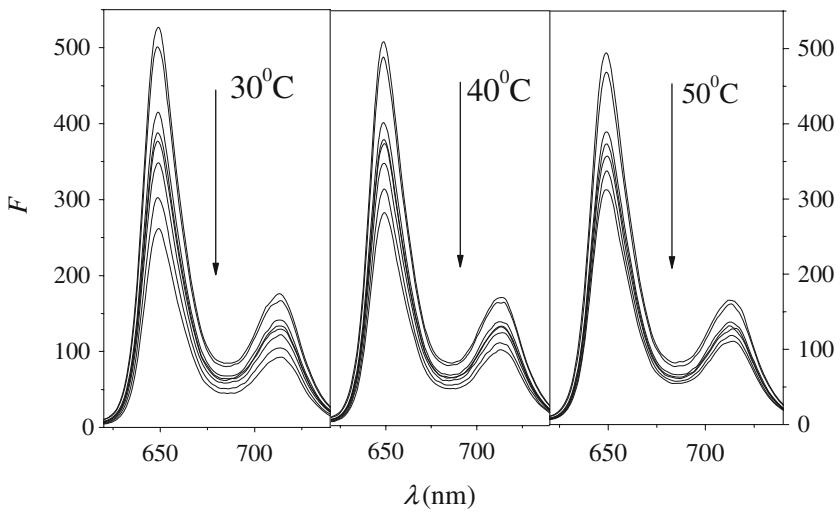

Fig. 7 The effect of AA on the fluorescence emission spectra of TPPyP. Conditions: $c_{TPPyP}=2.5\times10^{-7}mol{\cdot}L^{-1}$, $\rho_{AA}=10\,\mu g{\cdot}mL^{-1}$, Excitation and Emission Slit is 7.5 nm. The addition of volume AA is 0 mL, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 0.6 mL, 0.8 mL respectively. Arrows indicate the directions of spectral changes

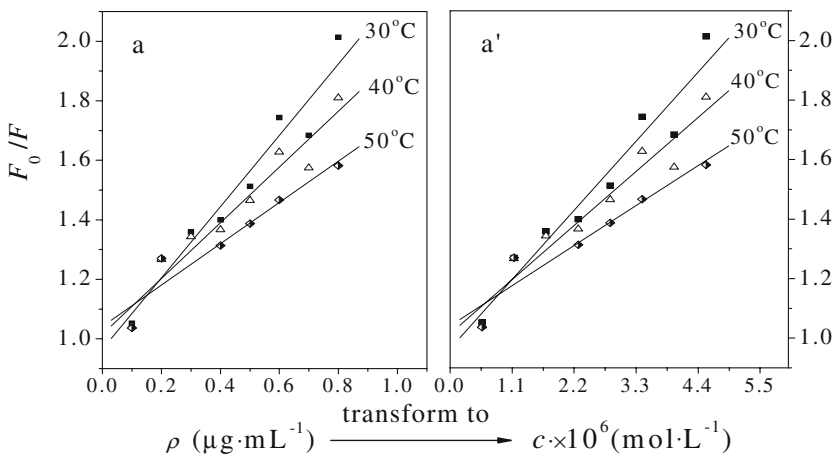

Fig. 8 The Stern-Volmer curves on fluorescence quenching of Porphyrins with concentration of AA

the concentration was found in calibration graph, and then converted to the content in tablets. Also, certain amount of standard solution was added in order to get the recovery value. The results were shown in Table 3.

It can be seen from the results in Table 3 that the relative standard deviation (RSD) is lower than 5% and the recovery is between 94.1% and 102.5%, proved that the method has good precision and accuracy. Therefore, the determination of AA by this method is reliable, sensitive and with good selectivity.

Study of quenching mechanism

Experimental method

The following solutions were successively added into a 10 mL colorimetric tube: 0.25 mL of 1.0×10^{-5}mol·L^{-1} TPPyP, a series of different volume of AA(10 μg·mL^{-1}), 0.50 mL CTMAB micro-emulsion and 1.50 mL pH4.52 B-R buffer. Then, the mixture was diluted to the mark with water and mixed thoroughly. After putting in 30 , 40 and 50 constant temperature water bath for about 10 min, the fluorescence intensity was monitored at 600~700 nm with fixed excitation wavelength at 414 nm. And excitation and emission slit widths were set at 7.5/7.5 nm. Fluorescence quenching spectra at different temperature were recorded.

Table 4 Regression equation and correlation coefficient

Curve	Temperature t/°C	Linear regression equation	Correlaation coefficient (r)
Fig. 7	30	F_0/F=0.9652+0.2110×10^6c (mol·L^{-1})	0.9705
	40	F_0/F=1.0155+0.1650×10^6c (mol·L^{-1})	0.9667
	50	F_0 / F=1.0418+0.1223×10^6c (mol·L^{-1})	0.9594

Results and discussion

It can be found from the above experiment that fixing the concentration of TPPyP, and the intrinsic fluorescence emission intensity reduced regularly with the concentration of AA increased, but the position and shape of fluorescence peak is unchanged. Fluorescence quenching spectra at different temperatures were shown in Fig. 7.

Fluorescence quenching processes is actually the processes that competed with luminescence process and shorten lifetime of excited state of luminescent molecular. The quenching processes could be the interaction between quencher and excited molecule of fluorescent material, or the interaction between quencher and ground state molecules of fluorescent material. The former is called dynamic quenching, and the latter is called static quenching. The interaction between fluorescence molecular and quencher and the property of quenching interaction can be respectively described as kinetic quenching constant K_{SV}(Stern-Volmer quenching constant) or static quenching binding constant K_{LB}.

It is difficult to decide the species of fluorescence quenching simply by measuring fluorescence intensity. Furthermore, the procedure is firstly assumed to be dynamic quenching, and the equation is described by eq. 1:

$$F_0/F = 1 + K_{SV}c_Q = 1 + K_q\tau_0 c_Q \quad (1)$$

Where F_0 and F are the fluorescence intensities in the absence and in the presence of quencher AA respectively,

Table 5 Binding parameters of AA with TPPyP at different temperature

Asymmetrical porphyrin	Temperature t/°C	K_{SV}/L·mol^{-1}
TPPyP	30	2.110×10^5
	40	1.650×10^5
	50	1.223×10^5

K_{SV} is the Stern-Volmer fluorescence quenching constant, c_Q is the concentration of quencher, τ_0 is average lifetime of molecule without quencher and K_q is quenching rate constant of bimolecule. The plot of F_0/F versus c_Q is constructed, and the Stern-Volmer figure at different temperatures are presented in Fig. 8.

Quenching rate constants can be obtained by the slope of quenching curves (Table 4), and the results are shown in Table 5. It can be see from Table 5 that, K_{SV} decreased when temperature rises, the phenomenon presents preliminary proof that the quenching is not caused by dynamic collision, but by the static quenching caused by the formation of complex.

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