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荧光猝灭分析中内滤效应的精确校正

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摘 要:研究了荧光猝灭分析中荧光强度的演变过程,发现样品对激发光的吸收分布会伴随内滤效应对荧光强度产生潜在影响.根据吸收分布影响荧光强度的物理机制,提出了利用荧光响应函数去除吸收分布影响的校正方法.通过分析五聚噻吩与富勒烯 C_{70} 之间在不同激发波长处光致电子转移引起的荧光猝灭,对校正效果进行验证,结果显示:单独对内滤效应进行校正后,各激发波长处的荧光猝灭率与 C_{70} 的吸收谱仍具有弱相关性,进一步对吸收分布的影响加以校正,所得荧光猝灭率则不再与 C_{70} 的吸收谱关联.表明在荧光猝灭分析时增加对吸收分布的校正可有效提高内滤效应的最终校正准确度.

关键词:光谱分析;荧光猝灭;校正;富勒烯;噻吩
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Method of Accurate Correction on Inner Filter Effects in Fluorescence Quenching Analysis

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Abstract: Via investigation on the evolution of fluorescence intensity in quenching process, it is discovered that the absorption distribution of exciting light can influence the fluorescence intensity in a potential way when the inner filter effects occur. According to the impact mechanism of absorption distribution on fluorescence intensity, a correction method based on fluorescence response function for removing the influence of absorption distribution is proposed. Photo induced fluorescence quenching of Quinquethiophene / fullerene (C_{70}) at different excitation wavelength is tested to inspect the correction effect. Experimental results demonstrate these quenching efficiencies obtained without correction on the influence of absorption distribution weakly correlated with the absorption spectrum of C_{70}, while the corrected ones are independent of them. Which means further correction on absorption distribution can effectively improve the final correction accuracy in fluorescence quenching analysis.

Key words: Spectral analysis; Fluorescence quenching; Correction; Fullerenes; Thiophene
OCIS Codes: 170.6280; 170.2520; 260.2510; 300.6170; 300.6390

0 Introduction

Fluorescence spectroscopy is widely applied in various scientific areas such as medicine[1-2], analytical chemistry[3], as well as environmental sciences[4-5]. However, the so-called Inner Filter Effect (IFE) still hinders its use as a reliable tool. For mixture solution, when the absorption spectrum of every component is overlapping each other the primary Inner Filter Effect (pIFE) will arise inevitably which would seriously interfere with the application of fluorescence analysis. Investigation on IFE was started in the last century. In recent years, its correction accuracy attracts researcher′s attention once again. Following the classical research methods, some improvements for IFE correction have been done[6-7], whereas most of them are too complex for application, the problem of IFE has not been solved fundamentally yet. It′s well known

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that failed correction of IFE will mislead researchers into false conclusions[8-9], however, for ordinary researchers, since the lack of a simple and practical correction method, many imprecise analyses still appeared in some literatures[10-11]. The IFE will hinder the application of spectroscopy in some areas seriously, especially for the investigation on organics, because most organic materials have a broad absorption range which is easy to induce the IFE. It is significant to find a simple and effective way to correct IFE properly.

In order to improve the correction accuracy, the mechanism of the IFE is examined in this work. We find that the Absorption Distribution of Exciting Light (ADEL) will also influence the fluorescence intensity when the IFE occurs. Based on this discovery, we propose a multi correction method which can satisfy the needs of accurate correction of IFE. Our work supplies a simple and effective method of accurate correction on IFE, which can help researchers to obtain the real information and to avoid making some wrong inference.

1 Theory

In fluorescence quenching analysis, the absorption of quencher on exciting and emission light will reduce the fluorescence intensity. This phenomenon is called IFE. Furthermore, the IFE, which refers to the absorption of exciting or emission light, includes pIFE and secondary Inner Filter Effect (sIFE). In the last century, some correction formulas of IFE had been presented by several research groups[12-13]. Their works were based on the ideal model with collimated exciting and emission beams, as shown in Fig. 1. The classical corrected fluorescence intensity is given by

$$F_{corr}=F_{obs}\times 10^{(a_{ex}+a_{em})/2} \tag{1}$$

where F_{obs} is the observed fluorescence intensity, F_{corr} represents the corrected value, a_{ex} and a_{em} are the values of absorbance of the solution per cm at λ_{ex} and λ_{em}, respectively.

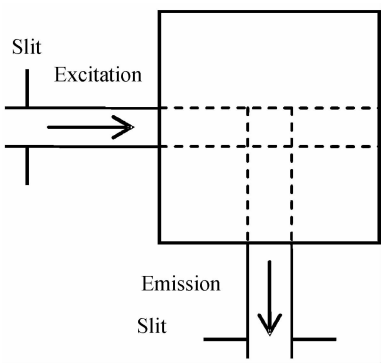

Fig. 1 Conventional ideal model for IFE correction (top view)

However, most commercial instruments adopted focusing optics to concentrate the exciting light beam. This will lead to the actual optical path away from the ideal model. The geometrical difference between real focusing beam and ideal collimated beam substantially determines that an accurate correction result for IFE could not be obtained via conventional correction method, especially under the situation with high absorbance.

Conventional correction methods only solved problem in some specific model. But it is impossible to build a model which can satisfy all type of instruments very well, because commercial spectrometers from different manufacturers have different optical parameters. Then, it is very difficult to realize precise correction on IFE by conventional methods. We advise that the problem should be solved not using any specific optical model but according to the investigation of the physical mechanism of IFE.

The IFE is induced by different mechanisms. In fluorescence quenching analysis, if the absorption spectrum of fluorophore and quencher is overlapping each other, the pIFE will arise inevitably, because the competitive absorption comes from the quencher will reduce the effective absorption of the exciting light of fluorophore and finally result in the decrease of fluorescence intensity. Different from the pIFE, the sIFE occurs in emission process if the emission spectrum of fluorophore overlaps with the absorption spectrum of quencher, which not only reduces the fluorescence intensity but also distorts the profile of emission spectrum. Usually, the fluorescence intensity of fluorophore (in pure solution) is measured firstly (marked as F_{obs1}), and the fluorescence intensity of this sample mixed with quencher (in mixed solution) is measured secondly (marked as F_{obs2}). When the inner filter effects occur, the data analysis directly using F_{obs1} and F_{obs2} will give rise to an artifact of a strong quenching efficiency. Let's have a look at the physical mechanism of IFE in mixed solution, first, the fluorophore is excited by excitation light and then emitting fluorescence (temporarily marked as F_1), due to the impact of pIFE (if it exist), F_1 will be less than F_{obs1}; second, F_1 is quenched (the fluorescence after quenching process temporarily marked as F_2); at last, due to the impact of sIFE (if it exist), F_2 will be weakened to a more smaller one, it is F_{obs2}. Obviously, the real quenching efficiency should be obtained via F_1 and F_2. However, F_1 and F_2 belong to process parameters, and can not be directly measured in experiment. According to the physical mechanism described above, we can indirectly get F_1 and F_2 via F_{obs1} and F_{obs2}.

Our correction method of pIFE and sIFE don't depend on specific optical model. In this way, the error

caused by the differences between real optical path and specific model can be avoided effectively. Further experimental study under the situation with high absorbance demonstrates that the individual correction only for IFE still cannot satisfy the needs of accurate correction. We find that the ADEL will also influence the fluorescence intensity as long as the pIFE occurs. This fact has been neglected in previous studies.

1.1 Correction on pIFE

Fluorescence intensity of a fluorophore is commonly expressed by the following equation [14]

$$F=2.3k\cdot\varphi_f(\lambda_{em})\cdot I_0(\lambda_{ex})\cdot\varepsilon(\lambda_{ex})\cdot\Delta l\cdot c \quad (2)$$

where F is the fluorescence intensity, k is an instrument parameter depending on geometrical and optical factors, $\varphi_f(\lambda_{em})$ is the quantum yield of fluorescence, $I_0(\lambda_{ex})$ is the intensity of exciting light input on the sample, $\varepsilon(\lambda_{ex})$ is the molar absorption coefficient of the fluorophore at λ_{ex}, Δl is the path length of the exciting light beam, and c is the molarity of the fluorophore.

Theoretically, the observed fluorescence intensity is proportional to the Effective Absorption of Exciting Light of Fluorophore (EAEF). So we can use another equation to describe the fluorescence intensity for convenience

$$F=\alpha\cdot k\cdot I \quad (3)$$

where α is a scale factor, k still represents the instrument parameter depending on geometrical and optical factors, and I is the EAEF.

Due to the influence of competitive absorption of quencher, the EAEF in pure solution and in mixed solution will be different, represented by I_{inP} and I_{inM}, respectively. Then the relationship for correction of pIFE can be expressed as

$$\frac{F_{corr1}}{F_{obs1}}=\frac{\alpha\cdot k\cdot I_{inM}}{\alpha\cdot k\cdot I_{inP}}=\frac{I_{inM}}{I_{inP}} \quad (4)$$

where F_{corr1} (corresponding to F_1) is the corrected intensity of pIFE.

For pure solution, I_{inP} can be calculated using the Beer-Lambert law. But for mixed solution, it is a little difficult to calculate the I_{inM}. We have developed a method to calculate I_{inM} [15]. According to literature [15], solution in sample cell is divided into many equal thin layers perpendicularly to the exciting light beam, and I_{inM} can be obtained by

$$\begin{cases} I_{inM}=I_0\cdot\dfrac{1+10^{-\Delta E_2}}{2}\cdot(1-10^{-\Delta E_1})\cdot \\ \quad\dfrac{1-10^{-n(\Delta E_1+\Delta E_2)}}{1-10^{-(\Delta E_1+\Delta E_2)}} \\ \Delta E_1=\varepsilon_1\cdot c_1\cdot\dfrac{l}{n};\ \Delta E_2=\varepsilon_2\cdot c_2\cdot\dfrac{l}{n} \end{cases} \quad (5)$$

where I_0 is the intensity of the exciting light, n is the number of thin solution layers, l is the path length of exciting light, ε_1 and ε_2 are the molar absorption coefficient of fluorophore and quencher at λ_{ex}, respectively, c_1 and c_2 are the molarity of fluorophore and quencher, respectively. The size of n will affects the accuracy of calculation results. Usually, n is equal to 100 or 1000. When $\Delta E_2<0.01$, the calculation error will be less than 1.2%.

According to Eq. (5) and Beer-Lambert law, Eq. (4) finally becomes

$$F_{corr1}=\frac{(1+10^{-\Delta E_2})(1-10^{-\Delta E_1})[1-10^{-n(\Delta E_1+\Delta E_2)}]}{2(1-10^{-n\Delta E_1})[1-10^{-(\Delta E_1+\Delta E_2)}]}\cdot F_{obs1} \quad (6)$$

where ΔE_1 and ΔE_2 are constants at λ_{ex}. With the absorption data of fluorophore and quencher, the pIFE can be corrected by Eq. (6).

1.2 Correction on sIFE

The sIFE occurs in emission process, which influences the measured spectrum F_{obs2} and can be corrected directly by the law of Beer-Lambert. When the exciting light beam inputs in the middle of the sample cell in horizontal direction, the correction equation can be expressed as

$$F_{corr2}(\lambda_{em})=10^{\varepsilon_2(\lambda_{em})c_2 l/2}\cdot F_{obs2}(\lambda_{em}) \quad (7)$$

where F_{corr2} (corresponding to F_2) is the corrected result of sIFE, and $\varepsilon_2(\lambda_{em})$ is a function of molar absorption coefficient changed with λ_{em}.

1.3 Correction on ADEL

For the situation with low absorbance, the attenuation of exciting light in sample cell can be negligible, and the intensity of emission light is equal everywhere consequently, as shown in Fig. 2 (a). In this case, the impact of ADEL can be neglected, and the response function k in Eq. (3) is treated as a constant. However, for the situation with high absorbance, the impacts of ADEL become stronger, and k is no longer a constant but a response function depending on ADEL. As shown in Fig. 2 (b), the fluorescence is not equal everywhere on the excitation path in sample cell. The ADEL depends on not only the concentration of sample but also the competitive absorption from quencher. Generally, commercial spectrometers use lenses to collect the emission light, and the optical axis is fixed at the center of sample cell. So the stronger the absorption of exciting light is, the smaller the response function k will be. In fluorescence quenching analysis, ADEL is mainly affected by the competitive absorption of quencher. Even if the fluorophore in pure solution and in mixed solution (mixed with quencher) have the same concentration, their ADEL are different, which finally affects the observed value of fluorescence intensity. Thus, when pIFE occurs, the impact of ADEL must be corrected simultaneously.

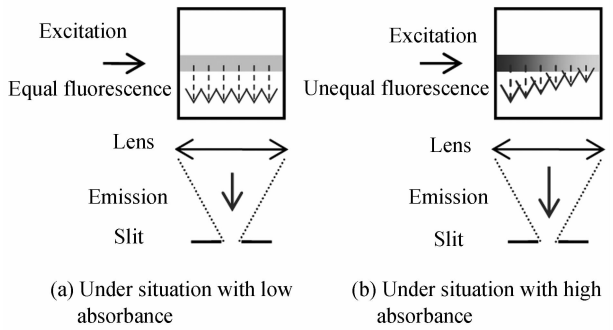

Fig. 2 Scheme of ADEL under the situation with low absorbance and high absorbance

The response function k can be obtained via experiments. Although the optical parameter of each spectrometer is not exactly the same, the response function k of each spectrometer is fixed. In order to obtain the response function k, we need a proper parameter to represent ADEL. There are many methods can be selected to express ADEL. In theory, the expression forms of ADEL do not affect the final correction result, but a reasonable choice can effectively reduce the amount of related calculation. In this paper, the solution in sample cell is divided into ten equal layers perpendicularly to the direction of exciting light, and the absorption percentage of exciting light of first layer (compared to total absorption) is used to represent ADEL. The ADEL in mixed solution can be calculated by Eq. (5). According to the rule above, the response function k can be obtained by calculating and normalizing the ratio of fluorescence intensity and effective absorption (F/I) at different concentrations. Once the response function k is obtained, the errors caused by ADEL can be corrected by

$$F_{\mathrm{corr3}}=\frac{k_2}{k_1}\cdot F_{\mathrm{corr1}} \tag{8}$$

where F_{corr3} is the corrected result of ADEL, k_1 is the corresponding value of response function related to pure solution, and k_2 is the corresponding value of response function related to mixed solution.

2 Experiment

Quinquethiophene (hereafter denoted as 5T) was selected as fluorophore. Fullerene C_{70} was selected as quencher. Fig. 3 shows the absorption spectrum of 5T and C_{70} as well as the fluorescence spectrum of 5T. Obviously, these three spectra overlap each other in a wide wavelength range. It can be predicated that the pIFE and sIFE will happen inevitably. The temperature of sample was controlled at 25 Centigrade degrees. All samples were not processed for deoxidization.

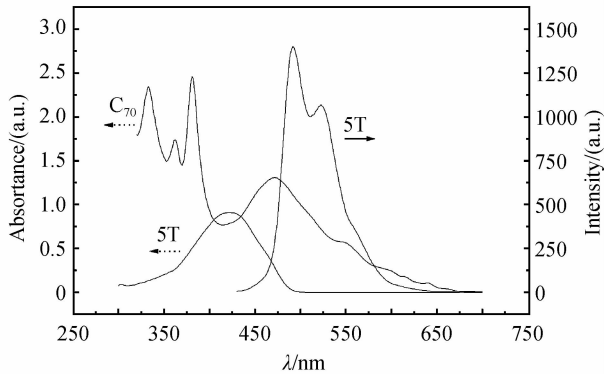

Fig. 3 Absorption spectrum of 5T (red line) and C_{70}, and fluorescence spectrum of 5T

Fullerene C_{70} (99.4%) was purchased from Beijing University, and 5T was obtained from the University of Jena. The molar concentrations of the two samples are 5×10^{-6} mol/L for 5T and 2.5×10^{-5} mol/L for C_{70}, respectively. The fluorescence and absorption spectra were measured by a fluorescence spectrophotometer (F-4500, Hitachi) and a UV-visible spectrophotometer (UV-2550, Shimadzu), respectively.

Usually, only one exciting wavelength is needed in fluorescence quenching analysis. However, we select multiple exciting wavelengths in the range from 360 nm to 450 nm in the experiment in order to investigate the impact of IFE and ADEL clearly. On the other hand, such experimental scheme is helpful for examining the corrected results of our proposed method.

3 Analysis and discussion

The fluorescence quenching between 5T and C_{70} is mainly caused by the photo-induced electron transfer[16]. According to related theory, their quenching efficiency will not be influenced by the wavelength of exciting light. In order to gain an insight into the impacts of IFE and ADEL, three kinds of methods for data processing were carried out.

First, the quenching efficiencies are obtained directly by the experimental data without any correction (the ratio of F_{obs1} and F_{obs2}), as shown in Fig. 4. These quenching efficiencies at each exciting wavelength are different, which does not correspond with the theoretical result of photo-induced electron transfer. One can find that the profile of these quenching efficiencies (solid triangle symbol) is similar to the absorption spectrum of C_{70}. The higher the absorbance of C_{70}, the larger the quenching efficiency. This phenomenon is mainly caused by IFE.

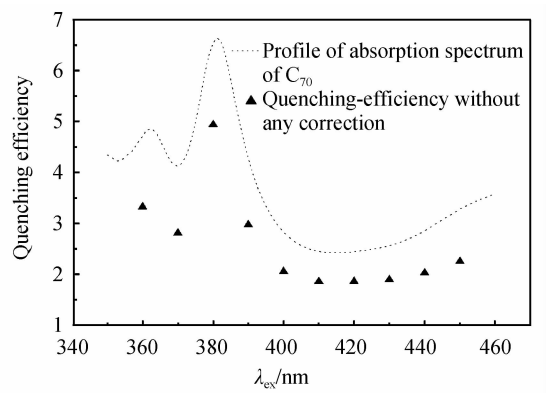

Fig. 4 Quenching efficiency of 5T/C_{70} without any correction

Then, the experimental data are corrected only for IFE. Fig. 5 shows the quenching efficiencies corrected on pIFE and sIFE (the ratio of F_{corr1} and F_{corr2}, solid rhombus symbol). Compared to previous data (hollow triangle symbol), these quenching efficiencies become lower and tend to be same. But their profile still displays a weak dependence on the absorption spectrum of C_{70}, which demonstrates that the single correction on IFE is not sufficient to remove the impact of IFE completely, especially when the exciting wavelength is in the range of high absorbance of quencher. According to the theoretical analysis described in 1.3, we can see that, except for IFE, ADEL will influence the fluorescence intensity via a potential way. So, further correction on ADEL must be carried to ensure the final accuracy of IFE correction.

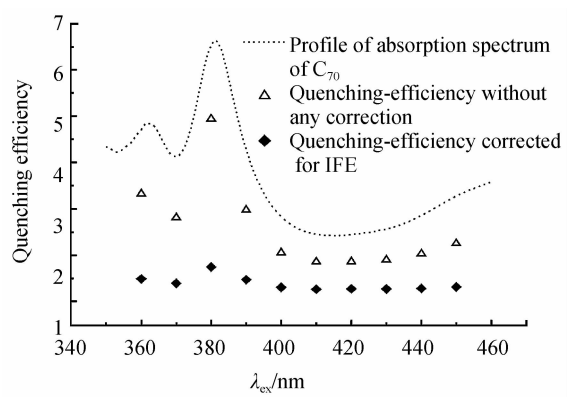

Fig. 5 Quenching efficiency of 5T/C_{70} only corrected on IFE

At last, the experimental data are corrected on both IFE and ADEL. As mentioned above, for a spectrometer, k is a fixed function only depending on its optical structure and can be obtained by experiment. The related data of k is shown in Fig. 6, where the vertical axis represents the normalized value of instrument response, and the horizontal axis represents the absorption percentage of first solution layer (as seen in 1.3). Determination on k valves should be performed carefully, which will affect the final correction results.

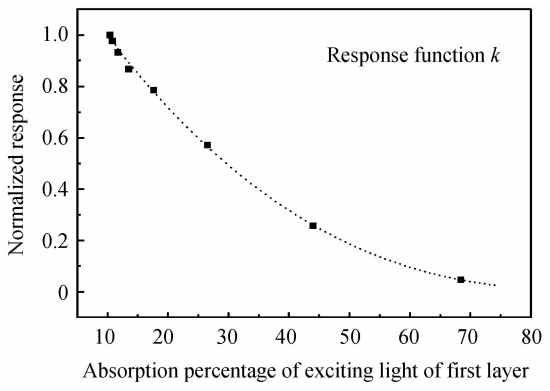

Fig. 6 Response function of spectrometer obtained by experiment

According to response function k, the impact of ADEL can be corrected by Eq. (8). The final result corrected on both IFE and ADEL (the ratio of F_{corr3} and F_{corr2}) is shown in Fig. 7. We can find that those solid dot symbols almost have no dependence on the absorption spectrum of C_{70}. The multi correction results correspond with theory very well, which means that the impact of IFE has been corrected completely.

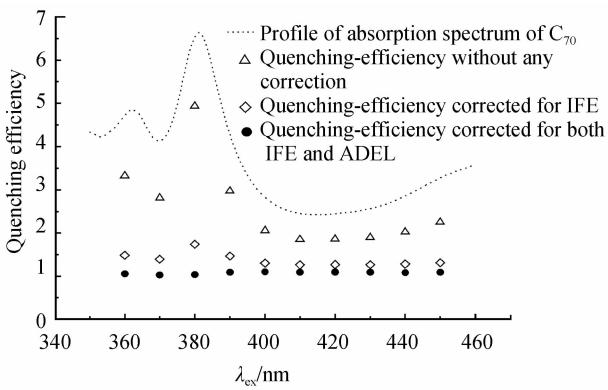

Fig. 7 Quenching efficiency of 5T/C_{70} corrected on both IFE and ADEL

Comparing Fig. 4, 5, 7, we can find that the IFE will induce a strong artifact of high fluorescence quenching if it is not corrected. Due to the potential influence of ADEL, single correction on IFE cannot ensure the correction accuracy especially under the situation with high absorbance. The quenching efficiency values single corrected for IFE do depend on the excitation wavelength, while the values multi corrected for both IFE and ADEL are independent of them. The latter is consistent with the theory of photo-induced electron transfer.

4 Conclusion

In this work, we demonstrated a new method of accurate correction on IFE. For the first time to the best of our knowledge, we find the ADEL will also influence the fluorescence intensity when the IFE occurs. So, in fluorescence quenching analysis, single

correction on IFE is not enough, the further correction on ADEL must be carried to ensure the final accuracy of IFE correction. The experimental results proved that the new method is very effective for IFE correction, even under the situation with high absorbance. This study provides researchers with a convenient way. With this multi correction method, one can easily finish the correction on pIFE, sIFE and ADEL separately or simultaneously in full spectral range.

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