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A highly sensitive resonance Rayleigh scattering method for the determination of bleomycinA_5 and bleomycinA_2 with some halofluorescein dyes

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Abstract

In a weak acidic medium, bleomycinA_5 ($BLMA_5$) and bleomycinA_2 ($BLMA_2$) can react with halofluorescein dyes such as erythrosine (Ery), eosin Y (EY), eosin B (EB) and Rose Bengal (RB) by virtue of electrostatic attraction and hydrophobic force to form ion-association complexes, which can result in the large-scale enhancement of resonance Rayleigh scattering (RRS) and the appearance of new RRS spectra. The increments of scattering intensity (ΔI) were directly proportional to the concentrations of bleomycin (BLM) in certain ranges. The detection limits for $BLMA_5$ and $BLMA_2$ ranged from 0.017 to 0.062 $\mu g\,ml^{-1}$. The Ery system had the highest sensitivity and its detection limit (3σ) was 0.017 $\mu g\,ml^{-1}$ for $BLMA_5$ and 0.018 $\mu g\,ml^{-1}$ for $BLMA_2$, respectively. Using Ery as a RRS probe, a new highly sensitive method for the determination of BLM anticancer drugs has been developed. It was applied in the determination of $BLMA_5$ and $BLMA_2$ in serum and urine samples. The recovery was from 99.0% to 103.0%. In this work, the RRS spectral characteristics of the binding products and the optimum conditions of the reaction were investigated. The mechanism of ion-association reaction and the reasons of enhancement of resonance light scattering were discussed.

Keywords: Resonance Rayleigh scattering; BleomycinA$_5$; BleomycinA$_2$; Halofluorescein dyes

1. Introduction

Bleomycin (BLM) is a class of anticancer antibiotics that can destroy duplication of DNA and is widely used for the clinical treatment of several neoplastic diseases including squamous cell carcinomas, non-Hodgkin's lymphomas, testicular carcinomas, and ovarian cancer. However, they exhibit bad renal and lung toxicity, and pulmonary fibrosis is a notable side effect [1]. Among bleomycin drugs, bleomycinA_5 ($BLMA_5$) and bleomycinA_2 ($BLMA_2$) produce the best anticancer effects and exhibit lower toxicity; hence they are used more frequently in clinical treatments. To take full advantage of these anti-tumor drugs and to decrease their toxicity, it is very necessary to quantitatively determine $BLMA_5$ and $BLMA_2$ in clinical analysis.

Many methods such as radioimmunoassay (RIA) [2,3], enzyme immunoassay (EIA) [4], microbiological assay [5], high-performance liquid chromatography (HPLC) [6–8], ultraviolet spectrophotometric (UV–vis) method [9] and oscillopolarography [10] and so on have been used for the determination of BLM. Immunoassay has high sensitivity and selectivity for BLM. However, the radioactive isotope may be harmful to one's health and its half-life limits the effective life of the instruments. The enzyme used in EIA also has a number of drawbacks such as instability, sensitivity to restrain and denaturalization. Microbiological assay needs a long time to culture bacteria. HPLC possesses high sensitivity but it also needs complex pretreatment and takes a long time to separate. UV–vis methods have been applied in the determination of BLM in ointment and suppository because of its simplicity. However, the sensitivities are not high enough to determine trace amounts of bleomycin in clinical analysis. So, it is significant to develop a more sensitive, simple and selective method for the determination of bleomycin.

Resonance Rayleigh scattering (RRS) is a new analytical technology developed in recent years and it has received much attention because of its sensitivity and simplicity. It has

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been applied to study biomacromolecules such as nucleic acids [11,12], proteins [13,14] and heparin [15,16], and has been used for the determination of some trace inorganic ions [17,18], organic compounds [19,20] and pharmaceuticals [21,22]. Our study showed that in pH 3–4 weak acidic medium, both $BLMA_5$ and $BLMA_2$ existed as univalent cation, and halofluorescein dyes such as Ery, EY, EB and RB existed as univalent anions (HL^-). They can bind with each other to form ion-association complexes by virtue of electrostatic attraction and hydrophobic force. As a result, not only the changes of absorption spectra and fluorescence quenching but also the significant enhancement of RRS intensity and new RRS spectra were observed. The scattering intensity increments (ΔI) of the binding products were directly proportional to the concentrations of bleomycin, which was the quantitative base for the determination of the two antibiotics. Among these systems, the Ery system displayed the highest sensitivity and the detection limit (3σ) was $0.017\,\mu g\,ml^{-1}$ for $BLMA_5$ and $0.018\,\mu g\,ml^{-1}$ for $BLMA_2$, respectively. The sensitivity of RRS method is several times higher than those of common UV–vis methods. In addition, the RRS method is simple, fast and selective. Therefore, this method can be used to determine the $BLMA_5$ and $BLMA_2$ in serum and urine samples.

In this study, the RRS spectral characteristics of four binding products were recorded. The optimum reaction conditions and the effects of foreign substances as well as their analytical applications were investigated. The mechanism of ion-association reaction and the reasons of the enhancement of RRS were discussed.

2. Experimental

2.1. Apparatus

A Hitachi F-2500 spectrofluorophotometer (Tokyo, Japan) was used to record the RRS spectra and fluorescence spectra and to measure the scattering intensity. A UV-VIS 8500 spectrophotometer (Tianmei, Shanghai, China) was used to record absorption spectra. A pHS-3C meter (Shanghai Dazhong Analytical Instrumental Plant, China) was used to adjust pH values.

2.2. Reagents

The stock solutions of $BLMA_5$ (Tianjing Taihe Medicine Plant, China) and $BLMA_2$ (Nippon Kayaku Co. Ltd., Tokyo, Japan) were prepared by dissolving 8.0 mg $BLMA_5$ and $BLMA_2$ in 100 ml of doubly deionized water. The stock solutions of halofluorescein dyes were all $1.0 \times 10^{-3}\,mol\,l^{-1}$ and the working solution was prepared by diluting the stock solution to $2.5 \times 10^{-4}\,mol\,l^{-1}$.

Britton–Robinson (BR) buffers were prepared by mixing $0.2\,mol\,l^{-1}$ NaOH and the mixture of $0.04\,mol\,l^{-1}$ H_3PO_4, H_3BO_3 and CH_3COOH in suitable proportions, and the pH values were adjusted with pH meter. All reagents were of analytical reagent grade and were used without further purification.

2.3. General procedures

As a first step, 1.0 ml of BR buffer solution, 0.5 ml of $2.5 \times 10^{-4}\,mol\,l^{-1}$ dye solution and appropriate amounts of bleomycin solution were added into a 10.0 ml volumetric flask. The resulting solution was diluted till the mark and then mixed thoroughly. The RRS spectra of the systems were recorded with synchronous scanning at $\lambda_{ex} = \lambda_{em}$ and the RRS intensity of the reaction product (I_{RRS}) and that of the reagent blank (I_0) were measured at the maximum RRS wavelength, $\Delta I_{RRS} = I_{RRS} - I_0$. The absorption and fluorescence spectra were also recorded.

3. Results and discussion

3.1. RRS spectra

The RRS spectra of dyes-$BLMA_5$ systems and dyes-$BLMA_2$ systems were found to be similar. Fig. 1A depicts the RRS

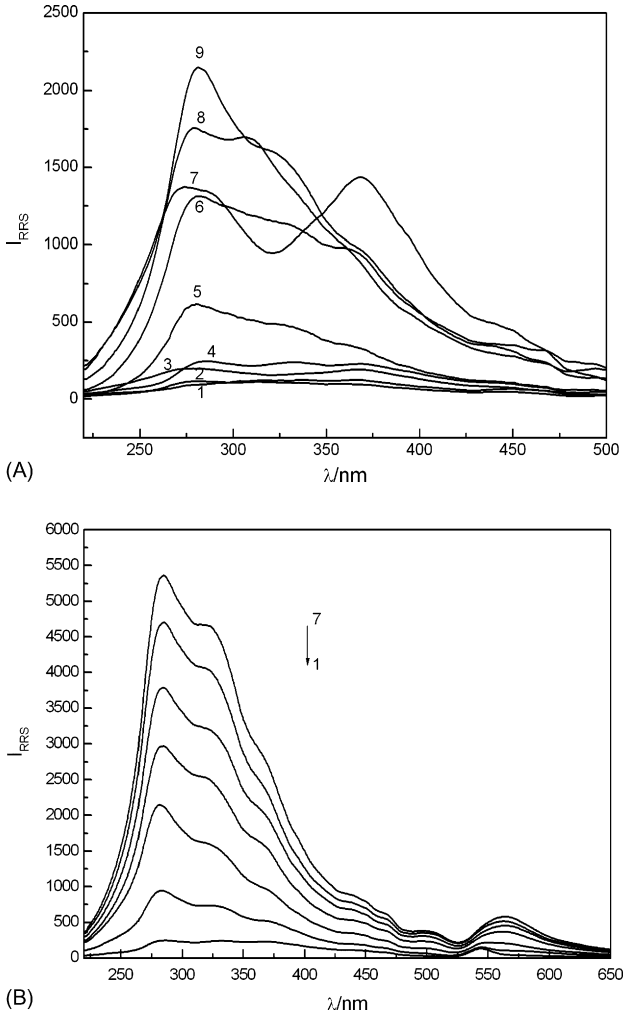


Fig. 1. RRS spectra. (A) The RRS spectra of binding products of halofluorescein dyes with $BLMA_5$. 1: $BLMA_5$, 2: EY, 3: EB, 4: Ery, 5: RB, 6: RB-$BLMA_5$, 7: EB-$BLMA_5$, 8: EY-$BLMA_5$, 9: Ery-$BLMA_5$. $C_{BLMA_5} = 0.32\,\mu g\,ml^{-1}$, $C_{dyes} = 1.25 \times 10^{-5}\,mol\,l^{-1}$. (B) The RRS spectra of binding products of Ery with $BLMA_5$ for various concentrations. 1–7: Ery-BLMA5 ($C_{BLMA_5} = 0$, 0.16, 0.32, 0.48, 0.64, 0.80, $0.96\,\mu g\,ml^{-1}$), $C_{dyes} = 1.25 \times 10^{-5}\,mol\,l^{-1}$.

spectra of dye-$BLMA_5$ systems. From Fig. 1A, it is clear that halofluoresceins have faint RRS peaks. The maximum wavelength (λ_{max}) of RRS is located at 270 nm for EY, 275 and 370 nm for EB, 280 nm for Ery and 275 nm for RB and their intensities are in the order of RB > EB > EY > Ery. When four dyes reacted with $BLMA_5$ to form ion-association complexes, RRS intensities enhance greatly. The EB system alone has two similar RRS peaks at 280 and 370 nm; all the rest of the binding products have maximum RRS peaks at 280 nm. The RRS intensity of the Ery system is the highest among the four systems, followed by the EY and EB systems, and then by the RB system. Fig. 1B shows that the enhancement of RRS intensity for the Ery-$BLMA_5$ system is directly proportional to the concentration of $BLMA_5$. So, the RRS method can be applied in the determination of $BLMA_5$. In addition, $BLMA_5$ and $BLMA_2$ have similar chemical properties, and their reaction conditions are similar; so, we decided to choose $BLMA_5$ as an example to investigate its interaction with four halofluorescein dyes.

3.2. Optimum reaction conditions

3.2.1. Effect of acidity

The effects of different buffer solutions on RRS intensities of four reaction systems were tested. The results showed that BR was better than other buffer solutions and the optimum pH ranges for the determination of $BLMA_5$ were 3.0–3.5 for EY, 3.4–4.1 for EB, 3.4–4.3 for Ery and 2.7–3.0 for RB, respectively (Fig. 2). So we chose pH 3.4 for EY, pH 2.9 for RB and pH 4.1 for Ery and EB as reaction acidity and the appropriate amount was 1.0 ml.

3.2.2. Effect of halofluorescein concentration

The experimental results showed that the RRS intensity (ΔI_{RRS}) reached maximum when the concentration of four halofluorescein dyes were 1.25×10^{-5} mol l^{-1} (Fig. 3A). If the concentrations of the dyes were lower than this, the reaction would be incomplete. On the contrary, if the concentrations were higher than this, the RRS signals would decrease because of the formation of dye dimers by self-aggregation. This was unfavorable to the ion-association reactions. Fig. 3B shows the absorption spectra of Ery. The dye concentrations varied from 5×10^{-6} to 1×10^{-3} mol l^{-1}. In water, when the concentration of Ery was 5×10^{-6} mol l^{-1}, λ_{max}^{abs} for Ery was 525 nm with a shoulder at 490 nm. As the concentration increased, the intensity of the shoulder peak at 490 nm increased. This was attributed to the existence of the dimers involving stacked monomers [23]. So the experiment concentration was 1.25×10^{-5} mol l^{-1} for halofluorescein dyes.

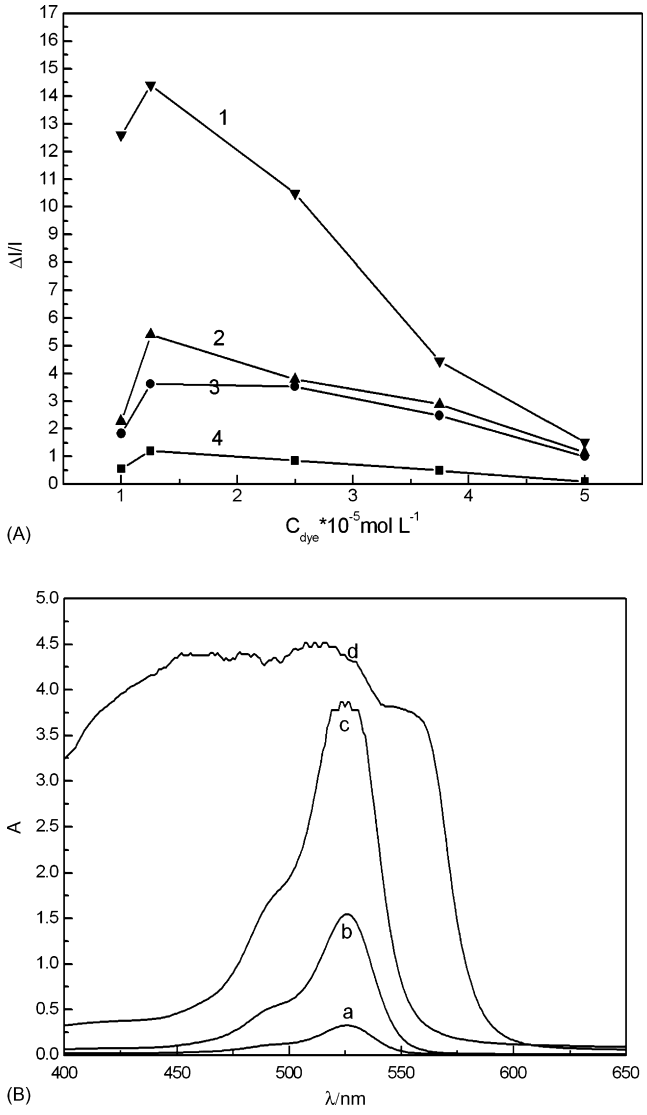


Fig. 3. Effect of concentration of dyes. (A) The RRS spectra. 1: Ery–$BLMA_5$, 2: EY-$BLMA_5$, 3: EB-$BLMA_5$, 4: RB-$BLMA_5$, $C_{BLMA_5} = 0.32$ μg ml^{-1}. (B) The absorption spectra of erythrosin C_{Ery} (a–d): 5×10^{-6}, 2×10^{-5}, 1×10^{-4}, 1×10^{-3} mol l^{-1}.

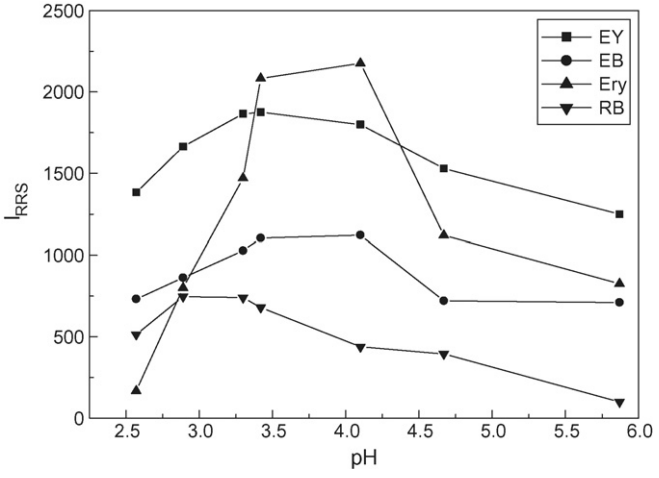


Fig. 2. Effect of acidity. $C_{dye} = 1.25 \times 10^{-5}$ mol l^{-1}, $C_{BLMA_5} = 0.32$ μg ml^{-1}.

3.2.3. Effect of ionic strength

The effect of ionic strength on the RRS intensity was investigated with NaCl solution (see Fig. 4). The RRS intensities decreased as the ionic strength increased. When [NaCl] = 0.10 mol l^{-1}, ΔI_{RRS} decreased 23.7% for the Ery system, 20.3% for the EY system, 52.2% for the EB sys-

Table 1
Related parameters for the calibration graphs and the detection limits for $BLMA_5$ and $BLMA_2$

System	Regression equation	Correlation coefficient (r)	Linear range ($\mu g\,ml^{-1}$)	Detection limit ($\mu g\,ml^{-1}$)
EY–$BLMA_5$	$\Delta I = 96.2 + 4292c$	0.9974	0.063–1.0	0.019
EB–$BLMA_5$	$\Delta I = -8.9 + 2753c$	0.9968	0.11–0.80	0.032
Ery–$BLMA_5$	$\Delta I = -7.04 + 5242c$	0.9966	0.056–1.2	0.017
RB–$BLMA_5$	$\Delta I = 95.6 + 1365c$	0.9986	0.21–1.2	0.062
EY–$BLMA_2$	$\Delta I = -12 + 4238c$	0.9985	0.064–0.80	0.019
EB–$BLMA_2$	$\Delta I = 15.2 + 3840c$	0.9950	0.076–0.40	0.023
Ery–$BLMA_2$	$\Delta I = 8.6 + 4777c$	0.9992	0.061–0.80	0.018
RB–$BLMA_2$	$\Delta I = -18.7 + 1796c$	0.9970	0.16–1.6	0.047

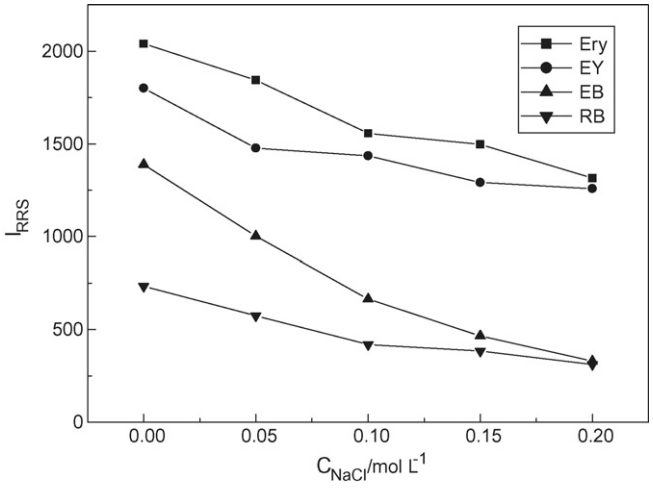


Fig. 4. Effect of ionic strength. $C_{dye} = 1.25 \times 10^{-5}\,mol\,l^{-1}$, $C_{BLMA_5} = 0.32\,\mu g\,ml^{-1}$.

tems and 42.9% for the RB system, respectively. So, the ion-association reaction should be under a low ionic strength condition. However, when $[NaCl] < 0.05\,mol\,l^{-1}$ the effect of ionic strength on the Ery system was smaller than on the rest of the systems. Therefore, the Ery system was found to be more suitable for the determination of BLM in some biologic samples.

3.3. Sensitivity of the method

Under optimum conditions, $BLMA_2$ and $BLMA_5$ reacted with four halofluorescence dyes to form complexes and RRS intensities were measured at their maximum wavelengths, separately. The calibration graphs of ΔI_{RRS} versus the concentration of BLM were constructed. Their linear ranges, correlation coefficients and detection limits are listed in Table 1. It was evident that the four halofluorescein dyes could be applied in the determination of BLM. Among the four systems, the Ery system had the highest sensitivity. The detection limits (3σ) for $BLMA_5$ and $BLMA_2$ were 0.017 and 0.018 $\mu g\,ml^{-1}$, respectively. Compared with those of common spectrophotometric methods and HPLC methods for the determination of BLM, the sensitivity of the RRS method was higher by two order of magnitude (see Table 2). This method proved to be more suitable for the determination of trace amounts of bleomycin.

3.4. Selectivity of the method

Taking the Ery-$BLMA_5$ system as an example, under optimum conditions, the effects of some coexisting substances on the determination were tested. The results are listed in Table 3. It can be seen that when the concentration of $BLMA_5$ is 0.8 $\mu g\,ml^{-1}$, 2000–8000 times of common metal ions such as K^+, Na^+, Mn^{2+}, NH_4^+, and Ca^{2+}, inorganic radical ions such as Cl^-, NO_3^-, SO_4^{2-}, Br^-, 3000–4000 times of saccharine, 50–125 times of amino acid, 15–38 times of HSA and BSA and 15–36 times of Cu^{2+}, Fe^{3+}, Mg^{2+} and Al^{3+} do not interfere with determination. So the selectivity of the method is good and the method can be applied to determine $BLMA_5$ and $BLMA_2$ in practical samples.

3.5. Analytical application

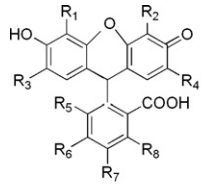
First, 2.0 ml of fresh serum sample (healthy human) and 4.0 ml of trichloroacetic acid were mixed thoroughly and centrifuged at 3000 rpm for 15 min and then 1.0 ml of supernatant fluid was diluted to 100.0 ml. Then, 1.0 ml of this solution was pipetted into a 10.0 ml volumetric flask and the concentrations of $BLMA_5$ and $BLMA_2$ were determined according to the general procedure. The recovery was tested by the standard addition method.

Subsequently, 5.0 ml of fresh urine sample (healthy human) was filtrated and 1.0 ml of filtrate was diluted to 10.0 ml. Then 1.0 ml of this solution was pipetted into a 10.0 ml volumetric flask and the concentrations of $BLMA_5$ and $BLMA_2$ were determined according to the general procedure. The recovery was tested by the standard addition method.

Next, 8.0 mg of $BLMA_5$ was injected into the vein of a rabbit. In 30 min, 5.0 ml of blood sample was phlebotomized and centrifuged at 3000 rpm for 15 min, then supernatant serum was pipetted and mixed with trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 15 min to eliminate protein. Excessive trichloroacetic acid and other organic impurities were removed by extraction using a ether. Finally, 0.5 ml of this solution was pipetted into a 10.0 ml volumetric flask and the concentration of $BLMA_5$ was determined according to the general procedure. The recovery was tested by the standard addition method. The results are listed in Table 4.

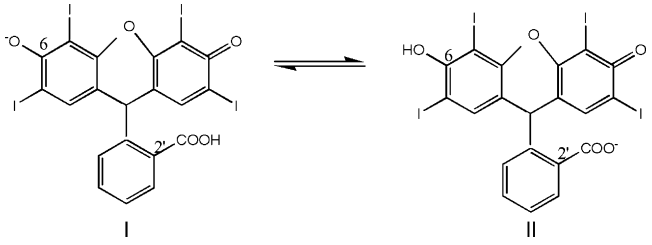
Table 2
Comparison of sensitivities of some methods for the determination of bleomycin

Method	Detect object	Detect limits ($\mu g\,ml^{-1}$)	Liner range ($\mu g\,ml^{-1}$)	References	Method	Detect object	Detect limits ($\mu g\,ml^{-1}$)	Liner range ($\mu g\,ml^{-1}$)	References
RIA	Bleomycin sulfate	0.008	0.02–0.1	[2]	Derivative spectrophotometry	$BLMA_5$	3	10–70	[9]
EIA	BLMs	25 pg/tube		[4]	UV-spectrophotometry	$BLMA_5$	8	25–88	[31]
HPLC	$BLMA_2$	0.02	0.05–5	[7]	UV-spectrophotometry	$BLMA_5$	5	15–39	[29]
Paired-ion HPLC	$BLMA_2$	2	5–50	[6]	UV-spectrophotometry	$BLMA_5$	10	30–70	[30]
Ion-pair RP-HPLC	BLMB2, $BLMA_2$	33	100–2000[1]	[27]	Oscillopolarography	$BLMA_5$	0.013	0.065–520	[10]
Bioassay	Bleomycin hydrochloride	3	10–40	[28]	RRS	$BLMA_5$, $BLMA_2$	0.017, 0.018	0.056–1.2, 0.061–0.80	This method



dyes	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Erythrosin	I	I	I	I	H	H	H	H
EosinY	Br	Br	Br	Br	H	H	H	H
EosinB	Br	Br	NO_2	NO_2	H	H	H	H
Rose Bengal	I	I	H	I	Cl	Cl	Cl	Cl

Scheme 1. Structure of halofluorescein dyes.



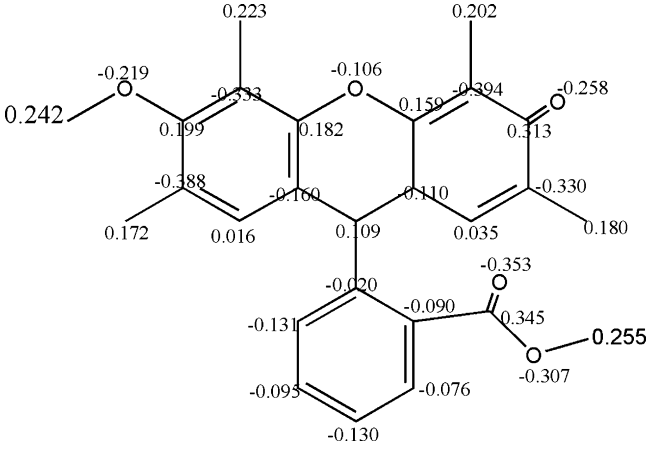
Scheme 2. Two species of HL^-.

3.6. The ion-association reaction of BLMAs with halofluorescein dyes

Ery, EY, EB and RB belong to an important class of acidic xanthenes dyes. Scheme 1 shows their molecular structures. Taking the $BLMA_2$–Ery system as an example, we investigated the interaction of halofluorescein dyes with BLM. The composition ratio of the ion-association complex for $BLMA_2$–Ery was 1:1, established by using Job's method of continuous variation and molar ratio method.

In pH 3–4 weak acidic medium, Ery mainly exists as a univalent anion (HL^-) [24,25], which has two possible species (see Scheme 2).

We use AM1 [26] method of quantum chemistry to calculate the negative charge distribution on the oxygen atom of 6-OH and 2′-COOH of Ery molecule (H_2L) (see Scheme 3) as −0.219 (for 6-OH) and −0.307 (for 2′-COOH), respectively. It shows that the hydroxyl at C-6 dissociates prior to the carboxyl group at C-2′. We also calculate their dissociation enthalpy, which is −285.5 kJ mol^{-1} (for 6-OH) and −165.5 kJ mol^{-1} (for 2′-COOH), respectively. The result shows that the species (*I*)



Scheme 3. Charge distribution of Ery molecule (H_2L).

Table 3
Effects of coexistent substances ($BLMA_5$ concentration: 0.8 μg ml^{-1})

Foreign substances	Ration foreign substance/$BLMA_5$	Change of RRS (%)	Foreign substances	Ration foreign substance/$BLMA_5$	Change of RRS (%)
DL-Aspartic acid	125	+2.5	K^+	8000	+2.5
L-Tyrosine	60	−4.0	Mg^{2+}	3, 15[a]	+5.0
D-Tryptophan	50	−4.3	NH_4^+	7000	+5.0
Glycin	70	−3.3	Mn^{2+}	1500	−1.5
Phenylalanine	125	−5.0	Na^+	4000	−2.4
Glucose	4600	−5.0	Cu^{2+}	4	+1.5
Sucrose	3000	−5.0	Ca^{2+}	3500	+5.0
Maltose	3000	−5.0	Fe^{3+}	5	+5.0
Lactose	3000	−5.0	Al^{3+}	36	+4.0
HAS	15	+4.0	Ba^{2+}	857	−5.6
BSA	38	+5.0	Cl^-	8000	+2.5
Urea	1500	+4.0	Br^-	5000	−1.0
NO_3^-	2000	+5.0	SO_4^{2-}	2000	−2.4

[a] The allowable ratio of foreign substance after adding sodium fluoride.

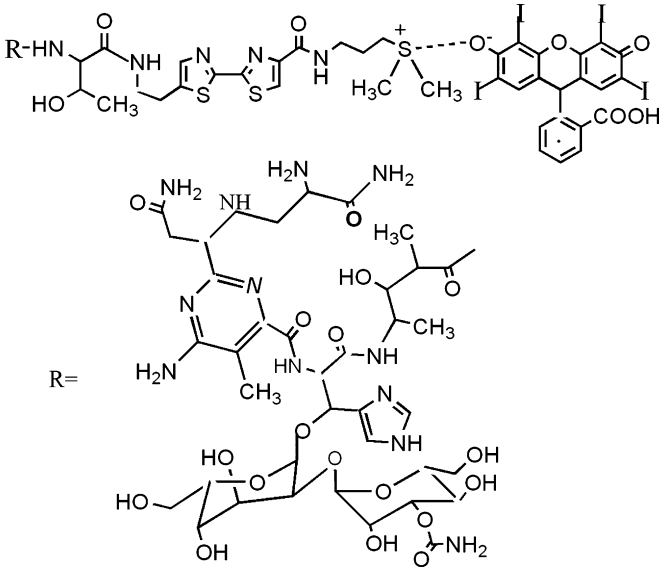
derived from the dissociation of 6-OH is more stable. So, in weak acidic medium, Ery mostly exists as a univalent anion species (*I*).

Under the same condition, the –S $(CH_3)_2$ groups connecting to the bithiazole moiety of DNA binding domain in $BLMA_2$ are positively charged and $BLMA_2$ exists as a univalent cation. Therefore, the structure of the binding product of $BLMA_2$ with Ery may be as follows (see Scheme 4).

3.7. Reasons for RRS enhancement

3.7.1. Resonance enhanced Rayleigh scattering

RRS is a scattering–absorption–rescattering process produced by the resonance of scattering and absorption when the wavelength of Rayleigh scattering is located at or is close to its molecular absorption band. In this case, the frequency of the electromagnetic wave absorbed by the electron is equal to that of scattering. Thus, it is certain that RRS spectral characteristics are closely related to the corresponding absorbance spectra. Fig. 5 shows the comparison of the RRS spectrum with the absorption spectrum of the $BLMA_5$–Ery complex. It is clear from Fig. 5 that the RRS spectrum is located at its absorption band and two RRS peaks (280 and 525 nm) are close to the corresponding absorption peaks (270 and 527 nm). Therefore, scattering intensity remarkably increases due to the resonance enhanced Rayleigh scattering effect.



Scheme 4. Molecular structure of $BLMA_2$–Ery complex.

3.7.2. Effect of polarization

According to the RRS formula deducted by Stanton:

$$I = \frac{16\pi^4 \rho_N I_0}{\lambda^4 r^2} |\bar{\alpha}|^2 \quad (1)$$

Table 4
Results for the determination of $BLMA_5$ and $BLMA_2$ in serum and urine samples

Sample	Found (μg ml^{-1})	Added	Added (μg ml^{-1})	Total found mean (n = 5) (μg ml^{-1})	R.S.D. (n = 5) (%)	Recovery (%)
Serum No. 1	ND[a]	$BLMA_2$	0.16	0.16	5.0	99
Serum No. 2	ND	$BLMA_5$	0.24	0.25	1.8	103
Urine No. 1	ND	$BLMA_2$	0.40	0.40	0.8	101
Urine No. 2	ND	$BLMA_5$	0.48	0.47	1.1	99
Rabbit serum[b]	0.32	$BLMA_5$	0.40	0.73	1.6	102

[a] ND: not detected.
[b] Detected after injection 30 min.

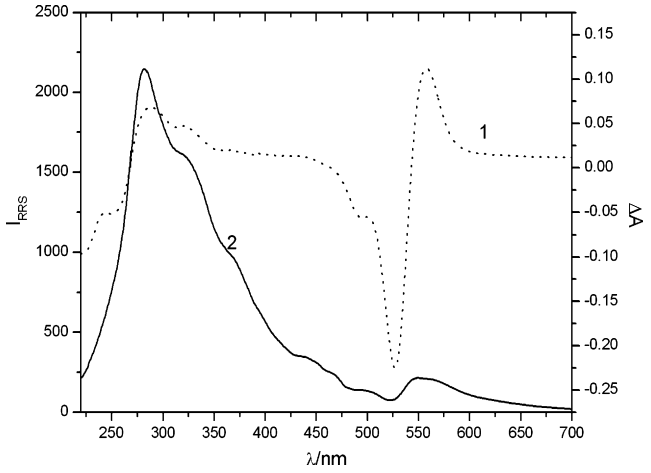


Fig. 5. Comparison with absorption and RRS spectra of $BLMA_5$–Ery complex $C_{dye} = 1.25 \times 10^{-5}$ mol l^{-1}, $C_{BLMA_5} = 0.32$ μg ml^{-1}. 1: absorption spectrum (measured against reagent blank), 2: RRS spectuum.

where ρ_N is the molecular density, I_0 the intensity of the incident light, λ the incident wavelength in the medium, r the distance from the molecule to the observer and $\bar{\alpha}$ is the molecular polarization ($\bar{\alpha}$ is composed of real part and imaginary part). It shows that when other parameters are constant, the scattering intensity is directly proportional to the polarization. So, the great increase of polarization is one of the important factors for the enhancement of RRS. According to the results calculated by the AM1 method, the average polarization of Ery^- and $BLMA_2^+$ is 335.15 and 720.69 a.u., respectively, while the average polarization of their binding product is 1134 a.u. Thus the remarkable increase of the average polarization after the interaction is a significant reason for the enhancement of RRS.

3.7.3. Enhancement of hydrophobicility

Before interaction, either BLM cation or halofluorescein anion has strong hydrophility and is easy to form hydrate. When they react with each other to form an ion-association complex, the hydrophobility enhances due to the neutralization of charges and the appearance of the liquid–solid interface. The formation of hydrophobic interface is profitable to the enhancement of RRS.

3.8. The energy transfer between light absorption, fluorescence and RRS

The formation of ion-association complex between BLM and halofluorescein results in the decrease of absorbance of halofluorescein dyes and the quenching of fluorescence. Figs. 6 and 7 show the absorption and fluorescence spectra of the $BLMA_5$–Ery system with the different concentrations of $BLMA_5$.

The relations between ΔA, ΔF and ΔI_{RRS} are shown in Fig. 8.

RRS is produced by the resonance of the Rayleigh scattering and light absorption with the same frequency. In this process, the enhancement of the scattering results from the absorbed light energy. Namely, part of the absorbed energy is transferred to the

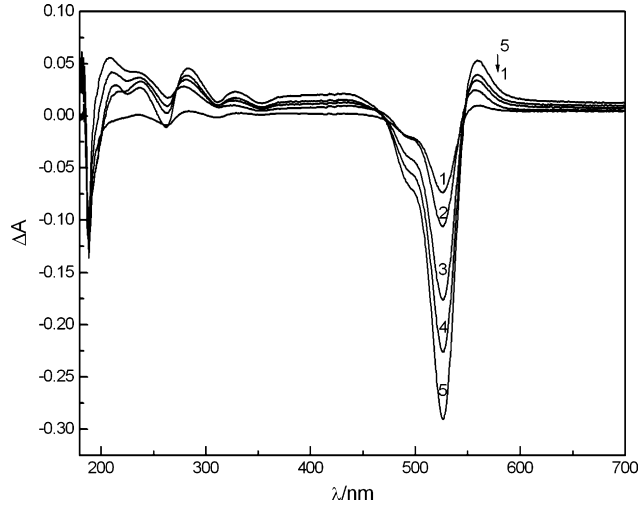


Fig. 6. Absorption spectra. [$BLMA_5$]: 1–5: 0.32, 0.48, 0.64, 0.80, 0.96 μg ml^{-1}, $C_{dyes} = 1.25 \times 10^{-5}$ mol l^{-1} (measured against reagent blank).

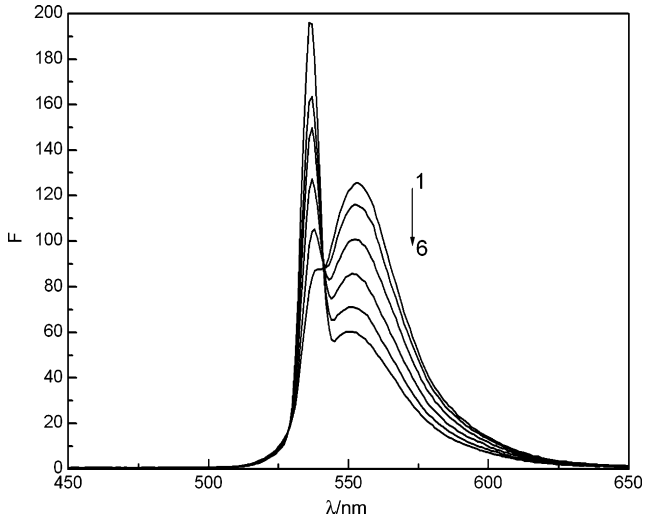


Fig. 7. Fluorescence spectra. 1: Ery, 2–6: $BLMA_5$–Ery system, [$BLMA_5$]: 0.4, 0.8, 1.2, 1.6, 2.0 μg ml^{-1}, respectively. $\lambda_{ex} = 535$ nm, slit width: 5 nm. $C_{dye} = 1.25 \times 10^{-5}$ mol l^{-1}.

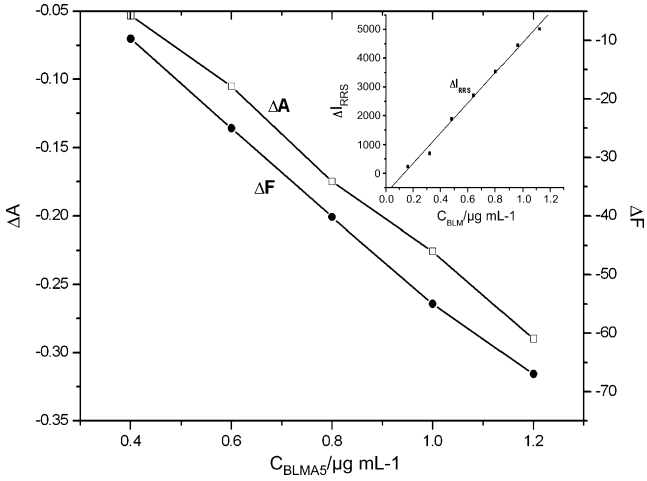


Fig. 8. Relationship of ΔA, ΔF and ΔI_{RRS} for $BLMA_5$–Ery ion-association complex. $C_{dye} = 1.25 \times 10^{-5}$ mol l^{-1}.

scattering by the resonance effect. So, the enhancement of the RRS and the decrease of absorbance occur simultaneously.

In the other side, molecules absorbed the energy and then released energy, the sum of the absorbed energy (E_A) should be equal to the sum of emission (fluorescence) energy (E_L), the scattering energy (E_{RLS}) and the non-radiative energy (E_N), that is,

$$E_A = E_L + E_{RLS} + E_N \quad (2)$$

Usually, the resonance light scattering is ignored in a transparent solution of small molecules. The fluorescence quenching is commonly ascribed to the energy transfer from fluorescence to the non-radiative energy (E_N). However, in some big molecules or hydrophobic ion-association complex systems, the scattering cannot be ignored. When the scattering is located at the fluorescence spectral band, energy transfer occurs between scattering and fluorescence. In such a condition, fluorescence quenching is not only ascribed to the transfer from radiative energy to non-radiative energy but also to the transfer from the fluorescence to the scattering, that is, part of the fluorescence is transferred to the light scattering by their resonance effect. It can be seen from Fig. 8 that the enhancement of RRS, the decrease of absorbance and the quenching of fluorescence occur synchronously.

4. Conclusions

In weak acidic medium, the antibiotics $BLMA_5$ and $BLMA_2$ react with halofluorescein dyes by virtue of electrostatic attraction and hydrophobic force to form 1:1 ion-association complexes. As a result, the RRS intensities of complexes enhance remarkably. The increments (ΔI) are directly proportional to the concentrations of bleomycin in a certain range. A new RRS method for determination of $BLMA_5$ and $BLMA_2$ has been developed. This highly sensitive, simple and fast method can be applied to determine trace amounts of $BLMA_5$ and $BLMA_2$ in serum and urine samples.

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