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ORIGINAL ARTICLE

Spectra, Stability and Labeling of a Novel Carbazole Derivative as a Fluorescent Turn-on DNA Probe

Xuening Fei[1,2] · Ran Li[1] · Dayong Lin[2] · Yingchun Gu[1,3] · Lu Yu[1]

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Abstract A novel fluorescent dye, 1-(6-carboxyhexyl)-4-(2-(9-ethyl-carbazole-3-yl) vinyl) quinolizinium bromide, was synthesized, and its structure was characterized by ^{1}H NMR, ^{13}C-NMR, IR and HRMS. The spectra properties of this dye in different solvents and under different pH value were invest'igated preliminarily, and the results showed that its fluorescent properties was affected by the polarity and the dipole moment of the solvent. The photostability and thermostability test results showed that its photoreduction rate constant was 1.64×10^{-5} mol/Min and its fluorescent intensity decreased little after heating at 80 °C for 6 h, suggesting the dye was quite stable. In the labeling experiment of BSA and DNA with the dye, the fluorescent all intensity increased with the addition of BSA and DNA. Specially, the dye showed an excellent turn-on effect upon binding with *ct*DNA.

Keywords Spectra properties · Fluorescent probe · DNA · Binding constant

✉ Yingchun Gu
jvgugugu@126.com; xueningfei@126.com

✉ Lu Yu
yulu@tcu.edu.cn

[1] Tianjin Chengjian University, No. 26 Jinjing Road, Xiqing District, Tianjin 300384, China

[2] Tianjin University, Tianjin 300072, China

[3] Institute of Chemistry Chinese Academy of Sciences, Beijing 100190, China

Introduction

Over the past decade, fluorescent dyes are used widely in bioimaging, especially for DNA detection [1, 2] due to the significant advantages of fluorescent dyes, such as relative stability, low aggregation and high affinity for some biological molecules [3–5]. However, in order to decrease the fluorescent background interference caused by the labeled organism, the stronger fluorescent intensity is required [6, 7]. It means the dye should exhibit a dramatic enhancement in fluorescent intensity upon binding to DNA and could be used as a turn-on fluorescent probe for DNA detection. So to design novel small molecules and study the interaction with DNA, to some extent, is one of the most important goals in modern medicinal and biological chemistry.

A number of dyes have been synthesized and applied successfully in fluorescent imaging technique and the detection of DNA in solution [8–10]. Some investigators believe that the positively charged heterocycles play an important role in the enhancement of the fluorescent intensity [11–13]. Thus, lots of positively charged dyes, such as benzooxazolinium, benzothiazolinium and quinolizinium derivatives have been successfully developed as fluorescent probes for DNA detection [14]. In this paper, we designed and synthesized a novel carbazole-based quinolizinium derivative. The probe exhibited very weak fluorescence in 10 mM Tris–HCl buffer (pH= 7.4), and showed excellent turn-on effect upon binding with calf thymus DNA hopefully. In addition, the absorption and fluorescence spectra in different solvents and under different pH value in aqueous solution were investigated preliminarily. The stabilities including photostability and thermostability were also studied. Finally, in order to assess its potential as DNA markers, the labeling properties of the dye with BSA and calf thymus DNA were compared.

Experimental

General

All chemical reagents and solvents were purchased and used without further purification. Fluorescence spectra were scanned on a Hitachi F-7000 spectrometer. The excitation and emission slits were 5 nm and the PTM voltage of the spectrometer were 700 V. The UV/Vis absorption spectra were recorded on a Perkin Elmer LAMBDA 35 spectrometer. ^{1}HNMR and ^{13}CNMR spectra were recorded on a Bruker (400 MHz) spectrometer. Chemical shifts were reported in parts per million (ppm) down field from TMS (tetramethylsilane) using DMSO-d6 as a solvent. HRMS spectra were recorded on a microTOF QII ESI-Q-TOF spectrometer. IR (KBr pellets) spectra were recorded on a Brucker Equinox-55 spectrometer. All chemicals were of analytical grade. The chemical purity is analyzed by HPLC on Laballiance PC4006A from America and the flow rate was 1.0 mL/min (4.6×250 mm, 5 μm, C18 reversed-phase column).

Preparation of Stock Solutions

For absorption or fluorescence measurements, DMSO were used for the solvent to obtain stock solutions (10 mL), then the stock solutions were diluted by aqueous solutions to the desired concentration (5×10^{-6} mol/L).

BSA stock solutions were prepared by dissolving the sample directly into PBS (pH=7.7) and then various amounts of stock solutions were used for dye-protein complexes. Finally, the dye was diluted to the desired concentration.

Calf thymus DNA were purchased from Sigma Chemical Co. and dissolved in 10 mM Tris–HCl buffer (pH=7.4). The purity of DNA was checked by monitoring the absorption spectrum and the ratio of the absorbance at 260 and 280 nm, A_{260}/A_{280}=1.83. The result was consistent with Ref [15]. The DNA concentration in Tris–HCl buffer were determined by the molar extinction coefficient ε=6600 M^{-1} cm^{-1} at 260 nm [16, 17].

Fluorescence Quantum Yields

The fluorescence quantum yields of the compound were determined by comparison with a standard solution with the following equation [18] to calculate relative fluorescence quantum yields:

$$\phi_X = \phi_S \cdot \left(\frac{n_X}{n_S}\right)^2 \cdot \frac{A_S}{A_X} \cdot \frac{F_X}{F_S}$$

In which the n_X and n_S are the refractive indexes of the sample and reference, F_X and F_S are the area of fluorescence spectra for the sample and reference respectively, and A_x and A_S are the absorbance for the sample and reference at the excitation wavelength respectively. The standard used in this study is Fluorescein in NaOH (0.1 mol/L) aqueous solution with a fluorescence quantum yield of 0.90 [19].

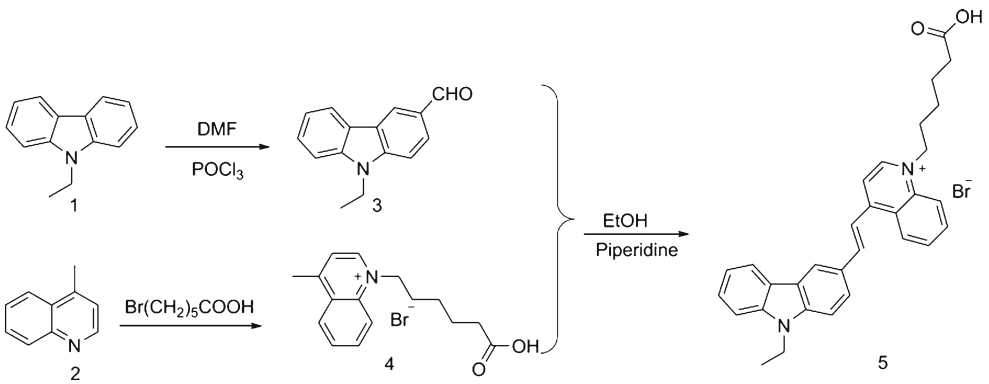
Synthesis

The detailed synthesis of compounds (3–5) and the illustration were shown in Scheme 1.

Synthesis of compound 3. The synthesis procedure of compound 3 was based on the Ref [20].

Synthesis of compound 4. Lepidine (2 g, 0.014 mol) and 6-Bromohexanoic acid (4.1 g, 0.021 mol) were stirred 6 h at 130 °C. After cooling down to room temperature, 20 mL of acetone was added to the reaction flask. The compound was yielded by filtration and washed with acetone several times. Yield: 85 %. Purity: 98.9 % (HPLC), ^{1}H NMR (400 MHz, DMSO-d6) δ=1.39–1.44 (m, 2H), 1.52–1.57 (m, 2H), 1.92–2.01 (m, 2H), 2.19–2.23 (t, J=9.60 Hz, 3H), 3.01 (s, 3H), 5.00–5.05 (m, 2H), 8.05–8.09 (t, J=8.0 Hz, 2H), 8.40 (d, J=11.60 Hz, 1H), 8.60 (d, J=11.20 Hz, 2H), 9.57 (d, J=8.00 Hz, 1H). ^{13}C-NMR (400 MHz, DMSO-d6) δ=19.98, 24.37, 25.76, 29.66, 33.89, 57.19, 119.87, 123.15, 128.37, 130.07,

Scheme 1 General procedure for the synthesis of compounds 3–5



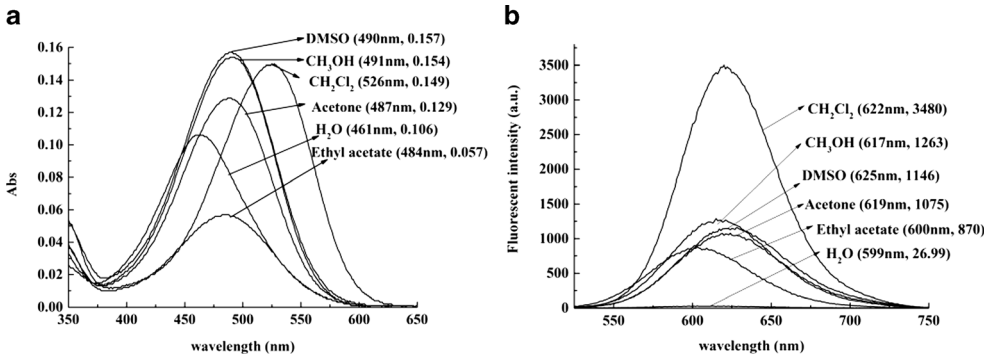


Fig. 1 The UV–vis spectra (**a**) and fluorescence spectra (**b**) of the dye in different solvent (5×10^{-6} mol/L)

134.57, 135.59, 137.21, 148.90, 159.03, 174.74. FTIR (KBr 1 %) ν_{max}=3433, 2939, 2563, 1724, 1616, 1602, 1529, 1406, 1371, 1232, 1209, 1161, 1055, 966, 856, 827, 763 cm^{-1}.

Synthesis of compound 5. A solution mixture of 3 (0.2 g), 4 (0.26 g) and piperidine (2 drops) in ethanol (30 mL) was heated to reflux overnight. After cooling down to room temperature, the organic solvent was removed by rotary evaporation. The residue was purified by silica-gel column chromatography to get compound 5. Yield: 30 %. Purity: 98.5 % (HPLC). ^{1}H NMR (400 MHz, DMSO-d_6) δ=1.39–1.52 (m, 5H), 1.60 (t, J=7.0Hz, 2H), 1.98–2.01 (m, 2H), 2.11 (t, J= 6.60 Hz, 2H), 4.53–4.58 (m, 2H), 4.99 (t, J=6.40 Hz, 2H), 7.36 (t, J=7.40 Hz, 1H), 7.58 (t, J=7.60 Hz, 1H), 7.73 (d, J= 4.20 Hz, 1H), 7.80 (d, J=4.40 Hz, 1H), 8.08 (t, J=7.60 Hz, 1H), 8.17 (d, J=4.20 Hz, 1H), 8.28 (t, J=7.80 Hz, 2H), 8.43 (q, J=24.80 Hz, 2H), 8.55 (d, J=4.60 Hz, 2H), 8.90 (s, 1H), 9.16 (d, J=4.40 Hz, 1H), 9.41 (d, J=3.00 Hz, 1H),11.80 (s, 1H). ^{13}C-NMR (400 MHz, DMSO-d6) δ=14.28, 24.64, 25.97, 29.57, 34.53, 37.78, 56.61, 110.24, 110.40, 115.75, 116.69, 119.52, 120.25, 121.11, 122.45, 122.78, 123.37, 126.98, 127.20, 127.33, 128.11, 129.30, 135.46, 138.32, 140.69, 141.78, 145.67, 147.39, 153.70, 175.45. FTIR (KBr 1 %) ν_{max}=3446, 2945, 2360, 1716, 1614, 1585, 1564, 1488, 1438, 1334, 1286, 1236, 1166, 1134, 960, 821, 748 cm^{-1}. HRMS (m/z): [M-Br]+ calcd for 463.2381, found 463.2380.

Results and Discussion

Spectra Properties of Compound 5

The Spectral Properties of Compound 5 in Different Solvent

In order to study the effect of different solvents on the UV–visible spectra properties and fluorescence spectra properties, the stock solutions of compound 5 were diluted to different solvents. The spectra results were shown in Fig. 1 and the spectral data were summarized in Table 1.

From Fig. 1a we can see the wavelength of compound 5 in CH_2Cl_2 was 526 nm, while the wavelength in other solvents were all less than 500 nm and the wavelength in H_2O was the lowest. In aprotic polar solvents, the maximum absorption wavelength of compound 5 was red-shifted with the increasing of solvent polarity. We can find that the absorbing intensity was stronger for the solvent with higher polarity, which is because the energy of the anti-bonding orbital and bonding orbital of the molecular are both reduced for the solvent with higher polarity.

The fluorescence spectra in different solvents were also recorded at the λ_{ex} of 490 nm and the results were shown in Fig. 1b and Table 2.

Figure 1b and Table 2 indicated that the compound possesses different maximum emission wavelength (λ_{em}) in different solvents, and the largest λ_{em} is obtained in CH_2Cl_2 solvent. In protic solvent, the λ_{em} is blue-shifted and fluorescent intensity decreased evidently with the increasing of the solvent polarity and it was the lowest in water solution. The possible reason is that the aggregation of compound 5 in water solution, thereby resulting in the increase of the nonradiative decay process and finally the obviously quenching occurred. In addition, with the increasing of the dipole moment and solvent polarity in aprotic solvents we could discover that the λ_{em} was red-shifted. The possible reason is that the solvent with larger polarity could result in the reduction of energy difference between excited state and ground state, which lead to a red shift of the dye.

We can see from Table 2 that the stokes shift in different solvents were different. The stokes shift were influenced by

Table 1 The solvent properties and UV–vis characters of compound 5

Solvent	nD^{20} [a]	f(n) [b]	λ_{max}/nm	ν [c]/10^{-3} cm^{-1}
CH_3OH	1.33	0.169	491	2.037
H_2O	1.34	0.173	461	2.169
Acetone	1.36	0.181	487	2.053
Ethyl acetate	1.37	0.184	484	2.066
CH_2Cl_2	1.42	0.202	526	1.901
DMSO	1.48	0.221	490	2.041

[a] Refractive index

[b] $f(n)=(n^2-1)/(2n^2+1)$

[c] $\nu=1/\lambda_{max}$

Table 2 The solvent properties and fluorescent characters of compound 5

Solvent	nD^{20} [a]	μ [b]	ε[c]	Δ f	λ_{max} (nm)	λ_{em} (nm)	Stokes shift	Φ[d]
CH_3OH	1.33	1.70	32.7	0.3080	491	617	126	0.0069
H_2O	1.34	1.82	80.1	0.3174	461	599	138	<0.001
Acetone	1.36	2.91	20.7	0.2838	487	619	132	0.0067
Ethyl acetate	1.37	1.88	6.02	0.2005	484	600	116	0.0213
CH_2Cl_2	1.42	1.20	8.9	0.2183	526	622	96	0.0193
DMSO	1.48	3.96	47.2	0.2630	490	625	135	0.0074

[a] Refractive index

[b] Dipole moment

[c] Dielectric constant

[d] Fluorescence quantum yield

two factors, refractive index and dielectric constant. The effect of solvents on fluorescent spectra could be stated by Lippert equation [21].

$$\Delta\nu = \nu_{em} - \nu_{abs} = \frac{2}{hc}\left(\frac{\varepsilon-1}{2\varepsilon-1} - \frac{n^2-1}{2n^2+1}\right)\frac{\mu^*-\mu}{a^3} + \text{constant}$$

$$\Delta f = \frac{\varepsilon-1}{2\varepsilon-1} - \frac{n^2-1}{2n^2+1}$$

Where ν_{abs} and ν_{em} are wavelengths of the excitation and emission respectively, so ν_{em} -ν_{abs} is stokes shift. The h is Planck constant and c is light speed. The a is the cavity radium that encapsulate the molecule. a is a constant when there is no hydrogen bond between the molecule and solvent. μ and μ* are the dipole moment of ground state and excited state. The Δf is called orientation polarizability.

The relationship between stokes shift and Δf of compound 5 in three different solvents were shown in Fig. 2. We can see that the stokes shift is approximately proportional to Δf, which is consistent with the Lippert equation. But for other solvents, there is a deviation between the relationship of Δν and Δf.

The Spectral Properties of Compound 5 in Different pH

The spectral properties of compound 5 with the concentration of 5×10^{-6} mol/L in aqueous solution were also measured in different pH ranging from 2 to 13. The results were shown in Fig. 3.

From Fig. 3, we can see the maximum absorption wavelength (λ_{max}), the maximum emission wavelength (λ_{em}) and the shapes of the spectra change little regardless of the different pH of the solution. But with the change of pH, the absorption and the fluorescent intensity were all changed obviously. The fluorescent intensity in basic solution was stronger than that in acid solution. In neutral solution, fluorescent intensity was the strongest, which is the same to the Ref [22]. One possible reason for this maybe due to the electronic configuration of compound 5 was different under different pH values. The other reason maybe the dye was more soluble in basic conditions, the dye aggregates can be soluble was increase so that the fluorescence intensities will be higher than in acid conditions.

The Spectral Properties of Compound 5 in Complex with ctDNA

We also studied the labeling of *ct*DNA with the dye (5×10^{-6} mol/L) in 10 mM Tris–HCl buffer (pH=7.4) and the results were shown in Fig. 4.

Figure 4 shows that the intensity of the compound was very weak without the addition of *ct*DNA. Interestingly, the intensity increased remarkably with the addition of *ct*DNA. One reason for this may be due to the change in the environment of compound 5 and the shielding properties of DNA. With the addition of *ct*DNA, the strong binding interaction between the cationic N-quinolizinium unit and the anionic phosphate of DNA were formed. The binding interaction can hinder the rotations around various bonds and thereby decreases the possible nonradiative process.

In addition, we defined the degree of signal enhancement as $(F-F_0)/F_0$, where F_0 and F are the fluorescent intensities at 602 nm in the absence and presence of DNA respectively, and

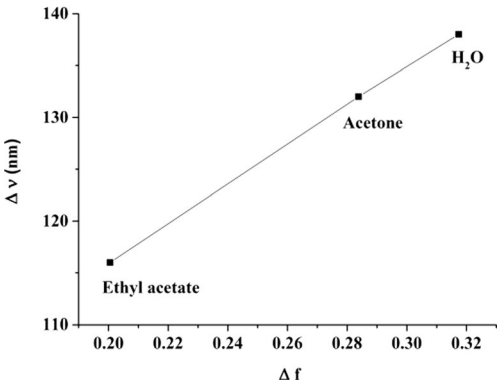


Fig. 2 The relationship between stokes shift and orientation polarizability (Δ f) of compound 6 expressed by the Lippert equation

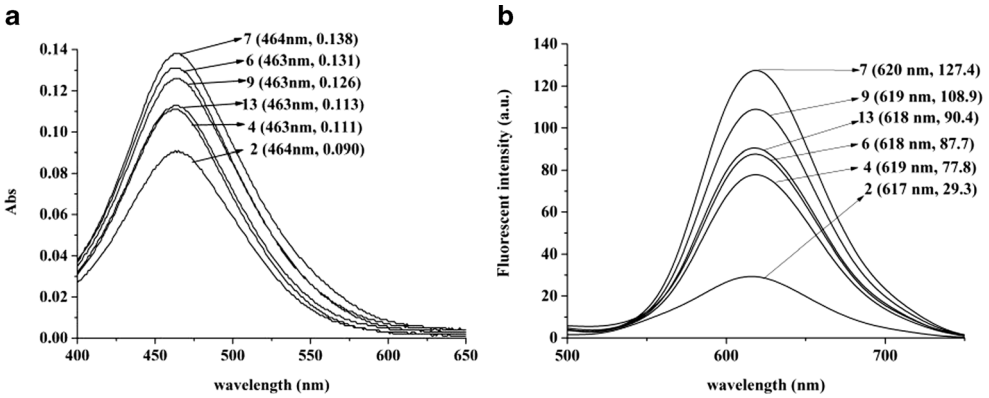


Fig. 3 The UV–vis spectra (**a**) and fluorescence spectra (**b**) of the dye in different pH (5×10^{-6} mol/L)

the fluorescence spectra of the probe in the presence of different concentrations of DNA were shown in Fig. 5.

Due to the intensity increased remarkably with the addition of DNA, the bonding association constant could be calculated by Benesi-Hildebrand equation [23]:

$$\frac{1}{F_x - F_0} = \frac{1}{F_{max} - F_0} + \frac{1}{F_{max} - F_0} \times \frac{1}{K[DNA]}$$

Where F_x and F_0 represent the fluorescent intensity of the probe in the presence and absence of DNA, respectively. F_{max} is the maximum fluorescent intensity of the probe in the presence of DNA. [DNA] is the concentration of DNA added. The equation could be simplified as:

$$\frac{F_{max} - F_0}{F_x - F_0} = 1 + \frac{1}{K[DNA]}$$

Plots of $(F_{max}-F_0)/(F_x-F_0)$ against [DNA] for the probe showed remarkable linear dependence (Fig. 6), justifying the validity of the used equation and hence confirming the one-to-one association between DNA and the probe. The binding constant (Ka) were determined from the slope of the corresponding plots, which came as 3.92×10^5 M^{-1}. The value was consistent with the previous reported [24].

The Spectral Properties of Compound 5 Labeled with Bovine Serum Albumin (BSA)

We studied the labeling of BSA with the dye (5×10^{-6} mol/L) and the results were shown in Fig. 7.

Different concentrations of BSA and the same concentration of dye were used to measure the fluorescence spectra. The results were shown in Fig. 7. It indicated that the fluorescent intensity of the compound increased with the addition of BSA, accompanied by a blue-shift of the maximum emission wavelength. There are two possible reasons for the increasing of fluorescent intensity. Firstly, with the addition of BSA, the compound was included into a hydrophobic cavity with lower polarity inside BSA molecule. The other reason is that the binding between BSA and the compound inhibited the rotational freedom of compound 5 and caused higher intensity. All these effects together caused the enhancement of the compound's fluorescent intensity. The blue shift of the maximum emission wavelength can be explained by the reduction of ground state energy of the compound when there is interaction between the dye and BSA. These results jointly confirmed that the compound can interact with BSA protein. But comparing with the reaction between the dye and DNA, the effect of the combination with BSA protein is not very satisfactory.

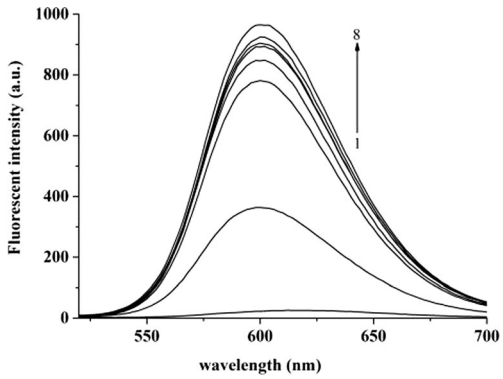


Fig. 4 The fluorescence spectra of compound 5 with different concentration of *ct*DNA (from 1 to 8: 0 μM, 4 μM, 8 μM, 12 μM, 16 μM, 20 μM, 24 μM, 28 μM). Excitation wavelength: 485 nm

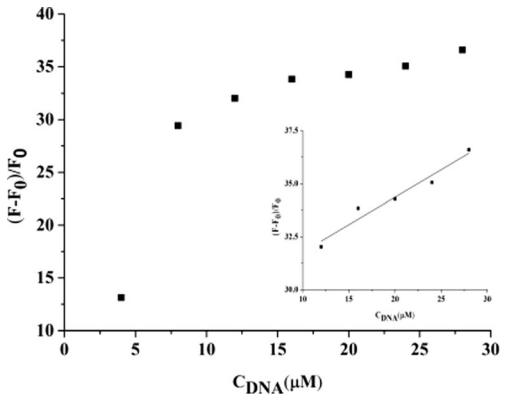


Fig. 5 The fluorescence spectra of the probe in the presence of varying concentrations of DNA. Inset: Linear range between $(F-F_0)/F_0$ and the *ct*DNA concentration (12–28 μM)

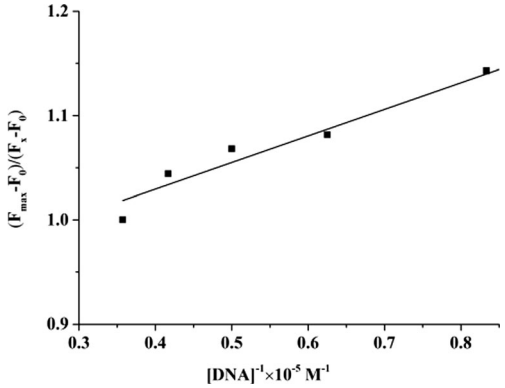


Fig. 6 The linearity of $(F_{max}–F_0)/ (F_x–F_0)$ against [DNA]

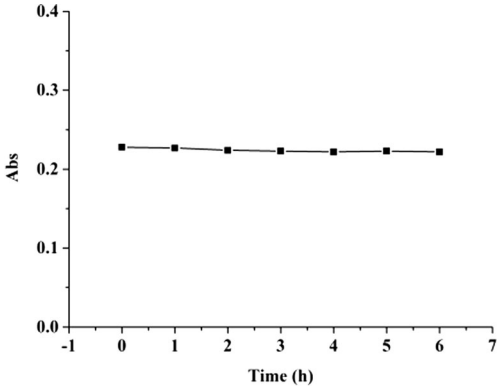


Fig. 8 The curve of photostability

Photostability of Compound 5

The photostability of fluorescent dyes has attracted a great attention of experts, because it plays an important role for their application as biomedical fluorescent labels. In this study, we used iodine-tungsten lamp (500 W) as the illuminant. The $NaNO_2$ aqueous solution (50 g/L) was used as the cold trap, which can decrease the effect on the stability caused by the strong heat from illumination. On the other hand, the solution could also filtrate most of the UV-lights, whose wavelength is shorter than 400 nm. The photostability experiment was performed in DMSO at room temperature without natural light [25]. The absorption intensity of the compound was measured after exposing to light with different time. The curve between the intensity at λ_{max} and the exposure time was shown in Fig. 8.

It can be seen that there is no prominent change after different exposure time. After irradiation of 6 h, the dye showed only 2.7 % photofading. It is indicated that the dye can be exposed to the strong light for a long time and still keep its intensity, which can make it as an excellent probe for application in many fields.

In addition, the rate of photooxidation could be calculated based on the photooxidation mechanism and counld be expressed as [26]:

$$d[Dye]/dt = k_1[Dye\,][O_2] \quad (1)$$

in which $[O_2]$ is a constant of about 3×10^{-4}mol/ L, so the following equation can be obtained:

$$-d[Dye]_0/dt = k[Dye] \quad (2)$$

$$\ln[Dye]_0/[Dye]_t = kt \quad (3)$$

In the equation, $[Dye]_0$ and $[Dye]_t$ were the original concentration of dye and the concentration after being illuminated for t hours, respectively. According to Lambert-Beer's law, there was a linear relation between concentration and the maximum absorption under low concentration. So Eq. (3) could be predigested as below:

$$\ln(A_0/A_t) = kt \quad (4)$$

In the equation, A_0 is the max absorbency of dye, A_t is the maximum absorbency after being illuminated and k is the photoreduction rate constant. The result was shown in Fig. 9. The photoreduction rate constant could then be obtained from the slope, the result is 1.64×10^{-5} mol/Min, which is

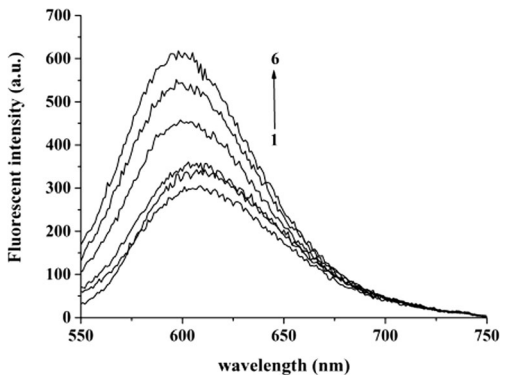


Fig. 7 The fluorescence spectra of compound 5 with different concentration of BSA (from 1 to 6: 0, 50, 100, 150, 200 and 250 mg/L). Excitation wavelength: 490 nm

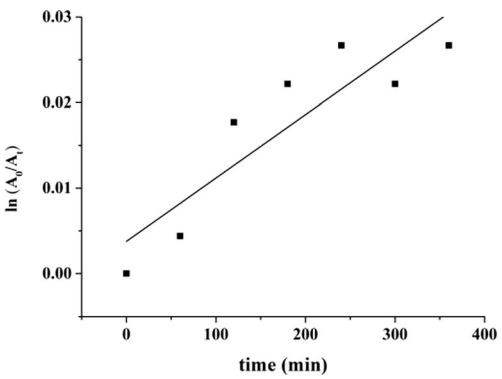


Fig. 9 The lightfastness of the dye

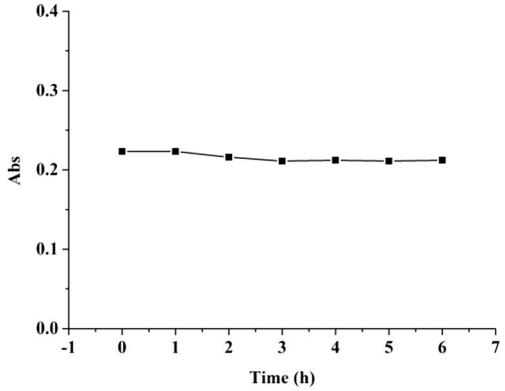


Fig. 10 The curve of thermostability

much little than those of cyanines [27, 28]. This lower photoreduction rate constant can be attributed to the trans structure. Because the trans structure is stable than cis structure [29].

Thermostability of Compound 5

The thermostability of the compound was measured at 80 °C in water bath. The concentration of the dye was 5×10^{-6} mol/L in DMSO. The absorption intensities of the compound after different time were measured to explore the thermostability. The results were shown in Fig. 10.

The results indicated that the absorption intensity reduced little after heating 6 h, so we can get that the thermostability of the dye solution is excellent.

Conclusion

In summary, a novel fluorescent dye, 1-(6-carboxyhexyl)-4-(2-(9-ethyl-carbazole-3-yl) vinyl) quinolizinium bromide, was synthesized and characterized by ^{1}H NMR ^{13}C-NMR, IR and HRMS. The photostability and thermostability were studied and the results showed that the compound was stable and can meet the need of the biological labeling. The properties of the dye in different solvents and in aqueous solution under different pH were also studied and the results showed that the dye is both sensitive to solvent and pH value. The dye was also used to label BSA and the results showed that the fluorescent intensity enhanced when labeling with BSA. Lastly, we studied the interaction of the dye with ctDNA. The results indicated that the dye give substantial increases in fluorescence on DNA binding and the binding constant was 3.92×10^{5} M^{-1}. These jointly proved that the dye could be used as a sensitive fluorescent turn-on probe for DNA.

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