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Determination of Trace Deoxyribonucleic Acid by Using Fluorescein Isothiocyanate-Phenosafranine as a Double-Luminescent Phosphorescence Probe

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Abstract Using Pb^{2+} as ion perturber, phenosafranine (PF) and fluorescein isothiocyanate (FITC) could emit strong and stable room temperature phosphorescence (RTP) signal on the filter paper, respectively. When they were mixed, the phenomenon that the RTP signal of PF and FITC enhanced significantly was found. And 1.12 ag DNA $spot^{-1}$ (sample volume was 0.40 µL, corresponding concentration was 2.8×10^{-15} g mL^{-1}) could cause the RTP signal of both PF and FITC to enhance sharply. The content of DNA was proportional to the ΔI_p of PF and FITC in the system at 634 and 659 nm. Thus, a new solid substrate room temperature phosphorimetry (SSRTP) for the determination of trace DNA was established by using FITC-PF as double-luminescent phosphorescence probe. The detection limit (LD) of this method calculated by $3S_b/k$ was 14 zg DNA $spot^{-1}$ for PF and 18 zg DNA $spot^{-1}$ for FITC, respectively, showing high sensitivity. It has been applied to the determination of trace DNA in practical samples and the analysis results were in accordance with those of fluorescence probe. The reaction mechanism of SSRTP for the determination of trace DNA was also discussed.

Keywords Deoxyribonucleic acid · Phenosafranine · Fluorescein isothiocyanate · Double-luminescent phosphorescence probe

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Introduction

DNA is not only the basic genetic material of all living beings, but also the main component of chromosomes [1], and it has close relationship with life activities and all kinds of metabolism [2]. Obviously, the determination of DNA content has an important significance [3]. With the development of science and technology, many new methods for the determination of DNA content emerged [4], such as spectrophotometry (LD: 4.0×10^{-9} g mL^{-1}) [5], fluorimetry (LD: 2.7×10^{-8} g mL^{-1}) [6], near-infrared fluorescence probe method (LD: 6.4×10^{-8} g mL^{-1}) [7], resonance light scattering spectroscopy (LD: 1.9×10^{-8} g mL^{-1}) [8], CdTe / CdS core-shell quantum dot fluorescent probe (LD: 2.0×10^{-8} g mL^{-1}) [9], CdS fluorescence probe method (LD: 1.8×10^{-8} g mL^{-1}) [10], Morin·SiO_2 phosphorescent probe method (LD: 1.5×10^{-12} g mL^{-1}) [11], polymerase chain reaction (PCR) technique [12] and so on. Recently, phosphorescent probes have become efficient tools which can explore the nature of micro-environment or biological macromolecules in organic medium (like configurational changes of nucleic acid and protein) and the reaction mechanism between them and drugs [13]. Phosphorescence has higher sensitivity, and biological molecules have no RTP emission in the near-

Fig. 1 Phosphorescence spectra of FITC-PF system (Cuvers 1–9 were emission spectra)
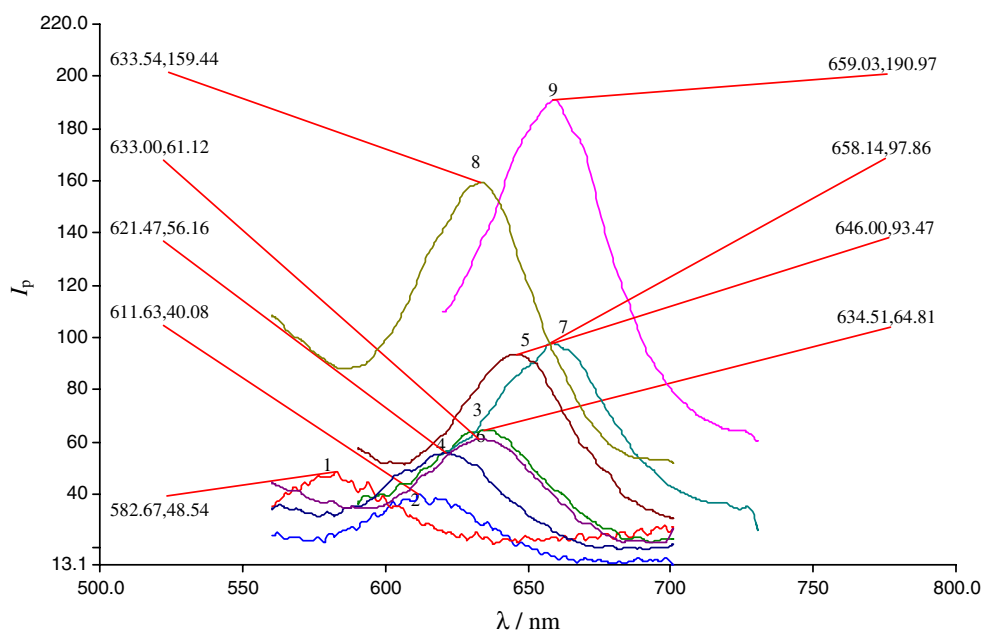
infrared long-wave region. Therefore, the quest and synthesis of a probe which can emit RTP in the long-wave region, to eliminate self-interference of biological molecules and to promote selectivity of RTP probe, has special significance to the study of biological molecules, especially, of the concentration and configurational changes of DNA. At the same time, it has a mutual complementation and verification with nucleic acid fluorescent probe [14]. In this paper, we developed a new double-luminescent phosphorescence probe which could emit strong and stable RTP in the long-wave region to further enhance the sensitivity, selectivity and flexibility of SSRTP for the determination of ultra-trace DNA and the research of life science.

In this experiment, we found that FITC-PF could interact with DNA, which resulted in the dramatical enhancement of RTP signal on filter solid substrate, and showed the superior characteristics of a double-luminescent phosphorescence probe. The optimal condi-

tions, analytical parameters, selectivity and the application of SSRTP for the determination of DNA were discussed using FITC-PF as a double-luminescent phosphorescence probe. Compared with a single-luminescent phosphorescence probe [11], the RTP signal of FITC or PF in FITC-PF double-luminescent phosphorescence probe could be used to determine DNA, which not only enhanced the flexibility of SSRTP, but also provided a sensitive, accurate and reliable analytical method to determine trace DNA in biological samples and promoted life science research.

Experimental

Apparatus and Reagents

A LS-55 luminescence spectrophotometer with a solid surface analysis apparatus (Perkin Elmer Corporation of

Table 1 The RTP characteristics of FITC-PF double-luminescent phosphorescence probe ($\Delta I_p = I_p - I_{p0}$, I_{p0} was the RTP intensity of reagent blank, I_p was the RTP intensity of the emission spectra)

System	λ_{em} (nm)	I_p	ΔI_p	$\Delta \lambda_{em}$	Measuring signal
1. Paper	582.7	48.5			
2. 1+0.15 mL PF	611.6	40.1			
3 .1+1.5 0 mL FITC	634.5	64.8			
4. 1+0.15 mL PF+1.50 mL FITC	621.5	56.2	16.1	9.9	PF
5. 1+0.15 mL PF+1.50 mL FITC	646.0	93.5	28.7	11.5	FITC
6. 4+1.0 fg DNA	633.0	61.1	4.9	11.5	PF
7. 5+1.0 fg DNA	658.1	97.9	4.4	12.1	FITC
8. 4+70.0 fg DNA	633.5	159.4	103.2	12.1	PF
9. 5+70.0 fg DNA	659.0	180.0	86.5	13.0	FITC

Table 2 The determination conditions

The measurement condition		The ΔI_p of FITC-PF-DNA system	Optimum
PF (mol L^{-1})	6.0×10^{-9}, 6.0×10^{-8}, 3.0×10^{-7}, 6.0×10^{-7}, 3.0×10^{-6}, 6.0×10^{-6}	10.9, 21.8, 32.4, 41.6, 42.7, 42.6	3.0×10^{-6} mol L^{-1}
(mL)	0.050, 0.10, 0.15, 0.50,1.0	26.7,35.8, 42.6, 42.5, 42.2	0.15 mL
FITC (mol L^{-1})	1.2×10^{-7}, 2.4×10^{-7}, 3.6×10^{-7}, 1.2×10^{-6}, 2.4×10^{-6}, 3.0×10^{-6}	20.6, 27.7, 32.6, 34.8, 35.7, 35.6	2.4×10^{-6} mol L^{-1}
(mL)	0.40, 0.60, 0.80, 1.00, 1.50, 2.00	20.1, 25.7, 30.1, 32.3, 35.6, 35.5	1.50 mL
Pb^{2+}(mol L^{-1})	0.30, 0.50, 0.70, 1.0, 1.20	27.3, 34.4, 38.6, 42.8, 42.7 (PF)	1.0 mol L^{-1}
		24.5, 29.3, 32.4, 35.9, 35.8 (FITC)	
pH of the reaction system	4.10, 5.10, 5.42, 5.76, 6.20	33.7, 35.8, 43.1, 43.0, 43.1 (PF)	5.42
		31.7, 33.4, 36.2, 36.1, 36.2 (FITC)	
Reaction temperature (°C)	30, 40, 60, 70, 80, 90	19.3 , 42.7, 33.8, 30.1, 29.8, 27.6 (PF)	40°C
		18.2 , 35.6, 31.8, 29.3, 19.6, 17.9 (FITC)	
Desiccation time (min)	5, 10, 15, 30, 40, 50	3.2, 11.6, 42.9 ,42.9, 42.9, 40.6 (PF)	15 min
		3.3, 10.9, 35.6, 35.6, 35.6, 33.2 (FITC)	
Solid substrate	Paper, PAM, NCM, ACM	42.9, 40.1, 36.4, 23.8 (PF)	Paper
		35.7, 33.6, 30.6, 23.8 (FITC)	
Ion perturber	Pb^{2+}, I$^-$, Hg^{2+}, Ag$^+$	42.8, 40.7, 32.4, 24.3 (PF)	Pb^{2+}
		35.7, 33.2, 29.6, 21.5 (FITC)	
Time of passing N$_2$ (min)	10, 15, 20, 25, 30	42.6, 42.4, 42.1, 43.3, 42.2 (PF)	Passing N$_2$ for 10 min
		35.7, 35.1, 35.3, 35.0, 35.6 (FITC)	
Time of not passing N$_2$ (min)	10, 15, 20, 25, 30	40.6, 41.4, 42.5, 39.3, 39.8 (PF)	
		31.7, 34.1, 35.6, 30.4, 29.6 (FITC)	
Standing time (min)	10, 20, 30, 40, 50, 60	31.6, 42.8, 42.9, 42.7, 42.9, 42.8 (PF)	20 – 60 min
		27.9, 35.6, 35.4, 35.6, 35.7, 35.6 (FITC)	

American, main parameters are: Ex. Slit: 10 nm; Em. Slit: 10 nm; scan speed: 1,500 nm min^{-1}), an AE240 electronic analytical balance (Mettler-Toledo Instruments Company Limited), infrared spectrophotometer (Necolet-360 Nicolet Company, USA) and a 0.50-μL (± 0.010 μL) flat head micro-injector (Shanghai Medical Laser Instrument Plant) were used.

CT-DNA working solution (Sigma Company): 1.0 mg mL^{-1} stock solution was prepared with water and placed at 4°C in the refrigerator for use; working solution: diluted 1.0 mg mL^{-1} stock solution to 10.00 and 1.00 (fg mL^{-1}) gradually with water; 1.0×10^{-4} mol L^{-1} PF solution; 1.0×10^{-4} mol L^{-1} FITC solution. All reagents were A.R. grade except that DNA was biological reagent. The water used was thrice-distilled water.

Filter paper, PAM, acetic cellulose membrane (ACM) and nitric cellulose membrane (NCM) were purchased from Luqiaosijia Biochemical Plastic Plant. They were cut into discs (Φ=15 mm) and a ring indentation (Φ=4.0 mm) was made at the center of each sheet with a standard pinhole plotter for use.

Experimental Method

To a 25-mL colorimetric tube, a certain amount of DNA, 1.50 mL of 4.0×10^{-5} mol L^{-1} FITC and 0.15 mL of 5.0×10^{-4} mol L^{-1} PF were added and diluted with water, and finally mixed homogeneously. The tube was kept at 40°C for 15 min, and then cooled by flowing water for 5 min. 0.40 μL of different concentrations of DNA and blank solution were suspended onto the center of the paper by a 0.50-μL micro-injection, and then dried at 90±1°C for 2 min. Then it was immersed in 1.0 mol L^{-1} Pb(Ac)$_2$ solution for 10 s, and dried at 90±1°C for 2 min. The phosphorescence intensity of test solution (I_{p_1}) and reagent blank (I_{p_0}) were directly measured at λ_{em}^{max}=633.5 nm (PF) or λ_{em}^{max}=659.0 nm (FITC). Then ΔI_p (= I_{p_1}–I_{p_0}) was calculated.

Table 3 Effects of coexistences (1.2 fg DNA mL^{-1}, 1.2 fg DNA mL^{-1}-X μg mL^{-1} coexistences (ions) were determined by the experimental method for 6 parallel determination, respectively, and the Er was calculated)

This method			Ref.[10]	Ref.[11]
Coexistent ions	The allowed concentration (μgmL^{-1})	Er(%)	The allowed concentration (μgmL^{-1})	The allowed concentration(μgmL^{-1})
Glysin	90	-2.4	8.0	72
Lysin	75	3.5	6.0	65
Arginine	70	3.6	5.0	57
Yeast RNA	15	-4.5	3.0	4.5
Ca^{2+}	15	4.4	4.0	4.8
Fe^{3+}	20	-4.6	2.8	11
Fe^{2+}	20	4.8	2.8	9
Co^{2+}	25	-2.8	6.0	12
Mn^{2+}	30	4.5	4.0	20
Cr^{3+}	20	-4.7		10
Isoleucine	70	3.3	5.5	57
Citric acid	30	4.4	4.5	15
Glucose	60	-3.9	10	48
Zn^{2+}	70	3.7	2.8	52
Mg^{2+}	90	2.6	4.8	72
Cu^{2+}	20	4.5	1.6	10
K$^+$	100	-2.3		80
NO$_3^-$	50	4.2		32
NO$_2^-$	50	4.1		28
PO$_4^{3-}$	60	3.8		40
CO$_3^{2-}$-	60	-4.1		42
Br$^-$	70	3.4		50
β-Cyclodextrin	10	4.8		6.5
Sucrose	10	5.0		3.6
SO$_4^{2-}$	60	3.8		40
SO$_3^{2-}$	50	3.7		30
S$_2$O$_3^{2-}$	40	-4.4		25
S^{2-}	30	4.5		20
Cl$^-$	100	2.1		80
Ac$^-$	80	-3.0		60
BSA	50	4.3		30

Infrared Spectra Analysis of FITC, PF and FITC-PF

To a 25-mL colorimetric tube, 15.0 mL of 4.0×10^{-5} mol L^{-1} FITC and 1.5 mL of 5.0×10^{-4} mol L^{-1} PF were added and diluted with water, and finally mixed homogeneously. The tube was kept at 40°C for 15 min, and then cooled by flowing water for 5 min. FITC-PF (salmon pink spot, R_f=0.43) was obtained by layer chromatography (chloroform : methanol : acetic acid=250: 25 : 1). Infrared spectra of FITC, PF and FITC-PF were scanned after the sample preparation by pressed disc method with KBr.

Results and Discussion

Phosphorescence Spectra of the FITC-PF System

The phosphorescence spectra of the FITC-PF system were scanned by the experimental method (Fig. 1, Table 1). Using Pb^{2+} as the ion perturber, PF and FITC could emit strong and stable RTP signal on filter paper solid substrate. And their λ_{em}^{max} were 611.6 nm and 634.5 nm, I_p values were 40.1 and 64.8, respectively. However, the RTP signals of PF and the FITC in the FITC-PF system were significantly enhanced, their λ_{em}^{max} were 621.5 nm and

Table 4 Analysis results of DNA in honey (n=6)

Samples	Measured value (ng mL^{-1})	Adding amount (ng mL^{-1})	Recovery (ng mL^{-1})	Recovery rate (%)	RSD (%)	Ref.[16] (ng mL^{-1})	RE (%)	Working wavelength
Honey A	0.114	0.010	0.0099	99.0	3.7	0.111	2.7	634 nm (PF)
	0.118	0.010	0.010	100.0	4.3	0.115	2.6	659 nm (FITC)
Honey B	0.128	0.010	0.010	100.0	3.6	0.130	−1.5	634 nm (PF)
	0.134	0.010	0.0099	99.0	4.5	0.137	−2.2	659 nm (FITC)
Honey C	0.135	0.010	0.0098	98.4	4.1	0.138	−2.2	634 nm (PF)
	0.140	0.010	0.010	99.7	3.5	0.144	−2.8	659 nm (FITC)
Honey D	0.151	0.010	0.0099	99.6	4.3	0.153	−1.3	634 nm (PF)
	0.156	0.010	0.010	100.2	4.1	0.159	−1.9	659 nm (FITC)
Honey E	0.163	0.010	0.0099	99.3	3.9	0.161	1.2	634 nm (PF)
	0.167	0.010	0.010	100.2	3.6	0.164	1.8	659 nm (FITC)
Honey F	0.180	0.010	0.0099	98.9	3.2	0.178	1.1	634 nm (PF)
	0.183	0.010	0.0099	99.1	3.7	0.180	1.7	659 nm (FITC)

646.0 nm, I_p values were 56.2 and 93.5, respectively, namely their λ_{em}^{max} red shifted for 9.9 nm and 11.5 nm, which prompted that there was a new compound formed. When 70.0 fg DNA was added, the RTP signal of PF and FITC in FITC-PF system were dramatically increased, their λ_{em}^{max} were 633.5 nm and 659.0 nm, I_p values were 103.2 and 86.5, with the λ_{em}^{max} red shifting for 12.1 nm and 13.0 nm, respectively. The reason might be that PF and FITC interacted with DNA to generate new materials, respectively. According to the relationship between RTP signal and DNA content, the possibility of SSRTP for the determination of DNA content was revealed using FITC-PF as a double-luminescent phosphorescence probe.

The Optimal Determination Conditions

For the system containing 0.48 ag DNA spot^{-1}, the effects of the concentration and volume of PF, FITC, acidity of reaction, temperature and time of reaction, solid substrate, standing time, the species of ion perturber and concentrations of Pb^{2+} on the ΔIp of the system were tested, respectively (Table 2). The results showed that the ΔIp of the system reached the maximum when 0.15 mL of 3.0× 10^{-6} mol L^{-1} PF、1.50 mL of 2.4×10^{-6} mol L^{-1} PF were

used, the pH of reaction system was 5.42, the reaction temperature was 40°C and the time was 15 min, time of passing N$_2$ was 10 min, paper as solid substrate as well as 1.0 mol L^{-1} Pb^{2+} was used.

Working Curve, Linear Range, LD and the Limit of Quantification (LOQ)

Under the optimal determination conditions, the content of DNA ranging from 0.0080 ag spot^{-1} to 1.12 ag spot^{-1} (corresponding concentration: 0.020–2.8 fg mL^{-1}, sample volume: 0.40 μL) had good linear relationship with the ΔI_p of the system (When the content of DNA were 0, 0.008, 0.16, 0.48, 0.80, and 1.12 ag spot^{-1}, the ΔI_p of the system were 0, 4.9, 14.3, 42.9, 71.4, 103.2 (for PF) and 0, 4.4, 11.9, 35.7, 59.5, 86.5 (for FITC), respectively.). When a FITC-PF double-luminescent phosphorescence probe was used to determine DNA content, the regression equations of working curve were ΔI_p=1.600+89.06 m$_{DNA}$ (ag spot^{-1}) and ΔI_p=1.391+74.39 m$_{DNA}$ (ag spot^{-1}); correlation coefficients (r) were 0.9987 and 0.9984; RSDs were 1.2–4.5% and 1.5%–3.4% (measured 0.020 and 1.12 ag spot^{-1} DNA for 7 parallel determination to calculate the RSD); LD were 2.8 ag spot^{-1} and 3.3 ag spot^{-1} (measured reagent
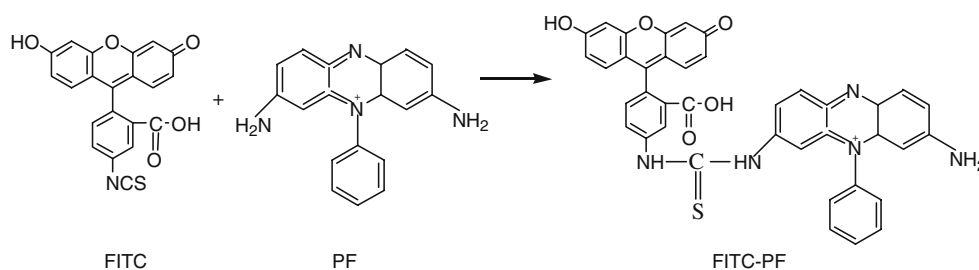
Fig. 2 Reaction between PF and FITC

Table 5 Infrared spectrum data of FITC, PF and FITC-PF (ν is stretching vibration; δ is in-plane bending vibration and w is out-plane bending vibration)

Sample	−OH	−C=O	−NCS	−C₆H₅	Phenazine ring					−NH₂	−N−CS−N−
	(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})	C=C (cm^{-1})	C—N (cm^{-1})	−C=N (cm^{-1})	CH (cm^{-1})	CH (cm^{-1})	(cm^{-1})	(cm^{-1})
FITC	ν: 3,345	ν: 1,732	ν: 2,050	ν: 1,607	ν: 1,610	ν:1,491					
				ν: 1,494	ν: 1 531						
				ν: 1,439							
PF							ν: 1,333	δ:1,073	w: 876	ν:3,321	
								δ:1,016	w: 831	ν: 3,177	
								δ : 947	w: 801	ν: 1,642	
									w: 747	δ: 1,193	
									w: 698		
FITC-PF	ν: 3,348	ν: 1,736		ν: 1,610	ν: 1,617	ν:1,496	ν: 1,338	δ:1,079	w: 836	ν:3,325	ν:1,380
				ν: 1,498	ν: 1 536			δ:1021	w: 808	ν: 3,182	
				ν: 1,445				δ : 952	w: 754	ν: 1,648	
									w:704	δ: 1,197	

blank for 11 parallel determination, calculated by 3Sb/k and Sb were 0.042 and 0.046, respectively.), LOQ were 9.2 ag spot^{-1} and 10.9 ag spot^{-1} (calculated by 10Sb/k), respectively. The results showed that this method was of good precision and high sensitivity.

Interference Experiment

Under the optimal conditions, the DNA content was measured by this method (1.2 fg mL^{-1}) and those in the literatures [14] (1.0 ng mL^{-1}) and [15] (0.10 µg mL^{-1}).

When the relative error (Er) was within ± 5%, the maximum allowed concentrations of coexistences are listed in Table 3.

The results showed that the maximum allowed concentrations of coexistences of this method were higher than those of the literatures [10, 11], which indicated high selectivity. And the maximum allowed concentrations of common cations (Mg^{2+}, K$^+$, Ca^{2+}), which might combine with the phosphate group (P) of DNA [15], were higher with less interference. While the maximum allowed concentrations of Mn^{2+}, Cr^{3+}, Fe^{3+}, Co^{2+} were lower, the

Fig. 3 Reaction between DNA and FITC-PF
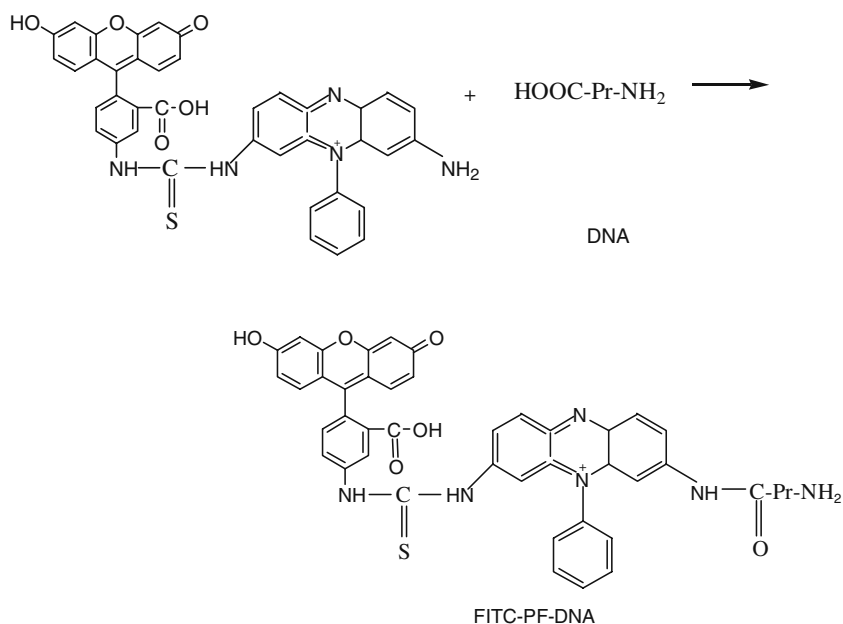
reason might be that they combined with the bases of DNA [15] and destroyed the hydrogen bonds, which led to acute changes in DNA structure. For the reason that the allowed concentrations of interference ions were higher than those in biological body, the interference of this method used to determine biological fluids was less. The allowed concentration of yeast RNA was 4.5 mg L^{-1}, so this method was suitable for the determination of RNA.

Sample Analysis

1.0 g (± 0.10 mg) A, B, C, D, E and F nectar were weighed and treated according to the method mentioned in Ref. [16], and diluted to 100 mL with water; 1.00 mL test solution were taken and diluted to 100 mL with water. Taking 1.00 mL test solution and measuring the DNA content of samples according to the experimental method and that in literature [16]. The results are listed in Table 4.

Seen from Table 4, no matter the working wavelength of FITC or that of PF in FITC-PF double-luminescent phosphorescence probe was used to determine the DNA content in honey A, B, C, D, E, F and G, the recovery rates (%) were within 99.0–100.2 and 98.4–100.0, RSDs (%) were in the context of 3.5–4.5 and 3.2–4.3, respectively, which showed that this method was of good flexibility, high accuracy and precision.

Reaction Mechanism

Using Pb^{2+} as perturber, both PF and FITC on filter paper solid substrate could emit strong and stable RTP signal after reacting at 40°C for 15 min; when they were mixed, the RTP signal of PF and FITC significantly enhanced with the λ_{em}^{max} red shifting for 9.9 nm and 11.5 nm, the reason might be that the -NCS [17] in FITC molecules reacted with the $-NH_2$ in PF molecules to produce new compounds FITC-PF (Fig. 2) which contained −NH−CS−NH− bond:

The infrared spectra of FITC, PF and FITC-PF was scanned in order to discussed the possibility that FITC reacted with PF to form FITC-PF. And the results are listed in Table 5.

The infrared spectra of FITC-PF kept the most characteristic adsorption peak of FITC and PF, but the stretching vibration peak of −NCS (ν: 2,050 cm^{-1}) in the range of 2,150–2,020 cm^{-1} disappeared and appeared a new stretching vibration peak of −N−CS−N− (ν: 1,380 cm^{-1}) in the range of 1,430–1,130 cm^{-1}. Thus, it could conclude that the −NCS of FITC reacted with $-NH_2$ to form the FITC-PF.

When 70.0 fg DNA existed, the RTP signal of PF and FITC in FITC-PF system dramatically increased with the λ_{em}^{max} red shifting for 12.1 nm and 13.0 nm, respectively. The reason might be the -COOH in H_2N-Pr-COOH (DNA) molecule reacted with the $-NH_2$ in FITC-PF to produce FITC-PF-DNA (Fig. 3).

According to the linear relationship between DNA content and the ΔI_p of the system, SSRTP can be used to determine DNA content using FITC-PF as a double-luminescent phosphorescence probe.

Conclusion

In this paper, FITC-PF double-luminescent phosphorescence probe was developed, a new SSRTP for the determination of trace DNA was established, DNA content in honey was determined by the emission wavelength of FITC and PF in double-luminescent phosphorescence RTP probe, respectively. The flexibility and applicability of SSRTP have been improved, showing broader application prospect. If FITC-PF double-luminescent phosphorescence probe is used to label antibodies and lectins, SSRTP immunization method or SSRTP affinity adsorption method for the determination of trace biological active substances (such as IgG, IgA, IgM, IgE, alkaline phosphatase, alpha-fetoprotein heterogeneity and so on.) will be established, showing the application prospect of phosphorescence probe.

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