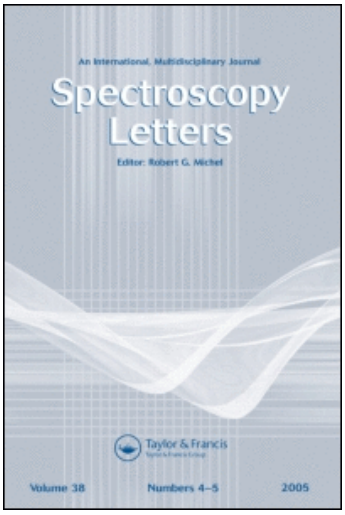

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Determination of Micro Amounts of Nucleic Acids Based on Shifting the Aggregate-Monomer Equilibrium of Fluorescent Dye

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Abstract: A sensitive spectrofluorimetric method for the determination of nucleic acids based on shifting the aggregate-monomer equilibrium of the fluorescent dye phenosafranine (PF) was proposed. Formation of aggregate induced by the pre-micellar aggregation of an anionic surfactant, sodium dodecyl sulfate (SDS), was observed. The possibility of using the in situ–formed aggregate as a fluorescent probe for nucleic acids was also studied. The results showed that the fluorescence intensity of the system increased dramatically when nucleic acids were added to the solution. The fluorescence enhancement effect was thought to be based on the nucleic acid-modulated shift of the aggregate-monomer equilibrium of PF in the anionic surfactant solution. Intercalation of the monomer in nucleic acids caused the dissociation of the aggregate and led to a very high fluorescence enhancement. It seemed that the aggregate molecules acted as a source of monomer molecules ready for interaction with nucleic acids. A linear dependence of fluorescence intensity enhancement on the concentration of fish sperm DNA (fsDNA) over the range 0.04–20 μg/mL fsDNA allowed sensitive quantification of fsDNA by a simple fluorescence method. The detection limit was 17 ng/mL and the RSD was 1.2% for 2 μg/mL fsDNA ($n = 7$). Furthermore, calibration graphs for calf thymus DNA (ctDNA) and yeast RNA were also obtained.

Keywords: Absorption spectra, anionic surfactant, fluorescence spectra, nucleic acids, phenosafranine

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INTRODUCTION

The quantitative determination of nucleic acids is of great importance in fundamental research and in clinical diagnosis.[1–3] However, it is difficult to detect nucleic acids by using their native fluorescence because of the poor fluorescence quantum efficiency ($\varphi_f = 4.0 \times 10^{-5}$),[4] and, therefore, extrinsic fluorescence probes are usually introduced during studies concerning nucleic acids. One of the most widely used probes is ethidium bromide (EB),[5] but it has the disadvantage of being a strongly carcinogenic compound, and this has inevitably limited its application. In the past few years, many studies have focused on new fluorescence probes for DNA exhibiting high sensitivity, selectivity, and safety.[6–10] Some synthetic cyanine heterodimers, such as thiazide orange homodimer (TOTO) and oxazide yellow homodimer (YOYO), are extremely sensitive probes compared with ethidium bromide and have replaced it for many purposes.[11,12] However, the limitation of these dyes is their high cost. In addition, TOTO and YOYO can induce the aggregation of DNA when the concentration of DNA exceeds 0.2 μg/mL.[11] The *in situ* aggregate formation of some acridine fluorescent dyes, such as acridine orange,[13] acridine yellow,[14] and rivanol[15] in presence of surfactant has been reported. Phenosafranine (PF), a phenazium dye, features a planar phenazine ring. To our knowledge, the formation of aggregates of this kind of fluorescent dye and its application in bioanalytical situations have not been reported. It was found that PF formed an aggregate in aqueous solution by the induction of the pre-micellar aggregation of an anionic surfactant. Furthermore, with the addition of DNA, the fluorescence intensity of the PF-SDS system was dramatically increased. We have therefore employed the dye aggregate as a fluorescent probe and developed a sensitive fluorimetric method for the determination of micro amounts of nucleic acids based on the DNA modulated shift of aggregate-monomer equilibrium of PF in the anionic surfactant solution. It was obvious that the dye aggregate played an important role to decrease background fluorescence of the system. The mechanism for the formation of aggregate in the presence of SDS and the fluorescence enhancement in the presence of nucleic acids were also studied. The method described in this paper gives a unique way of eliminating the natural fluorescence of the dye and, thus, increasing the sensitivity of nucleic acid quantification.

MATERIALS AND METHODS

Reagents

Calf thymus DNA (ctDNA), fish sperm DNA (fsDNA) and yeast RNA were purchased from Sigma co. (St. Louis, MO, USA) and used without further purification. The purity of the final DNA preparation was checked by monitoring

the absorption spectrum and the ratio of the absorbance at 260 nm and 280 nm. The ratio was 1.88, indicating the DNA was free from protein.[16] The concentrated stock solutions of DNA were directly prepared in double-distilled water until it was dissolved at a final concentration of DNA of 100 μg/mL. The stock solutions were stored at 4°C and used in 5 days. Sodium lauryl sulfate (SDS), sodium lauryl sulfonate (SLS), sodium dodecylbenzone sulfonate (SDBS), cetyltrimethylammonium bromide (CTMAB), and phenosafranine (PF) were analytical-grade reagents from Shanghai No. 3 chemical plant Shanghai, (China). PF was used after being recrystallized from water.

Other reagents were at least analytical grade and used without further purification. Double-distilled water was used throughout.

Apparatus

All fluorescence measurements were made with Hitachi F-4500 Fluorescence Spectrophotometer equipped with a l-cm quartz cell (Kyoto, Japan). The absorption spectra was performed on a Hitachi U-3010 spectrophotometer (Kyoto, Japan) by using a 1-cm quartz cell. All pH measurements were made with pHS-3C pH meter (Shanghai, China).

Standard Procedure

For the investigation of the interaction between PF and surfactants, portions of PF solution and surfactant solution were brought in a 10 mL standard flask and diluted to the mark with water.

For the investigation of the interaction between PF-SDS and DNA, 1.0 mL of PF solution (1×10^{-4} mol/L), 1.0 mL of SDS (1.0×10^{-2} mol/L), and 1.0 mL Tris-HCl buffer solution (pH 8.0) were transferred to a 10-mL standard flask. A known volume of nucleic acids standard solution was added, diluted to the mark with water, and mixed. The relative fluorescence intensity at 568 nm was measured with excitation at 528 nm.

RESULTS AND DISCUSSION

The Interaction of PF and Anionic Surfactant and its Absorption Spectra Study

Many fluorescent dyes[17,18] form dimers in aqueous solution when they are at high concentration. In general, the aggregation of dye molecules cannot be observed in diluted solution, but on addition of surfactant, some phenomena, such as intermolecular energy transfer[19] and aggregation of dye molecules,[15] can appear.

Similar characteristic of PF was observed as above. Figure 1 reveals the absorption spectra changes of PF in the presence of various concentration of SDS. There are three typical absorbance maxima.

At 1.0×10^{-5} mol/L, PF exists in pure water solution in the form of a monomer with an absorbance maximum at 530 nm. In the presence of SDS, when the concentration of SDS was lower than 3.0×10^{-4} mol/L, the absorbance of PF at 530 nm decreased, but a new blue-shifted peak located at 515 nm was observed when the concentration of SDS was increased to 1.0×10^{-3} mol/L accompanied with the appearance of an isobestic point. It may be assumed that the absorbance maximum at 515 nm is produced by the aggregate molecules, induced by the pre-micellar aggregation of anionic surfactant, whereas the isobestic points are indicative for an equilibrium between the momomer and aggregate. When the concentration of SDS was still made higher, a red-shifted peak located at 535 nm was observed and the absorbance increased. Finally, the absorbance peak leveled off as the SDS concentration was higher than its critical micellar concentration (CMC, 8.1×10^{-3} mol/L).[15]

Fluorescence Spectra Study on PF in the Presence of Anionic Surfactant

The variation of the fluorescence intensities as a function of surfactant concentration is shown in Fig. 2. In pure water solution, PF exhibited a strong

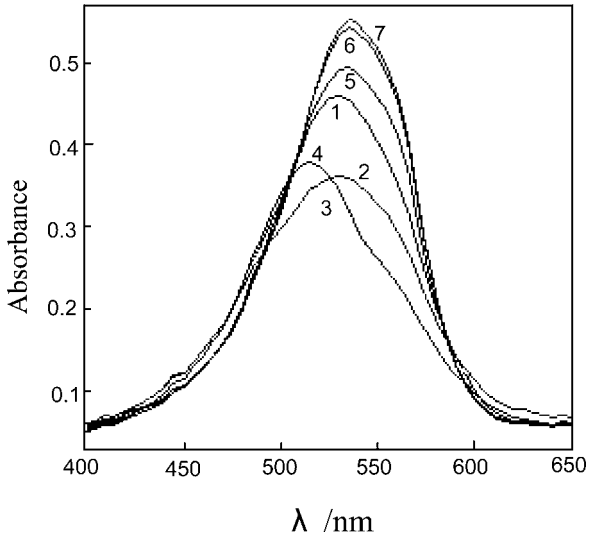

Figure 1. The visible spectra of PF-SDS system. The concentration of PF at 1.0×10^{-5} mol/L and SDS at (mmol/L): 1, 0; 2, 0.1; 3, 0.3; 4, 1.0; 5, 3.0; 6, 4.5; 7, 6.0.

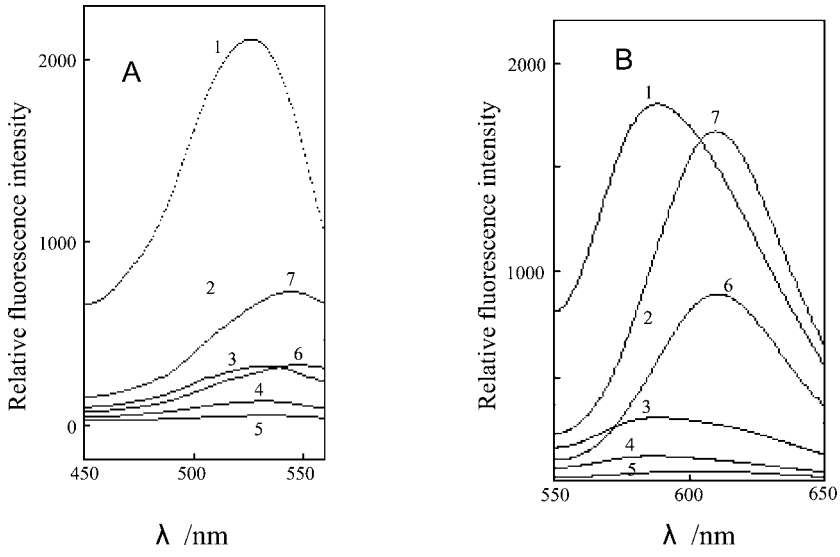

Figure 2. The fluorescent excitation (A) and emission (B) spectra of PF-SDS systems. The concentration of PF at 1.0×10^{-5} mol/L and SDS at (mmol/L): 1, 0; 2, 0.1; 3, 0.2; 4, 0.5; 5, 1.0; 6, 2.5; 7, 3.0.

fluorescence at 568 nm, but the fluorescence intensity was quenched by adding of SDS, and the quenchment resulted in a linear Stern-Volmer plot when the concentration of SDS was low, which is shown in Table 1. With the increase of the SDS concentration, the fluorescence intensity decreased dramatically. This result showed that PF formed an aggregate in the presence of SDS, based on the pre-micellar aggregation of SDS. The fluorescence quantum yield of aggregate was extremely low, and this caused the fluorescence quenchment of monomer PF.

When concentration of SDS was around 8.0×10^{-4} to 2.0×10^{-3} mol/L, in this region the quenching of the fluorescence intensity of the system became maximum and finally did not change with the increase of SDS

Table 1. The regression equations for PF fluorescence quenching

Surfactant	$C_{PF}/\text{mol L}^{-1}$	Equation ($c/\text{mol L}^{-1}$)	Correlation coefficients
SDS	1.0×10^{-5}	$F_0/F = 1.1 + 8.6 \times 10^{-3}c$	0.994
SLS	1.0×10^{-5}	$F_0/F = 1.0 + 1.4 \times 10^{-2}c$	0.994
SDBS	1.0×10^{-5}	$F_0/F = 1.0 + 9.8 \times 10^{-3}c$	0.995

PF, phenosefranine; SDS, sodium lauryl sulfate; SLS, sodium leuryl sulfonate SDBS, sodium dodecylbenzone sulfonate.

concentration. This indicated that almost all of the PF molecules did form aggregate.

When concentration of SDS was in the range of 2.2×10^{-3} mol/L to 4.0×10^{-3} mol/L, PF molecules were gradually solubilized into the micelles, resulting in the dissociation of the nonfluorescent aggregate. Therefore, the fluorescence intensity of the system was enhanced again along with the increase of the concentration of SDS, and at the same time a red shift in emission wavelength (626 nm) and a red shift in excitation wavelength (547 nm) were observed.

When the SDS concentration reached its CMC, almost all PF aggregate dissociated. The fluorescence intensity increased to a maximum and remained constant.

Similar behaviors were observed in PF/SLS and PF/SDBS system. But the aggregation could not be observed when anionic surfactant was replaced by cationic surfactant CTMAB. For nonionic surfactant Trition X-100, we failed to observe the formation of aggregate, although micellar enhanced fluorescence existed as expected.

Interaction Between PF-SDS System and Nucleic Acids

Absorption Spectra Study

When the interaction between PF-SDS system and nucleic acids was discussed, the experimental conditions were controlled to a stage that was favorable to the formation of aggregate in particular in the PF-SDS system, unless described otherwise.

Shown in Fig. 3 are the absorption spectra of PF-SDS in the presence of various concentrations of DNA. It can be seen that the absorbance of PF-SDS decreased when DNA was added, and the absorption maximum at 515 nm showed a red shift to 542 nm. The spectral changes could be explained in that the equilibrium between aggregate-momomer in the PF-SDS system was affected by PF intercalating in DNA.[20] The observed absorption maximum of 542 nm in the presence of DNA suggested that the dissociation of aggregate was induced by PF intercalating in DNA, which led to the increase of absorbance of the monomer.

Fluorescence Spectra Study

The interaction between the PF-SDS system and DNA was further probed by fluorescence spectrometry. Figure 4 shows the results for the PF-SDS system in the presence of various concentration of DNA. It can be seen that a very low fluorescence emission was detected in the absence of DNA, and a significant fluorescence enhancement was observed upon the addition of DNA into the

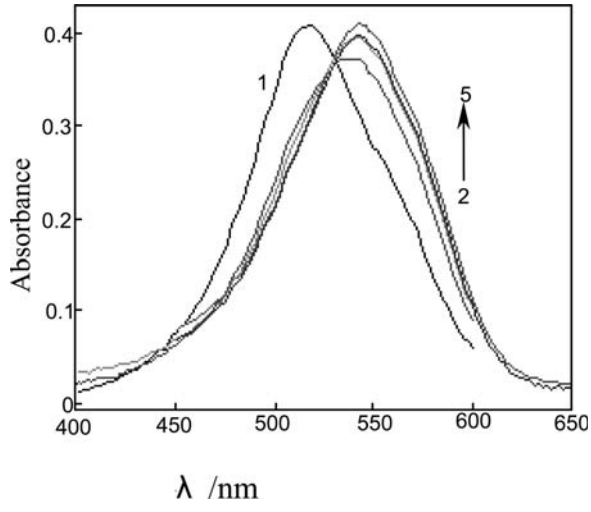

Figure 3. Absorption spectra of PF-SDS system in the presence of fsDNA. PF at 1.0×10^{-5} mol/L, SDS at 1.0×10^{-3} mol/L, and fsDNA (μg/mL) at 1, 0; 2, 60; 3, 100; 4, 140; 5, 160.

system. The fluorescence changes showed good linear relationship with the concentration of DNA (as shown Fig. 5); the linear relative coefficient was $r = 0.995$. The inherent fluorescence intensity of the free dye was eliminated by formation of the aggregate, and, therefore, the sensitivity of determination was improved.

The Studies of Experiment Conditions

The Influence of Concentration of PF

When the concentration of SDS was fixed at a level that was favorable for the formation of aggregate, namely 1×10^{-3} mol/L, the fluorescence intensity of PF-SDS system ΔF ($=F - F_0$) was examined as a function of PF concentration. The results showed that a maximum and constant value of ΔF was observed over the PF range of 4.0×10^{-6} to 1.5×10^{-5} mol/L. In this work, a PF concentration of 1.0×10^{-5} mol/L was recommended.

The Influence of pH

The effect of pH on the fluorescence enhancement of PF-SDS system in the presence of fsDNA was also investigated. When the ionic strength was very small, there was little effect on the interaction between PF aggregate and

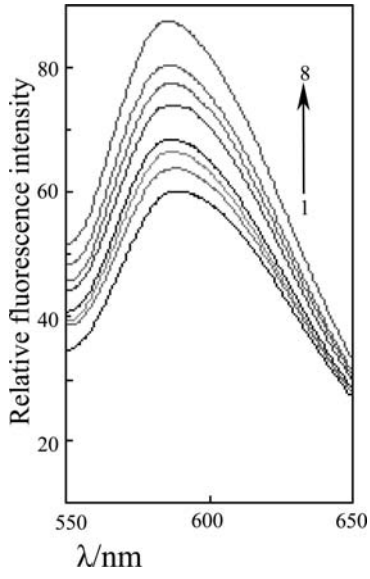

Figure 4. The fluorescent spectra of the PF-SDS-fsDNA system. The concentration of PF at 1.0×10^{-5} mol/L and SDS at 1×10^{-3} mol/L, $\lambda_{ex} = 528$ nm, $\lambda_{em} = 568$ nm, EX = EM = 5.0 nm. The concentration of fsDNA at (μg/mL): 1, 0; 2, 0.20; 3, 0.40; 4, 0.60; 5, 1.00; 6, 1.20; 7, 1.40; 8, 2.00.

DNA when pH was changed from 4.0 to 10.0. The larger slope under the condition of pH 8.00 indicated a more sensitive determination and, therefore, pH 8.00 modulated by Tris-HCl was selected.

Salt Effect

The effect of ionic strength on PF-SDS and PF-SDS-DNA systems was tested by the addition of a strong electrolyte NaCl. The results showed that when NaCl was added to the systems, the fluorescence intensity increased dramatically. This indicated that a high-salt medium could considerably induce the dissociation of PF aggregate causing a large fluorescence enhancement. Therefore, a low-salt medium should be maintained in order to obtain a lower background.

The Influence of Incubation Time

The effect of incubation time on the fluorescence intensity of PF-SDS systems in the presence or absence of DNA was investigated. It can be seen that the fluorescence intensity increased and reached a certain level after incubating for 15 min. In this work, an incubation time of 15 min was recommended.

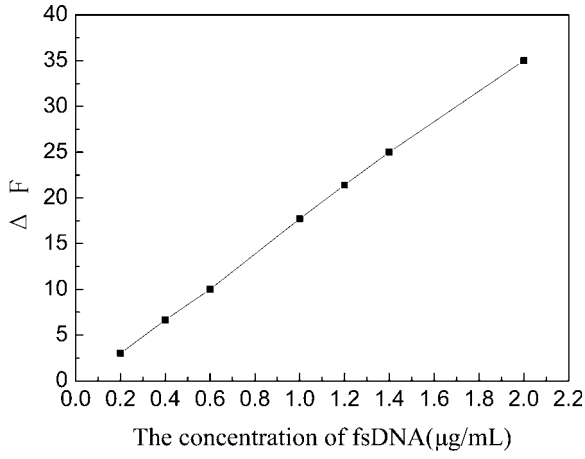

Figure 5. The response curve between fluorescence enhancement and the concentration of fsDNA.

Interference of Foreign Substances

The interference of foreign substances was tested, and the results are given in Table 2. It can be seen from Table 2 that most of the substances showed little interference except for several transition metal ions or heavy metal ions such as Cu(II) and Hg(II). However, the contents of these ions are usually very

Table 2. Tolerence of foreign substances ($\times 10^{-4}$ mol L^{-1})

Foreign substance	Maximum concentration	Relative error caused (%)
Na^+, Cl^-	1.0	+2.9
Ca^{2+}, Cl^-	1.0	−2.4
Mg^{2+}, Cl^-	1.0	−3.1
Pb^{2+}, NO_3^-	0.03	+2.1
Cu^{2+}, Cl^-	0.02	−3.7
Hg^{2+}, NO_3^-	0.002	−2.9
Ni^{2+}, NO_3^-	0.03	−3.1
Fe^{3+}, NO_3^-	0.5	+2.4
HSA	5 μg/mL	+3.6
BSA	5 μg/mL	+4.7
L-tryptophan	5 μg/mL	−3.9
L-tyrosine	10 μg/mL	−3.1
L-leucine	10 μg/mL	+1.1
L-glycine	10 μg/mL	−2.4

The interference tests were performed in the presence of 1 μg/mL fsDNA.

Table 3. The regression equations for nucleic acids

	Nucleic acids		
	fsDNA	ctDNA	Yeast RNA
C_{SDS} (mol L^{-1})	1×10^{-3}	1×10^{-3}	1×10^{-3}
C_{PF} (mol L^{-1})	1×10^{-5}	1×10^{-5}	1×10^{-5}
Calibration graph (c: mg/L)	$\Delta F = -0.7 + 18.4c$	$\Delta F = -3.1 + 57.4c$	$\Delta F = -1.2 + 25.3c$
Linear range (mg/L)	$0.04 \sim 20$	$0.06 \sim 18$	$0.12 \sim 20$
Detection limit (ng/mL)	17	21	36
r	0.995	0.998	0.998

small in most real samples; in any case, they can easily be removed during the processing of samples.

Calibration Graphs and the Analysis of Synthetic Samples

The calibration graphs for different kinds of nucleic acids were constructed by performing the standard procedure under the optimum conditions, and the results are given in Table 3. There are good linear relationships between ΔF $(=F - F_0)$ and the concentration of nucleic acids in a wide range (where F and F_0 are the fluorescence intensities in the presence and absence of DNA). The relative standard deviation was 1.2% for 2 μg/mL fsDNA ($n = 7$).

Table 4. Determination results of synthetic samples

Nucleic acid	Concentration (μg/mL)	Main interference	Found (μg/mL)	Recovery (%, n = 5)	RSD (%)
ctDNA	0.5	Cr^{3+}, Pb^{2+}, Ca^{2+}	0.51	$98.2 \sim 102.1$	1.1
ctDNA	2	BSA, Mg^{2+}	1.99	$97.3 \sim 105.6$	2.8
fsDNA	1.0	HSA, Mg^{2+}	0.99	$99.7 \sim 100.1$	2.4
fsDNA	15	HSA, γ-IgG	14.98	$96.3 \sim 104.3$	1.4
Yeast RNA	5.0	γ-IgG, Hg^{2+}	5.02	$96.7 \sim 105.3$	2.0
Yeast RNA	0.5	BSA,L-cystine	0.48	$93.7 \sim 100.1$	2.7

The concentration of foreign substances: Cr^{3+} 1.0 ng/mL; Pb^{2+} 0.5 ng/mL; Ca^{2+} 1.0×10^{-5} mol/L; Mg^{2+} 2.0×10^{-5} mol/L; Hg^{2+} 0.1 ng/mL; bovine serum albumin (BSA) 1.0 μg/mL; human serum albumin (HSA) 1.0 μg/mL; human γ-IgG 1.0 μg/mL; L-cystine 0.5 ng/mL.

With the calibration of curves, six synthetic samples were determinated simultaneously under the same conditions. The determination results are listed in Table 4. As Table 4 shows, all the results are satisfactory.

CONCLUSIONS

Based on the ability of nucleic acids to shift the aggregate-monomer equilibrium of PF, a method for the quantitative determination of nucleic acids was proposed. The sensitivity and accuracy of the method are satisfactory, and its applicability has been shown. The method described in this paper gives a unique way of eliminating the natural fluorescence of the dye and thus increasing the sensitivity of nucleic acid quantification.

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