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Determination of nucleic acids based on shifting the association equilibrium between tetrasulfonated aluminium phthalocyanine and Acridine Orange

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Based on the ability of nucleic acids to shift the association equilibrium of the ion-association complex of Acridine Orange and tetrasulfonated aluminium phthalocyanine, thus leading to an increase in the phthalocyanine fluorescence, a method is suggested for the fluorimetric determination of nucleic acids. Investigations were carried out on the spectral characteristics, order of addition of reagents, selection of the buffer system, effect of pH, influence of reaction time, effect of salt, the usage of reagents, interference of foreign substances and the effect of different acridine derivatives. Under the optimum conditions, the calibration graphs for the determination of calf thymus DNA (CT DNA), salmon DNA (SM DNA) and yeast RNA were linear over the ranges 0.04–1.2, 0.04–1.2 and 0.1–1.2 μg cm^{-1}, respectively. The detection limits for CT DNA, SM DNA and RNA were 17, 24 and 98 μg cm^{-3}, respectively. The relative standard deviation (n = 6) was within 4.6% for the detection of samples. The method was applied to the determination of *Staphylococcus aureus* DNA and the result was in agreement with that achieved by a UV method.

The quantitative determination of nucleic acids is of great importance in fundamental research and in clinical diagnosis. However, it is difficult to detect nucleic acids by using their native fluorescence because of the poor fluorescence quantum efficiency ($\Phi = 1.0 \times 10^{-5}$), and, therefore, extrinsic fluorescent probes are usually introduced during studies concerning nucleic acids. Ethidium bromide (EB), Acridine Orange (AO), 4′,6-diamidino-2-phenylindole (DAPI), Hoechst 33258, and propidium iodide are conventionally used as fluorescent stains and reagents for nucleic acids analysis, and are still widely applied at present.[1] A promising development in the synthesis of a new generation of fluorescent stains for the detection of nucleic acids is the discovery of symmetric and asymmetric cyanine dimers.[2] These cyanine compounds exhibit unique characteristics such as low background fluorescence and high affinity for nucleic acids.

Although the fluorescent probes mentioned above have been successfully employed in practical applications, some drawbacks still exist. For example, EB a classical fluorescent reagent, plays an important role in the study of nucleic acids, but its intrinsic emission may cause significant interference when the concentration of nucleic acids is at a low level. In addition, it is also a known carcinogen; thus, special measures are required during its handling and in the processing of any waste. Although dimeric cyanine compounds are excellent probes for nucleic acids, the difficulty of synthesis, lack of stability and high cost prevent them from being widely applied. Hence, it is worthwhile to develop new reagents or methods with practical and economic considerations for the detection of nucleic acids and considerable effort has been devoted to this task. For instance, a trivalent lanthanide cationic complex was found to be a selective probe for DNA;[3] the aluminium–8-hydroxyquinoline complex showed obvious response to both DNA and RNA;[4] 9,10-anthraquinone-2-sulfonate and vitamin K_3 were used as photochemical probes for nucleic acids;[5,6] methods based on the enhancement effect of nucleic acids on resonance light scattering were presented;[7,8] a method based on the ability of nucleic acids to shift the dimer–monomer equilibrium of AO was proposed;[9] the aggregation of two oppositely charged porphyrins was utilized for the determination of nucleic acids;[10] and palladium–porphyrin was used as a room-temperature phosphorescent probe of DNA.[11]

Fluorescent reagents emitting in the long-wavelength region have attracted great interest because of their distinct characteristics.[12] Fluorescent metal phthalocyanine compounds with a paramagnetic atom as the central coordinating atom are well known members of this family. Metal phthalocyanines and their derivatives, first used as dyes in industry, have been widely applied in many ‘high-tech’ areas;[13] some of them have since been developed as new probes for nucleic acid hybridization analysis,[14] fluorescence immunological assay,[15] detection of strong acids,[16] and determination of globin and albumin in human serum.[17]

In this work, a water-soluble derivative of aluminium phthalocyanine, tetrasulfonated aluminium phthalocyanine (abbreviated to AlS_4Pc hereafter), which is chemically stable and emits in the red-region with a high fluorescence quantum yield, was applied to form an ion-association complex with the cationic dye, AO. It was observed that the fluorescence of AlS_4Pc was dramatically quenched by AO, but evidently recovered when nucleic acids were present. Based on this phenomenon, a method for the quantification of nucleic acids was proposed and applied to the analysis of practical samples with satisfactory results. This work provides a new methodology for the detection of nucleic acids with the advantages of reducing the interference from background fluorescence and scattered light, and avoiding the risk of dealing with harmful chemicals. In addition, the reagents used are readily available.

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Experimental

Fluorescence spectra and relative fluorescence intensities were measured on a Model 650-10S fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with a xenon lamp, dual monochromators, a red-sensitive photomultiplier (R928), a 1 × 1 cm quartz cell and a functional recorder. The spectral bandpass for both excitation and emission was set at 5 nm. The absorption spectra were acquired on a Beckman 7400 (Beckman, Fullerton, CA, USA) spectrophotometer. A HM-20E pH-meter (TOA Electronics, Tokyo, Japan) was used for accurate adjustments of pH.

Reagents

All chemicals used were of analytical-reagent grade or the highest available purity. All aqueous solutions of the reagents were made up in distilled water that had been processed with an ion-exchange resin.

Salmon DNA and calf thymus DNA were obtained from Sino-American Biotechnology, Shanghai, China; the purity was checked by UV spectrophotometry, giving a value of $A_{260}/A_{280} \approx 1.8$. Yeast RNA was purchased from Shanghai Institute of Biochemistry, Chinese Academy of Science. Stock solutions of nucleic acids were prepared by directly dissolving commercially available salmon DNA, calf thymus DNA and yeast RNA in water at a concentration of 1.1 mg cm^{-3}; the solutions were divided into several parts, then stored in plastic centrifuge tubes at −20 °C. Working solutions were prepared by diluting the stock solutions to a concentration of 100 μg cm^{-3}, then stored at −4 °C.

AlS_4Pc was synthesized and purified according to the procedures described previously.[18] The identity of the product was confirmed by polyamide thin-layer chromatography and by its UV/VIS, fluorescence and IR spectra. A stock solution of AlS_4Pc was prepared by dissolving solid AlS_4Pc in water at a concentration of 1.0×10^{-3} mol dm^{-3}. A working solution of AlS_4Pc was prepared by diluting the stock solution to a concentration of 1.0×10^{-4} mol dm^{-3}. Both the stock and working solutions can be stored at room temperature. It was observed that the AlS_4Pc solution was very stable; the stock solution could be used after one year of storage.

Procedure

To a 10 cm^3 flask were added the reagents in the following sequence: 100 μl of 1.0×10^{-4} mol dm^{-3} AlS_4Pc, 0.8 cm^3 of 1.0×10^{-5} mol dm^{-3} AO, 1.0 cm^3 of pH 7.6 buffer (hexamethylenetetramine-HCl buffer) and a certain volume of a standard solution of nucleic acid. The mixture was diluted to 10.0 cm^3 with water and shaken until homogeneous. The relative fluorescence intensities of solutions without (F_0) and with (F) nucleic acid were measured. The excitation wavelength was set at 615 nm and the emission wavelength at 688 nm.

Results and discussion

Molecular structure of AlS_4Pc and absorption spectrum of the AO–AlS_4Pc system

Phthalocyanine compounds are artificially synthesized porphyrins, whose pyrrole moieties are stretched out by condensation with extra benzene rings. When each benzene ring in aluminium phthalocyanine contains a sulfonic acid group substituent, the resulting compound is AlS_4Pc, a strongly fluorescent compound with a negative charge (Fig. 1). The introduction of four hydrophilic substituents greatly enhances the solubility of aluminium phthalocyanine, making AlS_4Pc water-soluble and applicable in aqueous solution.

The absorption spectrum of the AO–AlS_4Pc system can be divided into three parts (Fig. 2): Part a, the Soret absorption band of AlS_4Pc; part b, the characteristic absorption of AO; and part c, the Q absorption band of AlS_4Pc. It can be seen that part a of the spectrum remains fairly consistent compared with parts b and c in which the spectrum is obviously changed in the presence of nucleic acids.

It was also found that the absorbance of the absorption spectrum in part b decreases gradually accompanied by a regular change in the spectral shape when the amount of nucleic acid in the system increases. In order to explore the reasons for the spectral changes in part b, absorption spectra of aqueous solutions with different AO concentrations but without nucleic acids were measured (see Fig. 3). It can be seen from Fig. 3 that the ratio of the maximum absorption for associated species to the maximum absorption for the monomer increases on increasing the concentration of AO in solution. As compared with Fig. 3, it was found that there is a similarity in the spectral change in part b, which means that the ratio of the maximum absorption for associated species of AO to the maximum absorption for the monomer increases gradually with an increase in the concentration of nucleic acids. The phenomena described above indicated that the association equilibrium between AlS_4Pc and AO could be shifted when nucleic acids were present. It is well known that AO is a fluorescent intercalating reagent for nucleic acids; thus, the interaction between nucleic acids and AO is strong; nucleic acids are capable of competition with AlS_4Pc for AO. As a result, AO molecules can be released from the association complex with

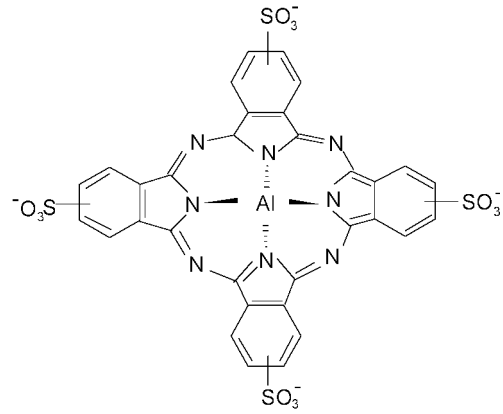

Fig. 1 Molecular structure of AlS_4Pc.

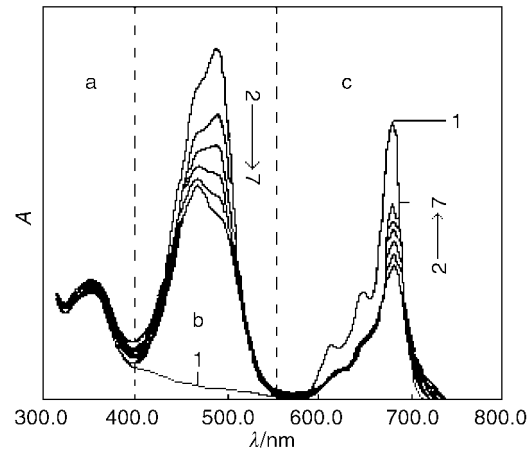

Fig. 2 Absorption spectra of the AlS_4Pc–AO system in the presence of nucleic acid (salmon DNA). [AlS_4Pc] = 1.0×10^{-6} mol dm^{-3}; [AO] = 8.0×10^{-7} mol dm^{-3}; concentration of nucleic acid for curves 1–7: 0, 0.04, 0.1, 0.2, 0.4, 0.8 and 1.6 μg cm^{-3}.

AlS_4Pc because of the interaction of AO with nucleic acids. The decrease in the spectral absorbance in part b of Fig. 2 may be attributed to the coupling of electronic motions in the intercalated AO and in the parallel planes of the nucleic acid bases which leads to a lowering of the transition probability of AO. The regular change in the spectral shape of AO may be a reflection of self-association of AO molecules, implying that it is possible for AO freed from AlS_4Pc to interact with nucleic acids in the form of a homodimer.

It was found that the introduction of AO led to a significant hypochromism in the Q band absorption of AlS_4Pc, but the absorbance recovered with the addition of nucleic acids. It was also found that the degree of recovery in the absorbance of AlS_4Pc showed a positive correlation with the amount of nucleic acid added (Fig. 2, part c), indicating that the association between AlS_4Pc and AO was weakened by the competitive binding of nucleic acids with AO. As for the fluorescence of AlS_4Pc, it was largely quenched in the presence of AO, but recovered when nucleic acids were added (Fig. 4); moreover, the recovery in the fluorescence of AlS_4Pc was proportional to the content of nucleic acids present in the system. This also supported the above-mentioned conclusion, that the association equilibrium between AlS_4Pc and AO was disturbed when nucleic acids were introduced. When nucleic acids were present, part of the associated AO migrated and interacted with the nucleic acids, resulting in an increase in the amount of free AlS_4Pc; hence, the fluorescence of AlS_4Pc recovered.

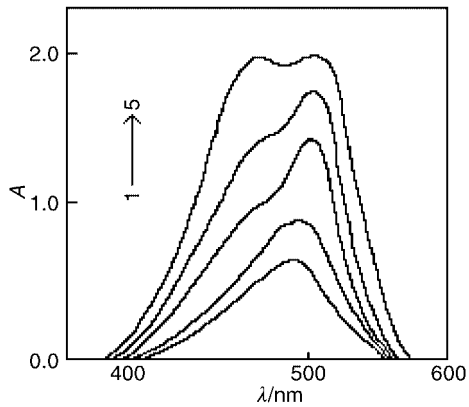

Fig. 3 Absorption spectra of AO in aqueous solution at different concentrations. Concentration of AO for curves 1–5: 2.0×10^{-6}; 6.0×10^{-6}; 10.0×10^{-6}; 20.0×10^{-6}; and 40.0×10^{-6} mol dm^{-3}.

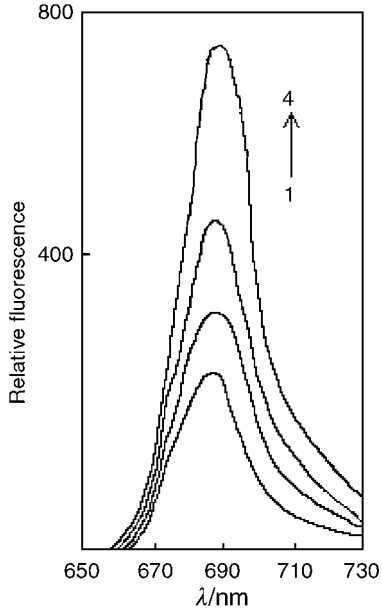

Fig. 4 Emission spectra of AlS_4Pc in AlS_4Pc–AO systems with different concentrations of nucleic acid (salmon DNA). 1: AlS_4Pc + buffer; 2: AlS_4Pc + AO + buffer; 3: AlS_4Pc + AO + buffer + 400 ng cm^{-3} salmon DNA; 4: AlS_4Pc + AO + buffer + 800 ng cm^{-3} salmon DNA.

Effect of the order of addition of reagents on the blank signal

An investigation was carried out in order to ascertain the best order for adding the reagents. The fluorescence intensity of the blank would be the smallest if the reagents were added in the correct order, such that the fluorescence of AlS_4Pc would be quenched the most by AO. Experiments showed that the smallest fluorescence signal of the blank could be achieved when the reagents were added in the order AlS_4Pc + AO + buffer (Table 1). This order for reagent addition was chosen in all subsequent experiments.

Selection of a buffer system

A phosphate buffer was first employed as the medium in the experiments, but the sensitivity and linear range of the calibration graph were unsatisfactory. Other buffer systems were then investigated. Hexamethylenetetramine-HCl buffer was finally chosen since it was found that the detection limit of the method in this buffer system was at least one order of magnitude lower than that in the phosphate buffer system. It was also found that Tris-HCl buffer was better than phosphate buffer, but not as good as hexamethylenetetramine-HCl buffer. We believe that the presence of large amounts of PO_4^{3-} ions (negatively charged) would inhibit the interaction of AO with nucleic acids, which may be the reason why phosphate buffer was not suitable for use in this work.

Effect of pH

An investigation was also performed into the influence of the pH of the hexamethylenetetramine-HCl buffer on the sensitivity of the method. The results showed that the best sensitivity was obtained with hexamethylenetetramine-HCl buffer of pH 7.6.

Influence of reaction time

The influence of reaction time on the fluorescence recovery of the system was also tested. Results showed that reaction time had little influence on the fluorescence recovery. The system easily reached equilibrium and remained stable. Detection was performed 5 min after the reagents had been mixed.

Effect of salt concentration

The effect of salt was examined. It was observed that the fluorescence of a blank solution increased markedly and the

Table 1 Effect of order of reagent addition on the degree of fluorescence quenching of the AO–AlS_4Pc system. [AlS_4Pc] = 1.0×10^{-6} mol dm^{-3}; [AO] = 8.0×10^{-7} mol dm^{-3}; hexamethylenetetramine-HCl buffer (pH 7.6)

Reagent addition order	Relative fluorescence intensity	Reagents addition order	Relative fluorescence intensity
Buffer + AO+AlS_4Pc	36	AO + AlS_4Pc + buffer	33
Buffer + AlS_4Pc + AO	34	AlS_4Pc + AO + buffer	27
AO + buffer + AlS_4Pc	38	AlS_4Pc + buffer + AO	34

sensitivity of the method decreased dramatically in a medium with a high concentration of salt. We believe this may be attributed to a decrease in the repelling effect among the phosphate anions on the backbone of nucleic acids in a medium containing large amounts of salt, which leads to a contraction of the double strand of the nucleic acids. The contraction is unfavorable for the interaction between AO and nucleic acids to occur, making the recovery in the fluorescence of AlS_4Pc smaller.

The usage of AO and AlS_4Pc

Experiments showed that the best sensitivity would be achieved when the final concentrations of AO and AlS_4Pc were set at 8.0×10^{-7} and below 1.0×10^{-6} mol dm^{-3}, respectively. However, the linear range of the calibration graph was shortened if the concentration of AlS_4Pc was too low. Taking both the sensitivity and the linear range of the calibration graph into account, 1.0×10^{-6} mol dm^{-3} was finally chosen for the concentration of AlS_4Pc.

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 tested showed little interference except for several transition metal ions or heavy metal ions such as Cu(II), Pb(II) and Hg(II). However, the contents of these ions are usually very small in most real samples; in any case, they can easily be removed during the processing of the samples.

Effect of different acridine compounds on the sensitivity

Acridine, Acridine Yellow and AO were chosen to examine the effect of using different acridine compounds on the sensitivity of the method. Structures of these compounds are shown in Fig. 5. The experimental results showed that the sensitivity of the method was in the order: AO >> Acridine Yellow > acridine, which reveals an obvious 'structure–function' relationship. For AO, the two positive tertiary amines are beneficial to the interaction with AlS_4Pc or nucleic acids. Furthermore, the hydrophobicity of the methyl groups is helpful for the planar coupling between AO and AlS_4Pc. As for Acridine Yellow, whose amino groups are in the free form, and acridine, in which there are no substituents, the interaction described above is much less strong than that of AO. Thus, a 'structure–function' effect occurred.

Table 2 Tolerance of foreign substances ($\times 10^{-4}$ mol dm^{-3}). +, −: refer to a positive or negative interference caused by the foreign substance on the results of the determination; the interference tests were performed in the presence of 2 μg cm^{-3} salmon DNA

Foreign substance	Tolerable content	Foreign substance	Tolerable content
Mg(II)	2.0 (−)	Cr(III)	0.25 (+)
Ca(II)	2.0 (−)	Al(III)	0.25 (−)
Co(II)	0.25 (−)	Hg(II)	0.00024 (−)
Mn(II)	0.5 (−)	NaCl	1.0 (+)
Cu(II)	0.0025 (+)	Glucose	56.8 (+)
Ba(II)	0.25 (+)	Adenine	2 (μg ml^{-1}, +)
Zn(II)	0.5 (−)	Cytosine	1 (μg ml^{-1}, +)
Pb(II)	0.05 (+)	Guanine	2 (μg ml^{-1}, −)
Cd(II)	0.5 (+)	Thymine	2 (μg ml^{-1}, −)
Fe(III)	0.9 (+)		

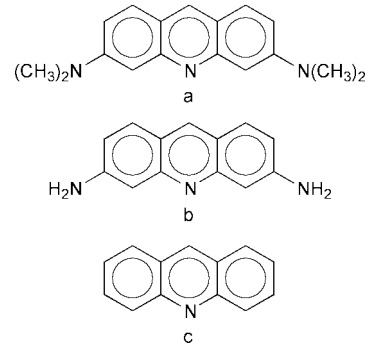

Fig. 5 Molecular structures of AO (a), Acridine Yellow (b), and acridine (c).

Table 3 Analytical parameters of the method

Nucleic acid	Calibration graph (x: μg ml^{-1})	Linear range/ μg ml^{-1}	Detection limit/ ng ml^{-1}	r
Salmon DNA	$y = 23.92 + 0.26x$	0.04–1.20	17	0.996
Calf thymus DNA	$y = 2.87 + 0.22x$	0.04–1.20	24	0.991
Yeast RNA	$y = 7.86 + 0.25x$	0.10–1.20	98	0.994

Table 4 Analytical result for a practical sample

Sample	Proposed method ($n = 6$)	RSD (%)	UV method
Staphylococcus aureus DNA/μg ml^{-1}	59.2	4.6	51.0

Calibration graphs and the analysis of practical 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. DNA obtained from *Staphylococcus aureus* by extraction according to the method of Rose[19] was determined and the result was compared with that obtained by a UV method[20] (Table 4). It can be seen that nucleic acid specificity, as has been noted in other methods, also exists in the proposed method. When the nucleic acid used as the standard and the nucleic acid to be determined come from different sources, specificity will be an issue. It is, therefore, recommended that the nucleic acid used as the standard should be the same as that being determined if a high degree of accuracy is required.

Conclusion

Based on the ability of nucleic acids to shift the association equilibrium between AO and AlS_4Pc, a method for the quantitative determination of nucleic acids is proposed. The sensitivity and accuracy of the method are satisfactory and its applicability has been shown. There are large numbers of compounds with positive or negative charges. Furthermore, it is not necessary for both of the compounds used to form an ion-association complex to be fluorescent. We therefore believe that it should be possible to find more pairs of compounds for the determination of nucleic acids according to the principle suggested in this work. This investigation introduces a new way of making use of the association equilibrium between negatively and positively charged dyes in biochemical applications.

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