Fluorescence spectral characteristics of novel asymmetric monomethine cyanine dyes in nucleic acid solutions

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Abstract Six new asymmetric monomethine cyanine dyes have been synthesized and their fluorescence characteristics in the presence of nucleic acids studied. The new dyes have no fluorescence of their own in water solutions upon excitation at 480 nm but they become strongly fluorescent in the presence of nucleic acids. The fluorescence maxima of the investigated dyes are found at 525–545 nm when bound to dsDNA and around 600 nm upon binding to RNA and ssDNA. Fluorescence quenching studies with increasing concentrations of NaCl indicate that the cyanine dyes have a mixed (intercalating and groove binding) type of interaction with dsDNA.

Key words: Fluorescence; Fluorescent dye; Fluorescent label; Intercalating agent; Nucleic acid; DNA; RNA

1. Introduction

Three decades ago LePecq and Paoletti showed that ethidium bromide forms strong fluorescent complexes with nucleic acids [1] and one decade later the same authors found that this dye intercalated within the base stacks of the DNA double helix [2]. Since that time ethidium bromide has almost entirely replaced other fluorescent dyes such as acridine orange, Hoechst 33258 and 33342 formerly used for biological and biomedical research. In recent years LePecq and coworkers have also shown that complexes between dsDNA and ethidium bromide homodimers are stable under agarose gel electrophoresis conditions and could be used for detection of nanogram amounts of dsDNA [3,4]. Recently similar studies have been carried out with another class of fluorescent dyes, the asymmetric polymethine cyanine dyes thiazole orange [5,6], homodimeric thiazole orange (TOTO-1) [7,8] and oxazole yellow homodimer (YOYO-1) [7]. These dyes demonstrated an even higher binding affinity to dsDNA in comparison with ethidium bromide, high quantum yield and extremely high (over 1000-fold) fluorescence enhancement on binding. A main advantage of the asymmetric cyanine dyes is their low (or absent) background fluorescence before binding to DNA which increases the sensitivity of detection of DNA bands by agarose electrophoresis to amounts as small as 5 pg dsDNA per band [9].

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Abbreviations: ds, double stranded; ss, single stranded; EtBr, ethidium bromide

During the last decade the fluorescent dyes have found application in many areas of the biological and biomedical fields (for details see [10]). The broad and multiple applications have raised new requirements for the physico-chemical and biological properties of the fluorescent dyes which triggered the synthesis of a new generation of fluorescent compounds.

Recently several papers and patents have been published for the synthesis of a variety of polymethine cyanine heterodimers [11] and asymmetric monomethine cyanine dyes [12–14].

In this paper we present our results on studying the interaction with nucleic acids of six new asymmetric monomethine cyanine fluorogenic dyes synthesized by the latter method.

2. Materials and methods

2.1. Chemical synthesis of the fluorogenic dyes

Six asymmetric monomethine cyanine fluorogenic compounds were synthesized as described [14]. The formulae and the abbreviations of the new dyes are shown in Fig. 1. Stock solutions were prepared by dissolving 1 mg of each dye in 1 ml of DMSO and subsequent dilution with double distilled water (about 25 ml for most of the dyes) to a final concentration of 10^{-4} mol/l.

2.2. Nucleic acid compounds

High polymeric salmon sperm dsDNA was purchased from Sigma Chemical Co. and bacteriophage M13 ssDNA was bought from New England BioLabs, Inc. Plasmid pBR322 and total *E. coli* RNA were purified from *E. coli* cells following standard procedures [15].

Synthetic oligonucleotides were prepared on a Cyclon 7300 (MilliGen) gene machine following the manufacturer's manual.

2.3. Fluorescence measurements

Corrected fluorescence spectra were scanned in a Perkin Elmer MPF44 spectrophotometer at a fluorescence excitation wavelength of 480 nm and an excitation and emission slit of 10 nm.

Fluorescence quantum yields (Q_f) were determined relative to thiazole orange (TO) [16].

3. Results

3.1. Absorption spectral studies

The asymmetric monomethine cyanine dyes used in this study were soluble in water and water-based solutions. Two maxima were observed in the absorption UV/Vis spectra in water at 280–320 nm and 480–520 nm respectively (for λ and ε values of the longest wavelength absorption band see Table 1). As seen from Table 1, the molar extinction coefficient corresponding to the first absorption maximum was quite high (50 000–80 000 M^{-1} cm^{-1}) for the six dyes. The absorption spectral analysis also showed that the presence of electron donor functional groups to both benzothiazole and quinoline

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moieties resulted in a red shift (about 15 nm) of the longest wavelength absorption maximum.

When the absorption spectra were taken in the presence of dsDNA a sharp decrease (about twice) in the molar extinction coefficient was observed for all dyes studied (see Table 1) whereas the position of the spectral maxima remained practically unchanged.

3.2. Fluorescence spectral analysis

The six dyes did not show any detectable fluorescence in water at the concentrations used in this study. However, a strong fluorescence appeared in the presence of nucleic acids and the fluorescence wavelength depended on the structure of the dyes used (Table 2 and Fig. 2).

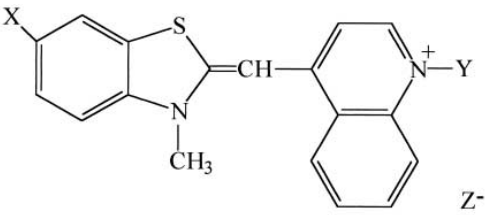
Five types of nucleic acids differing with respect to chemical composition (DNA or RNA), size, secondary structure (double stranded or single stranded) and conformation (linear or supercoiled) were used to study their interference with the fluorescence spectra of the asymmetric monomethine cyanine dyes. As seen from Table 2, the fluorescence maxima for the six dyes were located between 600 and 620 nm in the presence of RNA and ssDNA. The same spectral behavior has also been observed for the dye TO but to the best of our knowledge no reports have been made on this type of dye. However, the fluorescence wavelengths were different when the dyes were bound to dsDNA. All six dyes gave an intensive fluorescence with a small Stock shift (20 nm) of wavelengths at 525–545 nm when bound to linear (salmon sperm) dsDNA but the location of the fluorescence maxima varied in a broader range in the presence of low molecular weight (oligonucleotide) dsDNA or supercoiled plasmid DNA. As seen in Table 2, a red shift (as compared with linear dsDNA) occurred when the dyes interacted with pBR322 DNA. For most of the dyes this shift was as high as 40–60 nm and for TOHET and TOHE it was 10–20 nm. Fluorescence maximum wavelengths for two of the dyes (TOMEHE and TOAC) were comparable with that of the single stranded nucleic acids. A considerable red shift (65–80 nm) was also observed when the compounds TOHE and TOHEC were complexed with the double stranded oligonucleotide. Although the effect on the fluorescence of the rest of the dyes was less significant (varying in the range from 10 nm to the blue, to 30 nm to the red) it has to be mentioned that in two of the cases (TOMEHE and TOHET) a hypsochromic shift of 10 nm was observed.

EtBr was used as a reference dye in these experiments. As seen from Table 2, its fluorescence spectrum remained almost unchanged when the five nucleic acid compounds were applied.

Table 1
Absorption spectral characteristics of the asymmetric monomethine cyanine dyes

Dye	λ_{abs}[a]	ε[a]	λ_{abs}+dsDNA[b]	ε+dsDNA[b]
TOHE	502	80 700	507	39 300
TOHEC	502	85 000	512	40 000
TOME	515	60 500	522	29 700
TOMEHE	516	77 900	523	38 000
TOHET	516	57 200	521	28 300
TOAC	515	68 500	522	33 600

λ: absorption maximum of the longest wavelength absorption band.
[a]The dyes were dissolved in water at a final concentration of 0.5×10^{-5} mol/l.
[b]The concentration of the dyes in water is as in (a) and the concentration of salmon sperm DNA is 2 μg/ml.



Dye*	X	Y	Z
TOHE	H	C_2H_4OH	ClO_4^-
TOHEC	Cl	C_2H_4OH	Br^-
TOME	CH_3O	C_2H_5	I^-
TOMEHE	CH_3O	C_2H_4OH	Br^-
TOHET	HOC_2H_4O	C_2H_5	I^-
TOAC	CH_3CONH	CH_3	ClO_4^-

Fig. 1. Structure of the asymmetric monomethine cyanine dyes. Abbreviations: TOHE, thiazole orange hydroxyethyl; TOHEC, thiazole orange hydroxyethylchloro; TOME, thiazole orange methoxy; TOMEHE, thiazole orange methoxyhydroxyethyl; TOHET, thiazole orange hydroxyethoxy; TOAC, thiazole orange acetyl.

Based on the above results the conclusion can be drawn that all the investigated dyes are capable of distinguishing between double stranded and single stranded polynucleotides.

When the investigated dyes were used for agarose gel staining, clear gels (with no fluorescence background) were obtained and the color of the bright bands depended on the dye and nucleic acid used. The two dyes TOHET and TOAC gave yellow fluorescence for dsDNA and a green color for the RNA and ssDNA (data not shown).

3.3. Factors affecting the fluorescence of the nucleic acid-dye complexes

As mentioned already, the asymmetric monomethine cyanine dyes used in this study were not fluorescent until nucleic acids were added to the solution. The quantum yields (Q_f) of nucleic acid-dye complexes varied between 0.1 and 0.3 which corresponded to a fluorescence enhancement of 1000–3000 times.

As seen in Fig. 3, the fluorescence intensity of the monomethine cyanine dye solutions depended proportionally on the concentration of nucleic acid and the linear areas of the graph could be used for quantitation of nucleic acids in solution.

To shed light on the mechanism of the binding of the asymmetric monomethine cyanine dyes to nucleic acids, the sensitivity of fluorescence towards increasing salt concentrations was also studied. Fig. 4 shows that salt concentrations higher than 0.25 M NaCl severely quenched the fluorescence of the TOHE-nucleic acid complex decreasing the fluorescence intensity of about 80%. However, a residual fluorescence (of about 20%) remained stable at NaCl concentrations up to 3 M. Similar results were obtained with the other five compounds studied (data not shown). Fig. 4 also shows that the fluorescence of EtBr (treated identically) was poorly affected by the increasing concentrations of NaCl.

4. Discussion

The six asymmetric monomethine cyanine dyes presented in

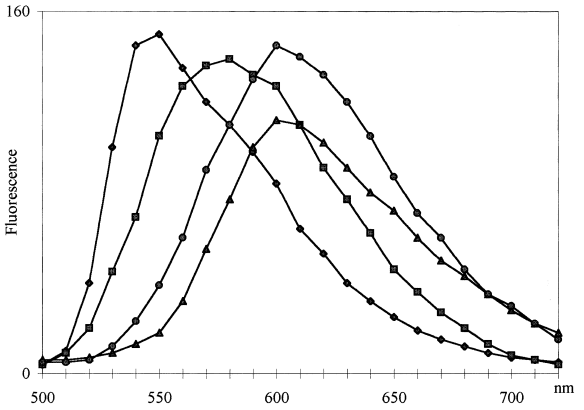


Fig. 2. Fluorescence spectra of the TOME dye in the presence of nucleic acids. Salmon sperm dsDNA (diamonds); plasmid pBR322 DNA (squares); bacteriophage M13 ssDNA (triangles); total *E. coli* RNA (circles).

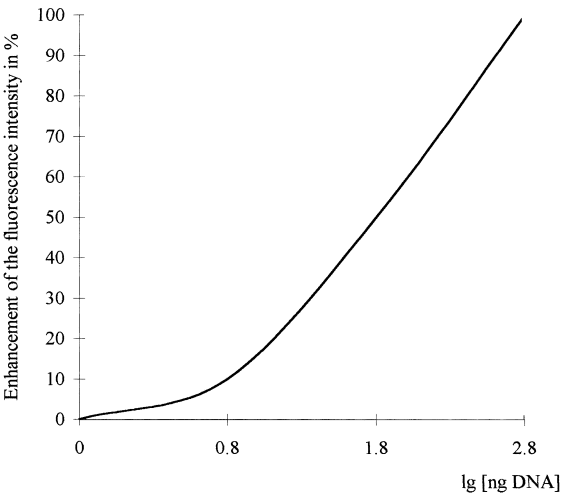


Fig. 3. Correlation between fluorescence intensity of TOHE and concentration of salmon sperm dsDNA. Water solutions of TOHE (0.5×10^{-5} mol/l) were supplemented with increasing concentrations of salmon sperm dsDNA.

this paper are fluorogenic dyes. The lack of fluorescence in the absence in nucleic acids is due to a free rotation around the internuclear bridge between the benzothiazole and quinoline rings as was shown for a similar type of benzoxazole dye [17]. The enhancement of the quantum yield and the appearance of strong fluorescence upon binding to nucleic acids is due to the strongly hindered rotation of the dye molecule.

There are three ways of binding of a noncovalent label to the nucleic acid molecule: (i) intercalation between the base stacks in the double helix, (ii) accommodation into the helical grooves and (iii) electrostatic (ion) interaction with the phosphate groups. This problem is well understood in the case of some antitumor drugs acting as DNA intercalators [18]. EtBr [2] is a typical example of an intercalating agent and the dye DSMI (*trans*-4-[4-(dimethylamino)stryryl]-1-methylpiridinium iodide) [19] of a groove binding compound.

To shed light on the mechanism of interaction of the dye with nucleic acids we studied both the spectral maximum shift [16,20] and the quenching effect of NaCl as recommended by Kumar et al. [19]. The red shift effect observed at the longest wavelength maxima in the absorption spectra was an indication of an intercalating interaction between the dyes and the dsDNA [21]. This conclusion was also supported by the residual fluorescence remaining stable even at extremely high salt concentrations (up to 3 M NaCl). However, unlike the DNA-EtBr complexes, the DNA-dye complexes in this case were less stable and dissociated at salt concentrations lower than 0.25 M NaCl. Similar results have been considered by others [19] as evidence for binding of the fluorescent substance to the minor groove of DNA.

DNA binding studies with dyes of the benzoxazole type (YOYO-1 and YO) have shown that both intercalation and groove binding took place [22]. We assume that the asymmetric monomethine cyanine fluorogenic dyes follow the same mechanism of interaction with dsDNA. This type of interaction, however, is impossible (or significantly restricted) with RNA and ssDNA. It could be speculated that in this case the dyes interact with nucleic acids electrostatically. This assumption is supported by experiments showing that the asymmetric monomethine cyanine dyes also become fluorescent in solution containing unstructured polyanions such as heparin, dextran sulfate etc. (unpublished results). Electrostatic (ionic) interaction might have taken place with dsDNA as well.

The six fluorogenic dyes presented here showed different spectral characteristics in dependence on the molecule structure and nucleic acid conformation of the dye. Although our results clearly showed that minor changes in the dye structure lead to significant changes in spectral and binding characteristics, we failed to find some firm correlation between fluorescent properties and chemical structures which would allow us to predict fluorescence characteristics and the type of interaction of new compounds of the same chemical group. To build

Table 2
Fluorescence maxima (nm) of asymmetric monomethine cyanine dyes in the presence of nucleic acids

Dye	dsDNA[a]		pBR322		ds(oligo)DNA	ssDNA[b]	RNA
	λ_{fl}	Q_f	λ_{fl}	Q_f	λ_{fl}	λ_{fl}	λ_{fl}
TOHE	525	0.21	545	0.33	605	620	605
TOHEC	525	0.18	580	0.27	590	600	600
TOME	542	0.12	580	0.13	840	605	605
TOMEHE	545	0.10	610	0.15	535	600	595
TOHET	540	0.11	550	0.17	530	600	605
TOAC	538	0.25	595	0.22	570	610	605
EtBr	595	–	605	–	595	610	605

[a]Salmon sperm.
[b]Bacteriophage.

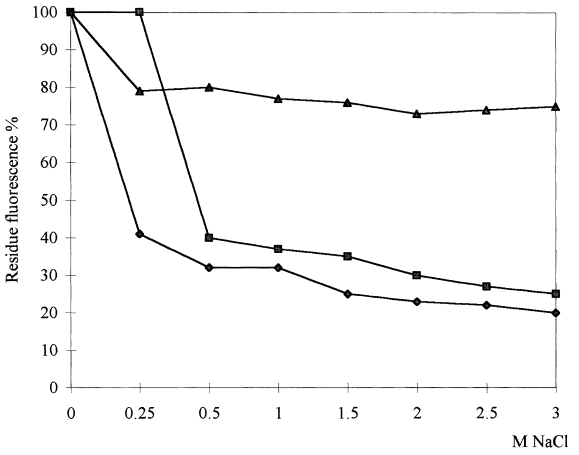


Fig. 4. Quenching effect of NaCl. Water solution of TOHE (1.3×10^{-5} mol/l) were supplemented with 6 μg/ml of salmon sperm dsDNA (diamonds) or 2.5 μg/ml of bacteriophage M13 ssDNA (squares). Triangles represent water solutions of EtBr (3.4×10^{-5} M) supplemented with 70 μg/ml of salmon sperm dsDNA.

up a reasonable structure/properties hypothesis, more experimental data with new fluorescent compounds are necessary, and these, we believe, will be accumulated soon.

The main conclusion drawn from the results presented in our study is that the asymmetric monomethine cyanine dyes are a prospective new class of fluorescent compounds for nucleic acid research. They have no fluorescence of their own, give clear solutions and agarose gels with no fluorescent background which increases the sensitivity of the fluorescence methods for analysis of nucleic acids. The spectral characteristics of some of the dyes (TOME and TOHET) are different depending on the conformation and the secondary structure of the nucleic acid which makes them prospective candidates for structural studies. In addition to their physico-chemical properties the new dyes are not or little mutagenic, as indicated by the Eims test (on bacteria) and the chromosome rearrangement test on yeast (data not presented).

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