New asymmetric monomethine cyanine dyes for nucleic-acid labelling:
absorption and fluorescence spectral characteristics

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Abstract

Absorption spectra and fluorescence properties of a series of newly synthesized asymmetric monomethine cyanine dyes are studied. The dyes carry one or two positive charges. They are devoid of their own fluorescence in solution and become fluorescent upon binding to nucleic acids only. The fluorescence maxima of the new dyes are localized between 530 and 650 nm. The wavelength and intensity of fluorescence are dependent on molecular structure of the dye, type of nucleic acid and the concentration of both nucleic acid and salts. Some of the dyes are capable of distinguishing between single-stranded and double-stranded (ds) polynucleotides giving fluorescence maxima localized at different wavelengths. Detection threshold for dsDNA for most of the dyes is comparable to that of ethidium bromide. The sensitivity of the dye-dsDNA complexes to NaCl concentrations show that the new dyes interact with dsDNA by both intercalation and electrostatically. ©2000 Published by Elsevier Science S.A. All rights reserved.

Keywords: Fluorescence; Fluorescent dyes; Fluorescent label; Intercalating agents; Monomethine cyanine dyes; Nucleic acids

Abbreviations: ds, double stranded; ss, single stranded; TMV, tobacco mosaic virus; TO, thiazole orange; DMSO, dimethylsulfoxide; DAPI, 4,6-diamino-2-phenylindol; QF, fluorescence quantum yield; TE, 10 mM Tris-HCl, pH 7.0, 1 mM EDTA

1. Introduction

Fluorescence dyes for nucleic acids labeling such as acridine orange [1], Hoechst 33258 and Hoechst 33342 [2]; ethidium bromide [3], DAPI (4,6-diamino-2-phenylindol) [4], etc. have been used in clinical laboratory and for research for many years [5]. In spite of their popularity, a general shortcoming of all these dyes is their own fluorescence determining a high fluorescence background. Ideal for nucleic acids labeling is the dye devoid of its own fluorescence in solution, having a high binding affinity to nucleic acids and illuminating after binding to the substrate only. These requirements are partly satisfied by the dye thiazol orange forming stable complexes with both DNA and RNA [6–8].

Recently we have studied spectral characteristics of a series of asymmetric monomethine cyanine dyes with no fluorescence in solution [9]. They become fluorescent (at 530–620 nm) after binding to nucleic acids and some of them are potent in distinguishing between double-stranded (ds) and single-stranded (ss) polynucleotides.

In this study, we are presenting the spectral properties of a new series of asymmetric monomethine cyanine dyes in relation with their interaction with nucleic acids. Some of the dyes carry a single positive charge and others are doubly charged in order to increase their affinity to anionic substrates.

2. Experimental details

2.1. Chemical synthesis and design of the fluorogenic dyes

The asymmetric monomethine cyanine dyes (Fig. 1) were synthesized as described before [10]. Stock solutions were prepared by dissolving of 1 mg of the dye in 1 ml DMSO and subsequent dilution with TE buffer (10 mM
Fig. 1. Asymmetric monomethine cyanine dyes.

Table 1

<table>
<thead>
<tr>
<th>Dye No.</th>
<th>Without DNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>With DNA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ&lt;sub&gt;abs&lt;/sub&gt; (nm)</td>
<td>ε (M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>λ&lt;sub&gt;abs&lt;/sub&gt; (nm)</td>
</tr>
<tr>
<td>1</td>
<td>510.5</td>
<td>73900</td>
</tr>
<tr>
<td>2</td>
<td>502.1</td>
<td>70900</td>
</tr>
<tr>
<td>3</td>
<td>512.5</td>
<td>80900</td>
</tr>
<tr>
<td>4</td>
<td>505.5</td>
<td>77500</td>
</tr>
<tr>
<td>5</td>
<td>507.5</td>
<td>51000</td>
</tr>
<tr>
<td>6</td>
<td>512.6</td>
<td>66300</td>
</tr>
<tr>
<td>7</td>
<td>512.6</td>
<td>68200</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dyes dissolved in TE buffer at a final concentration of 0.5 × 10<sup>-5</sup> mol/l.

<sup>b</sup>Dyes dissolved in TE buffer as in (*) in the presence of salmon sperm dsDNA at a concentration of 2.2 μg/ml.

3. Results and discussion

3.1. Chemical structure and design of the fluorogenic dyes

In our previous paper [9] we have shown that minor changes in the structure of the monomethine cyanine dyes result in significant changes in their properties as nucleic acids markers. This tempted us to create a new series of the same class of dyes (see Fig. 1) and study their spectral characteristics. They carry new substituents to the basic heterocycles and some of them (compounds No. 6 and 7) bear an additional (thiazole) heterocycle as an end group. As seen in Fig. 1, all dyes are positively charged, bearing one (No. 5 and 6) or two (No. 1–4 and 7) charges, respectively. The latter were designed especially to evaluate the significance of the electric charge for the interaction of the dyes with nucleic acids.

3.2. Absorption spectral studies

The longest wavelength absorption maximum of all compounds studied were located between 500 and 525 nm and their molar absorptivity values were between 50 000 and 81 000 M<sup>-1</sup> cm<sup>-1</sup> (Table 1). Absorption spectra in water solutions showed that the insertion of new substituents to the quinoline moiety shifted the absorption maximum bathochromically by 5–7 nm in comparison with the substitutions to the benzothiazole ring.

The UV–VIS spectra of the dye-DNA complexes showed that the interaction with dsDNA led to a slight bathochromic shift of about 2–10 nm and a hypochromic effect of 10–25% affecting the longest wavelength absorption maximum (Table 1). On the contrary, when the dyes were bound to a single-stranded oligonucleotide (20-mer) the same maximum was shifted hypsochromically (blue shift) by 25–40 nm and the hypochromic effect reached 40–60%. In the presence of tRNA or TMV RNA the longest wavelength maximum was also shifted to the blue by 20–30 nm and its intensity slightly decreased. It is known from literature.
that the bathochromic shift and hypochromism affecting the longest wavelength absorption band indicates that the dye is accommodated into the grooves of the double-helix [11–13].

3.3. Fluorescence spectral studies

The newly synthesized monomethine cyanine dyes are devoid of their own fluorescence in TE buffer and distilled water at room temperature, except for the compound No. 4 whose $Q_f$ value is less than 0.001. However, all they become fluorescent upon binding to polynucleotides. Our study showed that both position and intensity of the fluorescence maxima depended strongly on the dye structure and the type of polynucleotide used.

All dyes showed similar fluorescence characteristics in distilled water or TE buffer (pH 7.0) in the presence of dsDNA (2 μg/ml). As seen in Table 2, their fluorescence maxima were located at 528–532 nm and their quantum yields were found in the range of 0.18–0.32, except for compounds No. 6 and 7 whose $Q_f$ was ten times lower. When a ssDNA (synthetic oligodeoxyribonucleotide −1.6 μg/ml) was substituted for dsDNA in the same solutions, the fluorescence quantum yields dropped to less than 0.02 and the fluorescence maxima of the dyes lied between 520 and 570 nm (Table 2). As seen from Table 2, two of the dyes (No. 6 and 7) were not fluorescent in the presence of ssDNA and the fluorescence maxima of two other dyes (No. 2 and 4) were shifted by approximately 30 nm in comparison with the dsDNA. This bathochromic shift makes it possible the latter two dyes to be used for distinguishing between dsDNA and ssDNA in solution.

Table 2 also shows that all dyes were fluorescent in the presence of tRNA and TMV RNA. In comparison with DNA, their fluorescence maxima (except for No. 5) were shifted bathochromically by about 100 nm and lay between 570 and 660 nm.

To shed light on the mechanism of interaction of the new monomethine cyanine dyes with dsDNA, the effect of two factors on their fluorescence properties - nucleic acid and salt concentrations, was studied.

Fig. 2 shows that both position and intensity of the fluorescence maximum of compound No. 4 was variable and strongly depended on the concentration of DNA in solution. Whereas at dsDNA concentrations higher than 1.8 μg/ml the fluorescence maximum was found at 530 nm, it was shifted up to 622 nm at lower dsDNA concentrations. The bathochromic effect was accompanied also by a dramatic decrease (of about 100 times) in the intensity of the same maximum. Similar results were obtained also with two other dyes, No. 2 and No. 5, whereas the position of the fluorescence maxima of compounds No. 1 and No. 3 did not depend on the DNA concentration. Unlike compounds

Table 2

<table>
<thead>
<tr>
<th>Dye No.</th>
<th>dsDNA$^b$</th>
<th>Oligo$^c$</th>
<th>tRNA$^d$</th>
<th>TMV RNA$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_f$ (nm)</td>
<td>$Q_f$</td>
<td>$\lambda_f$ (nm)</td>
<td>$Q_f$</td>
</tr>
<tr>
<td>1</td>
<td>532.0</td>
<td>0.28</td>
<td>535.6</td>
<td>0.019</td>
</tr>
<tr>
<td>2</td>
<td>526.8</td>
<td>0.18</td>
<td>564.2</td>
<td>0.019</td>
</tr>
<tr>
<td>3</td>
<td>531.6</td>
<td>0.26</td>
<td>538.2</td>
<td>0.018</td>
</tr>
<tr>
<td>4</td>
<td>530.0</td>
<td>0.32</td>
<td>563.8</td>
<td>0.018</td>
</tr>
<tr>
<td>5</td>
<td>529.0</td>
<td>0.27</td>
<td>528.0</td>
<td>0.021</td>
</tr>
<tr>
<td>6</td>
<td>536.0</td>
<td>0.006</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$Monomethine cyanine dyes are dissolved in TE buffer as in Table 1.

$^b$Salmon sperm dsDNA at a concentration of 2 μg/ml.

$^c$Oligodeoxyribonucleotide (20 nm long) at a concentration of 1.6 μg/ml

$^d$E. coli total tRNA at a concentration of 2 μg/ml.

$^e$Tobacco mosaic virus RNA at a concentration of 1.5 μg/ml; (–), no fluorescence.

Fig. 3. Fluorescence intensity (at 530 nm) of compound No. 1 as a function of the dsDNA concentration. Salmon sperm dsDNA is dissolved in TE buffer at increasing DNA concentrations and the fluorescence intensity is measured and presented in arbitrary units (a.u.).

Fig. 4. Fluorescence intensity of compound No. 5 as a function of NaCl concentration. Solutions of dye No. 5 alone (circles) or in the presence of salmon sperm dsDNA at a concentration of 1.38 μg/ml (squares) in TE buffer are adjusted to varying concentrations of NaCl. The fluorescence intensities are measured at the fluorescence maxima of the dye-dsDNA complex (530 nm) and of the free dye No. 5 (620 nm).

No. 2, 4 and 5, the fluorescence intensity of compounds No. 1 and 3 decreased linearly on diminishing the dsDNA concentration (Fig. 3). The detection threshold of compound No. 1 for dsDNA at 532 nm was determined to be 70 ng/ml.

The effect of NaCl concentration on fluorescence was studied with the dye No. 5. As shown in Fig. 4, the fluorescence maximum was shifted hypsochromically from 620 nm in distilled water to 530 nm in 0.02 M NaCl. The intensity of the same maximum increased with increasing the NaCl concentration up to 0.04 M and then gradually decreased upon the further increase of the NaCl concentrations. It should be mentioned, however, that some residual fluorescence remained even at NaCl concentrations higher than 3 M. The fluorescence maximum of compound No. 5 in NaCl solutions (only) was located at 620 nm (Fig. 4). Although similar results were obtained with the dyes No. 2 and No. 4, the fluorescence intensity of compound No. 4 approached its maximum at 0.18 M NaCl which was probably due to the presence of two positive charges in its molecule. The fluorescence quantum yield of compound No. 4 in the presence of dsDNA was also dependent on salt concentration. Thus, whereas the Qf value for the dye-dsDNA complex (1.38 μg/ml dsDNA) in distilled water was 0.012 (at 620 nm) it raised up to 0.08 in 0.18 M NaCl at the same concentration of dsDNA (with a fluorescence maximum at 530 nm).

Dye No. 4 has been used also for studying the interference of the type of cation in solution with the fluorescence properties of the dye-dsDNA complexes. Our results showed...
that the elevation of NaCl concentration up to 0.18 M led to an eight fold increase in the fluorescence intensity, whereas the same molarities of CaCl₂ or Tris-HCl resulted in only two fold increase.

Recently we have shown that monomethine cyanine dyes interact by both electrostatic binding and intercalation into the minor groove of the dsDNA [9]. Salt and DNA concentration dependences of the fluorescence of the new monomethine cyanine dyes presented in this study implies the same mechanism of interaction with dsDNA.

It is known from literature that in glycerol solutions the freedom of internal molecular rotations is restricted and the pseudo-immobilized fluorescent dyes behave as intercalated into dsDNA [14]. Taking into consideration this phenomenon we have also studied the fluorescence spectra of the new monomethine cyanine dyes in glycerol. We found that all dyes became fluorescent at 530–540 nm and that both the position and intensity of their fluorescence maxima were similar to those typical for the dye-dsDNA complexes.

4. Conclusions

Main advantage of the new asymmetric monomethine cyanine dyes is the absence of their own fluorescence in water solutions. They become fluorescent in the presence of nucleic acids and their fluorescence quantum yields are satisfactory high. Fluorescence maxima of four of the compounds (No. 1 to 4) in the presence of dsDNA are located at around 530 nm while in the presence of RNA they lie at 600–660 nm. The fluorescence wavelength dependence on the nature and secondary structure of the nucleic acids makes it possible to use some of the dyes for distinguishing between ssDNA and dsDNA (No. 2 and 4) and between DNA and RNA (all except for No. 5). The new fluorogenic dyes will find application in gel electrophoresis of nucleic acids and also in developing new methods for their quantitation and visualization in cytological preparations.

Acknowledgements

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References