Homodimeric monomethine cyanine dyes as fluorescent probes of biopolymers

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Abstract

The fluorescence properties of newly synthesized homodimeric monomethine cyanine dyes in the presence of biopolymers are investigated. They do not fluoresce in TE buffer and bidistilled water but become strongly fluorescent ($Q_F=0.3–0.9$) in the region 530–650 nm when bound to dsDNA and ssDNA. The detection limit of dsDNA is about 1.7 ng/ml. Some of dyes studied are able to distinguish between dsDNA and ssDNA, RNA, BSA in solution and gel electrophoresis. The influence of different factors (temperature, pH and viscosity of the medium, presence of histone) on the formation of the dye–biopolymer complexes is investigated. The results of steady-state and dynamic fluorescence measurements concerning the different types of binding between dyes and biopolymers show that the new dyes are applicable in molecular biology as highly sensitive fluorescence labels. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Fluorescence detection of nucleic acids is a basic part of many procedures in molecular biology. Le Pecq and Paoletti [1,2] proposed the first fluorescence label for nucleic acids — ethidium bromide. The disadvantage of ethidium bromide is its fluorescence in the unbound state, as it coincides spectrally with the fluorescence of the ethidium bromide–nucleic acid complex. This decreases the signal-to-background ratio and thus diminishes the possibility to detect very small amounts of nucleic acids. In recent years many other compounds have been investigated that form highly fluorescent complexes with biopolymers [3–9] and thus might be used in clinical laboratory [10].

In previous papers we investigated the spectral characteristics of two series of newly synthesized monomethine cyanine dyes in the presence of different types of nucleic acids suggesting two possible types of interaction: electrostatic binding and intercalation into the minor groove of dsDNA [11,12]. The experimental results presented in these papers show that increasing the number of positive charges in the dye molecule gives rise to enhanced binding to polynucleotides and leads to higher fluorescence intensity of the dye–biopolymer complexes. On the other hand it has been shown [13] that dimeric forms of TO and YO (TOTO and YOYO) bind much stronger to dsDNA in comparison to the monomers TO and YO, respectively. This fact is explained by the increased binding strength owing to the fact that the dimeric forms of the dyes are bis-intercalators [7]. The binding is further stabilized by electrostatic interaction of the bis-cationic linker with the minor groove of the DNA.

Recently [14] we described the synthesis of new homodimeric monomethine cyanine dyes (HMCD) with four positive charges (Fig. 1) as well as their fluorescence properties in the presence of dsDNA and ssDNA. It was shown that they have no fluorescence of their own in solution (except for compounds 2 and 4 whose fluorescence quantum yield, $Q_F$, is less than 0.001) and become strongly fluorescent ($Q_F=0.3–0.9$) in the presence of dsDNA and ssDNA. All compounds studied are able to
2.2. Biopolymers

The following biopolymers are used in the study: herring sperm DNA (native (ds) and heat-denatured (ss)) (Sigma), tRNA from brewer’s yeast (Boehringer Mannheim), dNTP (Boehringer Mannheim), BSA (Sigma) and total histone from thymus isolated at non-denaturing conditions (gift by Dr. Eva Pascheva).

2.3. Absorption and fluorescence measurements

Absorption spectra are scanned on a Specord M40 (Karl Zeiss, Jena) UV–Vis spectrophotometer and the fluorescence spectra (excitation at 480 nm) on a Perkin-Elmer MPF44 spectrofluorimeter. The emission spectra are corrected using a standard Tungsten lamp, whereas the excitation spectra are corrected with Rhodamine B. The fluorescence quantum yield ($Q_F$) is determined relative to that of the dye TO ($Q_T=0.2$) [4]. The fluorescence decay curves (10,000 counts in the maximum, time resolution = 0.1 ns/chan) are measured at room temperature on a nanosecond Single Photon Counting spectrofluorimeter System PRA 2000, using a nitrogen filling flash lamp with $\lambda_{ex}=313$ nm and a detection wavelength corresponding to the maximum of the fluorescence band. The natural lifetime $\tau$ is estimated by a standard deconvolution procedure. The accuracy of the fit is controlled by the weight residuals, the autocorrelation function of the residuals and reduced $\chi^2$. Steady-state irradiation is carried out using a medium-pressure mercury lamp (Tungsram HGO 125 W).

3. Results and discussion

3.1. Absorption spectral studies

The longest wavelength absorption bands of the studied HMCD are at 505–506 nm [14]. A bathochromic shift of 15–20 nm is observed when they are bound to dsDNA. The intensity of the maximum diminishes by approximately 40%. Similar effects are obtained also for other dyes bound to dsDNA [11,12]. They are taken as an evidence for intercalation, as is the case in other investigations [15–17].

3.2. Fluorescence spectral studies

As mentioned in Ref.[14] the studied HMCD do not fluoresce in destilled water and TE buffer (pH 7.5) except for compounds 2 and 4, where $Q_F$ is less than 0.001. A strong emission arises when the dyes are bound to dsDNA or ssDNA. The fluorescence maxima are between 529 and 533 nm in the case of dsDNA and in the region 570–650 nm in the presence of ssDNA; the $Q_F$ values are in the range 0.30–0.94 depending on dye structure and type of DNA. The fluorescence intensity of
the dye complexes with tRNA, BSA and dNTP is much lower (about 10 times). The fluorescence characteristics of HMCD in the presence of different biopolymers are given in Table 2. As can be seen from these data, the position of the fluorescence maxima of the dye–biopolymer complexes depends strongly on the type of biopolymer used. In comparison to dsDNA the fluorescence maxima in the presence of tRNA and BSA are shifted bathochromically by about 100 nm (except for the dye N1-BSA complex) and lie between 570 and 630 nm. Hence all studied HMCD are able to distinguish between dsDNA and ssDNA, tRNA or BSA in solution.

The fluorescence spectra of dye N3–dsDNA (5 µg/ml) and dye N3–tRNA (6.4 µg/ml) complexes are shown in Fig. 2. As can be seen from the figure, the fluorescence intensity of the complex with tRNA is about 10 times lower than this of the corresponding complex with dsDNA. Furthermore, two fluorescence maxima at around 530 and 630 nm are observed in solution of dye N2 in TE buffer containing a mixture of 0.6 µg/ml dsDNA and 6.4 µg/ml tRNA (Fig. 3). Hence the investigated HMCD even permit the analysis of mixtures of DNA and RNA.

The detection limits for dsDNA and tRNA with dye N2 are 1.7 and 18 ng/ml, respectively.

The HMCD are used for agarose gel electrophoresis staining. Clear gels (with no fluorescence background) are obtained, which is an advantage of these dyes. The color of the bright bands depends on the dye structure and the nucleic acid used. Compound N1 gives yellow–green fluorescence for dsDNA and a orange for RNA (data not shown). The dye–polynucleotide complexes are stable during electrophoresis.

All dyes investigated are not stable in solution upon steady state irradiation with a medium-pressure mercury lamp (Tungsram HGO 125 W). After 4 h of irradiation they are completely decomposed. However, on binding to dsDNA their photostability increases dramatically: the fluorescence intensity remains unchanged even after 10 h of irradiation with the mercury lamp or when kept for at least 1 week in direct sunlight. These properties of the investigated HMCD make them suitable for fluorescence measurements that extend over long time periods.

As in the case of the monomethine cyanine dyes studied earlier [11,12] the observed large difference between the positions of the fluorescence maxima when HMCD are bound to dsDNA and to ssDNA, tRNA or BSA gives reason to assume two types of interactions between these dyes and biopolymers. Most probably in the case of dsDNA the dye intercalates between the two strains of the nucleic acid, while in the presence of tRNA, ssDNA and BSA an electrostatic binding takes place.

To prove the assumption for intercalation of the HMCD into dsDNA their fluorescence spectra in glycerol are measured. It is known from the literature [18] that in glycerol, owing to its high viscosity, the possibility of intramolecular rotations leading to radiationless deactivation of excited states is restricted. Therefore the pseudo-immobilized dyes behave similarly as when intercalated into dsDNA. We found that the position and intensity of the HMCD fluorescence in glycerol practically coincide with those of the HMCD–dsDNA complexes (in equimolar concentration). The concentration of HMCD in glycerol was the same as in TE buffer or bidistilled water: 1×10⁻⁶ M.

The electrostatic interaction between HMCD and biopolymers can be imitated by fluorescence measurements in solutions of the dyes in bidistilled water at different NaCl concentrations (0.12, 0.25, 0.5, 1 and 1.5 M). We found that all HMCD, although non-fluorescent in salt-free water, fluoresce in the presence of NaCl at 620–630 nm. In most cases, the fluorescence maxima of HMCD in the presence of tRNA, BSA and ssDNA, are in the same region (Table 1). This leads us to assume that most probably an electrostatic binding takes place. If 5 µg/ml dsDNA are added to solutions containing 1.0 or 1.5 M NaCl and HMCD the fluorescence maximum shifts to the blue reaching the region 530–540 nm, where also the emission...
of the dye–dsDNA complex occurs. Hence, even the extremely high alkaline character of the medium does not prevent the intercalation of the dyes into the minor groove of dsDNA.

Table 2 presents the fluorescence lifetimes of compounds N2 and N3 in the presence of different biopolymers. The fluorescence decay curves are fitted to the multiexponential linear function

\[ I(t) = \sum_{i} A_i \exp(-t/\tau_i), \]

where \( A_i \) are relative amplitudes of different lifetimes \( \tau_i \) (Table 2).

In the cases of dye N2 as well as of N3 in the presence of dsDNA, where it is supposed that the complex dye–dsDNA is due to intercalation of the dye into the minor groove of the double stranded nucleic acid, fluorescence decay curves are fitted by double exponential linear function. The fluorescence lifetimes are of same order of magnitude for both complexes: around 2 and 15 ns (see Table 2). In the presence of tRNA and BSA where the interactions between the dyes and the biopolymers are most probably electrostatic the fluorescence decay curves of dye N2 are fitted with good precision by a three-exponential linear function, while that of N3 is double exponential. The obtained three-exponential function for compound N2 could be attributed to the very low fluorescence of the dye, while compound N3 has no fluorescence of its own. The calculated fluorescence lifetimes in these cases are considerably lower: about 0.4 and 5 ns. The observed significant difference between the fluorescence lifetimes in the presence of dsDNA and tRNA/BSA supports the assumption for the formation of two different types of emitting complexes depending on the nature of the biopolymer. In order to confirm this hypothesis additional lifetime measurements of N2 in glycerol, which imitates the intercalation of the dye into the minor groove of dsDNA and in the presence of 0.5 M NaCl (where only an electrostatic interaction could take place) are carried out. The obtained fluorescence lifetimes in glycerol are alike to these of the dye–dsDNA complex, while in NaCl their values are very close to these of dye–tRNA or dye–BSA complexes.

In acidic (HClO\(_4\), pH 2.5) solutions containing HMCD and dsDNA, where the studied HMCD remain stable, the position of the fluorescence maxima lie between 620 and 660 nm. This experimental result shows that, contrary to alkaline medium which does not prevent the intercalation of the dyes into dsDNA, at pH 2.5 the intercalation is not possible because of hydrolyzation of dsDNA to nucleosides. The increase of pH values up to 5 leads to the appearance of a fluorescence maximum at 530–540 nm typical for the HMCD–dsDNA complexes.

The fluorescence maxima of HMCD in the presence of different nucleosides are between 630 and 680 nm (Table 3), i.e., in the region when the fluorescence in strongly acidic medium is observed.

As can be seen from Table 3, a strong dependence between the chemical structure and the fluorescence properties of the studied dyes in the presence of dNTP is observed. The dye N1 does not fluoresce with all dNTP. The dye N2 fluoresces with dGTP and UTP and does not fluoresce in the presence of dCTP and dTTP; the dye N3 does not fluoresce only with dCTP, while the dye N4 has fluorescence in the presence of dATP, dCTP and dGTP, but does not fluoresce with dTTP and UTP.

The influence of temperature on the formation of the dye–dsDNA complexes was also investigated. When a solution containing a dye N3 and dsDNA complex in bidistilled water is rapidly heated to 95°C the fluorescence maximum at 535 nm disappears and a new one at 630–640 nm arises. The latter maximum characteristic for the dye–ssDNA complex (see Table 1) has a 10 times lower intensity than that at 535 nm. The dye is stable at 95°C.

**Table 1**

<table>
<thead>
<tr>
<th>Dye</th>
<th>Biopolymer</th>
<th>( \tau_1 ) (ns)/A(_1)</th>
<th>( \tau_2 ) (ns)/A(_2)</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>DsDNA</td>
<td>1.57/93%</td>
<td>14.87/77%</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>TRNA</td>
<td>0.31/78%</td>
<td>4.21/22%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>0.33/82%</td>
<td>4.67/18%</td>
<td>1.13</td>
</tr>
<tr>
<td>N2</td>
<td>DsDNA</td>
<td>3.12/92%</td>
<td>13.08/8%</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>TRNA</td>
<td>0.4/76%</td>
<td>3.9/21%</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>0.4/74%</td>
<td>4.21/15%</td>
<td>32.5/11%</td>
</tr>
</tbody>
</table>

* Decay curve is fitted by biexponential decay function.

**Table 2**

<table>
<thead>
<tr>
<th>Dye</th>
<th>Biopolymer</th>
<th>( \tau_1 ) (ns)/A(_1)</th>
<th>( \tau_2 ) (ns)/A(_2)</th>
<th>( \tau_3 ) (ns)/A(_3)</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>DsDNA</td>
<td>1.57/93%</td>
<td>14.87/77%</td>
<td>–</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>TRNA</td>
<td>0.31/78%</td>
<td>4.21/22%</td>
<td>–</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>0.33/82%</td>
<td>4.67/18%</td>
<td>–</td>
<td>1.13</td>
</tr>
<tr>
<td>N2</td>
<td>DsDNA</td>
<td>3.12/92%</td>
<td>13.08/8%</td>
<td>–</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>TRNA</td>
<td>0.4/76%</td>
<td>3.9/21%</td>
<td>18.0/1%</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>0.4/74%</td>
<td>4.21/15%</td>
<td>32.5/11%</td>
<td>0.99</td>
</tr>
</tbody>
</table>

* Decay curve is fitted by biexponential decay function.

**Table 3**

<table>
<thead>
<tr>
<th>Dye</th>
<th>Biopolymer</th>
<th>( \lambda_2 ) (nm)</th>
<th>( \lambda_3 ) (nm)</th>
<th>( \lambda_4 ) (nm)</th>
<th>( \delta ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N2</td>
<td>633.9</td>
<td>–</td>
<td>652.2</td>
<td>–</td>
<td>648.8</td>
</tr>
<tr>
<td>N3</td>
<td>653.4</td>
<td>654.4</td>
<td>–</td>
<td>641.6</td>
<td></td>
</tr>
<tr>
<td>N4</td>
<td>662.6</td>
<td>–</td>
<td>640.2</td>
<td>677.0</td>
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</table>

* No fluorescence observed.
The slow lowering of temperature (passing from 95 to 42°C and 30 to 18°C) leads to the appearance again of the fluorescence at 535 nm and at the same time the maximum at 630–640 nm remains. On the basis of this experimental results one could assume that two different complexes, dye–dsDNA and dye–ssDNA, are present in solution after cooling, showing that the renaturation of DNA is incomplete.

On the contrary, when the temperature of solution containing a dye N3–dsDNA complex was gradually increased (19, 39, 59, 69, 79, 89 and 95°C) the fluorescence maximum at 535 nm shifts slightly to the red (with about 15 nm) and the fluorescence intensity diminishes (see Fig. 5). No increase of the fluorescence maximum at 630–640 nm was observed. The rapid lowering of the temperature from 95 to 30°C leads to increase of the fluorescence intensity, which becomes of the same order of magnitude as that of the unheated solution. Hence, it could be concluded that dsDNA can not be completely dissociated at this conditions. The observed decrease of the fluorescence intensity (see Fig. 4) could be attributed to the following: the slow heating weakens the hydrogen bonds between the two chains of DNA and eventually leads to their separation. At this situation the freedom of intramolecular rotations in the dye molecule increases giving rise to the radiationless deactivation of the fluorescent excited state.

If a solution of dsDNA in bidistilled water is heated to 95°C, when denaturation of dsDNA takes place, and then dye N3 is added, the only fluorescence maximum observed is at 630–640 nm; typical for the complex dye–ssDNA (electrostatic interaction). After cooling to 19°C the position of the fluorescence maximum remains unchanged. Hence, the electrostatic binding to the ssDNA dye hinders the possibility for renaturation of DNA.

When the dye is added to dsDNA preliminarily heated to 95°C and slowly cooled to 19°C, there are two maxima in the fluorescence spectrum: one at 535 nm (dye–dsDNA complex) and another at 630–640 nm (dye–ssDNA complex). This is most probably due to the presence of both ds- and ssDNA in solution.

Histones are basic proteins in chromatin bearing positive charges. That is why they do not fluoresce in the presence of HMCD, which also bear positive charges. The histones interact directly with the polynucleotide chain in the DNA binding sites.

As was mentioned before, the dye N3 fluoresces in the presence of dsDNA at around 540 nm (Fig. 5). When histone is added to an already formed complex dye N3–dsDNA an additional band with maximum at 630–640 nm appears. Most probably in this case the histone removes part of intercalated dye making possible electrostatic interaction between the dye and DNA also. If the “complex” consists of dsDNA and histone and to it is added dye there is only one fluorescence maximum at 620 nm. The reason for this experimental result could be the strong binding of histone to dsDNA which does not allow the dye to intercalate into the minor groove (Fig. 5).

4. Conclusions

The investigated HMCD are devoid of fluorescence of their own, but become strongly fluorescent ($Q_F = 0.3–0.9$) in the region 540–650 nm after binding to ds and ss nucleic acids in solutions with equimolar concentration of dyes and DNA. The formed complexes are stable upon irradiation with UV light for more than 10 h, at heating to 95°C, within a large range of pH. The obtained experimental results give the possibility to follow the melting of dsDNA as well as its reassociation and hydrolysis. The types of interaction of HMCD with biopolymers, intercalation into the minor groove and electrostatic binding, are studied using experiments in the presence of salt, positive charged histone, measurements in glycerol and fluores-
cence lifetime measurements. Some of the studied dyes are able to distinguish between dsDNA and ssDNA, RNA and BSA in solution and at gel electrophoresis. This property is their great advantage compared to other fluorescence labels used.

5. Abbreviations

$A_i$ relative amplitudes of decay with lifetime $\tau_i$
BSA bovine serum albumin
DMSO dimethylsulfoxide
ds double stranded
HMCD homodimeric monomethine cyanine dyes
$Q_F$ fluorescence quantum yield
ss single stranded
TE 10 mM Tris–HCl (pH 7.5), 1 mM EDTA
tRNA transport RNA
$\lambda_{ex}$ excitation wavelength
$\lambda_F$ fluorescence maximum
$\tau_F$ fluorescence lifetime

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References