Stability, specificity and fluorescence brightness of multiply-labeled fluorescent DNA probes

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ABSTRACT

In this work, we studied the fluorescence and hybridization of multiply-labeled DNA probes which have the hydrophobic fluorophore 1-(ε-carboxypentenyl)-1-ethyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonate (Cy3) attached via either a short or long linker at the C-5 position of deoxyuridine. We describe the effects of labeling density, fluorophore charge and linker length upon five properties of the probe: fluorescence intensity, the change in fluorescence upon duplex formation, the quantum yield of fluorescence (Φf), probe–target stability and specificity. For the hydrophilic dye Cy3, we have demonstrated that the fluorescence intensity and Φf are maximized when labeling every 6th base using the long linker. With a less hydrophilic dye, a labeling density this high could not be achieved without serious quenching of the fluorescence. The target specificity of multiply-labeled DNA probes was just as high as compared to the unmodified control probe, however, a less stable probe–target duplex is formed that exhibits a lower melting temperature. A mechanism that accounts for this destabilization is proposed which is consistent with our data. It involves dye–dye and dye–nucleotide interactions which appear to stabilize a single-stranded conformation of the probe.

INTRODUCTION

Fluorescent DNA probes for in situ hybridization are now commonly used in a wide spectrum of applications such as the determination of mRNA distributions on the cellular and subcellular level (1,2), monitoring gene expression (3,4), detecting gene deletions and translocations (5–7), detecting chromosome translocations (8) and the mapping (9–11) and sequencing of genes. Increasing the detection sensitivity would allow even greater application of this method. The main factors that determine the sensitivity of a fluorescent DNA probe are its brightness, target specificity and the stability of the probe–target duplex. Previously, DNA or RNA probes were either chemically labeled with only a single fluorophore on the 5′ end of the oligonucleotide (12,14) or were labeled by enzymatic means, such as nick translation or in vitro transcription, which incorporates dye-modified nucleotides for, on average, 4% of the bases (15,16). Current technology now allows multiple fluorescent labels to be directly attached to the oligomer by chemical means which may lead to brighter probes (17–19). However, increasing the labeling density of the fluorophore has been shown not to necessarily increase the sensitivity of the probe (20), since it also is known to quench fluorescence, reduce the extinction coefficient of the dyes (21,22) and decrease the stability of the probe–target duplex (17,23). The amount of destabilization is dependent upon the site used for attaching the dye. Some locations for covalent dye attachment, such as the sugar, via a modified 2′-aminonucleoside (24), or the phosphate backbone, via the use of phosphorothioates (25), have been shown to result in significant duplex destabilization even at a low degree of dye incorporation.

In this work, we have synthesized a series of DNA probes that are multiply labeled with either the disulfonated, anionic fluorophore Cy3, or a mono-sulfonated, zwitterionic Cy3 analogue, Cy3NOS. The oligonucleotides were 27mers of thymidine with dye-labeled deoxyuridine analogues evenly spaced throughout the oligonucleotide. Fluorophores were covalently attached to linkers at the C-5 position of deoxyuridine analogues where the effect upon hybridization should be minimal (17). Two linker lengths were studied. By using the 27dT sequence for our probes, dyes could be attached to the oligonucleotide without any sequence constraints and the duplex stability of the probes could be compared directly by determination of the melting temperature (T_m). The probe specificity was measured through ΔT_m measurements, defined as the difference between T_m values of the probe annealed to DNA target with respect to when annealed to a single-base mismatched target (26).

We report the effect of labeling density, fluorophore charge and linker length upon fluorescence intensity, the change in fluorescence upon duplex formation, the quantum yield of fluorescence (Φf), and probe-target stability and specificity.

MATERIALS AND METHODS

Dye synthesis and characterization

The active N-hydroxysuccinimidyl (NHSS) esters of Cy3 and Cy3NOS (Fig. 1A) used to label the oligonucleotides were synthesized according to the procedure of Mujumdar (27). Cy3NOS was obtained by using the non-sulfonated intermediate 1-(ε-carboxypentenyl)-2,3,3-trimethylindolenium iodide (a generous gift of Dr Mark Reddington) to produce the zwitterionic Cy3 analogue. The crude Cy3NOS was purified on a C18 reverse phase column using 15% MeOH as the eluant. Salts that co-eluted

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The Cy3NOS had an extinction coefficient (ε) of 135,000 l/mmol cm at its λmax 546 nm and a Φf of 0.046 in 10% MeOH determined against a Rhodamine 6G standard in EtOH. The dyes were converted to the NHS ester (27), dissolved in dry dimethylformamide (DMF) and stored at 4°C over activated 3 Å molecular sieves until use. The water solubility of the Cy3NOS carboxylic acid was found to be 4.53 × 10−4 M (0.249 g/l) at room temperature as determined by absorbance. For Cy3 carboxylic acid, the water solubility was >0.34 M (234 g/l).

**Oligonucleotide synthesis, labeling and purification**

The modified bases were introduced into the oligonucleotides using the phosphoramidites amino-modifier C2dT and amino-modifier C6dT (Glen Research). The fluorophores were attached with Cy3 and Cy3NOS. The structures of Cy3 and Cy3NOS were confirmed by 1H NMR. The Cy3NOS had an extinction coefficient (ε) of 150,000 and a Φf of 0.04 respectively (27). The dyes lead to a distribution of multiply-labeled probes (Fig. 2). The slowest running band in each lane is the fully labeled oligonucleotide.

The probes were purified by polyacrylamide gel electrophoresis (PAGE) on 20% acrylamide gels containing 7 M urea and 5% bisacrylamide (28). The slowest running band was excised and the oligomer was extracted by the ‘crush-and-soak’ method as described (28) with the exceptions that the elution buffer contained no SDS and the elution was carried out at room temperature overnight. The oligomers were recovered on a Sep-Pak C18 column (28). Three consecutive gels were required to obtain a pure probe. Unlabeled probes 42–66, as well as the unmodified oligonucleotides 27dT, 27dA and 26A1G, were also purified by PAGE, though only a single run was performed. A single band was observed by UV shadowing and excised.

MALDI mass spectrometry was used to confirm the structures of both a Cy3-labeled probe (probes 46) and a Cy3NOS-labeled probe (56NOS). For probe 46, the expected molecular weight was 11219 and the observed m/z ratio found was 11178. For probe 56NOS, the expected molecular weight was 11585 and the observed m/z ratio found was 11589. The results were within 0.36 and 0.012% of the calculated molecular weights respectively.

**Extinction coefficient of probes**

The extinction coefficients (ε) of the probes were determined in 10 mM Tris–HClO at pH 7.0 according to the method of Cantor and Tinoco (29). The experiments were performed in duplicate for each labeled probe and the results were within 2%. The results are listed in Table 1. The extinction coefficients at 260 nm used immediately added to the oligonucleotide solution, which was vortexed and kept overnight at room temperature. For probes 42 and 46, 20 dye equivalents per amino group were used. For the 52/56 and 62/66 probes, 30 and 40 dye equivalents per amino group were used respectively. For probe 56NOS, a 90 nmol sample of unlabeled probe 56 was taken up in 360 µl of Buffer A. To this, 255 µl of Cy3NOS NHS ester in dry DMF, equivalent to 30 dyes per amino group, was added and the reaction mixture was vortexed for 10 min. After labeling with either Cy3 or Cy3NOS, the free dye was removed from the labeled oligomer by gel-filtration through a 20 cm column of BioGel P4 (Bio-Rad) equilibrated with 0.1 M triethylammonium acetate.

Generally, 85% labeling was observed per amino group which leads to a distribution of multiply-labeled probes (Fig. 2). The slowest running band in each lane is the fully labeled oligonucleotide. The successively faster bands correspond to the n–1, n–2 and n–3 partially labeled probes, where n is the number of modified bases present.

The probes were purified by polyacrylamide gel electrophoresis (PAGE) on 20% acrylamide gels containing 7 M urea and 5% bisacrylamide (28). The slowest running band was excised and the oligomer was extracted by the ‘crush-and-soak’ method as described (28) with the exceptions that the elution buffer contained no SDS and the elution was carried out at room temperature overnight. The oligomers were recovered on a Sep-Pak C18 column (28). Three consecutive gels were required to obtain a pure probe. Unlabeled probes 42–66, as well as the unmodified oligonucleotides 27dT, 27dA and 26A1G, were also purified by PAGE, though only a single run was performed. A single band was observed by UV shadowing and excised.

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Figure 3. Absorbance spectra of probe 66 annealed to poly[dA] from 10 to 70°C showing that the extinction coefficient of the dyes is sensitive to the conformation of the probe. The absorbance at 551.4 nm drops abruptly between 30 and 40°C as probe 66 passes through the transition from being double-stranded to being single-stranded. The single-stranded probe shows no such transition (inset).

for the nucleotides were 9110 (pdT), 15300 (pdA) and 11800 (pdG) all in l/mol·cm (30). The \( \varepsilon \) of C2dT and C6dT were taken as 119% of dT (Hugh Mackie, Glen Research, personal communication) yielding a value of 10800. For pdT and pdG, the literature values listed are for 268 nm (pH 7) and 255 nm (pH 1) respectively. These were extrapolated to 260 nm, pH 7.0, by determining the ratio of the absorbance at 268 nm (pH 7) and 255 nm (pH 1) for pdT and pdG to the absorbance of the nucleotides at 260 nm, pH 7.0. With multiple dyes attached, there is a significant contribution by the dyes to the absorbance at 260 nm. Therefore, the absorbance of the dyes at 260 nm was taken into account (4900 and 9000 l/mol·cm per dye attached for Cy3 and Cy3NOS respectively).

Table 1. The extinction coefficients of the probes, in l mol\(^{-1}\) cm\(^{-1}\)

<table>
<thead>
<tr>
<th>Probe</th>
<th>( \varepsilon_{260} \times 10^{-3} )</th>
<th>( \varepsilon_{552} \times 10^{-3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unlabeled Cy3-labeled Cy3-labeled</td>
<td>Cy3-labeled</td>
</tr>
<tr>
<td>27T</td>
<td>2.15</td>
<td>–</td>
</tr>
<tr>
<td>27dA</td>
<td>3.63</td>
<td>–</td>
</tr>
<tr>
<td>26A1G</td>
<td>3.60</td>
<td>–</td>
</tr>
<tr>
<td>42</td>
<td>2.44</td>
<td>2.64</td>
</tr>
<tr>
<td>46</td>
<td>2.49</td>
<td>2.66</td>
</tr>
<tr>
<td>52</td>
<td>2.45</td>
<td>2.69</td>
</tr>
<tr>
<td>56</td>
<td>2.50</td>
<td>2.72</td>
</tr>
<tr>
<td>62</td>
<td>2.47</td>
<td>2.77</td>
</tr>
<tr>
<td>66</td>
<td>2.52</td>
<td>2.82</td>
</tr>
</tbody>
</table>

Due to differences between the Cy3 and Cy3NOS dyes, the extinction coefficients of probe 56NOS are significantly different at 260 and 550 nm, 2.78 and 5.10 \( \times 10^{-3} \) respectively.

Melting curves

Melting curves were obtained on an Aviv 14DS UV-vis Spectrophotometer (Aviv) fitted with a thermolectric temperature-controlled cell holder. The cuvette jacket temperature was monitored concurrently with the absorbance at 260 and 551.4 nm.

The wavelength 551.4 nm was chosen because it was found to be an isosbestic point for probe 66 when single-stranded (Fig. 3, inset). Absorbance spectra of probe 66 in 100 mM NaCl, 10 mM Na cacodylate pH 7.0, 25°C (Buffer B) were taken from 10 to 70°C at 10°C intervals from which the isosbestic wavelength was determined. By choosing to monitor this wavelength during the melting curve determination of double-stranded probes, the change in the absorbance of the dyes should be due to conformational changes in the DNA and not due to the effect of changes in temperature upon the dye itself.

The melting curves were determined as follows: 0.63 nmol of both probe and target (either the matching target 27dA or the mismatching target 26A1G) were combined and dried by speedvac. The sample was taken up in 550 \( \mu \)l Buffer B to yield a total strand concentration of 2.29 \( \times 10^{-6} \) M. The solution was warmed to 50°C and gradually cooled to 4°C where it was maintained until the melting experiment was performed. Samples (0.5 ml) of annealed probe were ramped at 1°C/min from 0 to 70°C, held for 2 min at 70°C and ramped back down to 0°C. Data were collected every 60 s, with each datum the average of 10 points collected over 1 s. The spectral bandwidth was 1 nm.

The lag between the true cuvette temperature and the monitored jacket temperature was corrected for by calibration using optical thermometry (31). In addition, melting curves were determined for Buffer B alone to account for any changes in buffer absorbance with temperature. These 260 and 551.4 nm melting curves were fitted to 4th order polynomials which were used to generate melting curves of the buffer at 260 and 551.4 nm for any given temperature ramp. These estimated curves were subtracted from
the melting curve of the probe, correcting for the change in background absorbance with temperature. The melting temperature \( (T_m) \) was determined by finding the inflection point of the absorbance curve at 260 nm from the derivative curve, \( dA/dT \), smoothed over 5 points.

Data for probe 66 is not listed as the probe bound irreversibly to a 0.2 µm Millex-GS filter (Millipore). Previous work in our lab with probe 66 using poly[dA] as the target indicated that the \( T_m \) and fluorescence data followed the same trend as probe 62.

**Quantum yield of fluorescence**

The \( \Phi_f \) of the hybridized probes were determined with a SPEX Fluorolog II spectrofluorometer (SPEX Industries, Inc.) fitted with a temperature controlled cuvette holder and using rhodamine 6G in EtOH as the standard according to O’Brien (32). The excitation wavelength was 514.5 nm which allows the entire emission spectra of the Rhodamine 6G, Cy3 and Cy3NOS to be observed. The emission was scanned from 516 to 800 nm with 1 nm increments, 1 s integration time with all slit widths at 0.5 mm. The annealed probes, previously used to determine the melting curves, were diluted with Buffer B such that their absorbances were 0.05 at 514.5 nm. Prior to the fluorescence measurements, the annealed probes were equilibrated at 21.5°C for 10 min. Fluorescence brightness was defined as the integrated peak area from 516 to 800 nm, correcting for probe concentration.

**Percent change in fluorescence upon hybridization**

To determine the effect of hybridization upon fluorescence, equimolar solutions of probe at 21.5°C, with and without poly[dA] target, were excited at 520.4 nm and the fluorescence emission was observed from 527.4 to 724.4 nm.

To ensure that the changes in fluorescence intensity measured were not due to changes in the extinction coefficient of the dyes caused by differences in probe hybridization, a second isosbestic point was determined at which the dye absorbance is the same regardless of whether the probe is single- or double-stranded (Fig. 3). By using this isosbestic point at 520.4 nm for the excitation wavelength, the fluorescence enhancement measurements should be a function of the \( \Phi_f \) only.

The percent change in fluorescence upon hybridization with target was determined as follows: 0.0546 nmol of probe was taken up in 4.2 ml of Buffer B, yielding a concentration of 1.30 × 10^{-8} M. From this solution, 2 ml were transferred to two matched quartz cuvettes. To one cuvette, 1.36 µl of poly[dA] (average length 300 nucleotides) was added. The dA concentration was 0.516 mM, which yielded a 1:1 nucleotide ratio of probe to target. The cuvettes were then sealed with teflon stoppers, shaken, warmed to 50°C and allowed to anneal for at least 6 h, slowly cooling to 4°C. The samples were excited at 520.4 nm and scanned from 527.4 to 724.4 nm, with the excitation and emission slit widths at 2 nm. Two measurements were taken for each probe before and after 50% dilution with buffer which was added directly to the cuvette. For probes 42, 52, 56 and 56NOS the experiments were repeated. The fluorescence determined was the peak area from 527.4 to 724.4 nm, which was associated with the transition from duplex to single-stranded DNA is reflected in the absorbance curve of the dye as well as the oligonucleotide absorbance at 260 nm.

The melting temperatures of the probes annealed to the matching target 27dA and to the mismatch target 26A1G were determined (Table 2). From these data, we make four observations: (i) each additional negatively-charged Cy3 reduces the \( T_m \) by −2°C; (ii) the zwitterionic Cy3NOS destabilizes the helix more than an equivalent number of Cy3 labels do; (iii) extending the linker length by four carbons does not appreciably alter the \( T_m \) and (iv) since \( \Delta T_m \) is the change in stability of the duplex when one mismatch is present (which is measure of probe specificity), the probe specificity does not change significantly with dye labeling.

The reduction in the \( T_m \) is not due to the incorporation of amino-modified uridine analogues per se; melting curve determinations for the six probes 42–66 indicated that there is no decrease in the \( T_m \) prior to labeling with Cy3 (data not shown).

**RESULTS**

**Duplex stability and specificity**

All the probes exhibited normal melting behavior. Figure 4 shows the melting curves for probes 42 and 62 which represent the low and high dye-labeling densities investigated in this study (four and six dyes per oligomer respectively). It is apparent that even when the labeling density on the probe is as high as every 5th base (probe 62), the duplex still melts in a cooperative manner with little broadening of the transition. That transition, however, has been shifted to a lower temperature compared to probe 42. In addition, the transition from duplex to single-stranded DNA is reflected in the absorbance curve of the dye as well as the oligonucleotide absorbance at 260 nm.

The fluorescence intensity of the hybridized Cy3-labeled probes initially increases as additional dyes are added (Table 2). Once six dyes are attached, however, the intensity decreases. The same trend is seen with the quantum yield of fluorescence (\( \Phi_f \)). Both the fluorescence and \( \Phi_f \) are relatively insensitive to the presence of a mismatch and the length of the linker.
The error in the \( T_m \), \( \Phi_f \) and fluorescence are 0.2, 0.01 and 0.1 respectively.

### DISCUSSION

**Mechanisms of duplex destabilization**

One of the conspicuous effects of multiple labeling of DNA oligonucleotides with the cyanine dyes is the degree to which it lowers the \( T_m \). The source of this destabilization appears to arise from dye–dye and dye–nucleotide interactions.

There is evidence that dye–dye interactions are present in the probes. The H-aggregate is the most common dye–dye aggregate which, in its simplest form, is a stacked dye dimer whose formation is characterized by a decrease in the monomeric dye absorption band and an increase in a non-fluorescent, blue-shifted H-band (33). A comparison was made between probes 56 and 56NOS when single- and double-stranded (Fig. 6). The increase in the 515 nm band relative to the 0→0 transition of the monomer absorption band is characteristic of the presence of dye dimer (21,33). Probe 56NOS shows a pronounced H-band compared to probe 56. This is not surprising considering the low water solubility of the Cy3NOS label which favors dye aggregation. As previously noted, the transition from duplex to single-stranded DNA is reflected in both the oligonucleotide and dye absorbance curves (Fig. 4). The drop in dye absorbance upon duplex melting is most easily explained by H-aggregate formation, since an increase in the H-band will be at the expense of the monomer absorption band. Since dimerization is favored when the probes are single-stranded (Fig.6), the decrease in dye absorbance should be centered around the \( T_m \), which is what is observed.

Dye dimer formation will affect the stability of the probe–target duplex. Dimer formation is an exothermic process, on the order

### Table 2.
The melting temperatures, \( \Phi_f \) and fluorescence brightness of the probes when bound to the target probes 27dA (match) or 26A1G (mismatch)

<table>
<thead>
<tr>
<th>Probe</th>
<th>( T_m ) (°C)</th>
<th>( \Delta T_m )</th>
<th>( \Phi_f )</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Match</td>
<td>Mis-match</td>
<td></td>
<td>Match</td>
<td>Mis-match</td>
</tr>
<tr>
<td>27T</td>
<td>49.7</td>
<td>44.9</td>
<td>4.8</td>
<td>–</td>
</tr>
<tr>
<td>42</td>
<td>41.5</td>
<td>37.0</td>
<td>4.5</td>
<td>0.09</td>
</tr>
<tr>
<td>46</td>
<td>41.6</td>
<td>37.0</td>
<td>4.6</td>
<td>0.09</td>
</tr>
<tr>
<td>52</td>
<td>40.5</td>
<td>35.7</td>
<td>4.8</td>
<td>0.10</td>
</tr>
<tr>
<td>56</td>
<td>40.6</td>
<td>35.4</td>
<td>5.2</td>
<td>0.11</td>
</tr>
<tr>
<td>56NOS</td>
<td>37.6</td>
<td>32.9</td>
<td>4.7</td>
<td>0.06</td>
</tr>
<tr>
<td>62</td>
<td>37.6</td>
<td>–</td>
<td>–</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Figure 5. Percent change in fluorescence observed for probes upon hybridization to target.

The 56NOS probe showed a 50% drop in fluorescence and \( \Phi_f \) compared to probe 56 even though the two probes share the same sequence, label density and linker length. Other than the additional sulfonate on Cy3, the labels themselves are very similar both structurally and spectriscopically. However, the presence of the additional sulfonate produces a large difference in water solubility.

Hybridization to target DNA significantly affects the fluorescence intensity of the probes (Fig. 5). The percent change in fluorescence upon duplex formation ranges from –35 to +20 for probes 42 and 66 respectively. Whether the fluorescence increases or decreases upon duplex formation depends upon labeling density, fluorophore charge and linker length. Probes that are labeled at low density, have short linkers, or are labeled with Cy3NOS, exhibit less fluorescence when hybridized. Only probes 56 and 66 show greater fluorescence in double-stranded structures.

Figure 6. Absorbance spectra of probes 56 and 56NOS when double- and single-stranded. The magnitude of the absorbance of the shoulder at 515 nm indicates the degree of dye dimer present. The spectra were normalized at the dye monomer band at 550 nm.
Figure 7. (A) The absorbance spectrum of probe 52 and its calculated composite spectrum when no dye–nucleotide or dye–dye interactions are present. The increase in the absorbance of the shoulder at 310 nm suggests that the presence of the dyes do perturb the nucleotide bases and that dye–nucleotide interactions are significant. (B) The absorbance spectrum of probe 56NOS and its calculated composite spectrum when no dye–nucleotide or dye–dye interactions are present. The more pronounced shoulder at 310 nm suggests that the zwitterionic dye Cy3NOS interacts more than the anionic dye Cy3 with the nucleotides. (C) Spectra of equimolar solutions of probe 56NOS when single-stranded and double-stranded. Note that the shoulder at 310 nm decreases upon addition of DNA target, suggesting that formation of the double helix disrupts the dye–nucleotide interactions. In addition, the double-stranded absorbance spectrum begins to look very similar to the composite spectrum of probe 56NOS when no dye–nucleotide or dye–dye interactions are present. The composite spectra were calculated by recording spectra of the probes when labeled with dyes and unlabeled; (i) the spectra of the free dyes were recorded and the baselines were corrected; (ii) the spectra of the unlabeled probes were normalized against the labeled probes to give spectra for identical probe concentrations; (iii) the spectra of the corrected dyes and unlabeled probes were added to give the composite spectra of 52 and 56NOS.

There are significant differences between the actual spectra of probes 56NOS and 52 and their respective composite spectra for which no dye–nucleotide or dye–dye interactions are present (Fig. 7A and B). The increase in the absorbance of the shoulder at 310 nm suggests that the presence of the Cy3 dyes do perturb the nucleotide bases and that dye–nucleotide interactions are significant. In Figure 7B, the more pronounced shoulder at 310 nm suggests that the zwitterionic dye Cy3NOS interacts more than the anionic dye Cy3 with the nucleotides. Comparing spectra of equimolar solutions of probe 56NOS when single-stranded and double-stranded (Fig. 7C), one notes that the shoulder at 310 nm decreases upon addition of DNA target. This is the behavior that one would expect since the formation of the double helix should disrupt the dye–nucleotide interactions. In addition, the double-stranded absorbance spectrum begins to look very similar to the composite spectrum of probe 56NOS when no dye–nucleotide or dye–dye interactions are present, indicating that duplex formation disrupts dye–dye, as well as dye–nucleotide, interactions.

Therefore, it appears that both dye–dye and dye–nucleotide interactions are present. If the dye–nucleotide and dye–dye interactions are strong enough, then the duplex should be destabilized by those interactions, which seems to be the case. The relative
contributions of the dye–dye and dye–nucleotide interactions to the destabilization of the hybridized probes will be probe-dependent. Dye–dye interactions will be favored at higher labeling densities and when longer linkers are used. Though dye dimers are known to quench fluorescence (33), it is possible that dye–nucleotide interactions may enhance it. It is known that increasing the planarity of cyanine dyes leads to an increase in the $\Phi_F$ (32). Therefore, if dye–nucleotide interactions stabilize a planar dye conformation, they may also enhance fluorescence. If this is true, then the balance between fluorescence quenching effects from dye–dye interactions and fluorescence enhancement effects from dye–nucleotide interactions may account for the complicated behavior seen in Figure 5.

CONCLUSION

In this study, we synthesized a series of multiply-labeled fluorescent oligonucleotides. We investigated the effect of labeling density, fluorophore charge and linker length upon fluorescence intensity, the change in fluorescence upon duplex formation, the quantum yield of fluorescence, probe–target stability and specificity to determine in vitro the best probe. Our work indicates that (i) the optimum in vitro probe is obtained when labeling with a hydrophilic dye such as Cy3 every 6th base using a long linker; (ii) the probe–target duplex formed is less stable for multiply-labeled fluorescent DNA probes due to dye–dye and dye–nucleotide interactions which appear to stabilize a single-stranded conformation of the probe; (iii) the target specificity is just as high as compared to the unmodified control probe.

In designing multiply-labeled fluorescent DNA probes, our work suggests that the primary concern should be to optimize the brightness of the probe while maintaining its ability to form a stable duplex. In optimizing probe brightness, one should begin with the design of the fluorophore itself since the optimum labeling density depends upon the fluorophore used. Future fluorophore development should focus on reducing the tendency of dyes to form these aggregates since it is dye–dye and dye–nucleotide interactions which contribute to quench fluorescence (33), it is possible that dye–nucleotide interactions may account for the complicated behavior seen in Figure 5.

REFERENCES


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