

Synthesis and Properties of Fluorescent NF- κ B-Recognizing Hairpin Oligodeoxyribonucleotide Decoys

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Intramolecular fluorescence quenching of cyanine dyes was investigated using a model hairpin oligonucleotide decoy encoding a NF- κ B p50 subunit binding site. Two types of hairpin oligonucleotides were synthesized: (1) 5'-(6-aminoethyl)- and 3'-(3-aminopropyl)-linked (**I**); (2) 5'-(6-aminoethyl)- and 3'-[3-(3-hydroxypropylthio)propyl]-linked (**II**). Oligonucleotide **I** was covalently modified using monofunctional either Cy3- or Cy5.5-*N*-hydroxysuccinimide esters. Using reverse-phase HPLC, mono- and dicyanineamide derivatives of **I** were isolated. Mono-Cy3-modified derivatives of **I**, but not the mono-Cy5.5-modified derivatives, showed a 2-fold higher Cy3 fluorescence intensity compared to the free dye. There was no detectable difference in fluorescence between the di-Cy3 derivative of **I** and the free dye at the same concentration. However, there was a 4-fold quenching of fluorescence in the case of the di-Cy5.5 derivative of the same hairpin oligonucleotide. The quenching of Cy5.5 fluorescence could not be explained by the interaction of Cy5.5 with nucleotide bases as demonstrated by incubating free Cy5.5 dye with oligonucleotides. The quenching effect was further investigated using an oligonucleotide bearing a cleavable 3'-amino-terminated linker bearing an S–S bond (**III**). After modification of the 5'- and 3'-end of oligonucleotide **III** with a Cy5.5 monofunctional hydroxysuccinimide ester, a 70–75% quenching of fluorescence was observed. Fluorescence was 100% dequenched after the reduction of S–S bond. The obtained result unequivocally demonstrates that the formation of intramolecular Cy5.5 dimers is the dominant mechanism of fluorescence quenching in symmetric dye–dye hairpin decoy beacons.

INTRODUCTION

The ability to attenuate the level of target gene expression in living cells allows individual biochemical pathways to be dissected (1) as well as alteration of the phenotype and, potentially, enables the attenuation of gene expression in vivo (2–6). The “knockdown” of gene expression could be achieved at transcription and post-transcription. In the first case, oligonucleotide hairpin decoys could be used (reviewed in ref 7). In the second case, siRNA (small interfering) (8, 9) and antisense oligonucleotides have been proven to be effective. The inhibitive effect of both DNA-based and RNA-based constructs depends to a large extent on their ability to penetrate into the cellular interior and on the rate of intracellular catabolism of short oligo deoxy- or ribonucleotides. Both endo- and exonuclease degradation severely limits the half-life of oligodeoxyribo- or oligoribonucleotides in vitro and in vivo. Nevertheless, the stability of oligonucleotides can be modified by applying a variety of synthetic strategies (10, 11), reviewed in refs 12–14). While recent research has proved that antisense oligoribonucleotides (especially siRNAs) are undoubtedly efficacious in vitro, they are susceptible to rapid degradation prior to interaction with target mRNA in vivo (15). Small interfering RNAs are also known to activate

interferon-mediated pathways resulting in a broad interferon-mediated gene upregulation (16, 17).

Hairpin oligonucleotide decoys are designed to block the transcription of a target gene by competing with genomic DNA for relevant transcription factors. For example, decoys bearing a NF- κ B recognition sequence, which binds the p50 subunit of a transcription factor, were amenable to modifications with reactive nucleotide analogues that enabled trapping of NF- κ B on the decoy and resulted in apoptosis (18–20). To assess the efficacy of intracellular penetration and to track their intracellular fate, oligonucleotides can be labeled with molecular probes to make them detectable by imaging techniques, either microscopic, or macroscopic for in vivo work (21–24). The goal of this work was to synthesize and characterize far-red fluorochrome-labeled decoys. Indocyanine far red fluorescent dyes have excellent photostability, are pH insensitive, and potentially could be detected in vivo. We anticipate that such labeled oligonucleotides will prove useful in elucidating mechanisms of hairpin decoy penetration into cells and imaging interactions between the decoy and target transcription activators.

MATERIALS AND METHODS

Materials. Oligodeoxyribonucleotides with 3',5'-diaminoalkyl linkers were synthesized at MGH Department of Molecular Biology, DNA Oligonucleotide Synthesis Core (Dr. Jay Klaren) and supplied in ammonia solution.

Triethylammonium acetate solution (2 M, TEAA), pH 7.0, was purchased from Glen Research (Sterling VA), 2-aminoethyl-2'-aminoethanethiosulfonate from Pierce-

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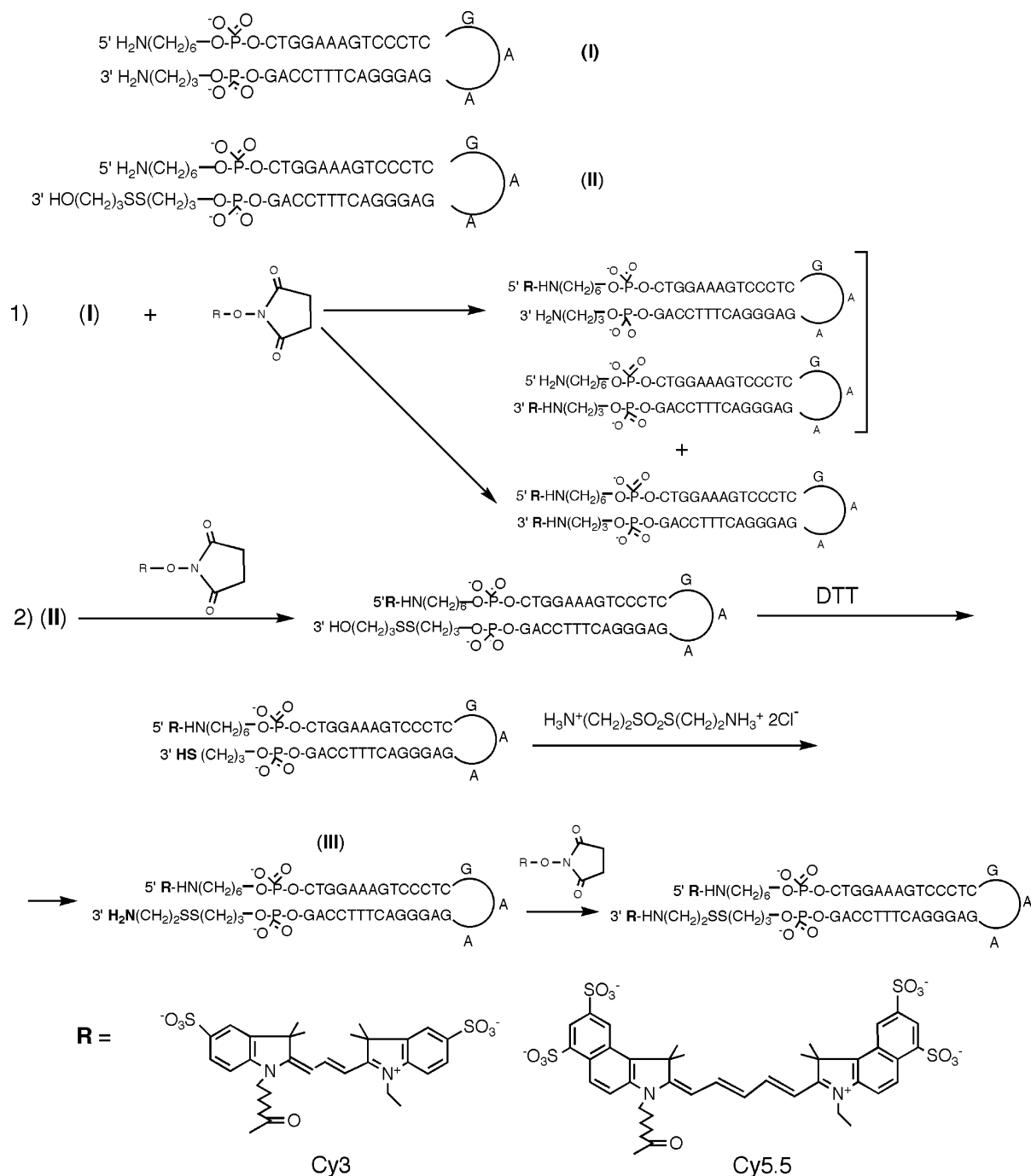


Figure 1. Scheme of fluorescently labeled hairpin oligonucleotide decoy synthesis. **I**, precursor (noncleavable, diamino terminated) oligonucleotide; **II**, oligonucleotide with a 3'-dithio linker; **III**, monomodified oligonucleotide with an amino-terminated dithio linker.

Endogen (Rockford, IL), Cy5.5 mono-*N*-hydroxysuccinimide ester (NHS) and Cy3 NHS from Amersham Biosciences (Piscataway, NJ). Micro Bio-Spin 6 columns (SSC buffer, pH 7.0) were obtained from Bio-Rad (Hercules CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Initial Purification of Oligonucleotides. The ion-pair reversed-phase HPLC method was used for the isolation and purification of modified oligodeoxyribonucleotides on a C18 column (Zorbax ODS 4.6 × 25 mm, DuPont). For HPLC linear gradient the following buffers were used: A, 2% acetonitrile in 0.1 M TEAA, pH 7; B, 50% acetonitrile in 0.1 M TEAA, pH 7. The column was eluted at 1 mL/min at room temperature. Purified oligonucleotides were concentrated in a centrifugal vacuum concentrator (SpeedVac, Savant).

Modification of Oligodeoxyribonucleotides Bearing 3'- and 5'-Aminoalkyl Linkers (Figure 1, Reaction 1). Ten nanomoles of the oligonucleotide d(pCTGGAAAGTCCCTCGAAGAGGGACTTTCAGp) bearing 3'- and 5'-aminoalkyl linkers was dissolved in 15 μL of 0.1 M NaHCO_3 . To this was added 7 μL of 62 mM Cy3-NHS (or 49 mM Cy5.5-NHS in DMSO), and the mixture was incubated at room temperature for 6–16 h in the dark. Then 40 μL of water was added, and the modified oligonucleotide was separated from excess dye by two consecutive separations on Micro Bio-Spin 6 columns according to the manufacturer's recommendations. After the second spin-chromatography, oligonucleotides were precipitated by standard ethanol–sodium acetate treatment and purified by HPLC as described above. The chromatography showed complete absence of free dyes

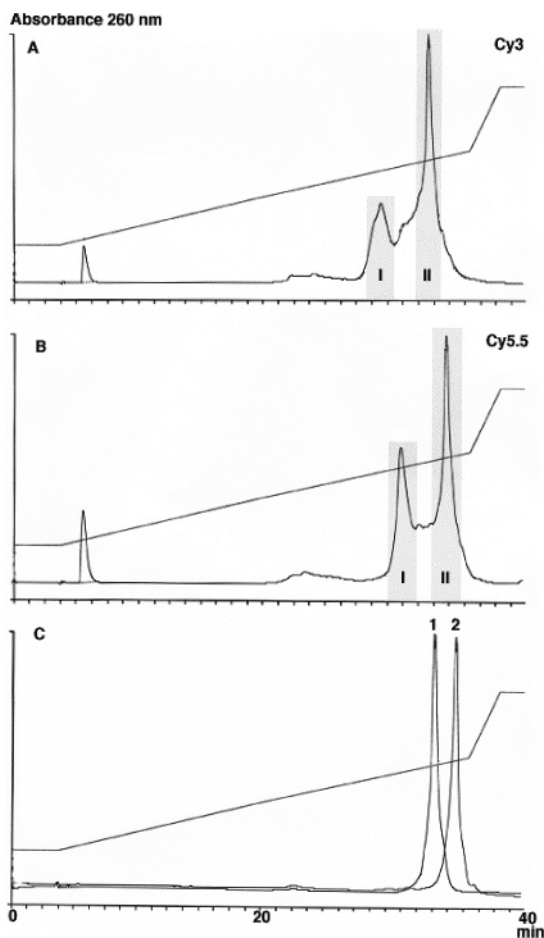


Figure 2. Preparative HPLC of Cy3 (A) and Cy5.5 (B) modified oligonucleotide **I**. Shaded peaks corresponding to mono (I) and di- (II)- modified oligonucleotides were collected for further analysis; (C) superimposed elution profiles of purified dicyanine-modified oligonucleotides: 1, Cy3-modified; 2, Cy5.5-modified oligonucleotide **I**.

and a disappearance of the peak corresponding to the nonlabeled oligonucleotide. Fractions corresponding to mono- and dimodified oligonucleotides were collected (Figure 2) and concentrated by evaporation using a centrifugal vacuum concentrator (final volume about 50–100 μ L). Oligonucleotides were precipitated in ethanol–sodium acetate by adding a 0.1 volume of 3 M sodium acetate, pH 5.5, followed by 5 volumes of cold ethanol. Labeled oligonucleotides were characterized using MALDI-mass spectrometry (Integrated DNA Technologies, Coralville, IA) and yielded for di-Cy3-labeled (**I**) ($M + H^+$) 11195.8 (calculated, 11207.0) and for di-Cy5.5 labeled (**I**) ($M + H^+$) 11775.1 (calculated, 11796.5). In calculations we assumed that acetate anion served as a counterion of the quarternized nitrogen in Cy dye residues.

Synthesis of Fluorescent Oligonucleotide Decoy Probes Bearing a Dithio Bond (Figure 1, Reaction 2). The initial compound used for the synthesis of dual-labeled probes was 5'-N-MeOTr-**II**, i.e. oligonucleotide d(pCTGGAAAGTCCCTCGAAGAGGGACTTTCCAGp) bearing asymmetric 5'-(6-MeOTr-aminohexyl)- and 3'-[3-(3-hydroxypropylthio)propyl] linkers (Figure 1). 5'-N-MeOTr-**II** was purified by HPLC on an octadecyl silica column (Zorbax ODS 5 μ m, DuPont) using a linear gradient of buffers A and B (10–100% B, 30 min) and was detritylated using 80% acetic acid for 1 h. Further synthesis included the following steps: (1) reacting the purified oligonucleotide with NHS esters of Cy5.5 or Cy3 dyes as described above; (2) isolating the monolabeled

oligonucleotide on the HPLC column and reducing the 3'-disulfide bond with dithiothreitol, resulting in the formation of an oligonucleotide bearing a 3'-(3-mercapto-propyl) linker. Briefly, 10 μ L of 1 M DTT was added to the monolabeled oligonucleotide dissolved in 100 μ L of 0.02 M NaHCO_3 . After a 1 h incubation at room temperature, the reduced oligonucleotide was precipitated by using ethanol–sodium acetate treatment; (3) reacting the above oligonucleotide with 2-aminoethyl-2'-aminoethane thiosulfonate. The reaction yielded oligonucleotide **III**, containing a 3'-[3-(2-aminoethylthio)propyl] linker (Figure 1). Briefly, the oligonucleotide was dissolved in 50 μ L of 0.2 M NaHCO_3 , the solution was cooled to 4 $^\circ\text{C}$, mixed with 15 μ L of 0.15 M 2-aminoethyl-2'-aminoethanethiosulfonate and incubated for 1 h at 4 $^\circ\text{C}$ followed by separation on a Micro Bio-Spin 6 column; (4) modifying oligonucleotide **III** with an *N*-hydroxysuccinimide ester of the reporter dye (Cy5.5 or Cy3); (5) isolating dual-labeled oligonucleotide by HPLC. Oligonucleotides were precipitated by adding 2% lithium perchlorate in acetone and redissolved in water. By MALDI-mass spectrometry the ($M + H^+$) was 11905.6 (calculated – 11893.8).

Interaction of Hydrolyzed Cyanine NHS-esters with Oligonucleotide Containing 3',5'-Diaminoalkyl Linkers. Three nanomoles of the oligonucleotide was dissolved in 25 μ L of solution containing hydrolyzed Cy5.5-NHS, and the resulting mixture was incubated at the same conditions as the standard Cy5.5-NHS oligonucleotide reaction mixture. To obtain hydrolyzed Cy5.5-NHS ester, 7 μ L of 49 mM Cy5.5-NHS in DMSO was dissolved in 18 μ L of 0.2 M NaHCO_3 , and the mixture was incubated for 36 h in the dark at room temperature.

Interaction of Hydrolyzed Cy5.5 NHS with DTT or Glutathione. To 35 μ L of hydrolyzed Cy5.5 were added 10 μ L of 1 M DTT (or 0.5 M reduced glutathione) and 10 μ L, 0.2 M NaHCO_3 , and the solution was incubated in the dark overnight.

The Reduction of Disulfide Bond in Dual-Labeled Oligonucleotides. To reduce the dithio bond in the oligonucleotide and to achieve dequenching of fluorescence, dual-labeled probes were diluted in 1–1.5 mL of PBS buffer (pH 7.5) and subjected to fluorimetry, followed by mixing with 15 μ L of 1 M DTT or with 30 μ L of 0.5 M reduced glutathione. The solutions were incubated overnight at room temperature followed by fluorimetry as described above.

DNase Digestion of Labeled Probes. Solutions of the oligonucleotides were prepared in magnesium-containing Dulbecco's PBS, pH 7, at the concentration of 20–100 μ M. Each solution was divided into aliquots. For each oligonucleotide, a half of the samples was treated with DNase I (10 U/mL, Stratagene, La Jolla CA) for 6 h at 37 $^\circ\text{C}$. Control samples were not treated with DNase. Absorbance and fluorescence at appropriate wavelengths were measured in DNase-treated samples and compared to the control samples.

Spectral measurements were performed at room temperature using a U-3000 and F-4500 Hitachi spectrophotometer and spectrofluorometer, respectively. The cyanine dyes and labeled oligonucleotides were diluted in 2 mL of PBS, pH 7.5, and absorbance and fluorescence spectra were recorded sequentially in quartz cuvettes.

RESULTS AND DISCUSSION

Tagging oligonucleotides with imaging probes provides a means of tracking these molecules in living cells and ultimately in vivo. For example, labeling of oligonucleo-

otides with radioactive isotopes has been used for non-invasive tracking of oligonucleotide biodistribution. This approach potentially enables localizing sites of target gene expression (reviewed in (25, 26)). Conjugation of oligonucleotides with cyanine dyes could result in highly useful "beacons" based on the effect of fluorescence quenching between the labeled proximal 3'- and 5'-termini. The quenching effect is abrogated by the dequenching of fluorescence upon the interaction with *in vitro* targets in structurally constrained stem-loop beacons as well as stemless beacons (reviewed in 27, 28). Moreover, it has been demonstrated that enzyme-specific dequenching of cyanine-labeled macromolecules can be imaged *in vivo* (29). In this report we investigated quenching and dequenching effect in hairpin oligonucleotide decoys using two commercially available cyanine dyes.

Purification of Initial Oligonucleotides. To obtain a first generation of quenched oligonucleotide decoy probes, we used d(pCTGGAAAGTCCCTCGAAGAGG-GACTTTCCAGp) as a precursor for synthesis of dye-labeled probes. This oligonucleotide was prepared to enable asymmetric modifications at the 3'- and 5'-termini using a protected 5'-(6-MeOTr-aminoethyl)-linker as well as a free amino group-containing 3'-(3-aminopropyl) linker (Figure 1). We assumed that above linkers would provide a means of conjugating the first dye (reporter or quencher) specifically to the 3'-amino group. The synthesis would be followed by (1) the isolation of the 3' mono Cy-dye labeled 5'-N-MeOTr-I fraction by HPLC; (2) detritylation of I by 80% acetic acid. Deprotected compound would then be used to attach the second dye (quencher or reporter) to the free 5'-amino group followed by HPLC purification. Contrary to our expectations, we found that the 5'-MeOTr-NH bond is unstable and hydrolyzes rapidly during the purification of I by HPLC in 0.1 M TEAA, pH 7.0, or by using the Poly-Pack cartridge method. Since partial (10–30%) detritylation of the 5'-amino group took place, oligonucleotide I could not be used for synthesis of probes dually labeled by two different dyes. Therefore, after HPLC isolation from the reaction mixture, oligonucleotide I was detritylated by 80% acetic acid, and the obtained oligonucleotide (HPLC elution time, 18.5 min) was used to synthesize probes dually labeled by Cy3 or Cy5.5 cyanine dyes.

Purification of dye-modified oligonucleotides from nonreacted dyes was attempted using three approaches: (a) by repeated ethanol–sodium acetate precipitation of oligonucleotide; (b) by gel filtration on NAP-10 cartridges; (c) by separating on Micro Bio-Spin 6 columns. We found that the particular dyes and oligonucleotides used in this work could not be efficiently separated using size-exclusion chromatography on a Sephadex G-25 column (NAP-10). A procedure involving separations on Micro Bio-Spin 6 columns followed by ethanol–sodium acetate precipitation of the oligonucleotides was clearly superior over other purification strategies. The mono- and dimodified derivatives could then be easily separated using a C18 HPLC column Figure 2 (A,B, peaks I and II). The isolated fluorescent dimodified oligonucleotides showed a very high purity (Figure 2, C) suitable for further spectral analysis and mass-spectrometry. Elution times on HPLC column were 27.0 min for the di-Cy3-modified oligonucleotide and 28.3 min for the di-Cy5.5-modified one.

Spectral Properties of Modified Oligonucleotide

I. To investigate spectral properties of cyanine dye-modified oligonucleotides, we initially performed a modification of hairpin oligonucleotide I with Cy3-NHS and

Cy5.5-NHS followed by the isolation of mono- and dicyanine derivatives of I using HPLC. The above dyes were chosen because of the advantageous photostability of Cy3 and the near-infrared "in vivo imaging" fluorescence range of Cy5.5 (29, 30). The initial experiments showed that the attachment of dyes to hairpin oligodeoxyribonucleotides with 3'-(3-aminopropyl), 5'-(6-aminohexyl)-linkers resulted in significant changes in the visible range of cyanine dye absorption when spectra of nonconjugated dyes were compared to the cyanine-modified oligonucleotides. First, we observed an increase of absorbance in the range of 500–525 nm in the case of Cy3 (Figure 3A) and in the range of 600–650 nm in the case of Cy5.5 after the conjugation (Figure 3B). Such spectral changes were previously attributed to a formation of dimers in Cy-modified proteins (31). In our case, such spectral changes were detectable only in the case of dimodified oligonucleotides as opposed to its mono-Cy derivative. There was no evidence of the formation of J-aggregates that absorb light at longer wavelengths. The increase of absorption at shorter wavelengths was especially prominent in the case of Cy5.5 and was less prominent for Cy3 derivatives (Figure 3). In addition, there was a small but detectable 3–5 nm-"red" shift of the fluorescence emission maximum. We also observed that covalent linking of a single Cy3 group to I causes a 100% increase of the fluorescence intensity. In the case of Cy5.5 fluorescence in the mono-derivatized oligonucleotide did not change appreciably whereas dimodified I showed a substantial quenching of Cy5.5 fluorescence which could be reliably measured using extensive enzymatic degradation of the oligonucleotide. As a result of DNase-mediated cleavage, fluorescence increased approximately 13 times. Additional control experiments that included incubating hydrolyzed cyanine dye esters with the oligonucleotide showed that the above fluorescence effects were specific for conjugated dyes and could not be explained by simple interaction of cyanine moiety with nucleotides (e.g. partial intercalation of cyanine group between the nucleotide pairs).

Experiments with Derivatives of Oligonucleotide II Containing a Cleavable Bond.

To prove that the observed self-quenching effects were caused by the interaction of two cyanine dye residues positioned in close proximity to each other (i.e. that hairpin conformation induces the dimerization of Cy5.5 dye), we designed a hairpin oligonucleotide II (HPLC elution time, 19.1 min) with a nucleotide sequence identical to that of I but containing a dithio bond at the 3'-terminus of the molecule (Figure 1). We hypothesized that the bond could be reduced and that the formed 3'-sulfhydryl could then react with 2-aminoethyl-2'-aminoethanethiosulfonate to form a 3'-amino linker bearing a dithio bond. The amino group could be then used for further modification of II with a second fluorophore or other probe to yield oligonucleotide III (Figure 2, HPLC elution time, 28.0 min). In oligonucleotide III that bears two Cy5.5 modifications, the 3'-dithio bond could be reduced using a 10–15 mM dithiothreitol (or glutathione). First, we excluded potential quenching of Cy dyes as a result of chemical interaction with DTT or glutathione. Second, we tested whether the reduction that cleaves the S–S bond results in the dissociation of the dimer and resultant dequenching of fluorescence. As expected, after DTT or glutathione treatment, we observed a change in absorbance in the range of 600–650 nm (Figure 4A) and a dequenching of the Cy5.5 fluorescence (Figure 4B and 4C). The ratio of fluorescence intensities measured after and before the treatment with DTT was in the range of 3.5 to 4. The latter quenching factor was close to that measured using

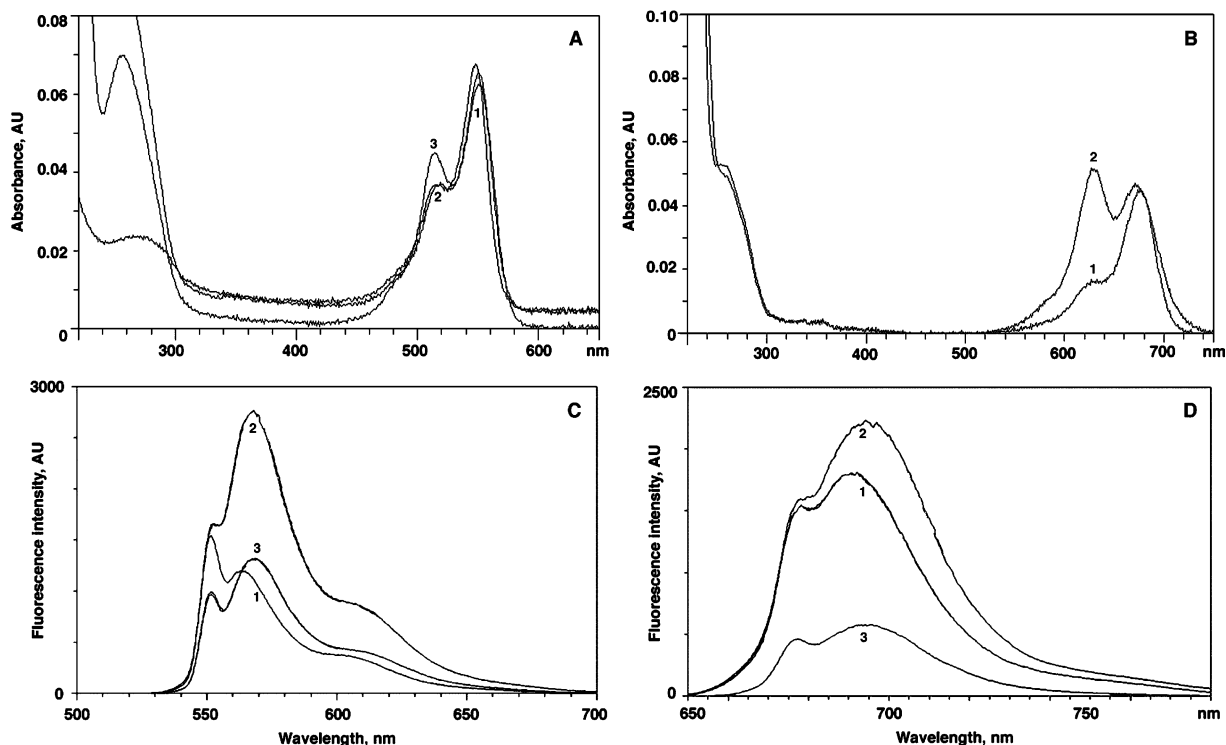


Figure 3. (A) Absorption and (C) emission spectra of 1, Cy3 dye; 2, mono-Cy3 derivative; and 3, di-Cy3 derivative of oligonucleotide I. (B) Absorption spectra of 1, mono-Cy5.5; 2, di-Cy5.5 derivative of oligonucleotide I, (D) Emission spectra of 1, Cy5.5; 2, mono-Cy5.5 derivative; and 3, di-Cy5.5 derivative of oligonucleotide I. The spectrum of di-Cy5.5 oligonucleotide I shows strong absorption at 629 nm due to the dye dimerization and no absorption due to J-aggregates.

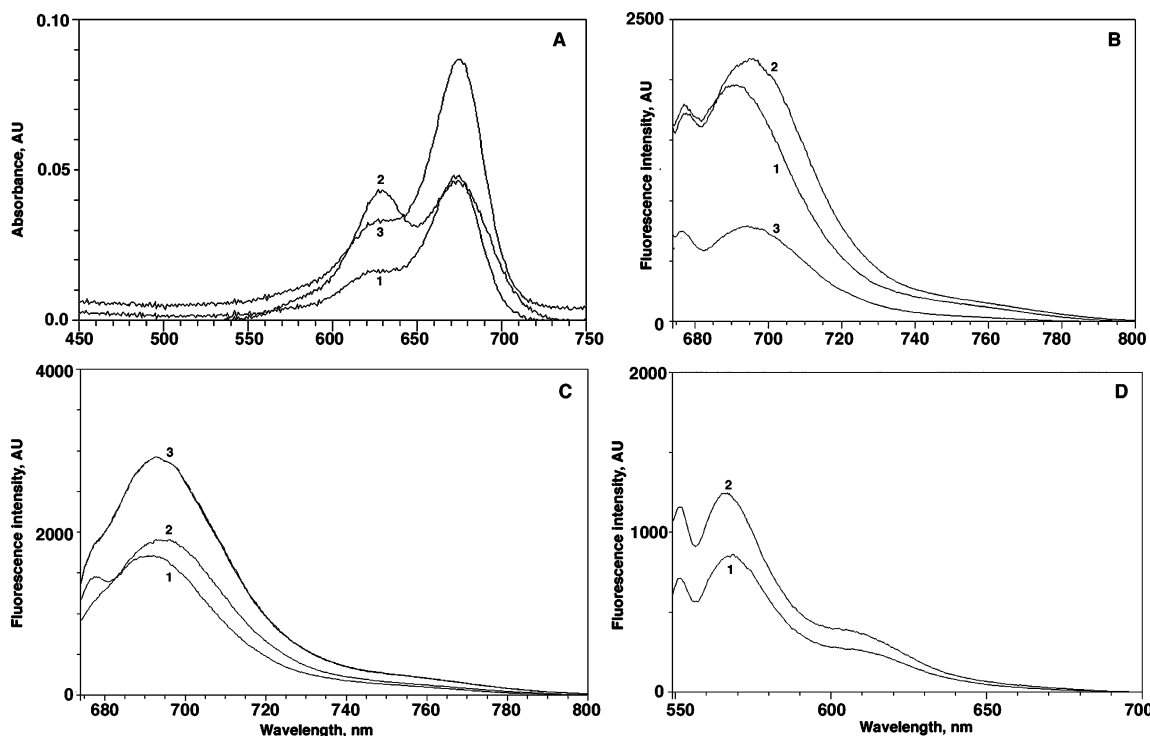


Figure 4. (A) Absorption spectra: 1, Cy5.5 in PBS; 2, dimodified oligonucleotide III before disulfide bond reduction with DTT; 3, after DTT treatment. (B) Emission spectra before DTT treatment: 1, Cy5.5; 2, mono-Cy5.5-modified oligonucleotide III; 3, di-Cy5.5-modified oligonucleotide III. (C) Emission spectra after disulfide bond reduction with DTT: 1, Cy5.5; 2, mono-Cy5.5-modified oligonucleotide III; 3, di-Cy5.5-modified oligonucleotide III. (D) Emission spectra of di-Cy3-modified oligonucleotide III: 1, before; and 2, after DTT treatment.

extensive DNase I-mediated degradation (5.4 times, Table 1). The absorbance spectrum reverted to the typical Cy5.5 shape following the reduction with a concomitant 1.8-fold increase at the maximum of absorbance because of the transition of 50% of the dimerized, blue-shifted

Cy5.5 molecules into the noninteracting pool of molecules. Interestingly, dye quenching in non-DTT-cleavable di-Cy5.5 decay was more efficient than that of in a cleavable one (Table 1). The observed difference in quenching factors (12.9 for 3'-Cy5.5-I-5'-Cy5.5 vs 5.6 for 3'-Cy5.5-

Table 1. Some Fluorescent Properties of Cyanine Dyes and Labeled Oligonucleotide Hairpin Probes

fluorophore/labeled oligonucleotide	excitation, nm	emission max., nm	quenching factor ^a
Cy3	548	563	1.0
Cy5.5	674	692	1.0
Cy3-I	548	568	— ^b
Cy3-I-Cy3	548	568	5.4
Cy5.5-I	674	695	1.1
Cy5.5-I-Cy5.5	674	695	12.9
Cy3-II	548	568	ND ^c
Cy3-III-Cy3	548	567	ND
Cy5.5-II	674	695	1.2
Cy5.5-III-Cy5.5	674	695	5.6

^a Quenching factor was calculated as a ratio of fluorescence intensities measured in a sample containing 20–100 μ M of labeled hairpin oligonucleotide decoy without DNase I to that of containing DNase I. ^b Fluorescence of oligonucleotide-conjugated Cy3 was higher than that of the free Cy3 by a factor of 2. ^c ND, not done.

III-5'-Cy5.5, Table 1) could be a result of differences in both linker length and structure in above constructs. Alternatively, thiol–disulfide exchange catalyzed by minute amounts of free thiols in oligonucleotide III dimodified preparation could result in partial dequenching of Cy5.5 dye.

The DTT treatment of di-Cy3-modified oligonucleotide lead only to a modest increase of fluorescence (1.4 times, Figure 4D). However, as noted before, in the case of Cy3 it is problematic to estimate the degree of true quenching of Cy3 conjugated to a hairpin nucleotide since the monomodified Cy3-oligonucleotide had higher fluorescence than free Cy3 dye at the same molar concentration (see Figure 4C). In the case of a monomodified oligonucleotide (i.e. Cy3-NH-oligonucleotide-S–S–CH₂CH₂-CH₂OH) disulfide reduction did not result in any appreciable changes of probe fluorescence (not shown).

In conclusion, we synthesized and tested fluorescent hairpin oligonucleotides bearing one or two fluorophores at their termini. Our results demonstrate that (1) there is a dimerization of cyanine dyes both in the case of dimodified Cy5.5 and dimodified Cy3-labeled oligonucleotides, (2) dimerization of the Cy5.5 fluorophore results in strong self-quenching, and (3) fluorescence quenching in dimodified Cy5.5 oligonucleotides could be reversed by dissociating one of the linkers between the oligonucleotide and one of the dyes. The observed effects and developed synthetic approaches are instrumental in further design of hairpin oligonucleotide beacons that would have reversible, quenched near-infrared fluorescence. We are currently refining the developed synthetic strategy for achieving highly termini-specific labeling with fluorescent probes and fluorescence quenchers.

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