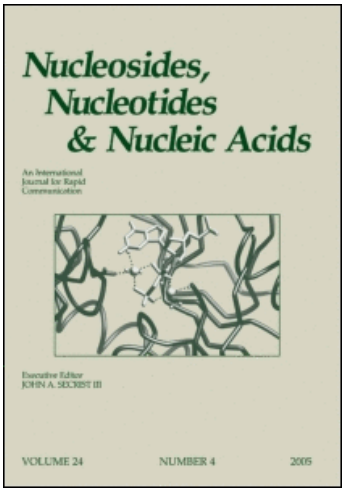

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SYNTHESIS AND BIOPHYSICAL STUDIES ON FLUORESCENTLY LABELED OLIGODEOXYRIBONUCLEOTIDES

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□ *Two highly fluorescent compounds, viz. 6-(6-isobutyrylamino-1,3-dioxo-1 H,3H-benzo [de]isoquinolin-2-yl)-hexanoic acid and 6-(6-dimethylamino-1,3-dioxo-1 H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid have been synthesized, characterized, and attached to 12-mer oligodeoxyribonucleotides at their 5′-end using suitable linker molecule. These labeled oligodeoxyribonucleotides have shown appreciable fluorescence even at 0.0019 μM concentrations. Thermal denaturation studies have shown comparatively higher Tm values when oligodeoxyribonucleotides are labeled. These labeled oligodeoxyribonucleotides have been purified on RP-HPLC utilizing their hydrophobicity and on polyacrylamide gel because of their easy detection due to fluorescence.*

Keywords Oligodeoxyribonucleotides; Fluorescent compounds; Polyacrylamide gel; Thermal denaturation studies

INTRODUCTION

Fluorescent probes are now in widespread use in various formats of DNA/RNA assays, which exploit the high affinity and specificity of nucleic acid hybridization and/or the possibility of target amplification.[1,2] Fluorescently labeled probes have a variety of applications,[3–5] including DNA sequencing,[6,7] fluorescence in situ hybridization (FISH),[8,9] gene arrays,[10] fluorescence resonance energy transfer (FRET),[11,12] PCR,[13,14]

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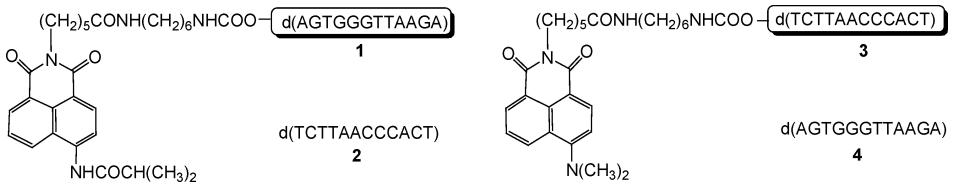

FIGURE 1 Fluorescently labeled oligodeoxyribonucleotides with their complementary sequences.

etc. Although in many cases the probes provide satisfactory performance in such assays, sensitivity of conventional fluorescent labels is less than optimal when compared with radioisotope and enzyme labels.[1,15]

Radioisotopes are the common labels but these are now been replaced by fluorophores due to some disadvantages like cumbersome nature of autoradiographic/scintillation counting method, isotope instability, health hazards, and disposal problems.[16]

Fluorophores represent an attractive class of reporter molecules since they are stable, directly detectable, and offer an opportunity for the simultaneous detection of multiple probes. Fluorescence is dependent on solvent, pH, temperature, and concentrations; so fluorescent probes can be used for studies of molecular interactions,[17–19] cellular functions, and biochemical processes,[20] etc.

Fluorescent molecules can be covalently attached by various enzymatic[21,22] or chemical methods[23,24] through various active sites on bases, sugars (3′, 5′), or phosphate units of oligonucleotides.[25,26] These molecules are attached at the end of spacer arms or side chains of nucleobases,[23] sugars, and oligonucleotides utilizing $-NH_2$ or –SH functional groups.[27] We have labeled oligodeoxyribonucleotides, having primary aliphatic amino group at 5′-terminus, with fluorophores of high sensitivity (Figure 1).

MATERIALS AND METHODS

Chemicals, Oligonucleotides, and Buffers

Carbonyldiimidazole, hexamethylenediamine, and dicyclohexylcarbodiimide were purchased from Sigma-Aldrich Co. (India). All other chemicals and solvents were purchased from Merck. Solvents were duly purified and dried before use. TLC was done on silica gel G (Merck, India), and compounds detected by UV light exposure and column chromatography on silica gel (60–120 μm; Merck). Oligonucleotide synthesis was carried out on Pharmacia LKB-Gene Assembler plus (Pharmacia Biosystems, Inc., NJ, USA). The following sequences were synthesized using standard β-cyanoethyl nucleosides phosphoramidite chemistry: *oligo-1*: DMT-AGT GGG TTA AGA and *oligo-2*: DMT-TCT TAA CCC ACT.

LCAA-CPG was used as a support for solid phase synthesis. The hybridisational studies were done in 0.01 M Na_2HPO_4, 0.01 M $NaH_2PO_4 \cdot 2H_2O$, 1.0 M NaCl buffer at pH 7.1.

Spectroscopy

Purification of oligonucleotides was carried out on HPLC Agilent 1100 series system consisting of a quaternary pump, diode array photometric detector, autosampler and chromatography was performed on a Supelcosil LC18 column (Sigma Aldrich, India) employing a UV-visible detector for fluorescence detection. The oligonucleotides were purified using the following solvent system: solvent A, 0.1 M CH_3COONH_4, pH 7.1; solvent B, CH_3CN; flow rate, 1 mL/minute and a linear gradient from 0 to 25% B in 25 minutes. Fluorescence spectra were recorded on Fluoromax-3 (Jovin Yvon Horiba, Tokyo, Japan) spectrofluorometer. Mass spectra were measured on a Applied Biosystems/MDS-Sciex API-100 instrument in ESI mode (Applied Biosystems, CA, USA). The melting temperature measurements were performed on a Perkin-Elmer Lambda Bio 20 UV-visible spectrophotometer fitted with a PTP-6 (Peltier Temperature Programmer (MA, USA)) device. ^{1}H NMR was recorded using DRX 300 instrument with $CDCl_3$ as solvent.

Synthesis

6-(6-Isobutyrylamino-1,3-dioxo-1 H,3 H-benzo[de]isoquinolin-2-yl)-hexanoic acid (I). 6-(6-Amino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl) -hexanoic acid (50 mg, 0.15 mmol), indigenously prepared,[26] was dried using anhydrous pyridine (3 × 10 mL), and dissolved in anhydrous pyridine (2 mL). Reaction mixture was cooled on ice bath. After 30 minutes, isobutyric anhydride (3 mL) was added and the reaction mixture was removed from ice bath. Reaction was left overnight for stirring. After 24 hours, reaction mixture was chilled on ice bath and cold water (2 mL) was added. After 15 minutes, it was concentrated and poured into water, washed with ether and left for crystallization. The title compound was obtained in 69% yield (273.2 mg). R_f, 0.3, (DCM); UV(MeOH): λmax 397 nm; ^{1}H NMR ($CDCl_3$, TMS = 0.00): 7.97–6.79 (d, 5 H, Ar-H), 3.2–1.29 (d, 8 H); 2.78 (d, H), 2.23 (s, 2 H); 1.19 (s, 6 H); ^{13} C NMR ($CDCl_3$): 18.0, 24.8, 26.8, 28.7, 35.8, 42.0, 111.5, 116.3, 120.4, 125.9, 126.7, 127.0, 130.3, 137.7, 138.3, 151.6, 165.9, 177.2; MS (EI) m/z: 396.443 (M^+); $C_{22}H_{24}N_2O_5$ C, 66.65; H, 6.10; N, 7.07; found: C, 66.25; H, 6.15; N, 6.9; ΔG°: 28.42 [kJ/mol]; ΔH°: -557.21 [kJ/mol].

6-(6-Dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (II). 6-(6-Amino-1,3-dioxo-1 H,3 H-benzo[de]isoquinolin-2-yl)-hexanoic acid (3.26 g, 10 mmol) was dissolved in dried DMF (10 mL) and chilled on ice bath. Tributylamine (4.8 mL, 20 mmol) was added and the reaction mixture was stirred. After 15 minutes, when reaction mixture was chilled sufficiently, methyl iodide (1.8 mL, 28 mmol) was added dropwise

and allowed to overnight stirring at room temperature. After 24 hours, dried ether (100 mL) was added and the reaction mixture was placed in refrigerator for 2 hours. It was then concentrated and extracted with DCM and water. Crystals were obtained in DCM fraction in 71% yield (251.34 mg). R_f, 0.8, (DCM:MeOH::9:1, v/v); UV(MeOH): λmax 268 nm; ^{1}H NMR ($CDCl_3$, TMS = 0.00):): 7.97–6.79 (d, 5 H, Ar-H), 3.2–1.29 (t, 8 H); 2.23 (s, 2 H); 2.85 (s, 6H); ^{13} C NMR ($CDCl_3$): 24.8, 26.8, 28.7, 35.8, 42.0, 44.0, 116.8, 121.0, 126.6, 127.7, 128.5, 134.4, 138.6, 161.3, 165.9, 177.0; MS (EI) m/z: 354.406 (M^+); Anal. calcd. for $C_{20}H_{22}N_2O_4$ C, 67.78; H, 6.26; N, 7.90; found C, 67.7; H, 6.19; N, 7.94; ΔG°: 183.88 [kJ/mol]; ΔH°: -330.91 [kJ/mol].

Labeling of Oligodeoxyribonucleotides

The oligodeoxyribonucleotides have been synthesized on Pharmacia LKB Gene Assembler Plus on 0.2 μmol scale using standard phosphoramidite protocol. The CPG attached 12-mer DMT- d(AGTGGGTTAAGA) was placed into a vial and the resin was treated with 3% trichloroacetic acid in DCM to remove DMT group and the resin was washed with anhydrous HPLC grade dioxane (3 × 3 mL). Carbonyldiimidazole (50 mg) dissolved in dioxane (1.0 mL) was added to it and the reaction was allowed to proceed for 30 minutes at room temperature with occasional shaking. After this, the solid support was washed well with dioxane (3 × 3 mL). The 5′-activated oligodeoxyribonucleotide[28] was then allowed to react with hexamethylenediamine (1.0 mL, 0.2 M) dissolved in dioxane:water (9:1) in the same reaction vessel. The reaction was allowed to proceed for 20 minutes at room temperature. Finally, the reagents were washed with dioxane and methanol and the support air-dried. Similarly, other oligodeoxyribonucleotide, d(TCTTAACCCACT), was also derivatised at its 5'-end to bear a primary amino group. These derivatised oligodeoxyribonucleotides were cleaved from support with ammonia (25%, 4 mL) for 10 hours at 55°C. Ammonia was removed under vacuum and the oligodeoxyribonucleotides were then purified by reversed phase HPLC on LC18 column using gradient buffer CH_3COONH_4, 0.1 M, pH 7.1 (A) and CH_3CN (B) having flow rate, 1 mL/minute and a linear gradient from 0 to 25% B in 25 minutes. The absorption was monitored at 260 nm for peak detection. The retention time observed for d(AGTGGGTTAAGA) (10 OD, 280 μg) was 9 minutes and for d(TCTTAACCCACT) (11 OD, 363 μg) was 8.2 minutes. The desired fractions were pooled and lyophilized.

p-Nitrophenyl ester[29] of fluorophores were prepared by dissolving the fluorophores (0.054 mM) separately in dry dioxane (2.0 mL) containing dry pyridine (0.2 mL), TEA (0.2 mL), and p-nitrophenol (0.054 mM, 7.56 mg). Dicyclohexylcarbodiimide (0.135 mM, 27.8 mg) was added and the reaction mixture was left for 2.5 hours with

occasional shaking. Dicyclohexyl urea was removed by filtration to obtain the p-nitrophenyl ester of 6-[6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl]-hexanoic acid and 6-(6-dimethylamino-1,3-dioxo-1 H,3 H-benzo[de]isoquinolin-2-yl)-hexanoic acid (yield 65%).

5'-Alkylamino oligodeoxyribonucleotides (6.5 OD and 8 OD for oligonucleotides A and B, respectively) were dissolved separately in 250 μL solutions of a mixture of 0.1 M $Na_2CO_3/NaHCO_3$ (pH 9.0). To these solutions, 500 μL solutions of p-nitrophenyl ester of fluorophores dissolved in a mixture of 1.0 M $Na_2CO_3/NaHCO_3$ buffer (pH 9.0):DMF:H_2O (5:2:3. v/v) were added, the reaction mixtures vortexed and wrapped with aluminium foil to prevent light exposure. The reaction mixture was stirred for 8 h at room temperature in dark. The labeled oligodeoxyribonucleotides were then passed through Sephadex G-25 (Sigma Aldrich, India) column using ethanol-water (8:2, v/v) as eluent. The filtrates containing fluorescently labeled oligodeoxyribonucleotides were collected, concentrated and resuspended in water (1.0 mL) and purified by reversed phase HPLC on LC18 column using gradient buffer CH_3COONH_4, 0.1 M, pH 7.1 (A) and CH_3CN (B) having flow rate, 1 mL/minute and a linear gradient from 0 to 25% B in 25 minutes. The absorption was monitored at 260 nm for peak detection. The retention time observed for labeled d(AGTGGGTTAAGA) was 16.5 minutes and for d(TCTTAACCCACT) was 15.8 minutes. The desired fractions were pooled and lyophilized. Finally, 150 μg (6 OD; 0.03 μmol) and 165 μg (7 OD; 0.035 μmol) labeled oligodeoxyribonucleotides were obtained. The synthetic procedure has been outlined in Figure 2. Analysis of

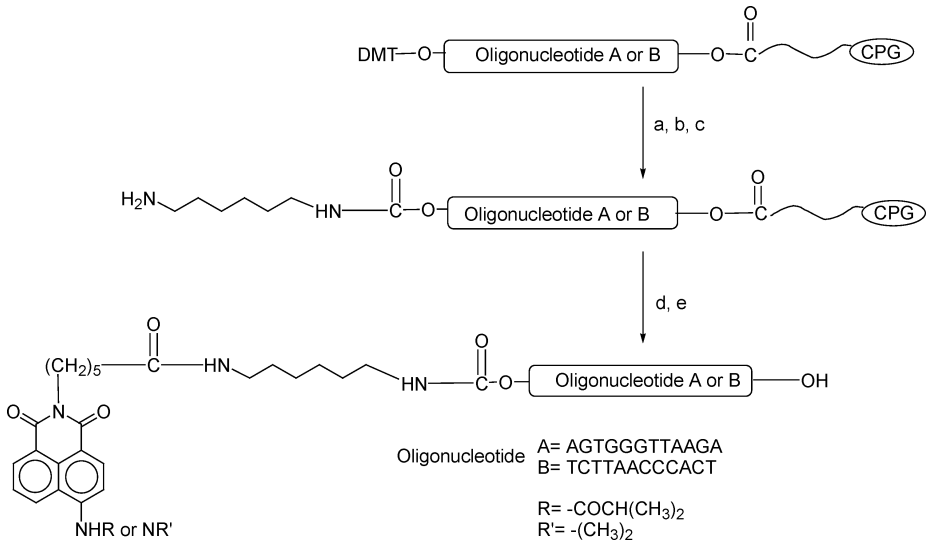

FIGURE 2 Flouresent labeling of oligodeoxyribonucleotides. a) 3% trichloroacetic acid in dichloromethane; b) carbonyldiimidazole dissolved in dioxane; c) hexamethylenediamine in dioxane:water; d) ammonia (25%, 4 mL) 10 hours at 55°C, e. p-nitrophenyl ester of fluorophore dissolved in $Na_2CO_3/NaHCO_3$ buffer(pH 9.0):DMF:H_2 O::5:2:3, v/v.

ESI-MS of labeled d(AGTGGGTTAAGA) and labeled d(TCTTAACCCACT), m/z = 4285.53 and m/z = 4034.67, respectively.

RESULTS AND DISCUSSION

We have synthesized two new fluorescent molecules and characterized them using different spectroscopic techniques. Fluorescence of these fluorophores has been studied in different organic and inorganic media. The fluorophores were dissolved separately in MeOH at 10 μM concentrations and their fluorescence was recorded (Figure 3). These fluorophores showed higher degree of fluorescence than several fluorophores synthesized in our laboratory[26] and, hence, have been chosen for labeling of oligodeoxyribonucleotides. A six-carbon chain of caproic acid acts as a spacer arm and a 15 atoms (13 carbon + 2 nitrogen) long chain has been utilised to attach fluorophores to oligonucleotides. This has been done to avoid direct interaction of fluorophores with oligonucleotides after their covalent attachment.

The coupling of these fluorophores with oligodeoxyribonucleotides through its activated ester is efficient and fluorophores **I** and **II** have been covalently attached to oligodeoxyribonucleotides bearing 5′-NH_2 function generated by the reaction between oligodeoxyribonucleotides and hexamethylene diamine mediated by CDI. Such amino protected oligodeoxyribonucleotides can be stored for longer time also. The DMT-oligodeoxyribonucleotides, amino linker bearing oligodeoxyribonucleotides and fluorescently labeled oligodeoxyribonucleotides were purified by HPLC on LC18 column using gradient buffer CH_3COONH_4, 0.1 M, pH 7.1 and CH_3CN and absorption monitored at 260 nm

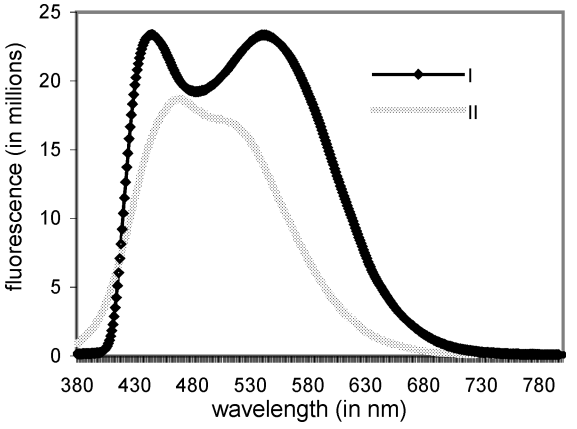

FIGURE 3 Fluorescence of fluorophores **I** and **II.** I: 6-(6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid; I: 6-(6-dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid.

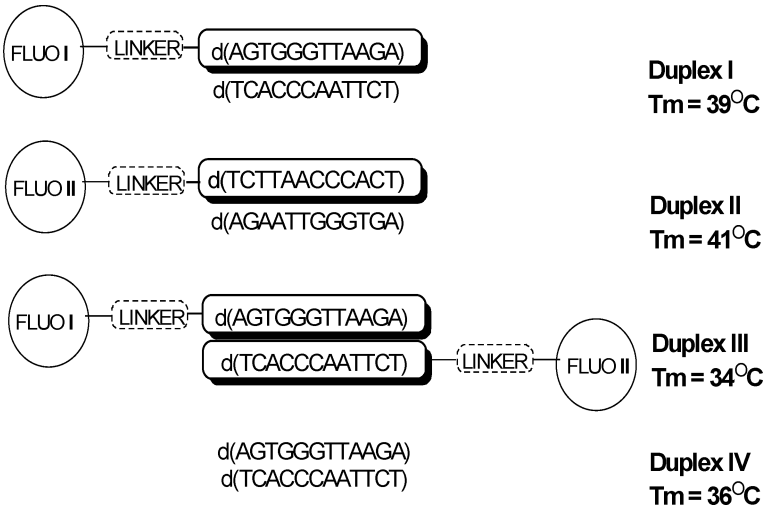

FIGURE 4 Duplexes I–IV.

for peak detection. The retention time observed were 10.7, 9, and 16.5 minutes for d(AGTGGGTTAAGA), 5′-NH_2-oligonucleotide and labeled oligonucleotide, respectively, and 10, 8.2, and 15.8 minutes for d(TCTTAACCCACT), 5′-NH_2-oligonucleotide and labeled oligonucleotide, respectively. Fluorescently labeled oligodeoxyribonucleotides also were monitored through UV detector of HPLC.

We have taken the following four duplexes for fluorescence and thermal denaturation studies. Duplex I is having fluorescently labeled d(AGTGGGTTAAGA), **1**, and its complementary strand d(TCTTAA CCCACT), **2**, while duplex II is having fluorescently labeled d(TCTTAA CCCACT), **3**, and its complementary strand, d(AGTGGGTTAAGA), **4**. Duplex III is having both labeled strands, **1** and **3**, while duplex IV is having both unlabeled strands, **2** and **4** (Figure 4).

The duplexes were formed by mixing the two strands, each having a concentration of 0.44 μM, in the hybridisation buffer (0.01 M Na_2HPO_4, 0.01 M $NaH_2PO_4.2H_2O$, 1.0 M NaCl, pH 7.1). The solutions were heated at 90 °C for 5 minutes and then cooled to room temperature during 10 minutes before measuring the melting temperatures. The melting temperature was determined as the maximum of the first derivative plots of the melting curves obtained by measuring absorbance at 260 nm against increasing temperature (0.5°C/minute) from 15 to 70 °C (Figure 5).

All melting temperatures are reported within uncertainity ±1°C, as determined from multiple experiments. The thermal denaturation studies showed that the singly labeled duplexes I and II melted at a slightly higher temperature (Tm; 39°C and 41°C, respectively) than the unlabeled duplex IV (Tm; 36°C), whereas the doubly labeled duplex III melted at slightly lower temperature (Tm; 34°C).

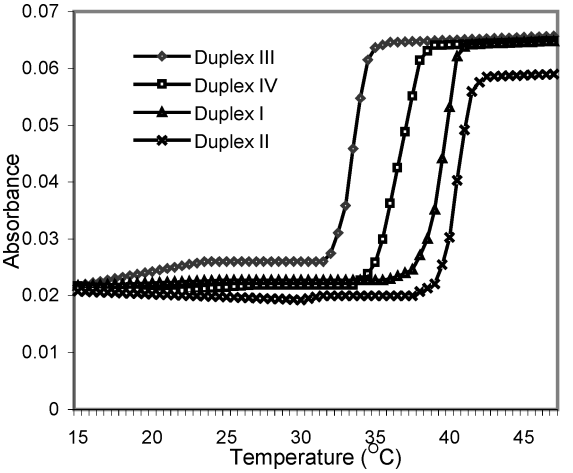

FIGURE 5 Tm graph of duplexes I–IV.

The fluorescence of these labeled duplexes was scanned in the range of 300–700 nm at room temperature (Figure 6a) and at varying temperature from 15 to 70°C as well (Figure 6b). Excitation wavelengths were fixed at 397 and 368 nm for duplex I and duplex II, respectively, and the duplex III was excited once at 397 nm (III) and then at 368 nm (III). The emissions were recorded at 475 nm.

The study showed that fluorescence of 6-(6-dimethylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid fluorophore increased 1.5 times when used in combination with 6-(6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid (III' in Figure 6a and 6b)

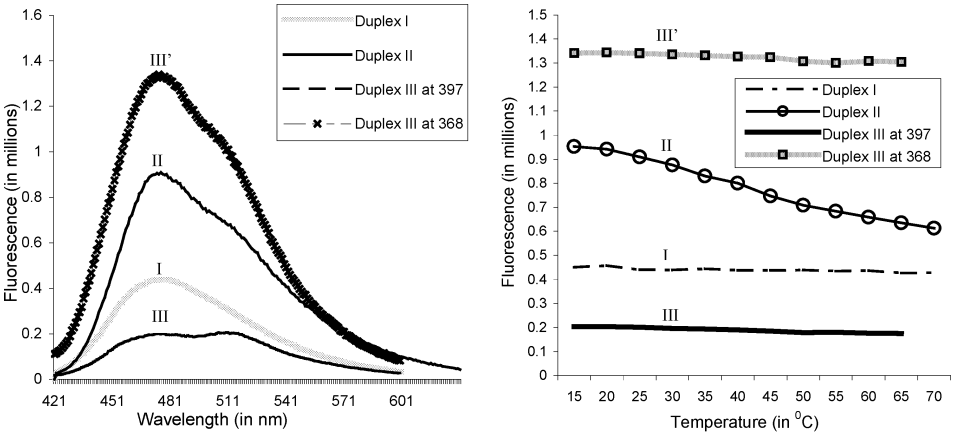

FIGURE 6 Graphs of fluorescence studies. a) Fluorescence of duplexes excited at their excitation wavelengths at 25°C; b) spectra of fluorescence of duplexes with increasing temperature at their emission wavelength.

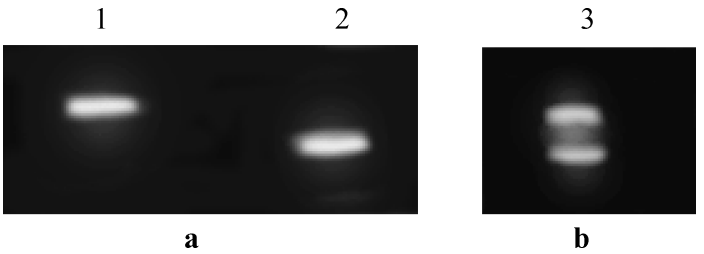

FIGURE 7 Polyacrylamide gel electrophoresis analysis of duplexes I and II. a) 20% Native PAGE; b) 20% (6 M urea) Denaturing PAGE; 1 is duplex II, 2 is duplex I, and 3 is duplex III.

as compared to its fluorescence observed in the case of duplex II at the same excitation wavelength, that is, 368 nm. Further the fluorescence of the 6-(6-isobutyrylamino-1,3-dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-hexanoic acid decreased to half when used in combination with 6-(6-dimethylamino-1,3-dioxo-1 H,3 H-benzo[de]isoquinolin-2-yl)-hexanoic acid fluorophore at its excitation wavelength 397 nm in comparison to its fluorescence in duplex I. This behavior may be due to proximity effect as the duplex III showed slightly lower Tm than the unlabeled duplex IV. However, such proximity effect is not observed in duplexes I and II.

An interesting observation was that the fluorescence of labeled oligodeoxyribonucleotide, **3**, increased when it formed a duplex II with its complementary sequence. This clearly has been supported by fluorescence measurement at varying temperature where the fluorescence was considerably decreased beyond its melting point, that is, 41°C where it existed as single stranded. This decrease in fluorescence may be attributed to temperature dependence of fluorescence emission. However, the fluorescence was almost unchanged in the case of duplexes I and III.

These labeled duplexes were run on 20% nondenaturing and 20% denaturing (6 M urea) polyacrylamide gel electrophoresis (PAGE). DNA was loaded in 6x TBE buffer with 0.25% bromophenol blue. Gel was run in 5× TBE buffer for 8 hours. The labeled oligodeoxyribonucleotides have showed lower electrophoretic mobility than unlabeled oligodeoxyribonucleotides. Oligodeoxyribonucleotide labeled with compound II moved faster on PAGE and also fluoresced more than Oligodeoxyribonucleotide labeled with compound I (Figure 7). The fluorescence intensity was deduced from the DNA loaded. A sample (3 μL) of initial concentration 0.005 μM was loaded and the approximate area of fluorescent spot was 3.85 mm^2 on the gel of thickness 2 mm).

Thus, we have developed highly stable and sensitive fluorophores and their fluorescence is not adversely affected by the ions normally present in biological systems. These molecules also increase the hybridizational affinity of labeled oligonucleotides when used separately, while a combination of these, lowered the hybridisational affinity. The labeled oligonucleotides were easily detected on PAGE as well on the basis of their fluorescent nature.

REFERENCES

1. Kricka, L.J. *Non-Isotopic DNA Probe Techniques*. Academic Press Inc: San Diego, CA, 1992.
2. Nath, J.; Johnson, K. Fluorescence in situ hybridisation (FISH): DNA probe production and hybridisation criteria. *Biotech. Histochem.* **1997**, 73, 6–22.
3. Okamoto, A.; Ichiba, T.; Saito, I. Pyrene-labeled oligodeoxynucleotide probe for detecting base insertion by excimer fluorescence emission. *J. Am. Chem. Soc.* **2004**, 126, 8364–8365.
4. Nakano, S.; Uotani, Y.; Uenishi, K.; Fujii, M.; Sugimoto, N. Strategies for introducing non-radioactive labels during the automated sequence analysis of nucleic acids. *Nucleic Acids Res.* **2005**, 33, 7111–7119.
5. Kurata, S.; Kanagawa, T.; Yamada, K.; Torimura, M.; Yokomaku, T.; Kamagata, Y.; Kurane, R. Fluorescent quenching based quantitative detection of specific DNA/RNA using a BODIPYTM FL-labelled probe or primer. *Nucleic Acids Res.* **2001**, 29, e34.
6. Igloi G.L. Strategies for introducing non-radioactive labels during the automated sequence analysis of nucleic acids. *Elec. J. Biotech.* **1998**, 1(1), 23–30.
7. Markiewicz, W.T.; Groger, G.; Rosch, R.; Zebrowska, A.; Markiewicz, M.; Klotz, M.; Hinz, M.; Godzina, P.; Seliger, H. A new method of synthesis of fluorescently labelled oligonucleotides and their application in DNA sequencing. *Nucleic Acids Res.* **1997**, 25, 3672–3680.
8. Johnson, K.L.; Nath, J. A review of fluorescence in situ hybridization (FISH): current status and future prospects. *Biotech. Histochem.* **1999**, 75(2), 54–78.
9. Iourov. I.Y.; Soloviev, I.V.; Vorsanova, S.G.; Monakhov, V.V.; Yurov, Y.B. An approach for quantitative assessment of fluorescence in situ hybridization (FISH) signals for applied human molecular cytogenetics. *J. Histochem. Cytochem.* **2005**, 53(3), 401–408.
10. van Hal, N.; Vorst, O.; van Houwelingen, A.; Kok, E.; Peijnenburg, A.; Aharoni, A.; van Tunen, A.; Keijer, J. The application of DNA microarrays in gene expression analysis. *J. Biotechnol.* **2000**, 78, 271–280.
11. Masuko, M.; Ohuchi, S.; Sode, K.; Ohtani, H.; Shimadzu, A. Fluorescence resonance energy transfer from pyrene to perylene labels for nucleic acid hybridization assays under homogeneous solution conditions. *Nucleic Acids Res.* **2000**, 28, e34.
12. Marras, S.A.E.; Kramer, F.R.; Tyagi, S. Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes, *Nucleic Acids Res.* **2002**, 30, e122.
13. Hukkanen, V.; Rehn, T.; Kajander, R.; Sjoroos, M.; Waris M. Time-resolved fluorometry PCR assay for rapid detection of herpes simplex virus in cerebrospinal fluid. *J. Clin. Microbiol.* **2000**, 38, 3214–3218.
14. Livak, K.; Flood, S.; Marmaro, J.; Giusti, W.; Deetz, K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.* **1995**, 4, 357–362.
15. Shan, G.; Huang, W.; Gee, S.J.; Buchholz, B.A.; Vogel, J.S.; Hammock, B.D. Isotope-labeled immunoassays without radiation waste. *Proc. Natl. Acad. Sci. USA* **2000**, 97(6), 2445–2449.
16. Hermanson, G.T. Bioconjugate Techniques. Academic Press: San Diego, CA, 1996.
17. Bloom, L.B.; Otto, M.R.; Beecham, J.M.; Goodman, M.F. Influence of 5′-nearest neighbors on the insertion kinetics of the fluorescent nucleotide analog 2-aminopurine by Klenow fragment. *Biochemistry* **1993**, 32, 11247–11258.
18. Weissleder, R.; Ntziachristos, V. Shedding light onto live molecular targets. *Nature Medicine* **2003**, 9(1), 123–128.
19. Erijman, E.A.; Jovin, T. FRET imaging. *Nature Biotech.* 21(11), 1387–1395.
20. Hiratsuka, T. *Gendai Kagaku* **1994**, 283, 20.
21. Owenius, R.; Österlund, M.; Lindgren, M.; Svensson, M.; Olsen, O.H.; Persson, E.; Freskgård, P.-O.; Carlsson, U. Properties of spin and fluorescent labels at a receptor-ligand interface. *Biophys. J.* **1999**, 77(4), 2237–2250.
22. Verma, S.; Eckstein, F. Modified oligonucleotides: Synthesis and strategy for users. *Ann. Rev. Biochem.* **1998**, 67, 99–134.
23. Horn, T.; Chang, C.A.; Urdea, M.S. An improved divergent synthesis of comb-type branched oligodeoxyribonucleotides (bDNA) containing multiple secondary sequences. *Nucleic Acids Res.* **1997**, 25, 4842–4849.

24. Guan-Sheng, J.; Burgess, K. Oligonucleotides with strongly fluorescent groups π-conjugated to a nucleobase: syntheses, melting temperatures, and conformation. *Bioorg. Med. Chem. Lett.* **2003**, 13, 2785–2788.
25. Dubey, K.K.; Singh, R.K.; Misra, K. A novel bifunctional fluorescent tag for use in molecular biology. *Indian J. Chem.* **1995**, 34B, 876–878.
26. Dubey, K.K.; Singh R.K.; Misra, K. A novel fluorescent tag for labelling of some antisense oligonucleotides. *Neurochemistry Internatl.* **1997**, 31(3), 405–412.
27. Agarwal, S. *Protocols for Oligonucleotide Conjugates,* Humano Press, Totowa, NJ, 1984, p 73.
28. Bonfils, E.; Depierreux, C.; Midoux, P.; Thuong, N.T.; Monsigny, M.; and Roche, A.C. Drug targeting: synthesis and endocytosis of oligonucleotide-neoglycoprotein conjugates. *Nucleic Acids Res.* **1992**, 20(17), 4621–4629.
29. Gait, M.J. *Oligonucleotide synthesis: a practical approach*. IRL Press: Oxford, 1984, 47–81.