Available online at www.sciencedirect.com
SCIENCE DIRECT®
Dyes and Pigments 60 (2004) 9–16
DYES and PIGMENTS
www.elsevier.com/locate/dyepig

Benzothioxanthene dyes as fluorescent label for DNA hybridization: synthesis and application

Ping Mao[a,d], Xuhong Qian[b,*], Huizhan Zhang[c], Wei Yao[a,d]

[a]*Institute of Pesticides & Pharmaceuticals, State key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China*
[b]*State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian, 116012, China*
[c]*State key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China*
[d]*Shanghai Key Laboratory of Chemical Biology, Shanghai, 200237, China*

Received 15 March 2002; received in revised form 11 May 2002; accepted 12 June 2003

Abstract

With naphthalimide as starting material, a new fluorescent benzothioxanthene dye with application for labeling oligodeoxynucleotide was prepared through imidation, deprotection and iodoacetylation. The benzothioanthene derivatives have DNA intercalation action and displayed a large Stokes' shift in Tris–HCl–H_2O buffer. The fluorescence intensity of the dye-labeled oligodeoxynucleotide, which was obtained through the connection of the dye to the sulphydryl functions of phosphate, was found to be sensitive to nucleobase mismatch in DNA sequence during hybridization, and so could be used as novel heterogeneous hybridization probes.

Keywords: Dye; Synthesis; Oligodeoxynucleotide; Fluorescence label; Intercalation; Heterogeneous hybridization

1. Introduction

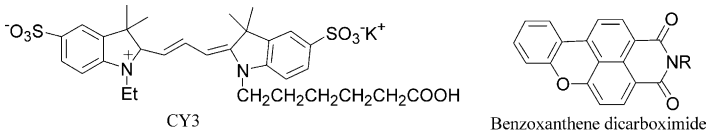
Fluorescent dyes have been frequently used for the analysis of nucleic acids through non-covalent binding (e.g. intercalation, groove binding and electrostatic attraction) or covalent binding with DNA. Cyanine dyes became ideal commercial fluorescent probes for many bioactive targets, such as antibodies, avidin, and DNA [1]. Recently, they have been widely used as fluorescent labels in DNA heterogeneous hybridization (e.g. in gene chips). The most famous cyanine dyes are CY3 (absorption: 550 nm, fluorescence: 570 nm in DNA system) and CY5 (absorption: 647 nm, fluorescence: 674 nm in DNA system). However, although they have high extinction coefficients, good water-solubility and low aggregation under labeling conditions, they display only low photostability, and very small Stokes' shift. In addition, they are not able to show an obvious response to one or two nucleobase mismatches in heterogeneous hybridization, as they bind with single or double strands of DNA through electrostatic attraction.

Naphthalimide is a famous fluorophore [2] and heterocyclic dyes derived from them are usually

* Corresponding author. Tel.: +86-21-64252945; fax: +86-411-3673488.
E-mail address: xhqian@dlut.edu.cn (X. Qian).

0143-7208/$ - see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/S0143-7208(03)00127-X

stable, fluorescent, and easily modified for interaction with other biomolecules through their imide moiety. They have been used as labels or probes for proteins, cells, lysosomes and other acidic organelles [2–4]. Benzothioxanthene dyes derived from naphthalimides, have been used for protein detection in the range of 10–20 ng, and are commercial available [2–4]. Up to now, naphthalimide dyes with strong fluorescence have not been used for labeling onto oligodeoxynucleotides or oligonucleotides. Here, we report the preparation and application of novel benzothioxanthene dyes as fluorescent labels for oligodeoxynucleotide (absorption: 462 nm, fluorescence: 585 nm in DNA system) derived from 1,8-naphthalimide, which have large Stokes' shift, high photochemical stability and DNA intercalation ability (Scheme 1).



Scheme 1. The structure of CY3 cyanine and benzoxanthene dicarboximide.

2. Results and discussion

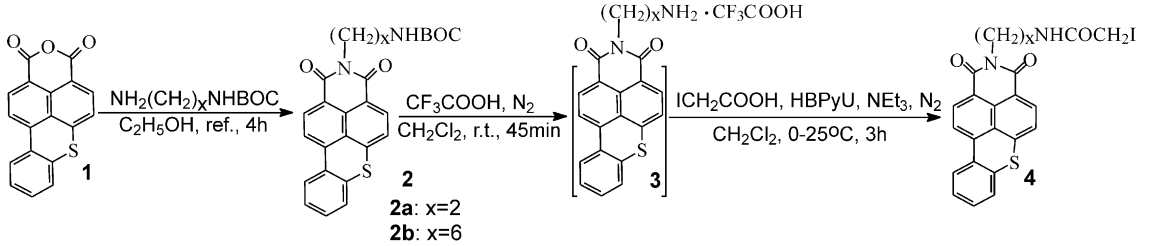
2.1. Preparation

A series of synthetic routes (Scheme 2) have were used for the preparation of dye **4a**, iodoacetyl aminohexyl-benzothioxanthene-3,4-dicarboximide, using benzothioxanthene **1** as starting material. In order to prevent the formation of a cyclo-byproduct in the imidation of the anhydride moiety with polymethylene diamine, one of the amino groups of the imidation agent was protected using the *N-tert*-butoxycarbonyl group. The preparation procedure included: the imidation of **1** with *N-tert*-butoxycarbonyl-polymethlene diamine in ethanol under reflux for 4 h to give **2** at a yield of 80–95%, which gave **3** through deprotection with trifluoroacetic acid in methylene chloride at room temperature for 45 min and followed acidation with iodoacetic acid to produce dye **4** in presence of triethylamine and the condensation agent HBPyU under nitrogen atmosphere. All of these derivatives were identified via ^{1}H NMR, EI-MS, IR and elemental analysis.

From Table 1 it is seen that the fluorescence maxima of the precursor benzothioxanthtne **1**, has an obvious red shift in polar solvent. Its fluorescence was strong in a hydrophobic environment, but very weak in a strong protic solvent or hydrophilic environment (e.g. ethylene glycol, Isopropanol/water 2:8), because of the possible formation of hydrogen bonding [6].

Compared with **1**, the fluorescence of **2a** and **2b** with a substituted amino polymethylene side chain at the imide group were weak (Table 2). This quenching was presumably due to the photo-induced intramolecular electron transfer (PET) between the excited fluorophore and the substituted amino group [7]. Therefore, we chose **2a** with relatively strong fluorescence (over **2b**) to synthesize **4a**.

The intercalation-induced fluorescence enhancement was a very important strategy in homogeneous



Scheme 2. The synthesis of benzothioxanthene derivatives for labeling.

hybridization [8–10], as the interior (between base pairs) of duplex DNA is also hydrophobic. We found that the intensity of fluorescence of **4a** was increased with the concentration of DNA due to intercalation, when mixed with calf thymus DNA in buffer. The binding coefficient (K) of **4a** was about 1.11×10^3 M^{-1} (excitation at 460 nm, fluorescence emission at 540 nm), while that of **1** was 4.26×10^3 M^{-1}.

2.2. Application

In DNA-sequencing by hybridization the fluorescent dye is attached via a spacer either to the 3′- or 5′-end of an oligodeoxynucleotide [11,12], internucleotidic phosphate [13,14], ribose moiety [8–10,15,16] or nucleobase [17–19], respectively. The labeling can be achieved either by their incorporation during chemical synthesis [20] or by post-synthesis modification [21] through an oligodeoxynucleotide with amino or sulphydryl linkers. The functional group of iodoacetamides could be employed to covalently label the phosphorothioate diester to produce the corresponding phosphorothioate triester [22]. Here, we carried out a direct coupling of the label onto the sulphydryl functions of the phosphate via a polyethylene bridge to obtain a new type of oligodeoxynucleotide fluorescent probe used for heterogeneous DNA hybridization.

The results for the coupling reaction was photographed on gel under ultra-violet light at 365 nm (Fig. 1), we could observe a light fluorescence in lane (3) which meant that only s-alkylation modified 14-mers oligodeoxynucleotide was successfully labeled. Compared with its precursor, **4a**, labeled oligodeoxynucleotide has a large red shift and a large Stokes' shift in fluorescence (absorption at 462 nm, fluorescence emission at 585 nm) in 10 mM Tris–HCl buffer (pH 7.5). Its fluorescence

Table 1
The spectra data of dye **1** in various solvents

Solvents	ε	UV λ^{uv}_{max} (log ε)	FL λ^{fl}_{max} (ϕ)[a]
Dioxohexane	2.2	450 (4.28)	508 (0.175)
Ethyl ether	4.3	498 (4.25)	502 (0.219)
Acetyl acetate	6	452 (4.27)	509 (0.186)
1,2-Dicholoethane	10	451 (4.29)	519 (0.161)
Acetone	20.7	451 (4.29)	518 (0.182)
Ethylene glycol	38.0	425 (3.78)	525 (0.068)
Iso-propanol/water (8:2)	30.7	451 (3.75)	528 (0.192)
Iso-propanol/water (2:8)	68.9	452 (3.40)	538 (0.020)

[a] $\lambda_x = 460$ nm, quantum yield based on quinine sulfate in 1.0 N sulfuric acid.

Table 2
Fluorescence data of some dyes before and after labeling on oligodeoxynucleotides

Compound	λ^{fl}_{max}/nm(ϕ)	$\lambda^{fl}_{max}-\lambda^{uv}_{max}$ (nm)	Stokes' shift (cm^{-1})
1	521(0.21)	65	2736
2a	520(0.160)	53	2182
2b	522(0.088)	60	2488
4a	521(0.161)	59	2451
4a[a]	540(0.015)	78	3126
Single strand DNA[a]	585 (0.010)	123	4551
Double strand DNA[a]	585(0.007)	123	4551

[a] $\lambda_x = 460$ nm, in 10 mM Tris–HCl–H_2O buffer (pH 7.5) containing DMSO (10^{-3}–10^{-4} M). $\lambda_x = 460$ nm, in absolute ethanol.

quantum yield was down to about 0.010, which might be due to fluorescence quenching from the strong stacking interactions [8].

The hybridization of the labeled oligodeoxynucleotides on the nylon film with complementary target oligodeoxynucleotide sequences was photographed under ultraviolet light at 365 nm, and analyzed quantitatively by using a fluorescence spectrometer. It was found that after hybridization the fluorescence intensity of the dye-labeled oligodeoxynucleotide at 585–590 nm increased with increasing concentration as shown in Fig. 2.

After washing any single strand oligodeoxynucleotide away (similar to the procedure in gene chips), heterogeneous hybridization on nylon film (Fig. 3) showed that the different matches obviously gave different fluorescence intensity responses on the visualization of nylon hybridization film, and the fluorescence was gradually quenched with the increasing of mismatch sites corresponding to the revival of stacking interaction from unmatched bases.

The fluorescence intensities were in the order: no mismatch > one mismatch > two mismatch (Table 3). For one nucleobase mismatch, the extent of fluorescence quenching for the mismatch near the 5′-terminus of the labeled strand was less than that of a mismatch at the 3′-terminus. The reason is that the fluorescence decrease with a mismatch near the 5′-terminus, where intercalation was likely, was strongly balanced by intermolecular intercalation-induced fluorescence increasing. The fluorescence was decreased to a smaller extent with a mismatch at the 3′-terminus, as the intercalation effect would not occur at the 3′-end.

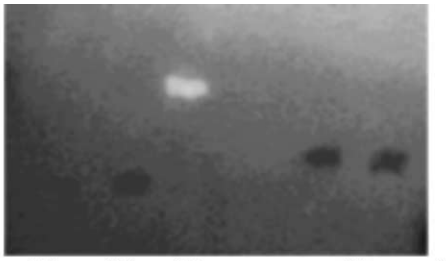


Fig. 1. The PAGE photo of oligo-label conjugate under light at 365 nm.

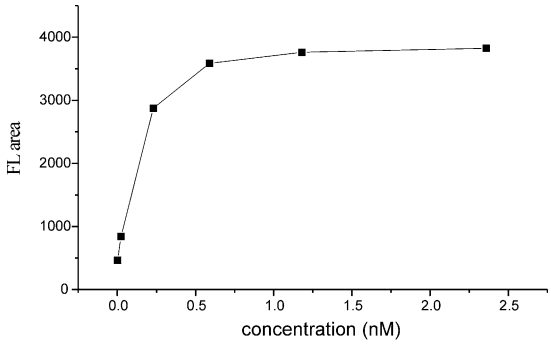


Fig. 2. Fluorescence integration area on hybridization nylon film verses concentration of duplex DNA after hybridization ($\lambda_x = 460$ nm).

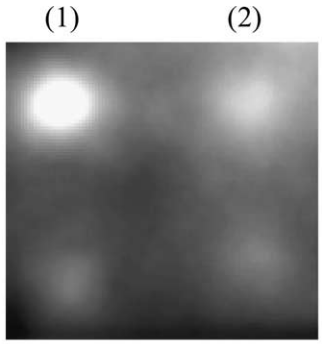


Fig 3. hybridization results of labeled oligodeoxynucleotides (at 2.36 nM) with target DNA sequence having base mismatch on nylon hybridization film ($\lambda_x = 365$ nm).

3. Experimental

Melting points were taken on a digital melting point apparatus WRS-1 made in Shanghai and it was uncorrected. Infrared spectra were recorded on a Nicolet FT IR-20SX, mass spectra on a Hitachi M80, ^{1}H NMR on a Bruker AM-300 or AM-500 using TMS as an internal standard. Combustion analysis for elemental composition was done on an Italy MOD.1106 analyzer. Absorption spectra were measured on Shimadzu UV-265; fluorescence spectra on a Perkin Elmer LS50 with quinine sulfate in sulfuric acid as the quantum yield standard. Commercial reagents and solvents were purchased from a standard chemical supplier and used without further purification.

3.1. Preparation of benzothioxanthene-3,4-dicarboxylic anhydride (1)

The synthesis was done according to literature [5]. **1:** m.p. >300 °C, ^{1}H NMR (d-DMSO, 300 MHz): δ 8.55 (m, 3H, 2-H, 6-H, 7-H), 8.36 (*d*, $J=8.0$ Hz, 1H, 2-H), 7.83 (*d*, $J=8.1$ Hz, 9-H), 7.56–7.70 (*m*, 3H, 10-H, 11-H, 12-H); EIMS (*m*/*z*%): 306($[M+2]^+$, 8.7), 304(M^+, 100).

3.2. Preparation of N-tert-butoxycarbonyl-aminoethyl-Benzoxanthene-3,4-dicarboximide (2a)

A solution of di-*tert*-butyl bicarbonate (2.45 g, 0.011 mol) in dioxane (30 ml) was added over a period of 2.5 h to a solution of 1,2-diaminoethane (5.25 g, 0.087 mol) in dioxane (30 ml). The mixture was allowed to stir for 22 h and the solvent was removed using a rotary evaporator. Water (50 ml) was added to the residue and the insoluble bis-substituted product was filtrated. The filtrate was extracted with methylene chloride (3×50 ml) and then washed with water. The methylene chloride was evaporated to yield *N*-tert-butoxycarbonyl-1, 2-ethylenediamine as a yellow oil.

Then, compound **1** (0.1 g, 0.33 mol) was stirred under reflux in anhydrous ethanol (5 ml) with *N*-tert-butoxycarbonyl-1, 2-ethanediamine (0.07 g, 0.40 mol) for 4 h. The liquor was cooled, filtered and the residue was dissolved in CH_2Cl_2 and then purified on column chromatography, using petroleum–dichloromethane (4:1) as eluant. The orange–yellow product was collected at a yield of 95%. mp. 232 °C, υ_{max} (KBr)/cm^{-1}: 3380, 2960, 2930, 1710, 1690, 1645, 1585, 1380, 760; ^{1}H NMR (500 Hz, $CDCl_3$): δ 8.62 (*d*, $J=8.0$ Hz, 1H, 2-H),

Table 3
Relative fluorescence intensity of labeled oligodeoxynucleotides with DNA having base-pair mismatch[a]

Hybrid	Mismatch number	FL.[c]
5′-dCTG GAT GTT CCT GC-3′ 3′-dGAC CTA CAA GGA $C_{(S)}$G-5′	0	0
5′-dCTG GAT GTT CCT GC-3′ 3′-dGAC CTA CAA GGA $C_{(S)}$G-5′-dye	0	1
5′-dCTG GAT GTT CCT<u>A</u>[b] *C-3′* 3′-dGAC CTA CAA GGA $C_{(S)}$G-5′-dye	1	0.77
5′-dCT<u>C</u> GAT GTT CCT GC-3′ 3′-dGAC CTA CAA GGA $C_{(S)}$G-5′-dye	1	0.70
5′-dCTG GA<u>A</u>GT<u>A</u>CCT GC-3′ 3′-dGAC CT A CA A GGA $C_{(S)}$G-5′-dye	2	0.50

[a] Fluorescence measurements were carried out on hybridization nylon films by solid-phase with the concentration of labeled oligodeoxynucleotides at 2.36 nM: the target DNA sequences are shown by italic.
[b] The mismatch base is underlined.
[c] Relative fluorescence intensity, $\lambda_x=460$ nm.

8.42 (*d*, $J=8.0$ Hz, 1H, 6-H), 8.21 (m, 2H, 1-H, 7-H), 7.50 (d, $J=8.0$ Hz, 1H, 9-H), 7.40 (m, 3H, 10–12-H), 4.36 (*t*, $J=5.7$ Hz, 2H, 1′-H), 3.54 (*m*, 2H, 2′-H), 1.33 (*s*, 9H,-C$(CH_3)_3$); EI-MS (*m*/*z*,%): 372 ($[M+H\text{-}Bu^tO]^+$, 19.9), 328 ($[M-NHCOBu^tO]^+$, 50.9), 317($[M+H-CH_2NHCOBu^tO]^+$, 31.5), 303 ($[M+H-CH_2CH_2NHCOBu^tO]^+$, 56.5) (Found: C, 67.26, H, 4.9, N, 6.28; requires: C, 67.41, H, 5.39, N, 5.82%).

3.3. Preparation of N-tert-butoxycarbonyl-aminohexyl-benzoxanthene-3,4-dicarboximide (2b)

The synthesis of *N-tert*-butoxycarbonyl-1, 6-hexylenediamine was similar to the above, 1,6-hexylenediamine was used instead of 1,2-ethylenediamine.

The synthesis of **2b** was similar to the above, with *N-tert*-butoxycarbonyl-1,6-hexylenediamine was used instead of *N*-tert-butoxycarbonyl-1,2-ethanediamine. The orange–red product was collected in a yield of 80%. **2b** mp. >300 °C, υ_{max} (KBr/cm^{-1}): 3360, 2960, 2925, 1690, 1650, 1590, 790, 760; EI-MS (*m*/*z*,%): 503($[M+H]^+$, 3.58), 429 ($[M-COBu^tO]^+$, 4.51), 318 ($[M-(CH_2)_5$-NHCOBO$]^+$, 27.2), 303 ($[M-(CH_2)_5NH$-NHCOBu$^tO]^+$, 86.5); ^{1}H NMR ($CDCl_3$): δ 8.62 (*d*, $J=8.0$ Hz, 1H, 2-H), 8.42 (*d*, $J=8.0$ Hz, 1H, 6-H), 8.21 (*m*, 2H, 1-H&7-H), 7.51 (*d*, $J=8.0$ Hz, 1H, 9-H), 7.40 (*m*, 3H, 10–12H), 4.18 (*t*, $J=7.4$ Hz, 2H, 1′-H), 3.11 (t, $J=7.4$ Hz, 2H, 6′-H), 1.74 (*m*, 2H, 2′-H), 1.60 (*m*, 6H, 3′–5′H), 1.44 (*s*, 9H-C$(CH_3)_3$) (Found: C, 69.42, H, 6.26, N, 5.38 requires: C,69.32, H,5.98, N,5.04).

3.4. Preparation of Iodoacetyl aminohexyl-Benzoxanthene-3,4-dicarboximide (4a)

Compound **2a** (300 mg, 0.67 mmol) was stirred under nitrogen in CH_2Cl_2 (5 ml) with trifluoroacetic acid (5 ml) at room temperature for 45 min. The solvent was removed using a rotary evaporator and the residue was dissolved in CH_2Cl_2 and removed again. This was repeated several times, then vacuum dried for 2 h until a violet–red solid was collected as intermediate **3a**.

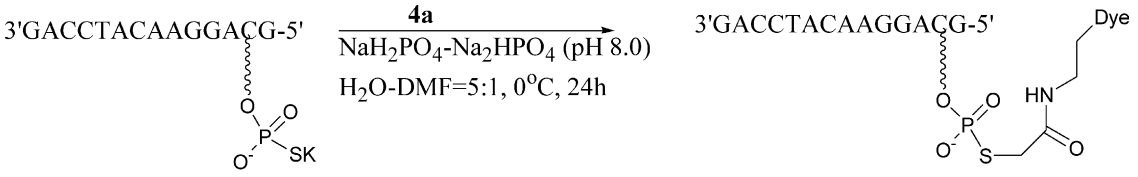
The intermediate **3a** (100 mg, 0.217 mmol), iodoacetic acid (44.5 mg, 0.239 mmol) and the condensation agent HBPyU (117 mg, 0.27 mmol) were put in a 25 ml flask wrapped with tinfoil. Dried CH_2Cl_2 (5 ml) was added under nitrogen and triethylamine (0.11 ml, 0.76 mmol) was slowly added under the ice–salt bath. After a few minutes, the bath was removed and the liquor stirred for 3 h. The solvent was removed by using a rotary evaporator and the residue was dissolved in CH_3Cl and purified by preparative thin layer chromatography on silica gel using petroleum–acetic ether (3/1, v/v) first and then dichloromethane–acetic ether (5/1, v/v) as eluent. The zone with $R_f=0.6$ was collected as product and gave a red solid. mp. >300 °C .υ_{max} (KBr/cm^{-1}): 3350, 2960, 2930, 2850, 1690, 1645, 1585, 760, 755; EI-MS (*m*/*z*,%): 388 ($[M+H-I]^+$, 4.93), 373 ($[M-CH_2I]^+$, 3.04), 327 ($[M+2-NHCOCH_2I]^+$, 82.21), 303 ($[M+H-CH_2CH_2NHCOCH_2I]^+$, 71.08); ^{1}H NMR($CDCl_3$): δ 8.60 (*d*, $J=8.0$ Hz, 1H, 2-H), 8.40 (*d*, $J=8.0$ Hz, 1H, 6-H), 8.20 (*m*, 2H, 1-H&7-H), 4.95 (*s*, 2H,-CH_2I), 4.52 (*t*, $J=5.2$ Hz, 2H, 1′-H), 3.83 (dd, $J=5.1$ Hz, 2H, 2′-H).

3.5. Intercalation of dyes to DNA

Solution (0.1 ml) of a compound in DMSO (10^{-3}–10^{-4} M) mixed with 0.1 M Tris–HCl buffer (pH 7.4) to 10 ml. Then, two groups of samples were prepared at a concentration of chemical at 10^{-5}–10^{-6} M, one contained calf thymus DNA (50 μM), the other contained no DNA but had the same concentration of chemical as control. The above solutions were shaken for 1 h at 25 °C. Fluorescence wavelength and intensity area of samples were measured under the following conditions: excitation: 450–460 nm, emission: 470–650 nm.

3.6. Fluorescent labeling of oligodeoxynucleotide thiophosphate diester with dye

A coupling reaction was carried out. A mixture (250 μl) of thiophosphate oligonucleotide salt (5OD), **4a** (0.5 mg), DMF (70 μl), and phosphorate sodium buffer solution (pH 8.0) (180 μl) was incubated at 50 °C for 24 h to label the oligodeoxynucleotide thiophosphate diester. The reactant was purified on a Sephadex G-50 column with



Scheme 3. The labeling of **4a** to oligodeoxynucleitide.

0.1 M TEAA as eluent. The purified oligo–label conjugate, formamide and indicator were mixed and sampled onto urea denatured PAGE (20%) under 25 V/cm for 1.5 h until the indicator moved out of the gel. The gel was carefully taken out and photographed under ultra-violet light at 365 nm (Scheme 3).

3.7. Hybridization

The target DNA sequences in a buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 25 °C) were denatured at 90 °C, cooled quickly and then spotted on fixed hybridization nylon film by micropipettes. The film was warmed at 80 °C, coiled in a hybridization tube and 1 ml prehybridization solution added. The tube was put in a water bath and prehybridized at 44 °C for 1 h. After that, the prehybridization solution was taken out. The fluorescently labeled oligodeoxynucleotide was dissolved in sterilized ddH_2O, denatured at 90 °C and cooled quickly which was put into, along with the hybridization solution, hybridization tube and hybridized at 44 °C for 4 h. Then, the hybridization solution was taken out, the film was first washed with 2×SSC and 0.1% SDS solution in hybridization tube at 44 °C twice, for 5 and 15 min, respectively. Finally it was washed twice with 0.1×SSC and 0.1% SDS solution in a petri dish at room temperature for 5 and 15 min, respectively. The film was observed under ultraviolet light at 365nm and could be photographed to observe the results.

4. Conclusions

A new fluorescent dye, iodoacetyl aminohexylbenzoxanthene-3,4-dicarboximide, for DNA hybridization was prepared through imidation, deprotection and iodoacetylation with benzothioxanthene as starting material. These benzothioanthene derivatives have DNA intercalation action and showed large Stokes' shifts in Tris–HCl–H_2O buffer. After condensation with the sulphydryl functions of the phosphate, the fluorescence intensity of the dye labeled on oligodeoxynucleotide was found to be sensitive to nucleobase mismatchs in the DNA sequence during hybridization.

Acknowledgements

The Ministry of Education of China, the National Natural Science Foundation of China and Science and Technology Commission of Shanghai supported this work.

References

[1] Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS. Bioconjugate Chemistry 1993;4:105.
[2] Hauland RP. Handbook of fluorescent probes and research chemicals6th ed. Eugene, USA: Molecular Probes, Inc; 1996.
[3] Middleton RW. J Heterocyclic Chem 1985;22:1567.
[4] Middleton RW. J Heterocyclic Chem 1986;23:849.
[5] Grayshan PH, Kadhim AM, Peters AT. J Heterocyclic Chem 1974;11:33.
[6] De Costa MDP, de Silva AP, Pathirana ST. Can J Chem 1987;65:1416.
[7] Yao W, Qian X. Dyes and Pigments 2001;48:43.
[8] Yamana K, Gokota T, Ozaki H, Nakano H, Sangen O, Shimidzu T. Nucleosides & Nucleotides 1992;11:383.
[9] Yamana K, Ohashi Y, Nunota K, Nakano H. Tetrahedron 1997;53:4265.
[10] Yamana K, Mitsui T, Hayashi H, Nakano H. Tetrahedron Lett 1997;38:5815.
[11] Thuong NT, Chassignol M. Tetrahedron Lett 1988; 29:5905.
[12] Tanaka T, Yamada Y, Ikehara M. Chem Pharm Bull 1988;36:1386.

[13] Tang S, Agrawal S. Nucleic Acids Res 1990;18:6461.
[14] Asseline U, Thuong NT. Nucleosides & Nucleotides 1992; 10:359.
[15] Smith LM, Fung S, Hunkapillar MW, Hunkapillar TJ, Hood LE. Nucleic Acids Res 1985;13:2399.
[16] Telser J, Cruickshank KA, Morrison LE, Netzel TL, Chan C-K. J Am Chem Soc 1989;111:7226.
[17] Sarfati SR, Pochet S, Guerreiro C, Namane A, Huynh-Dinh T, Igolen J. Tetrahetron 1987;43:3491.
[18] Roget A, Bazin H, Teoule R. Nucleic Acids Res 1989; 17:7643.
[19] Sarfati SR, Namane A. Tetrahedron Lett 1990;31:2581.
[20] MacKellar C, Graham D, Will DW, Burgess S, Brown T. Nucleic Acids Res 1992;20:3411.
[21] Agrawal S. Methods in molecular biology, vol. 26. In: Agrawal S, editor. Protocols for oligonucleotide conjugates. NJ: Humana Press; 1995.
[22] Fidanza JA, Ozaki H, McLaughlin LW. J Am Chem Soc 1992;114:5509.