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Tethered thiazole orange intercalating dye for development of fibre-optic nucleic acid biosensors

Xiaofeng Wang, Ulrich J. Krull*

Chemical Sensors Group, Department of Chemistry, University of Toronto, Erindale Campus, Room 4060, 3359 Mississauga Road North, Mississauga, Ont., Canada L5L 1C6

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Abstract

Single-stranded DNA (ssDNA) oligonucleotide in solution, or that is immobilized onto a surface to create a biosensor, can be used as a selective probe to bind to a complementary single-stranded sequence. Fluorescence enhancement of thiazole orange (TO) occurs when the dye intercalates into double-stranded DNA (dsDNA). TO dye has been covalently attached to probe oligonucleotides (homopolymer and mixed base 10mer and 20mer) through the 5′ terminal phosphate group using polyethylene glycol linker. The tethered TO dye was able to intercalate when dsDNA formed in solution, and also at fused silica surfaces using immobilized ssDNA. The results indicated the potential for development of a self-contained biosensor where the fluorescent label was available as part of the immobilized oligonucleotide probe chemistry. The approach was shown to be able to operate in a reversible manner for multiple cycles of detection of targeted DNA sequences.

Keywords: Fluorescence; Intercalation; Thiazole orange; DNA; Biosensors; Hybridization

1. Introduction

DNA detection technologies can generally be classified as belonging in two categories; biosensors that are selective for one or a few nucleic acid sequence targets, or arrays that may be used for parallel DNA hybridization analysis, directly yielding sequence information from genomic DNA fragments. Biosensor and array devices are predicated on the use of immobilized single-stranded DNA (ssDNA) or nucleic acid analogues as probe molecules. Many methods of detection of hybridization have been proposed. The most popular approaches include techniques rooted in electrochemical, plasmon resonance, acoustic wave, electrostatic, interferometric and luminescent processes [1–9]. A very sensitive method is that based on fluorescence [2]. Fluorescent dyes that can selectively associate with double-stranded DNA (dsDNA) to undergo an enhancement of quantum yield offer a method for detection of hybridization without use of labeled DNA, and provide for an improved level of selectivity that is based on the structure of the hybrid rather than just the presence of DNA. This report focuses on the conjugation of an intercalating dye to the ssDNA probe to create a biosensor that does not require external addition of fluorescent indicators.

The concept of conjugation of markers to nucleic acid oligomers for the purpose of detection of hybridization is not new, and recent examples can be found in methods using tethered fluorescent probes on

* Corresponding author. Tel.: +1-905-828-5437; fax: +1-905-828-5425.
E-mail address: ukrull@utm.utoronto.ca (U.J. Krull).

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DNA [4], methods using tethered fluorescent probes on peptide nucleic acids [5], and tethered electrochemical probes such as ferrocene as the marker [6]. A wide variety of markers, including Cy3 and Cy5 as commonly encountered in micro-array devices, have been attached to nucleic acids. The attachment of a label to a target molecule is not of interest in the work presented herein. Rather the goal is to prepare the immobilized nucleic acid probe molecule so that it is associated with a fluorescent indicator that is sensitive to the structure of dsDNA.

Selectivity of detection is in part based on immobilization of short ssDNA of carefully chosen nucleotide sequence on the surface of a fiber. Further selectivity is achieved by use of fluorescent intercalating agents, which can only bind to dsDNA. Intercalation of a dye into the double helix causes a substantial increase in fluorescence intensity, and the increase in fluorescence is used to detect the presence of complementary DNA (cDNA) in various sample solutions. The addition of dye to stain dsDNA for detection is cumbersome experimentally because this necessitates a number of steps in methodology. A mutagenic dye must be added to the sample solution, followed by mixing and incubation to allow for kinetics of intercalation. Waste disposal is also a concern. A better approach would be to anchor the dye near the ssDNA so that it is readily available for intercalation should hybridization occur. Furthermore, denaturation of the hybridized DNA would free the dye and regenerate the ssDNA, so that the sensor chemistry could be fully reversible for analyses of a number of samples. Dye can be attached to ssDNA by a short tether [4,5]. The dye cannot intercalate into ssDNA, and the emission of the dye is relatively low. Once hybridization occurs, the dye intercalates rapidly because this process no longer requires diffusion due to the relative proximity of the dye to the site of hybridization. The increase of fluorescence from the dye can be used quantitatively for determination of the concentration of cDNA.

Demonstration of the use of tethered thiazole orange (TO) dye for applications involving peptide nucleic acid probe molecules in bulk solution has been previously reported [5]. As a preliminary step in the development of a biosensor, we had earlier examined this strategy for development of sensor chemistry by linking ethidium bromide with an alkyl chain to dT_{20} that had been immobilized to a fused silica optical fiber [4]. A fiber optic spectrofluorimeter with an optical layout based on an epifluorescence microscope provided an excellent combination of sensitivity and ease of use. The fiber acted as a waveguide, and fluorophore that associated with any hybrids along the surface of the fiber reported the degree of hybridization. Some fluorescence was recaptured by the fiber, and the light that was carried back through the fiber was separated from excitation radiation by a dichroic mirror, and was then detected by a photomultiplier tube [4,7]. This early work confirmed that the analytical advantages of improvement of speed of detection could be achieved [4], but the work did not consider the limitations of use of an alkyl tether, the length of the tether, or the sensitivity of the fluorescent dye in terms of intensity change upon intercalation.

The work has now been extended to consider another important intercalating dye, and to consider some of the issues that are associated with tether chemistry and length. A significant body of the current understanding of DNA intercalating agents has come from work using TO [5,8]. An important property of TO is the observation of an increased (up to 100-fold) fluorescence intensity upon intercalation to dsDNA, which is at least a 10-fold improvement in comparison to use of ethidium bromide. The change of fluorescing behavior of TO is due to the restriction of rotation around the methine bond between the two heterocyclic systems in the molecule. An oligonucleotide with tethered TO has been used to investigate the potential for development of a quantitative and reversible DNA biosensor. The dye was coupled to ssDNA by means of a tether based on polyethylene glycol.

2. Experimental

2.1. Reagents

Reagent grade solvents were from Sigma–Aldrich (Mississauga, Ont.) and were further purified or dried by standard laboratory protocols. Reagents for DNA synthesis were from Dalton Chemical Laboratories Inc. (Toronto, Ont.). Anhydrous acetonitrile (VWR, Toronto, Ont.) was pre-dried by distillation from P_2O_5 and redistilled from CaH_2 under dry argon. Tethahydrofuran (BDH, Toronto, Ont.) was pre-dried

over CaH_2, filtered and distilled immediately prior to use from sodium metal (Aldrich)/benzophenone (Aldrich). Water was double-distilled in glass, treated with diethyl pyrocarbonate (Aldrich) and autoclaved. Molecular biology grade polyacrylamide gel electrophoresis (PAGE) reagents and apparatus were obtained through Bio-Rad (Hercules, CA). Silica gel (Toronto Research Chemicals, Toronto, Ont.) had a particle size of 30–70 μm.

2.2. Apparatus

DNA synthesis on controlled pore glass (CPG) beads and silica fiber substrates was done using a PE-ABI 392-EP DNA synthesizer (Perkin-Elmer Applied Biosystems, Foster City, CA) and relied on the well-established β-cyanoethylphosphoramidite method as described previously [7]. Plasma cleaning was done using a Harrick PDC-32G plasma cleaner (Harrick Scientific Corporation, Ossining, NY). All oligonucleotides were purified by PAGE. The molecular weights of tethered oligonucleotides were determined using a Voyager-DE™ STR Biospectrometry™ Workstation (PerSeptive Biosystem, Foster City, CA).

^{1}H-NMR spectra were recorded on a Varian Mercury 300 NMR spectrometer. Spectra were recorded at room temperature unless specified. The mass spectra were determined on a VG-Analytical 70-SE spectrometer. Fluorescence emission experiments in solution were done using a Perkin-Elmer LS-50B Luminescence Spectrophotometer (λ_{ex} = 480 nm, at 20 °C). Fluorescence melting experiments were done using a PTI Fluorescence System (Version 1.41, Photon Technology International, Lawrenceville, NJ). Fluorescence from optical fibers was collected using a custom-built spectrofluorimeter which has been described elsewhere [10]. Studies of nucleic acid hybridization in solution were done by measuring absorbance at 260 nm with a Hewlett-Packard 8452A spectrophotometer interfaced to a PC by means of an HPIB interface (HP82335, Hewlett-Packard).

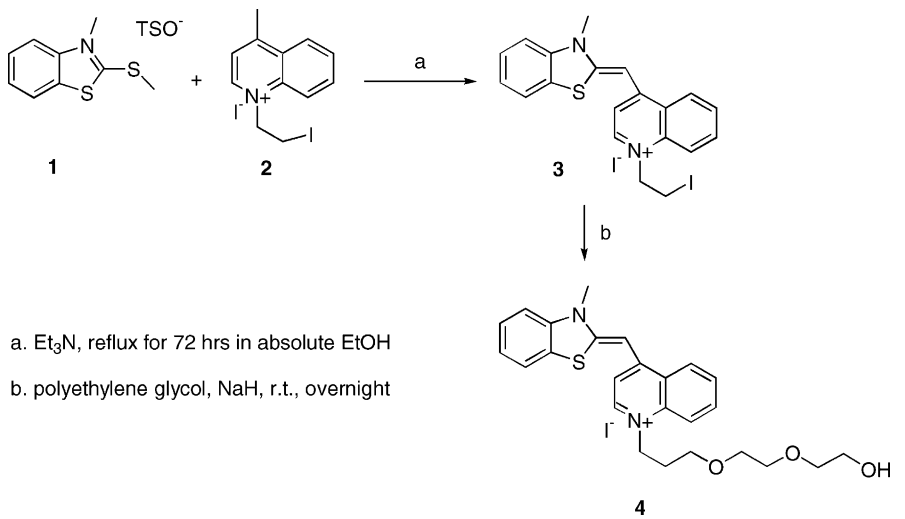
2.3. Procedures

2.3.1. Dye synthesis

Preparation of TO dyes is shown in Scheme 1. The intermediate cyanine dye **3** was synthesized by condensation of benzothiazole derivative **1** and the quinolinium compound **2** in the presence of Et_3N in absolute ethanol [11,12]. This was subsequently reacted with 1 equivalent of polyethylene glycol linker in the presence of NaH to give compound **4**.

2.3.2. Preparation of fused-silica optical fibers

The jacket material surrounding the fused silica fibers (400 μm core diameter, 3M PowerCore™ Series Optical Fiber, FT-400-URT, supplied by Thor



Scheme 1. (a) Et_3N, reflux for 72 h in absolute EtOH; (b) polyethylene glycol, NaH, room temperature, overnight.

Labs Inc., Newton, NJ, USA) was mechanically removed by use of an appropriately sized Micro-Strip® fiber-optic stripping tool (Thor Labs Inc.). Optical fiber pieces 48 mm in length were then made by use of a wedge-shaped diamond scribe tool (Thor Labs Inc., Newton, NJ), which provided optical quality surfaces at both termini of each fiber segment under visual inspection at 40× microscope.

The fused silica fiber segments and CPG were cleaned before surface modification by the following method of Kern and Poutinen [13]. The fiber substrates were added to a 1:1:5 (v/v/v) solution of 30% ammonia /30% H_2O_2/de-ionized water and gently agitated at 80 °C for 5 min. The substrates were then recovered, washed with water and then added to a 1:1:5 (v/v/v) conc. HCl/30% H_2O_2/de-ionized water for 5 min at 80 °C for 5 min with gentle agitation. The substrates were then washed with water (3× 30 ml), methanol (3× 30 ml), chloroform (3× 30 ml), and diethyl ether (3× 30 ml), respectively, dried over P_2O_5 under vacuum until required.

2.3.3. Derivatization of fiber optic surface

Synthesis of 17-dimethoxytrityloxa-3,6,9,12,15-pentaoxa-1-heptadecanol (DMT-HEG).

To 5.6 ml (21 mmol) of hexaethylene glycol in 5 ml absolute pyridine was added 7.1 g (21 mmol) of dimethoxytrityl chloride in 10 ml absolute pyridine under Ar. The mixture was stirred overnight. At this point TLC analysis (hexanes:ethyl acetate 1:1) indicated the complete absence of dimethoxytrityl chloride. The mixture was diluted by 600 ml of CH_2Cl_2, washed by 5% $NaHCO_3$ (2× 100 ml), water (2× 100 ml) and brine (150 ml). The solvent was removed and the residue was purified by flash chromatography to yield 2.94 g (5.04 mmol) DMT-HEG (24% yield).

^{1}H-NMR (200 MHz, $CDCl_3$) δ = 3.22 (t, 2H), 3.51–3.74 (m, 22H), 3.78 (s, 6H), 6.81 (d, 4H), 7.19–7.47(m, 9H).

2.3.4. Functionalization of substrates with 3-glycidoxypropyltrimethoxysilane (GOPS)

The cleaned fused silica substrates were suspended in a solution of xylene/3-glycidoxypropyltrimethoxysilane (GOPS)/diisopropyl ethylamine (100:30:1 (v/v/v)) The mixture was stirred at 80 °C for 24 h under Ar. The substrates were then washed with methanol (2× 50 ml), CH_3Cl (2× 50 ml), Et_2O (2× 50 ml) successively, dried and stored under vacuum and P_2O_5 at room temperature until required.

2.3.5. Linkage of DMT-HEG onto GOPs functionalized substrates

To 500 mg (20 mmol) of NaH suspended in 10 ml anhydrous pryidine was added 1 g (1.7 mmol) of DMT-HEG dissolved in 10 ml. The mixture was stirred at room temperature for 2 h under Ar. The reaction mixture was filtered through a sintered glass frit under a positive pressure of Ar into a round bottom flask containing the GOPS functionalized substrates. The mixture was gently agitated on an oscillating platform for 8 h under Ar. Then the substrates were washed with methanol (3× 20 ml), water (3× 20 ml), methanol (3× 20 ml), CH_2Cl_2 (3× 20 ml), Et_2O (3× 20 ml), respectively. The DMT-protected HEG functionalized substrates were dried and stored under vacuum and over P_2O_5.

2.3.6. Capping of unreacted silanol and hydroxyl functionalities with chlorotrimethylsilane (TMS-Cl)

The dried substrates were suspended in a solution of TMS/pyridine (1:10 (v/v)) for 16 hat room temperature under Ar. The substrates were subsequently recovered and washed with pyridine (3× 20 ml), methanol (3× 20 ml), and Et_2O (3× 20 ml), respectively. The substrates were stored under vacuum and over P_2O_5 at room temperature.

2.3.7. Solid phase phosphoramidite synthesis of tethered oligonucleotides

For the final coupling step (attaching TO dye to the 5′ end through various polyethylene glycol linkers), the following procedure was followed. After the synthesis of final base, equal volumes of (2-cyanoethoxy) bis(diisopropylamino)-phosphine (1 M, 0.5 mmol for pre-packed CPG column from Dalton and 1.0 mmol for fiber optic column) and tetrazole (0.5 M, 0.25 mmol for pre-packed CPG column from Dalton and 0.5 mmol for fiber optic column) in CH_3CN were added to the support by using pre-programmed synthesis cycles. Then the support was removed from the synthesizer and the mixture of tetrazole (0.5 M in CH_3CN, 0.5 mmol for pre-packed CPG column from Dalton and 1.0 mmol for fiber optic column) and TO dye (**4**, 0.1 M in DMF, 0.25 mmol for pre-packed CPG column from Dalton and 0.5 mmol for fiber

optic column) were added to the support by syringe over a period of 18 h under Ar. After washing the support with CH_3CN, the mixture of Cap A and Cap B (20 ml, 1:1) was added to the column followed by the oxidant (20 ml, 0.1 M I_2 in a solution of 1:10:40 H_2O/pyridine/THF) to finish the oxidation step. The conjugate was then washed with CH_3CN and CH_2Cl_2, deprotected and removed from the support as necessary. PAGE was used for purification of the conjugated oligonucleotides. This final coupling step had an average 50% yield.

TO derivative **4** was synthesized (Scheme 1) and covalently attached to oligonucleotides through the phosphate at the 5′ terminus via diethylene glycol linker. The oligonucleotide sequences that were conjugated to the dye were:

b1 5′-TTT TCT TCT T-3′ (polypyrimidines)
b2 5′-TTT TTT TTT TTT TTT TTT TT-3′ (polyT)
b3 5′-TTT TTT TTT TTT TTT TTT TT-$(OCH_2CH_2O)_6$-fiber

The TO-ssDNA conjugates that were synthesized were:

B1 TO-$OCH_2CH_2OCH_2CH_2O$-5′-TTT TCT TCT T-3′
B2 TO-$OCH_2CH_2OCH_2CH_2O$-5′-TTT TTT TTT TTT TTT TTT TT-3′
B3 TO-$OCH_2CH_2OCH_2CH_2O$-5′-$(T)_{20}$-$(OCH_2CH_2O)_6$-fiber
B4 TO-$OCH_2CH_2OCH_2CH_2O$-5′-TTC TCT CTT TTT TTT-3′-$(OCH_2CH_2O)_6$-fiber

The cDNA included mixed **dG_{20}**: 5′-GGC CCA AGA AGA AAA GCG CG-3′, which was designed to be “partially complementary” to **B1** and **b1**, and **cB4**: 5′-AAA AAA AAG AGA GAA-3′.

2.3.8. Characterization of tethered oligonucleotides in solution and on fiber optic surface UV absorbance studies

A quartz cuvette (1 cm × 1 cm × 4 cm) that was sonicated in methanol, plasma-cleaned and then treated with Sigmacote® (Sigma–Aldrich) was used for all absorbance and fluorescence measurements. The experiments were done using 1× PBS buffer. For the free dye absorbance experiments, 1 equivalent of ssDNA (dT_{20}) or dsDNA (dA_{20}·dT_{20}) was mixed with free TO dye solution and incubated at room temperature for at least 20 min in the dark. For the tethered dye experiments, 1 equivalent of ssDNA (dA_{20}) was mixed with the tethered oligonucleotide buffer solution and incubated at room temperature for at least 20 min in the dark.

2.3.9. Fluorescence studies

Fluorescence measurements were done using 1× PBS buffer, at pH 7.0, with DNA concentrations of approximately 1 μM. All measurements were done using slit widths of Ex/Em 8 nm/8 nm and a scan speed of 500 nm/min. Samples were introduced into a 1.5 ml cell that was thermally isolated with a water jacket. Temperature was controlled with a recirculating water bath. Emission spectra were recorded with λ_{ex} of 480 nm. Absorption at 480 nm never exceeded 0.05 a.u., so that inner filter effects were negligible [14].

2.3.10. Thermal studies

Temperature dependence studies by absorbance at 260 nm were done using 1× PBS (1.0 M NaCl, PO_4^{n-}, pH 7), and a concentration of DNA of 0.62 μM. The temperature was scanned in a range of ca. 5–80 °C using a temperature ramp rate of 0.1–0.2 °C min^{-1}. All analyses were done at least twice.

Thermal studies of fluorescence from DNA in solution were done in 1× PBS buffer at a concentration of 1.5 μM (TO ∼ dT_{20}:dA_{20} 1:1) by monitoring fluorescence at 525 nm (λ_{ex} of 490 nm) over the temperature range of ca. 15–80 °C using a temperature ramp rate of 0.14 °C min^{-1}.

3. Results and discussion

There are a variety of compounds that have been discovered or synthesized that intercalate with DNA. A common structural feature of such intercalating agents is that they have a conjugated aromatic ring and a relatively rigid frame. In addition, the cationic nature of these molecules also appears to be important in DNA binding affinity. TO contains a benzothiazole ring covalently linked to a quinoline ring through a monomethine bridge. Free TO molecules show very low fluorescence in solution, and in the presence of ssDNA in solution. In the presence of dsDNA the fluorescence of TO sharply increases. The nature of this emission enhancement is due to a restriction of

rotation around the methine bond between the two heterocyclic systems in the molecule [15].

A homogeneous hybridization assay in solution can provide generation of an increase of fluorescence intensity upon hybridization when TO dye is covalently attached to oligonucleotides [16,17]. The use of polyethylene oxide as a tether should provide excellent physical flexibility so that the dye can move easily between solution and dsDNA. It is also clear that polyethylene oxides are useful in biosensor applications because they tend to reduce (in contrast to alkyl chains), non-selective binding of peptides, proteins and lipids that could be present as contaminants in real-world samples [18].

An earlier series of fluorescence experiments using dsDNA in solution has confirmed that the 3–4 base pairs at each of the terminal ends of short strands of dsDNA are not as tightly hybridized as those base pairs in the center of the strand [19]. This work established that the minimum length of a tether must allow the dye to extend into the dsDNA to at least the first 3–4 bases to achieve optimal energetics for stabilization of intercalation.

3.1. UV absorption spectroscopy

The absorption of **4** in hybridization buffer (1× PBS) as a function of increasing concentration of dye showed linearity ($r^2 = 0.998$) over the range from 0 to 16 μM. The $\varepsilon^{TO} = 7.1 \times 10^3\ M^{-1}\ cm^{-1}$ at 260 nm [20] was used for calculation of the concentration of conjugated oligonucleotide-TO dye. There was a red shift in the absorption maxima upon interaction of the conjugate with free DNA in solution (Table 1). The shift was largest for the conjugate when it interacted with complementary ssDNA (12 nm). This shift was likely due to solvent relaxation effects. The increase of molar absorptivity indicated a structural change of the TO dye when interaction occurred with oligonucleotides.

Table 1
Steady state absorption determined for 4 when free in solution or complexed to ssDNA or dsDNA

	ε ($M^{-1}\ cm^{-1}$)[a]	λ_{max} (nm)[b]
Free TO	9870	504
Free TO + dT_{20}	14635	504
Free TO + AT_{20}	15774	508
B2	12269	514
B2 + dA_{20}	13650	514

[a] ±3%.
[b] ±1 nm.

3.2. Fluorescence characterization

The fluorescence intensities for TO-ssDNA conjugates in solution and from free TO dye in solution with ssDNA showed very low emission (see Fig. 1A–C). A 20-fold fluorescence enhancement was seen for experiments that used free TO in the presence of fully complementary $dA_{20}{\cdot}dT_{20}$. A much lower six-fold enhancement of fluorescence was observed when the conjugates (**B1** and **B2**) reacted with their complementary sequences (Fig. 1B and C). In both cases, the enhancement of fluorescence was consistent with DNA hybridization and associated binding of dye. It is known that at concentrations in the low micromolar range, there are potentially multiple modes of binding by intercalating dyes [21], not all of which would result in significant enhancement of fluorescence. The observation that there was no substantial change of fluorescence when the probe **B1** was mixed with dA_{20} (1:1 and 1:2, molar ratio), where there were two base pair mismatches, provided further evidence that the TO tethered to the oligonucleotide probe was interacting with dsDNA by intercalation (Fig. 1B).

3.3. Thermal effects

The effect of temperature on the emission intensity of the conjugate in solution was examined. The fluorescence intensity of the conjugate mixed in an equimolar ratio with complementary ssDNA was observed to decrease smoothly over a temperature range of about 25–75 °C (Fig. 2). Ultimately, the fluorescence intensity reduced to a magnitude that was similar to that measured for the single-stranded conjugate. The melt temperature of about 58 °C was similar to that observed for a similar chemical systems in the absence of dye and tether [22], suggesting that the presence of the tether and the dye do not significantly alter the energetics of the hybrid.

A solution absorptivity experiment suggested that the melt temperature of the conjugate in the presence of fully complementary target might be a few degrees

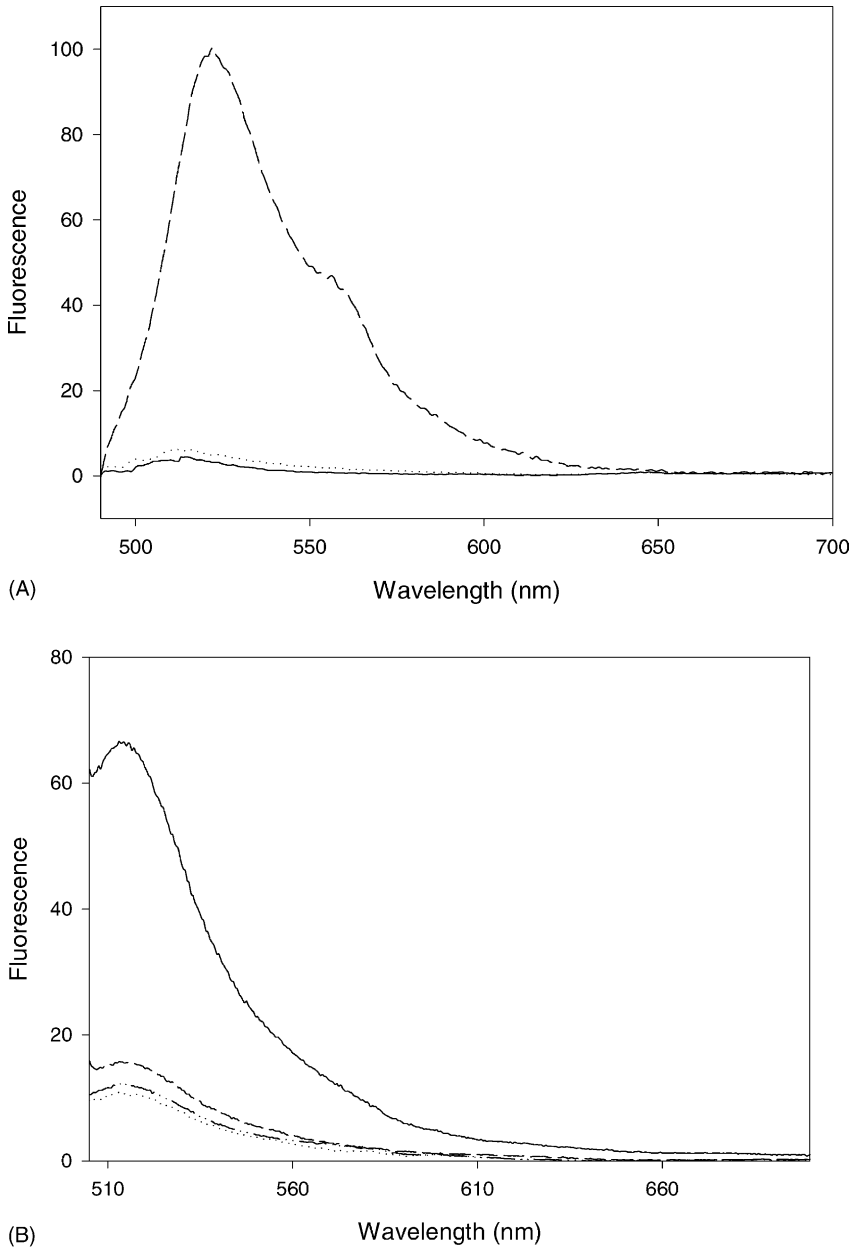


Fig. 1. (A) Fluorescence of free TO dye in the presence of various DNA samples (—) fluorescence of free TO dye (1 μM in 1× PBS buffer), (···) fluorescence of free TO dye with dT_{20} (1:1, 1 μM in 1× PBS buffer), (- - -) fluorescence of free TO dye with dsDNA dA_{20}·dT_{20} (1:1, 1 μM in 1× PBS buffer); (B) Fluorescence of oligonucleotide with tethered TO dye (**B1**) in the presence of various forms of DNA fluorescence of **B1** with mixed dG_{20} (1:1, 0.62 μM in 1× PBS buffer) (···) fluorescence of **B1** with dA_{20} (1:1, 0.62 μM in 1× PBS buffer); (- - -) fluorescence of **B1** with dA_{20} (1:2, 0.62 μM in 1× PBS buffer); (- · - ·) fluorescence of **B1** (0.62 μM in 1× PBS buffer); (C) Fluorescence of oligonucleotide with tethered TO dye (**B2**) in the presence of various forms of DNA fluorescence of **B2** with dA_{20} (1:1, 0.62 μM in 1× PBS buffer), (···) fluorescence of **B2** with dT_{20} (1:1, 0.62 μM in 1× PBS buffer); (- - -) fluorescence of **B2** (0.62 μM in 1× PBS buffer).

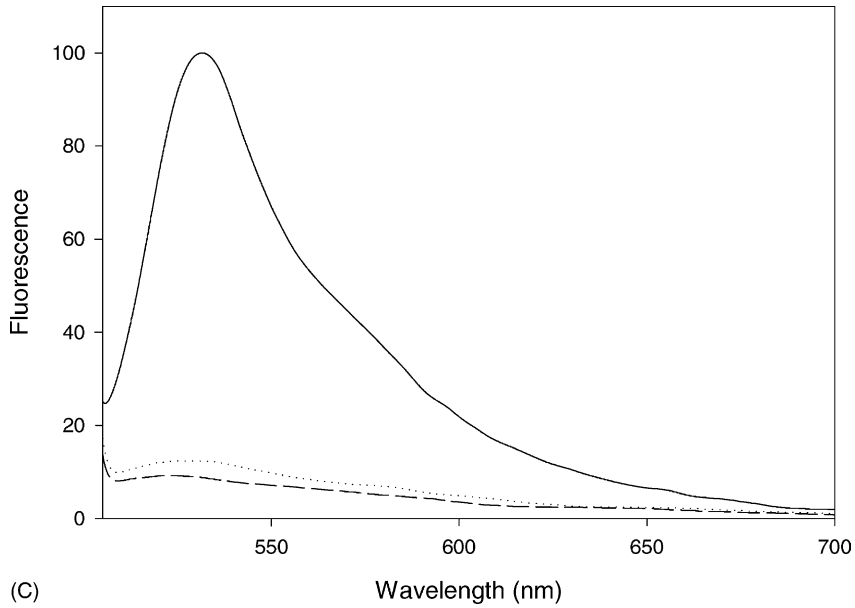


Fig. 1. (*Continued*).

less than the identical chemical system in the absence of the dye and tether. The thermal stability of the conjugated dye system was similar to that without the conjugate. Importantly, the absorption melt profiles showed deviations in the smoothness of the melt curve which could not be attributed to a melt of a duplex, and suggested that there may be aggregates or higher order associations of nucleic acid strands [23] (Fig. 3). The absorption experiment provided results that were complementary to those based on fluorescence. The

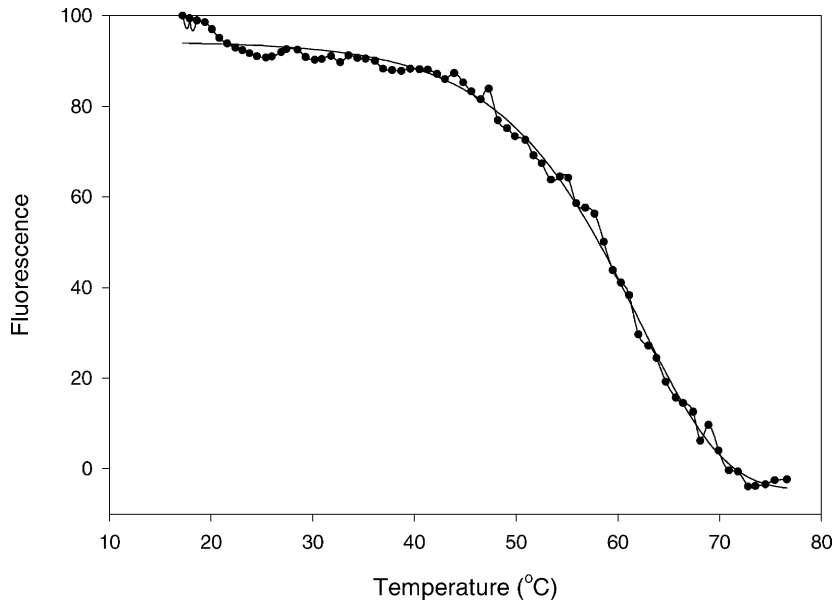


Fig. 2. Fluorescence melt curve of **B2** with dA_{20} (530 nm) (1:1, 1.5 μM in 1× PBS buffer).

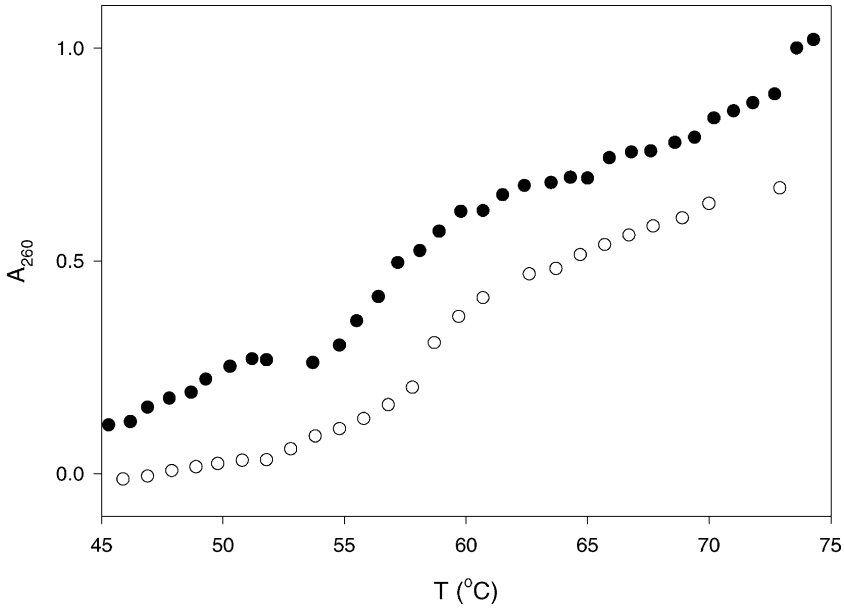


Fig. 3. Melting curve of duplex (●) **B2** with dA_{20} (1:1, 0.62 μM in 1× PBS); (○) dA_{20}·dT_{20} (1:1, 0.62 μM in 1× PBS).

absorption experiments directly probed the structure of associated DNA whereas the fluorescence experiments relied on an indicator dye to bind to DNA and indirectly report DNA structure. To extract more information from fluorescence experiments, the concentration of the probe-to-target DNA ratios were varied by titration.

3.4. Titrations for investigation of binding stoichiometry

The fluorescence intensity changes seen at one concentration of free TO with increasing concentrations of dA_{20}·dT_{20} followed a smooth and relatively linear relationship to about 0.8 mole fraction of added dsDNA.

Fluorescence titrations were completed to examine the effect of altering the ratios of TO-ssDNA conjugate in relation to complementary target in solution (Fig. 4A and B). The results for the titrations using complementary homopolymers of oligonucleotides show that multiple modes of binding interactions were likely present. The presence of A–T sequences would not normally have resulted in parallel-stranded triplexes [24], but the presence of tethered dye may have altered the stability of the oligonucleotide interactions and promoted formation of complexes of higher order. It is also possible that minor groove ligands such as TO might bind to a triplex structure [25]. It would appear that both duplex and also higher order complexes could be formed during the titration. The early portions of the titrations suggested that the presence of complementary sequences resulted in fluorescence increases. A control experiment indicated that no significant change in fluorescence intensity was observed after correction for dilution when starting with **B2** and then adding the non-complementary sequence dT_{20}. The appearance of a fluorescence intensity maximum as dA_{20} was added to **B2** conjugate (Fig. 4A) suggested that a higher order complex could be forming. The trends of the other titration curve (Fig. 4B) confirmed that the fluorescence intensity changes were based on competitive events. For example, it is known that there are differences in partitioning of an intercalator into a duplex structure and a triplex structure, and that there is more exclusion from the triplex structure [26]. Unambiguous interpretation of the data of Fig. 4 becomes complicated in terms of assignment of stoichiometry as both duplex and higher order structures could be present simultaneously.

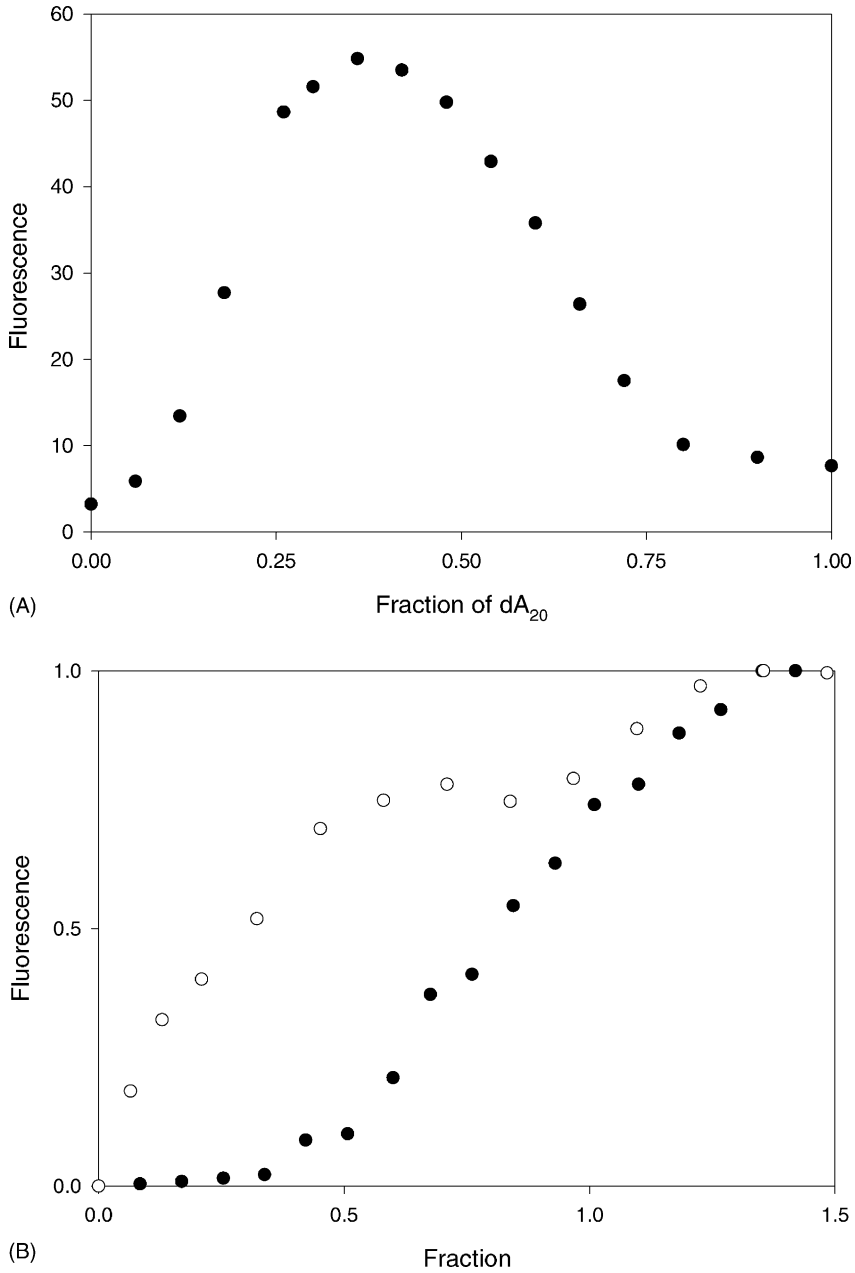


Fig. 4. (A) Fluorescence titration of **B2** by dA_{20} (529 nm) [**B2**]=0.62 μM in 1× PBS buffer. (B) Fluorescence titration (possible formation of multiplex) (529 nm). Symbols: (●) titration of $dA_{20}{\cdot}dT_{20}$ by **B2** [$dA_{20}{\cdot}dT_{20}$] = 0.62 μM in 1× PBS; (○) titration of **B2** by $dA_{20}{\cdot}dT_{20}$ (1:1, 0.62 μM in 1× PBS).

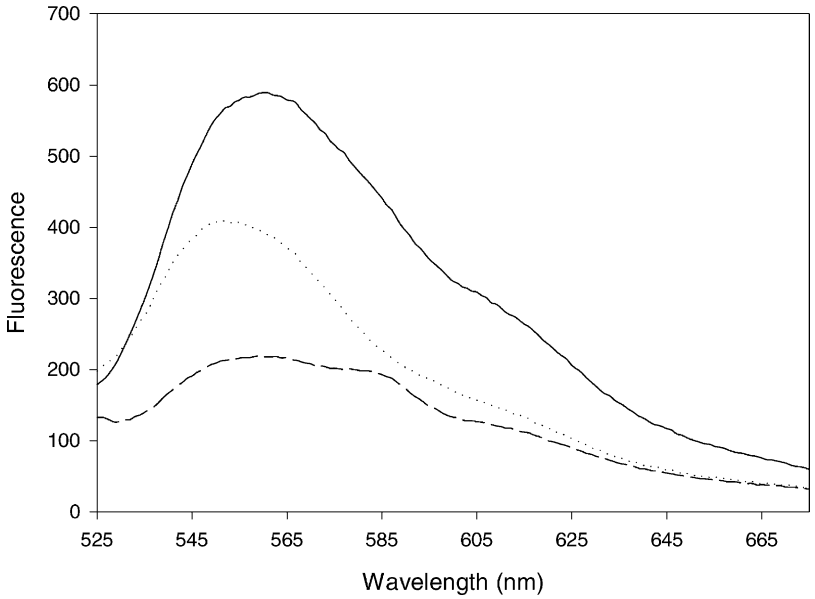


Fig. 5. Fluorescence of immobilized tethered dye on fiber optic surface (**B3**) (—) fluorescence emission of **B3** in the presence of dA_{20} (1 μM in 1× PBS buffer), (- - -) fluorescence emission of **B3** in 1× PBS buffer, (· · ·) fluorescence emission of **B3** in the presence of its fully non-complementary sequence dT_{20} (1 μM in 1× PBS buffer).

Furthermore, there were different quantities of TO in solution (therefore different relative fluorescent intensities) depending on whether dA_{20} or **B2** was the initial sample to be titrated. Compounding difficulties even more was the fact that TO dyes were able to aggregate to polyanions [27], and it is possible that some higher order aggregates may have been present.

The results that are presented in Fig. 4 serve as a caution in terms of selection and design of probe sequences. The problem of formation of mixed multiplicities of DNA associations encountered in using dA_{20} and **B2** as a model system was not observed for the mixed nucleobase system **B1**. The interaction of **B1** with its complementary target showed a titration curve that was similar to the one for the interaction of free TO with dA_{20}·dT_{20}.

3.5. TO-ssDNA conjugate immobilized onto optical fibers

The fluorescence response from fibers that were coated with **B3** (TO at the 5′ end, exposed to buffer) was examined. The response time to reach maximum signal after single stranded target was added was in the range of 10–60 s. Fig. 5 shows wavelength results that are typical of the spectra that were observed. There was a large red shift of emission wavelength (λ_{max} 530–561 nm) when dA_{20} was added, suggesting substantial solvent relaxation at the interface of the fiber. Concurrently, the fluorescence enhancement showed a six-fold increase above the background level in the absence of any target. This result was similar to that shown in Fig. 1C for experiments that were done in solution. A more suitable experimental control is based on the fluorescence change caused by addition of non-complementary ssDNA, as shown in Fig. 5. The results indicated that adsorption of the non-cDNA had a significant effect on the environment of the tethered TO. This appeared to be largely an electrostatic phenomenon where the positively charged dye interacted with the relatively concentrated DNA at the solid interface. An example of the limitation that this can pose is shown in Fig. 6, showing response for complementary and non-complementary target. The non-selective adsorption was largely eliminated by moving to high salt (3× PBS), with the concurrent advantage being that the dsDNA stability was improved. The background intensity effect produced by

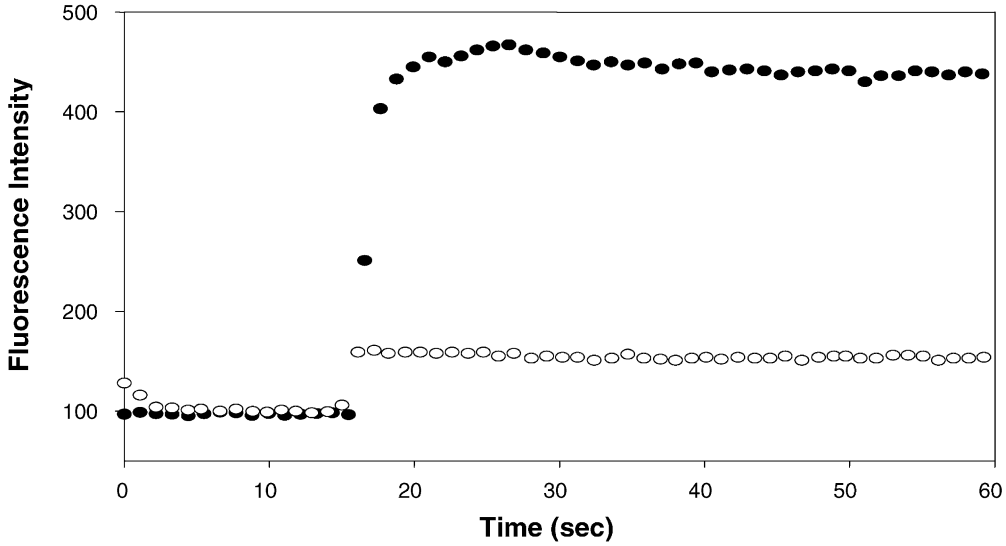


Fig. 6. Time dependent response of a fiber optic sensor. Symbols: (●) fluorescence of **B4** in the presence of **cB4** at 555 nm in 1× PBS; (○) fluorescence of **B4** in the presence of fully non-complementary 5′-TTC TCT CTT TTT TTT-3′ at 555 nm in 1× PBS.

non-cDNA could be reduced to less than 10% of that shown in Fig. 5. The relationship of salt concentration to stringency was also dependent on the density of immobilized DNA [22,28], and experiments are now underway to optimize the density so that appropriate salt concentration can be selected to achieve hybrid stability and minimize adsorption of the tethered dye.

The optical fibers could be re-used numerous times (dependent upon experimental conditions), and provided calibration curves for varying concentrations of

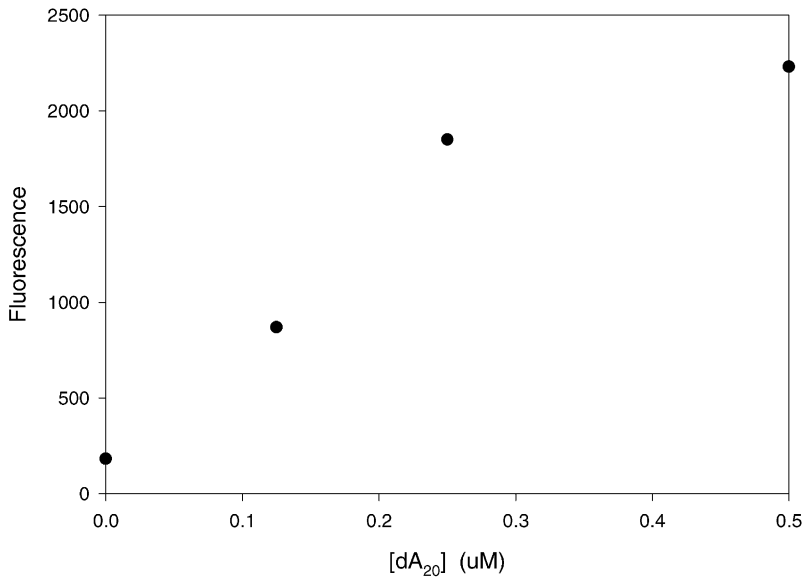


Fig. 7. Calibration curve of **B3** in the presence of dA_{20} with various concentration at 560 nm, 1× PBS buffer (560 nm).

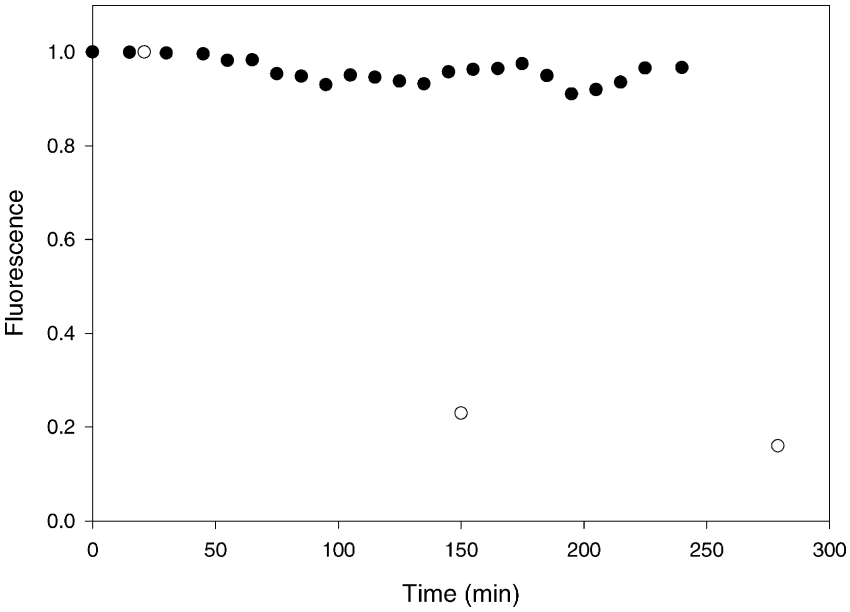


Fig. 8. Photobleaching experiment (●) fluorescence of **B2** in the presence of dA_{20} at 529 nm (1:1, 0.62 μM in 1× PBS); (○) fluorescence of **B3** in the presence of dA_{20} at 560 nm ($[dA_{20}] = 0.62$ μM in 1× PBS).

cDNA as shown in Fig. 7. However, the immobilized conjugate demonstrated another undesirable property, and that was sensitivity to photobleaching. Fig. 8 indicates that while photobleaching caused signal reduction by 15% for TO-ssDNA conjugate in solution over a period of 4 h. Once the conjugate was immobilized and irradiated in the TIRF configuration, reduction of signal reached 80% in 4 h. This problem was effectively ameliorated by using gated excitation and detection in the fiber optic spectrofluorimeter. The occasional interrogation of the fiber sensor for a period of a second with polling in a cycle of tens of seconds or longer was used to reduce photobleaching to a negligible effect over a period of 8 h.

4. Conclusions

This preliminary report indicates that the association of an intercalating dye to ssDNA by means of a tether molecule that permits the dye to reach into an area of stability in a hybrid, provides a method for labelling that might be suitable for development on a self-contained biosensor. The labelled ssDNA can function for detection of hybridization in solution, and also when immobilized at a solid interface. Issues that could potentially limit practical applications will include non-selective adsorption and photobleaching effects. Further work must now consider the influence of the presence of a tether on the availability of ssDNA and the stability of dsDNA. The work must also consider the density of immobilized ssDNA, because proximity could provide potential for molecular ‘cross-talk’ and fluorescence quenching if long tethers are used. Exploration of the analytical advantages offered by the use of a tether to localize the dye will then be explored, and will include study of how the system can provide for reproducible background fluorescence from dye in solution, to permits normalization of background signals in terms of monitoring photobleaching, drift in excitation source intensity, and drift in detector sensitivity.

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