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Synthesis and fluorescence studies of thiazole orange tethered onto oligonucleotide: development of a self-contained DNA biosensor on a fiber optic surface

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Abstract—Thiazole orange dyes were derivatized with ethylene glycol linkers of various lengths, and were covalently linked to the 5′ end of the oligonucleotides after solid-phase synthesis. The labeled oligonucleotides exhibited enhanced fluorescence upon hybridization to complementary DNA sequences at the surfaces of optical fibers, providing for a self-contained labeling strategy. It was determined that the melt temperatures of DNA hybrids using one mixed polypyrimidine base oligonucleotide sequence were dependent on the length of the tethers, and that the melt temperature could be shifted by more than 10 °C when tethers were introduced.

The design and synthesis of single-stranded DNA (ssDNA) probe molecules that are labeled with fluorescent dyes has been a subject of interest in the area of microarray and other genetic detection systems.[1] A topic that has received some attention is associated with the tethering of dyes that show enhanced fluorescence intensity upon hybridization.[2] Single-stranded DNA oligonucleotide derivatives having such properties would provide the basis for development of hybridization assays suitable for the determination of complementary DNA and RNA targets.

To stain double-stranded DNA (dsDNA) using an intercalating dye in bulk solution necessitates use of extra reagents in analytical protocols and extra steps in methodology such as washing to remove the adsorbed dye. A different approach is to anchor the dye near or on the ssDNA so that it is readily available for intercalation upon hybridization, improving the speed of development of the fluorescence signal. The detection chemistry could also be made reversible for analyses of a number of samples as denaturation of the hybridized DNA would free the dye and regenerate the probe.

We have been interested in developing various DNA biosensors using a total internal reflection fluorescence configuration with optical fibers as a platform for immobilization of ssDNA. Fused silica is well suited for optical experiments throughout the UV–visible spectral range, and such fibers possess a relatively flat and homogeneous surface that is amenable to modification.[3] Individual optical fibers may be functionalized with different oligonucleotide probes. In some cases, fibers can bundled together to form an array.[4] Hybridization can be monitored by the appearance of increased fluorescence intensity when using intercalating dyes, and this has been achieved using fibers that are coated with short ssDNA probes that are complementary to the target sequences that are in a sample solution. Such DNA biosensors can be used to detect sequences of DNA made by PCR or those are cut by ultrasound from genomic materials,[5] and provide for detection systems that can be indicative of specific diseases, pathogens, or events that are related to the natural function of DNA such as transcription.[6]

Our previous work has shown that oligonucleotides that are covalently linked to thiazole orange (TO) exhibit significantly enhanced fluorescence intensity upon hybridization to complementary DNA in solution.[7] Thiazole orange contains a benzothiazole ring covalently linked to a quinoline ring through a monomethine bridge.

Keywords: Thiazole orange; Optical fiber; Biosensor; DNA; Hybridization.
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The fluorescence quantum yield of free TO molecules is very low in aqueous solution. In the presence of dsDNA, the fluorescence intensity of TO 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.[8,9] Gel electrophoresis and fluorometric titration studies indicated a linear fluorescence intensity increase with the length of the DNA fragment of naturally occurring DNA, suggesting no base specific interactions between TO and dsDNA.[10] A homogeneous hybridization assay based on the interaction between TO and dsDNA can provide a significant fluorescence signal enhancement.[11]

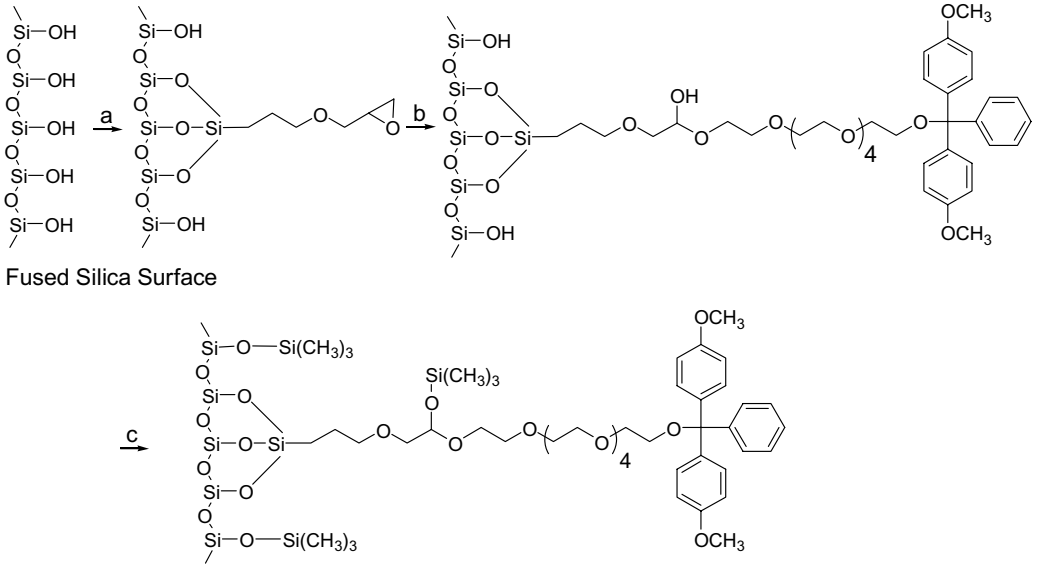
We describe here the solid-phase synthesis of ssDNA on fused silica optical fibers, where thiazole orange derivatives incorporate glycol-based linkers of various lengths at 5′-terminus of the oligonucleotide. Glycol-based tethers have been previously invoked for investigations involving both DNA[12] and RNA complexes.[13] The hydrophilic polyether chain may offer adequate flexibility to facilitate intercalation between a TO derivative and dsDNA. The goal of the present work was to investigate the influence of the tether length to overcome limitations in the process of intercalation with dsDNA caused by steric hindrance and to reduce interference from neighboring immobilized sequences. This synthetic strategy provides a route for the development of DNA biosensors for nucleic acid-based diagnostics.

Hexaethylene glycol (HEG) was selected as a linker to attach ssDNA probe molecules to the fused silica surfaces. It has been reported that HEG linkers can provide a hydrophilic fluid environment that is suitable for hybridization between immobilized ssDNA strands and target strands that are free in solution.[14] Growth of ssDNA from the linker is done by solid-phase synthesis, and the density of immobilized ssDNA can be templated by the quantity of HEG that is immobilized.[15] For example, glycidoxypropyltriethoxysilane (GOPS) can be used to activate fibers, followed by control of the reaction time of HEG (dimethoxytrityl-HEG) to place different densities of linker onto the surface. Covalently linked Si–O bonds are stable in mildly basic deprotection condition, which helps to retain the ssDNA sequence on the surface of optical fibers.

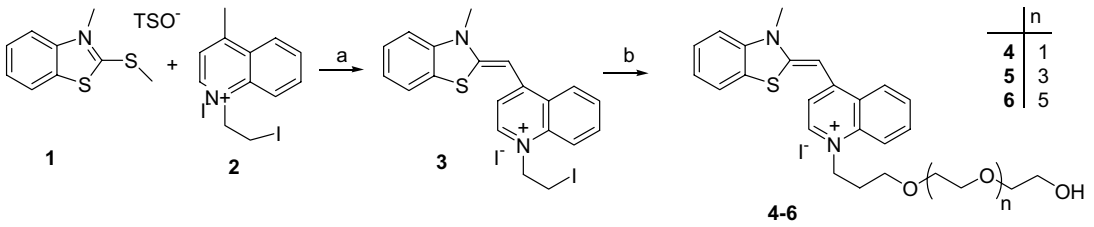
The two-stage method developed by Kern and Puotinen[16] was used for cleaning of fused silica fibers. After washing the substrates with a 1:1:5 (v/v/v) solution of 30% NH_4OH/30% H_2O_2/H_2O and 1:1:5 (v/v/v) solution of concd HCl/30% H_2O_2/H_2O, respectively, the hydroxyl groups on silica surface were activated by reaction with GOPS followed by coupling with 17-dimethoxytrityl-oxa-3,6,9,12,15-pentaoxa-1-heptadecanol linker (DMT-HEG). Any unreacted hydroxyl groups were then capped by chlorotrimethylsilane (TMS-Cl). The DMT-protected hydroxyl terminus was then ready for the solid-phase phosphoramidite synthesis of ssDNA using an automated synthesizer (Scheme 1).

Preparation of thiazole orange derivatives is shown in Scheme 2. Condensation of benzothiazole derivative **1** and the quinolinium compound **2** in the presence of Et_3N in absolute ethanol gave the intermediate cyanine dye **3** in 49% yield.[17] Unreacted starting materials were removed during recrystallization by acetone/ether (1:1). **3** was subsequently reacted with 1 equiv of polyethylene glycol linkers of various chain lengths in the presence of NaH to give compounds **4**, **5**, and **6**, respectively.[18]

Mixed polypyrimidine base ssDNA sequences immobilized onto fused silica substrates were prepared using phosphoramidite protocols and an ABI-392 DNA synthesizer using a modified procedure.[19] Several procedures for attaching fluorescent dyes to the 5′-end of an oligonucleotide during solid-phase synthesis have been reported. They usually use a protected amine, thiol, or



Scheme 1. Immobilization onto fiber optic surface. Reagents and conditions: (a) xylene/DIEA, 3-glycidoxypropyltrimethoxysilane (GOPs), 80 °C; (b) NaH, pyridine, 17-dimethoxytrityloxa-3,6,9,12,15-pentaoxa-1-heptadecanol (DMT-HEG), rt; (c) chlorotrimethylsilane/pyridine, rt.



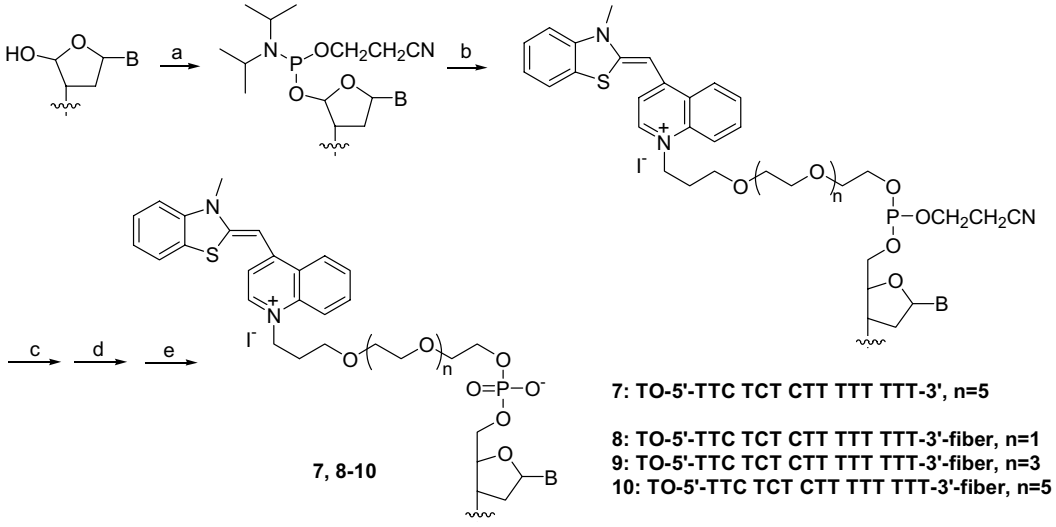
Scheme 2. Synthesis of thiazole orange dye derivatives. Reagents and conditions: (a) Et_3N/EtOH, reflux; (b) NaH, DMF, reflux.

carboxyl at one end of a spacer and a phosphoramidite at the other end. A nucleophilic linker is ready for preparation of a conjugate after appropriate deprotection.[20,21] The synthesis of phosphoramidites that contained cyanine derivative with various linkers initially proved to be problematic because products of the phosphitylation are unstable. A 'reverse' protocol was then developed to synthesize TO tethered oligonucleotides[22,23] (Scheme 3). Phosphitylation was accomplished by adding (2-cyanoethoxy)bis(diisopropylamino)-phosphine with *N*,*N*-diethylamine at the end of automated synthesis. This provided support-bound oligonucleotide with a 5′-terminal hydroxyl group. The final coupling step was done by adding TO derivatives **4**, **5**, or **6** in the presence of tetrazole, followed by capping and oxidation. The deprotection of primary amine groups on nitrogenous bases was done by means of treatment with 0.05 M K_2CO_3 in anhydrous methanol (48 h) in order to minimize cleavage of immobilized conjugates from fiber surfaces. The final coupling step gave an average 40–50% yield of tethered sequence.[24]

After immobilizing oligonucleotides that contained tethered TO labels (TO-ssDNA) onto a fiber optic surface, the fluorescence response from the coated fibers was examined using an intrinsic mode fiber optic spectrofluorimeter (Ar laser λ_{ex} at 488 nm, dichroic mirror cut-off at 505 nm, λ_{em} at 555 nm).[25] Figure 1 shows spectral results that are typical for the TO-ssDNA probe molecules. There was a red shift of the emission maximum (λ_{max} 530–560 nm) and a substantial five to sevenfold increase in fluorescence intensity when the complementary sequence was introduced to the sensor surface. The emission wavelength shift suggested that substantial environmental changes were experienced by the TO at the interface of the fiber under conditions that were conducive to hybridization. Similar fluorescence intensity enhancements were observed when a solution containing complementary sequences was added to free (i.e., not surface immobilized) TO-ssDNA such as **7** at 1:1 molar ratio in aqueous buffer solution.

In our previous work[26] we completed molecular simulations using INSIGHT II, which is based on a self-consistent force field calculation (Accelrys, Inc., San Diego, CA). Energy calculations were done using a system based on 20mer ssDNA that was linked to TO using polyethers. The labeled ssDNA was allowed to hybridize to form duplex with a fully complementary sequence. The linker length was varied from $n = 0$ to 8, and the results indicated that hexaethylene glycol (HEG, $n = 5$) represented the tether length that achieved the most stable energetics in modeling that included intercalation of the dye.

Table 1 lists a series of related TO-ssDNA probes on fiber surface, which were used to experimentally evaluate the influence of the length of the linkers on the stability



Scheme 3. Synthesis of thiazole orange dye tethered sequence on fiber optic surface. Reagents and conditions: (a) (2-cyanoethoxy)bis(diisopropylamino)-phosphine, *N*,*N*-diethylamine, CH_3CN; (b) tetrazole, thiazole orange derivatives **4**, **5**, or **6**; (c) *N*-methyl imidazole, acetic anhydride; (d) I_2, H_2O; (e) deprotection by 0.05 M K_2CO_3 in absolute MeOH or NH_4OH, 48 h.

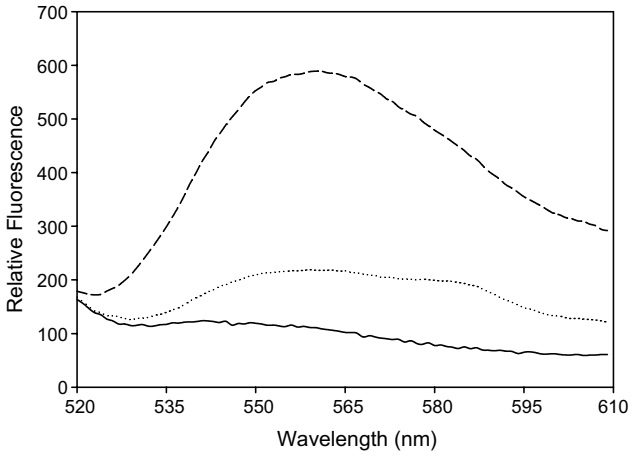


Figure 1. Fluorescence spectra of **8** in the presence of various DNA samples (excitation at 488 nm, 30 °C): (—) fluorescence of free **8** in 1 × PBS; (···) fluorescence of **8** with **5′**-AAA AAA AAC AGA GAA-3′ (single-base mismatch) (1 mL 0.62 μM in 1 × PBS, hybridization time: 5 min, then washed with 2 mL 1 × PBS buffer); (---) fluorescence of **7** with **5′**-AAA AAG AGA GAA **3-′** (complementary sequence) (1 mL 0.62 μM in 1 × PBS, hybridization time: 5 min, then washed with 2 mL 1 × PBS buffer).

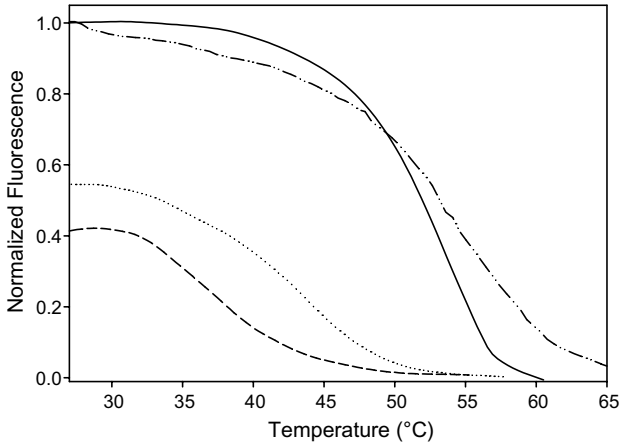


Figure 2. Thermal denaturation profiles for different linker lengths. The influence of length on the stability of the intercalation between tethered TO and dsDNA is apparent in the form of shifts in melt temperature: (---) **8** with complementary DNA in 1 × PBS; (···) **9** with complementary DNA; (—) **10** with complementary DNA; (–··) **11** with complementary DNA in the presence of free TO derivative **3** (0.05 μM in 1 × PBS, 1:1). Experimental conditions: complementary DNA: 5′-AAA AAA AAG AGA GAA-3′, 0.05 μM in 1 × PBS; excitation at 488 nm, emission at 560 nm; hybridization time 5 min, followed by washing with 2 mL 1 × PBS buffer; temperature ramp was 0.8 °C/min.

of the intercalation between the dye and dsDNA. At room temperature the stability of the hybrids was influenced by the presence of the tethered TO, and this was quantitatively investigated by observing changes of fluorescence intensity upon hybridization (Fig. 2). A systematic evaluation of thermal denaturation profiles indicated that an increase of the length of the tether induced an increase in the thermal stability of the TO-dsDNA complex. The trends of these results were consistent with predictions from the molecular simulation of energy minimization. Interestingly, the thermal denaturation profile of **10** in the presence of complementary DNA was similar to that observed from the non-tethered probe **11** when in mixture with complementary DNA and untethered TO derivative **3**. These results suggest that polyether is a suitable linker, which when adjusted in length, could provide sufficient flexibility so that the energetics of untethered and tethered dyes could be similar. Practical experiments and modeling both suggested that a linker based on hexaethylene glycol (**10**) provided the most thermodynamically stable hybrid, while still minimizing the length of the tether to avoid possible interference with the neighboring sequences.

The hybridization of complementary and single-base mismatch oligonucleotides to the optimized sensor system (**10**) was investigated by collection of fluorescence intensity over a period of time. Figure 3 shows the hybridization process of **10** with complementary oligonucleotide (5′-AAA AAG AGA GAA-3′), single-base mismatch oligonucleotide (5′-AAA AAA AAC AGA GAA-3′), and in 1 × PBS without the presence of oligonucleotide.[27] There was sevenfold fluorescence intensity enhancement when the complementary sequence was introduced to the sensor surface, when compared to the signal that was observed when using a sequence containing a single base pair mismatch. The differential fluorescence enhancement upon hybridization with sequences of different complementarity suggests potential applications to distinguish SNPs in nucleic acid diagnosis. Chemical regeneration of the sensor was performed at a 30 °C hybridization temperature by pumping 1 mL 90% formamide in TE buffer through the sample cell. The sensor was reusable for multiple cycles of detection of target.

In conclusion, a series of oligonucleotides that were modified by tethering of TO using different lengths of glycol-based tethers were successfully synthesized on fused silica surfaces. Fluorescence data indicated that the immobilized labeled TO-ssDNA sequences retained hybridization affinity for complementary ssDNA even

Table 1. Immobilized oligonucleotides

8	TO-(O)$_3$-5'- TTC TCT CTT TTT TTT-3'-fiber optic
9	TO-(O)$_4$-5'- TTC TCT CTT TTT TTT-3'-fiber optic
10	TO-(O)$_6$-5'- TTC TCT CTT TTT TTT-3'-fiber optic
11[a]	5'-TTC TCT CTT TTT TTT-3'-fiber optic

[a] Synthesis of **11** followed the same protocol as other tethered probes (without the final dye coupling step).

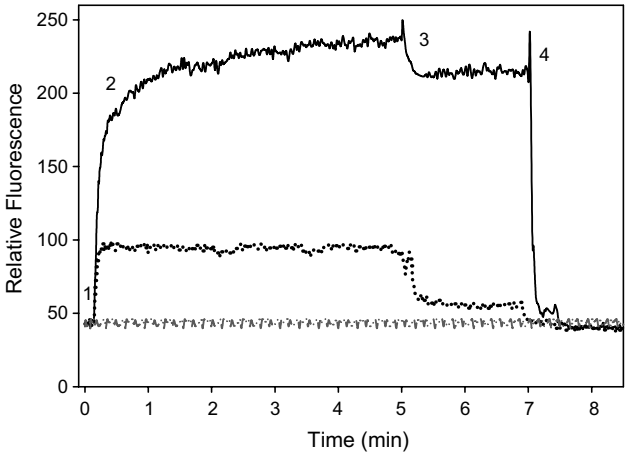


Figure 3. Fluorescent intensity signal during different steps of a hybridization assay cycle for **10**: (1) equilibration; (2) hybridization; (3) washing; (4) regeneration. (—) hybridization with complementary sequence (5′-AAA AAA AAG AGA GAA-3′); (···) hybridization assay with single-base mismatch sequence (5′-AAA AAA AAC AGA GAA-3′); (-···-) fluorescence of **10** in 1 × PBS (excitation at 488 nm, emission at 560 nm; hybridization time: 5 min, then washed with 2 mL 1 × PBS buffer; sensor was regenerated by the treatment with 90% formamide in TE buffer; 30 °C).

when using relatively long HEG tethers at the 5′ end of the probe molecules. Selectivity was sufficient for determination of SNPs. An interesting observation was that the polyether tether length had some impact on the melt temperature. The results suggest that further research is warranted to determine if it may be possible to adjust the effective duplex melting temperature by use of these tethers so that some combinations or arrays of immobilized nucleic acid films in a system can be made to have similar T_m even if they have some differences in oligonucleotide length and sequence. Such a finding would provide opportunity for a method to optimization simultaneous analysis of many interfacial hybridizations, which would facilitate quantitative high throughput screening capacity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.01.030.

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24. To characterize the yield of tethered sequence, synthesis was carried out GOPS-functionalized controlled-pore glass (CPG) with well-defined surface area, in tandem with the oligonucleotide synthesis on the optical fiber surface. The conjugates were then cleaved from the surface by means of exposure to concentrated NH_4OH for 3 h, lyophilized, and redissolved in water. An estimate of the efficiency of the final coupling step could be determined by comparing the ratio of the free oligonucleotide (failed coupling product) to the conjugated oligonucleotide, as could be quantified by the reverse phase HPLC isolation procedure.
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27. Sample (0.5 uM in 1 × PBS) delivery time 30 s, hybridization time 5 min, washing time 2 min at 1 mL/min with 1 × PBS. The experiment was run at 30 °C.