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A fluorescent molecular switch for room temperature operation based on oligonucleotide hybridization without labeling of probes or targets

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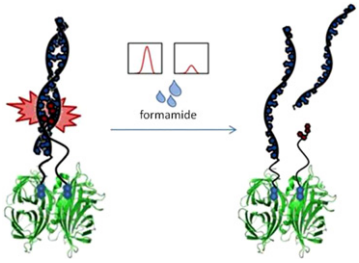
HIGHLIGHTS

- A molecular switch triggered by oligonucleotide hybridization is evaluated.
- Chaotrope suppresses undesired electrostatic binding of intercalating dye.
- Chaotrope concurrently allows single base mismatch discrimination.
- Operation at room temperature and in solid-phase assay is reported.

GRAPHICAL ABSTRACT



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ABSTRACT

A molecular switch was prepared by self-assembly. Neutravidin served as a template that allowed for a biotinylated probe oligonucleotide to be placed adjacent to a biotinylated long-chain linker that was terminated with thiazole orange (TO). Hybridization of probe oligonucleotide with target to form double-stranded DNA resulted in intercalation of the adjacent TO probe. This was a reversible process that could be tracked by fluorescence intensity changes. Formamide was used as a denaturant for double-stranded DNA, and could be used to depress thermal denaturation temperatures. In this work formamide had a dual function, providing for control of hybridization selectivity at room temperature, while concurrently ameliorating non-specific adsorption to improve signal-to-noise when using thiazole orange as a fluorescence signalling agent to determine oligonucleotide hybridization. Room temperature single nucleotide polymorphism (SNP) discrimination for oligonucleotide targets was achieved both in solution and for molecular switches that were immobilized onto optical fibers. In solution, a concentration of 18.5% formamide provided greater than 40-fold signal difference between single-stranded DNA and double-stranded DNA, in contrast to only a 2-fold difference in the absence of formamide. Selectivity for SNP determination in solution was demonstrated using targets of varying lengths including a 141-base PCR amplicon. The improved signal-to-noise achieved by use of formamide is likely due to preferential displacement of dye molecules that are otherwise electrostatically bound to the polyanionic nucleic acid backbone.

1. Introduction

Nucleic acid hybridization of single-stranded probes with targets is routinely used in various analytical strategies for the determination of genetic markers associated with disease and identification of organisms. The use of intercalating dyes to detect hybridization combines the utility of a fluorescent label with an additional element of selectivity that ideally is capable of distinguishing between single-stranded DNA (ssDNA) and double-stranded (dsDNA) [1,2]. In this respect, thiazole orange (TO) has experienced a recent resurgence as a preferred fluorescent probe for DNA diagnostics and imaging. TO has been incorporated into several different scaffolding structures, including: polyamidoamine (PAMAM) dendrimers to provide increased signal by

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covalently binding multiple fluorescent dyes [3], folate molecules for selective tumor cell recognition [4], and oligonucleotides (both as nucleobase substitutes and appended fluorescent tags) to create 'on/off' fluorescent probes for applications in imaging and detection [5–9]. Suppression or enhancement of effects that are associated with aggregation of TO have been spatially controlled using nucleic acid hybridization, and this has been demonstrated to provide analytical signals for diagnostics [7,10]. Recent work by our group has described a novel molecular switch assembly that makes use of TO. This is based on a Neutravidin central scaffold that localizes a biotinylated probe oligonucleotide adjacent to a biotinylated long chain linker that is terminated with TO intercalating dye. Upon hybridization, the TO dye becomes intercalated, and the resulting change in fluorescence can be used to determine hybridization without any need for labeling of target oligonucleotide [11]. In this new work, concurrently improved selectivity and signal-to-noise for oligonucleotide targets has been demonstrated at room temperature for the new molecular switch.

Many bioassays that rely on nucleic acid hybridization strive to discriminate single base-pair mismatches between probe and target oligonucleotide sequences [12]. DNA duplex stability can be used to identify mismatches that are present in a target and can be altered by either thermal or chemical means. Many fluorescence assay methods are predicated on the use of thermal denaturation to differentiate between fully complementary and mismatched target oligonucleotides [13,14]. However, data obtained from thermal scans must then be corrected for variation of fluorescence intensity as a function of temperature. The sample analysis process typically requires slow temperature changes (typically 0.1–0.5 °C min^{-1}) or long hybridization times in order to achieve equilibration of the system at the time of measurement [15]. To overcome this limitation of speed, kinetic methods are being developed to achieve more rapid analysis times [16]. As an alternative, chemical methods to increase selectivity have begun to grow in popularity [15,17]. Formamide and urea are able to destabilize DNA duplexes by competing with Watson–Crick base pairing [18]. In particular, formamide can lower the thermal melt temperature (T_m) of a duplex through disruption of hydrogen bonding motifs or by alterations of hydration patterns of dsDNA [18]. Because formamide does not chemically react with nucleic acids at room temperature, it is frequently used to lower T_m values for dsDNA in PCR reactions to avoid DNA degradation. The lowering of the T_m of oligonucleotide duplexes using formamide is reported to range from 0.60 to 0.72 °C/% formamide in solution [19–21], or 2.4–2.9 °C $mole^{-1}$ formamide [18] for DNA duplexes of lengths greater than 800 base pairs. Fuchs et al. [17] have recently reported an average lowering of T_m for immobilized oligonucleotides (16-mer) of 0.58 ± 0.05 °C/% formamide. Formamide, in conjunction with our molecular switch design, has been used to achieve sufficient selectivity to determine a SNP at room temperature.

The monomeric form of TO shows a distinct advantage over the more commonly used intercalating dye ethidium bromide since TO shows a much larger fluorescence enhancement in the presence of dsDNA, and also shows preferred selectivity between ssDNA and dsDNA [22–24]. Monomeric TO is advantageous over its homodimeric bis-intercalative derivative TOTO, since TOTO has similar affinity for single- and double-stranded DNA. Despite the fluorescence intensity differences associated with interaction of TO with ssDNA and dsDNA, there is still significant fluorescence associated with TO association to ssDNA, and this has been shown to be even more pronounced with the biotinylated TO derivatives [11]. TO binds to dsDNA by intercalation, but also to both dsDNA and ssDNA by electrostatic interaction of the cationic charge of the dye with the negatively charged polyanionic backbone of DNA. In this work, it has been demonstrated that the contributions to the overall fluorescence intensity arising from non-intercalative modes of association of TO with oligonucleotides is ameliorated by the presence of formamide in solution. The use of formamide concurrently allows improved control of SNP discrimination of the molecular switch. SNP discrimination using 19-mer and 34-mer oligonucleotide targets and for a 141-base β-actin PCR amplicon generated using symmetric PCR was examined. Time-resolved fluorescence decay measurements of fully complementary and mismatched duplexes in solution provided further insight into the changes in microenvironment that were associated with the addition of formamide.

2. Experimental

2.1. Reagents and oligonucleotides/PCR amplicons

All chemicals were reagent grade or better and used without further purification. Reagents used for thiazole orange synthesis and synthetic details for dye synthesis are described elsewhere [11]. Formamide (99.5%) was from Sigma–Aldrich (Oakville, ON, Canada). Neutravidin was from Thermo Scientific (Rockford, IL, USA). Reagent-grade toluene, ethanol, acetone, isopropanol, methanol, dichloromethane, and anhydrous diethyl ether were from EM Science (Toronto, ON, Canada). The oligonucleotide sequences presented in Table 1 were obtained from Integrated DNA Technologies (Coralville, IA, USA), with the exception of the β-actin PCR amplicon which was produced in-house. Oligonucleotides were dissolved in 0.1 M PBS buffer at pH 7.4 (100 mM PBS, 20 mM NaCl).

Clinically relevant sequences were used for the work, and the probe and target oligonucleotides are listed in Table 1. Probe A was complementary to a portion of the *Homo sapiens* survival motor neuron protein coding gene (SMN 1) [13]. Probe B was complementary to a portion of the *Homo. sapiens* β-actin gene and Probe C contained a single base pair mismatch (bpm). The targets for Probe A included fully complementary sequences (both a 19 base pair target equal in length to the probe and a 34-mer 'long' target) and sequences containing one or three base pair mismatches. The two sequences that were non-complementary to the Probe A sequence were a T_{20} homopolymer and a mixed base sequence which corresponded to a portion of the *Salmonella enterica* invasion protein A coding gene (inv A) [25,26]. The target sequence for Probe B was a 141-base pair PCR amplicon associated with the *Homo sapiens* β-actin gene. All dilutions were prepared with 0.1 M PBS buffer. Solutions containing a 1:1 ratio of Probe A and the different target oligonucleotides were heated at 95 °C for 5 min and slowly cooled to room temperature to generate dsDNA. Formamide was added to buffered solutions according to percentages and times indicated with the data.

2.2. PCR amplification of β-actin gene fragments

A 141-base pair PCR product was amplified from β-actin template DNA using the following primer pair; 5′-TCA CCC ACA CTG TGC CCA TC-3′ (forward primer) and 5′-GTG GTG GTG AAG CTG TAG CC-3′ (reverse primer). Symmetric PCR amplification was performed using a pre-programmed thermal cycle with: a preheating (95 °C for 5 min) step followed by 45 cycles (at 95 °C for 60 s, 61 °C for 30 s, and 72 °C for 30 s), and a final extension at 70 °C for 10 min in 100 μL of reaction mixture containing 0.02 μg mL^{-1} of template DNA, 1× buffer (10 mM Tris–HCl (pH 8.0), 50 mM KCl, 0.08% Nonidet P40), 1.5 mM $MgCl_2$, 0.5 μM of each primer, 25 nM dNTPs, and 2.5 units of Taq polymerase (Fermentas Life Sciences Canada Inc, Burlington, ON). PCR products of 141 bp were run on agarose gels (1%, w/v) that were pre-stained with SYBR Gold (0.8×). Gels were run at 100 V in 1× TE buffer (100 mM Tris, 2 mM EDTA) for 60 min and examined