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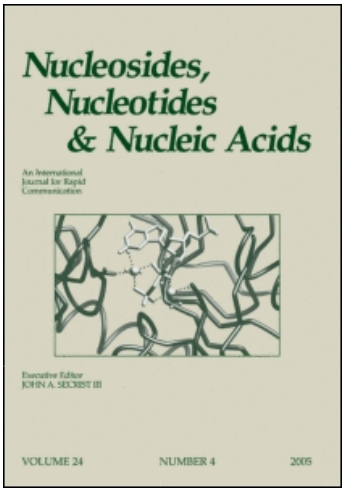

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A Simple Fluorescent Biosensor for Theophylline Based on its RNA Aptamer

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A SIMPLE FLUORESCENT BIOSENSOR FOR THEOPHYLLINE BASED ON ITS RNA APTAMER

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□ *Theophylline is a potent bronchodilator with a narrow therapeutic index. A simple fluorescent biosensor that detects clinically relevant theophylline concentrations has been developed using the well-characterized theophylline binding RNA aptamer. Hybridization of the RNA aptamer to a fluorescently labeled DNA strand (FL-DNA) yields a fluorescent RNA:DNA hybrid that is sensitive to theophylline. The biosensor retains the remarkable selectivity of the RNA aptamer for theophylline over caffeine and is sensitive to 0–2 μM theophylline, well below the clinically relevant concentration (5–20 mg/L or ~10–50 μM). Adding a dabcyl quenching dye to the 3′-terminus of the fluorescently labeled DNA strand yielded a dual-labeled DNA strand (FL-DNA-Q) and increased the dynamic range of this simple biosensor from 1.5-fold to 4-fold.*

Keywords RNA aptamer; Fluorescent biosensor; Theophylline

INTRODUCTION

Theophylline is a bronchodilator used in the treatment of acute and chronic asthmatic conditions. Unfortunately it has a narrow therapeutic index (5–20 mg/L or ~10–50 μM) and above this range the drug is toxic, causing tachycardia, nausea, arrhythmia, and convulsions.[1] Theophylline's

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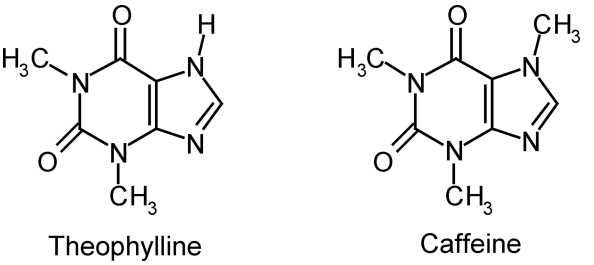

FIGURE 1 Structures of theophylline and caffeine.

narrow therapeutic index requires vigilant monitoring during clinical treatment to prevent these toxic side effects. Although its use in industrialized nations has decreased over the last 2 decades in favor of inhaled corticosteroids and β-andrenergic agonists, it remains the most commonly prescribed asthma drug worldwide due to its low cost and effectiveness.

Numerous methods have been developed for measuring serum theophylline concentrations including gas and liquid chromatography, ultraviolet spectrometry, and immunoassays.[2] Analysis of serum levels of theophylline is complicated because theophylline structurally is similar to other members of the xanthine family such as caffeine (Figure 1).

The most common clinical diagnostic assay for theophylline is based on a monoclonal antibody. This antibody displays only a moderate level of selectivity (up to 500-fold) for theophylline over caffeine and theobromine (a dimethyl xanthine found in chocolates). As a result of this moderate selectivity, these immunoassays may produce false positive results for theophylline toxicity in patients who ingest caffeine. A better assay for theophylline would require a molecule (an antibody or otherwise) capable of a higher level of discrimination between theophylline and caffeine.

In vitro selection is a combinatorial technique that isolates RNAs with high affinity and selectivity for a particular target from a large random pool of RNA sequences by an iterative process of selection and amplification.[3,4] RNA sequences derived by in vitro selection, called RNA aptamers, are good candidates for development into clinical biosensors because they typically possess both high affinity and high selectivity for their targets. In vitro selection has identified RNA aptamers for target molecules ranging from small molecules to proteins.[5,6]

One of the most successful examples of an RNA aptamer for a small molecule is the RNA aptamer for theophylline. Jenison reported an RNA sequence that displayed a high level of affinity for theophylline ($K_d < 0.5\ \mu M$) and amazing selectivity for theophylline over caffeine (~10,000–20,000-fold better binding to theophylline).[7] The consensus theophylline-binding RNA sequence contains a 15 nucleotide motif that is required for high-affinity ligand binding (Figure 2a). Within the 15 nucleotide consensus

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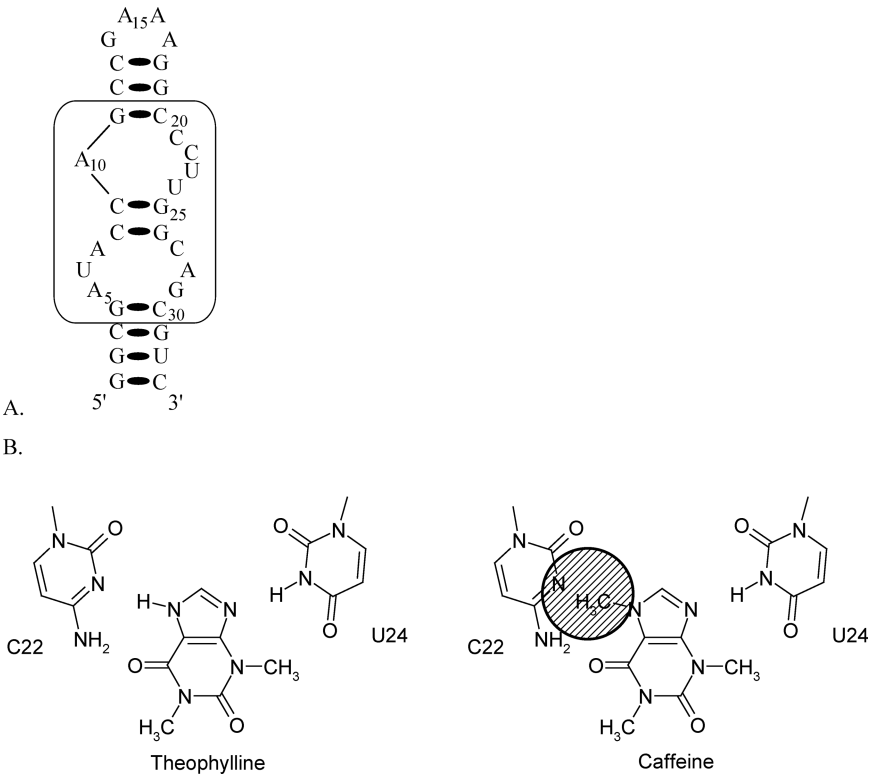

FIGURE 2 a) The RNA aptamer sequence used in this study. Boxed nucleotides indicate the consensus RNA required for theophylline binding. Residue 27, which can be cytosine or adenosine, is cytosine in this work. b) Hydrogen bonding in the C22-Theophylline-U24 base triple. The N7-methyl group prohibits caffeine from binding in the theophylline pocket.

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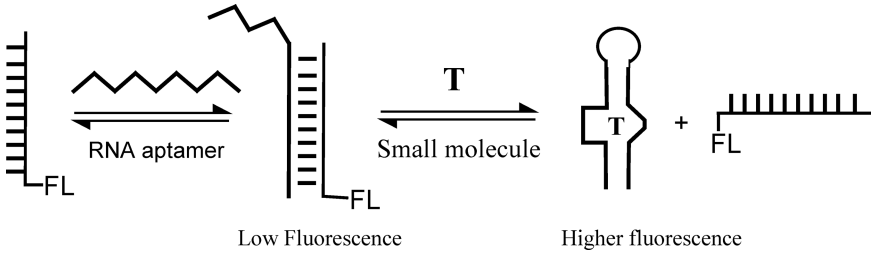
region for theophylline binding, 14 nucleotides are absolutely conserved, and one nucleotide is semi-conserved (position 27 can be an A or C).

The NMR structure of the RNA: theophylline complex revealed a well-ordered binding pocket where an intricate network of hydrogen bonds and stacking interactions locked the theophylline into the complex.[8–10] The structural basis of selectivity between theophylline and caffeine arises due to the formation of a unique base-triple interaction between theophylline and two conserved RNA nucleotides. Theophylline forms hydrogen bonds with residues C22 and U24 whereas the N7 methyl group of caffeine makes an RNA-caffeine complex sterically unachievable (Figure 2b). Stacking of the C22-theophylline-U24 base triple between 2 other base triples (A7-C8-G26 and U6-A28-U23) stabilizes the core of the RNA-theophylline complex, along with other stacking and hydrogen-bonding interactions.

The isolation and characterization of the theophylline-binding RNA aptamer provides a new basis for a theophylline biosensor. Instead of basing the biosensor on the recognition of theophylline by an antibody, the selective recognition of theophylline by the theophylline binding RNA



SCHEME 1 The indirect detection of drug binding by the formation of an RNA:drug complex at the expense of an FL-DNA:RNA hybrid.

aptamer could be the basis for a novel, RNA-based biosensor. Several other labs have either utilized the theophylline aptamer in some sort of biosensor or as a modular signaling element.[11–15] Biosensors based on other RNA aptamers also have been described recently.[16–23]

We have developed an indirect fluorescent biosensor for theophylline (Scheme 1). After hybridizing a short fluorescent DNA strand to the RNA aptamer sequence, the addition of theophylline to the FL-DNA:RNA hybrid duplex results in the formation of an RNA: theophylline complex and release of the FL-DNA strand. This biosensor system responds to clinically relevant concentrations of theophylline and shows a high level of selectivity for theophylline over caffeine. The dynamic range of this simple biosensor is significantly enhanced through the use of a dual-labeled probe (FL-DNA-Q) in place of the FL-DNA probe strand.

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EXPERIMENTAL

Materials and Reagents

All salts and buffers were obtained from commercial suppliers and were used without further purification. Tris (free base), EDTA, NaCl, $MgCl_2$, triethyl ammine, acetic acid, and HEPES were all purchased from Aldrich. Acetonitrile (HPLC grade) was obtained from Fisher Scientific Inc. (Chino, CA, USA). RNAse free water (also called nanopure water) was purified to greater than 18 megaohms (MΩ) using either a Barnstead or Millipore water purification system. All solutions were sterile filtered through 0.2 μm filters to remove any bacterial contamination and generate RNAse free stock solutions.

Theophylline, caffeine, 1-methylxanthine, 3-methylxanthine, and theobromine (3,7-dimethylxanthine) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). while 1,3,7-trimethyluric acid, was from Sigma Chemical Co. (Milwaukee, WI, USA). All analogs were at least 98–99% pure. Solutions of 20 mM of all drug analogs were prepared in RNAse free water.

TABLE 1 DNA oligonucleotides used for the melting temperature study

Theophylline RNA	5′- GGC CAU ACC AGC CGA AAG GCC CUU GGC AGC GUC-3′
10-mer DNA	3′- CCG CTA TGG T -5′
12-mer DNA	3′- CCG CTA TGG TCG -5′
15-mer DNA	3′- CCG CTA TGG TCG GCT -5
20-mer DNA	3′- CCG CTA TGG TCG GCT TTC CG -5′

DNA Purification and Quantitation

DNA oligonucleotides were purchased from Integrated DNA Technologies Inc. (IDT Coralville, IA, USA). FL-DNA strands contained the 5′ fluorescein label. The FL-DNA probe (5′-FL-TGG TAT CGC G-3′) and other DNA sequences were purified on a Shimadzu (Columbia, MD, USA) HPLC system equipped with a reverse phase column (Haisil 100 C18, 5 μM beads, 250 × 4.6 mm) and a single wavelength UV-visible detector at 260 nm.

The samples were injected in TEAA buffer (100 mM triethyl ammonium acetate, pH 7.6) and a gradient of 0–20% acetonitrile was applied over 30 minutes to elute the DNA. The maximum column pressure was ~1200 psi with a flow rate of 1.0 mL/min. Typically, 1% of the total DNA was injected as an analytical trace, followed by 2 preparative traces containing ~50% of the crude DNA each. The peak of interest was collected, frozen and, lyophilized overnight to remove solvent. The samples were reconstituted in 300 μL TE buffer (10 mM tris, 1 mM EDTA, pH 8.0 or 7.5 as indicated) and quantitated by UV-visible spectroscopy at 260 nm.

The 10-nucleotide dual-labeled DNA strand (FL-DNA-Q) was modified at the 5′ terminus with fluorescein and at the 3′ terminus with a dabcyl (4-(4-dimethylaminophenylazo) benzoic acid) label. The strand 5′-FL-GAC GCT GCC A-Q-3′ was purchased from IDT, Inc., purified by HPLC and quantitated by UV-visible spectroscopy at 260 nm.

RNA Preparation

RNA was prepared by in vitro transcription as described previously.[24,25] An initial 40 nucleotide RNA transcript was cleaved in trans by a hammerhead RNA to produce the desired 33 nucleotide RNA with a homogenous 3′ termini. RNA was gel purified on 20% denaturing PAGE and RNA stock solutions were prepared in TE buffer and quantitated by UV-visible spectroscopy at 260 nm.

Melting Temperatures Measurements

DNA strands (see Table 1) were annealed to their complimentary strands and placed in a Uvikon XS (Research Instruments International,

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San Diego, CA, USA) UV-visible spectrophotometer equipped with a Peltier temperature control unit. The final DNA concentration was 0.5 μM of each strand and the buffer was 50 mM HEPES (pH 7.4), 300 mM NaCl, 5 mM $MgCl_2$. Absorption at 260 nm was monitored as a function of temperature as the samples were heated from 20°C to 90°C at a rate of 1°C/minute. Data was analyzed in kaleidagraph to determine the T_m value, corresponding to the temperature at which 50% change in A_{260} was observed.

Fluorescence Titrations

The fluorescence hybridization and drug titration experiments utilized either a Horiba FluoroMax-2 (Horiba Jobin Yvon, Edison, NJ, USA) or a Photon Technology International Inc. QM-2 (PTI, Lawrenceville, NJ, USA) spectrofluorimeter. Either quartz or disposable plastic cuvettes were used and the temperature was maintained at 25°C with a circulating water bath. Data was collected and analyzed by the built-in fluorimeter software and MS Excel. Samples were excited at 494 nm (λ_{exc}) and fluorescence intensity recorded at 520 nm (λ_{max}). Fluorescence intensity data typically were normalized to the initial intensity of a control sample. Fractional changes in fluorescence intensity were then plotted as a function of the titrant added.

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Fluorescence Hybridization Experiments

The following procedure was employed to insure that the fluorescing species (e.g. FL-DNA) was not diluted over the course of the titration. A 25 μL sample of 10 μM FL-DNA was mixed with 100 μL of 10x standard binding buffer solution and the volume was adjusted to 500 μL with RNAse-free water. This solution was then split into a 480 μL (a) fraction and a 20 μL (b) titrant fraction. 480 μL of nanopure water was added to the 480 μL FL-DNA fraction and it was placed in the fluorimeter cell. The titrant solution (b) was prepared by adding 20 μL of DNA or RNA at high concentrations (40 μM in 1x buffer) to the 20 μL fraction of FL-DNA solution. This scheme yielded a final concentration of 0.25 μM FL-DNA in both fractions (a) and (b). Fraction (a) was then heated to 90°C and annealed. At this point the fluorescence was measured giving the zero point of the titration. After each addition of an aliquot of the titrant (fraction b), the solution was heated to 90°C for 2 minutes and allowed to cool back to room temperature before the measurement of fluorescence.

Titration with Theophylline and Other Xanthines

Both hybridization as well as drug titration experiments with the dual-labeled probe (FL-DNA-Q) were performed as described for single-labeled (FL-DNA) probes. The concentration of the FL-DNA: RNA hybrid duplex was maintained at 0.25 μM in both the initial sample (960 μL) and

the added titrant (40 μL) to avoid dilution effects as discussed above for the hybridization experiments. When monitoring the response of the fluorescent biosensors to theophylline in the presence of a background level of caffeine, caffeine was added to the initial fluorimeter and titrant solution.

RESULTS AND DISCUSSION

Choosing Fluorescent Probes

In choosing the fluorescent DNA probes for a simple hybridization biosensor, the main regions on the theophylline binding RNA aptamer of interest were those nucleotides involved with theophylline binding. As shown in Figure 2, the 15 nucleotides that are required for theophylline binding are located centrally within a 33 nucleotide RNA sequence. By selecting 10 nucleotide DNA sequences complimentary to either the first 10 or the last 10 nucleotides of the RNA (FL-DNA and FL-DNA-Q respectively), the DNA probes could easily overlap the conserved core of the RNA (Figure 3).

The complimentary DNA strands were chemically modified with the fluorescein dye at their 5′ termini through an amide linkage. Fluorescein was selected as the fluorescent probe since it is commercially available and absorbs and emits light in the visible region (λ_{exc} = 494 nm and λ_{emi} = 520 nm). For the FL-DNA-Q dual-labeled probes, the 3′-terminus also was modified with dabcyl, which provides efficient quenching of fluorescein emission through resonant energy transfer.[26, 27]

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Melting Temperature of Hybrid Duplexes Related to the Fluorescent Theophylline Sensor

Our indirect biosensor is based on the competition for the RNA between theophylline and a complimentary DNA probe (Scheme 1). In order to insure that the RNA:theophylline complex would be more thermodynamically stable than the RNA:DNA hybrid duplexes, we undertook melting temperature (T_m) studies of relevant DNA:DNA and DNA:RNA duplexes.

DNA probes of 12, 15, and 20 nucleotide lengths were constructed by extending the complimentary regions of the 10-nucleotide FL DNA probe (Table 1). For these T_m studies the DNA strands were not modified with fluorescein. Because of significant problems in measuring the T_m of the RNA:DNA hybrid duplexes, we also investigated the T_m of a related set of DNA:DNA duplexes. This construct was the "perfect-length duplex," with the FL-DNA sequence hybridized to its exact DNA compliment, whereas the DNA:RNA construct where the FL-DNA probe sequence was hybridized to the 33-nucleotide RNA aptamer was known as the "duplex with a tail."

As expected, the melting temperature increased with length within each series of duplexes (see Figure 4 and Table 2). Although the absorbance of

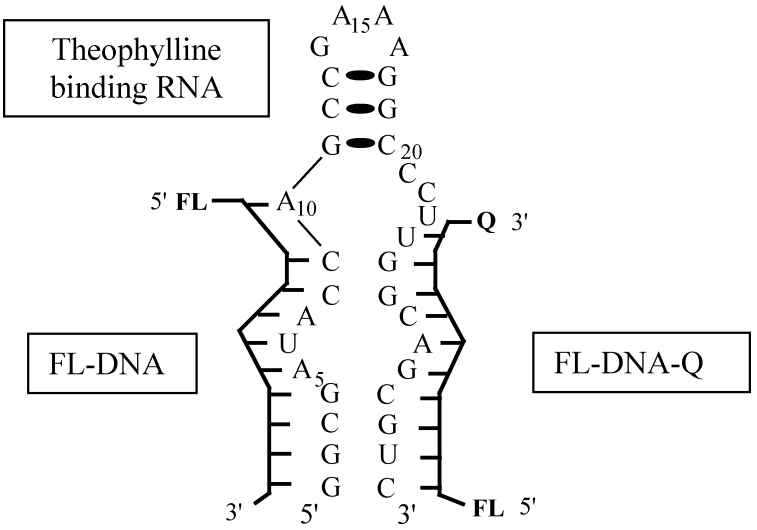

FIGURE 3 A schematic illustrating the complimentarity of the FL-DNA probes to the 5′ and 3′ termini of the theophylline binding RNA. Data for the FL-DNA and the FL-DNA-Q strands are presented in this article. FL = 5′-fluorescein label, Q = 3′-dabcyl label.

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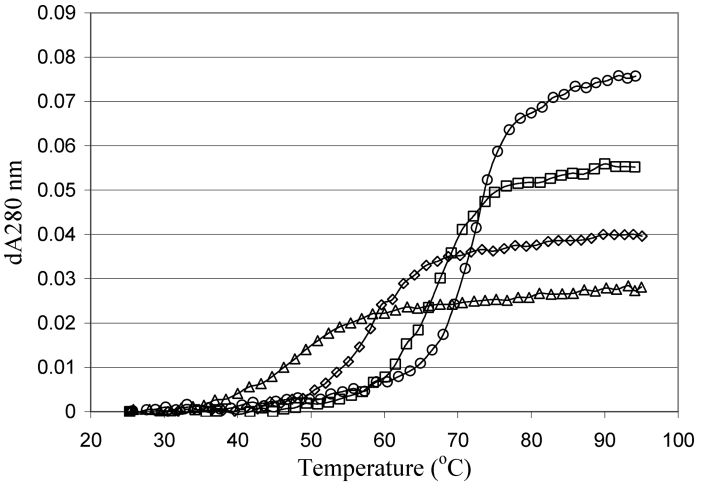
the single-stranded "tail" region prohibited accurate measurement of the melting temperatures for the DNA:RNA hybrids, the trend in T_m values appeared similar to that observed for perfect length duplexes. Importantly, the 10 nucleotide long probes designed for this study were stable at room temperature ($T_m \sim 40–50°C$), but were significantly less stable than the literature value for the RNA:theophylline complex (72°C). The 20 nucleotide DNA:DNA duplex melted at approximately the same temperature as the T_m of the RNA:theophylline complex. In the future, CD spectroscopy may help confirm the T_m values of the duplexes containing the single stranded "tail" regions.

Hybridization of FL-DNA Probes to the RNA Aptamer for Theophylline

The theophylline-binding RNA aptamer is a 33-nucleotide sequence that forms a relatively stable hairpin structure, even in the absence of theophylline. Hybridization titration experiments were carried out to determine whether the free RNA aptamer would hybridize to a complimentary DNA probe and to establish how the formation of a DNA: RNA duplex would change the fluorescence intensity of the labeled DNA probe strand (FL-DNA or FL-DNA-Q).

The single stranded FL-DNA has a constitutive level of fluorescence, and when base-paired to a complimentary strand (DNA or RNA) the fluorescence was expected to decrease, due to quenching of the fluorescent



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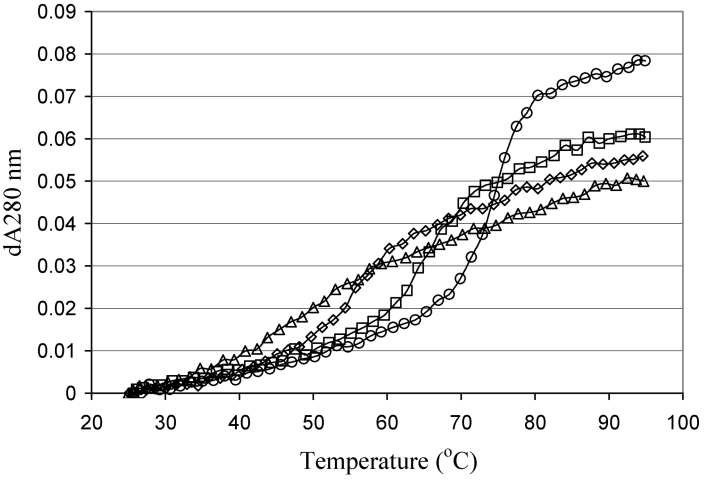

(b)

FIGURE 4 Melting temperature profiles for the series of (a) perfect length DNA:DNA duplexes and (b) DNA:RNA duplexes with tails. Solutions were at 0.5 μM DNA duplex, 50 mM HEPES buffer (pH 7.3), 300 mM NaCl, 5 mM $MgCl_2$. Absorbance was monitored as a function of temperature from 25°C to 95°C, 1°C/minute. In each profile, a single trace for the 10-mer (triangles), 12-mer (diamonds), 15-mer (squares), and 20-mer (circles) DNA are shown.

signal. Figure 5 shows the results of a typical hybridization titration of an FL-DNA strand that is complimentary to the 5′-end of the RNA aptamer. As the FL-DNA strand was titrated with the RNA aptamer, the resulting FL-DNA:RNA hybrid duplex displayed decreased fluorescence intensity. Similarly, hybridization of the FL-DNA to a 10 nucleotide complimentary DNA strand also showed a reduction in fluorescence intensity. The decrease in fluorescence intensity stabilized when one equivalent of the

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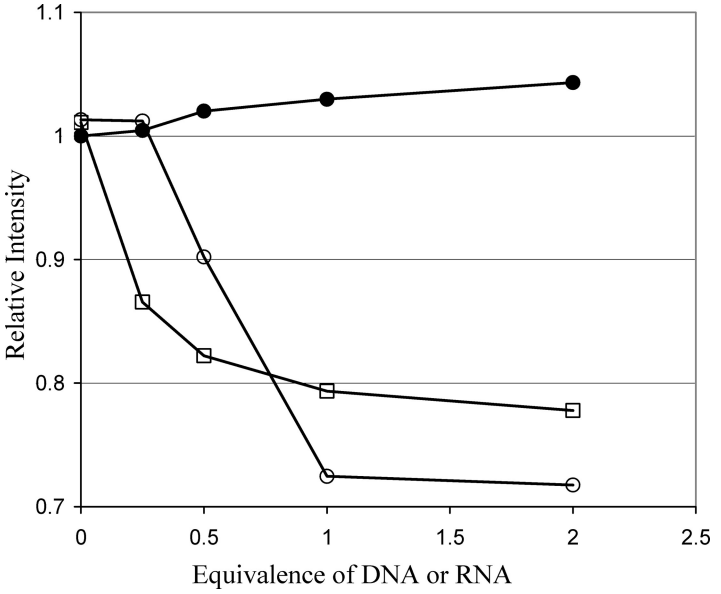

FIGURE 5 The hybridization of FL-DNA to the theophylline RNA aptamer or a complimentary DNA strand. Squares and triangles represent titration of FL-DNA with the RNA aptamer for theophylline or a complimentary DNA strand respectively, while diamonds represent the control titration of FL-DNA with buffer.

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complimentary DNA or RNA aptamer was added. Similar results were observed with a 15 nucleotide version of the FL-DNA as well as fluorescent DNA probes complimentary to the 3′ end of the RNA. The quenching of the fluorescein signal is likely due to the proximity of the fluorescein label to guanine residues of the RNA in the FL-DNA:RNA duplex.

Titrations with Theophylline, Caffeine, and Other Analogs

The FL-DNA:RNA hybrid duplexes were titrated with the asthmatic drug theophylline or its analogs. There were two questions that were to be answered: First, upon addition of theophylline to the RNA:DNA hybrid, would the RNA aptamer dissociate from the fluorescent DNA probe and form an RNA: drug complex? Such a shift would require that the RNA

TABLE 2 Melting temperature (T_m) data for a series of DNA:DNA perfect length duplexes relevant to the indirect fluorescent biosensor for theophylline. T_m values listed in °C and are the average of 2 trials

Length	T_m
10 mer	46.4
12 mer	56.6
15 mer	66.3
20 mer	71.0

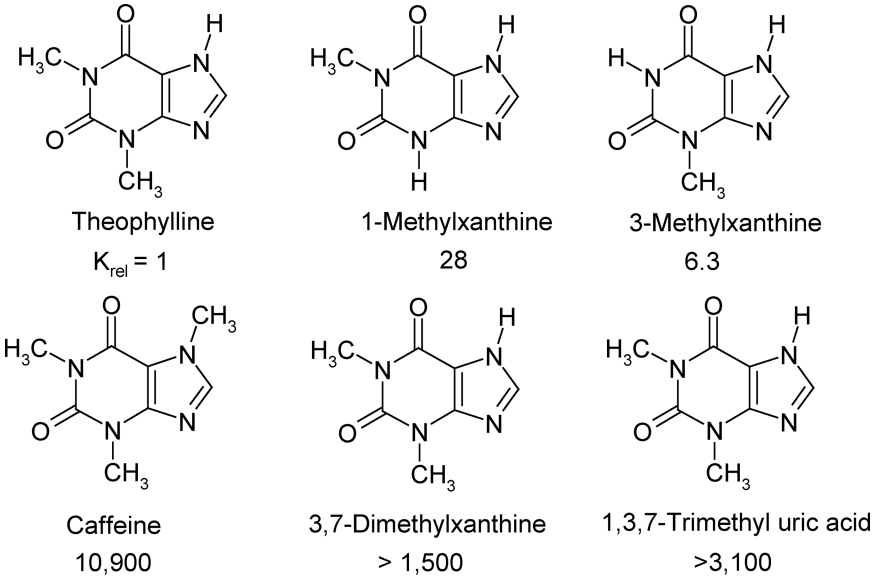

FIGURE 6 Xanthine analogs and their published relative dissociation constants (K_{rel}) for the theophylline binding RNA aptamer. K_{rel} is the ratio of the K_d for a particular analog divided by the K_d for theophylline ($K_d = 0.32\ \mu M$). Data taken from Jenison et. al.

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aptamer have a higher affinity for theophylline than the complimentary DNA probe. The formation of this RNA:drug complex could be measured indirectly by the recovery of fluorescence intensity of the DNA probe. Second, would the titration of the RNA:DNA hybrids with theophylline analogs (e.g., caffeine) reflect the published selectivity of the theophylline binding RNA aptamer for theophylline over caffeine?

When the FL-DNA:RNA hybrid was titrated with the drug theophylline, an increase in fluorescence intensity was observed (Figure 7). This change in intensity indicated that the RNA: drug complex was formed at the expense of the FL-DNA:RNA hybrid duplex. Addition of theophylline gave a 1.5-fold increase in fluorescence over the range of 1–5 μM theophylline. This increase in fluorescence corresponded to the amount of fluorescence quenching due to the hybridization of the FL-DNA probe.

The xanthine derivatives shown in Figure 6 were selected for competition studies to probe the selectivity of the biosensor. In contrast to the dose-dependent response to theophylline, the fluorescence intensity of the FL-DNA:RNA hybrid remained unchanged when titrated with caffeine (Figure 7). This indicates that the RNA aptamer showed a higher affinity for the FL-DNA probe than for caffeine, a result in agreement with the 10,000–20,000-fold higher affinity of the RNA for theophylline than caffeine. Furthermore, when the FL-DNA: RNA hybrids were titrated with theophylline in the presence of a high concentration of caffeine (500 μM),

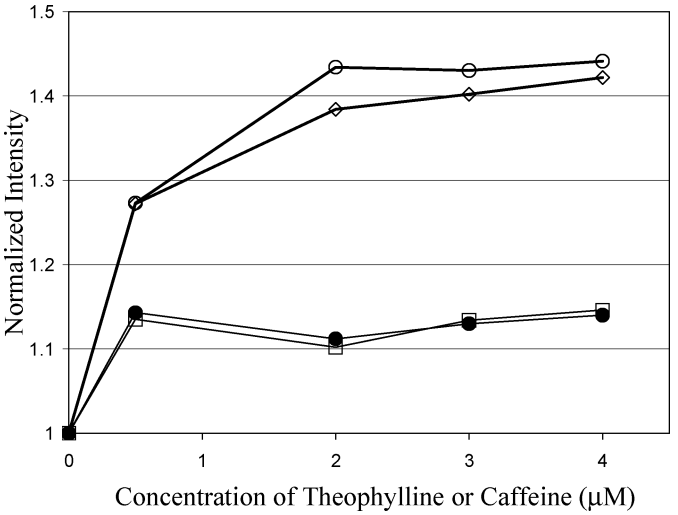

FIGURE 7 Titration of the FL-DNA:RNA hybrid with theophylline alone (squares) or with theophylline in the presence of 500 μM caffeine (squares). The titration with caffeine (circles) and a buffer control (diamonds) are also shown. The fluorescence intensity has been normalized to the first point in each respective titration.

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the response to theophylline mirrored the response to theophylline in the absence of caffeine.

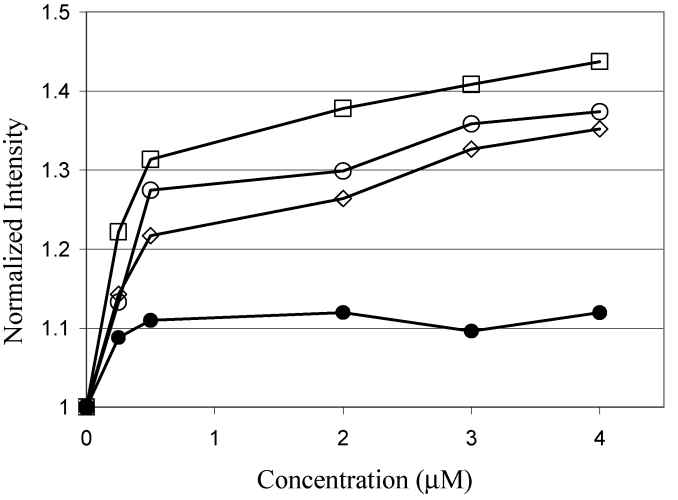
Besides caffeine, there are numerous methylxanthines, including the metabolites of both theophylline and caffeine, that could possibly interfere with an assay for theophylline. Titration with theophylline-like derivatives 1-methylxanthine and 3-methylxanthine showed that the theophylline RNA aptamer was responsive to these derivatives, however not with the same intensity as theophylline (Figure 8a). The responses corresponded to the relative dissociation constants (K_{rel}) shown in Figure 6.

In contrast to the response to theophylline compounds, the biosensor showed poor affinity for the caffeine-like derivatives 3, 7-dimethylxanthine (theobromine) and 1, 3, 7-trimethyluric acid (Figure 8b). Theobromine showed about half the response as theophylline and trimethyluric acid had a minimal response, similar to caffeine.

The biosensor shows promising selectivity, retaining the high affinity of the RNA aptamer for theophylline-like derivatives and low affinity for caffeine like analogs.

Dual Labeled Probes

One problem with the fluorescent theophylline biosensor is its limited dynamic range. Hybridization of the FL-DNA strand to the RNA leads to about a 1.5-fold decrease in fluorescence intensity and therefore, addition of theophylline to this FL-DNA:RNA hybrid could offer at most a 2-fold recovery of fluorescence. Three methods of improving the dynamic range



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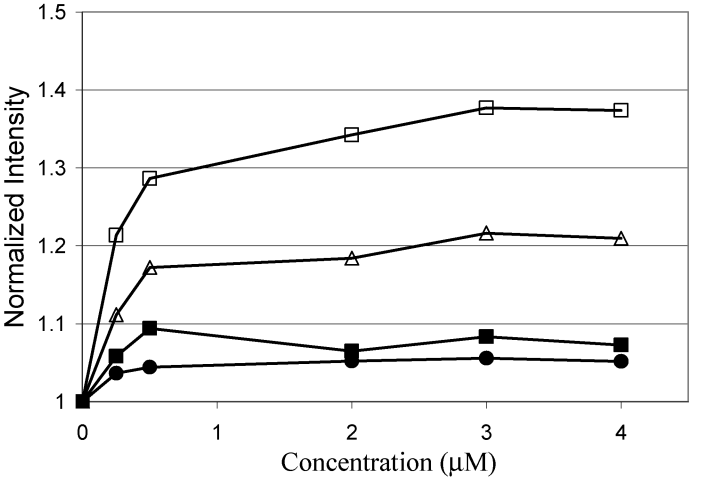

(b)

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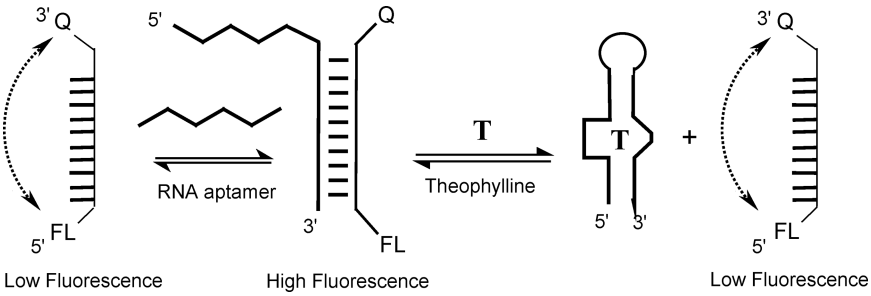
FIGURE 8 Titration of the FL-DNA: RNA hybrid duplex with theophylline-like analogs (a) or caffeine-like analogs (b). Both graphs show the response to theophylline (squares) and buffer (closed circles). Graph (8a) also has 3-methylxanthine (circles) and 1-methylxanthine (diamonds), while Graph (8b) also has 3,7-dimethylxanthine (theobromine, triangles) and 1,3,7-trimethyluric acid (filled squares). The fluorescence intensity has been normalized to the first point in each titration.

of the biosensor were hypothesized. First, one could make the free FL-DNA brighter by using a different dye than fluorescein. However, even with a brighter free FL-DNA, the ratio in fluorescence of the free FL-DNA to the FL-DNA:RNA hybrid might still remain about a 1.5-fold difference.

A second option to improve the dynamic range of the biosensor was to make the FL-DNA:RNA hybrid species quench better. Although it is



SCHEME 2 A fluorescent biosensor based on hybridization of a dual labeled DNA probe (FL-DNA-Q) to an RNA aptamer. Addition of theophylline should cause a decrease in fluorescence as the FL-DNA-Q strand is released.

known that local sequence can have a profound effect on the quenching efficiency,[28] there are no predictable means of designing the FL-DNA strands so that they will be more quenched when hybridized to RNA. Better quenching in the DNA:RNA hybrid also might be accomplished through the chemical modification of the RNA aptamer strand with a quenching dye. However, this would require that the RNA be chemically synthesized and not simply transcribed, a significant limitation.

A third, perhaps nonintuitive, option for increasing the dynamic range would be to make the free DNA probe *darker* through the addition of a quenching dye such as dabcyl (4-(4-dimethylaminophenylazo) benzoic acid) as a second label, creating a dual-labeled DNA probe (FL-DNA-Q). When the fluorescein and dabcyl dyes come into close proximity the dabcyl efficiently quenches fluorescein emission through an energy transfer mechanism.[28] However, when the probe strand base pairs with either complimentary DNA or RNA, the fluorescein and dabcyl are physically separated and fluorescence would be recovered.

The use of a dual-labeled DNA strand (FL-DNA-Q) would reverse the expected changes in fluorescence–the free FL-DNA-Q would *not* fluoresce but the hybrid FL-DNA-Q:RNA hybrid would fluoresce (Scheme 2). Addition of theophylline would cause a decrease in fluorescence because the FL-DNA-Q strand would become free again and be able to quench itself. This approach has the advantage of maintaining an unmodified RNA, while offering the potential for a sizable increase in dynamic range.

This use of dual-labeled probes is reminiscent of the molecular beacon approach to hybridization detection, where the fluorescent and quenching dyes are placed at the 5′ and 3′ termini of a hairpin loop structure.[29] Molecular beacons were designed to increase the sensitivity of hybridization-based biosensors and have been reported to increase the detection of DNA-RNA hybrids at least 10-fold over single fluorescent labeled DNAs. Similar dual-labeled probe and molecular beacon approaches are the basis for a number of recent nucleic acid based biosensors.[20,30–32]

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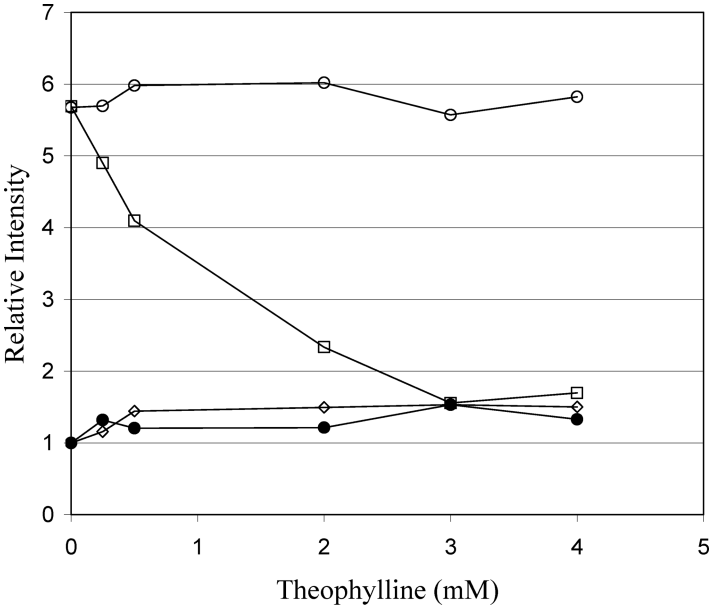

FIGURE 9 Theophylline titration of FL-DNA-Q:RNA hybrid (squares) and FL-DNA-Q:DNA hybrid (circles). As a control, titrations of the FL-DNA-Q strand alone with theophylline (diamonds) and buffer (closed circles) also are shown. All fluorescent intensities were normalized to the fluorescence of the free FL-DNA-Q strand.

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We constructed our 10 nucleotide dual-labeled DNA probes without a hairpin-forming stem region in order to avoid having to balance the stability of the molecular beacon against the stabilities of the DNA:RNA duplex and the RNA:theophylline complex. Hybridization experiments with the dual-labeled probe DNA strands showed that upon the addition of complimentary DNA or RNA, the fluorescence of the FL-DNA-Q strand increased approximately 4-fold (data not shown). Similar to hybridization experiments with singly labeled FL-DNA strands, the change in fluorescence was saturated at about one equivalent of complimentary DNA or RNA strand.

Upon titration of the FL-DNA-Q:RNA hybrid with the drug theophylline, a 4-fold decrease in fluorescence intensity was observed, indicating that the RNA:drug complex was formed at the expense of the FL-DNA-Q:RNA hybrid (Figure 9). In contrast, when theophylline was titrated into a solution of a FL-DNA-Q:DNA hybrid, no change in intensity was observed. To ensure that the fluorescence of the free dual-labeled probe was not influenced by the addition of theophylline, an additional control was performed wherein the dual-labeled probe alone was titrated with theophylline. As expected, the addition of either theophylline to the dual-labeled probe alone yielded no change in fluorescence.

Although the use of the FL-DNA-Q strand increased the dynamic range of the biosensor, the 4-fold difference was actually less than expected.

The difference in fluorescence between the hybridized and single-stranded forms of the FL-DNA-Q strand likely is limited by 2 factors: the short length of the FL-DNA-Q strand allows residual quenching in the hybridized form and the 3′-dabcyl dye label provides incomplete quenching in the single-stranded form.

Using standard helical parameters for an A-form helix, the 5′-terminus and 3-terminus are only separated by 27 Å (10 base pairs x 2.7 Å rise/base pair), which is only barely beyond the Forster radius for efficient quenching of the fluorescein–dabcyl pair (25 Å). Thus, even in the hybridized state, there may be substantial quenching of the fluorescein. Using a longer FL-DNA-Q strand may lead to less quenching in the hybridized state and more substantial dynamic range in the biosensor. As for the incomplete quenching in the single stranded form, the data in Figure 9 illustrates that the FL-DNA-Q strand is not completely quenched when in single-stranded form, again limiting dynamic range. A more efficient quenching dye could alleviate this limitation to the dynamic range of the biosensor.

Both adjustments to the FL-DNA-Q strand—increasing length and improving the efficiency of the quenching dye—will not only allow for a greater dynamic range, but also allow the system to operate at lower absolute concentrations of RNA aptamer (<0.25 uM). The lower concentration of RNA aptamer would allow the lower limit of detection to be decreased.

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CONCLUSIONS

Based on the results presented here, the simple biosensor based on the theophylline-RNA aptamer is promising. Indirect detection of RNA: drug complexes through the activation and or quenching of a fluorescent DNA probe shows promise as a simple and sensitive biosensor scheme.

Using a 10-nucleotide long FL-DNA probe made the FL-DNA: RNA hybrid less thermodynamically stable than the the RNA: drug complex. Upon addition of between 1 μM and 5 μM theophylline to the FL-DNA:RNA hybrid, a 1.5-fold increase in fluorescence was observed due to the formation of the RNA:drug complex and release of the FL-DNA strand. The limit of detection of this simple biosensor is significantly lower than the clinical range of theophylline concentrations (10–20 mg/L or 25–50 μM). This is an optimal feature for development into a clinical assay with biological samples such as serum or blood plasma, where a 10-fold dilution of the sample is not uncommon.

When the FL-DNA:RNA hybrid is titrated with caffeine, there is no change in the fluorescence, indicative of the poor affinity of caffeine for the theophylline-RNA aptamer. Additionally, our biosensor still responds to theophylline in the presence of high concentrations of caffeine (500 μM). The biosensor showed a good response to the theophylline-like derivatives

1-methylxanthine and 3-methylxanthine and minimal response to caffeine-like derivatives 3,7-dimethylxanthine (theobromine) and 1,3,7-trimethyluric acid. This selectivity would allow for the quantification of theophylline without interference from caffeine and its metabolites. Clinically, a theophylline biosensor based on its RNA aptamer could reduce false positive results that are problematic with biosensors based on monoclonal antibodies.

Initially, a 1.5-fold change in fluorescence intensity was observed in our fluorescent biosensor based on the single-labeled FL-DNA probe. The dynamic range of the fluorescent theophylline biosensor was increased to a 4-fold response when a dual labeled probe containing 5′-fluorescein and 3′-dabcyl labels was used. We currently are optimizing the identity of the quenching dye as well as the length, placement and concentration of FL-DNA-Q probes in order to maximize the dynamic range of this simple fluorescent biosensor for theophylline. An optimized version of the biosensor for theophylline would be amenable to analysis of theophylline in plasma or serum.

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