Dimeric carbocyanine dye and nucleic acid aptamer mediated detection of food borne toxin

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Food borne toxins represent a serious challenge to the advances in the operation of the food industry with respect to food processing and distribution. While selective identification of the toxins can be easily accomplished using standard chromatographic and mass spectrometric methods, these are not conducive for use in the field. In this work, we use nucleic acid aptamers in a label-free strategy for detection of specific food borne toxins. Our approach relies on the non-covalent association of a dimeric carbocyanine dye with the quadruplex secondary structures formed by aptamers specific for certain toxins. The presence of a specific toxin Bisphenol A in solution leads to displacement of the dye that manifests as a loss of fluorescence. The work presented here represents an interesting application of the de-aggregation induced fluorescence enhancement of dimeric carbocyanine dyes. Sub-micromolar levels of Bisphenol A can be detected using the aptamer-dimeric carbocyanine dye mediated approach. Further, the strategy is specific towards the detection of toxins based on the nucleic acid aptamers used in the assay.

**Keywords:** Nucleic acid aptamers, dimeric carbocyanine dye, G-quadruplex, fluorescence sensing, food borne toxins

Proper functioning of the public food distribution system, food processing, and hospitality industry directly affect the day-to-day activities of the common person. In the current age, greater access to packaged and processed foods has made human populations more prone to food borne toxins. Ingestion of food borne toxins contributes to the spread of food borne diseases\textsuperscript{1}. The detection of specific toxins is usually accomplished by analytical chromatographic and mass spectrometric approaches. The instrument-intensive nature of such methods precludes their wider application and use in the field. Immunology-based methods or biosensors offer the promise of assay development that does not necessarily depend on high-end infrastructure. However, these are constrained by difficulties associated with antibody generation towards toxins, short shelf life of antibodies and batch to batch variation in their efficacy\textsuperscript{2}. Methods that are conducive to be used by the common citizen and that can be easily adapted for unskilled personnel are expected to contribute immensely towards food safety considerations.

In this context, nucleic acid based biosensors are simple, rapid and inexpensive and in contrast to antibody based biosensors, can be easily synthesized, modified and regenerated\textsuperscript{3,4}. One particular class of emerging biosensors uses aptamers as their bioreceptors\textsuperscript{5,6}. Aptamers (which can be considered as nucleic acid analogs of antibodies) are single-stranded oligonucleotides, DNA or RNA, capable of binding broad range of targets, such as small molecules, proteins, and even specific types of cells\textsuperscript{5,7,8}. A major challenge underlying the use of aptamer based biosensors is the development of general methods to convert the highly specific molecular recognition between aptamers and their targets into detectable signals\textsuperscript{9}. The conjugation of aptamers with chromophores has been explored to address this issue\textsuperscript{10}. Aptamers labelled with optical probes have been used for setting up detection strategies and assays\textsuperscript{11,12}. Persistent drawbacks of such approaches include the high cost of synthesis of specific nucleic acid conjugates and interference of the optical probes with aptamer-target interactions. We have previously examined commercially available chromophores for their ability to perform direct transduction of the aptamer-target recognition event into straightforward changes in fluorescence signals\textsuperscript{13}.

In this work, we use a quadruplex-selective dimeric carbocyanine dye for the selective fluorescence
detection of the toxin Bisphenol A (BPA). We have recently reported the ability of a dimeric carbocyanine dye to form well-defined aggregates in a solution that are disrupted in the presence of specific quadruplex DNA. The dimeric carbocyanine dye aggregates are fluorescence-quenched and disruption of these aggregates is accompanied by a large fluorescence increase. A large proportion of nucleic acid aptamers are known to fold into quadruplex secondary structures. We examine the ability of our dimeric carbocyanine dye to reveal the presence of a specific toxin based on their ability to bind quadruplex-forming nucleic acid aptamers. A combination of UV-visible, fluorescence, and CD spectroscopy is used to characterize the interactions between the aptamer, toxin, and dye. Our results indicate that presence of toxin concentrations above a specific threshold is sufficient to disrupt the quadruplex-dye association thereby leading to lower fluorescence. The loss of fluorescence intensity can be directly correlated to the concentration of toxin present. Since the nucleic acid aptamer for each toxin is highly selective, the fluorescence quenching is only observed when the cognate aptamer-toxin combinations are present. Our results highlight an interesting application of dimeric carbocyanine dyes for their ability to respond to changes in aggregation behaviour and holds promise for the development of detection assays for food borne toxins that can be widely applied.

Results and Discussion

The BPA aptamer is known to fold into a quadruplex topology. The quadruplex conformation of the aptamer has been exploited in several detection methodologies that have been set up for BPA. In this regard, we have recently developed a dimeric carbocyanine dye towards the selective recognition of quadruplexes. The dimeric dye (Figure 1), was found to self-assemble into extended H-aggregates with characteristic blue-shifted absorbance maximum. Specific G-quadruplexes were found to disrupt the dye aggregates due to the presence of suitable sites of interaction for dye binding. We first studied the interaction of dye with the BPA aptamer (Table I) in the presence and absence of target BPA, by UV-visible spectroscopy. Interaction of the dye with the aptamer alone resulted in the formation of quadruplex-associated H-dimers, as indicated by the absorbance band at 530 nm (Figure 2). Interestingly, dye is known to form extended H-aggregates that absorb in the range of 430 – 450 nm. The absence of such H-aggregates of in the presence of BPA aptamer is indicative of dye-binding to the aptamer. Equimolar amounts of target BPA led to the emergence of an absorbance band at 430 nm and concomitant lowering of absorbance at 530 nm. These changes in the absorbance spectrum of in the presence of BPA point to the ability of the target BPA to disrupt the quadruplex topology and therefore block the binding sites of the aptamer making it less accessible for dye binding. The behavior of in the presence of BPA aptamer with or without BPA target was further studied by fluorescence spectroscopy. As shown in Figure 3, the fluorescence of is enhanced more than 1400-fold in the presence of the BPA aptamer compared to the dye alone. Notably, the emission intensity of drops by more than 2-fold when the BPA target is introduced. This observation is consistent with the formation of H-aggregates of and decrease in the amount of aptamer-bound H-dimers. The lower fluorescence of in the presence

Table I — DNA sequences used in this work

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>1</td>
<td>BPA Aptamer</td>
<td>5′-CGG GTG GGT GGT CAG GTG GGA TAGT CTC CCG TTC GCA ACG CAT CAC GGG TTC GCACCA-3′</td>
</tr>
<tr>
<td>2</td>
<td>OTA Aptamer</td>
<td>5′-GATCGGGAGCGTGGCCTGAAGGGAGCATCGGGACA-3′</td>
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of the aptamer and target supports the hypothesis that aptamer binding to the target BPA results in the lesser availability of binding sites for the dye which spontaneously assemble into H-aggregates. To contextualize the effects of BPA binding on the quadruplex topology of the aptamer, we tested the interaction of 1 with the BPA aptamer in the presence of small amounts of Li⁺. Li⁺ is known to destabilize DNA quadruplexes. Presence of Li⁺ results in a lower emission of 1 that is comparable to the fluorescence of 1 in the presence of BPA (Figure 3). Thus, the quadruplex topology is clearly critical for binding of 1, and unavailability or disruption of the quadruplex in the presence of BPA target prevents binding of the dye ultimately leading to loss of fluorescence. We performed CD spectroscopy to further substantiate the correlation between the behavior of dye 1 and the quadruplex topology in presence and absence of target BPA. The BPA aptamer folds into parallel quadruplex based on the positive and negative CD bands at 280 and 240 nm, respectively (Figure 4). The intensity of these bands is significantly reduced in the presence of BPA target and presence of Li⁺. These observations are consistent
with the disruption of the quadruplex conformation of aptamer by the BPA target. The presence of dye 1 does not alter the quadruplex conformation or its disruption by BPA (data not shown). We performed experiments to identify the sensitivity of our assay. The fluorescence of 1 was measured in the presence of BPA aptamer with variable amounts of BPA target. 0.5 µM of BPA was clearly found to elicit fluorescence quenching of the dye (Figure 5). Lower amounts of BPA were found to produce quenching of fluorescence within 10-15% of the control that lacks BPA. Finally, the selectivity of our approach was tested using another food-borne toxin Ochratoxin A (OTA). Since the BPA aptamer is selective for BPA, the presence of OTA is not expected to result in fluorescence quenching of the dye. This is consistent with our observations as shown in Figure 6. Interestingly, the fluorescence of 1 remains quenched in the presence of OTA aptamer with and without the OTA target. Thus, the fluorescence turn-off of 1 observed in the presence of BPA aptamer and the cognate target is specific to that aptamer alone. These results emphasize the promising ability of the dimeric carbocyanine dye 1 to indicate the formation of a specific aptamer-target complex and by extension enable the identification and quantification of that target.

**Materials and Methods**

The dimeric carbocyanine dye 1 was synthesized and used as described previously. All reagents used for the synthesis of 1 were purchased from Sigma-Aldrich, Bangalore, India. The dye was purified by recrystallization and dye stock solutions were prepared in MeOH and diluted whenever experimentation was necessary. Dye samples were stored at 4°C. Extinction coefficients used for

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**Figure 4** — CD spectra of BPA aptamer with dye 1 and BPA

**Figure 5** — Fluorescence of BPA aptamer + 1 in presence of variable amounts of BPA
determination of dye concentration was $\varepsilon_{550} = 31800 \text{ M}^{-1} \text{ cm}^{-1}$. DNA oligonucleotides used in this work were purchased from Sigma Chemical Company (Bangalore, India). Extinction coefficients calculated by the nearest neighbour method were used in conjunction with absorbance measurements for determination of oligonucleotide concentrations. DNA samples in all cases unless otherwise specific contained 2 µM of strands and were prepared in 10 mM phosphate buffer $pH$ 7.0 containing 10 mM KCl. Samples containing Li$^+$ had 9 mM KCl and 1 mM LiCl. The total volume of samples was made up to 1 mL using DNase-free water. The aptamer sequences were first annealed before being used in the assays. Briefly, samples containing requisite oligonucleotides concentrations were first heated to 90°C and were allowed to gradually cool to RT.

**Experimental Section**

**Detection of Bisphenol A:** Samples containing 2 µM of the aptamer for BPA in 10 mM KCl and 10 mM phosphate buffer $pH$ 7.0 were prepared and annealed overnight. 2 µM of BPA was added to the sample and incubated for 15 min. This was followed by addition of Dye 1. The final concentration of Dye 1 was ensured to be 2 µM. Addition of the dye was ensured to result in very little change in volume of the overall sample (<5%). The addition of the dye was followed by incubation at RT for 15 min. Samples with Ochratoxin A (OTA) were prepared by using 2 µM of OTA in place of BPA as per the sample preparation above. UV-visible spectra of the samples were recorded on a JASCO V-750 spectrophotometer at 25°C. Fluorescence spectra on the same samples were recorded on a Horiba Fluorolog spectrofluorimeter by excitation at 540 nm. Instrument slit widths were controlled by the operating software and were kept consistent at 4 nm. Experiments with variable amounts of BPA were performed using the same procedure as above but with different concentrations of the toxin. CD spectra of G-rich oligonucleotides samples were performed on a JASCO J-815 spectropolarimeter with Peltier system temperature controller.

**Conclusion**

A dimeric carbocyanine dye 1 has been used for assaying the presence of the toxin Bisphenol A. The approach relies on fluorescence quenching of the dye in the presence of the cognate aptamer-target complex. Sub-micromolar levels of BPA can be selectively detected using the current method. Work towards extending the strategy towards other quadruplex-forming aptamers is currently ongoing in our laboratory.

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**References**