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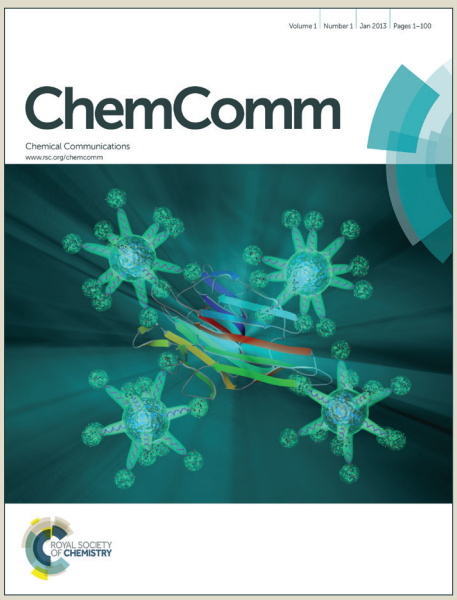

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

A Label-free Fluorescent Molecular Switch for DNA Hybridization Assay Utilizing a G-quadruplex-selective Auramine O

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Received (in XXX, XXX) XthXXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

A sensitive and selective assay of DNA is developed by utilizing a signal transduction strategy with rational redesign of the hairpin structured G-quadruplex molecular switch (G4-MS) assembled with auramine O (AO). By monitoring the change of the fluorescent signal, we could identify and further quantitatively determine the target DNA in the samples.

The sensing of nucleic acids is of particular interest and importance for biomedical research because a large number of genetic diseases, such as cancer and mitochondrial diseases, have been revealed to be associated with single-nucleotide polymorphisms (SNPs).[1,2] Nucleic acid analytes, including SNPs, have been specifically measured by adapting molecular beacons (MBs), in which a synthetic oligonucleotide is functionalized with two chromophores, most often at its ends because MBs recognize their targets with higher specificity than linear DNA probes.[3-5] Hybridization with a complementary target DNA strand induces a conformational change of MBs that changes the interaction of the two chromophores, giving rise to a specific fluorescent signal that is used for detection.[6]

Despite considerable progress over the past two decades, the inflexibility of the stem constructed by Watson-Crick base pairs and the need of labels remain the two major challenges for the MBs-based techniques. Several methods have been attempted to solve the problem of inflexibility of the stem, such as using metal-ligation interaction,[7-9] triplex hairpin-shaped structure,[10,11] bipyrene functionalized oligonucleotide and γ-cyclodextrin[12] and dual-labeled G-quadruplex-shaped structure as the stems (called G-quadruplex molecular beacons, G4-MBs).[13-15] Despite all the efforts made in every investigation, these methods have their limitations, such as the use of toxic metal ions of metal-ligation interaction, and the difficulty of designing the competitor to form the triplex-stem etc. G4-MBs can control the stem stability by easily varying the concentrations of K^+ or Na^+ in the medium, but the probes were still dual labelled as the traditional MBs. However, the necessity of fluorophore-quencher pair optimization and sophisticated probe synthesis, purification and cost limited its application as common biosensing approaches. Furthermore, labeling on the 5' and/or 3' end may suppress the binding. G-quadruplex ligands represent a new class of anticancer agents.[16] Although many G-quadruplex binders have been developed, many of these small organic molecules have cell toxicity, insufficient structure selectivity versus duplex DNA, and poor water solubility. Therefore, development of G-quadruplex binders with good performance, not only interacting strongly with their target but also exhibiting high selectivity for G-quadruplex versus duplex DNA, is still a challenge. Synthesis of novel binders requires complex synthesis steps and the binding performance cannot be predictable. Another way is to screen from the existing molecules, especially the optical dyes because of their remarkable photophysical properties.[17-20] Thus, development DNA determination with flexibility of the stem, and in the same time label free, low cost, facile preparation and certain efficiency is desirable.[20,21]

Herein, a sensitive and selective assay of DNA has been developed by utilizing a new signal transduction strategy with rational redesign of the hairpin structured G-quadruplex molecular switch (G4-MS) assembled with auramine O (AO). In the absence of capture probe, AO itself was almost nonfluorescent in Tris-HCl buffer.[22] In contrast, the fluorescence of AO increases to a large extent in the presence of capture DNA, suggesting the formation of G-quadruplex.[23-28] The resulting fluorescence response of the reversible noncovalent complex of capture probe and AO has laid the foundation for the development of the fluorescent sensing platform for nucleic acid target described herein. The principle of our strategy for DNA hybridization assay was illustrated in Scheme 1. The noncovalent, label free fluorescent G4-MS was constructed based on the

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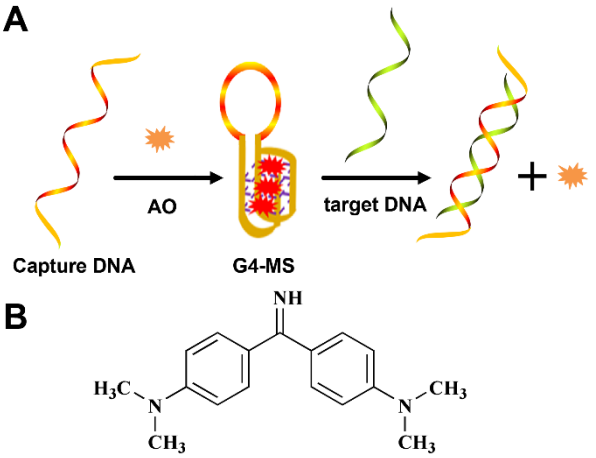

Scheme 1 (A) Schematic representation of the formation of G4-MS and the interaction between G4-MS and target DNA. (B) Chemical structure of AO.

following designs: (1) the label free nonstructured single-stranded DNA (ssDNA) has short G-rich segments both at 3´ and 5´ termini which can form the 3 quartets stem of G4-MS and 12 nucleotides in the middle of the ssDNA forming the loop of G4-MS that is specific to the target and serves as the capture probe; (2) AO as a novel G-quadruplex inducer and stabilizer whose fluorescence can be enhanced greatly via binding to G-quadruplex and serves as the signal transducer. The fluorescence of the reaction containing G4-MS was quenched intensively upon the addition of complementary target DNA (ctDNA) because the AO-G-quadruplex affinity is lower compared to that of the analyte-G4-MS complex, and the hairpin structure is converted to rigid DNA duplex species, releasing free AO, thus transduce the DNA hybridization into fluorescent signal and the fluorescence quenching of G4-MS can be effectively measured.

The optical properties with absorption of AO binding toward Human Telomeric G-quadruplex (htDNA) were firstly investigated. In the absence of htDNA in Tris-HCl buffer (10 mM, pH 7.4), AO showed a distinct absorption pattern with a maximum peak at 430 nm (Fig. S1). Upon gradual introduction of htDNA to the AO solution, the absorption maximum band of AO shifted bathochromically to 450 nm, an indication of strong interaction between AO and htDNA (Fig. S1B),[23] suggesting a π-π interaction between the guanine bases of G-rich DNA and AO.[23] It is known that certain monovalent cations (such as K^+, Na^+ and NH_4^+) can stabilize G-quadruplex, we rationalize that AO/G-quadruplex complex has a similar trend but with stronger change in its absorption spectra in the presence of potassium (Fig. S1A).

To investigate the interaction of AO and DNA with different conformational structures, we characterized the fluorescence properties of AO in the presence of different conformational DNA, ssDNA, dsDNA and G-quadruplex DNA under the identical experimental condition, respectively. As shown in Fig. 1A (inset), in the presence of ssDNA, the fluorescence of AO increased to a slight extent (ca. 15-fold, curve c) because the interaction is typically weak and only electrostatic in nature as reported.[29] The fluorescence of AO increased to a larger extent (ca. 26-fold, curve b) in the presence of dsDNA, which might be caused by the excimer formation similar to the observations made

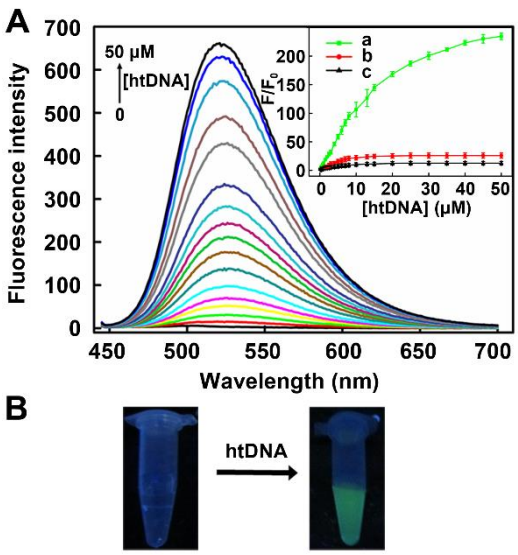

Fig. 1 (A) Fluorescence titration of 10 μM AO in Tris-HCl buffer solutions by different concentrations of htDNA in the presence of 50 mM K^+. Inset: Fluorescence intensity enhancement (F/F_0) of AO (10μM) at 515 nm plotted against different concentrations of ssDNA (c), dsDNA (b) and G-quadruplex (a), respectively. F and F_0 are fluorescence intensities of DNA solutions with and without htDNA, respectively. (B) Optical photographs recorded under 365 nm UV light irradiation for AO aqueous solution in the absence and presence of 10 μM htDNA, respectively.

by our lab and others with the dye Thioflavin T (ThT) and dsDNA.[30-32] The fluorescence of AO increased to a most remarkable extent (ca. 240-fold, curve a) in the presence of G-quadruplex, showing a strong interaction with the aid of K^+.

We then tested the fluorescence properties of AO and the AO-htDNA complex. Upon gradual addition of htDNA to the AO solution in the presence of monovalent cation (such as K^+), the emission intensity was remarkably enhanced and the intensity increases to a saturation level at [htDNA]/[AO] > 8 (Fig. 1A). A plot of F/F_0 versus [htDNA]/[AO] (where F and F_0 are emission intensities with and without htDNA) shows a fluorescence enhancement up to a 240-fold increase at [htDNA]/[AO] ratios > 8:1, indicating the high sensitivity of the measurements for AO and htDNA interaction. The remarkable enhancement of the emission intensity is a strong indication of the formation of AO and htDNA complex, suggesting that AO may work as the G-quadruplex inducer and stabilizer with the aid of the monovalent cation. Specifically, we here hypothesize that htDNA would form a G-quadruplex complex in the presence of AO, and the nonradiative channel in excited AO would be closed and the quantum yield of the confined AO would increase appreciably to become fluorescent because AO in the complex would be restricted to rotate freely.

With 365 nm UV illumination, the original non-emissive solution shows intense green fluorescence (Fig. 1B), indicating that AO can induce G-quadruplex formation even without monovalent cation. As far as we know, it is rare that small molecules can induce G-quadruplex formation without the aid of monovalent cation. As shown in Fig. S2, the fluorescence of AO/G-quadruplex complex in the absence of K^+ was indeed

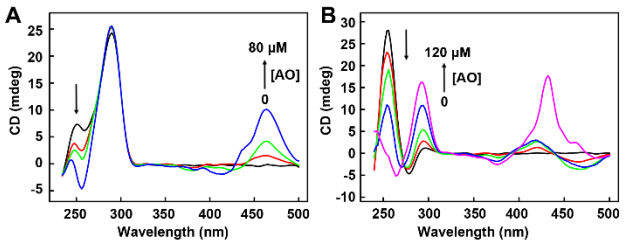

Fig. 2 CD absorption response of htDNA in Tris-HCl buffer upon the addition of AO in the presence (A) and absence (B) of 5mM K^+.

weaker than that with the presence of K^+, indicating that the higher increase in fluorescence emission observed upon K^+ addition might be due to the formation of more stable G-quadruplex in the presence of K^+ ions. These results further suggest that AO itself could be the G-quadruplex inducer and stabilizer even without the aid of monovalent cations, thereby enabling AO to serve as a "light-up" fluorescent binder and stabilizer of G-quadruplex.

To validate that the observed fluorescence increase is due to the formation of G-quadruplex structure of htDNA caused by AO, Circular Dichroism (CD) experiments were further conducted.[33,34] The G-quadruplex has characteristic patterns in CD spectra: for a parallel topology, there is a positive peak near 260-265 nm with a minimum one near 245 nm, whereas for an antiparallel topology, there are positive peaks at 245 nm and 295 nm with a minimum one around 260-265 nm. As observed in Fig. 2A, htDNA folded from the mixed (parallel and antiparallel) quadruplex into antiparallel quadruplex upon the addition of AO into htDNA solution in the presence of 5 mM K^+ and showed the typical positive CD peaks at 265 nm and 295 nm. However, in the absence of K^+, htDNA folded into the antiparallel topology upon the addition of AO into htDNA solution and showed positive CD peaks at 245 nm and 295 nm and a negative peak at 265 nm (Fig. 2B).

Taken together, the above data strongly suggest that G4-MS can be utilized in an efficient alternative approach for DNA hybridization assay. The potential application of this concept was further investigated by performing DNA titration experiments. Fig. 3A shows the quantitative detection of DNA by using G4-MS upon introducing different concentrations of complementary target DNA (ctDNA). With the titration of G4-MS with ctDNA, a substantial quench was observed by increasing the concentrations of ctDNA from 20 nM to 50 μM. The quenching efficiency was defined as $(F_0-F)/F_0$, where F_0 and F are the emission intensities of G4-MS without and with the analytes, respectively. The quenching efficiency decreased when the ctDNA concentration was higher than 50 μM and then reached a plateau (larger than 74%) beyond that point (inset in Fig. 3A), and higher ctDNA concentration produced only small intensity variation. This result suggests that the binding of target DNA by the loop sequence of G4-MS can unfold the stem structure through formation of a duplex species, and constitute the basis for fluorescent assay of DNA hybridization using this protocol. Quantification of ctDNA could be then estimated by measuring the relative emission intensities F_0/F. The linearity of the response calculated by the linear plot of relative emission intensities F_0/F versus ctDNA

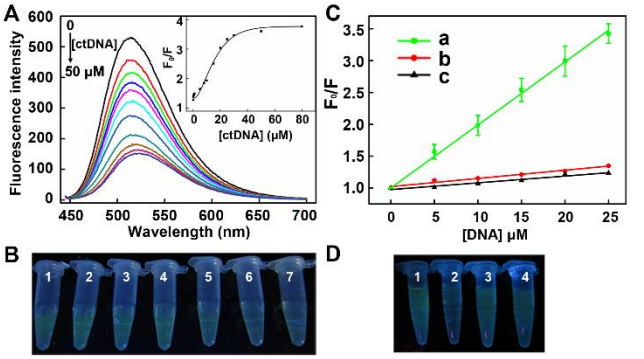

Fig. 3 (A) Fluorescence response of G4-MS at different target DNA concentration. Inset: The quenching efficiency with various concentrations of ctDNA. (B) Photographs of G4-MS illuminated with 365 nm UV light in the absence (1) and presence of different concentrations of ctDNA (from left to right, the concentrations of the ctDNA were 0 μM, 0.02 μM, 1 μM, 5 μM, 10 μM, 15 μM and 20 μM, respectively). (C) Selectivity of the sensing method against smDNA and random DNA at the different concentrations of target DNA (ctDNA, smDNA and rDNA). (D) Photographs of G4-MS illuminated with 365 nm UV light in the absence (1) and presence of ctDNA (2), smDNA (3) and rDNA (4) at the concentration of 15 μM.

concentration was in the range of 20 nM to 30 μM (r =0.993) and the limit of detection was 6 nM according to $3s_B$. This proposed method illustrated the feasibility for assay of DNA hybridization (Fig. 3A inset).

Moreover as demonstrated in Fig. 3B, the effect of different concentrations of ctDNA on the fluorescence of G4-MS could also be conveniently detected by the naked eye, which provided a facile method for visual detection of DNA. Representative photographs (Fig. 3B) were taken upon the illumination by 365 nm UV light of the G4-MS sample solution in the absence (left) or presence (right) of different concentrations of ctDNA (tubes 1-7 from left to right, the concentrations of the ctDNA were 0 μM, 0.02 μM, 1 μM, 5 μM, 10 μM, 15 μM and 20 μM, respectively). By addition of different concentrations of ctDNA, the fluorescence of G4-MS was substantially quenched. Such sensing platform generated bright green color emission that could be readily recognized by protected naked eye.

Three different target DNA sequences, i.e., the complementary target DNA (ctDNA), the single base-mismatched DNA (smDNA) and noncomplementary target DNA (random DNA, rDNA), were used to investigate the selectivity of the assay. Each of target DNA (ctDNA, smDNA and rDNA) was added in the reaction solution at the same concentrations respectively, and their corresponding optical changes were monitored. Fig. 3C shows the comparison of the G4-MS fluorescence decrease induced by ctDNA, smDNA and rDNA, respectively. As shown in Fig. 3C, emission intensities were reduced more greatly for different concentrations of ctDNA (Curve a) than noncomplementary (Curve c) or single base mismatch (Curve b). Fig. 3D gives the photographs of G4-MS illuminated with 365 nm UV light in the absence or presence of different targets at the concentration of 15 μM. These results clearly demonstrated that G4-MS still kept its high selectivity of hairpin structured probe for the object and was quite able to

discriminate mismatch DNA sequences even of a single base-difference.

To test the feasibility of the proposed strategy, the real-time fluorescence responses of G4-MS upon additions of subsequent ctDNA was recorded. As illustrated in Fig. S3, the fluorescence intensity of the sample containing G4-MS but without any addition of ctDNA was about 205 au. After 5 μMctDNA was introduced into the sample for 5 min, the fluorescence signal of the sample was dramatically reduced to about 90 au.

In summary, we for the first time demonstrated the ability of traditional fluorescent dye AO to bind and stabilize G-quadruplex selectively over ssDNA and dsDNA with enhanced fluorescence. Based on the high fluorescent complex of AO-G-quadruplex, we developed a novel, label-free, simple but specific fluorescent detection strategy for DNA detection. With a rational redesign, AO as a new G-quadruplex stabilizer and binder functioned to produce a fluorescent ensemble which worked as stem for the proposed hairpin structured molecular switch and provided efficient signal transduction once the fluorescent molecular switch binds to target DNA. By tuning the middle sequence (e.g. an aptamer for ATP) of the G4-MS, the proposed design could have general applicability and thus become a versatile detection scheme.

This work was supported by the National Science Foundation of China (21175028, 21375022, 21105063, 21175091), Innovation Scientists and Technicians Troop Construction Projects of Henan Province, Program for Innovative Research Team in Science and Technology in University of Henan Province (2012TRTSTHN018) and Natural Science Foundation of Henan Province (112300410297, 142300410314).

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