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"Molecular beacon"-directed fluorescence of Hoechst dyes for visual detection of Hg(II) and biothiols and its application for a logic gate†

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A molecular beacon (MB)-like DNA hairpin biosensor based on the reversible directing fluorescence of Hoechst dyes was developed for fluorescent detection of Hg^{2+} and biothiols and furthermore for logic operation.

Molecular beacons (MBs) are dually labeled single-stranded DNA (ssDNA), which are internally quenched (*i.e.* fluorescence resonance energy transfer, FRET) as a result of the proximity between a fluorophore (donor) and a quencher (acceptor) tagged at either end.[1] Traditional MBs have been employed in a wealth of applications, but always require dual labeling, which will inevitably result in some complicated or expensive operations.[2] Moreover, the quencher may not thoroughly quench fluorescence, which gives rise to a high background signal. To address this, in a series of studies, many new types of MB-like probes solely labeled with fluorophores at one end have been developed for detecting diverse targets, in which nanomaterials are used as quenchers.[3] However, this type of MB-like probe is totally dependent on the particular structural and photophysical features of nanomaterials. Therefore, it is highly desirable to exploit label-free fluorescent techniques. Recently, many research efforts have been devoted to the development of label-free and convenient methods for fluorescent detection of varied target molecules.[4] The label-free and straightforward fluorescent probes may offer certain advantages over traditional MBs, because they are simple, facile and economic without the need for covalent labeling of fluorophores/quenchers on the nucleic acids.

Hoechst dyes are part of a family of blue fluorescent dyes used as cell permeable nucleic acid stains.[5] The dyes can bind to the minor groove of double-stranded DNA (dsDNA) with a preference for sequences rich in adenine (A) and thymine (T). Although the dyes can bind to all nucleic acids, AT-rich dsDNA enhances fluorescence considerably.[6] This sequence-dependent fluorescence-enhancing property would provide potential to devise novel label-free probes upon integration with certain designed functional DNA sequences and their target molecules, however, to the best of our knowledge, there is no report on it. Thymine (T) has been show to be one of the most specific ligands for Hg^{2+}, forming a T-Hg^{2+}-T complex with strong affinity and high selectivity.[3c,d,7] Enlightened by the above facts, we envisaged that designed AT-rich ssDNA can be directed to form a stem-loop hairpin with an Hg^{2+}-induced T-Hg^{2+}-T complex, which can provide a medium for enhancing fluorescence of Hoechst dyes for label-free detection of Hg^{2+}. In this work, we report that the fluorescence of Hoechst dyes is lit up by addition of an Hg^{2+}–ATprobe hairpin (ATprobe: 5′-ATATTATTATCCCCCCT-TATTTTTTT-3′, which was predicted by the popular structure-prediction program Mfold, Table S1, ESI†). The specific interaction of Hoechst dyes with the Hg^{2+}–ATprobe hairpin leads to a considerable fluorescence enhancement, which is sensitive and selective to Hg^{2+}. Furthermore, we employed the Hg^{2+}–ATprobe–Hoechst complex as a novel detection system to sensitively and selectively monitor biothiols (Cys, GSH and Hcys), which act as a destroyer toward T-Hg^{2+}-T structure through thiol–Hg^{2+} interactions.[3c]

Scheme 1 presents the homogeneous strategy of using an Hg^{2+}–ATprobe hairpin to modulate the fluorescence of Hoechst dyes for the reversible fluorescent detection of Hg^{2+} and biothiols. Hg^{2+}-directed formation of a stem-loop hairpin provides a harbor to accommodate Hoechst dyes as signal moieties. The free Hoechst dye displays weak fluorescence, but underwent a marked fluorescence enhancement (*i.e.*, the fluorescence "on" state) upon binding to the hairpin-like probe resulting from the Hg^{2+}-induced conformational alteration in ATprobe. Besides, biothiols effectively removed Hg^{2+} from the complex of T-Hg^{2+}-T to destroy the hairpin-like structure, causing the Hoechst dyes switch to the fluorescence "off" state again. In this case, the ATprobe–Hoechst solution can be used as a selective indicator for Hg^{2+} and biothiols.

The circular dichroism (CD) measurement was firstly applied to test the conformational alteration in the ATprobe toward Hg^{2+} and following Cys (a model biothiol), respectively. Fig. S1A (ESI†) shows a significant change in the ellipticity (mdeg) of the ATprobe in the presence of Hg^{2+} resulting from altering the conformation of the ATprobe through the formation of T-Hg^{2+}-T complexes. A control experiment was also conducted to examine the CD spectra of the

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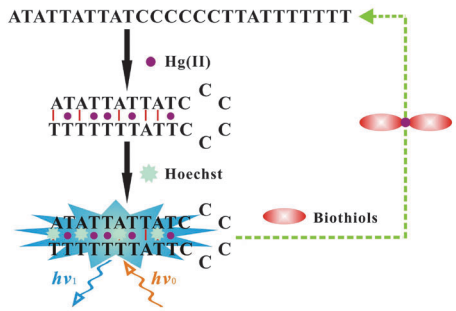

Scheme 1 Schematic representation of the use of an Hg^{2+}–ATprobe hairpin to regulate fluorescence of Hoechst dyes and its application for reversible fluorescent monitoring of Hg^{2+} and biothiols (Cys, Hcys and GSH).

PMprobe (a perfectly matched DNA hairpin) (Fig. S1A, ESI,† red line). Upon the following addition of Cys (Ser as a control), the former CD spectra of the ATprobe treated with Hg^{2+} caused the recovery to its default ellipticity in the absence of Hg^{2+} (Fig. S1B, ESI,† green line). And as expected, there is lack of signal change in the presence of Cu^{2+} for the ATprobe (Fig. S1C, ESI†). The above resultant shifts of CD spectra of the ATprobe toward Hg^{2+} and following Cys give direct evidence for our design principle.

Upon addition of an Hg^{2+}–ATprobe hairpin, the solution of Hoechst dyes shows a marvelous fluorescent enhancement compared to the sole presence of an ATprobe (Fig. 1A and Fig. S2, ESI†), which indicated that the fluorescence of these dyes is related to the Hg^{2+}-induced allosteric effect of the ATprobe. Considering the appreciable changes in fluorescence properties of ATprobe–Hoechst solution in response to Hg^{2+}, the potential of developing a novel fluorescent probe for determination of Hg^{2+} was assessed. We first optimized the optimal stoichiometric ratio of the ATprobe and Hoechst dyes for sensing Hg^{2+} (Fig. S3, ESI†). Unless noted otherwise, the following experiments were all carried out under the optimal condition (*i.e.*, 2 μM ATprobe and 0.01 mg mL^{-1} Hoechst dye used). As shown in Fig. 1A, upon addition of increasing concentrations of Hg^{2+} from one stock solution to the ATprobe–Hoechst solution, a visual readout can be obtained under the 365 nm UV lamp excitation. These results were further confirmed by fluorescence spectroscopy. A gradual increase in the fluorescent intensity (FI) was clearly detected with the addition of an increasing concentration of Hg^{2+} to the ATprobe–Hoechst solution (Fig. 1B). It can be seen that the FI value is sensitive to the concentration of Hg^{2+}, the fitting range is from 0 to 7 μM with a one-phase association equation ($Y = 0.15 + 0.97 \times [1 - \exp(-0.24X)]$, regression coefficient $R^2 = 0.99$, where Y is the FI ratio at 456 nm and X is the concentration of Hg^{2+}) (Fig. 1C). Using the ATprobe–Hoechst solution, Hg^{2+} could be detected at concentrations as low as 5 nM based on three times signal-to-noise level of the blank sample (3σ). This sensitivity is better than that reported previously using a FAM-labeled fluorescent probe (40 nM),[8] and shows the same performance as the sensitive electrochemical method (10 nM)[9] or the colorimetric method (10 nM).[7e] To test the selectivity, other metal ions (10-fold higher concentration) were examined under the same conditions as in the case of 1 μM Hg^{2+}. As shown in Fig. 1D and E, an obvious fluorescent enhancement of ATprobe–Hoechst solution can be obtained with the treatment of Hg^{2+} compared to other metal ions. We also studied the possible applicability of the sensor for the direct measurement of Hg^{2+} in real samples. The results are listed in Table S2 (ESI†). The recovery of the added known amounts of Hg^{2+} to the water samples measured by the sensor was in general larger

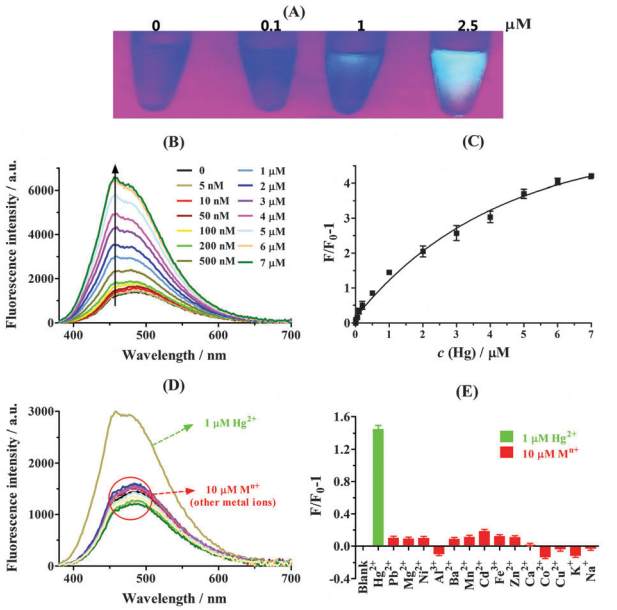

Fig. 1 (A) Visual detection of Hg^{2+} (0, 0.1, 1 and 2.5 μM) based on our proposed method. The picture was taken under a 365 nm UV lamp excitation using a digital camera. (B) Fluorescent response of ATprobe–Hoechst in solution upon addition of varied concentration of Hg^{2+} with an excitation wavelength of 360 nm. (C) Plot of the fluorescence ratios ($F/F_0 - 1$) *vs.* the increasing concentrations of Hg^{2+} of the same data. (D) Selectivity analysis for Hg^{2+} detection. Spectra represent the ATprobe–Hoechst in solution in the presence of 1 μM Hg^{2+} and 10 μM other metal ions including Pb^{2+}, Mg^{2+}, Ni^{2+}, Al^{3+}, Ba^{2+}, Mn^{2+}, Cd^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, K^+ and Na^+. (E) Bars represent the fluorescence ratios ($F/F_0 - 1$) ATprobe–Hoechst in solution in the presence of 1 μM Hg^{2+} and 10 μM other metal ions.

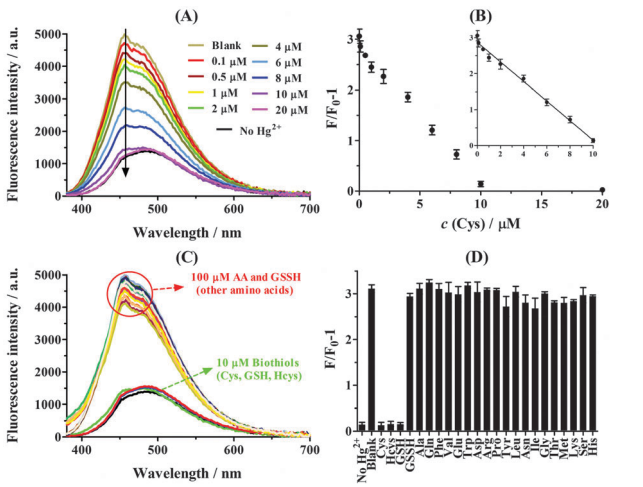

Fig. 2 (A) Fluorescence response of ATprobe–Hoechst–Hg^{2+} solution (5 μM Hg^{2+} added) to Cys. The fluorescence emission spectra are depicted for various Cys concentrations with an excitation wavelength of 360 nm. (B) Plot of the fluorescence ratios ($F/F_0 - 1$) *vs.* the increasing concentrations of Cys of the same data. Inset: magnification of the plot of the fluorescence ratios ($F/F_0 - 1$) corresponding to the Cys concentration in the range 0–10.0 μM. (C) Selectivity analysis for Cys detection. The fluorescence emission spectra are shown for Cys, Hcys, GSH, GSSH and other 19 amino acids. (D) Bars represent the fluorescence ratios ($F/F_0 - 1$) ATprobe–Hoechst–Hg^{2+} solution in the presence of 10 μM biothiols (Cys, Hcys, GSH), 100 μM GSSH, and 100 μM other 19 amino acids.

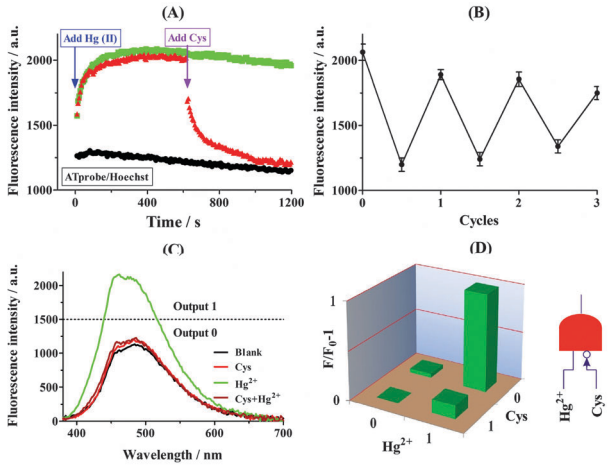

Fig. 3 (A) Time-course of the Hg^{2+}/Cys-facilitated reversible fluorescence changes in the ATprobe–Hoechst sensing system. (B) Reversible switching of the described sensing system between the ''on'' and ''off'' states through the addition of Hg^{2+} (0.5 μM) and Cys (1 μM). (C) Fluorescence spectra of the ATprobe–Hoechst sensing system in the presence of different inputs: Cys (0 μM) + Hg^{2+} (0 μM) (black line), Cys (1 μM) + Hg^{2+} (0 μM) (red line), Cys (0 μM) + Hg^{2+} (0.5 μM) (green line) and Cys (1 μM) + Hg^{2+} (0.5 μM) (brown line). (D) Fluorescence ratios ($F/F_0 - 1$) of the ATprobe–Hoechst sensing system in the presence of different inputs and an illustration of the INHIBIT logic gate.

than 95%, which indicated that the present method is promising in practical application with great accuracy and reliability.

In view of the ability of Cys to grab Hg^{2+} from T-Hg^{2+}-T complexes, we further investigated the feasibility of the quantitative detection of Cys on the basis of the above ATprobe–Hoechst solution containing Hg^{2+}. Fig. 2 illustrates that after adding Cys with increasing concentrations, a gradual FI decrease in the ATprobe–Hoechst–Hg^{2+} solution was observed. The results clearly verified the competition mechanism between the Cys and the ATprobe for Hg^{2+}. The detection limit was estimated to be 0.1 μM using ATprobe–Hoechst–Hg^{2+} solution. The ATprobe–Hoechst–Hg^{2+} platform is suitable for Cys detection against other chemically close amino acids sensitively and selectively (Fig. 2C and D). Apart from Cys, we also utilized the ATprobe–Hoechst–Hg^{2+} solution to monitor other biothiols including GSH and Hcys (Fig. S4, ESI†). In addition, we studied the possible applicability of the sensor for the direct measurement of Cys in real samples including urine and serum (Table S3, ESI†).

For an appealing generation of molecular logic systems, in recent years, various DNA logic gates have been engineered.[7f,10] In this work, the Hg^{2+}/Cys-facilitated fluorescence changes in the ATprobe–Hoechst sensing system can occur within 20 min (Fig. 3A) and reversible switching of the systems between the "on" and "off" states through the alternating addition of Hg^{2+} and Cys (Fig. 3B) can be observed. Additionally, the sensing system enabled the design of a DNA logic system based on Boolean logic. For input, we defined the presence of Hg^{2+} or Cys as 1 and their absence as 0. Fluorescence change of ATprobe–Hoechst was defined as the output (1 or 0) for the logic gate (Fig. 3C). The possible input combinations and output were listed and an illustration for the INHIBIT logic gate was presented (Fig. 3D).

In this study, we have developed a molecular beacon (MB)-like DNA hairpin biosensing platform using Hoechst dye as a signal indicator, and demonstrate its feasibility in the application of fluorescent detection of Hg^{2+} based on the Hg^{2+}-thymine chemistry and furthermore for biothiol monitoring. The emission from Hoechst dyes responded to the target molecule *via* the reversible structure change of the MB-like DNA hairpin. This novel "mix and measure" MB-based probe design offers many advantages, including simplicity of preparation and manipulation compared with other methods that employ specific strategies including tedious procedures,[7b,e,9] and the need for labels,[7b–d,f,8] *etc.* A Hoechst-indicated MB-based biosensing platform was developed for Hg^{2+} and biothiol recognition in a label-free format, and a new concept for performance of logic systems demonstrated with a Hg^{2+}/Cys-driven fluorescent DNA INHIBIT logic gate. This is the first application of "molecular beacon"-directed fluorescence of Hoechst dyes, and there is still much room for its further application upon integration with various technologies (*e.g.*, nanotechnology, aptamers, *etc.*).

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