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Label-free fluorescent assay of ATP based on an aptamer-assisted light-up of Hoechst dyes†

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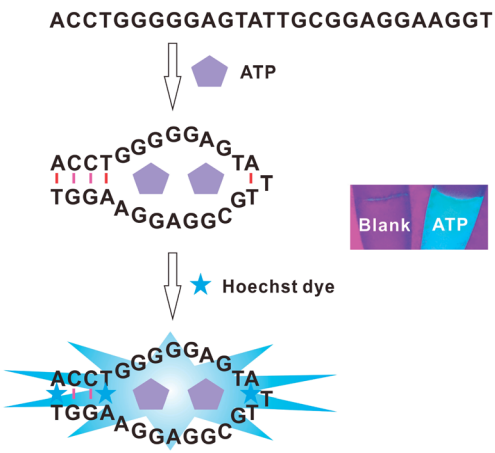
A label-free fluorescence assay was developed for light-up detection of ATP in a visual-readout format based on the aptamer-directing fluorescence of Hoechst dyes.

Hoechst dyes are a group of AT-selective minor groove binding blue fluorescent dyes which are cell permeable nucleic acid stains.[1] The fluorescence of Hoechst dyes can be enhanced considerably upon binding to AT-rich double-stranded DNA (dsDNA).[2] Some label-free probes have been developed based on this sequence-dependent fluorescence-enhancing property, where certain designed functional DNA sequences and their target molecules were incorporated.[3] Zhu *et al.* developed a label-free aptamer-based sensor for the detection of L-argininamide using Hoechst 33258,[3a] Sarpong and Datta demonstrated that nucleic acid-binding dyes (*e.g.* Hoechst 33258, *etc.*) can act as efficient indicators of aptamer–target interactions,[3b] and Zhou *et al.* devised an optical aptamer sensor for cocaine using minor groove binder (Hoechst 33342) based energy transfer.[3c] Aptamers are artificial functional nucleic acids (DNA or RNA) that are isolated through an *in vitro* selection process or systematic evolution of ligands by exponential enrichment (SELEX) to specifically bind to cells, proteins or low-molecular-weight inorganic or organic substrates.[4] Because of their specificity and good binding constants, aptamers have been used in the development of various assays for the detection of small molecules, heavy metals, proteins, cancer cells *etc.*[5]

Recently, our group revealed that a designed AT-rich ssDNA could provide a medium for enhancing the fluorescence of Hoechst dyes for label-free detection of Hg^{2+}.[3d] This was based on a Hg^{2+}-induced T–Hg^{2+}–T complex[6] to direct the designed AT-rich ssDNA to form a step-loop hairpin. Moreover, a novel graphene oxide (GO)-based fluorescent "on/off" switch was developed to visually "turn on" detection of sequence-specific DNA and "turn off" detection of exonuclease with sensitivity and selectivity in a single step in a homogeneous solution, utilizing "molecular beacon"-hosted Hoechst dyes (HMB) as signal indicators and GO as an excellent energy acceptor to efficiently quench the fluorescence of the HMB dyes in a label-free format.[7]

We describe herein our ongoing efforts to develop a facile and label-free assay for ATP, in which the aptamer-assisted light-up of Hoechst dyes acts as signal indicators (Scheme 1). As shown in Scheme 1, the ATP aptamer can assemble a hairpin-like structure when challenged with ATP (*i.e.* target-dependent adaptability of aptamers). ATP-directed formation of a hairpin-like structure (dsDNA with specific A–T base pairing) provides a docking site to accommodate Hoechst dyes as a signal moiety. The free Hoechst displays weak fluorescence, but undergoes a



Scheme 1 Schematic representation of the use of target–aptamer recognition to regulate the fluorescence of Hoechst dyes and its application for light-on detection of ATP.

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marked fluorescence enhancement upon binding to the hairpin-like probe with specific A–T base pairing resulting from the ATP-induced conformational alteration of its aptamer (Apt). In this case, the Apt–Hoechst solution can be used as a selective indicator for ATP.

As shown in Fig. 1, the as-prepared Apt–Hoechst solution exhibits fluorescence emission at 506 nm with excitation at 360 nm. However, in the presence of ATP, the fluorescence of the Apt–Hoechst solution was found to be enhanced. This is attributed to the ATP-mediated conformational alteration of its aptamer, resulting in its self-folding to facilitate the docking of Hoechst dyes into the resultant A–T base pairing, leading to considerably enhanced fluorescence. Considering the appreciable changes in fluorescence enhancement of the Apt–Hoechst solution by ATP, the potential of developing a novel fluorescent assay for the determination of ATP was assessed.

The different concentrations (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 7.5, 10.0 and 20.0 mM) of ATP from one stock solution were added to the Apt–Hoechst solution. A gradual increase in the fluorescence intensity (FI) was clearly detected with the addition of increasing concentrations of ATP to the Apt–Hoechst solution (Fig. 1A). The ATP-stimulated FI increase of the Apt–Hoechst solution was rapid. In the presence of 2 mM ATP, the assay exhibited a nearly saturated signal within 2 min (Fig. S1†), which is faster than previously reported using a silver nanocluster-based label-free fluorescence probe.[8] It can be seen that the FI value is sensitive to the concentration of ATP. The fitting range is from 0 to 20 mM with a Boltzmann sigmoidal equation ($Y = -5.276 + 14.623/[1 + \exp(1.940 - X)/3.254]$) and regression coefficient $R^2 = 0.997$, where Y is the FI ratio at 506 nm and X is the concentration of ATP. Additionally, a linear equation can be obtained from the concentration range of 0 to 5 mM ($Y = 1.106X + 0.102$, $R^2 = 0.993$) (Fig. 1B). Using the Apt–Hoechst solution, ATP could be detected at concentrations as low as 0.05 mM based on a three times signal-to-noise level of the blank sample (3σ). This detection limit was better than that previously reported using a silver nanocluster-based label-free fluorescence probe.[8]

To test selectivity, competing stimuli including CTP, GTP and UTP at different molar levels were examined under the same conditions as in the case of ATP (Fig. 2A). It was found that ATP results in an obvious change in the fluorescence of the Apt–Hoechst solution, while there was a nearly negligible fluorescent change in the presence of other stimuli. Moreover, as shown in Fig. 2B, upon addition of ATP and competing stimuli (CTP, GTP and UTP) to the Apt–Hoechst solution, a visual readout could be obtained under UV lamp excitation at 365 nm. These results were further confirmed by fluorescence spectroscopy (Fig. S2†). The results demonstrated the excellent selectivity of this approach applied for ATP detection over competing stimuli. Since there are many artificial functional nucleic acids (*e.g.* aptamers) that are reported to selectively bind with a wealth of targets, it can be envisioned that the proposed method in our work may have the potential to be applied widely, if AT-rich regions can be found or introduced into the sequence of functional nucleic acids.

As the evidence now seems strong that ATP is released in large amounts from urothelial cells in response to distention, stress, and inflammation,[9] urinary ATP quantification can be an

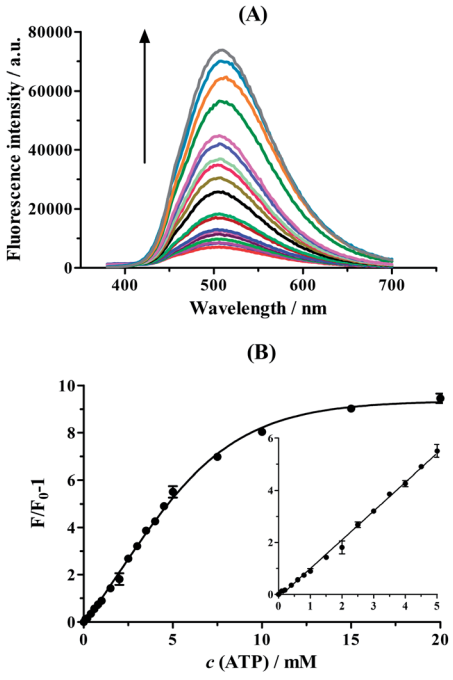

Fig. 1 The fluorescence response of an Apt–Hoechst solution to ATP. (A) The fluorescence emission spectra are shown for various ATP concentrations: 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 7.5, 10.0 and 20.0 mM; (B) the plot of the fluorescence ratio ($F/F_0 - 1$) *vs.* the increasing concentrations of ATP for the same data.

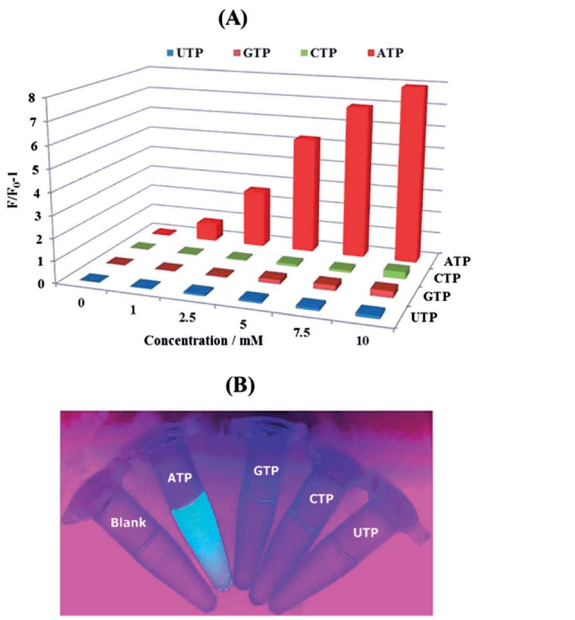

Fig. 2 Selectivity analysis for ATP detection. (A) The bars represent the fluorescence ratio ($F/F_0 - 1$) of the Apt–Hoechst solution for ATP, CTP, GTP and UTP with concentrations of 0, 1.0, 2.5, 5.0, 7.5 and 10.0 mM. (B) Visual discrimination of ATP, GTP, CTP and UTP (5.0 mM ATP and 10 mM competing stimuli) based on our proposed method. The picture was taken under handheld UV lamp excitation at 365 nm using a digital camera.

Table 1 Determination of ATP in real samples using the Apt–Hoechst solution

Detected (mM)	Added (mM)	Found (mM)	Recovery (%)
Not found	2.00	1.91	95.5
Not found	2.50	2.47	98.8
Not found	3.00	2.97	99.0

important issue in the diagnosis of urinary tract diseases. Therefore, we also studied the possible applicability of the sensor for the direct measurement of ATP in real samples. Under the experimental conditions, the proposed method was applied to analyze ATP in artificial urine samples. The results are listed in Table 1. The recovery of the added known amounts of ATP to the 50% artificial urine samples measured by the sensor was in general greater than 95%, which indicated that the present method is promising in practical applications with great accuracy and reliability.

In conclusion, we have successfully demonstrated that the aptamer-directing fluorescence of Hoechst dyes can be used as a label-free signal indicator for inexpensive, simple, rapid, and selective detection of ATP. This novel "mix and measure" label-free assay design offers many advantages, including simplicity of preparation and manipulation compared with other methods that employ specific strategies including tedious procedures, and the need for labels, *etc.*

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