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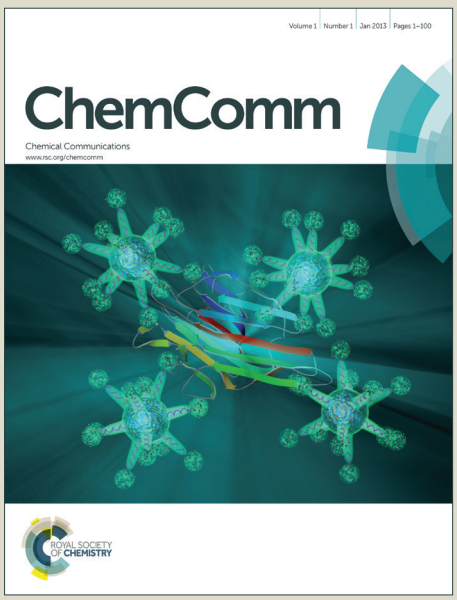

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Triplex molecular beacon for sensitive recognition of melamine based on abasic-site-containing DNA and fluorescent silver nanoclusters

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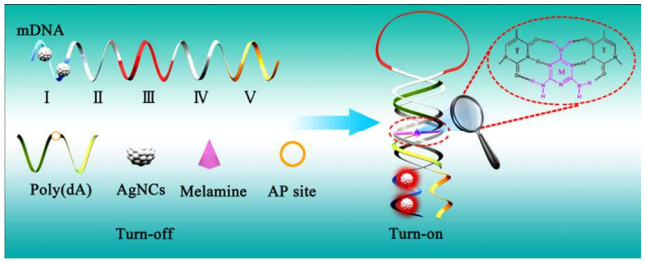
A melamine aptamer derived from abasic-site-containing triplex molecular beacon (tMB) was designed and developed for sensitive recognition of melamine by integrating tMB and fluorescent silver nanoclusters (Ag NCs).

The study on molecular recognition of biomolecules can contribute greatly to the understanding of cellular processes, clinical diagnosis and drug screening. In the past decades, aptamers have been developed using systematic evolution of ligands by exponential enrichment (SELEX) technique for specific recognition of biomolecules including small molecules, proteins, cells, etc.[1, 2] The recent advance using an abasic site (AP site) in DNA or RNA duplexes as an active cavity to design aptamers has opened a new avenue for selective molecular recognition.[3, 4] AP sites, one of the most common forms of DNA damages, can be artificially incorporated into DNA or RNA duplexes to provide a hydrophobic vacancy for a ligand to bind the nucleobase through pseudo-base pairing. The fluorescence of the label-free ligands can increase or decrease upon the binding, which can be utilized for recognition of nucleic acids and small molecules based on selective base recognition or competitive strategies.[5] This concept avoids the use of large pool of nucleic acids for aptamer selection and provides a label-free fluorescent method for biosensor development.

Molecular beacons (MBs), which are hairpin-like DNA structures with a fluorophore and a quencher at their termini, have attracted much attention in the application of nucleic acid analysis with the characteristics of convenience and high sensitivity.[6] However, the dual-labeling procedure is expensive and time-consuming, thus severely limits the optimization of MBs sequences for achieving high selectivity and sensitivity. To alleviate this problem, To alleviate this problem, couples of novel strategies have been testified for MB design, including single-labeled MBs, label-free MBs, etc.[7] Recently with the development of triplex technology,[8] this issue can be addressed by the introduction of triplex-forming oligonucleotides (TFOs) in the stem of MBs to form triplex MBs (tMBs), since the stability of the triplex stem can be modulated by adjusting the length of non-labeled TFOs, and thereafter the detection performance can be optimized with the use of only one dual-labeled sequence.[7a] On the other hand, organic fluorophores labeled often suffer from the problem of photobleaching. To overcome the limitation, fluorescent nanomaterials were utilized as candidates in the labeling of MBs with the advancement of nanotechnology. Particularly, fluorescent nanoclusters with different functions have been actively applied in various chemical and biological detections. [9]



Scheme 1 A fluorescent turn-on strategy for recognition of melamine based on AP site-containing tMB and DNA-templated silver nanoclusters.

Melamine, a toxic contaminant for food and water sources, has gained increased attention in recent years. Biosensors for sensitive detection and accurate quantification of melamine are highly desired.[10] Herein, a strategy for sensitive and specific melamine recognition was developed with the combination of tMB aptamer and fluorescent nanoclusters. As shown in Scheme 1, to design the aptamer, an AP site was introduced into a short poly(dA) sequence. A 58 nt oligonucleotide designated as mDNA are composed of five parts and region II and IV can hybridize with poly(dA) sequence to form a hairpin-like triplex through Watson-Crick and Hoogsteen base pairing of thymine (T) and adenine (A). Melamine can locate in the AP site by hydrogen bonds interaction between thymine and melamine. By shortening the length of poly(dA) sequence, the stability of the triplex was weakened. However, it can be enhanced upon the binding of melamine. In such a manner, melamine-modulated structure transition allows sensitive detection of melamine. Different from the AP site-based duplex aptamers previously reported which utilized fluorescent ligands,[3-5] here we employed DNA-templated silver nanoclusters (DNA/AgNCs) as signal indicators. DNA/AgNCs were synthesized in the cytosine (C)-rich sequence on one terminal of mDNA (region I). They displayed weak initial fluorescence emission which was significantly enhanced with proximity to guanine (G)-rich overhang fragment (region V)

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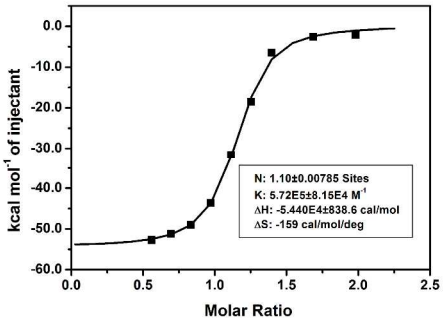

Fig. 1 Isothermal calorimetric titration for melamine binding to tMBs. A solution of melamine (2 mM) was added to the DNA solution (150 μM) buffered at pH 7.4 (10 mM PB, 2 mM $Mg(NO_3)_2$, 100 mM $NaNO_3$). Temperature was 20 °C.

located on the other end of mDNA as a consequence of triplex formation. In this way, a label-free and turn-on strategy for recognizing melamine was successfully developed. The fluorescent assay exhibited the advantage of high sensitivity and cost-effective. Moreover, it is also applicable for the recognition of other molecules if the sequences of tMB are rationally designed.

Isothermal titration calorimetry (ITC) experiment was firstly performed to characterize the binding of melamine to tMB by titrating melamine into the DNA solution. To ensure an efficient binding of melamine to triplex, a short mDNA-s sequence (5′-*TTTTTTTTTT*CAGGCA*TTTTTTTTTT*-3′, italic bases constitute the stem of tMB) without the appended tails for fluorescence sensing was used to form triplex with a long poly(dA) sequence of 11A (5′-AAAAAXAAAAA-3′, X denotes the AP site of spacer C3). A notable exothermic heat of the binding reaction was recognized from the ITC titration. The associated heats were plotted in Fig.1 against the molar ratios of melamine to tMB and the solid line in the figure denotes the calculated fits of data assuming a single set of identical sites. A binding constant of $(5.7\pm0.8)\times10^5$ can be obtained from the fitting, indicating a high binding affinity of tMB aptamer for melamine. Therefore, melamine-modulated tMB formation was utilized for the study of melamine recognition.

mDNA sequence (5′-**CCCTTAATCCCC***TTTTTTTTTT*CAGGCA*TTTTTTTTTT*GGGTGGGGTGGGGTGGGG-3′, italic bases denote region II and IV constituting the stem of tMB with poly(dA); bold bases denote region I for Ag NCs synthesis; underlined bases denote region V for fluorescence enhancement of Ag NCs) with two appended fragments on the terminals was utilized for Ag NCs synthesis. Fig.2A shows the TEM image of the Ag NCs prepared by using mDNA as template. Ag NCs exhibited uniform dispersion with averaged diameter around 2 nm. Since the sensing strategy is based on the formation of triplex hybridized by mDNA-Ag and poly(dA) sequence with melamine, it is important to optimize the length of poly(dA) sequence containing AP site in order to get a sensitive response. We designed five poly(dA) sequences of different lengths from 11 adenines to 7 adenines and examined their fluorescence response in the absence and presence of melamine, respectively. As shown in Fig.2B-2F, the initial fluorescence emission of mDNA-Ag itself was relatively weak. With the addition of poly(dA) with different lengths, fluorescence intensity increased in different

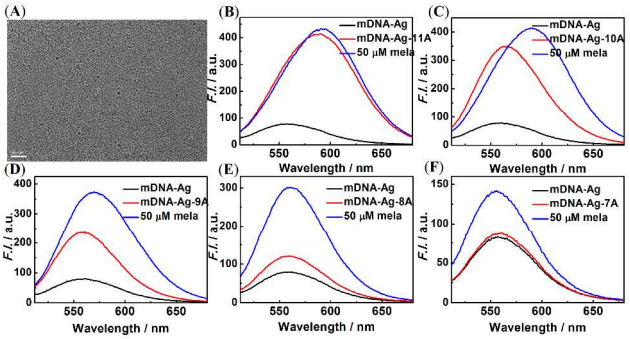

Fig. 2 (A) TEM image of the mDNA-Ag NCs. Scale bar: 50 nm. (B-F) Fluorescence spectra of mDNA-Ag (black line), mDNA-Ag and poly(A) sequences of different length from 11A to 7A in the absence (red line) and presence of 50 μM melamine (blue line). Fluorescence were monitored at excitation wavelength of 492 nm at 20 °C. Conditions: 1 μM mDNA-Ag and poly(A); buffer solution: 10 mM PB, 2 mM $Mg(NO_3)_2$, pH 8.

extent with the greatest increment for sequence 11A and the smallest for sequence 7A. Longer poly(dA) sequence tended to bind mDNA-Ag with higher affinity. Therefore, sequence 11A can hybridize with mDNA-Ag even in the absence of melamine to form triplex. By contrast, the shortest sequence 7A can't induce the structure transition of mDNA-Ag. However, upon the addition of melamine with the same concentration, there was only a slight increase in the fluorescence intensity for sequence 11A but the biggest enhancement for sequence 8A. In the presence of melamine, sequence 7A leaded to fluorescence enhancement but the extent was smaller than that of sequence 8A. Moreover, along with the trends of fluorescence intensity increment, we noticed a red shift of the emission peaks in different degrees upon the formation of triplex. This phenomenon was in good agreement with the literature report.[9d] It could be most likely attributed to the different distance between G-rich part and DNA/Ag NCs. By comparison, we used sequence 8A which showed lower affinity to mDNA-Ag but the highest response for melamine sensing for further experiments.

Circular dichroism (CD) spectra, which can reflect the change of DNA structure and conformation, was measured to confirm the formation of tMB. As shown in Fig.3A, melamine as a small molecule had no CD signal, and the CD spectrum of mDNA displayed a positive band and a negative band centered at 275 nm and 240 nm respectively due to the spatial conformation of single oligonucleotide. Upon the addition of sequence 8A, the negative band at 240 nm increased and showed red shifts of 5 nm. A new negative peak appeared nearby 210 nm, which has been considered as a characteristic feature of triplex formation.[11] This indicates that some of 8A sequences can hybridize with mDNA to form tMB in the absence of melamine, and it is in accordance with the slight augment of fluorescence upon the addition of sequence 8A to mDNA-Ag. When melamine was added, the intensity of the characteristic negative peak at 210 nm increased. Meanwhile, the negative band at near 245 nm increased and the positive band at about 275 nm decreased slightly. The change of DNA conformation in the system of mDNA/8A suggests that triplex MBs can form in higher degree in the presence of melamine by the interaction of mDNA with sequence 8A and melamine. This was confirmed by the observation that the fluorescence of DNA/Ag NCs was enhanced significantly by

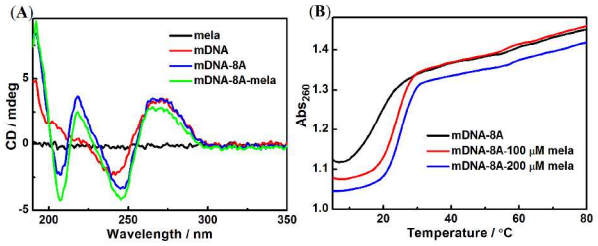

Fig. 3 (A) CD spectra of melamine (black line), mDNA (red line), and mixture of mDNA and sequence 8A in the absence of melamine (blue line) and presence of 75 μM melamine (green line). Concentrations of DNA were 5 μM. (B) UV melting curves of mDNA/8A complex (2 μM) in the absence (black line) and in the presence of 100 μM (red line) and 200 μM (blue line) melamine, respectively. UV absorbance was measured at 260 nm in a solution containing 10 mM PB (7.4), 2 mM $Mg(NO_3)_2$ and 100 mM $NaNO_3$.

proximity to G-rich part induced by melamine. Taken together, the CD and fluorescence data consistently verified the formation of tMB in the presence of melamine and supported the sensing principle of melamine by our strategy.

To evaluate the contribution of melamine to the stability of triplex, the UV melting curves of complexes were recorded and the results were shown in Fig.3B. All three curves were monophasic, indicating direct melting of the looped triplex to its constituent single-strand DNAs, i.e., mDNA and 8A. mDNA/8A complexes showed T_m value of 18.5 ±0.4°C, indicating low thermal stability of mDNA/8A and most of them were dissociated at 20°C. This was consistent with the fluorescence data that there was a little increase of fluorescence when sequence 8A was added into mDNA-Ag solution at 20°C. The melting temperature of mDNA/8A complex in the presence of 100 μM melamine increased up to 23.8±0.2°C, 5.3°C higher than the T_m of the complex in the absence of melamine, suggesting that melamine was vital for triplex formation and increased the stability of triplex significantly. Increasing melamine concentration further to 200 μM, the T_m increased correspondingly to 25.1±0.2°C. All the results proved that the fluorescence enhancement in this strategy was caused by the binding of melamine and the consequent tMB formation.

We then investigated the melamine recognition at the optimized condition (see Fig. S1, ESI for optimization). The fluorescence response and fluorescence intensity changes (F/F_0) upon the addition of melamine are shown in Fig. 4, where F and F_0 represent for the fluorescence intensities of mDNA-Ag/8A in the presence and absence of melamine, respectively. The system had a sensitive response to 0.05 – 200 μM of melamine. In contrast, negligible fluorescence intensity change was detected after addition of different concentration of melamine to mDNA-Ag in the absence of sequence 8A (Fig 4B). These results confirmed that the increase of fluorescence in this system was ascribed to the formation of tMB hybridized by mDNA-Ag and 8A in the presence of melamine. Despite the absence of covalent interaction between melamine and mDNA/8A, melamine bound to mDNA/8A with high affinity by pseudo-base pairing with two thymine bases opposite the AP site via three hydrogen bonds (Scheme 1), resulting in the sensitive response for melamine. In addition, we investigated the selectivity of the proposed method towards melamine detection, which involved several structural analogs of melamine and some other potential interfering

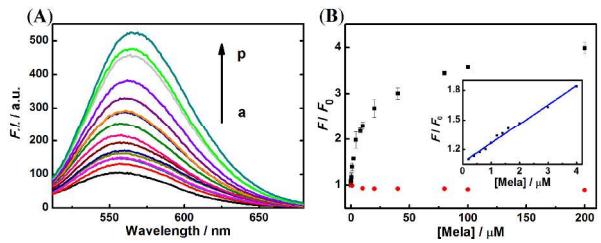

Fig. 4 (A) Fluorescence emission spectra of tMB hybridized by mDNA-Ag and sequence 8A at 20°C in the presence of various melamine concentrations (from **b** to **p)**: 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 8, 10, 20, 40, 80, 100, 200 μM. Curve **a** (black line) represents the fluorescence of mDNA-Ag. (B) The relationship between the fluorescence intensity and the concentration of melamine (black, square). Control experiment (red, round): response of melamine without sequence 8A. Inset is the linear response of melamine at low concentrations.

substances in real sample analysis (Fig. S2, ESI). Compared with the significant fluorescence increase for melamine addition, there were only negligible changes for other species, indicating the high selectivity for melamine. Furthermore, the stability of DNA/Ag NCs (mDNA-Ag) was also studied (Fig. S3, ESI). No significant changes (<5%) were observed for both the fluorescence emission of mDNA-Ag itself and its response to melamine for at least 5 days, confirming good stability of DNA/Ag NCs system.

Melamine contamination in foods can cause malnutrition or even severe kidney damage. Therefore, sensitive detection of melamine becomes urgent and vital in food industry. We applied this strategy to detect melamine in milk sample. By performing standard addition experiments, good recoveries were obtained (Table 2, ESI), indicating that the method is promising in real sample analysis.

In summary, we have designed an aptamer for melamine based on abasic-site-containing tMB, and then constructed a label-free and turn-on strategy for melamine analysis with the signaling of DNA-templated Ag NCs. The triplex structure provided an appropriate site for melamine binding with high binding affinity, thus allowing rational design of melamine sensing strategy. The binding of melamine resulted in great fluorescence enhancement of Ag NCs appended on the tMB. All results consistently confirmed that the new platform can be used for melamine recognition with high sensitivity, and implied it was of good potential for melamine analysis in real samples. The label-free and turn-on strategy is also promising in future for recognition of other molecules if the tMB sequences are rationally designed.

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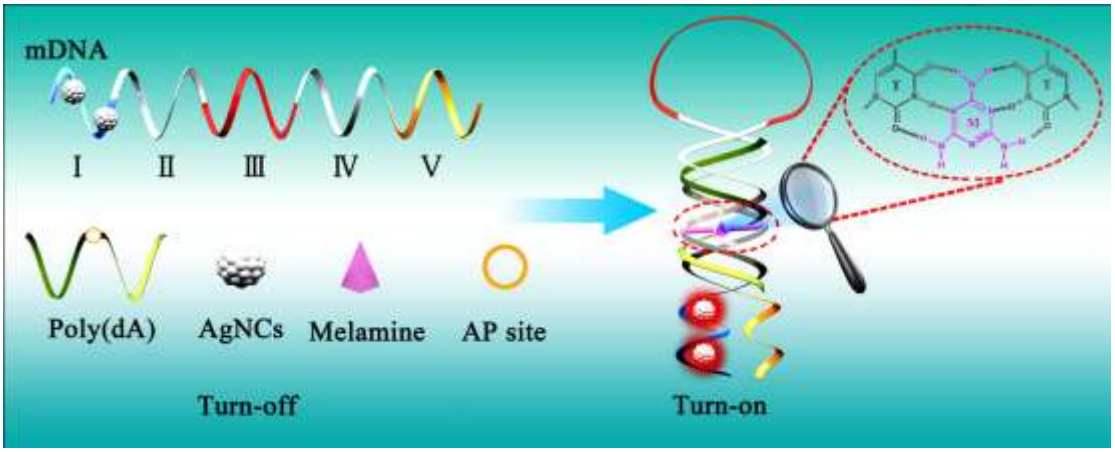

A label-free and turn-on strategy based on triplex molecular beacon for fluorescence melamine recognition with the signaling of Ag NCs was developed.