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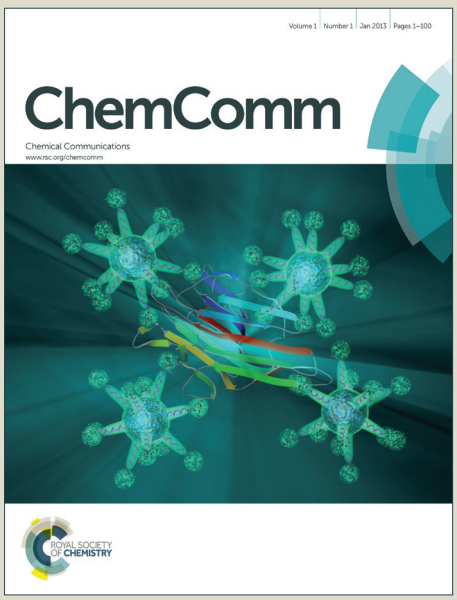

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ARTICLE TYPE

A new mode to light up adjacent DNA-scaffolded silver probe pair and its application for specific DNA detection

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Via fluorescence enhancement of proximity-dependent DNA-scaffolded silver nanocluster probe pair and exonuclease III-mediated signal amplification, we present a new fluorescence turn-on mode and apply for specific DNA detection.

Recently, molecular-scale noble-metal nanoclusters (*e.g.*, Ag, Au, Cu, and Pt), consisting of several to tens of metal atoms, have drawn much attention in the fields of chemistry, biology, and materials.[1] In contrast to nanoparticles (sizes greater than ~2 nm), noble-metal nanoclusters with dimensions (sizes smaller than ~2 nm) comparable to the Fermi wavelength of conduction electrons, exhibit strong light absorption through electronic transitions between molecule-like energy levels and possess the appearance of size-dependent strong fluorescence emission upon photoexcitation in the UV-visible range.[2] As promising substitutes for organic dyes and quantum dots, they have gradually become as a novel type of robust fluorophores characterized due to the attractive features of excellent brightness and photostability, subnanometer size, good biocompatibility, high quantum yield, and water solubility.

Among these nanoclusters, fluorescent silver nanoclusters (AgNCs) have received an explosion of interest, especially water-soluble DNA-scaffolded fluorescent AgNCs (DNA/AgNCs). Since DNA/AgNCs are highly fluorescent, more photostable, and cost-effective to synthesize, they have found in a wide range of applications such as cellular imaging, biological labelling, and chemical/biological sensing. In a typical synthesis protocol, DNA/AgNCs are facilely produced at room temperature via sequentially mixing silver nitrate ($AgNO_3$), cytosine-rich oligonucleotide, and a reducing reagent of sodium borohydride ($NaBH_4$),[3] resulting in bright fluorescence emission throughout the visible to near-infrared range by simply programming the template sequences.[4] Usually, these highly fluorescent DNA/AgNCs have been directly utilized as fluorophore labels in many biosensor designs.[5] However, Yeh and co-workers recently reported an interesting phenomenon that darkish DNA/AgNCs could be transformed into bright red-emitting clusters with ~500-fold fluorescence enhancement when placed in proximity to guanine-rich (G-rich) DNA sequence. [6] On the basis of this discovery, a few fluorescence turn-on biosensors have been developed by manipulating the distance between the DNA/AgNC and G-rich DNA. [7]

Inspired by the fluorescence light-up phenomenon of DNA/AgNC via hybridization upon G-rich DNA activator, herein we demonstrated a novel fluorescence turn-on mode based on the adjacent DNA/AgNC probe pair. We found that there is a stronger fluorescence enhancement when placing two darkish DNA/AgNCs together to form a probe pair through their complementary linkers than that of placing darkish DNA/AgNC close to G-rich DNA activator. As a proof-of-principle work, we took advantage of this interesting feature and applied for fluorescence turn-on detection of sequence-specific DNA via combining exonuclease III (Exo III)-aided target recycling strategy for signal amplification.

The working principle of the proposed assay is depicted in Fig. 1. The reaction system consists of hairpin-shaped Probe A, linear Probe B, and Exo III. Probe A is designed with three components, a nucleic acid segment (denoted in brown) complementary to the target DNA at 3' terminus, a nucleic acid segment (denoted in green) for hybridization with partial Probe B, and an AgNC-nucleation segment (denoted in red) at 5' terminus. Probe B is designed with another AgNC-nucleation segment (denoted in purple) and a short nucleic acid segment (denoted in yellow) complementary to partial Probe A (green part). It should be noted that Probe A is stable in the presence of Exo III due to a rational design of an Exo III-resistant 3'-protruding terminus. Exo III is a multifunctional exonuclease, which can specifically catalyse the stepwise removal of mononucleotides from the blunt or recessed 3'-hydroxyl terminus of a duplex DNA, and is practically inactive towards duplex DNA with protruding 3' terminus (more than 4 nt) or single-strand DNAs.[8] Due to its unique enzymatic property, Exo III is utilized to achieve selective enzymatic digestion of Probe A to destroy its stem-loop structure upon the hybridization with target DNA and to realize the signal amplification in this work. In the absence of target DNA, Probe A remains stable stem-loop structure and its product Probe A/AgNC can't effectively form a probe pair with Probe B/AgNC since the complementary segment in Probe A (green part) is blocked by stable self-hybridization. Thus, the reaction solution exhibits weak fluorescence signal from the darkish AgNCs. In the presence of target DNA, Probe A and target DNA form a double-stranded structure with unique characteristic of 3'-blunt terminus at Probe A and 3'-overhang terminus at the target DNA. Exo III specifically recognizes the duplex region, and then selectively digests the Probe A from its 3' terminus, liberating the intact target DNA. The released target DNA hybridizes with a new Probe A to trigger another Probe A-digestion cycle. After enzymatic reaction, the resultant Probe A is

used as template to synthesize AgNC, which exhibits weak fluorescence intensity. Upon addition Probe B-templated AgNC (Probe B/AgNC), as-prepared Probe A/AgNC is linked with Probe B/AgNC together to form a probe pair through the complementary sequences (denoted in green and yellow), resulting in a bright fluorescence emission in solution. Correspondingly, the produced fluorescence signal is directly proportional to the amount of target DNA present, thus the target DNA of interest can be quantitatively determined.

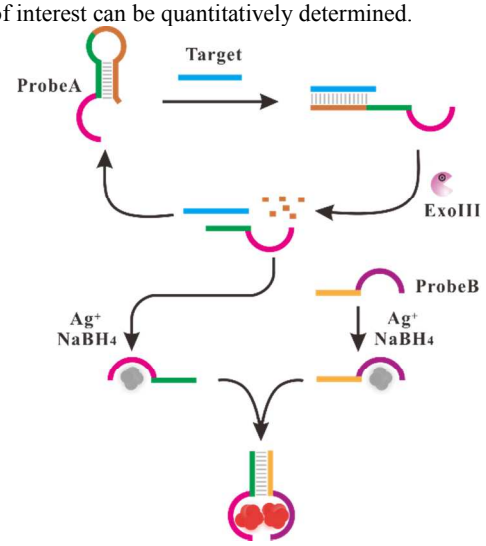

Fig. 1 Schematic illustration of the working principle of the proposed proximity-dependent darkish DNA/AgNC probe pair for fluorescence turn-on detection of sequence-specific DNA via coupling Exo III-assisted signal amplification strategy.

Prior to specific DNA detection, we first performed a series of control experiments to verify that adjacent darkish DNA/AgNC probe pair has a stronger fluorescence enhancement than G-rich-proximity-induced fluorescence activation. According to the published AgNC-nucleation sequences in the reported literatures,[7d,9] five probe (named as Probe 1, Probe 2, Probe 3, Probe 4, and Probe 5) were rationally designed (see Table S1 in ESI for the detailed sequences). Probe 1, Probe 2, Probe 3, and Probe 4, each containing a AgNC-nucleation sequence[9] and a complementary linker sequence for the formation of probe pair, were used as templates to synthesize DNA/AgNCs. Probe 5 contains a reported G-rich sequence [6] and a complementary linker sequence. As described in Fig. S1 and S2 in ESI, two experimental conditions for the preparation of proximity-dependent DNA/AgNC probe pair were investigated, including reaction buffer for DNA/AgNC synthesis and incubation time for the formation of DNA/AgNC probe pair. Briefly, the aqueous DNA/AgNC solution was prepared by adding $AgNO_3$ to the tested probe containing AgNC-nucleation sequence in MOPS buffer followed by reduction with $NaBH_4$. The as-prepared DNA/AgNCs were then mixed together and kept in the dark at room temperature for 80 min for the formation of probe pair. As expected, the AgNCs scaffolded by Probe 1, Probe 2, Probe 3, and Probe 4, all displayed very weak fluorescence emission with excitation at 560 nm, respectively (dotted curves, Fig. 2). We studied the fluorescence enhancement by placing the as-formed DNA/AgNC in proximity to the specific enhancers. Consistent with previous findings,[6] the fluorescence of Probe 1/AgNC solution upon the addition of Probe 5 could be enhanced with a bulk ratio over 500-fold due to G-rich overhang-activated fluorescent AgNCs (green curve, Fig. 2). Meanwhile, a brighter red fluorescence emission from Probe 1/AgNC solution was also clearly observed when adding Probe 2/AgNC solution (red curve, Fig. 2). The fluorescence intensity of the Probe 1/AgNC and Probe 2/AgNC probe pair was increased ca. 3-fold than that of Probe 1/AgNC and Probe 5. A similar phenomenon was also observed in AgNC probe pair made from Probe 3/AgNC and Probe 4/AgNC, which contained different AgNC-nucleation sequences (blue curve, Fig. 2). In addition, the as-formed proximity-dependent DNA/AgNC probe pair was characterized by TEM. As shown in Fig. S3, the size of DNA/AgNC probe pair was obviously larger than DNA/AgNC alone, which provided the supporting evidence of the formation of adjacent DNA/AgNC probe pair. At present, we can't fully explain the fluorescence enhancement mechanism on the adjacent darkish DNA/AgNCs. We hypothesize that it is possible due to the surface-enhanced fluorescence on metal nanoclusters, which needs further verification.

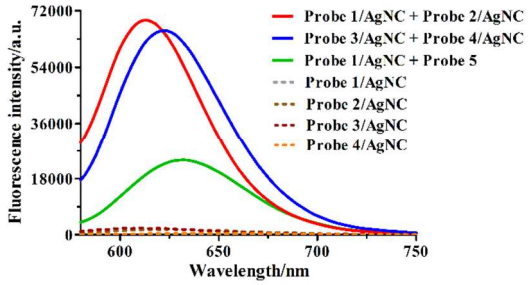

Fig. 2 Investigation on the fluorescence enhancement of darkish DNA/AgNC using different proximity modes, including hybridization with G-rich overhang, and the formation of proximity-dependent DNA/AgNC probe pair. The fluorescence emission spectra were recorded from 580 nm to 750 nm under excitation at 560 nm.

To demonstrate the feasibility of the proposed Exo III-aided DNA detection assay via proximity-dependent DNA/AgNC probe pair, we selected a synthetic DNA sequence (named as T1, see Table S1 in ESI) as a model to optimize the experimental conditions. Three crucial factors including the stem length in Probe A, the amount of Exo III, and Exo III-mediated enzymatic reaction time were investigated. We found the hairpin-shaped probe with 9-bp stem, 10 U Exo III, and 100 min of Exo III-assisted reaction time provided the best performance for the specific T1 detection (see Fig. S4, Fig. S5, and Fig. S6 in ESI for detailed information). Under the optimum experimental conditions and according to the experimental procedures described in ESI, we investigated the sensitivity of the proposed method by detecting T1 at different concentrations. As shown in Fig. 3A, a gradual increase in the fluorescence emission spectrum is clearly observed with an increase in T1 concentration from 10 nM to 600 nM. Figure 3B inset illustrates that the fluorescence intensity at peak value of 610 nm exhibited a good linear relationship with T1 concentration in the dynamic range from 40 nM to 200 nM. The fitting equation is $Y = 79.54\ X + 2401.8$, with a correlation coefficient R^2 of 0.9911. These results confirm that our proposed method has the potential for quantitative detection of DNA target. In addition, we spiked different amounts of T1 to test the accuracy of the proposed method. For an unknown

concentration of sample, the concentration was calculated from the above linear equation. The results are summarized in Table S2, which shows that recoveries were in the range of 94.47~103.05%.

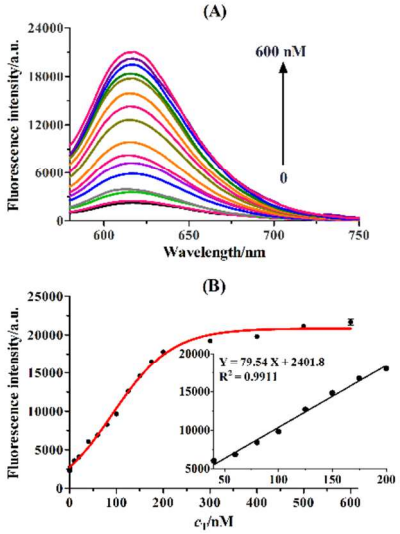

Fig. 3 Sensitivity investigation of the proposed method. (A) Fluorescence emission spectra responses to excitation at 560 nm in the presence of T1 at different concentrations (0, 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, 300, 400, 500, and 600 nM). (B) Plot of the relationship between the fluorescence intensity at the peak value of 610 nm and T1 concentration. Inset shows the linear part from 40 nM to 200 nM.

We further conducted the study on the sequence-specificity of the proposed method. Four kinds of targets including the perfect match target (T1), 1-base mismatch target (T2), 2-base mismatch target (T3), and 3-base mismatch target (T4) were selected (see detailed sequence information in Table S1 in ESI). Each target was tested at two concentrations of 20 nM and 100 nM. Fig. 4 exhibits the comparison result of (F/F_0–1) values from four-tested target DNAs. It is observed that only the perfectly complementary T1 target gave the highest values. These results clearly suggested that the proposed assay developed in this work allows a good sequence specificity to discriminate single nucleotide polymorphism.

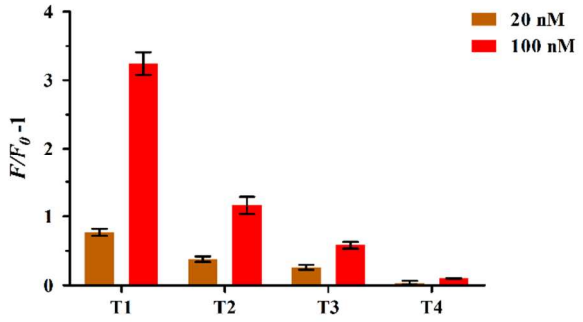

Fig. 4 Sequence-specificity investigation of the proposed method upon the different DNA targets. Bar representing fluorescence ratio (F/F_0–1) values from the different inputs of T1, T2, T3, and T4 with the same concentration of 20 nM and 100 nM, respectively. The (F/F_0–1) values were employed to evaluate the performance, where F and F_0 are the fluorescence intensities at 610 nm obtained from the presence and the absence of tested DNA targets, respectively.

In summary, we for the first time have demonstrated a new fluorescence enhancement mode based on adjacent DNA/AgNC probe pair. Compared to the G-rich overhang-proximity-induced fluorescence activation, our reported fluorescence light-up DNA/AgNC probe pair displays a better performance on fluorescence enhancement with a brighter fluorescence emission. Coupling Exo III-mediated signal amplification strategy, we successfully applied such an adjacent DNA/Ag NCs fluorescence light-up system for the specific DNA detection with good selectivity and sensitivity. We believe our findings expand the future applications of DNA/AgNCs and provide a new concept for development of fluorescence turn-on biosensors.

Notes and references

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