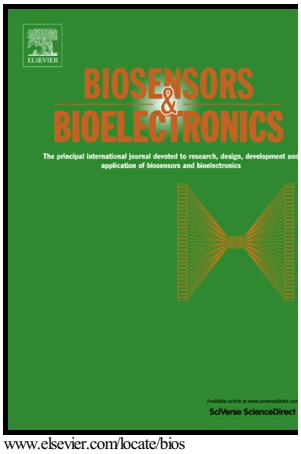

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A Label-Free Fluorescent Molecular Beacon Based on DNA-Ag Nanoclusters for the Construction of Versatile Biosensors

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Abstract

In this paper, we developed a simple, low-cost and sensitive DNA sequences detection biosensor based on a label-free molecular beacon (MB) whose DNA hairpin structure terminal has a guanine-rich sequence that can enhance fluorescence of silver nanoclusters (Ag NCs). Without hybridization between hairpin probe and target DNA, the Ag NCs presented bright fluorescence for the proximity of guanine-rich sequences (GRSs). After binding with target DNA, the hairpin shape was destroyed which results in a decrease of the Ag NCs fluorescence intensity. With this biosensor, we detected three disease-related genes that were the human immunodeficiency virus (HIV) gene, hepatitis B virus (HBV) gene and human T-lymphotropic virus type I (HTLV-I) gene. The detection limits based on S/N of 3 were 4.4 nM, 6.8 nM and 8.5 nM for HIV gene, HBV gene and HTLV-I gene, respectively. Our sensor was also of high selectivity and could distinguish even one nucleotide mismatched target.

Keywords: DNA detection, Silver nanoclusters, Molecular beacons

1. INTRODUCTION

Molecular beacons (MBs) are hairpin-shaped nucleic acid probes with fluorophore and quencher at two ends of the probes, which become fluorescent upon binding to a complementary sequence or target resulting in the disruption of the stem−loop structure and an increase in separation distance between the dual labels(Tyagi and Kramer 1996). Since their discovery in 1996, MBs have presented great activity in various applications, such as DNA and RNA detection (Tyagi and Kramer 1996; Tyagi et al. 2000; Zhang et al. 2001), the monitoring of living systems (Bratu et al. 2003; Medley et al. 2005; Perlette and Tan 2001; Santangelo et al. 2004; Tyagi et al. 1998), the investigation of enzymatic processes (Biggins et al. 2000; Li et al. 2000b; Tang et al. 2003; Tang et al. 2005), the design of biosensors (Fang et al. 1999; Li et al. 2001), the study of protein–DNA interactions (Fang et al. 2000; Li et al. 2000a), and the fabrication of biochips (Fang et al. 1999; Wang et al. 2002; Yao and Tan 2004). However, they still present some challenges, for example, conventional MBs usually are required to be labeled with two non-native moieties (Bonnet et al. 1998; Bonnet et al. 1999; Yang et al. 2005), leading to not only a high cost of running but also potentially more complex processes such as separation and purification. Furthermore, their sensitivity is also limited due to the incomplete quenching (Tyagi et al. 1998). To overcome these shortages, DNA-template silver nanoclusters have been developed as a new class of fluorophores (Guo et al. 2009a; Gwinn et al. 2008; Han and Wang 2012; LisaáPhipps 2012; Petty et al. 2011; Petty et al. 2004; Richards et al. 2008; Sengupta et al. 2008; Sharma et al. 2010; Vosch et al. 2007; Zheng et al. 2007) that have been utilized in the analysis of various biological molecules (Guo et al. 2009a; Guo et al. 2009b; Lan et al. 2011; Lan et al. 2010; Yang and Vosch 2011; Zhou et al. 2011).

Silver nanoclusters (Ag NCs) as a form of novel nanomaterials have attracted great attentions for their small sizes and novel features (Lu and Chen 2012; Shang et al. 2011; Wilcoxon and Abrams 2006). They have been shown to have great superiorities over common organic dyes and quantum dots, including high photostability, subnanometre size, non-toxicity and remarkable biocompatibility (de Souza 2007; Lin et al. 2009; Vosch et al. 2007). In recent years, oligonucleotide-template Ag NCs have caught many eyes for their facile synthesis, tunable fluorescence emission, and high photostability (Richards et al. 2008; Sharma et al. 2010). They are used to analyze thiol compounds (Huang et al. 2011), metal ions (Guo et al. 2009b; Lan et al. 2010; Wilcoxon and Abrams 2006), proteins (Sharma et al. 2011), and single-nucleotide mutation (Guo et al. 2009a). Werner and coworkers discovered an interesting phenomenon that the fluorescence of DNA−Ag NCs can be enhanced when Ag NCs were in proximity to guanine (G)- or thymine (T)-rich DNA (Yeh et al. 2010). On the basis of this, certain “turn-on” fluorescent assays for biologic molecules detection (Li et al. 2012; Yeh et al. 2010) and logic gates (Huang et al. 2012; Li et al. 2013) have been developed and constructed. This “turn-on” phenomenon may provide the possibility for analysis of a broader class of targets through a variety of methods.

In this study, we report a facile and versatile label-free fluorescent molecular beacon based on a DNA-Ag nanoclusters probe to detect disease-related genes. The probe is tagged with DNA template Ag NCs and guanine-rich sequences (GRSs) at two terminals serving as signal reporter with a loop whose sequence was complementary to target DNA. In the absence of target DNA, the probe has a hairpin-shape leading to the enhancement of Ag NCs fluorescence because the Ag NCs is in proximity to GRSs. After adding target DNA, the hairpin loop opens which keeps Ag NCs away from GRSs. This results in the decrease of fluorescence. To test the applicability of our approach, we choose three disease-related genes, the immunodeficiency virus gene (HIV), Hepatitis B virus gene (HBV) and human T-lymphotropic virus type I (HTLV-I), as our target.

2. EXPERIMENTAL

2.1.Materials and Reagents

All DNA samples were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China), and their sequences are listed in Table S1. They were stocked in pure water and quantified using UV-vis absorption spectroscopy with the following extinction coefficients ($\varepsilon_{260\ nm}$, $M^{-1}\ cm^{-1}$) for each nucleotide: A = 15 400, G = 11 500, C = 7400, T = 8700 (Li et al. 2011; Zhang et al. 2011). Silver nitrate ($AgNO_3$) and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were all of analytical reagent grade and used as received. Milli-Q water (18.2 MΩ) was used throughout. UV-vis absorption spectroscopy was recorded on a Cary 500 Scan UV-vis-NIR spectrophotometer (Varian). Fluorescence emission spectra were performed at room temperature on a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., France). The fluorescence spectra were recorded at room temperature by irradiating at 563 nm.

2.2.Preparation of Fluorescent Silver Nanoclusters (Ag NCs)

The 50 μM solution of hairpin probes were diluted with sodium phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4, 100 mM CH_3COONa, 5 mM $Mg(CH_3COO)_2$, pH 7.5) and annealed to room temperature for 1 hour after being heated at 90 ℃ for 10 minutes. A 1 mM solution of $AgNO_3$ was then added into the hairpin probe. After 0.5 h at room temperature, 1mM $NaBH_4$ was added into the mixture followed by vigorous shaking for 1 min. Final concentrations were 2 μM in hairpin probe, 12 μM in $AgNO_3$, and 12 μM in $NaBH_4$. The reaction mixture was kept in the dark at room temperature for 4 h before use.

2.3.Detection of Target DNA

In a typical DNA assay, the prepared 2 μM Ag NCs were hybridized with different concentrations of target DNA in sodium phosphate buffer at 37 ℃ for 2

hours. The final concentration of hairpin probe was 0.2 μM. To investigate the selectivity of our probe, the prepared 2 μM Ag NCs were mixed with 50 μM target DNA or their corresponding analogs in sodium phosphate buffer. The mixtures were hybridized at 37 ℃ for 2 hours. To optimize the number of base pairs in the hairpin probe stem, target DNA with final concentration of 50 μM was added to 2 μM hairpin probes and incubated at 37 ℃ for 2 hours. The final concentration of all hairpin probes and target or their corresponding analogs for all tests but typical assays was 0.2 μM and 0.5 μM.

3. RESULTS AND DISCUSSION

Scheme 1 demonstrates the principle of DNA analysis. When adding target DNA (T), the hairpin loop opens driving Ag NCs away from GRSs. This leads to the decrease of the original enhanced Ag NCs fluorescence intensity. As shown in Fig. 1, with the absence of target DNA, the probe with G rich overhang (P) presents a bright emission band at 623 nm when excited at 563 nm. This is much higher than that without G-rich overhang (C) due to the proximity of GRSs. After hybridizing with target DNA, the Ag NCs fluorescence fades as the probe and target forms a double strand (P-T) which induces the Ag NCs and GRSs to move away from each other. Since the probe C had no G rich overhang, when hybridized with target and formed a double strand (C-T), the Ag NCs fluorescence changes little which may be caused by the change of Ag NCs micro environment.

Scheme 1.

Fig. 1.

To optimize the DNA probe design, we investigate the Ag NCs fluorescence intensity and the fluorescence signal-difference-to-signal (ratio of the signal difference and the signal) with different stem lengths. As shown in Fig. 1B, the fluorescence intensity and S/N (signal to noise ratio) increase with the length of hairpin stem. With more bases in the stem, the hairpin structure is more stable and Ag NCs becomes much closer to GRSs. This results in a brighter fluorescence emission. In summary, the probe with 6 base stem is chosen as our test probe.

Fig. 2.

To detect the immunodeficiency virus (HIV) gene as a model, different concentrations of target HIV DNA sequence are mixed with 0.2 μM hairpin probe solution. As expected, we find that the original enhanced fluorescence is weakened when increasing the concentration of target HIV gene (Fig. 2). According to the results of fluorescence spectra, in the absence of target DNA the Ag NCs fluorescence presents a bright red emission at 623 nm. Upon the addition of an increasing amount of target DNA, the fluorescence intensity decreases gradually. Fig. 2B shows the relationship between the target DNA concentration and fluorescence intensity, indicating a dependence of Ag NCs fluorescence on target DNA concentration. From this figure, we observe that the fluorescence decreases significantly as target HIV DNA concentration increases from 10 nM to 2 μM. The inset shows the calibration curve for quantitative study of target DNA, the fluorescence intensity is linearly

dependent on the target DNA concentration in the range from 10 nM to 200 nM (R=0.999). The limit of detection is estimated to be 4.4 nM based on S/N of 3. Each datum was the mean of three replicates (N=3) and the error bars represent the standard deviations of the measurements.

Fig. 3.

To examine the selectivity of the sensing system, similar DNA strands with only one (T1), two (T2), four (T4) nucleotide mismatch and the completely (Ta) mismatched DNA strands are also investigated. The sequences are shown in Table S1. Fig. 3 shows the normalized Ag NCs fluorescence intensity of probe, target DNA and other mismatched DNA strand. The normalized fluorescence intensity of the target HIV gene decreases significantly compared to the probe. This is because T can hybridize with the probe and switch off the hairpin loop which forces the Ag NCs to separate from the GRSs. In contrast, other mismatched DNA leads to the decrease in fluorescence to a lesser degree. The result shows that our approach can be used to identify specific DNA, even distinguishes at one nucleotide mismatched level.

To demonstrate that our sensing system can be applied to detect a wide range of disease-related DNA, we design the part of the probe for target hybridization to analyse hepatitis B virus (HBV) gene and human T-lymphotropic virus type I (HTLV-I) gene. As shown in Fig. S2 and Fig. S4, it is found that the Ag NCs fluorescence intensity decreases gradually with the increasing concentrations of target HBV and HTLV- I DNA. The fluorescence has a response range from 5 nM to 2 μM. The inset plots the calibration curve for quantitative study of target DNA. The fluorescence intensity decreases linearly over the target DNA concentration range from 5 nM to 200 nM, with the linear correlation coefficient of 0.995 for HBV gene and 0.990 for HTLV-I gene. The detection limit is estimated to be 6.8 nM and 8.5 nM for HBV and HTLV-I based on S/N of 3, respectively. Each datum was the mean of three replicates (N=3) and the error bars represent the standard deviations of the measurements.

Fig. S3 and Fig. S5 reveals the selectivity of the probes and the effects of hairpin stem on the analysis of HBV and HTLV-I gene. As shown in Fig. S3A and Fig. S5A, the complementary target DNA can reduce the fluorescence intensity remarkably. However other similar DNA strands have less effect than complementary target. All these results suggest that the hairpin-guanine-rich probe shows a high selectivity. From the results in Fig. S3B and Fig. S5B, to achieve the maximum difference after the addition of target, with the consideration of the fluorescence intensity of silver clusters, 5 base pairs in the stems are optimized for both two hairpin probes. Under the influence of certain complex factor, such as the lengths of stem, the loop sequences and the unhybridized nucleotide residues, these two detection systems present different performances on the changes of the stem lengths of the probe.

4. CONCLUSIONS

Reason

In summary, we develop a facile and versatile label-free fluorescent molecular

beacon to analyse disease-related DNA based on GRSs enhanced Ag NCs fluorescence changes from the hybridization of target and molecular beacons. Using this method, HIV gene, HBV gene and HTLV-I gene are detected with a limit of detection of 4.4 nM, 6.8 nM and 8.5 nM, respectively. Our probe shows a high selectivity towards the target HIV, HBV and HTLV-I DNA over other similar strands. Comparing the Ag NCs fluorescence intensity and changes of the analysis system, hairpin probes with 6 or 5 base pairs in the stem are optimized for the detection of target DNA. Our method can provide a sensitive and selective platform for DNA analysis, which has a wide applicability through changes in the hybridization part with target DNA.

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Scheme Titles

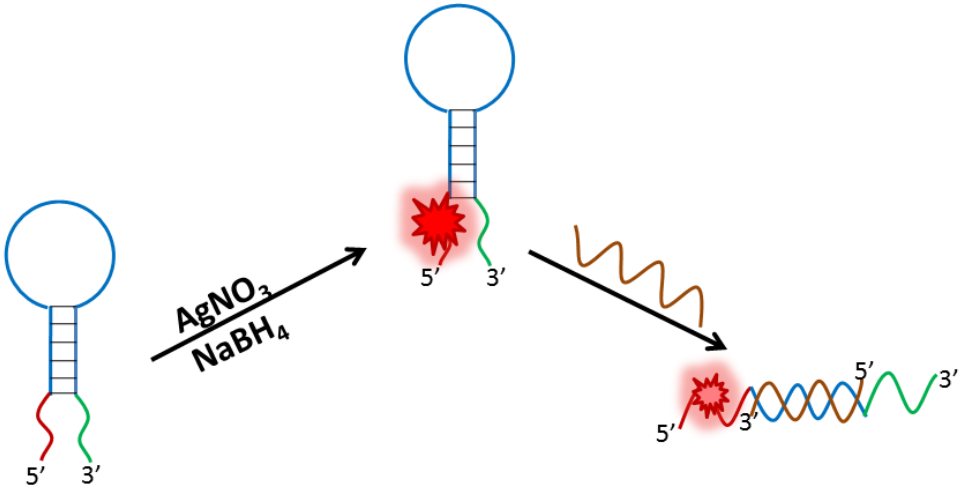
Scheme 1. Schematic representation of analysis of DNA based on the hybridization of target and molecular beacons induced fluorescence changes of Ag NCs whose fluorescence was enhanced by G-rich sequences.

Figure captions

Fig. 1. (A) Fluorescence responses of 0.2 μM probes with/without guanine-rich sequences before/after addition of 0.5 μM target HIV gene. (B) Stem sequence optimization of hairpin probe for HIV analysis. F_0 is the fluorescence intensity of P. F is the fluorescence intensity of P-T. Pn represents that there are n bases in the probe stem. The heights of the bars represent the average of three replicates (N = 3), the error bars represent one standard deviation from the average.

Fig. 2. Analysis for HIV gene: (A) Fluorescence responses to the addition of various concentrations of target DNA. (B) The dependence of the fluorescence intensity on the DNA concentration. The inset shows the linear dependence of fluorescence intensity on the DNA concentration, the linear range is from 10 nM to 200nM. Each datum was the mean of three replicates (N=3) and the error bars represent the standard deviations of the measurements.

Fig. 3. Selectivity of the hairpin probe (0.2 μM) for analysing target HIV DNA T (0.5 μM), over other mismatched DNA: T1 (one-base mismatched target), T2 (two-base mismatched target), T4 (four-base mismatched target), and Ta (absolutely mismatched target). The heights of the bars represent the average of three independent measurements (N = 3), the error bars represent one standard deviation from the average.



name	Sequences(5′-3′)
Ag NCs template	CCCTTAATCCCC
Guanine-rich sequences	GGGTGGGGTGGGGTGGGG
HIV gene	GCTAGAGATTTTCCACACTGACT
HBV gene	AGTTACTCTCTTTTTTGCCTTCTGA
HTLV-I gene	GAGTATTTGCGCATGGCCTGGAGGA

Scheme1.

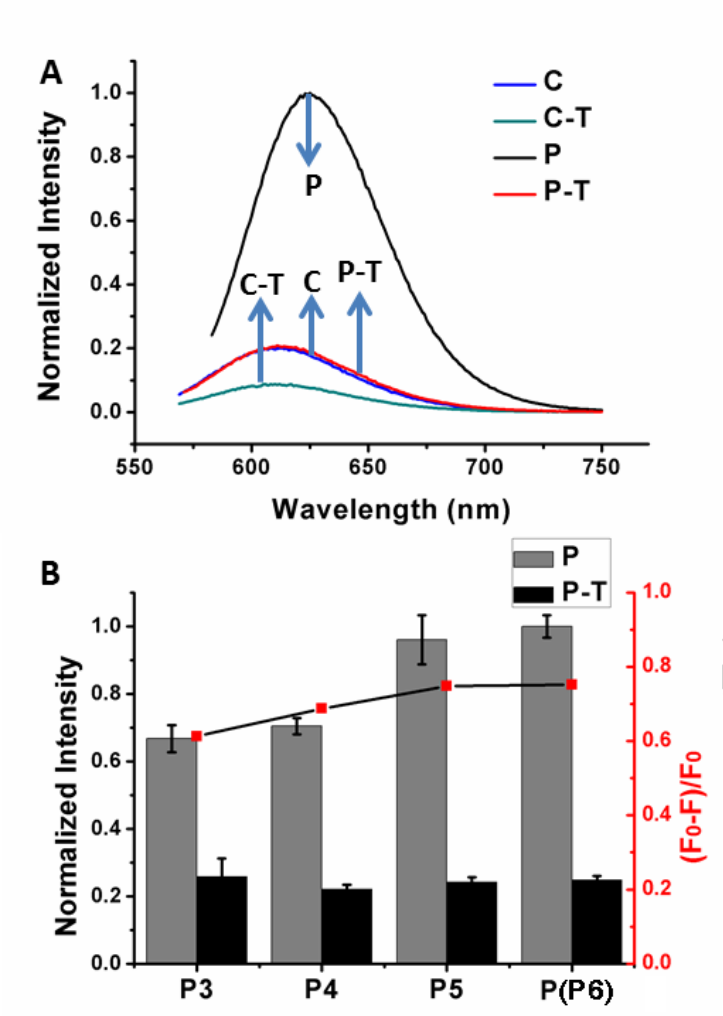


Fig. 1

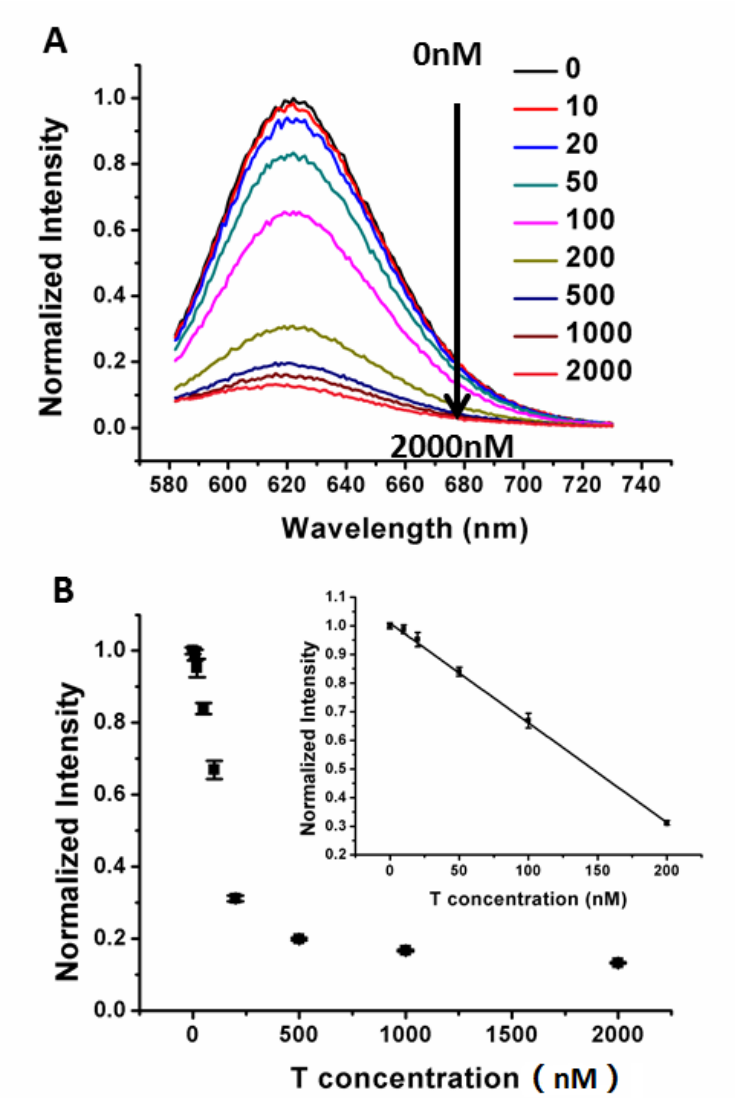


Fig. 2

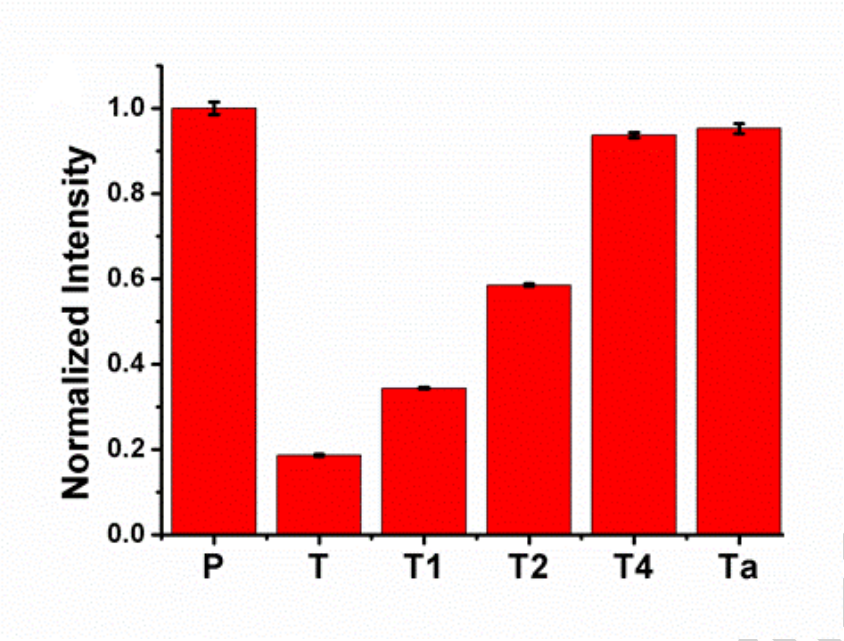


Fig. 3

Highlights

1) We developed a DNA sequences detection biosensor based on a label-free molecular beacon (MB) whose DNA hairpin structure terminal has a guanine-rich sequence that can enhance fluorescence of silver nanoclusters (Ag NCs). DNA template Ag NCs serving as signal reporter can simplify the operation and reduce the cost which is caused by the employment of fluorescent label.
2) We choose three different disease-related genes that are the human immunodeficiency virus (HIV) gene, hepatitis B virus (HBV) gene and human T-lymphotropic virus type I (HTLV-I) gene as our detected target.
3) The method has a detection limit as low as nanomolar.
4) The method shows high selectivity and can distinguish one nucleotide mismatched target.