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Label-free fluorescent molecular beacon based on a small fluorescent molecule non-covalently bound to the intentional gap site in the stem moiety

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ABSTRACT

A label-free fluorescent molecular beacon (MB) based on a fluorescent molecule, 5,6,7-trimethyl-1,8-naphthyridin-2-ylamine (ATMND) which is non-covalently bound to the intentional gap site in the stem moiety of the label-free MB, was developed. In the absence of a cDNA, ATMND fluorescence is significantly quenched because it binds to the unpaired cytosine at the gap site by hydrogen bonding. As a result, the label-free MB shows almost no fluorescence. Upon hybridization with cDNA, the label-free MB undergoes a conformational change to destroy the gap site. This results in an effective fluorescent enhancement because of the release of the ATMND from the gap site to the solution. Fluorescence titration shows that ATMND strongly binds to the cytosine at the gap site ($K_{11} > 10^6$). Circular-dichroism spectroscopy indicates that the binding of ATMND at the gap site of the stem moiety does not induce a significant conformational change to the hairpin DNA. Under optimal conditions, the fluorescent intensity of the label-free MB increases with an increase in cDNA concentration from 50 nM to 1.5 μM. A detection limit of 20 nM cDNA was achieved. A single mismatched target ss-DNA can be effectively discriminated from cDNA. The advantage of the label-free MB is that both its ends can be left free to introduce other useful functionalities. In addition, the label-free MB synthesis introduced in this paper is relatively simple and inexpensive because no label is required.

1. Introduction

The sequence-specific detection of DNA hybridization has attracted considerable interest in a wide range of areas, including molecular diagnostics, environmental monitoring, and antibioterrorism [1]. Because of the continued demand for sensitive and selective DNA probes, many kinds of DNA probes have been developed in recent years through various molecular-engineering strategies. These include adjacent probes [2], TaqMan probes [3], linear binary fluorescence resonance energy transfer (FRET) probes [4], the nucleic acid stain SYTO [5], GFP-fused RNA-binding proteins [6], quenched autoligation probes [7], and molecular beacons (MBs) [8,9]. Among these DNA probes, MBs have attracted much attention because of their stability, unique functionality, and molecular specificity [10,11].

Conventional MBs are single stranded (ss) oligonucleotide probes that possess a stem-and-loop structure (also called hairpin structure) [12]. The loop portion of an MB is complementary to a target ss DNA, whereas the stem is formed by 5–7 bp from two complementary arm sequences that are on either end of the MB. A fluorophore is attached to the end of one arm, while a quencher is attached to the end of the other arm. The stem maintains a close proximity to the two moieties, causing the fluorescence to be quenched by FRET. When a MB hybridizes with its cDNA, it undergoes a spontaneous conformational reorganization with the opening of the stem, leading to fluorescent restoration. Their unique target recognition and signal transduction capabilities have led to applications in many biochemical and biological assays, including quantitative PCR [13], protein–DNA interactions [14,15], multiplex genetic analysis [16], and mRNA detection in living cells [17]. Since the first appearance of MBs in 1996, they have been continuously improved. The new types of MBs that have been developed are wavelength shifting [18], enzymatic amplification [19], and electrochemical signal transduction [20]. Novel fluorophores and quenchers, such as gold nanoparticles [21], superquenchers [22], pyrene derivatives [23], conjugated polymers [24], and Cu^{2+} complexes [25], are also becoming increasingly attractive for various applications. Recently, fluorescent hairpin

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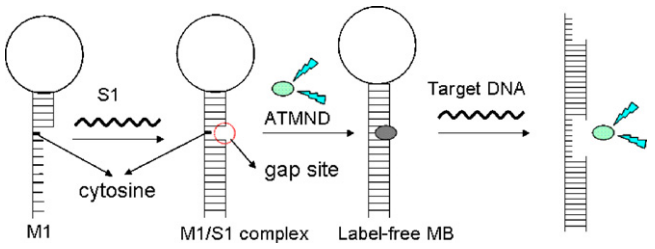

Scheme 1. Schematic illustration of the detection of a target DNA using a label-free MB (named GSMBs). The small fluorescent molecule (ATMND) was bound to the unpaired base (cytosine here) at a gap site in the stem moiety of a hairpin DNA (M1/S1 complex). It formed the label-free MBs accompanied by fluorescent quenching of ATMND. Upon hybridization to the target DNA, fluorescence was restored because of conformational reorganization that forces the stem apart and releases ATMND to the solution.

oligonucleotides, called "quencher-free MBs (QF-MBs)", have been found to function as MBs even without the attachment of additional quencher moiety [26–28]. The QF-MB fluorescence is quenched by nucleobases, and this is called "nucleobase quenching or base quenching" [29]. An important advantage of mono-labeled MBs or free-labeled MBs is that either one or both ends are free for further modifications [30]. While the resulting sensor systems are, in many cases, highly sensitive and even allow the detection of a single nucleotide mismatch, the system still requires specific labeling with probes, such as fluorescent dyes. Therefore, design of an alternative MB-based optical approach that avoids oligonucleotide labeling should be of general interest and widely applicable.

Although DNA-intercalating dyes have been used to detect double stranded DNA in solution, as a simple and label-free probe design [31,32], this method suffers from intrinsic limitations in the case of MBs. This is because dyes can intercalate in the stem moiety of the MB to produce a high background. Some small fluorescent molecules can bind to unpaired nucleobases via hydrogen bonding, and this is accompanied by fluorescent quenching of small molecules. To improve the binding of small molecules to the unpaired base, some kinds of unique binding pockets that are opposite from the unpaired base in the DNA duplex, such as a basic site, bulge site, and gap site, have been intentionally constructed [33–36]. As part of a general program aimed at developing label-free MBs, we investigated whether the applicability of non-covalently bound small fluorescent molecules as signal molecules for MBs.

A simple approach is presented for preparing label-free MBs (GSMBs) as a signal reporter for target sensing. A small fluorescent molecule is hydrogen bonded to an unpaired base at the intentional gap site in the stem moiety of the label-free MBs (Scheme 1). The label-free MBs are tested as simple optical probes that can be used as biosensors.

2. Materials and methods

2.1. Chemicals and reagents

All oligodeoxynucleotides used in this study were obtained from Sangon Biological Engineering Technology (Shanghai, China), and the sequences are shown in Table 1. The oligodeoxynucleotide concentration was determined from the molar extinction coefficient at 260 nm using a UV spectrophotometer (UV-2450, Shimadzu Corporation, Japan) [37]. Annealing of M1 and S1 strands to form a M1/S1 complex was done by heating the solution at 95 °C for 5 min and then slowly cooling to room temperature, followed by refrigeration for 30 min to ensure the formation of hairpin structures and a gap site in the M1/S1 complex.

5,6,7-Trimethyl-1,8-naphthyridin-2-ylamine (ATMND) was purchased from Enamine Ltd., Ukraine. Sodium cacodylate was obtained from Alfa Aesar. Unless otherwise stated, all measurements were done in 10 mM cacodylic acid sodium salt buffer (pH 7.0) containing 100 mM NaCl and 1 mM EDTA. Other chemicals and reagents were commercially available and were of analytical grade. Water was obtained from Millipore Milli-Q purification system.

2.2. Fluorescence measurements

A Varian Cary Eclipse fluorescence spectrophotometer equipped with a thermoelectrically temperature-controlled holder was used. The fluorescence spectra were recorded between 370 and 500 nm upon excitation at 350 nm, using a slit width of 0.5 and 2.0 mm each, and a scan speed of 100 nm/min.

2.3. Circular dichroism spectroscopy

All circular dichroism (CD) measurements were performed on a Chirascan Instrument (Applied Photophysics Ltd., UK) at 20 °C using a quartz cylindrical cell of 1 mm path length with the temperature adjusted using a circulating bath. Individual 200 μL samples were prepared containing ATMND and/or DNA. Spectra were recorded at a bandwidth of 1.0 nm and measured at every 0.2 nm over a range of 210–360 nm.

2.4. Detection of PCR products

A 115-bp sequence (5′-ATAATCCACCTATCCAGTAGGAGAAA TCTATAAAAGATGGATAATCCTGGGATTAAATAAATAGTAAGAATGTA TAGCCCTACCAGCATTCTGGACATAAGACAAGGACCAAA-3′) in the *gag* region of the human immunodeficiency virus type 1 (HIV-1) genome was amplified by PCR in a DNA Thermal Cycler (Eastwin Life Sciences, Inc., China) using oligonucleotide primers as described by Wabuyele and Vo-Dinh [38]. The forward primers (5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′) and the reverse primer (5′-TTTGGTCCTTGTCTTATGTCCAGAATGC-3′) of the *gag* region were used. PCR amplification was carried out in 50 μL of 100 mM Tris–HCl buffer (pH 8.3) containing 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 10 ng of 115-bp templates sequence described above, 1.25 units of Ex-Taq (TaKaRa), and 0.5 μM each of the forward and reverse primers. Amplification was achieved with an initial denaturation step of 95 °C for 2 min, followed by a 30-cycle process that includes a denaturation step for 15 s at 95 °C, an annealing and extension step at 60 °C for 60 s, and a final extension at 72 °C for 2 min. After PCR amplification, the PCR product was

Table 1
Design of probes and target oligonucleotides.

Name	Sequence	Notes
M1	3′-*CCTGCCACGCTCCGC***C**CCTAGC*TCTAAATCACTATGGTCGC*GCTAGG-5′	
S1	3′-*GCGGAGCGTGGCAGG*-5′	
cDNA	3′-GCGACCATAGTGATTTAGA-5′	Perfect match
TM	3′-GCGACCATANTGATTTAGA-5′ (N = A, T or C)	Single base mismatch
TM2	3′-GCGTCCATAGTGATTAAGA-5′	Two bases mismatch
TM4	3′-GCGTCCATGATGATTAAGA-5′	Four bases mismatch
TN	3′-CCGTCCCCAGTGATTAAGT-5′	Non-complementary

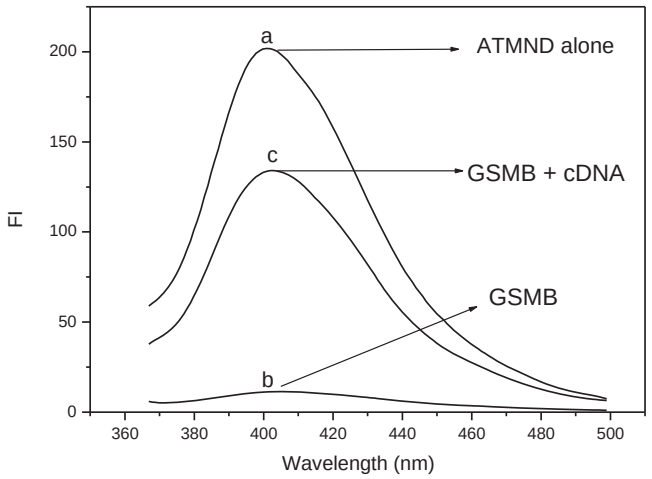

Fig. 1. Fluorescence spectra of ATMND before (a) and after bound to M1/S1 complex to form GSMB (b). (a) ATMND alone (2.0 μM); (b) GSMB (ATMND (2.0 μM) in the presence of M1/S1 complex (2.0 μM)). Spectra (c) is fluorescence spectra of GSMBs (M1/S1/ATMND complex, 2.0 μM) in the presence of target cDNA (1.5 μM). Sample solutions were adjusted to pH 7.0 with 10 mM sodium cacodylate containing 100 mM NaCl and 1 mM EDTA. Excitation wavelength is 350 nm. Temperature is 20 °C.

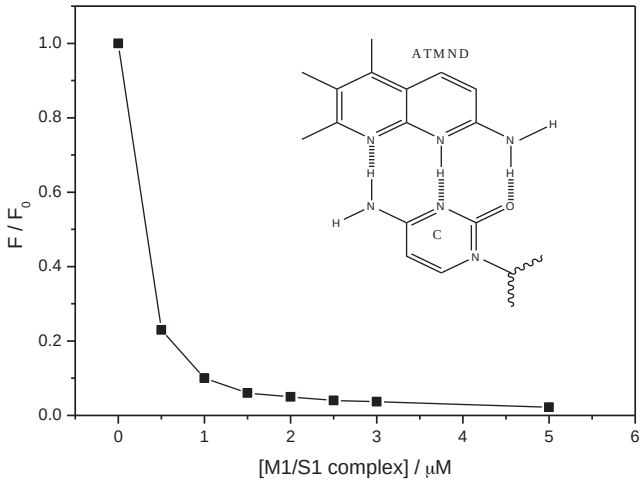

Fig. 2. Non-linear regression analysis of the fluorescence-titration curve based on a 1:1 binding isotherm model. [AMND] = 1 μM. Inset: possible binding structure of the 1:1 complex between ATMND and cytosine.

denatured by a rapid chilling to obtain single-stranded DNA. The reaction solutions were buffered to pH 7.0 with 100 mM sodium cacodylate containing 1.6 mM EDTA. In a control experiment, a random 115 bp templates sequence was used for the PCR amplification, other experimental conditions were as same as described above.

The HIV-1 GSMB probe (sequence of loop moiety is 3′-TTTTCCATTTCCATACATAT-5′, other moieties were as same as that of the GSMB described above) was used. For the detection of PCR products of the *gag* region of the HIV-1 genome, the HIV-1 GSMB probe solution was added into the resulting PCR products solution, and made final concentration of HIV-1 GSMB probe reach 2 μM.

3. Results and discussion

3.1. Label-free MB design

As illustrated in Scheme 1, the label-free MB (GSMB) is composed of a small fluorescent molecule (ATMND), a DNA strand containing 47 nucleotides (M1), and a DNA strand containing 15 nucleotides (S1). Strand S1 is complementary to a 15 mer sequence (15 bases written in italic in M1) at the 3′-end of M1 [39]. The M1/S1 complex, containing a gap site opposite from cytosine, a hairpin structure (underlined bases of M1) consisting of a 6-base-pair stem, and a 19-base loop sequence (underlined bases and written in italic of M1) [40], was formed by annealing the solution containing M1 (2 μM) and S1 (2 μM). ATMND (2 μM) was then added to the M1/S1 complex solution to form the M1/S1/ATMND complex. This was the designated free-label MB, which is named as GSMB. ATMND was expected to bind strongly to the unpaired cytosine at the gap site in the M1/S1 complex, resulting in the quenching of its fluorescence [41]. Upon addition of a complementary target ss-DNA, an open hairpin structure was expected to release ATMND from GSMB. This results in an emissive response of ATMND fluorescence.

3.2. GSMB characterization in solution

To demonstrate the feasibility of the principle shown in Scheme 1, we evaluated the initial background fluorescence of GSMBs in the absence of cDNA as well as the fluorescence change after target addition. Fig. 1 shows the fluorescence spectra of ATMND (2 μM) in 10 mM sodium cacodylate buffer solution (pH 7.0) containing 100 mM NaCl and 1 mM EDTA. In the absence of the M1/S1 complex, ATMND exhibits a strong emission band with a maximum at 401 nm (spectrum a). However, ATMND exhibits significant quenching of its fluorescence upon addition of the M1/S1 complex (spectrum b); the fluorescence intensity of ATMND decreases from 201 u to 11 u. A little decrease in the fluorescence intensity of ATMND is observed in the presence of an ss M1, S1, or fully matched duplexes (2 μM) (data are not shown). A small fluorescent molecule can bind with the unpaired base via hydrogen bonding in a DNA duplex. This induces fluorescence quenching of the molecule [36]. These results suggest that ATMND is incorporated into the gap site in the stem moiety of the M1/S1 complex and binds to cytosine through hydrogen bonding.

The response of GSMBs upon cDNA addition was examined. As shown in Fig. 1, ATMND fluorescence intensity recovers greatly by 13-fold after the addition of 1.5 μM target cDNA (Fig. 1, spectrum c). The increase in fluorescence intensity upon addition of the target can be assigned to the recovery of ATMND fluorescence. This is due to the release of ATMND to the solution caused by the hybridization of target cDNA to the loop section of GSMBs and effective opening of the hairpin structure.

In order to quantitatively determine the affinity of ATMND to cytosine at the gap site in the stem moiety of the M1/S1 complex, the fluorescence titration for ATMND binding was then investigated. As shown in Fig. 2, the resulting changes in the fluorescence intensity at 401 nm give a distinct titration endpoint at a 1:1 ratio of [M1/S1 complex]/[ATMND], indicating that ATMND forms a stable 1:1 complex with M1/S1 complex at the gap site. The 1:1 binding constant K_{11}, as calculated by the titration curve [42], reaches $8.1 \times 10^6\ M^{-1}$ under the present experimental conditions. Although ATMND does not covalently bind to DNA, it shows high binding affinity to the M1/S1 complex by pseudo-base pairing with cytosine at the gap site via three hydrogen bonds [43].

For the label-free MB-based fluorescent "signal-on" method, achieving the maximum sensitivity was the key consideration. As a basic principle, the sensitivity of MBs can be improved by either increasing the fluorescence intensity of the fluorophore or enhancing the efficiency of the quencher [44]. A small fluorescent molecule can bind to an unpaired base in a cooperative fashion, that is, hydrogen bonding with an unpaired base and stacking with nucleobases flanking the binding site. Accordingly, the fluorescence-quenching efficiency of ATMND after binding to an unpaired cytosine at the gap site in an M1/S1 complex may be affected by the neighboring bases,

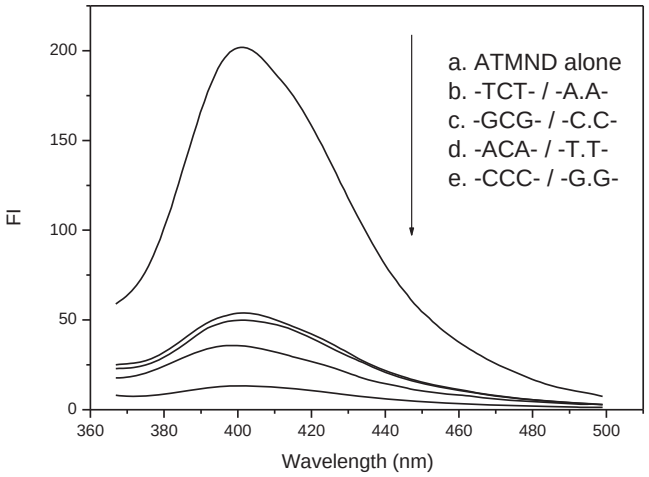

Fig. 3. Fluorescence spectra of ATMND (2.0 μM) in the presence of 2.0 μM M1/S1 complex with different flanking bases.

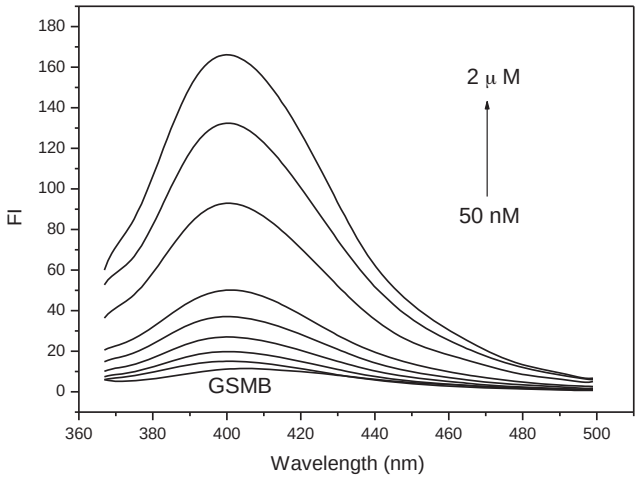

Fig. 5. Fluorescence spectra of a GSMB after hybridization with (from up to down) 2.0, 1.5, 1.0, 0.5, 0.3, 0.2, 0.1, 0.05, and 0 μM of cDNA, respectively.

although complementary hydrogen bonds were formed between ATMND and the cytosine base. To examine the effect of flanking bases on the fluorescence quenching of ATMND, we investigated the sequence-dependent binding of ATMND to a single cytosine at the gap site in the stem moiety of the M1/S1 complex. As shown in Fig. 3, the strongest quenching is observed when ATMND bound with the M1/S1 complex having G–G base pairs at both 5′ and 3′ sides of a gap site (Fig. 3, spectrum e), where the fluorescent intensity at 401 nm is quenched by as much as 95% in the presence of 2.0 μM of the M1/S1 complex. For other flanking bases (Fig. 3, spectra b–d), the observed fluorescence quenching was relatively moderate and followed in the order of T–T (74%) < C–C (76%) < A–A (83%). These results suggest that the quenching efficiency of ATMND fluorescence after binding to cytosine at the gap site in an M1/S1 complex is affected by the neighboring bases, although complementary hydrogen bonds play a crucial role in the binding of ATMND to the cytosine base. Therefore, Gs at both 5′ and 3′ sides of a gap site were chosen in our GSMBs.

The CD spectra in the UV range can be used to monitor the conformational transition of DNA. To determine if conformational changes occur upon binding of ATMND to the gap site at the stem moiety of an M1/S1 complex, CD experiments were performed for the M1/S1 complex before and after ATMND binding (Fig. 4). As shown in Fig. 4a, in the absence of ATMND and cDNA, the M1/S1 complex displays a positive peak at 275 nm and a negative peak at 250 nm, which is characteristic of a helix of the stem moiety in a β-conformation [45]. After the addition of ATMND, no noticeably induced CD was observed in the M1/S1/ATMND complex (GSMBs) (Fig. 4b), indicating that binding of ATMND to the cytosine base at the gap site of the stem moiety does not induce a significant conformational change of the M1/S1 complex. Therefore, it does not affect the hybridization dynamics of the target DNA. After hybridization with cDNA, the negative peak at 250 nm increases in height without a shift in the peak position (Fig. 4c). The change in the intensity of the CD peak at 250 nm is associated with formation of the helix. Thus, it would be reasonable to suggest that the increase of CD peak at 250 nm is due to the formation of longer helix by the hybridization of cDNA with the loop of the GSMBs [46].

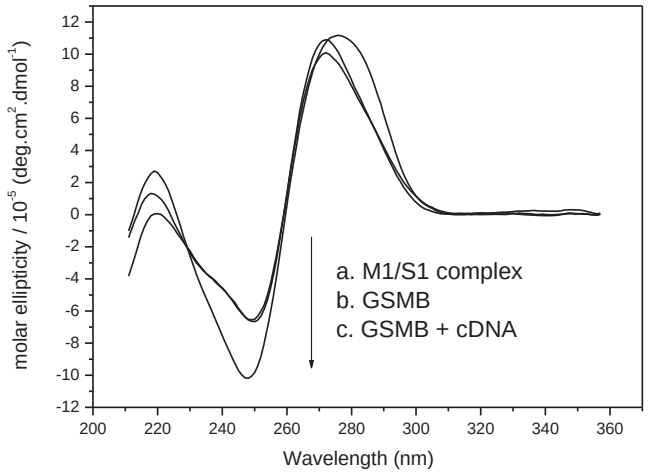

Fig. 4. CD spectra of M1/S1 complex in the (a) absence and (b) presence of ATMND. (c) CD spectra of GSMBs in the presence of target cDNA. [M1S1] = 10.0 μM, [ATMND] = 20.0 μM and [cDNA] = 10.0 μM.

3.3. Detection of target DNA using label-free MB

Because the fluorescent-quenching efficiency is dependent on the concentration of the M1/S1 complex and the ATMND, we optimized both concentrations. A concentration of 2 μM was chosen to achieve the good sensitivity. Under optimal conditions, calibration curves were obtained based on the average of three replicate measurements (Fig. 5). As expected, signal enhancements were observed to be proportional to the cDNA concentration in the range of 50 nM to 1.5 μM. The linear regression equation is $I = 11 + 81c$ (μM) ($r = 0.9923$) with detection limits of 20 nM. The reproducibility of the measurements, estimated as the relative standard deviation of seven measurements at 1.0 μM cDNA with different GSMBs, is less than 5%.

The selectivity of MB is generally attributed to the conformation of the stem-loop structure, and this correlation is also observed for the GSMBs reported here. The selectivity of GSMB is investigated by measuring the fluorescence of GSMBs after hybridization with 1.5 μM cDNA, 1.5 μM single-base-mismatched DNA (TM), 1.5 μM two bases of mismatched oligonucleotide (TM2), 1.5 μM four bases of mismatched oligonucleotide (TM4) and 1.5 μM non-cDNA (TN), respectively. All results are displayed in Fig. 6. For the non-cDNA (TN), almost no response was observed. (data are not shown). The GSMBs responded to the single-base mismatch, but the response was significantly smaller than for cDNA (Fig. 6). Hybridization of the GSMBs with its cDNA and single-base-mismatched DNA in the solution displays a 13-fold and a 4-fold enhancement, respectively, in emission intensities relative to that observed for background fluorescence of the GSMBs. Therefore, the total discrimination factor is 3.3 for the recognition of a single-base mismatch. This indicates a

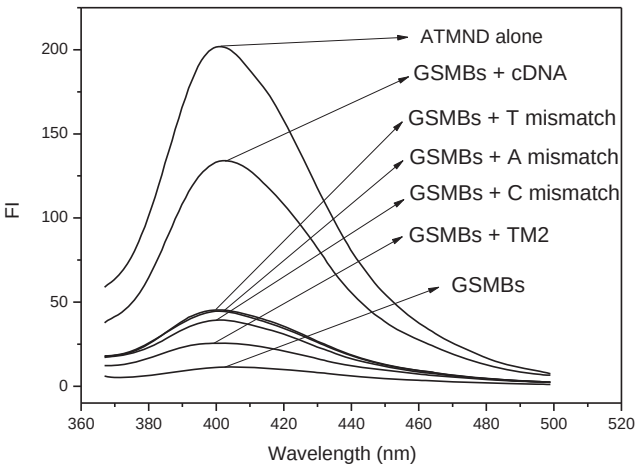

Fig. 6. Fluorescence response of GSMBs to cDNA, single-base-mismatched DNAs (TM, 3′-GCGACCATANTGATTTAGA-5′) and two-base-mismatched DNAs (TM2). From down to up: GSMBs, TM2, TM with N = C, TM with N = A, TM with N = T, cDNA, and ATMND alone, respectively. [GSMB] = 2.0 μM, [DNA] = 1.5 μM and [ATMND] = 2.0 μM.

high selectivity for the target sequence. The two-base mismatched oligonucleotide (TM2) gave about one-fifth the signal of a perfect matched sequence. The response from the four-base mismatched oligonucleotide (TM4) is negligible, just like the response from the complete noncomplementary oligonucleotide.

To illustrate the usefulness of the GSMB for DNA detection, the GSMB was used to detect the PCR products of a partial sequence of the HIV-1 gene in a homogeneous solution, results are shown in Fig. 7. Background fluorescence of the HIV-1 GSMB probes is show in Fig. 7 (curve a). After hybridized to PCR products of the *gag* region of the HIV-1 genome, the fluorescence signal was significantly enhanced, as shown in Fig. 7 (curve c). Addition of the HIV-1 GSMB probe solution into PCR products obtained from control experiment, however, only a little increase of the fluorescence signal was observed (Fig. 7, curve b). This indicates that the significant enhancement of fluorescence was caused by the hybridization of the PCR products to the HIV-1 GSMB probes. Therefore, the GSMB can be used to detect PCR products.

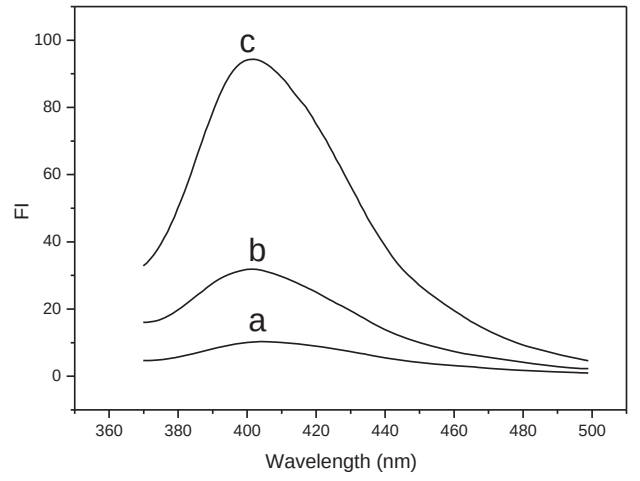

Fig. 7. Fluorescence detection of PCR products of the *gag* region of the HIV-1 genome. (a) Fluorescence of GSMBs; (b) fluorescence of GSMBs after added into PCR products obtained from control experiment; (c) fluorescence of GSMBs after added into PCR products of the *gag* region of the HIV-1 genome.

4. Conclusion

In summary, we have demonstrated the feasibility of the label-free MBs (named GSMBs) to detect nucleic acid sequences, based on non-covalent binding between a small fluorescent molecule and a hairpin DNA containing a gap site in the stem moiety. The label-free "signal-on" MBs that require no covalent labeling with fluorophores and a quencher unit showed a significant response to the target cDNA. The fluorescent intensities of the label-free MBs increased upon hybridization with the target cDNA, whereas a little increase of fluorescence was obtained upon addition of single-base-mismatched DNA sequences. Therefore, the label-free MBs in this study are useful probes that can distinguish their target and single-base-mismatched DNA sequences. Unlike conventional MBs, both ends of the label-free MB in this study are free for the introduction of other useful functionalities. Furthermore, the label-free MB synthesis is relatively simple and inexpensive because no label is required.

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