



Fluorescent detection of single nucleotide polymorphism utilizing a hairpin DNA containing a nucleotide base analog pyrrolo-deoxycytidine as a fluorescent probe

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ABSTRACT

A novel fluorescent method for the detection of single nucleotide polymorphism (SNP) was developed using a hairpin DNA containing nucleotide base analog pyrrolo-deoxycytidine (P-dC) as a fluorescent probe. This fluorescent probe was designed by incorporating a fluorescent P-dC into a stem of the hairpin DNA, whose sequence of the loop moiety complemented the target single strand DNA (ss-DNA). In the absence of the target ss-DNA, the fluorescent probe stays a closed configuration in which the P-dC is located in the double strand stem of the fluorescent probe, such that there is weak fluorescence, attributed to a more efficient stacking and collisional quenching of neighboring bases. In the presence of target ss-DNA, upon hybridizing the ss-DNA to the loop moiety, a stem-loop of the fluorescent probe is opened and the P-dC is located in the ss-DNA, thus resulting in strong fluorescence. The effective discrimination of the SNP, including single base mismatch ss-DNA (A, T, G) and double mismatch DNA (C, C), against perfect complementary ss-DNA was achieved by increased fluorescence intensity, and verified by thermal denaturation and circular dichroism spectroscopy. Relative fluorescence intensity had a linear relationship with the concentration of perfect complementary ss-DNA and ranged from 50 nM to 3.0 μ M. The linear regression equation was $F/F_0 = 2.73 C (\mu\text{M}) + 1.14$ ($R = 0.9961$) and the detection limit of perfect complementary ss-DNA was 16 nM ($S/N = 3$). This study demonstrates that a hairpin DNA containing nucleotide base analog P-dC is a promising fluorescent probe for the effective discrimination of SNP and for highly sensitive detection of perfect complementary DNA.

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1. Introduction

Single-nucleotide polymorphisms (SNPs) are the most common sequence variations in the human genome [1,2] and thus taken as attractive markers or tools for the identification of disease-causing genes for early diagnosis [3] and key enablers in realizing the concept of personalized medicine [4]. Current methods for SNP genotyping, including discrimination and detection of SNPs, rely on a wide variety of allelic discrimination methods (e.g., primer extension [5], hybridization [6], ligation [7], and enzymatic cleavage [8]) in connection with detection methods (e.g., fluorescence [9,10], mass spectroscopy [11], electrochemical method [12], and chemiluminescence [13]). Although these methods are sensitive and specific, some of them require tedious assay processes [14], expensive instruments (e.g., mass spectrometer) [11], and tight control over experimental conditions (e.g., temperature) [15]. Fluorescence detection method for SNP genotyping has been receiving much attention because it is simple, rapid and sensitive [16].

Much effort has been devoted to the development of fluorescent detection methods for SNP genotyping with high sensitivity, reproducibility, simplicity, including the development of new molecular recognition such as specific single strand DNA containing an apurinic or apyrimidinic site (AP site) [17] or bulge site [18], the synthesis of new fluorescent labels/quenchers with high efficiency [19,20], and the exploration of appropriate design strategies. For the exploration of appropriate design strategies, fluorescently labeled SNP discrimination methods using TaqMan [21,22] and MBs as fluorescent probes [23] have been established for wide applications. However, the probe ss-DNA needs to be labeled with the fluorescent tags including fluorophore and quencher in these methods, and cleavage enzyme is needed in TaqMan method. For molecular recognition compounds, Saito's group and Tera-mae's group successfully developed a series of fluorescent SNP discrimination methods using specific single strand DNA (ss-DNA) containing an apurinic or apyrimidinic site (AP site) or bulge site as molecular recognition probes [24–28]. However, the sensitivities of these newly developed methods are limited due to their signal-off approaches.

In recent years, fluorescent nucleoside base analogs for sensing ss-DNA and proteins based on DNA hybridization and aptamer-

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target binding are of great interest on account of their use of two function groups, the molecular recognition element and signal producer. Fluorescent nucleoside base analogs, such as 2-aminopurine, pyrrolo-deoxycytidine (P-dC), and 3-methylisoxanthopterin are non-natural nucleotide bases that closely resemble naturally occurring purine or pyrimidine base structures and can be incorporated as quasi-intrinsic probes into oligonucleotides using standard automated synthetic methods [29]. Li et al. [30] designed aptamer-based fluorescent biosensors for thrombin and adenosine using the aptamers as the molecular recognition elements and a fluorescent nucleotide analog-modified competitor oligonucleotide as the signal transduction element. Park et al. [31] developed signal-on aptasensors for thrombin employing unmodified DNA aptamers and signaling DNA probes containing P-dC. Katilius et al. [32] reported that fluorescent nucleotide analogs as aptamers were used for sensing thrombin including 2-aminopurine, 4-amino-6-methylpteridine, or 3-methylisoxanthopterin. Turro et al. [33] designed a novel hairpin DNA modified with 2-aminopurine at the loop and P-dC at the stem that enhanced sensitivity for the detection of complementary DNA, which provides a novel perspective in the design of biosensors. However, a probe containing fluorescent base analogs for SNP discrimination has not been reported to date.

The aim of this work is to develop a simple fluorescent method for discrimination and detection of SNPs. A hairpin DNA probe containing a nucleotide base analog, P-dC, was designed as a fluorescent probe (FP). Scheme 1 presents a schematic diagram of the discrimination and detection of SNPs using the designed FP containing nucleotide base analog P-dC in the stem of the hairpin DNA probe. In the absence of target ssDNA, FP maintains its hairpin conformation and P-dC is in double strand DNA, led to weak fluorescence intensity. This is attributed to the fact that a more efficient stacking and collisional quenching of neighboring bases occurs [29,33]. In the presence of target ssDNA, FP is opened and P-dC is in single strand, and thus resulting in strong fluorescence. Depending on hybridizing with different type of target ssDNA including perfect complementary (pc) ssDNA and mismatch ssDNA, different characteristic change of its fluorescence response is obtained. Based on this principle, we developed a simple method for the detection of SNPs. The principle behind the proposed method and its performance in discrimination and detection of SNPs are presented in this paper.

2. Materials and methods

2.1. Chemicals and reagents

The particular gene used as the target and one mismatch gene in this work were selected from the research work reported by Tyagi et al. [23], and two mismatch genes in which the mutation site is the same as the Ref. [23] were designed. The hairpin DNA was designed based on the molecular beacon (MB) sequence reported by Tyagi et al. [23] with slight modifications. Sequences of the FP and target ss-DNA are as follows:

FP: 5'-GCGAGAAGTTAAGACCTATGCT (P-dC) GC-3'

Target DNA

Perfect complementary DNA (pc DNA): 5'-CATAGGTCTTAACCTT-3'

Single base mismatch DNA1 (sm1): 5'-CATAGGTATTAACCTT-3'

Single base mismatch DNA2 (sm2): 5'-CATAGGTTTAACCTT-3'

Single base mismatch DNA3 (sm3): 5'-CATAGGTCTTAACCTT-3'

Double base mismatch DNA (dm): 5'-CATCGGTCTTAACCTT-3'

All ss-DNA segments were synthesized by Takara Biotechnology Co. (Dalian, China) and used without further purification. The stock and working solutions of the ss-DNA segments were prepared

with testing buffer composed of 10 mM Tris-HCl-5 mM MgCl₂-1 mM EDTA (pH 8.0). Stock solution was kept in frozen before use. Millipore Milli-Q water (18.2 MΩ cm) was used in all experiments. Unless otherwise indicated, all reagents and solvents were purchased in their highest available purity and used without further purification.

Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Varian, U.S.A.). Melting curve measurements were performed on a UV-vis spectrophotometer (UV-2450, Shimadzu Corporation, Japan), while circular dichroism (CD) spectra were measured on a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd., England).

2.2. Fluorescence measurement

150 μL of 5.0 μM FP was mixed with 150 μL of target DNA in fixed concentration and then incubated at 75 °C for 5 min, followed by slow cooling to room temperature and refrigeration at 4 °C for 30 min to form FP/target DNA duplexes. Then, 100 μL of the refrigerated solution was transferred into a 1 mm × 1 cm quartz cuvette, and the fluorescence spectrum was recorded at an excitation wavelength of 340 nm and an emission wavelength ranging from 360 to 610 nm. For the determination of perfect complementary (pc) DNA, the concentration of the pc DNA was quantified through the ratio of fluorescence intensity F/F_0 , where F_0 is the fluorescence intensity of the FP at 443 nm before hybridization and F is the value after hybridization.

2.3. Melting temperature (T_m) measurement

A total of 3.0 μM duplex of FP and ss-DNA was prepared by mixing the same volume and same concentration of FP and target DNA. Absorbance of the FP and DNA duplex was then measured at 260 nm using a UV-vis spectrophotometer equipped with a thermoelectrically temperature-controlled micro-multicell holder (eight cells, optical path length = 1 mm). Temperature range was 5–80 °C with a heating rate of 0.5 °C/min. The resulting absorbance versus temperature curves was differentiated to determine T_m values.

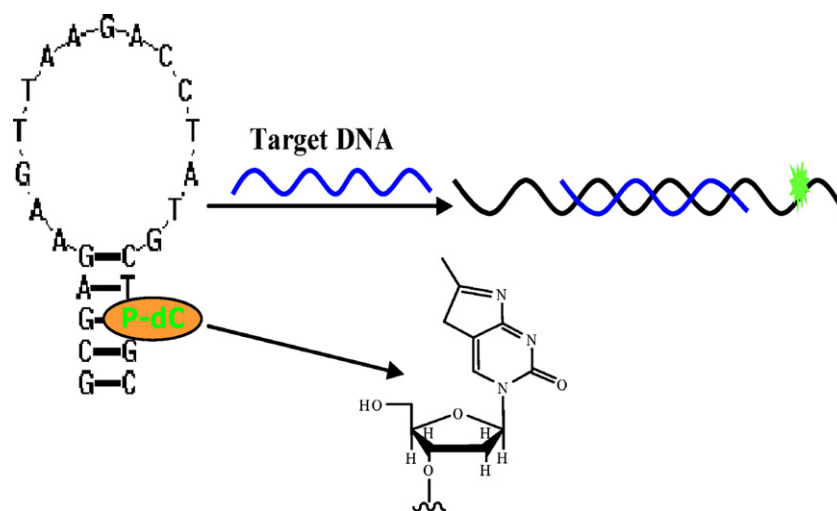
2.4. CD Spectroscopic measurement

DNA samples (20 μM) were prepared by mixing the same volume and same concentration of FP and target DNA, which were then hybridized using PCR instrument with following temperature program: 75 °C for 5 min, cooling to 5 °C, and incubation at 4 °C overnight. CD spectra of the DNA samples were recorded using a 1-mm quartz cell at a scanning speed of 100 nm/min with a response time of 2 s and a scanning range of 230–310 nm at room temperature. Values reported are the average of three scans.

3. Results and discussion

3.1. Discrimination of single nucleotide polymorphism

Fig. 1 shows the fluorescence spectra of the designed FP in the absence/presence of pc DNA. From Fig. 1, it can be seen that the maximum emission wavelength of FP in the absence/presence of target pc DNA is ~447 nm. This is nearly equal to that reported by Turro [33,34]. Furthermore, the fluorescence intensity of FP at 460 nm upon addition of 0.5 μM pc DNA (Fig. 1, curve b) and 1.0 μM pc DNA (Fig. 1 curve c) is 2.53-fold and 4.17-fold higher than that in the absence of pc DNA. These results suggest that the proposed approach using the designed FP is potentially applicable to the development of a fluorescence method for the detection of target pc DNA.



Scheme 1. Schematic diagram of fluorescent detection of single nucleotide polymorphism utilizing a hairpin DNA containing pyrrolo-deoxycytidine (P-dC) as a fluorescent probe.

Fluorescence spectra of the FP in the absence/presence of target DNAs including pc and mismatch DNAs are shown in Fig. 2. Fluorescence intensity at 447 nm in the presence of 5.0 μM pc DNA (curve b) is 6.1 fold higher than that in the absence of target DNA (curve a). In addition, fluorescence intensity is 3.8-fold for sm1 (A), 2.8-fold for sm2 (T), 2.5-fold for sm3 (G), and 1.1-fold for dm(C, C) compared with in the absence of any target DNA. Moreover, curve (c) is much different from curve (d) and curve (e). This is attributed to the fact that the binding ability with target DNA containing A is stronger than that containing T due to effective stacking with two A's flanking the mutation site G in FP, whereas such effective stacking disappears for the complex between G and T. In addition, the binding ability of GG mismatch formed between the FP and target DNA containing G is smaller than that of A and T mismatch.

This result indicates that the stacking interaction in the binding event at hairpin probe plays an importance function, in accordance with the appearance of the selectivity, and suggests that the FP have the different binding ability to different target nucleotide. To clearly illustrate the selectivity of the proposed method, a selectivity coefficient α defined by Tan [35] was utilized in this study.

$$\alpha = \frac{(S/B)_{i,j}}{(S/B)_{i,k}}$$

where S is fluorescence intensity obtained in the presence of FP (i) or target DNA ($k = \text{pc DNA}; j = \text{sm1–3, dm}$), and B is that of FP. The

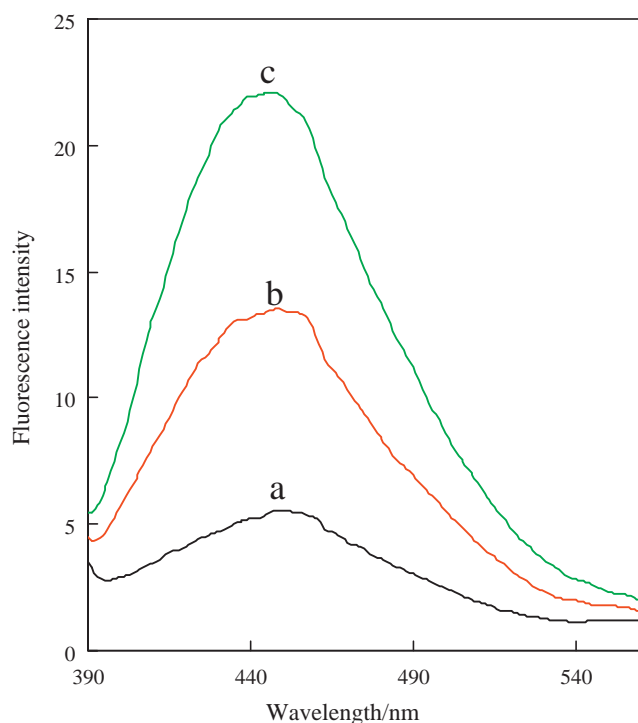


Fig. 1. Fluorescence spectra of FP with excitation at 340 nm. (a) 5.0 μM FP alone, (b): a + 0.5 μM pc DNA and (c) a + 1.0 μM pc DNA.

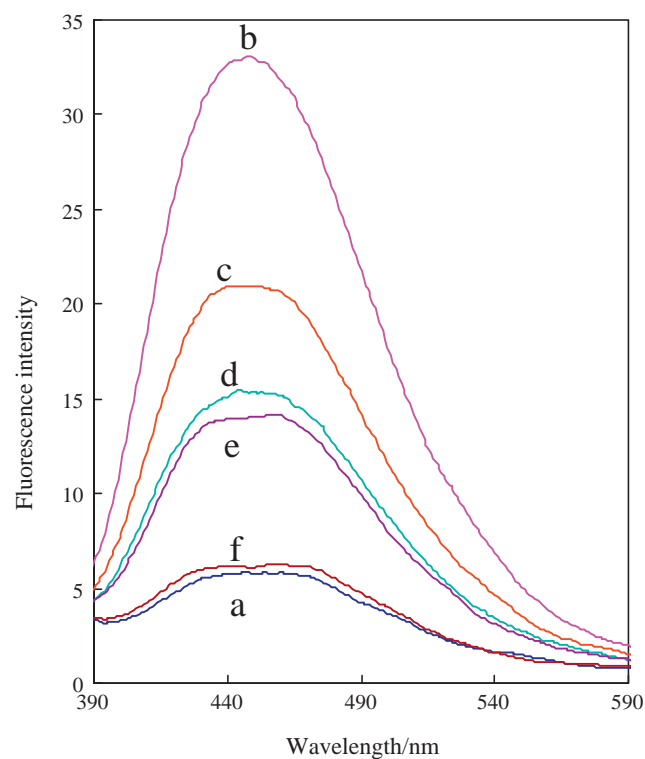


Fig. 2. Fluorescence spectra of FP with excitation at 340 nm in 5.0 μM FP. (a): blank, (b) 5.0 μM pc DNA, (c) 5.0 μM sm1 (A), (d) 5.0 μM sm2 (T), (e) 5.0 μM sm3 (G) and (f) 5.0 μM dm (C, C).

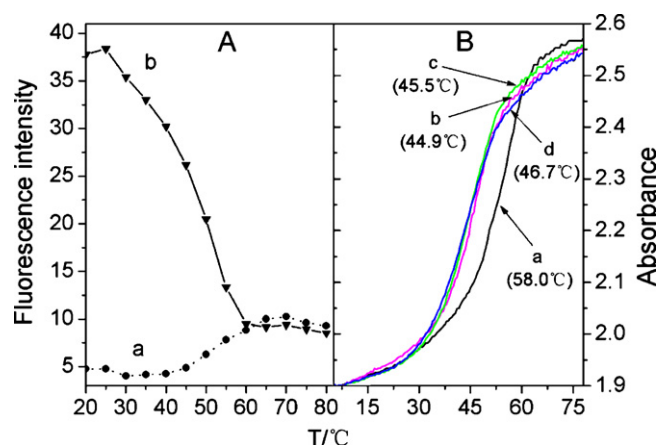


Fig. 3. (A) Dependence of fluorescence intensity on melting temperature and (B) UV melting curves of duplexes obtained after the FP hybridized with pc DNA and single base mismatch DNA, respectively.

A: (a) 5.0 μM FP alone and (b) 5.0 μM duplex of FP and pc DNA.

B: (a) FP + pc DNA, (b) FP + sm1 DNA, (c) FP + sm2 DNA

selectivity of FP for the pc-DNA target was used as the standard ($\alpha = 1$). It should be noted that a lower selectivity coefficient implies a better selectivity of the probe. The selectivity coefficient of FP is 0.624 for sm1 (A), 0.468 for sm2 (T), 0.404 for sm3 (G), and 0.188 for dm (CC). The α (0.404) for single-mismatch DNA (C) over pc DNA (G) obtained in this work is lower than that (0.764) reported using a molecular beacon in which the fluorescence of fluorescein is quenched by 4-(4-dimethylaminophenylazo)benzoic acid [23], and is also lower than that (0.472) using single-wall carbon nanotube as quencher for the same fluorophore [35]. This indicates that the designed FP has good ability to discriminate between pc DNA and mismatch DNA. Effective discrimination of SNPs using FP, therefore, is evident.

In order to understand the conformation change of the designed FP, thermal denaturation profiles of FP for P-dC were measured. Fig. 3A shows the dependence of the fluorescence intensity of FP on temperature in the absence (a) and in the presence (b) of pc DNA. Fluorescence intensity in the absence of pc DNA maintains at a nearly stable value within 20–40°C, and then slowly increases at 40–65°C, and afterwards become constant again. These findings concur with those reported by Turro [33]. This can be attributed to the conformational change that the designed FP undergoes because of the temperature changes – that is, FP exists in a closed state at low temperature and exists in an opened state at high temperature. From curve (b) in Fig. 3A, it can be seen that fluorescence intensity is ~7.5-fold higher than that in the absence of pc DNA at 20–25°C, due to the formation of a duplex of FP and pc DNA, where P-dC in the stem of FP stays in an un-hybridized state. Fluorescence intensity sharply decreases when temperature is increased from 25 to 60°C because the increased temperature results in FP transitions from double duplex to single strand random coil, accompanied by and a decrease in quantum yield of P-dC [33]. At temperatures higher than 60°C, fluorescence intensity is maintained at a constant value. This is because the duplex of PB and pc DNA completely dissociates, thus leading to the return of FP into its hairpin state. This also suggests that the designed FP can be used in the discrimination of SNP at room temperature.

T_m measurements were performed to further understand the conformation change that the designed FP undergoes during discrimination of SNP with temperature changes. Fig. 3B shows the effect of pc DNA and single base mismatch DNA on the UV melting curve of FP. Curve (a) is different from the curves (b–d), which were measured at temperatures within 30–60°C. The T_m of duplexes of FP and target DNA segments can be calculated from data in Fig. 3B

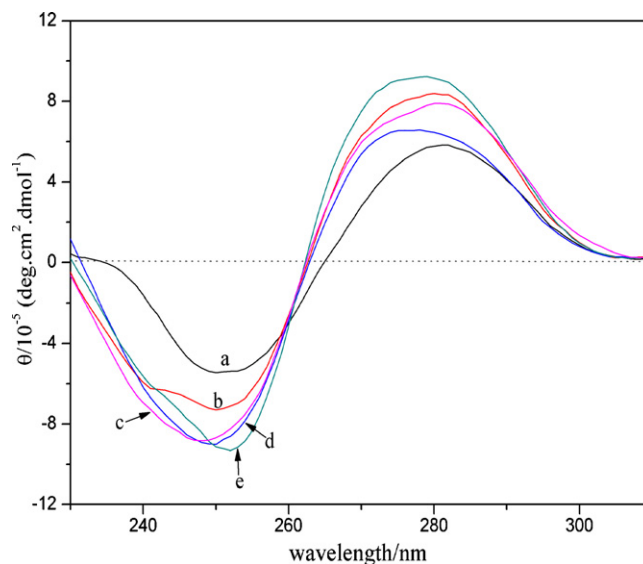


Fig. 4. CD spectra of FP and FP after hybridized to target DNA in a 10 mM Tris–HCl buffer (pH 8.0) containing 5 mM MgCl₂, 1 mM EDTA. (a) FP, (b) FP + pc DNA, (c) FP + sm1 DNA, (d) FP + sm2 DNA and (e) FP + sm3 DNA.

and the results are inserted in Fig. 3B. T_m for single-mismatch DNA segments range from 44.9 to 46.7°C, which is 11.3–13.1°C lower than that for pc DNA (58.0°C). This is consistent with the restoration ratio of fluorescence intensity, thus indicating that the stability of the duplex formed by FP and pc DNA is greater than that of the duplex by FP and single base mismatch DNA. Further, this suggests that discrimination of SNP using the designed FP can be performed at room temperature.

Differences in conformational changes that FP undergoes when it binds to pc DNA and single-mismatch DNAs were also demonstrated by CD (Fig. 4). CD spectra displayed a positive peak at ~280 nm, a negative peak at ~250 nm, and a cross-over point from positive to negative intensity at ~260 nm. These peaks are typical characteristics of standard B-form DNA [36]. After hybridization of FP with target DNAs, the negative peak height at ~250 nm increased, which may be attributed to the formation of longer double helix from 5 to 15 base pairs. Furthermore, the negative peak height of the helix after hybridization of pc DNA with FP (curve b in Fig. 4) at ~250 nm noticeably changed, as compared with those by the hybridization of single-mismatch DNA segments (curves c–e in Fig. 4).

The discrimination of SNPs using the designed FP can be supported by predicting the duplex formation free energy using soft MeltWin 3.5. Duplex formation free energy (ΔG) between FP and pc DNA is –12.32 kcal/mol, which is lower than that of the duplex of FP and mismatch DNA segments (ΔG is –10.61, –9.56, and –9.99 kcal/mol for sm1, sm2, and sm3, respectively). These predicted results concur with the data obtained from fluorescence spectra, T_m , and CD spectra.

3.2. Fluorescence determination of target DNA

Fig. 5 shows the fluorescence spectra of FP in the presence of different concentrations of pc DNA. The insert reveals the dependence of the ratio of fluorescence intensity F/F_0 on the concentration of pc DNA. Fluorescence intensity increases with increasing concentration of pc DNA within 50 nM–3.0 μM. The linear regression equation is $F/F_0 = 2.73 C (\mu\text{M}) + 1.14$ ($R = 0.9961$) and the limit of detection obtained is 16 nM ($S/N = 3$). The relative standard deviation of seven measurements in 1.0 μM pc DNA was 3.6%, indicating that the developed method has good reproducibility.

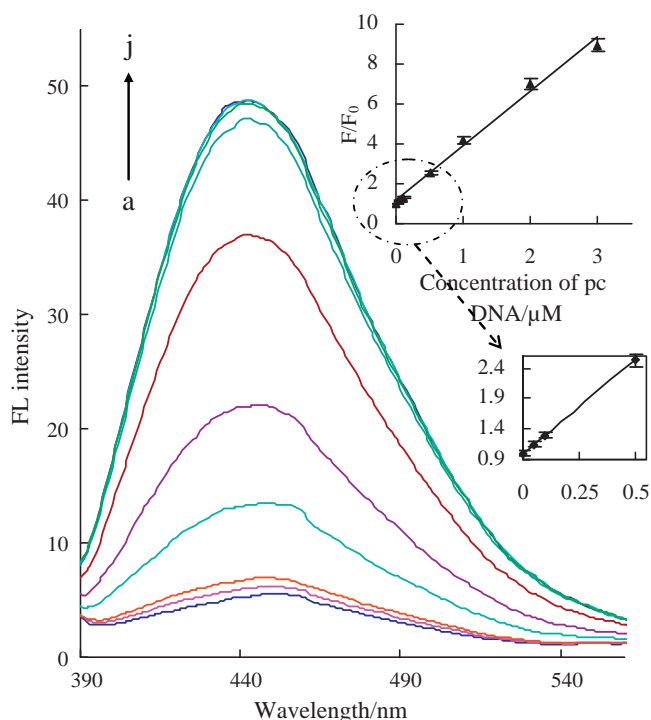


Fig. 5. Fluorescence spectra of FP in the presence of different concentrations of pc DNA. (a) 0 μM , (b) 0.05 μM , (c) 0.1 μM , (d) 0.5 μM , (e) 1.0 μM , (f) 2.0 μM , (g) 3.0 μM , (h) 4.0 μM , (i) 5.0 μM and (j) 6.0 μM . Concentration of FP is 5.0 μM and excitation wavelength is 340 nm.

Binding constant K of the FP (P) with target DNA (T) was calculated according to the method reported by Connors [37]. Eq. (1) described the dependence of the ratio of fluorescence intensity F/F_0 on the concentration of target DNA (see Supporting material).

$$\frac{F}{F_0} = 1 + \frac{Kk_c}{k_p} [T] \quad (1)$$

Therefore, the plot of $\frac{F}{F_0} - 1$ versus $[T]$ should give a straight line and K can be gained from the value of the slope. If the concentration of T is much higher than that of P , a maximum value of fluorescent intensity F_{\max} can be obtained: $F_{\max} = k_c[PT] = k_c[P]_t$. This equation, when combined with Eq. (2) (see Supporting material), becomes $F_{\max}/F_0 = k_c/k_p$.

Fig. 6 shows the dependence of $\frac{F}{F_0} - 1$ on the concentration of target DNAs. A straight line can be obtained when concentration is between 0 and 3.0 μM pc DNA. The slope obtained from this straight line is 2.796. Using $F_{\max} = 48.54$, $F_0 = 5.268$, and the obtained slope, binding constant K was calculated to be $0.296 \times 10^6 \text{ M}^{-1}$. This value is approximately one order lower than that from the pc double duplex of 15 base pairs [38,39]. The hybridization of target DNA to the designed FP performs two reactions: a disassociation reaction in the stem of the FP and a binding reaction in the loop of the FP with target pc DNA. The incorporation of P-dC in the stem moiety of the FP may effect thermodynamic of the FP, and the relative low binding constant of the FP designed in this work results in an effective discrimination of SNPs.

Similarly, the calibration experiments of the FP with mismatch DNAs were also performed. The result is shown in Fig. 6. From Fig. 6, it can be seen that the relative fluorescence intensity has a linear relationship with the concentration of mismatch ss-DNA, and ranged from 100 nM to 4 μM for sm1, 50 nM to 4.0 μM for sm2, 50 nM to 4.0 μM for sm3 and 500 nM to 7.0 μM for dm, respectively. The linear regression equations were $F/F_0 = 2.34C (\mu\text{M}) + 0.036$ ($R = 0.999$) for sm1, $F/F_0 = 1.91C (\mu\text{M}) + 0.008$

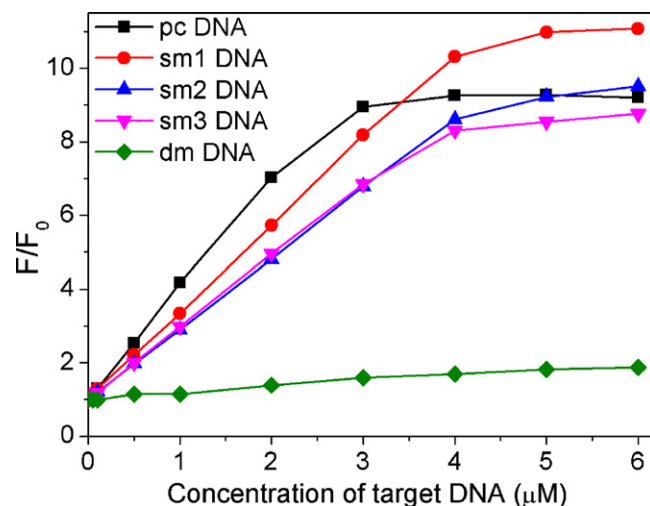


Fig. 6. Fluorescence intensity ratios of FP at 447 nm in a titration with target DNAs. Concentration of FP is 5.0 μM and excitation wavelength is 340 nm.

($R = 0.999$) for sm2, $F/F_0 = 1.87C (\mu\text{M}) + 0.053$ ($R = 0.998$) for sm3 and $F/F_0 = 0.182C (\mu\text{M}) + 0.002$ ($R = 0.984$) for dm, respectively. The binding constants K of the designed FP with mismatch DNAs were calculated from the data of Fig. 6 according to the Eq. (1), to be $K(\text{sm1}) = 0.244 \times 10^6 \text{ M}^{-1}$, $K(\text{sm2}) = 0.202 \times 10^6 \text{ M}^{-1}$, $K(\text{sm3}) = 0.216 \times 10^6 \text{ M}^{-1}$, $K(\text{dm}) = 0.09 \times 10^6 \text{ M}^{-1}$, respectively. These results indicate that the FP designed has the good discriminating ability for SNP relative to C base.

4. Conclusion

This study was able to develop a new approach for the discrimination of SNPs using a designed FP that is comprised of a hairpin DNA containing nucleotide base analog P-dC. By incorporating fluorescent base analogs into the signaling probe, a general and cost-effective way to develop a fluorescent method for the discrimination of SNPs with high selectivity towards the DNA targets was successfully established. Furthermore, a simple and highly sensitive method for the fluorescent detection of pc DNA and mismatch DNAs was developed. This work was able to demonstrate that a hairpin DNA containing P-dC is a promising fluorescent probe for the effective discrimination of SNP and for highly sensitive detection of pc DNA. A higher number and higher fluorescence efficiency nucleotide base analogs in the stem of the modified hairpin that can be used as a fluorescent probe could be designed to increase the sensitivity of the proposed method for SNP genotyping. The new strategy for fluorescent biosensing could find wide application in a diverse range of areas, such as drug discovery, clinical diagnostics, and environmental monitoring by using aptamer as probe.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.02.005.

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