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DNA quantification based on FRET realized by combination with surfactant CPB

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ABSTRACT

In this work, we developed a novel DNA quantitative analysis based on fluorescence resonance energy transfer (FRET) realized by combination with a surfactant CPB. The approach was capable of detecting long-stranded DNA in a separation-free format. A sandwich-type FAM-c-DNA-t-DNA-r-DNA-TAMRA conjugate was first formed by the capture probe tagged with FAM, the reporter probe tagged with TAMRA and the target DNA through hybridization. The donor (FAM) and the acceptor (TAMRA) were bridged to afford a FRET system. Subsequently, an addition of the cationic surfactant CPB to the system resulted in a substantial change of the microenvironment and an effective condensation of DNA strands. Consequently, without altering the component of the double strands, an enhanced acceptor fluorescence signal from FRET was achieved and a quantification of the target DNA containing 30 bases was enabled. Under the optimal experimental conditions, an excellent linear relationship between the increase of acceptor fluorescent peak area and the target DNA concentration was obtained over the range from 1.0×10^{-7} to 3.0×10^{-9} mol L^{-1}. The proposed approach offered adequate sensitivity for the detection of the target DNA at 1.0×10^{-9} mol L^{-1}.

1. Introduction

There is clinical evidence that certain genes are closely related to the occurrence and extension of several types of common human cancers. Detection of these cancer genes and their mutated products has become a powerful tool for the clinical diagnosis of cancers and gained great interest among cancer researchers. KRAS is one of the most frequently mutated oncogenes and encodes for a guanine nucleotide-binding protein, which acts as a signal transducer. Furthermore, KRAS may harbor a number of point mutations resulting in cancers, particularly at codon 12 [1–5]. Due to the role of KRAS in cancer diagnosis and therapy, it has necessitated the development of a quantitative detection method for the mutated KRAS.

Nowadays there are many approaches for quantitative detection of nucleic acids. Among them, the conventional PCR relies on cumbersome, semi-quantitative amplification of target DNA. Both DNA microarrays and northern blotting require the separation of unbounded probes from the solid substrate, while molecular beacons with the stem-loop structure have the drawbacks of poor stability and high false positive signals.

Fluorescence resonance energy transfer (FRET) generates dual fluorescence emissions from a single excitation light. By utilizing these fluorescence signals, calculation of actual distance between donor and acceptor, quantitative analysis and monitoring of the conformation (or structure) change of biomolecules can be accomplished [6–8]. Based on FRET systems, the detection of nucleic acids and other biomolecules could employ the two fluorescence signals to identify false positive signal immediately. Moreover, the analyses are on a nanometer scale with great space-time resolution [2,6,9,10]. Although FRET acts as a "spectroscopic ruler", in practice, it is a short ruler with a limited dynamic range, normally 2–8 nm for one-step FRET [11–14]. For systems using DNA strands as the FRET linker, the transfer distance is dependent on the number of bases between the donor and the acceptor, which directly determines FRET efficiency. Therefore, the number of the bases for interval needs to be carefully designed. Ju et al. [15] examined the FRET properties of the donor–acceptor spacing from 1 to 10 bases. The fluorescence intensity of the donor and acceptor increased with the separation due to reduced quenching, which was sufficiently large to compensate for the decrease in FRET efficiency. Subsequently, Hung et al. [16] studied 2–16 intervening bases between the FRET pair and demonstrated that the optimal energy transfer occurred at 8 bases. However, for two probe–target hybrids, the optimal spac-

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ing for FRET varied from 5 or 6 bases for the two molecular beacons designed by Tsourkas et al. [17] to 8 bases for the fast DNA detection technique designed by Chen et al. [13]. Due to the strong dependence of FRET on separation distance, its applications in detecting long-stranded DNA are limited.

For the past few years, studies on FRET systems for long range have been growing steadily. With a multi-step energy transfer, the available distances that could be measured were prolonged with the number of fluorophores in the chain [10,14,18,19]. Sauer and co-workers [20–23] achieved the multi-step FRET by using several different fluorophores, which were covalently attached to the DNA and arranged in a cascade-like photonic wire. From the initial donor to the final acceptor, up to 40 bases worked as a scaffold. On the other hand, as a relay between the input and output fluorophores, an array of identical fluorophores could avoid significant energy loss through homotransfer [14,24–26], leading to the longest range of 50 bases [14]. Although it alleviates the limitation of distance, the multi-step FRET requires the exact position for a serial of emission spectra and results in the added steps of energy loss as well as the drawback of decreased directionality. So far, it has not been applied to quantify long-stranded DNA.

DNA is a kind of semi-flexible and highly charged polyelectrolyte that could strongly interact with cationic surfactants, causing significant compaction of the DNA strand. When mixed in solution, DNA and cationic surfactant will spontaneously combine by means of electrostatic and hydrophobic interactions. As a result, the DNA conformation transforms from elongated coil to compact globule in bulk [27–29]. It has been proved that cationic surfactant interacts with DNA by a combination of initial electrostatic interaction that followed by a cooperative binding of surfactant ligand to the same DNA molecule, driven by hydrophobic forces [30]. The reorganization would stabilize the complex and favor the DNA condensation process, giving rise to the conformational transition to globule state [30–33].

By taking advantage of the surfactant-induced conformational changes of DNA molecules, a new method to achieve long range FRET was established, which could be used to detect long-stranded targets without separation of the unhybridized probes. The sandwiched conjugate formed an effective FRET donor–acceptor ensemble. The addition of cationic surfactant CPB shortened the distance between the donor and acceptor to satisfy an efficient energy transfer. Consequently, the quantification of the target DNA containing 30 bases was successfully accomplished with a detection limit of 1.0×10^{-9} mol L^{-1}.

2. Experimental

2.1. Reagents

6-Carboxyfluorescein tagged capture DNA (FAM-c-DNA): 5′-CAG CTC CAA CTA CCA-3′-FAM, 6-carboxy-tetramethylrhodamine tagged reporter DNA (TAMRA-r-DNA): TAMRA-5′-TCT TGC CTA CGC CAA-3′, target DNA1 (t-DNA1) for 20 bases spacing: 5′-TTG GCG TAG GCA AGA TTT TTT TTT TTT TTT TTT GGT AGT TGG AGC TG-3′, t-DNA2 for 30 bases spacing: 5′-TGG TAG TTG GAG CTG TTG GCG TAG GCA AGA-3′ (codon 7 to 16 of KRAS, codon 12 GGT to GTT mutation) and t-DNA3 for 35 bases spacing: 5′-TGG TAG TTG GAG TTT TTC TGT TGG CGT AGG CAA GA-3′ were purchased from Jinsite Biotechnology (Nanjing, China). Cetyl pyridine bromide (CPB) was obtained from Shanghai Chemical Reagent Company (Shanghai, China). TE buffer (pH 7.5) consisted of 1.0×10^{-2} mol L^{-1} Tris–HCl and 1.0×10^{-3} mol L^{-1} EDTA. The hybridization buffer (pH 7.4) consisted of 1.0×10^{-2} mol L^{-1} Tris–HCl, 1.0×10^{-3} mol L^{-1} EDTA and 5.0×10^{-2} mol L^{-1} NaCl. All chemicals were of analytical grade and the solvents were purified using a MilliQ apparatus.

2.2. Apparatus

All the fluorescence measurements were performed on a Hitachi F-4500 spectrofluorimeter (Hitachi, Japan).

The CD spectra were recorded in a Jasco J-810S circular dichroism chiroptical spectrometer (JASCO, Japan).

2.3. Methods

DNA stock solutions were first diluted with the TE buffer (pH 7.5) and mixed in the hybridization buffer (pH 7.4) to probe DNA of 2.0×10^{-7} mol L^{-1} and target DNA of 1.0×10^{-7} mol L^{-1}. The mixture was denatured at 95 °C for 5 min, annealed at 72 °C for 15 min, and subsequently incubated at 37 °C overnight to form the FAM-c-DNA-t-DNA-r-DNA-TAMRA conjugate. Then the surfactant CPB was added to a level of 8.0×10^{-4} mol L^{-1} and 1 h later, fluorescence measurements were performed. An excitation and emission slit width of 10 nm was used. The emission spectra were obtained by setting the excitation wavelength at 470 nm and recording the emission wavelength from 490 to 650 nm.

3. Results and discussion

3.1. Formation of the FRET system and its fluorescence properties

The proposed assay method employed a pair of linear oligonucleotide probes, one with a donor fluorophore (FAM) and the other with an acceptor fluorophore (TAMRA). Probes were designed to hybridize to adjacent regions on the same target in order to bridge the two fluorophores. FAM emission and TAMRA excitation overlapped well and the both emission peaks were well separated from each other, enabling them to act as a valid FRET donor–acceptor pair (Fig. 1). In fluorescence measurements, emission intensity was monitored by exciting at 470 nm, where FAM was powerfully excited while TAMRA was scarcely excited.

As shown in Fig. 2A, when the intervening bases between donor and acceptor decreased from 35 to 20, FRET did not occurred in aqueous solution. In this case, the sandwiched FAM-c-DNA-t-DNA-r-DNA-TAMRA conjugate kept helical DNA conformation, which existed as anionic conjugated polymers of rigidity (3.4 nm for every 10 bases) with the distance between FAM and TAMRA exceeding the range for FRET. Therefore, energy transfer could not be observed. While with the distance decreased, the donor emission intensity dropped because of the quenching [15,16]. On the other hand, in the presence of the cationic surfactant CPB, the donor fluorescence intensity was significantly weakened with the obvious

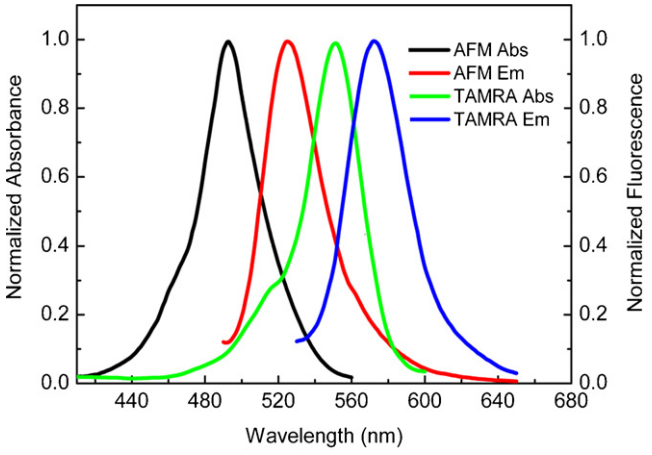

Fig. 1. Normalized absorption and emission profile of FAM and TAMRA.

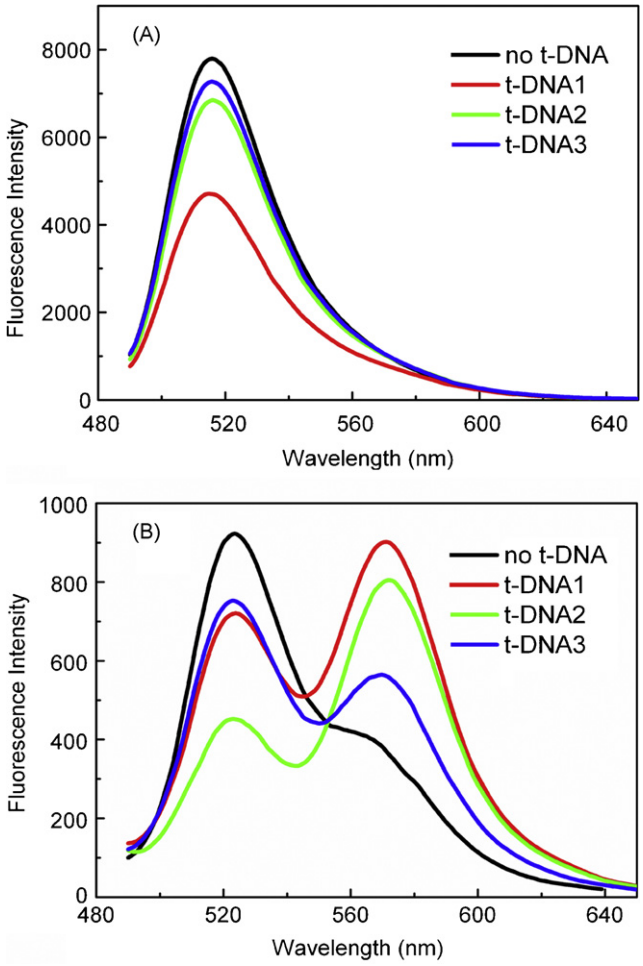

Fig. 2. Fluorescence emission spectra of FRET system in the absence (A) or presence of 8.0×10^{-4} mol L^{-1} CPB (B). The spectra were of system without t-DNA and with t-DNA for intervals of 20 bases (t-DNA1), 30 bases (t-DNA2) and 35 bases (t-DNA3) respectively. The concentration of probe DNA and t-DNA were 2.0×10^{-7} and 1.0×10^{-7} mol L^{-1} respectively.

enhancement of the acceptor fluorescence intensity. Moreover, the acceptor fluorescence enhancements decreased with the increase of the base interval (Fig. 2B). This demonstrated that a FRET process from FAM to TAMRA occurred with the addition of CPB.

A comparison between Fig. 2A and B clearly showed that the addition of CPB to the DNA solution resulted in FAM fluorescence quenching by more than 80%, accompanied by a red shift of FAM emission maximum from 515 to 525 nm. This indicated that the environment of FAM in the DNA/CPB complex suffered a significant change. Based on this, it was concluded that CPB and DNA interacted with each other, inducing the alteration of the DNA conformation. A change in conformation by condensing DNA led to a change in the distance between the two fluorophores, facilitating the energy transfer. From the CD spectrum (Fig. 3) in aqueous solution, one positive peak at 280 nm and one negative peak at 250 nm were the characteristics of double-stranded DNA in B conformation. In the presence of CPB, the intensity of both peaks decreased to be nearly invisible, suggesting that the negative charge of DNA was neutralized and the DNA conformation was transformed. The compaction of DNA was driven by the attractive electrostatic interaction between different parts of DNA strand and multivalent counterions offered by CPB that self-assembly aggregated in the vicinity of DNA. As a result, the originally expanded DNA strands were condensed to bring donor and acceptor into close approximation and realize FRET under the impact of hydrophobic interaction.

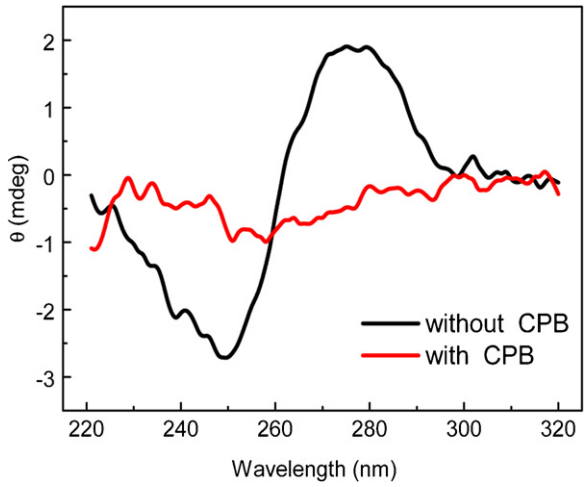

Fig. 3. CD spectra of the DNA system without and with CPB of 8.0×10^{-4} mol L^{-1}. The concentration of probe DNA and target DNA were 4.0×10^{-6} and 2.0×10^{-6} mol L^{-1} respectively.

3.2. Effect of CPB on the FRET system

3.2.1. Effect of different CPB addition sequences

To find the optimal CPB addition sequence for the FRET system, three parallel experiments were conducted by adding CPB prior to DNA denaturization, before or after incubation overnight respectively. It was found that if CPB was added before DNA hybridization was completed (the first two cases), FRET between FAM and TAMRA could not be observed. This could demonstrate that CPB hindered DNA normal hybridization and the sandwiched DNA conjugate did not form. Thus energy could not be transferred to the acceptor. Distinctively as depicted in Fig. 2B, FRET was apparently effective with the addition of CPB after the incubation, illustrating that CPB could help realizing the FRET between donor and acceptor bridged by the target DNA. Therefore, CPB was added after DNA had been incubated overnight in the assay.

3.2.2. Effect of CPB concentration

Changes of the TAMRA fluorescence emission were studied by adding CPB from 0 to 1.75×10^{-3} mol L^{-1} at probes 2.0×10^{-7} mol L^{-1} and targets 1.0×10^{-7} mol L^{-1} (Fig. 4). The effect of CPB concentration on the fluorescence enforcement of TAMRA was investigated using $\Delta F = (F_1 - F_0)$ as standard. F_1 and

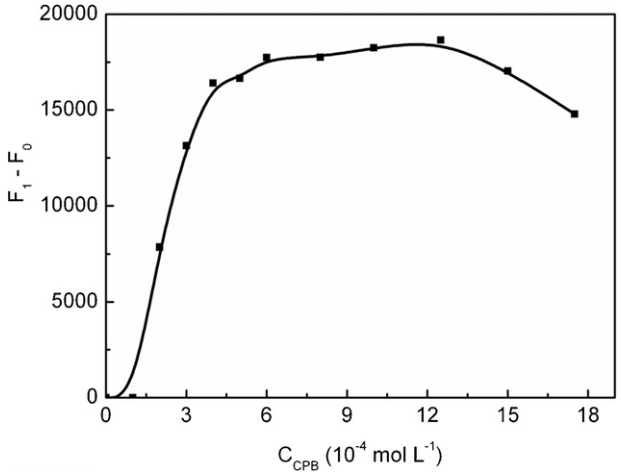

Fig. 4. Effect of the CPB concentration on ΔF. The concentration of probe DNA and t-DNA were 2.0×10^{-7} and 1.0×10^{-7} mol L^{-1} respectively.

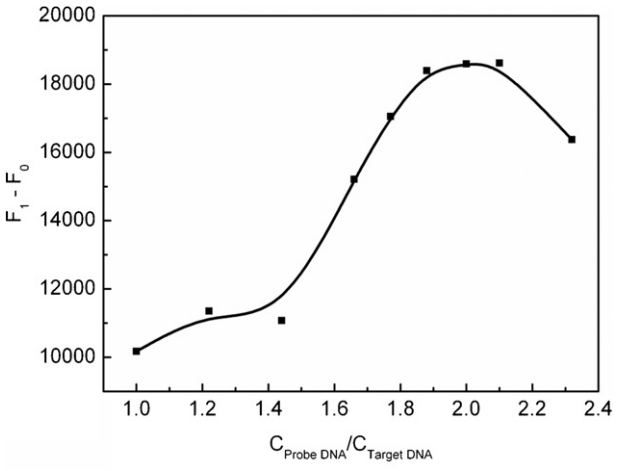

Fig. 5. Effect of $C_{Probe\,DNA}/C_{Target\,DNA}$ on ΔF. The concentration of t-DNA was 1.0×10^{-7} mol L^{-1} and CPB was 8.0×10^{-4} mol L^{-1}.

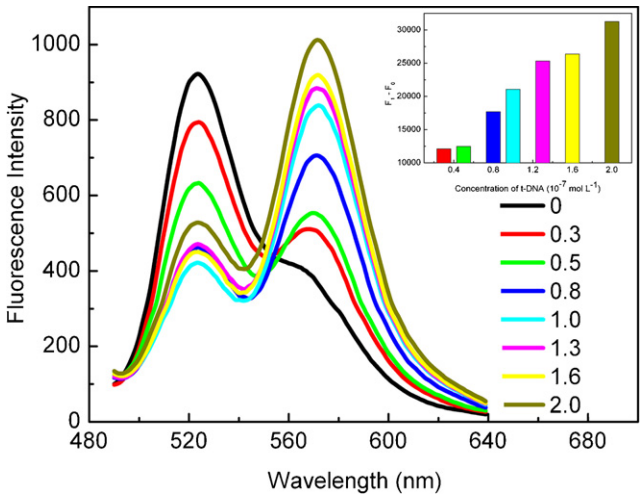

Fig. 6. Effect of the t-DNA concentration ($\times 10^{-7}$ mol L^{-1}) on fluorescence intensity. The insert histogram represented ΔF values at different t-DNA concentration. The concentration of probe DNA was 2.0×10^{-7} mol L^{-1} and CPB was 8.0×10^{-4} mol L^{-1}.

F_0, obtained from the least squares fitting procedure, were areas under acceptor peaks in positive and negative experiments respectively. Hence, ΔF could reflect the energy transferred from FAM to TAMRA. It was noted that ΔF increased with the CPB concentration below 4.0×10^{-4} mol L^{-1} and changed little from 4.0×10^{-4} to 1.25×10^{-3} mol L^{-1}, and then ΔF began to decrease. A possible reason was that, for CPB lower than 4.0×10^{-4} mol L^{-1}, not all DNA interacted with CPB to the greatest degree because of the insufficient negative charge from CPB. Consequently, there were free/unbound DNA strands coexisting with surfactant-saturated DNA strands [33]. In this case, the transferred energy was enhanced as the CPB concentration increased. When all DNA strands were saturated with the surfactant in bulk, any further surfactant added to the solution could not influence ΔF. The effect of the CPB concentration on ΔF could further prove that the changes of the fluorescence signal were due to the combination of negative DNA strands and positive CPB, leading to the DNA structural transition and an effective FRET from donor to acceptor. Finally, a CPB concentration of 8.0×10^{-4} mol L^{-1} was selected in this assay.

3.3. Effect of the probe/target concentration ratio on the FRET system

For conventional DNA detection, excess probes would make false positive signal, so that it was necessary to keep probes and targets equivalent or separate the free probes [2,13,17]. In our approach, the excess probes did not cause any false positive signal since the contributions from the direct excitation of acceptor and the FRET between unhybrided probes were small enough to be subtracted by taking ΔF as signal. This enabled that a separation-free format could be achieved for t-DNA detection.

To enhance the signal intensity, the effect of the probe and target concentration ratio on ΔF has been investigated (Fig. 5). It was apparent that ΔF trended to increase for a probe/target ratio ranged from 1.0 to 1.9. When the ratio ranged from 1.9 to 2.1, ΔF did not change and reached a maximum, where the t-DNA was fully hybridized. A probe/target ratio of 2.0 was used in the assay, which could guarantee that t-DNA was completely hybridized and a maximum fluorescence signal was obtained without any increase of background signal.

3.4. Detection of t-DNA based on acceptor fluorescence signal

Fig. 6 revealed the typical dependence of emission spectra on the t-DNA concentration from 0 to 2.0×10^{-7} mol L^{-1}. With the increase of the t-DNA concentration, the fluorescence intensity of donor was continuously quenched while that of acceptor was continuously enhanced (the change of ΔF shown in the insert histogram). At each probe/target ratio, the fluorescence intensity of acceptor was dependent on the total amount of double-stranded DNA that linked FAM and TAMRA. As there was no special t-DNA in negative experiments, DNA hybridization was absent, resulting in the lowest emission intensity of acceptor. The amount of double-stranded DNA increased as t-DNA concentration increased, corresponding to the increase of donor–acceptor pairs that could generate FRET signal.

Under the optimized conditions described above, it was found that this FRET system produced a sensitive and selective response to the t-DNA concentration. Over the range of 1.0×10^{-7} to 3.0×10^{-9} mol L^{-1}, an excellent linear relationship between ΔF and the t-DNA concentration was observed as shown in Fig. 7 and the detection limit was determined as 1.0×10^{-9} mol L^{-1} (3 times the standard deviation above the blank). The linear regression equation was determined to be $Y = 324 + 1.81 \times 10^{11}\,C$ ($R = 0.9996$). In summary, based on FRET realized by the addition of CPB, the long-stranded DNA detection was achieved in a sandwiched hybridization mode.

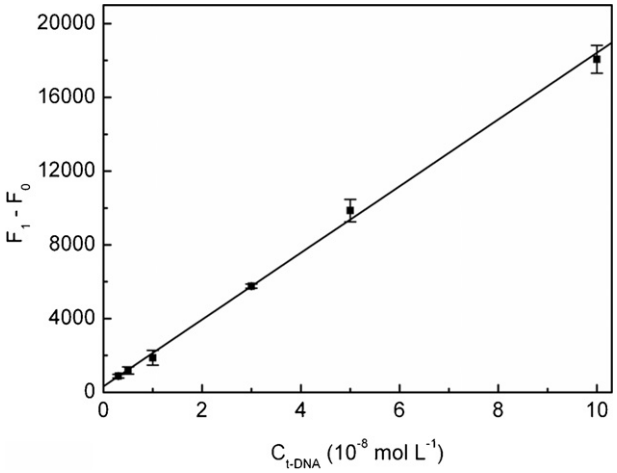

Fig. 7. Relationship between ΔF and the concentration of t-DNA.

4. Conclusions

A novel DNA detection method based on FRET was achieved. The proposed method was demonstrated to have unique advantages, including a separation-free format and expanded applications for detecting long-stranded targets. To assemble the FRET system, oligonucleotides bridged the donor and acceptor through sandwiched hybridization. Then the condensation of DNA strands was driven by attractive interactions between DNA and the surfactant CPB, generating an effective FRET from FAM to TAMRA. The acceptor fluorescence signal and the analyte concentration showed an excellent linear relationship in the range from 1.0×10^{-7} to 3.0×10^{-9} mol L^{-1}. Using the conventional dyes and apparatus, this new approach could be applied to detect long-stranded DNA that was unable to be quantitatively analyzed with FRET in aqueous solution.

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