RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Linear DNA probe as alternative to molecular beacon for improving sensitivity of homogenous fluorescence biosensing platform for DNA detection using target-primed rolling circle amplification

Fulin Zhou, Baoxin Li* and Jiyuan Ma

Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry & Chemical Engineering, Shaanxi Normal University, Xi'an 710062, China

*Fax: +86-29-81530727. E-mail: libaoxin@snnu.edu.cn (B. Li)

Abstract

Herein, we report a simple and homogenous fluorescence method for ultrasensitive DNA detection. It is based on rolling circle amplification (RCA) and fluorescence resonance energy transfer (FRET). As an alternative to molecular beacon (MB), linear single-labeled DNA probe was used in this RCA-based fluorescence strategy for DNA detection. The performance of linear DNA probes was compared with that of MB probe in RCA-based fluorescence strategy. The results showed that linear DNA probe could effectively avoid the fluorescence quenching between neighboring signal probes, which would significantly improve the sensitivity of RCA-based fluorescence strategy. This method exhibited a high sensitivity toward target DNA with a detection limit of 0.7 aM, which was about 100-fold lower than that of the RCA-based fluorescence strategy with MB as signal probe. This method provides a simple, isothermal, and low-cost approach for sensitive detection of DNA and holds a great potential for early diagnosis in gene-related diseases.

Introduction

Nucleic acid analysis is critical in molecular diagnostics, genetics therapy, forensic investigation, and biomedical research.[1,2] The disease-related nucleic acids are often found only in trace amounts in biological extracts, and seeking a convenient and cost-effective method for probing ultralow

concentrations of specific single stranded DNA (ssDNA) has risen to become a great challenge in recent years.[3,4] Target amplification is usually employed to achieve high sensitivity.[5,6] As the classical target amplification method, polymerase chain reaction (PCR) can amplify one oligonucletide target, improving the sensitivity to the extent that the single-molecule detection limit is often realized.[7] However, PCR requires precise temperature control and multiple primer design, which increases the experimental cost and complexity; PCR is prone to false positives arising from artifactual amplification (e.g., of primer dimers, etc.).[8] In recent years, various alternative strategies of PCR have been developed to improve the detection sensitivity and adaptability, such as rolling circle amplification (RCA), hybridization chain reactions, and target recycling amplification. Among these methods, RCA has become increasingly popular for the quantitative detection of nucleic acids and indirect detection of other targets.[9–15] RCA is an isothermal enzymatic process, where a short DNA primer is amplified to form a long ssDNA in the presence of a circular DNA template and special DNA polymerases. Unlike PCR, RCA can be conducted at a constant temperature (room temperature to 37 ºC) in solution. The RCA product is a concatemer containing tens to hundreds of tandem repeats that are complementary to the circular template. A single molecule can be amplified hundreds to thousands of times of template as a fundamental characteristic of RCA.

The power, simplicity, specificity and versatility of RCA technique have made it an attractive tool for biomedical research.[14,15]

In the RCA-based biosensing platform, the target quantification is achieved through the quantification of RCA product. The most common way to analyze RCA product is gel electrophoresis separation and radioactive-band measurements, which suffers from high labor intensity and inherent safety problems (e.g., health hazard and waste disposal problems).[16] To address the problem, some alternative methods have been developed, including fluorescence,[10,11,16,17] colorimetry,[12,18] surface-enhanced Raman scattering spectroscopy,[19] bioluminescence[20] and electrochemical method [21]. Recently, molecular beacon (MB), one very popular fluorescent DNA probe, has garnered much attention in the RCA-based biosensing platform.[22–27] MB is specifically designed short of nucleic acid that folds into a hairpin of stem-and-loop structure. In the absence of the target, the stem structure brings the quencher and fluorophore into close proximity, and thus the MB is "closed" or "off" as the result of fluorescence resonance energy transfer (FRET). In contrast, target binding to the MB loop region induces conformational changes and results in the opening of the hairpin, and thus the fluorescence is turned "on".[28] In RCA-based biosensing platform with MB as signal probe, RCA product with thousands of repeated DNA domains makes many MBs open and restore their fluorescence due to the hybridization between the

RCA product and MB.[22–27] However, RCA product is different from most DNA molecules present in a typical biological sample because RCA product constitutes tens of kilobases long single-stranded tandem repeated copies of the sequence, and folds into random coils in solution.[19, 29–31] The coiled RCA product can serve as a template for periodic binding of MB because of complete complementary of MB to each repeated sequence of the RCA product. This results in the opening of numerous MBs. For each MB on the RCA product, the effective spatial separation of the fluorophore and the quencher increases fluorescent signal. But the coil structure of RCA product can shorten the distance between fluorophore and the quencher of neighboring MBs, resulting in FRET between the donor and acceptor of neighboring MBs. This would decrease the sensitivity of the RCA-based biosensing platform with MB as signal probe.

To address this problem, we herein develop a novel RCA-based fluorescence biosensing strategy for detection of DNA. In this strategy, the two short linear DNA probes, labeled at the end with fluorophore and quencher, respectively, are used as signal probes for quantification of RCA product. The designed strategy was depicted in **Scheme 1**. The strategy used a preliminarily synthesized circular DNA as recognition probe for hybridization with DNA target, and the DNA target then primed a RCA reaction to generate a long product with tandem repeated sequence.

The RCA product could be hybridized with thousands of fluorophore-labeled linear probe and quencher-labeled linear probe, thus allowing detection of DNA target. In this strategy, the fluorophore-labeled probe and quencher-labeled probe self-assembled in head-to-head fashion on the coiled RCA product, thereby leading to efficient FRET between fluorophore and quencher. The performance of linear DNA probes was compared with that of MBs in the RCA-based fluorescence biosensing strategy. The present linear DNA probes method was turn-off model, and the coil structure of RCA product did not influence signal readout. However, the MBs method was turn-on model, and the FRET between the donor and acceptor of neighboring MBs due to the coil structure of RCA product would decrease the sensitivity. The results showed that the linear DNA probes were more suitable for highly sensitive detection of DNA in the RCA-based strategy. In this DNA detection system, the strategy of combination of RCA and linear DNA probe could significantly improve the sensitivity, and the detection limit was estimated to be 0.7 aM (3σ). In addition, the linear DNA probe is single-labeled. So, the method is simple and low-cost.

Experimental

Chemicals and materials

All oligonucleotides designed in this study were commercially

synthesized by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China), and purified by high performance liquid chromatography (HPLC). The sequences of oligonucleotides were listed in Table S1 (Supporting Information). The oligonucleotide stock solutions (20 μM) were prepared in 100 mM phosphate buffered saline (PBS) (100 mM KCl, 20 mM $MgCl_2$, pH 7.4) and diluted to the desired concentration with the same PBS. Before use, the oligonucleotide solution was heated to 90 °C for 5 min and cooled slowly over a 10 min period to room temperature to unwind the single-strand oligonucleotide. T4 DNA ligase and deoxynucleotides (dNTPs) were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Phi29 DNA polymerase was obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). All other chemicals were of analytical-reagent grade and obtained from standard reagent suppliers. Millipore Milli-Q water (18 $M\Omega\ cm^{-1}$) was used in all experiments.

Ligation and rolling circle amplification reactions

Firstly, 20 μL phosphorylated padlock DNA probe (1×10^{-12} M) was mixed 20 μL target DNA (varying concentrations), and the mixture was incubated at 37 °C for 1 h. Then, 4 μL T4 DNA ligase solution (1 U/μL) and 5 μL 10 × ligation buffer (pH 7.5, 660 mM Tris-HCl, 66 mM $MgCl_2$, 100 mM dithiothreitol (DTT), and 1 mM ATP) were added into the above solution. The ligation was performed for 16 °C for 1 h. After ligation, T4

DNA ligase was inactivated by heating the reaction mixture at 55 °C for 10 min. The resulting mixture could be used directly or stored at 4 °C. For circle amplification reaction, 4 μL Phi29 DNA polymerase (1 U/μL), 8 μL dNTPs (2.5 mM) and 40 μL 2.5×Phi29 buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 60 mM KCl, 10 mM DTT.) were successively added into the ligation reaction. The polymerization reaction was carried out at 37 °C for 150 min. Subsequently, the reaction was terminated by incubating at 65 °C for 10 min to inactivate Phi29 polymerase.

Fluorescence measurement

10 μL RCA amplification product was mixed with 10 μL fluorophore-labeled probe (3×10^{-11} M) and 10 μL quencher-labeled probe (6×10^{-11} M), and then diluted to a final volume of 200 μL with 100 mM PBS (pH 7.4). After incubation for 1 h at 37 °C, the fluorescence spectra were measured by using a Hitachi F-4600 fluorometer (Tokyo, Japan) at the excitation wavelength of 480 nm, and the spectra were recorded in the range from 500 nm to 570 nm. Slit widths for the excitation and emission were set at 10 nm and 10 nm, respectively. The fluorescence emission intensity was measured at 518 nm. The target DNA concentration was quantified by the fluorescence intensity. The background signal (F_0) was the fluorescence intensity of the system in the absence of target DNA.

Results and discussion

Principle of RCA-based fluorescence biosensing of DNA

The strategy for RCA-based homogenous fluorescence detection of DNA is depicted in **Scheme 1**. We designed the padlock DNA probe, and the probe contains two domains: hybridization region (red) and detection region (blue). The red portions in the 5′- and 3′- terminal of the padlock DNA probe are designed to be complementary to the target DNA in a head-to-tail fashion. Therefore, in the presence of target DNA, the padlock probe can be specifically ligated and circularized with the help of T4 DNA ligase, indicting that the specificity of the proposed assay depends on the ligation reaction. Subsequently, circularization template can be gained, and in the presence of Phi29 DNA polymerase and dNTPs, the RCA process is triggered-on, leading to the isothermal autonomous synthesis of a long tandem repeated ssDNA. In order to quantify the RCA product, two short linear DNA probes (P_1 and P_2) are designed. P_1 is labeled with 6-carboxy-fluorescein (FAM) at the 5′-termini, and P_2 is labeled with Black Hole Quencher (BHQ_1) at the 3′-termini. As the signal probes, P_1 and P_2 are complementary to the RCA product in each repeat sequence. So, the RCA product can hybridize with FAM-labeled P_1 and BHQ_1–labeled P_2 in head-to-head fashion. As a result, FAM and BHQ_1 bound on the coiled RCA product are closed to each other, which can lead to efficient FRET between FAM and BHQ_1 and cause a decrease of FAM fluorescence intensity at 518 nm. In contrast, in the absence of target

DNA, no circularization template and no RCA product can be gained, and no FRET occurs in the system because FAM labeled on P_1 and BHQ_1 labeled on P_2 are far away from each other. Therefore, a sensitive "signal-off" fluorescence biosensing of DNA is achieved based on RCA.

The feasibility of this proposed method is validated by fluorescence spectral characteristics. As shown in **Figure 1**, in the absence of the target, the fluorescent intensity is strong (curve a), indicating that FRET did not occur. Once 1×10^{-11} M target DNA is added into the system, the fluorescence intensity significantly decreases (curve b). The control experiments showed that T4 DNA ligase and Phi29 DNA polymerase themselves did not influence the fluorescence intensity of FAM labeled on P_1. In the presence of T4 DNA ligase or Phi29 DNA polymerase alone, target DNA addition did not result in the fluorescence decrease. The results strongly indicated that only target DNA can trigger the ligation and RCA reaction to generate RCA product for the amplified FRET response.

In order to check the sensitivity of the RCA-based fluorescence strategy for DNA detection with linear DNA probes, the common RCA-based approach with MB probe was designed for comparison. As shown in **Scheme 2**, the RCA product can hybridize with the loop of MBs, resulting in separation of FAM (fluorophore) and BHQ_1 (quencher). Thus, the MBs restore their fluorescence. We also measured the fluorescence

spectra in the presence and absence of 1×10^{-11} M target DNA (**Figure 2**). In this "signal-on" strategy, the fluorescence intensity increased from 330 to 500 **(Figure 2)**, and the signal-to-noise ratio (F/F_0, F and F_0 are the fluorescence signals in the presence and absence of target DNA, respectively.) at 518 nm for 1×10^{-11} M target DNA was about 1.52. In contrast, for the same concentration of target DNA, the fluorescence intensity in the signal-off strategy (**Scheme 1**) decreased from 2300 to 800 **(Figure 1)**, and F_0/F was about 2.88. Obviously, the sensitivity of the "signal-off" strategy with linear DNA probes was much higher than that in the "signal-on" strategy with MB probes.

In order to explore the reason of the different sensitivity in both fluorescence strategies, we detected DNA using direct fluorescence method without RCA, as shown in **Scheme S1** and **S2**. The fluorescence responses to 1×10^{-11} M target DNA (**Figure S1** and **S2**) were much lower than that in the strategies with RCA (**Figure 1** and **2**), which indicated the amplification function of RCA. But, without RCA process, the sensitivity in the "signal-off" strategy (**Figure S1**) was almost the same as in the "signal-on" strategy (**Figure S2**). The experimental results showed that the RCA would cause the sensitivity difference in the both RCA-based strategies. Different from a short DNA chain, RCA product is tens of kilobases long single-stranded tandem repeated copies of the sequence, and folds into random coils in solution.[19, 29–31] In the "signal-on" strategy,

MB sequence is complementary to the RCA product in each repeat sequence, and the coiled RCA product can serve as a template for periodic binding of MB, resulting in open of numerous MBs. Because of effective spatial separation of FAM and BHQ_1 of intra MB, MBs can restore their fluorescence. But, the coil structure would shorten the distance between FAM and BHQ_1 of neighboring MBs, and lead to FRET of inter MBs. As a result, the sensitivity of the signal-on strategy would significantly decrease. However, the strategy with linear DNA probes is signal-off, and the FRET can not decrease the sensitivity and even can enhance the sensitivity.

To further validate that the distance between neighboring MBs can affect the fluorescence response of MB, we designed seven DNA chains with different length: cMB–cMB, cMB–A_3–cMB, cMB–A_5–cMB, cMB–A_{10}–cMB, cMB–A_{15}–cMB, cMB–A_{20}–cMB, and cMB–A_{30}–cMB. cMB is complementary sequence of MB. A_3, A_5, A_{10}, A_{15}, A_{20} and A_{30} refer to containing 3, 5, 10, 15, 20, 30 adenines in sequence, respectively. The sequences of oligonucleotides were listed in Table S2 (Supporting Information). Each of DNA chains contains two cMBs, and two cMBs are separated with 3~30 adenines base. PolyA can link the two cMBs. We controlled the nucleotides number of polyA to tune the distance between two cMBs. Thus, each of these DNA chains can hybridize with two MBs, and the distances between the two MBs bound on the seven DNA chain

are different. We measured the fluorescence spectra of MB in the presence of these seven DNA chains, respectively. As shown in **Figure 3A**, the MB/cMB hybridization can restore the fluorescence of system, and the fluorescence intensity decreases with decreasing the distance between two MBs in the range of 0~30 base space. The results suggest that FRET of inter MBs occurs in the system and the FRET efficiency depends on the distance between two MBs. On the other hands, the results (**Figure 3B**) showed that the phenomenon did not occur when linear DNA probe (P1 and P2) replaced MB. Theses experiments further confirmed that the FRET of inter MBs decreased the sensitivity of the RCA-based strategy with MB as signal probe. Linear DNA probe could effectively avoid the fluorescence quenching between neighboring signal probes, which is favorable to improve sensitivity of the RCA-based fluorescence strategy.

Optimization of assay conditions

In order to achieve an optimal sensing performance, experimental parameters affecting RCA reaction and quantification of RCA product were optimized, including the of amount of T4 DNA ligase and Phi29 DNA polymerase, and the concentrations of padlock probe, dNTP substrates and signal probes, and the incubation time. The experimental results showed that the optimal concentrations or amounts of padlock

probe, T4 DNA ligase, Phi29 DNA polymerase, and dNTP substrates were 1 pM, 4 U, 4 U and 2.5 mM, respectively (Figure S4 to S10, Supporting Information). In theory, more complementary copies of the padlock probe are generated with the elongation of RCA reaction time, and stronger signal amplification will be produced. So, the effect of RCA reaction time on the fluorescence signal was examined. The fluorescence intensity increased quickly with increasing reaction time, and nearly reached a plateau after 150 min. This might be attributed to the fact that the RCA reaction had reached equilibrium caused by exhaust of RCA substrates or inactivation of phi29 DNA polymerase.[24] Therefore, 150 min was used as the optimum time for RCA reaction. This time was in agreement with the reported RCA reaction time of 1–3 h.[19] The concentration of P_1 and P_2 signal probes is another important factor in this assay system. When the concentration of FAM-labeled P_1 and BHQ_1-labeled P_2 was too low, the response of the system was very small. The possible reason was that RCA product might not hybridize with sufficient P_1 and P_2, and some repeats in RCA product would not simultaneously bind FAM-labeled P_1 and BHQ_1–labeled P_2. Too high concentration of P_1 and P_2 would lead to high background signal, which would reduce the sensitivity. The optimal concentrations of P_1 and P_2 were 30 pM and 60 pM, respectively.

Sensitivity of the homogeneous RCA-based fluorescence detection of

DNA

Under the optimized conditions, experiments were carried out by adding target DNA with different concentrations into the system to evaluate the sensitivity of the RCA-based fluorescence assay. The results showed (Figure 4) that when the DNA concentration increased from 1.0×10^{-18} to 1.0×10^{-16} M, the fluorescence intensities continuously decreased. With the measurement of the fluorescence intensity, we quantitatively analyzed the fluorescence response with the concentration of target DNA. As shown in **Figure 5**, the fluorescence intensity change (F_0–F) of the system was a good linear fit to the concentration of target DNA in the range of 1 aM to 0.1 fM. The detection limit was calculated to be 0.7 aM by evaluating the average response of the blank plus 3 times the standard deviation. Furthermore, for comparison, we also measured the fluorescence response of the RCA-based strategy with MB as signal probe (**Scheme 2**). The results showed that the target DNA could be detected quantitatively in the range from 0.3 fM to 3 fM (**Figure S3**, Supporting Information), and the detection limit was 0.1 fM, which was about 100-fold higher than that obtained in the RCA-based fluorescence strategy with the linear DNA probe as signal probe. The high sensitivity of this proposed method might be attributed to two factors: (1) high amplification efficiency of RCA and (2) effectively avoiding FRET of inter MBs on the coiled RCA product.

Selectivity of the homogeneous RCA-based fluorescence detection of DNA

The specificity of this proposed method for detecting DNA was investigated by testing the fluorescence response to nine kinds of DNA sequences, including complementary target DNA (T), single-base mismatched DNA (T1), two-base mismatched DNA (T2), and the noncomplementary DNA (Tn) at the same concentration of 0.1 fM. For single-base mismatched DNA, the position of the mismatched base are at five different sites: the 3'-end position ($T1^a$), the 5'-end position ($T1^b$), the middle position where target DNA hybridized with 3'-termini of padlock probe ($T1^c$), and the middle position where target DNA hybridized with 5'-termini of padlock probe ($T1^d$). For two-base mismatched DNA, the position of the two mismatched base are at three different sites: the middle position ($T2^a$), the 3'- and 5'-end position ($T2^b$), and other position ($T2^c$). **Figure 6** shows the fluorescence intensity changes with target DNA and other mismatched DNA strands. The results illustrated that the completely complementary target DNA (T), T1, T2 and Tn were distinctly discriminated under the same detection conditions. It was worth noting that the specificity of assay was largely affected by the ligase for sealing the termini of the padlock probe ($T1^c$, $T1^d$, and $T2^a$). In addition, the method could discriminate the site of the single mismatched base ($T1^a$ and $T1^b$). The extension of primer (target DNA) started from its 3'-end. If

the 3'-end of the primer is not hybridized with the padlock probe, the efficiency of amplification reaction would be greatly reduced, and even lead to the RCA failed completely. All in all, the good selectivity of the assay was attributed to the high specificity of hybridization reaction, ligation reaction, and amplification reaction.[32] These results indicated the high specificity of this proposed strategy.

Conclusion

A simple RCA-based fluorescence strategy for ultrasensitive DNA detection has been demonstrated in this study. In this proposed strategy, the two short linear DNA probes, FAM-labeled P_1 and BHQ_1–labeled P_2, are used as signal probes for readout of RCA product. Linear DNA probes can obviate the fluorescence quenching of neighboring signal probes. With highly efficient amplification and excellent signal readout, the present method could achieve a detection limit as low as 0.7 aM, which is far more sensitive than that of the RCA-based fluorescence strategy with MB as signal probe, and holds a great potential for early diagnosis in gene-related diseases. In addition, this strategy offers a convenient "mix-and-detect" protocol for homogeneous assay, which avoids the separation and washing steps. Given the simplicity and sensitivity, single-labeled linear DNA probe promises to be a general applicable signal probes in RCA-based fluorescence method for detection of various targets including DNA, microRNA, and aptamer-binding molecules.

Acknowledgments

This work was supported financially by the National Natural Science Foundation of China (No. 21275096), Shaanxi Provincial Natural Science Foundation (No. 2013SZS08-Z01), and Program for Innovative Research Team in Shaanxi Province (No. 2014KCT−28).

REFERENCES

1 B. J. Zou, Y. J. Ma, H. P. Wu and G. H. Zhou, *Angew. Chem., Int. Ed.,* 2011, **50**, 7395−7398.

2 F. Wang, J. Elbaz, R. Orbach, N. Magen and Willner, I. *J. Am. Chem. Soc*., 2011, **133**, 17149−17151.

3 D. Li, S. Song and C. Fan, *Acc. Chem. Res*., 2010, **43**, 631−641.

4 H. Zhang, F. Li, B. Dever, X.-F. Li and X. C. Le, *Chem. Rev*., 2013, **113**, 2812−2841.

5 N. Tomita, Y. Mori, H. Kanda and T. Notomi, *Nat. Protoc*., 2008, **3**, 877−882.

6 J. Zhou, Q.-X. Wang and C.-Y. Zhang, *J. Am. Chem. Soc.,* 2013, **135**, 2056−2059.

7 Y. S. Lie and C. J. Petropoulos, *Curr. Opin. Biotechnol.,* 1998, **9**, 43–48.

8 P. Gill and A. Ghaemi, *Nucleosides Nucleotides Nucleic Acids,*

2008, **27**, 224−243.

9 W. Zhao, M. M. Ali, M. A. Brook and Y. Li, *Angew. Chem., Int. Ed*., 2008, **47**, 6330−6337.

10 J. Yin, P. Gan, F. Zhou and J. Wang, *Anal. Chem.,* 2014, **86**, 2572−2579.

11 J. Ge, L.-L. Zhang, S.-J. Liu, R.-Q. Yu and C. Xia, *Anal. Chem.,* 2014, **86**, 1808−1815.

12 F. Wang, C.-H. Lu, X. Liu, L. Freage and I. Willner, *Anal. Chem.,* 2014, **86**, 1614−1621.

13 Y. He, D. Chen, M. Li, L. Fang, W. Yang, L. Xu and F. Fu, *Biosens. Bioelectron.,* 2014, **58**, 209−213.

14 M. M. Ali, F. Li, Z. Zhang, K. Zhang, D. K. Kang, J. A. Ankrum, X. C. Le and W. Zhao, *Chem. Soc. Rev.,* 2014, **43**, 3324−3341.

15 T. Kobori and H. Takahashi, *Anal. Sci.,* 2014, **30**, 59−64.

16 Y. Cheng, X. Zhang, Z. Li, X. Jiao, Y. Wang and Y. Zhang, *Angew. Chem. Int. Ed*., 2009, **48**, 3268–3272.

17 J. Yin, P. Gan, F. Zhou and J. Wang, *Anal. Chem*., 2014, **86**, 2572−2579.

18 L. H. Tang, Y. Liu, M. M. Ali, D. K. Kang, W. A. Zhao and J. H. Li, *Anal. Chem.,* 2012, **84**, 4711–4717.

19 C.-Y. Zhang and J. Hu, *Anal. Chem.,* 2010, **82**, 8991–8997.

20 S. Bi, T. Zhao, B. Luo and J. J. Zhu, *Chem. Commun.,* 2013, **49**,

6906–6908.

21 D. Wang, L. Hu, H. Zhou, E. S. Abdel-Halim and J.-J. Zhu, *Electrochem. Commun*., 2013, **33**, 80–83.

22 S. Bi, B. Ji, Z. Zhang and J.-J. Zhu, *Chem. Sci.,* 2013, **4**, 1858–1863.

23 L. Zhang, J. Zhao, J. Jiang and R.-Q. Yu, *Chem. Commun*., 2012, **48**, 8820–8822.

24 Y. Zhao, L. Qi, F. Chen, Y. Dong, Y. Kong, Y. Wu and C. Fan, *Chem. Commun*., 2012, **48**, 3354–3356.

25 Z. Zou, Z. Qing, X. He, K. Wang, D. He, H. Shi, X. Yang, T. Qing and X. Yang, *Talanta,* 2014, **125**, 306–312.

26 P. Liu, X.-H. Yang, Q. Wang, J. Huang, J.-B. Liu, Y. Zhu, L.-L. He and K.-M. Wang, *Chin. Chem. Lett.* 2014, in press, http://dx.doi.org/10.1016/j.cclet.2014.05.002.

27 C. Ma, W. Wang, Q. Yang, C. Shi and L. Cao, *Biosens. Bioelectron.,* 2011, **26**, 3309–3312.

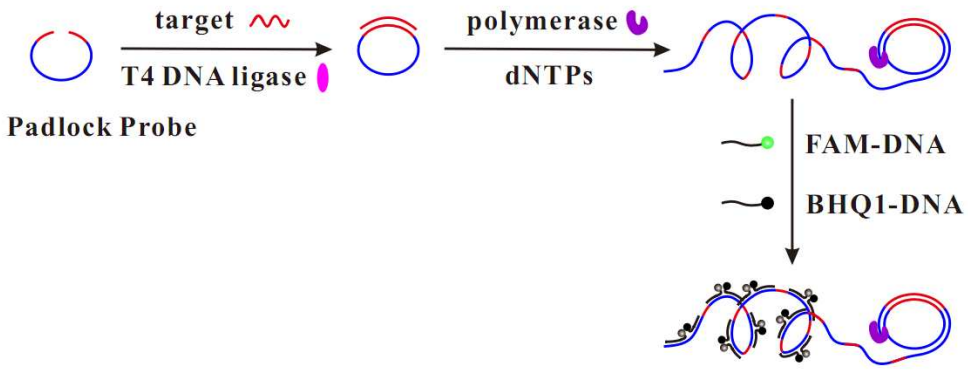
28 S. Tyagi and F. R. Kramer, *Nat. Biotechnol*., 1996, **14**, 303–308.

29 G. A. Blab, T. Schmidt and M. Nilsson, *Anal. Chem*., 2004, **76**, 495–498.

30 T. Zardán Gómez de la Torre, M. Strömberg, C. Russell, J. Göransson, M. Nilsson, P. Svedlindh and M. Strømme, *J. Phys. Chem. B,* 2010, **114**, 3707–3713.

31 Z. Cheglakov, Y. Weizmann, A. B. Braunschweig, O. I. Wilner and I. Willner, *Angew. Chem. Int. Ed*., 2007, **47**, 126–130.

32 H. Liu, L. Li, L. Duan, X. Wang, Y. Xie, L. Tong, Q. Wang and B. Tang, *Anal. Chem.,* 2013, **85**, 7941−7947.



Scheme 1. Schematic illustration of the strategy for RCA-based homogenous fluorescence detection of DNA with linear DNA probe as signal probe.

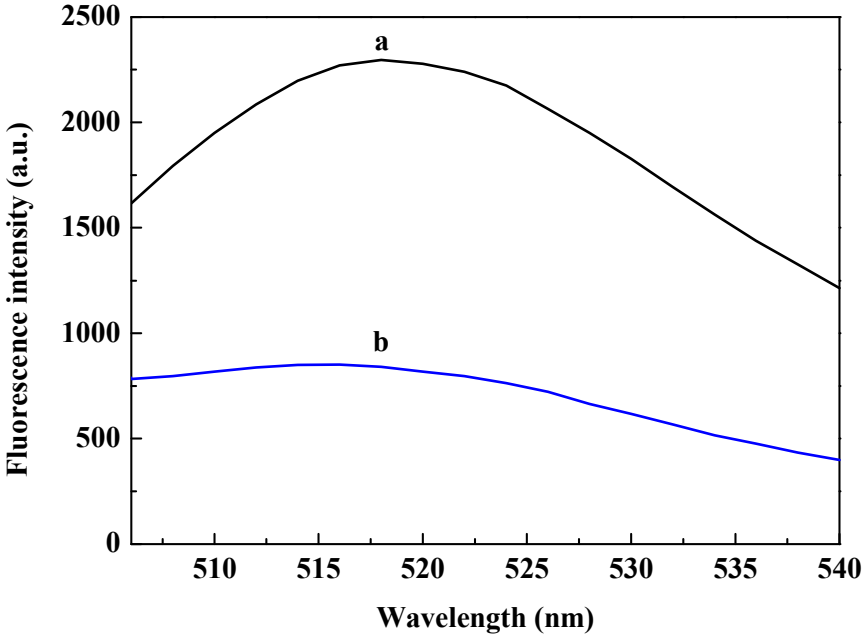
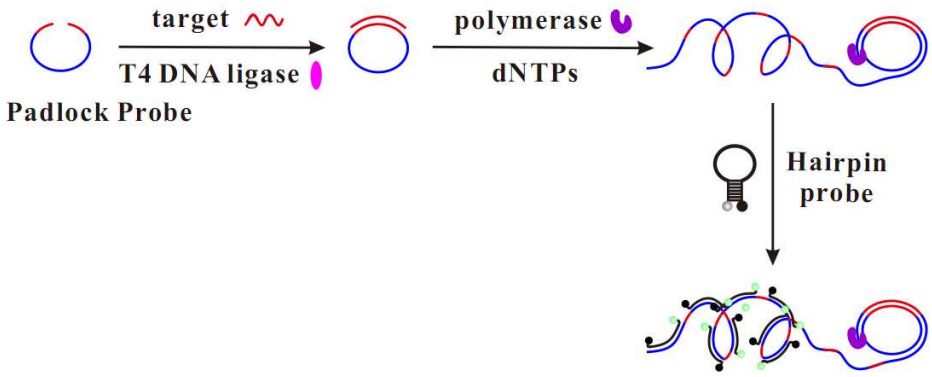


Figure 1. Fluorescence spectra of the RCA-based fluorescence system with linear DNA probe as signal probe in the absence (a) and presence (b) of 1×10^{-11} M target DNA. Experimental conditions: 20 μL Padlock probe (1.0×10^{-8} M), 20 μL target DNA (1.0×10^{-11} M), 4 μL T4 DNA ligase (1 U/μL), 4 μL phi29 DNA polymerase (1 U/μL), 8 μL dNTPs (2.5 mM), RCA reaction time 150 min, 10 μL FAM-DNA (0.1 μM), 10 μL BHQ_1-DNA (0.2 μM).



Scheme 2. Schematic illustration of the strategy for RCA-based homogenous fluorescence detection of DNA with MB (hairpin probe) as signal probe.

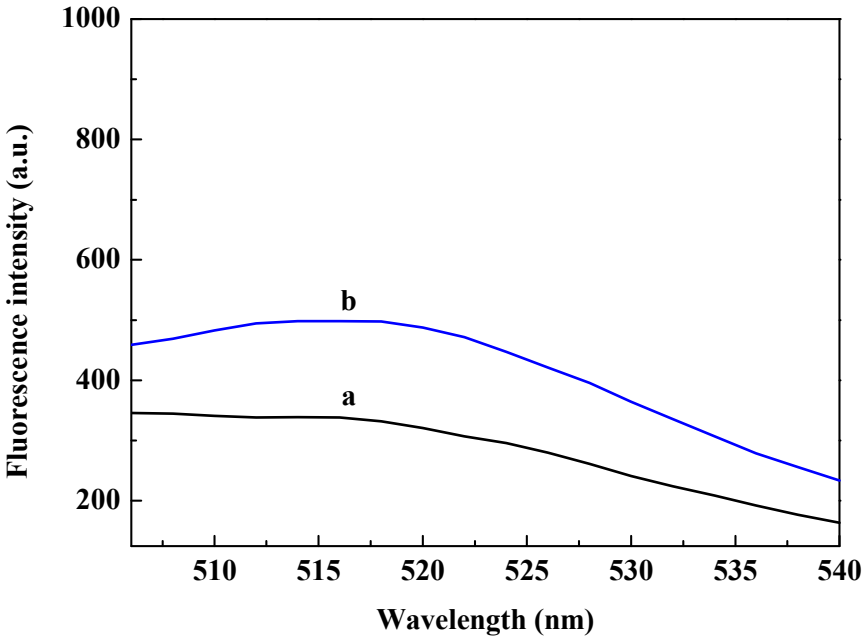


Figure 2. Fluorescence spectra of the RCA-based fluorescence system with MB as signal probe in the absence (a) and presence (b) of 1×10^{-11} M target DNA. Experimental conditions: 20 μL Padlock probe (1.0×10^{-8} M), 20 μL target DNA (1.0×10^{-11} M), 4 μL T4 DNA ligase (1 U/μL), 4 μL phi29 DNA polymerase (1 U/μL), 8 μL dNTPs (2.5 mM), RCA reaction time 150 min, 10 μL molecular beacon (0.1 μM).

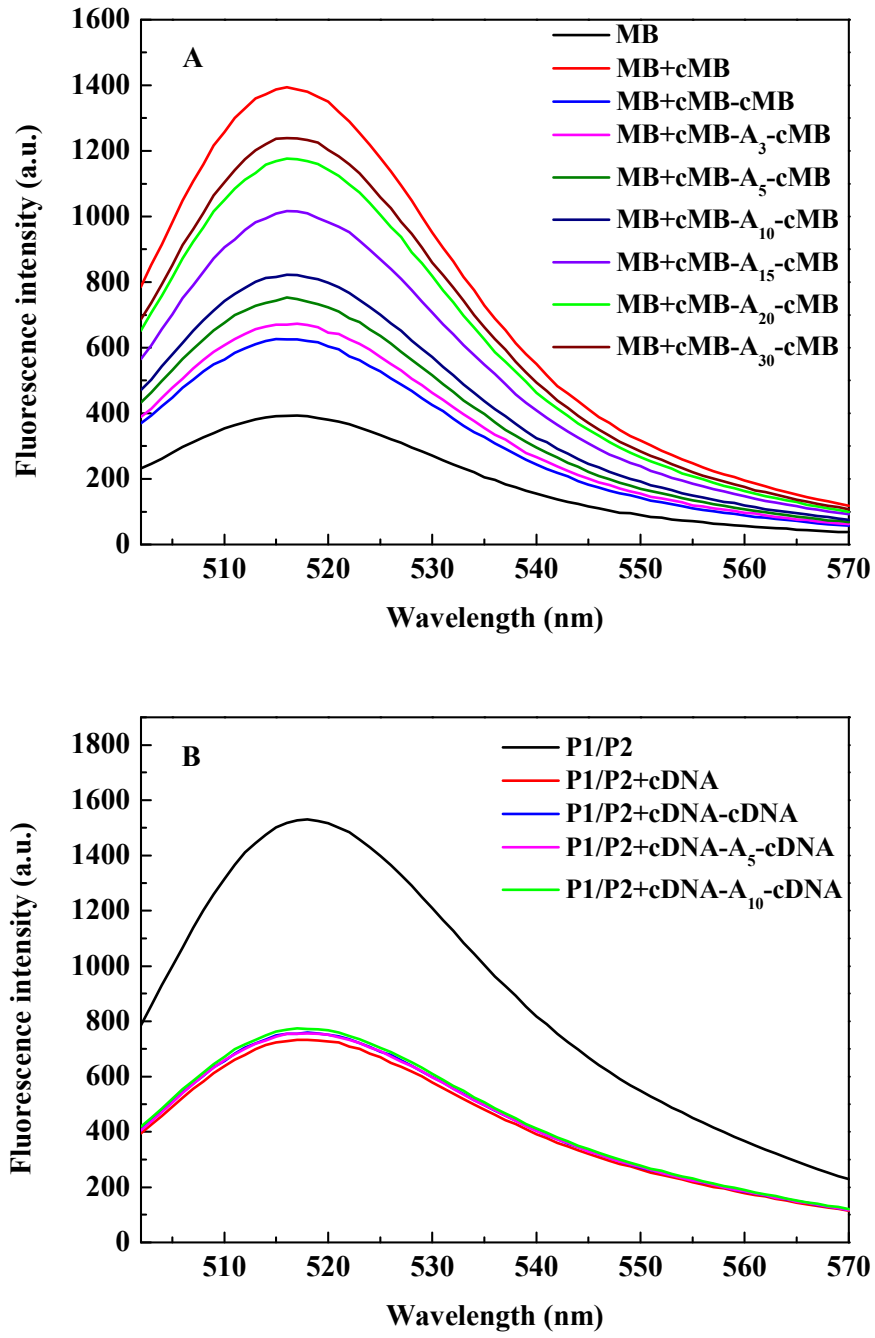


Figure 3. (A) Fluorescence spectra of 0.4 μM MB under different conditions and (B) fluorescence spectra of 0.4 μM P1/P2 under different conditions. The concentration of cMB and cDNA (complementary sequence of P1/P2) was 0.4 μM, and the concentration of other DNA was 0. 2 μM.

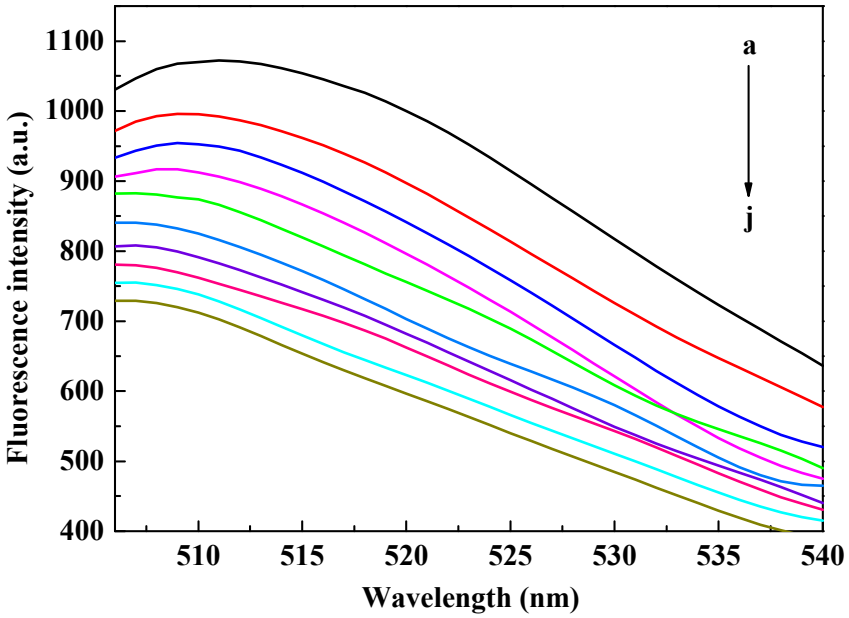


Figure 4. Fluorescence spectra for the determination of DNA at different concentrations. The concentration of DNA from curve a to curve j was 0, 1.0×10^{-18}, 3.0×10^{-18}, 5.0×10^{-18}, 7.0×10^{-18}, 1.0×10^{-17}, 3.0×10^{-17}, 5.0×10^{-17}, 7.0×10^{-17}, and 1.0×10^{-16}, respectively. Inset: the response of F_0/F to the DNA concentration.

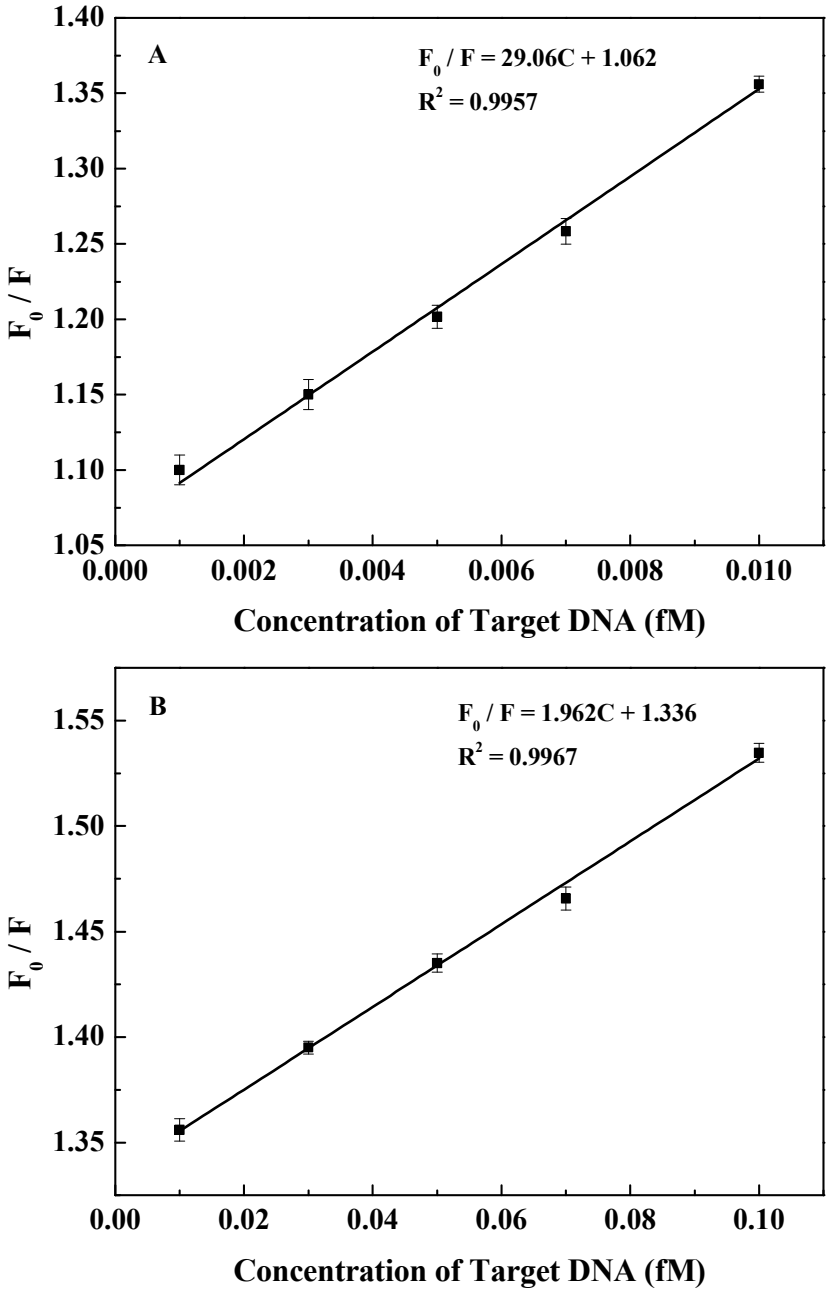


Figure 5. Linear relationship between the ratio of F_0 to F (F_0/F) and target DNA concentration from 0.001 to 0.01 fM (A) and 0.01 to 0.1 fM (B). Error bars represent the standard deviations of three independent measurements. Experimental conditions: Padlock probe 1 pM, T4 DNA ligase 4 U, phi29 polymerase 4 U, dNTPs (2.5 mM) 8 μL, RCA reaction time 150 min, FAM-DNA 30 pM, BHQ_1-DNA 60 pM.

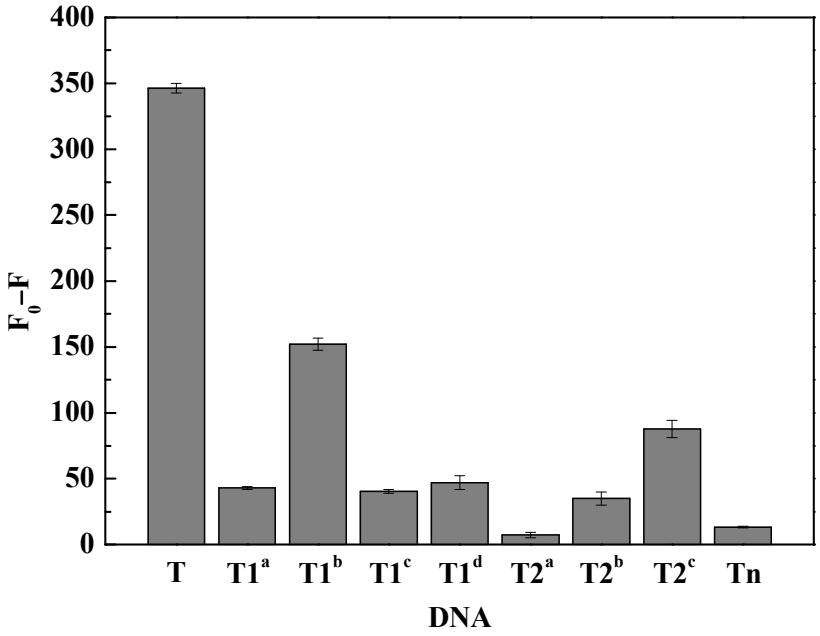
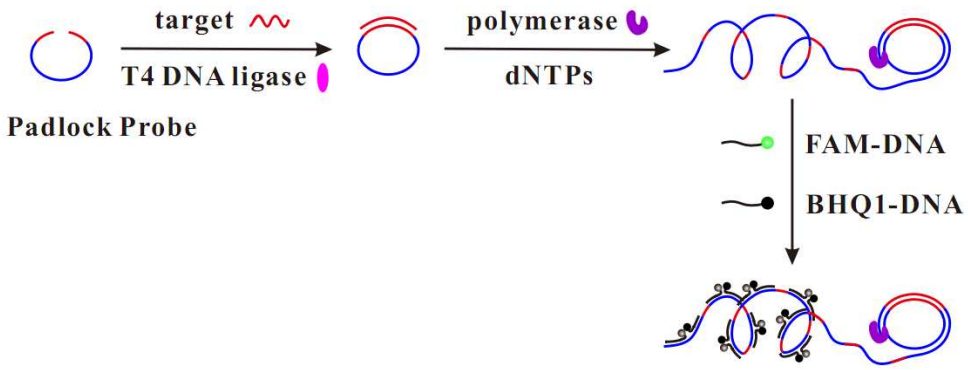


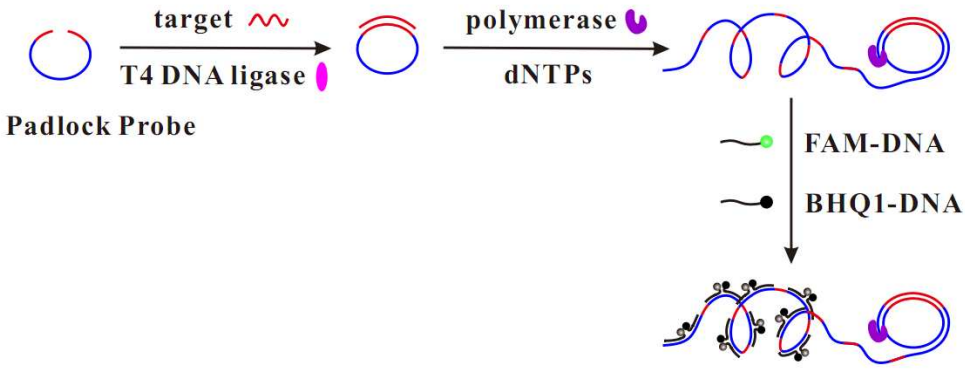
Figure 6. Specificity of the assay for DNA detection by hybridizing probe DNA with different target DNA: completely complementary target DNA (T), single-base mismatched DNA ($T1^a$, $T1^b$, $T1^c$, $T1^d$), two-base mismatched DNA ($T2^a$, $T2^b$, $T2^c$), and the non-complementary DNA (Tn). Experimental conditions: Padlock probe 1 pM, T4 DNA ligase 4 U, phi29 polymerase 4 U, dNTPs (2.5 mM) 8 μL, RCA reaction time 150 min, FAM-DNA 30 pM, BHQ_1-DNA 60 pM. Target DNA is 0.1 fM.

for TOC only



Linear single-labeled DNA probes are used in this RCA-based fluorescence strategy for DNA detection, which could effectively avoid the fluorescence quenching between neighboring signal probes when hairpin probe as signal probe and significantly improve the sensitivity of RCA-based fluorescence strategy.

for TOC only



Linear single-labeled DNA probes are used in this RCA-based fluorescence strategy for DNA detection, which could effectively avoid the fluorescence quenching between neighboring signal probes when hairpin probe as signal probe and significantly improve the sensitivity of RCA-based fluorescence strategy.