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Cascade Signal Amplification Strategy for Sensitive and Label-free DNA Detection Based on Exo III-catalyzed Recycling Coupled with Rolling Circle Amplification

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A sensitive and label-free fluorescence assay for DNA detection has been developed based on cascade signal amplification combining exonuclease III (Exo III)-catalyzed recycling with rolling circle amplification. In this assay, probe DNA hybridized with template DNA was coupled onto magnetic nanoparticles to prepare a magnetic bead-probe (MNB-probe)/template complex. The complex could hybridize with the target DNA, which transformed the protruding 3' terminus of template DNA into an blunt end. Exo III could then digest template DNA, liberating the MNB-probe and target DNA. The intact target DNA then hybridized with other templates and released more MNB-probes. The liberated MNB-probe captured primer, circular DNA and then initiated rolling circle amplification (RCA) reaction, realizing a cascade signal amplification. Using this cascade amplification strategy, a sensitive DNA detection method was developed which was superior to many existing Exo III-based signal amplification methods. Moreover, N-Methyl mesoporphyrin IX, which had a pronounced structural selectivity for G-quadruplex, was used to combine with the G-quadruplex RCA products and generate fluorescence signal, avoiding the need of any fluorophore-label probes. The spike and recovery experiments in human serum sample indicated that our assay also had a great potential for DNA detection in real biological samples.

Introduction

The development of sensitive methods for DNA sequences detection has attracted substantial research efforts because of their broad applications in clinical diagnosis, gene therapy and mutation analysis.[1] Among different approaches, fluorescent detection combined with signal amplification is one of the most popular DNA assay methods. Because fluorescence assay offers stability, robustness, and detection sensitivity. Amplified detection can amplify trace amounts of DNA to levels that are detectable and further improve the sensitivity of fluorescence assay.[2] So far, several fluorescent amplification techniques have been developed for sensitive detection of DNA including DNAzyme-mediated amplification,[3] nanoparticle-based assays,[4] enzyme-assisted amplification[5] and so on. Among these techniques, enzyme-assisted amplification attracts much attention because of the high signal amplification efficience. For example, nicking endonuclease signal amplification (NESA), a newly developed enzyme-assisted amplificaton method, leads to the generation of more than 1000 nicked probes from one target in 2 hours.[6] However, nicking endonuclease requires target DNA with a specific sequence for enzyme recognition, which limits the versatility.

To widen the signal amplification methods in analyzing the target sequence without the specific recognition unit, Exonuclease III (Exo III)-based target recycling has received much attention.[7] Exo III catalyzes the stepwise removal of mononucleotides of duplex DNA from its 3'-termini irrespective of the sequence.[8] Therefore, Exo III-based signal amplification provides a more ideal candidate for universal amplification detection of DNA. However, nearly all of these reported studies were involved in fluorophore-label probes such as the stem-loop hairpin molecular beacons[9] or displacing probes.[10] These labelled probes always brought about high cost and operation complexity. Moreover, the labelled fluorophore might not be completely quenched, which increased the background. Although an Exo III based amplification method which could generate label-free signal was developed,[11] that method showed poor sensitivity. Approach for label-free as well as sensitive DNA detection is still in great demand.

Herein, we proposed for the first time a sensitive and label-free cascade signal amplification strategy which combined Exo III-catalyzed recycling with rolling circle amplification (RCA). RCA is an isothermal DNA replication technique which can be preformed in homogeneous system and on the surface of magnetic bead.[12] It can generate a large number of tandem DNA repeats in hours.[13] By combining RCA with Exo III-catalyzed recycling, the developed technique presented per DNA recognition event to numerous long cascade fluorescent tags for assay readout. Moreover, N-methyl mesoporphyrin IX (NMM), which could selectively bind to G-quadreplex structure instead of other DNA forms, was employed to interact with RCA products

and generate label-free fluorescent signal.[14] This cascade signal amplification strategy offered high sensitivity due to primary amplification via Exo III-aided target recycling and followed by a secondary RCA amplification and a tertiary fluorescence significant from the interaction between NMM and G-quadruplex. In addition, the label-free technique allowed easy implementation with simple instrumentation. It was, thus, a very powerful tool for highly sensitive and label-free DNA detection.

Experimental section

Chemicals

Materials and measurements. Exo III, T4 DNA ligase, Phi29 DNA polymerase and dNTPs were obtained from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). N-methyl mesoporphyrin IX (NMM) was purchased from Porphyrin Products (Logan, UT). Streptavidin-MNBs (350 nm diameter, 1.343 g/mL, aqueous suspension containing 0.1 % bovine serum albumin, 0.05 % Tween-20, and 10 μM EDTA at a concentration of 3.324×10^{11} beads/mL) were purchased from Bangs Laboratories Inc. (Fishers, IN). Tween-20 from Sigma (St. Louis, MO), Tris (> 99.8 %) from Amresco Inc. (Solon, OH) and other chemicals (analytical grade) obtained from standard reagent suppliers were used in the work without further purification.

As shown in Table 1, all oligonucleotides were synthesized by Shanghai Sangon Bilogical Engineering Technology&Service Co. Ltd (Shanghai, China).

TTL buffer consisted of 0.10 M Tris-HCl (pH 8.0), 0.1 % Tween-20 and 1.0 M LiCl. Reaction buffer consisted of 10 mM Tris–HCl (pH 8.0), 75 mM KCl and 10 mM $MgCl_2$.

MNB-probe preparation

The MNB-probe was prepared as follows: firstly, the mixtures of bio-probe DNA (0.5×10^{-6} M) and temple DNA (0.5×10^{-6} M) were heated at 90 ℃ for 5 min and incubated at 37 ℃ for 2 h. After that, 1μL of streptavidin-MNBs suspension was washed five times with 200 μL of TTL buffer to remove surfactants.

Table 1 Oligonucleotide sequences (5'-3') used in the assay[a]

Name	Sequence
Probe DNA	CTCTTCGAGGG*TTTTGGG*TTTTGGGTTTTG*GG*AGCTA-biotin
Template DNA	*AAAACCCAAAACCCAAAACCC*GCGACGAG TCACAACAG
Primer DNA	CCCAAAACCCTCGAAGAGTTAGGATCGT GTGGTT
Circular template DNA	p-GAT CCT AAC CCA ACC CGC CCT ACC CAA AAC CCA ACC CGC CCT ACC CAA AAC CCA ACC CGC CCT ACC CAA CCA CAC
Target DNA	CTGTTGTGACTCGTCGCAATAAC
T1	CTGTT**C**TGACTCGTCGCAATAAC
T2	CTGTT**CA**GACTCGTCGCAATAAC
T3	CTGTT**CAC**ACTCGTCGCAATAAC
T4	CTGTT**CACT**CTCGTCGCAATAAC

[a]Bold letters indicate the mismatched bases. Different types and underlines indicate different hybridization regions.

Then, the DNA mixtures were mixed with the MNBs and incubated at 37 ℃ for 2 h. At last, the MNB-probe was washed once with reaction buffer (10 mM Tris–HCl, 75 mM KCl, 10 mM $MgCl_2$, pH 8.0) to remove the excess DNA.

Exonuclease III-aided recycling process

Before the Exo III-aided recycling process, varying concentrations of target DNA were added to the MNB-probe solution and incubated at 37 ℃ for 0.5 h. After that, 0.25 μL (200 U/μL) Exo III and 10 μL 10 × reaction buffer were added. The recycling process was performed at 37 ℃ for 2 h. At last, the MNB-probe was washed twice with 150 μL of reaction buffer, then 90 μL of Mg-K buffer was added for the next step.

Rolling circle amplification

10 μL of 0.5 μM primer DNA was added to the MNB-probe suspension and incubated at room temperature for 1.5 h. The resulting MNB-probe-primer DNA was washed once with 150 μL of reaction buffer. 10 μL of 2.0 μM circular DNA template was added to the MNB-probe-primer DNA biocomplexes solution and incubated at 37 ℃ for 30 min, followed by adding 0.5 μL of T4 DNA ligase and incubating at 37 ℃ for 1 h. Then, 5 U phi29 DNA polymerase, 20 μL 10 mM dNTPs, and 10 μL 10 × reaction buffer were added to the resulting biocomplexes and incubated at 37 ℃ for 2 h. After that, 4 μL NMM (7.5×10^{-5} M) was added to the solution and incubated at 37 ℃ for 0.5 h.

Fluorescence measurements

Fluorescence was measured by using a Hitachi F-4500 spectrofluorimeter (Hitachi, Japan) at the room temperature. Slit widths for the excitation and emission were set at 10 nm and 10 nm, respectively. The excitation wavelength was 399 nm. The fluorescence emission intensity was measured at 607 nm.

Fluorescent imaging

Fluorescent imaging was performed with an Olympus IX81 fluorescence microscope (Tokyo, Japan) equipped with a high-numerical-aperture 60 × (1.45 NA) oil-immersion objective lens, a mercury lamp source, a mirror unit consisting of a 470-490 nm excitation filter (BP470-490), a 505 nm dichromatic mirror (DM 505), a > 580 nm emission filter (IF 580), and a 16-bit thermoelectrically cooled EMCCD (Cascade 512 B, Tucson, AZ, USA).

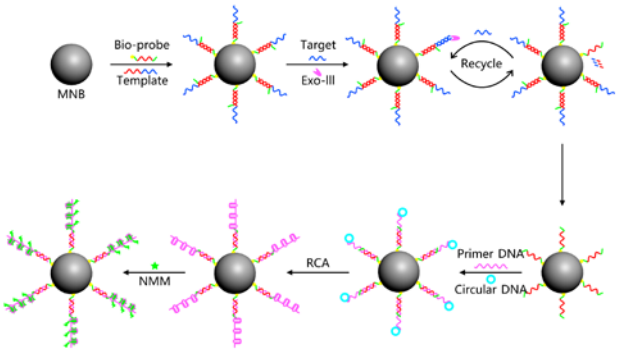
Gel electrophoresis

0.7 % agarose gel electrophoresis was carried out to verify the RCA reaction. The electrophoresis was carried out in 1 × TAE (pH = 8.0) at 120 V constant voltage for about 2 h at room temperature.

Results and discussion

Principle of the cascade signal amplification strategy

Scheme 1 depicted the principle of the cascade signal amplification strategy. Biotinylated probe DNA (p-DNA) which hybridized with template DNA was immobilized onto MNBs to form the MNB-probe/template complex. In the absence of target DNA, the template DNA had a protruding 3'-termini and could not be digested by Exo III which cleaved double-strand DNAs



Scheme 1 Schematic illustration of cascade signal amplification strategy combining exonuclease III-catalyzed recycling with rolling circle amplification.

from blunt or recessed 3'-termini. Upon the introduction of target DNA, the hybridization of template and target DNA led template to have a blunt 3'-terminus, so Exo III could stepwise hydrolyze template DNA from its 3'-terminus. It should be noticed that even though perfectly hybridized with template, the target still had 6-nt single-stranded sequence at its 3'-terminus to prevent the hydrolysis by Exo III. With the stepwise removal of mononucleotides from template, target and p-DNA were liberated. The released target DNA then hybridized with another template, leading to a target recycling process and liberating more MNB-probes. After this recycling process, primer DNA was added and captured by MNB-probe. Circular template was annealed to the primer DNA. Ligation by DNA ligase led to the circularization of the circular template. After that, RCA process was initiated by Phi29 DNA polymerase and synthesized a long repeated G-quadruplex sequence. After the addition, NMM strongly interacted with the G-quadruplex product, bringing a great fluorescence enhancement.

Verification of the cascade signal amplification strategy

To verify the feasibility of the Exo III-aided target recycling process, fluorescent imaging was performed to confirm the duplex digesting and p-DNA releasing process on the MNB via intercalating fluorophore SYBR Green I into the MNB-probe. As shown in Fig. 1A, in the absence of target, the double-stranded structures of p-DNA/template duplex allowed the intercalation of SYBR Green I and then a fluorescence signal could be detected (Fig. 1A-1). Subsequently, upon the addition of target and Exo III, template DNA hybridized with target DNA and then was digested by Exo III, releasing target and liberating p-DNA. The transition of double-stranded to single-stranded DNA led to an obvious decrease of fluorescence dots amount (Fig. 1A-2). These results indicated that target DNA acted as a trigger of the Exo III catalyzed recycling process which could lead to the liberation of MNB-probes. The feasibility of the RCA process was also verified. As shown in Fig. 1B, the RCA process on the MNB-probe was verified by detecting the fluorescence intensity of RCA product. MNB-probe was used to capture primer DNA and circular DNA, by which the RCA product was successfully generated and an obvious signal enhancement was observed (Fig. 1B RCA). Without primer DNA, MNB-probe and circular DNA could not initiate a RCA process, let alone signal amplification

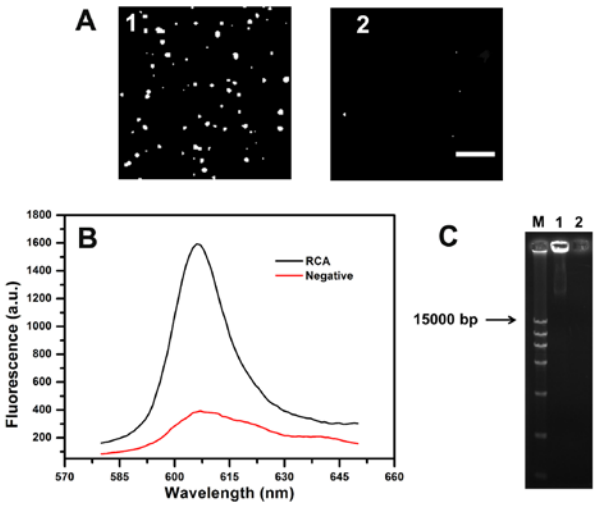


Fig. 1 (A) The fluorescence imaging of the Exo III-catalyzed recycling process on the MNB-probe. Target DNA was 10 nM. Scale bar, 10 μm. (B) The fluorescence emission spectra of RCA process on the MNB-probe. Primer DNA was 5.0×10^{-8} M, circular template DNA was 2.0×10^{-7} M. (C) Agarose gel (0.7 %) electrophoresis images obtained after the RCA process.

(Fig. 1B negative). Moreover, As shown in Fig. 1C, electrophoresis experiment was performed to further prove the RCA process. Compared with the negative control experiment (Lane 2) which displayed no band, the RCA process generated product which showed extremely low mobility (Lane 1). This phenomenon indicated that the RCA product had a high molecular weight and could lead to enormous signal amplification. Time-depent fluorescence response under different conditions were also carried out to further demonstrate the feasibility of our assay (see Fig. S1, ESI†).

In addition, to demonstrate the signal amplification capacity of our cascade strategy, we compared the signal intensity of cascade assay with that of the RCA process (see Fig. S2, ESI†). The result clearly demonstrated that this cascade signal amplification assay had a more powerful amplification capacity than one-step amplification assay.

Optimization of the reaction conditions

The fluorescence intensity was determined by both Exo III-catalyzed recycling and RCA process. In order to achieve an optimal sensing performance, experimental parameters affecting Exo III recycling and RCA were optimized, including the dosage of Exo III, the concentration of primer DNA and circular DNA, as well as the concentration of NMM. Considering that different target concentration might cause different influence to our strategy and need different optimal experiment conditions, we chose low (5.0×10^{-11} M), middle (5.0×10^{-10} M) and high (1.0×10^{-8} M) concentration of target DNA to optimize the experiment conditions. It should be noticed that F and F_0 occured in the article represented for the fluorescence signals in the presence and in the absense of target DNA, respectively.

Exo III played a key role in the first signal amplification process, so the dosage of Exo III was investigated. Other experimental parameters were set as: 5.0×10^{-8} M of primer DNA, 2.0×10^{-7} M of circular DNA and 3.0×10^{-6} M of NMM.

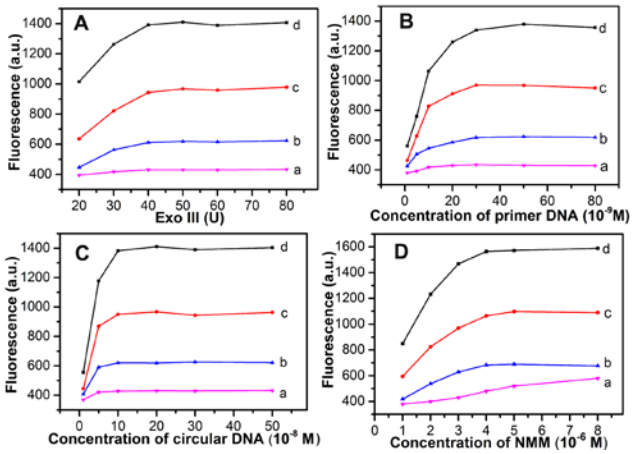


Fig. 2 The effects of Exo III dosage (A), concentration of primer DNA (B), concentration of circular DNA (C) and concentration of NMM (D) on the fluorescence response corresponding to different concentrations of target DNA: no target DNA (a), 5.0×10^{-11} M (b), 5.0×10^{-10} M (c), 1.0×10^{-8} M (d).

Experiments indicated that the fluorescence signal intensity was obviously influenced by Exo III (Fig. 2A). The fluorescence intensity increased significantly with the increasing dosage of Exo III. After peaked at 2.0×10^{-7} M, the ratio of fluorescence intensity reached a plateau. Thus, 50 U of Exo III was chosen to achieve the best experiment platform.

Furthermore, the concentration of primer DNA and circular DNA used in RCA process were optimized. We first optimized the concentration of primer DNA. Other experimental parameters were set as: 50 U of Exo III, 2.0×10^{-7} M of circular DNA and 3.0×10^{-6} M of NMM. As shown in Fig. 2B, the fluorescence intensity increased significantly with the increasing concentration of primer DNA. After peaked at 5.0×10^{-8} M, the ratio of fluorescence intensity reached a plateau. Thus, 5.0×10^{-8} M was chosen as the optimum concentration of the primer DNA. The similar experiment result was observed in optimizing the concentration of circular DNA (Fig. 2C), which peaked at 2.0×10^{-7} M. Thus, 2.0×10^{-7} M was chosen as the best concentration (other experimental parameters: 50 U of Exo III, 5.0×10^{-8} M of primer DNA and 3.0×10^{-6} M of NMM).of circular DNA.

Moreover, the concentration of NMM was also investigated. Before the investigation, other experimental parameters were set as: 50 U of Exo III, 5.0×10^{-8} M of primer DNA and 2.0×10^{-7} M of circular DNA. As shown in Fig. 2D, the fluorescence response in the presence of target reached its maximum with 3.0×10^{-6} M. Then, the increasing negative signal caused the decrease of S/N ratio. Thus, 3.0×10^{-6} M was chosen as the optimal concentration of NMM.

Sensitivity of the cascade signal amplification strategy

To assess the sensitivity of this cascade signal amplification strategy, a series of different concentrations of target DNA were measured. As shown in Fig. 3A, the fluorescence intensity increased with an increasing concentration of target from 0 to 10 nM, revealing a target concentration dependent response. This phenomenon implied that more templates were digested with the increase of target concentration, freed more MNB-probes to generate larger amount of RCA products which finally increased the signal intensity significantly. Fig. 3B showed the relationship

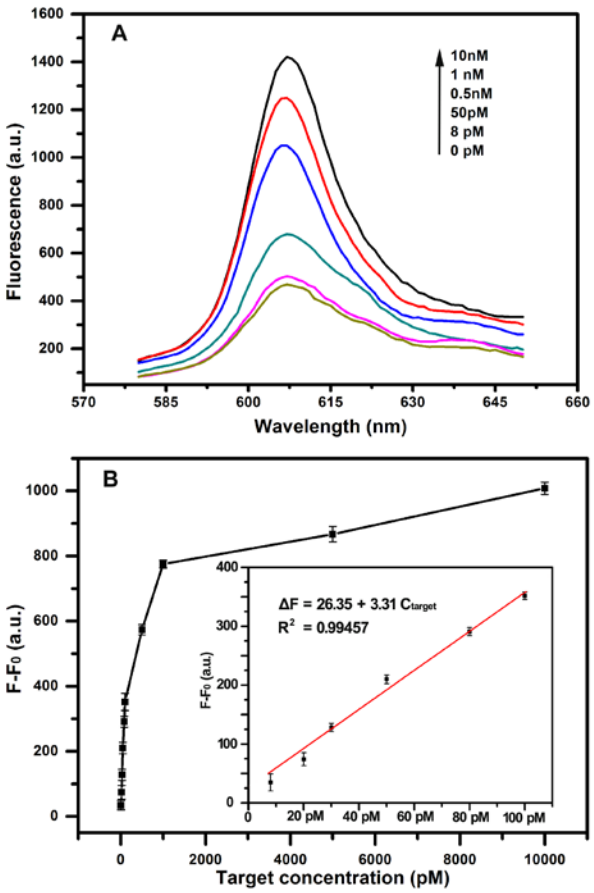


Fig. 3 (A) Fluorescence emission spectra of the cascade signal amplification strategy in the presence of various concentrations of perfectly matched target DNA. (B) The relationship between fluorescent intensity and concentration of target DNA. Insert: linear relation between the fluorescence intensities at 607 nm and target concentration.

between the signal intensity at 607 nm and the concentration of target DNA. Under the optimal conditions, the fluorescence intensity increased proportionally to the concentration of target DNA within the range from 8 pM to 100 pM, with a detection limit of 3.2 pM (3σ/slope). The sensitivity of this cascade signal amplification method was superior to many existing Exo III-based signal amplification methods[15] and comparable to other signal amplification strategies.[16]

In addition, the precision and repeatability of this signal amplification strategy were assessed by calculating the relative standard deviation (RSD) of intra- and inter- assay (n=3). The RSD with the same batch were 4.0 %, 3.8 % and 3.3 % at the target concentration of 1.0×10^{-10} M, 5.0×10^{-11} M and 1.0×10^{-11} M, respectively. The RSD of batch-to-batch measurement were 5.2 %, 4.7 % and 3.6 % at the above-described target concentrations in three repetitive assays. The results implied good precision and repeatability of this amplification strategy.

Selectivity of designed signal amplification strategy

In order to evaluate the specificity of this strategy, we then challenged our assay to different mismatched targets. As shown in Fig. 4, the fluorescence intensity decreased significantly with the increasing mismatched bases. Great signal intensity was obtained on the addition of perfectly matched target. It was about

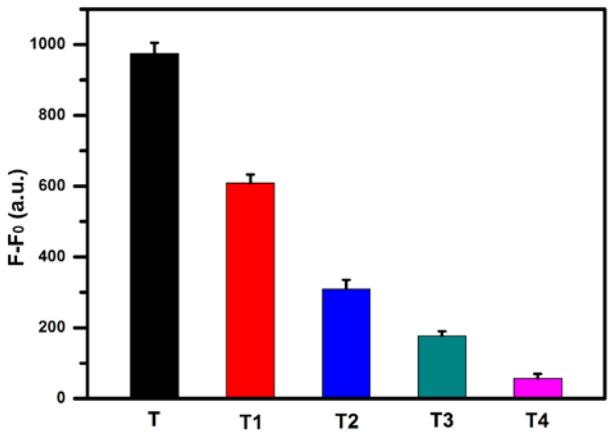


Fig. 4 Selectivity of the cascade signal amplification strategy. Target DNA and other mismatched DNA were all 10 nM.

1.9 times and 3.8 times higher than that for one-base mismatched and two-base mismatched targets at the same concentration. Moreover, four-base mismatched target showed nearly no response. The result implied that this method not only had a good selectivity, but also had a potential usage in distinguishing different mismatched DNA targets. The single-base mismatched target in a high concentration might have certain interference to the detection of low-abundant target DNA. This problem could be solved by some pretreatments to generate more targets, for example, PCR[17].

Feasibility in complex biological matrices

To demonstrate the feasibility of our strategy in complex biological matrixes, we performed spike and recovery experiments in human serum sample. The human serum sample was first diluted in 1:1 ratio with reaction buffer and then spiked with target at three concentrations (1.0×10^{-11} M, 5.0×10^{-11} M, and 1.0×10^{-10} M). From Fig. 5, the fluorescence intensity obtained from serum sample decreased slightly compared to that from reaction buffer. The concentration of target in serum sample was determined by using calibration method and the recovery

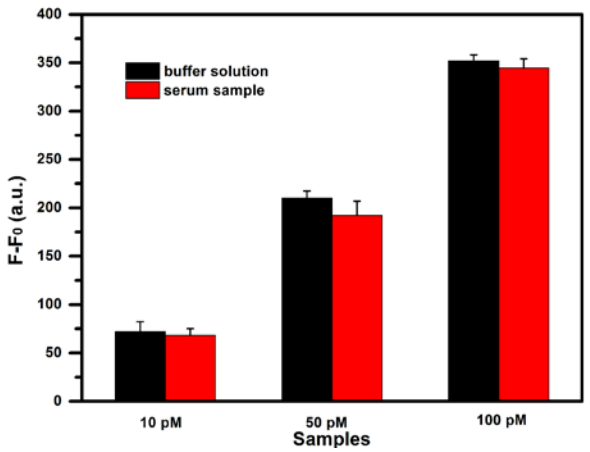


Fig. 5 Fluorescence signal of the cascade signal amplification strategy corresponding to different concentrations of target DNA (1.0×10^{-11} M, 5.0×10^{-11} M, and 1.0×10^{-10} M) in buffer solution and serum sample, respectively.

was calculated to be 95 % ± 2 %. The result indicated that our assay had a great potential for DNA detection in real biological samples.

Conclusions

In summary, a sensitive and label-free DNA detection approach has been developed herein based on cascade signal amplification consisted of Exo III-catalyzed recycling and rolling circle amplification. Compared with traditional Exo III-based signal amplification methods, this cascade signal amplification strategy exhibited a higher signal amplification efficiency and improved the detection sensitivity significantly. Moreover, the G-quadruplex RCA product could produce a fluorescence signal by simply adding NMM, without the need of fluorophore-labeled oligonucleotide probes. The MNBs used in our assay made it easy to collect target DNA from complex sample by magnetic separation, which made this approach suitable for sequence detection in real samples. Furthermore, as Exo III does not require specific recognition site, this strategy can be directly expanded for different target sequences detection by changing the sequence of template DNA, which shows a great potential in bioanalytical application, gene therapy and clinical diagnosis.

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Notes and references

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† Electronic Supplementary Information (ESI) available: additional detials for the experiment procedure and supplementary figures. See DOI: 10.1039/b000000x/

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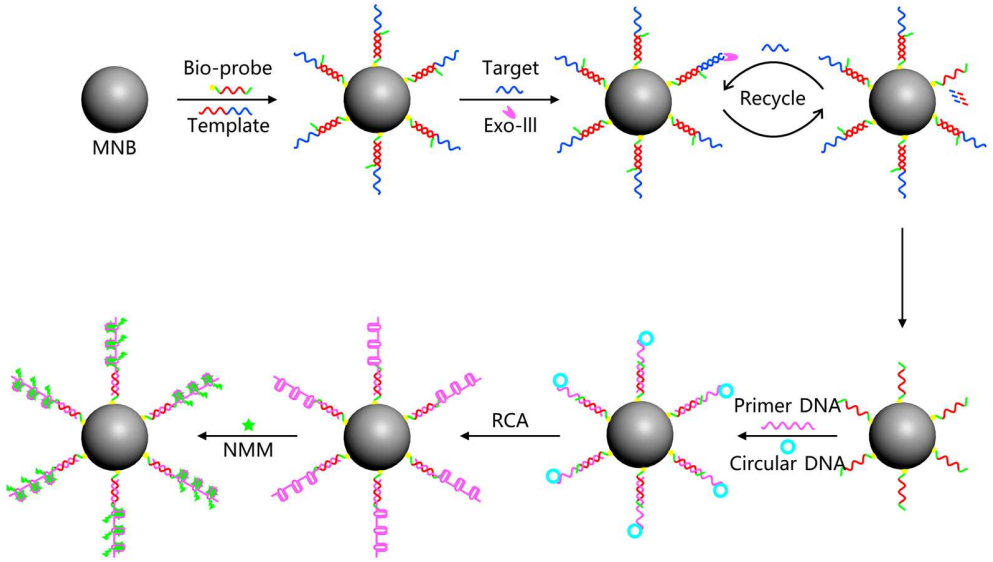
Table of contents:



A sensitive and label-free DNA detection method was developed based on cascade amplification combining exonuclease-III recycling with rolling circle amplification.