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Rapid and Label-Free Monitoring of Exonuclease III-Assisted Target Recycling Amplification

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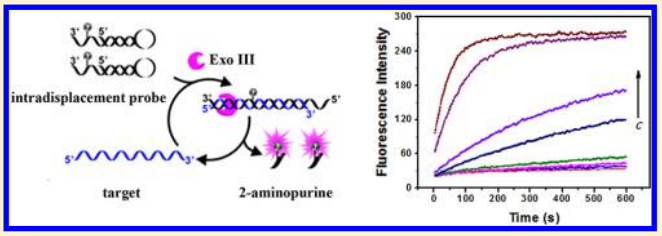
ABSTRACT: Target recycling-oriented amplification has been widely applied for sensitive detection of DNA, RNA, and proteins due to its successful overcoming the inherent limitation of target-to-signal ratio of 1:1 in the traditional hybridization assay. Exonuclease III (Exo III) is usually used as the cleavage enzyme in the target recycling-oriented amplification because of its easy availability, high catalytic activity, and wide applicability. Even though Exo III is assumed to be double-stranded DNA (dsDNA) specific exonuclease in most literature, its cleavage of single-strand DNA (ssDNA) does occur, resulting in the target-independent degradation of probes. Herein, we design an intramolecular displacement probe with the capability of resistance to the nonspecific digestion of Exo III and fast hybridization kinetics. Through the substitution of 2-aminopurine for adenine in the intramolecular displacement probes, we develop a rapid and label-free approach to monitor Exo III-assisted target recycling amplification. We further demonstrate that this method can be used for the detection of DNA and proteins with excellent specificity and high sensitivity. Importantly, this method can be extended to rapid, label-free and multiplexed detection of various nucleic acids, proteins, and small molecules using different kinds of fluorescent nucleotide analogues and specific aptamers.



Contrary to the traditional hybridization assay with the target-to-signal ratio of 1:1, target recycling-oriented amplification makes one target function as a "catalyst" to interact with multiple probes, achieving great signal amplification.[1] So far, a variety of nucleases such as Rnase H,[2] nicking endonuclease,[1,3] flap endonuclease,[4] DNase I,[5] duplex-specific nuclease,[6] endonuclease IV,[7] exonuclease III,[8] and lambda exonuclease[9] have been employed as the cleavage enzymes in the target recycling-oriented amplification for sensitive detection of DNA, RNA, and proteins. Among them, exonuclease III (Exo III) is frequently used because of its easy availability, high catalytic activity, and wide applicability.[10] More importantly, Exo III can selectively digest the 3′-end of double-stranded DNA (dsDNA).[8b,11] Contrary to the nicking endonuclease-based target recycling amplification which requires specific target sequence,[1,3] Exo-III assisted target recycling amplification does not require any specific recognition sequence, making it an ideal candidate for the development of universal detection platforms. Exo III-assisted target recycling has been applied for sensitive detection of various biomolecules in combination with molecular beacons,[8b,12] semiconductor quantum dots,[8c] and grapheme oxide,[8d,13] but the requirement of fluorescently labeled probes and functionalized particles increases the experimental cost and the design complexity.

It is generally assumed that the preferred substrate of Exo III is dsDNA instead of single-stranded DNA (ssDNA) in the Exo III-assisted target recycling amplification.[10a,14] However, recent research demonstrates that ssDNA might be digested by Exo III as well,[15] which may cause high background noise and low target turnover due to the unspecific digestion of probes and target molecules by Exo III. To solve this issue, a variety of approaches,[16] such as the use of linear DNA probe labeled with a fluorophore and a quencher,[16b] molecular beacon,[17] and intermolecular displacement probe,[18] have been tried, but molecular beacon and the linear DNA probe labeled with a fluorophore and a quencher are very expensive and difficult to design,[17] and the intermolecular displacement probe suffers from slow hybridization kinetics.[18] Here, we develop an intramolecular displacement probe with the capability of fast hybridization kinetics and resistant to nonspecific digestion of Exo III. Through the substitution of 2-aminopurine for adenine in the intramolecular displacement probes, we develop a rapid and label-free approach for sensitive and specific detection of DNA and proteins by monitoring Exo III-assisted target recycling amplification.

MATERIAL AND METHODS

Materials. Exonuclease III, NEBuffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.0), and NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9) were purchased from New England Biolabs (Ipswich, MA, USA). Exo III was diluted to 1 U/μL with the dilution buffer

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(50 mM Tris−HCl, pH 8.0, 10 mM DTT, and 0.5 mg/mL BSA) immediately before use. All oligonucleotides (Table 1)

Table 1. Sequences of the Oligonucletides[α]

note	sequence (5′-3′)
linear probe	GTC TAT GCG TGAp ACT G
intermolecular displacement probe	5′-GTC TAT GCG TGAp ACT G-3′ 3′-C*A*G* ATA CGC ACT-5′
intramolecular displacement probe	CGC ATG TCT ATG CGT GApA CTG
target without 3′-protrusion	CAG TTC ACG CAT AGA C
target with 9 nt 3′-protrusions[β]	CAG TTC ACG CAT AGA CTA GGT AAA G
target with 15 nt 3′-protrusions	CAG TTC ACG CAT AGA CGG TTG GTG TGG TTG G
target with 29 nt 3′-protrusions	CAG TTC ACG CAT AGA CAG TCC GTG GTA GGG CAG GTT GGG GTG ACT
target with 3′-phosphorothioate modification	CAG TTC ACG CAT A*G*A* C
target with single-base mismatched[γ]	CAG TTC AC***A*** CAT A*G*A* C
target with single-base mismatched[δ]	CAG T***G***C ACG CAT A*G*A* C
target with two-base mismatched	CAG T***G***C AC***T*** CAT A*G*A* C
random sequence	CTA CGC CGA ATC C*T*A* C
chimeric probe	CAG TTC ACG CAT AGA CGG TTG GTG TGG TTG G

[α]The Ap indicates the 2-aminopurine substitution. [β]The target with 9 nt 3′-protrusion is defined as the target with 9 nt ssDNA domain at the 3′ terminal after the hybridization of target with probe. [γ]This target contains a single-base mismatched that hybridizes with the stem of intramolecular displacement probe. [δ]This target contains a single-base mismatched that hybridizes with the 3′-protrusions of intramolecular displacement probe. The underlined letters of probes are the complementary sequence of target. The asterisk indicates the phosphorothioate modification. The italic bold letters of the target are the mismatch bases. The underlined letters of the chimeric probe are the recognition sequence of thrombin.

were synthesized and purified by Sangon Biotechnology Co. Ltd. (Shanghai, China). Thrombin (lyophilized powder, 2000 units/mg) and horseradish peroxidase (HRP) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Shanghai Excell Biology, Inc. (Shanghai, China). Hemoglobin was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Milli-Q water (Millipore, Bedford, MA, USA) was used in all experiments.

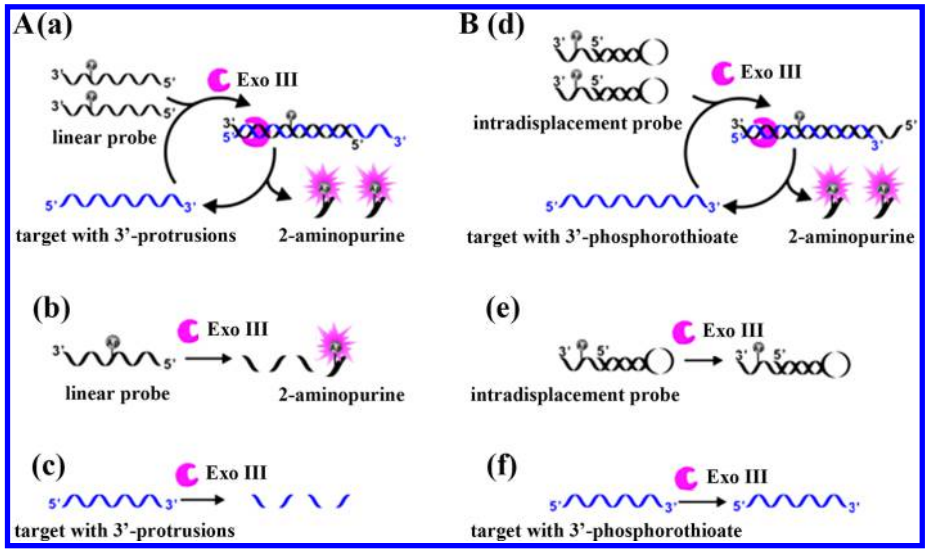
Detection of DNA with Exo III-Assisted Target Recycling Amplification. 2-Aminopurine fluorescence was measured on a spectrofluorometer (F-4600, Hitachi) equipped with a circulating water bath. Exo III-assisted target recycling amplification was monitored by recording the time course of reaction. The excitation wavelength of 310 nm and emission wavelength of 365 nm were used for the fluorescence measurement. The linear probes, intermolecular displacement probes, and intramolecular displacement probes were prepared in Tris buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA), heated to 95 °C for 5 min, and followed by gradual cooling to room temperature for 1 h. In the Exo III-assisted target recycling amplification, 100 nM probes were incubated with 1.67 nM target in NEBuffer 1 at 30 °C for 5 min, and the time course was recorded after adding 1.0 μL of 1 U/μL Exo III. To measure the steady-state kinetic parameters of Exo III toward different DNA substrates, different-concentration DNA was incubated in NEBuffer 1 at 30 °C for 5 min. The reaction was initiated by adding 1.0 μL of 1 U/μL Exo III, and the enzymatic activity was monitored by the spectrofluorometer. Initial reaction rates were measured from the time course curves and converted to 2-aminopurine concentration using a standard curve of 2-aminopurine obtained under the identical conditions.[19] Steady-state kinetic parameters were calculated by fitting the data with the Michaelis−Menten equation using ORIGIN 8.5 (Micirocal Software, Northampton, MA). For DNA detection, different-concentration target DNA was incubated with 100 nM intramolecular displacement probe respectively, and the fluorescence of 2-aminopurine was measured at 37 °C.

Detection of Protein with Exo III-Assisted Target Recycling Amplification. A 20 μM chimeric probe was mixed with 2.67 μM thrombin in NEBuffer 4 at 37 °C for 30 min to allow the complete binding of the chimeric probe with thrombin, and then 5 μL of reaction solution was incubated with 1.0 μL of 1 U/μL Exo III for 5 min, followed by measurement with the same experimental procedures for DNA detection except for the replacement of NEBuffer 1 with NEBuffer 4. Bovine serum albumin (BSA), horseradish peroxidase (HRP), and hemoglobin were used as the negative controls.

RESULTS AND DISCUSSION

Principle of Exo III-Assisted Target Recycling Amplification. In the conventional Exo III-assisted target recycling amplification (Scheme 1A, part a), the formation of dsDNA through the hybridization of the target with the linear probe leads to the digestion of dsDNA by Exo III and subsequently the release of the target, which can further hybrid with new linear probes repeatedly, leading to the digestion of abundant dsDNAs by Exo III and the release of abundance free 2-aminopurine. However, Exo III can digest not only dsDNA but also ssDNA,[15] leading to not only the increase of background noise due to the digestion of DNA probes by Exo III (Scheme 1A, part b) but also the decrease of target turnovers due to the digestion of target molecules by Exo III (Scheme 1A, part c). To solve this issue, we developed an intramolecular displacement probe which contained a hairpin probe with 3′-protrusions (Scheme 1B, part d). In the presence of target DNA, the short competitor in the stem of the probe is displaced, and a complementary dsDNA with a blunt 3′ terminus is formed. Exo III can preferentially digest this dsDNA with k_{cat}/K_m of 141.7×10^5 $mol^{-1}\cdot L\cdot s^{-1}$ (Table 2), leading to the release of 2-aminopurine and the target. The released target can hybridize with the new intramolecular displacement probes repeatedly, releasing abundant 2-aminopurine and achieving great signal amplification. Importantly, the use of 2-aminopurine enables the label-free monitoring of Exo III-assisted target recycling amplification. Unlike the redox- and fluorescence-labeled probes,[20] 2-aminopurine can be simply incorporated into the position of adenine in DNA probes and has no significant inhibition on the exonuclease activity.[19] In addition, 2-aminopurine exhibits no distinct fluorescence when incorporated into DNA strands but enhanced fluorescence when free in solution,[19,21] thus providing a simple readout for Exo III-assisted target recycling amplification. Most importantly, unlike the conventional fluorophore reporter[20] and molecular beacon,[22] the quenching of 2-aminopurine fluo-

Scheme 1. Label-Free Monitoring of Exo III-Assisted Target Recycling Amplification[a]



[a](A) In the conventional Exo III-assisted target recycling amplification (a), the digestion of linear ssDNA by Exo III leads to both high background noise (b) and the degradation of target molecules (c). (B) In the proposed Exo III-assisted target recycling amplification (d), the use of intramolecular displacement probe can significantly reduce the background noise (e), and the use of 3′-terminus phosphorothioate modification can prevent the degradation of target molecules (f).

Table 2. Steady-State Kinetic Parameters of the Hydrolytic Reactions of Exo III with Different Kinds of Probes

DNA	K_m (μM)	V_{max} (nM·s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mol^{-1}·L·s^{-1})
linear ssDNA probe	0.57	1.37	0.38	6.7×10^5
dsDNA with a blunt 3′ terminus	0.12	6.07	1.70	141.7×10^5
intermolecular displacement probe	0.028	0.025	0.007	2.5×10^5
intramolecular displacement probe	0.060	0.050	0.014	2.3×10^5

rescence results from the stacking interaction of 2-aminopurine with the adjacent nucleobases,[23] thus no extra quencher is required. Notably, previous research demonstrated that target DNA with phosphorothioate modification could be resistant to the digestion of Exo III.[15,24] Therefore, the use of intramolecular displacement probe and the target with 3′-phosphorothioate modification can significantly reduce both the background noise (Scheme 1B, part e) and the degradation of target molecules (Scheme 1B, part f) in Exo III-assisted target recycling amplification.

Design of Intramolecular Displacement Probe. Previous research demonstrated that the intermolecular displacement probe with 3′-protrusions exhibited a slow hybridization rate[25] and that the intramolecular hybridization was more stable than the intermolecular hybridization.[26] To obtain both fast hybridization kinetics and low background noise, we developed an intramolecular displacement probe (Scheme 1, part d). This intramolecular displacement-based probe consists of two regions: a target recognition sequence at the 3′-end and a short competitor sequence at the 5′-end (Scheme 1B, part d). The short competitor is partially complementary to the recognition region, which has no significant inhibition effect on the hybridization of the target with a probe.[26] Because intramolecular hybridization is more stable than the intermolecular hybridization,[26a] the length of competitor (5 nt) should be shorter than that of the intermolecular displacement probe (>12 nt) in order to acquire the fast hybridization kinetics. In the absence of target DNA, the competitor DNA will hybridize with the recognition sequence, forming a hairpin structure with a loop length of 4 nt which is much shorter than that of 15−30 nt in the molecular beacon.[22] Since the stability of the hairpin structure is inversely proportional to the loop length,[27] the hairpin probe with a short loop will be resistant to the target-independent degradation of ssDNA probes by Exo III (Scheme 1B, part e), leading to extremely low background noise.

To demonstrate the superiority of the intramolecular displacement probe to both the linear probe and the intermolecular displacement probe in Exo III-assisted target recycling amplification, the fluorescence of 2-aminopurine was monitored in the absence (Figure 1A) and in the presence of target DNA (Figure 1B), respectively. As shown in Figure 1, a high background noise is observed in the presence of linear probes (Figure 1A, curve a), while no distinguishable background noise is observed in the presence of both intermolecular displacement probes (Figure 1A, curve b) and intramolecular displacement probes (Figure 1A, curve c), suggesting that Exo III is capable of digesting only the linear probes. This is also confirmed by the steady-state kinetic parameters for the digestion of linear probes, intermolecular displacement probes, and intramolecular displacement probes by Exo-III (Table 2). The value of k_{cat}/K_m is usually used as a parameter to compare the relative rates of enzyme acting on different substrates. As shown in Table 2, there is no significant difference in the value of k_{cat}/K_m between the intermolecular displacement probes ($k_{cat}/K_m = 2.5 \times 10^5$ mol^{-1}·L·s^{-1}) and the intramolecular displacement probes ($k_{cat}/K_m = 2.3 \times 10^5$ mol^{-1}·L·s^{-1}). In contrast, the k_{cat}/K_m of linear probes ($k_{cat}/K_m = 6.7 \times 10^5$ mol^{-1}·L·s^{-1}) is approximately 3-fold higher than that of intermolecular displacement probes and intramolecular displacement probes, suggesting that linear probes tend to be digested by Exo III.

In the presence of target DNA, the fluorescence signal obtained by the intramolecular displacement probe (Figure 1B,

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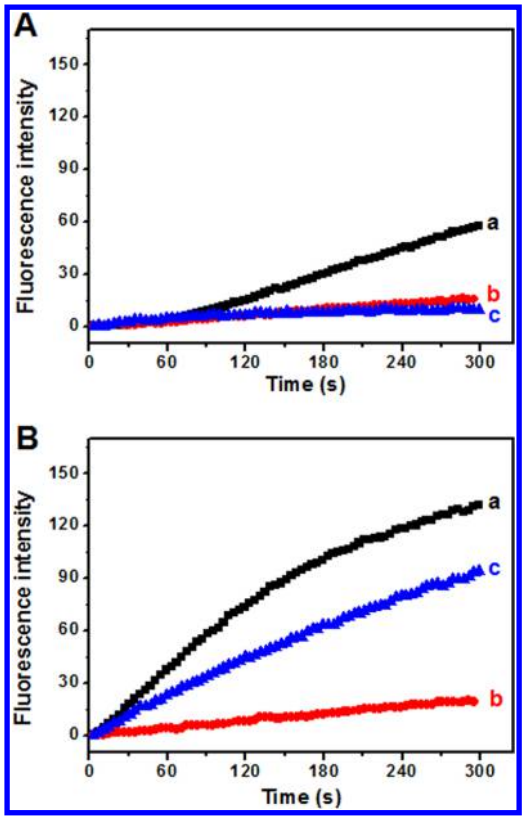


Figure 1. Time courses of 2-aminopurine fluorescence obtained by the linear probe (a), the intermolecular displacement probe (b) and the intramolecular displacement probe (c) in the absence (A) and in the presence of target DNA (B), respectively. Each probe concentration is 100 nM, and the target concentration is 1.67 nM.

curve c) increases more quickly than that by the intermolecular displacement probe (Figure 1B, curve b), suggesting that the intramolecular displacement probe has faster hybridization kinetics than the intermolecular displacement probes due to a shorter competitor sequence of 5 nt in the intramolecular displacement probe than that of 12 nt in the intermolecular displacement probe.[26a]

To further evaluate the proposed Exo III-assisted target recycling amplification, the time courses of 2-aminopurine fluorescence obtained by the intramolecular displacement probe in response to the target with 3′ phosphorothioate modification and various lengths of 3′-protrusions were investigated, respectively. As shown in Figure 2, no fluorescence signal is observed in the presence of buffer (Figure 2, cure a) and the target without 3′-protrusions (Figure 2, cure b). In contrast, a distinct fluorescence signal is observed in the presence of the target with 3′-protrusions (Figure 2, curves c, d, and e). Notably, the fluorescence signal enhances with the increase in the length of 3′-protrusions (Figure 2, curves c, d, and e), and the fluorescence signal in response to the target with 29 nt protrusions (Figure 2, curve e) reaches almost the same level in response to the target with 3′-phosphorothioate modification (Figure 2, curve f). Therefore, just like the target with 3′-phosphorothioate modification which cannot be digested by Exo III,[15,24] the target with long 3′-protrusions length can be resistant to the nonspecific degradation of Exo III (Figure 2, curves e and f). Moreover, the target DNA with long-length 3′-protrusions should be a good option in the practical applications.

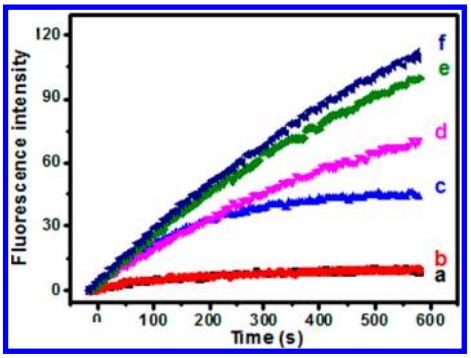


Figure 2. Time courses of 2-aminopurine fluorescence obtained by the intramolecular displacement probe in response to buffer (a), the target without 3′-protrusions (b), the target with 9 nt 3′-protrusions (c), 15 nt 3′-protrusions (d), 29 nt 3′-protrusions (e), and 3′-phosphorothioate modification (f). Each target concentration is 1.67 nM.

Detection of Target DNA with Exo III-Assisted Target Recycling Amplification. To demonstrate the feasibility of the proposed method for DNA detection, the detection specificity and sensitivity were investigated, respectively. As shown in Figure 3A, the fluorescence of 2-aminopurine obtained by the intramolecular displacement probe increases quickly with time and reaches a plateau within 5 min, which is much shorter than that of 30−60 min in the conventional Exo III-assisted amplification.[8b,12] This rapid signal response can be attributed to the fast hybridization kinetics endowed by the intramolecular displacement probe (Figure 1B). Notably, a linear correlation is obtained between the log initial reaction rate (V) and the log target concentration (C) over a range of 4 orders of magnitude (Figure 3B). The correlation equation is $\log V = 0.6672 \log C + 5.3286$ with a correlation coefficient of 0.9865, where V is the initial reaction rate (s^{-1}), and C is the DNA concentration (molar), respectively. This result is in agreement with the report that the initial reaction rate of nuclease-assisted target-recycling amplification is proportional to the target concentration,[1,4c] suggesting that the target DNA can be rapidly and accurately quantified by measuring the initial reaction rate. The detection limit of the proposed method can reach 10 pM, which has improved by as much as 3 orders of magnitude as compared with that of 2-aminourine-based molecular beacons,[28] and is also comparable with that of doubly labeled molecular beacons.[8b] The improved sensitivity might be attributed to the low background noise and the reduced target degradation (Scheme 1B). In addition, the proposed method has significant advantages of rapid, label-free, and low-cost.

To investigate the specificity of the proposed method, the detection of a perfect matched target, the targets with a single-base mismatched, two-base mismatched, and the random sequence was investigated, respectively. As shown in Figure 3C, the perfect-matched target (Figure 3C, a) can be well distinguished from both single-base mismatched (Figure 3C, b and c), two-base mismatched target (Figure 3C, d), and the random sequence (Figure 3C, e), suggesting the high specificity of the proposed method for DNA detection. Notably, the initial reaction rate of the intramolecular displacement probe in response to the target with a single-base mismatched that hybridizes with the stem of the intramolecular displacement probe is 0.17 $nM \cdot s^{-1}$ (Figure 3C, b), much higher than that of 0.05 $nM \cdot s^{-1}$ in response to the target with a single-base mismatched that hybridized with the 3′-protrusions of the

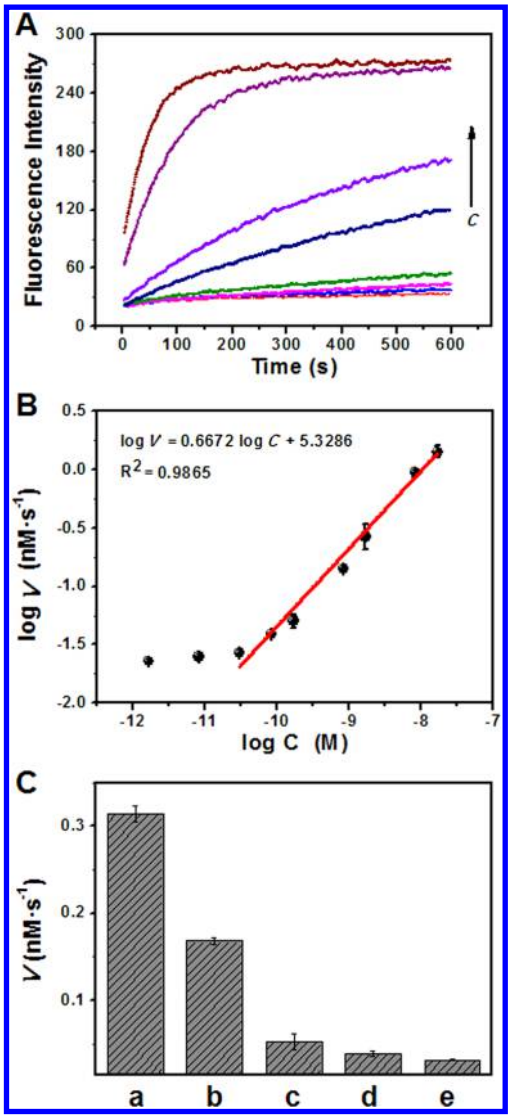
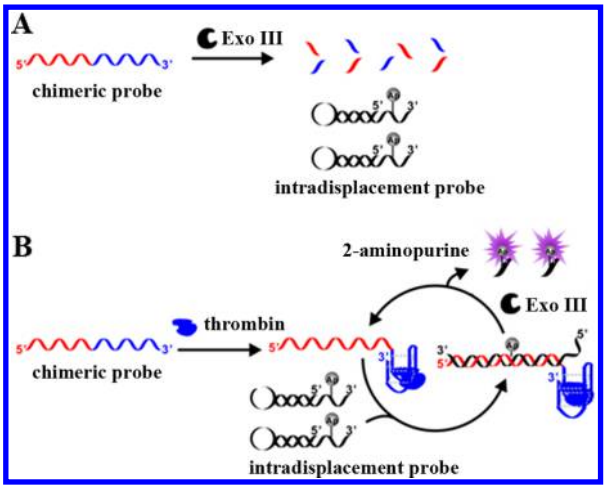


Figure 3. (A) Time courses of 2-aminopurine fluorescence obtained by the intramolecular displacement probe in response to various concentrations of target DNA. The DNA concentration from bottom to top is 1.67, 8.35, 30, 83.5, 167, 835, 1670, 8350, and 16700 pM, respectively. (B) Linear relationship between the log initial reaction rate (V) and the log target concentration. (C) Initial reaction rates of the intramolecular displacement probe in response to the perfect-matched target (a), the target with a single-base mismatched that hybridizes with the stem of intramolecular displacement probe (b), the target with a single-base mismatched that hybridized with the 3′-protrusions of intramolecular displacement probe (c), the target with two-base mismatched (d), and the random sequence (e). Error bars show the standard deviation of three experiments.

intramolecular displacement probe (Figure 3C, c). This result can be well explained by the fact that the intramolecular displacement hybridization is initiated at the ssDNA domains of hairpin probe,[29] suggesting that the intramolecular displacement probe can even discriminate the mismatched position of targets with a single-base mismatched.

Detection of Proteins with Exo III-Assisted Target Recycling Amplification. The proposed method can be further extended to the detection of protein using an aptamer-based intramolecular displacement probe. As a principle-of-concept, we designed a chimeric probe which consisted of an aptamer recognition domain[30] and a target recycling reaction domain for thrombin detection (Scheme 2). As shown in the Scheme 2A, in the absence of thrombin, the free chimeric

Scheme 2. Schematic Illustrations of Thrombin Detection Using Aptamer-Based Intramolecular Displacement Probe in the Absence (A) and in the Presence of Thrombin (B)



probes are completely degraded by Exo III and cannot initiate the Exo III-assisted target recycling amplification, thus no fluorescence of 2-aminopurine is observed. However, in the presence of thrombin, the binding of thrombin with the recognition domain of the chimeric probe prevents the target recycling reaction domain from being digested by Exo III. The protected chimeric probe can hybridize with the intramolecular displacement probe and form a dsDNA with a blunt 3′ terminus, which can be digested by Exo III, leading to the release of both 2-aminopurine and the chimeric probe. The released chimeric probe can hybridize with the new intramolecular displacement probes and initiate new digestion events repeatedly, releasing abundant 2-aminopurine and achieving great signal amplification (Scheme 2B). As a result, thrombin can be quantified rapidly and sensitively by monitoring the fluorescence of 2-aminopurine in a homogeneous way.

As shown in Figure 4A, the fluorescence of 2-aminopurine obtained by the intramolecular displacement probe shows no obvious changes in the absence of thrombin but increases quickly with time in the presence of thrombin. Notably, a linear correlation is obtained between the initial reaction rate (V) and the thrombin concentration (C) in a range from 0.4 to 32 nM (Figure 4B). The correlation equation is $V = 0.0485C - 0.0131$ with a correlation coefficient of 0.9962, where V is the initial reaction rate (s^{-1}), and C is the thrombin concentration (nanomolar), respectively. The detection limit of the proposed method can reach 0.2 nM, which has improved by as much as 2 orders of magnitude as compared with that of the conventional aptamer-based method,[31] and is also comparable with that of nicking endonuclease-assisted recycling amplification.[32] However, nicking endonuclease-assisted recycling amplification requires a doubly labeled probe which increases the experimental cost and the design complexity.[32] Moreover, the proposed method can specifically distinguish thrombin from bovine serum albumin (BSA), horseradish peroxidase (HRP), and hemoglobin. As shown in Figure 4C, the initial reaction rate (V) of the intramolecular displacement probe in response to thrombin is about 6-fold higher than that in response to

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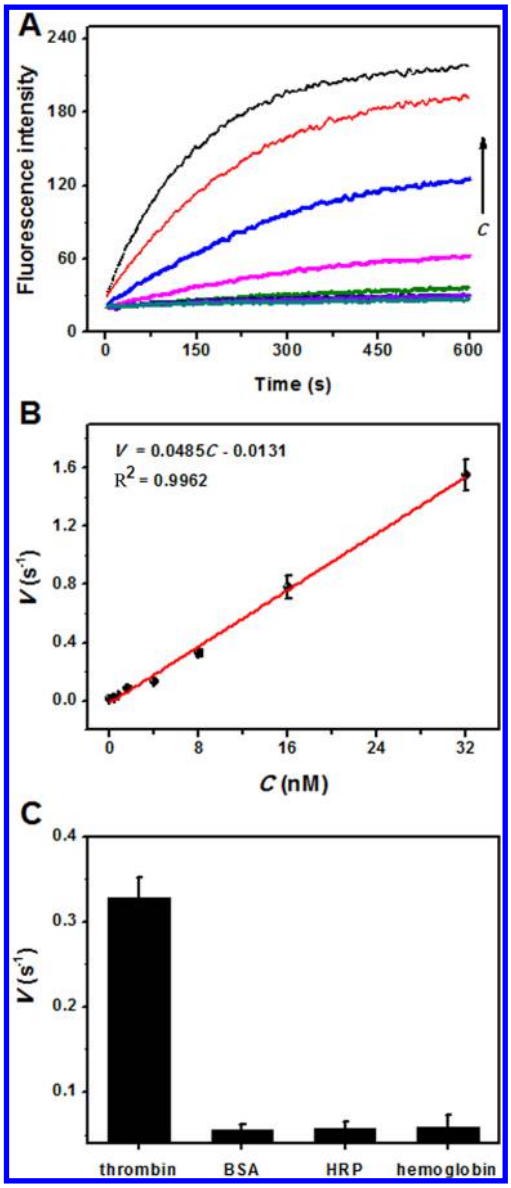

Figure 4. (A) Time courses of 2-aminopurine fluorescence obtained by the intramolecular displacement probe in response to various concentrations of thrombin. The thrombin concentration from bottom to top is 0, 0.4, 0.8, 1.6, 4.0, 8.0, 16.0, and 32.0 nM, respectively. (B) Linear relationship between the initial reaction rates (V) and the thrombin concentrations. (C) Initial reaction rates of the intramolecular displacement probe in response to thrombin, BSA, HRP, and hemoglobin. Error bars show the standard deviation of three experiments.

BSA, HRP, and hemoglobin. This excellent specificity can be attributed to the selective binding of aptamer to thrombin, which protects the chimeric probes from being digested by Exo III. These results demonstrate that the proposed method can be used for rapid and label-free detection of low-abundant protein specifically. Moreover, this assay might provide a universally rapid and label-free platform for the detection of various proteins and even small molecules by simply changing the recognition domain of the chimeric probe to the corresponding aptamer sequence.[33,34]

CONCLUSION

In conclusion, we have developed an intramolecular displacement probe with the capability of resistance to nonspecific digestion of Exo-III and fast hybridization kinetics. Through the substitution of 2-aminopurine for adenine in the intramolecular displacement probes, we develop a rapid and label-free approach for sensitive and specific detection of DNA and proteins by monitoring Exo-III assisted target recycling amplification. Our method has significant advantages of simplicity without the requirement of fluorescent-labeled probes or extra quenchers, rapidity, resistance to nonspecific degradation of Exo-III, excellent specificity, and high sensitivity over the conventional target recycling amplification. Importantly, this method can be further extended to rapid, label-free, and multiplexed detection of various nucleic acids, proteins, and small molecules using different kinds of fluorescent nucleotide analogues and specific aptamers.[33−35]

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Notes

The authors declare no competing financial interest.

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