

Biosensing by Tandem Reactions of Structure Switching, Nucleolytic Digestion, and DNA Amplification of a DNA Assembly**

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Abstract: ϕ 29 DNA polymerase (ϕ 29DP) is able to carry out repetitive rounds of DNA synthesis using a circular DNA template by rolling circle amplification (RCA). It also has the ability to execute 3'–5' digestion of single-stranded but not double-stranded DNA. A biosensor engineering strategy is presented that takes advantage of these two properties of ϕ 29DP coupled with structure-switching DNA aptamers. The design employs a DNA assembly made of a circular DNA template, a DNA aptamer, and a pre-primer. The DNA assembly is unable to undergo RCA in the absence of cognate target owing to the formation of duplex structures. The presence of the target, however, triggers a structure-switching event that causes nucleolytic conversion of the pre-primer by ϕ 29DP into a mature primer to facilitate RCA. This method relays target detection by the aptamer to the production of massive DNA amplicons, giving rise to dramatically enhanced detection sensitivity.

DNA amplification is a valuable method in genomics, molecular diagnosis, chemical biology, and DNA nanotechnology. Along with the polymerase chain reaction,^[1] an isothermal DNA amplification technique known as rolling circle amplification (RCA) has recently attracted great attention.^[2,3] RCA involves elongation of a DNA primer over a circular DNA template by DNA polymerases with strand-displacement ability and high processivity, such as ϕ 29 DNA polymerase (ϕ 29DP).^[4] These polymerases can continuously dislodge newly synthesized DNA strands from the circular template, making it available for many rounds of copying. The product of RCA is extremely long single-stranded (ss) DNAs with thousands of repeating units.^[2,3]

Owing to its amplification power and operational simplicity, RCA has become a popular DNA amplification technique.^[5–9]

Nature has evolved DNA polymerases into impressive enzymes with multiple functions. For example, ϕ 29DP is capable of carrying out 3'–5' exonucleolytic digestion of ssDNAs (but not double-stranded DNAs),^[10] along with its DNA polymerization and strand-displacement functions.^[4] The nucleolytic activity, common among DNA polymerases, has been evolved to proofread DNA replication in vivo.^[11] However, this property is rarely explored for in vitro applications.

Herein we report a biosensing strategy that makes full use of ϕ 29DP in combination with structure-switching aptamers.^[12] This approach uses a circular template (CT), a pre-primer (PP), and an aptamer probe (AP) to create a DNA assembly with two DNA duplexes, as illustrated in Figure 1 a.

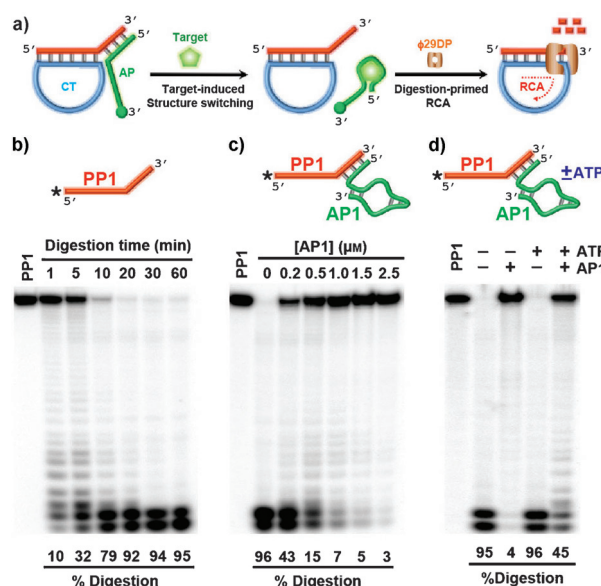


Figure 1. a) Working principle of stimuli-responsive, digestion-primed RCA. Digestion of 1 μ M radioactive PP1 with 0.1 U μ L^{−1} ϕ 29DP b) alone for 0–60 min, c) in the presence of 0–2.5 μ M AP1 for 30 min, and d) in the presence of 1.5 μ M AP1 and 0.5 mM ATP. The reaction mixtures were analyzed by 20% dPAGE.

In the absence of the target for the aptamer, the PP–AP duplex prevents ϕ 29DP from performing 3'–5' digestion of PP. The presence of the target, however, triggers release of the aptamer from the DNA assembly, exposing the 3' element of PP for nucleolytic trimming. This in turn converts PP into a mature primer to enable RCA. Our approach, therefore, rationally integrates the structure-switching ability of DNA

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aptamers with the nucleolytic and polymeric functions of ϕ 29DP. This design converts target detection to the production of massive DNA amplicons, giving rise to dramatically enhanced detection sensitivity. This can therefore overcome the problem of relatively poor detection sensitivity that is typical of structure-switching aptamers.^[12a]

Using the well-known anti-ATP DNA aptamer,^[13] we assessed the digestion of PP in the absence and presence of AP. The AP and PP for ATP detection are named AP1 and PP1 (sequences of the DNA molecules used for this work are provided in the Supporting Information, Table S1). As shown in Figure 1b, more than 90 % of PP1 (1 μ M) was degraded by ϕ 29DP (0.1 units μ L⁻¹) within 30 min. However, degradation of PP1 was decreased to 3 % in the presence of 2.5 μ M AP1 (Figure 1c). The results indicate that AP1 can indeed block nucleolytic digestion of PP1 by ϕ 29DP through AP1–PP1 duplex formation.

It is interesting to note that AP1 is rather resistant to nucleolytic digestion by ϕ 29DP, as less than 5 % was digested after 60 min (Supporting Information, Figure S1), compared to 96 % for PP1 under the same conditions (Figure 1a). This indicates that the aptamer has a structure that is resistant to exonucleolytic digestion by ϕ 29DP, consistent with the reported hairpin structural model of the aptamer.^[13b]

We examined the effect of ATP on PP1 digestion, expecting that ATP would induce AP1 release from the AP1–PP1 duplex by structure switching.^[12] Indeed, addition of ATP (0.5 mM) led to significantly increased cleavage of PP1 in the presence of AP1 (45 %, vs. 4 % without ATP; Figure 1d). In contrast, no significant change was observed in PP1 digestion when GTP was supplied (Supporting Information, Figure S2). This result shows that AP1 release is ATP-dependent.

We next investigated the digestion of PP1 (1 μ M) in the presence of CT1 (0–2.5 μ M; Figure 2a). The PP1 digestion pattern was changed when CT1 was supplied: with increasing concentrations of CT1, the amount of small digestion products was reduced whereas the quantity of mid-range fragments (denoted MRF) was increased (Figure 2a). This

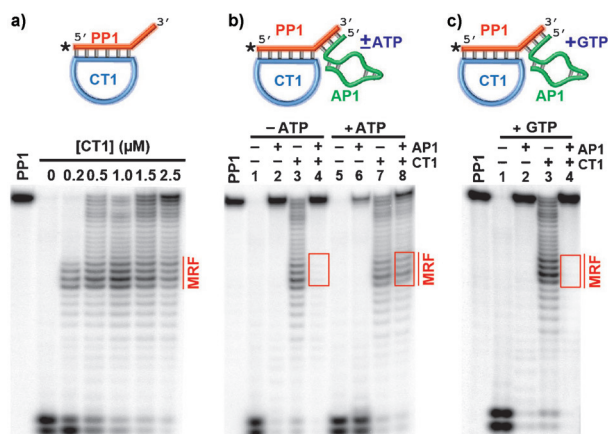


Figure 2. Digestion of 1 μ M radioactive PP1 with 0.1 U μ L⁻¹ ϕ 29DP for 30 min in the presence of a) 0–2.5 μ M CT1, b) 1 μ M CT1, 1.5 μ M AP1 and 0.5 mM ATP, c) 1 μ M CT1, 1.5 μ M AP1 and 0.5 mM GTP.

observation is consistent with the expectation that the unpaired region of PP1 was trimmed by ϕ 29DP.

The digestion pattern of PP1 within the PP1–AP1–CT1 assembly was then studied. In the absence of ATP, PP1 was protected from exonucleolytic digestion by ϕ 29DP, as no MRF were observed (Figure 2b, lane 4, red box). However, addition of ATP resulted in trimming of the exposed 3'-end, reflected by the appearance of MRF (Figure 2b, lane 8, red box). When ATP was replaced by GTP, MRF disappeared (Figure 2c, lane 4, red box).

The results from Figure 1 and 2 allow us to conclude that: 1) ϕ 29DP can digest ss PP1; 2) formation of the PP1–AP1 duplex blocks PP1 digestion; 3) addition of ATP promotes release of AP1 from the tripartite assembly; and 4) ϕ 29DP trims the exposed ss fragment of PP1, converting it into the mature primer.

To show that nucleolytic trimming of PP1 can result in a mature primer that can initiate RCA, we carried out the RCA reaction with the PP1–CT1 hybrid. As a control, we performed the same reaction with I–PP1–CT1, a modified PP1 containing an inverted dT at the 3'-end. This modification should render I–PP1 completely resistant to digestion by ϕ 29DP. Indeed, it was found that ϕ 29DP was incapable of degrading I–PP1 (Supporting Information, Figure S3a). Agarose gel analysis indicated that RCA product (RP) was produced when PP1 was incubated with CT1, dNTPs, and ϕ 29DP (Supporting Information, Figure S3b). However, RP was not observed when I–PP1 was used to replace PP1. These results show that successful trimming of PP1 by ϕ 29DP is a prerequisite for RCA.

We next examined the ATP-promoted RCA reaction of the PP1–AP1–CT1 assembly. Three events were expected to occur: 1) ATP-promoted structure switching, 2) exonucleolytic trimming of PP1 by ϕ 29DP, and 3) RCA by ϕ 29DP. As discussed below, we separated the structure-switching event (mixing the DNA assembly with ATP) from the primer trimming and RCA events (mixing ATP/DNA solution with ϕ 29DP/dNTPs). The results are shown in Figure 3. The first 6

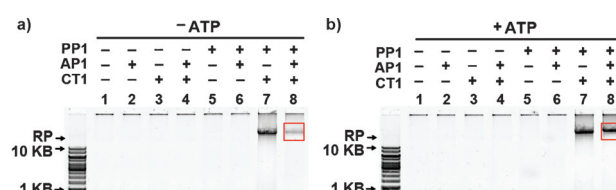


Figure 3. Agarose gel analysis of RP from RCA reactions of PP1, CT1, and AP1 in the a) absence and b) presence of ATP.

lanes of each panel serve as negative controls (RCA should not occur when PP1 or CT1 is omitted). Each lane 7 serves as a positive control (RCA should occur when both PP1 and CT1 are provided but AP1 is omitted). The final lane of each panel serves as the ATP-dependence test. As expected, no RP was observed in any of the negative controls but was found in the two positive controls. More importantly, the presence of ATP indeed resulted in significantly more RP production: the RP band in lane 8 of Figure 3b is much more intense than the

same band in Figure 3a (indicated by the red boxes). In theory, RP should not have been observed in the absence of ATP. However, it is known that the DNA aptamer can also bind dATP.^[13] Therefore, the small amount of RP in the absence of ATP is likely to have originated from the nucleolytic trimming-RCA step where dATP was supplied as part of the dNTPs needed for DNA amplification. This is also the reason that we separated the structure-switching step from the trimming and RCA step.

To demonstrate that ligand-responsive RCA was a general feature for structure-switching aptamers, we investigated another aptamer system. We designed a new DNA aptamer probe, AP2, based on a reported aptamer that binds human platelet-derived growth factor (PDGF).^[14] To prevent the degradation by ϕ 29DP, AP2 was modified with an inverted dT at the 3'-end (named I-AP2) as this aptamer does not have an intrinsic structure resistant to nucleolytic digestion by ϕ 29DP.

The tripartite assembly is made of I-AP2-PP2-CT1. Digestion of radioactive PP2 was carried out under various conditions and the results are nearly identical to the ATP system (Figure 4a). Briefly, in the absence of I-AP2 and CT1,

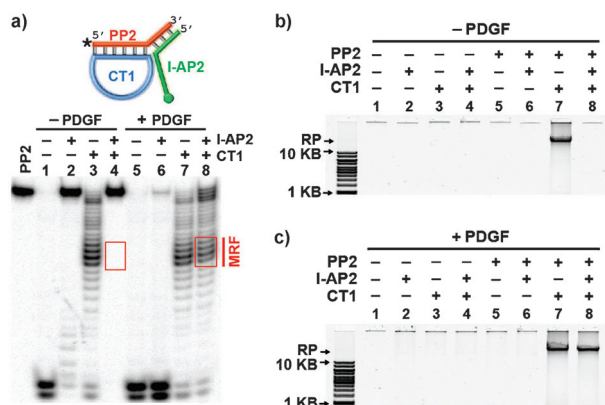


Figure 4. a) Digestion of 1 μ M radioactive PP2 with 0.1 U/ μ L ϕ 29DP for 30 min in the presence of 1 μ M CT1, 1.5 μ M I-AP2, and 100 nM PDGF. b), c) Agarose gel analysis of RP in RCA reaction mixtures containing various combinations of 1 μ M PP2, 1 μ M CT1, and 1.5 μ M I-AP2 in the absence and presence of 100 nM PDGF.

PP2 was fully digested (lanes 1 and 5). When I-AP2 was provided but CT1 was omitted, PP2 was very much protected in the absence of PDGF (lane 2) but largely digested in the presence of PDGF (lane 6). However, when CT1 was provided but I-AP2 was omitted, PP2 was partially digested into MRF both in the absence (lane 3) and presence (lane 7) of PDGF. More importantly, when both I-AP2 and CT1 were provided, PP2 was fully protected in the absence of PDGF (lane 4), but trimmed into MRF in the presence of PDGF (lane 8, red box).

The results of the RCA reaction of the I-AP2-PP2-CT1 assembly are shown in Figure 4b,c. In contrast to the ATP system, structure switching, nucleolytic trimming, and RCA reactions can be performed simultaneously. As expected, in the absence of PDGF, the RCA reaction was arrested (Figure 4b, lane 8; lanes 1–7 serve as various controls, as in

the case of the ATP system). However, RP was observed upon addition of PDGF (Figure 4c; lane 8). Control experiments with other proteins (BSA, thrombin, and IgG) and a mutant DNA aptamer (I-AP2M) demonstrated that the RCA reaction was dependent both on the matching target for the aptamer (Supporting Information, Figure S4a) and the specific aptamer sequence (Supporting Information, Figure S4b). These results demonstrate that stimuli-responsive, digestion-primed RCA can be generally adopted for structure-switching aptamers. We also analyzed the production of RP in response to increasing concentrations of PDGF. As shown in Figure 5a, as low as 10 pM can be detected by agarose gel analysis.

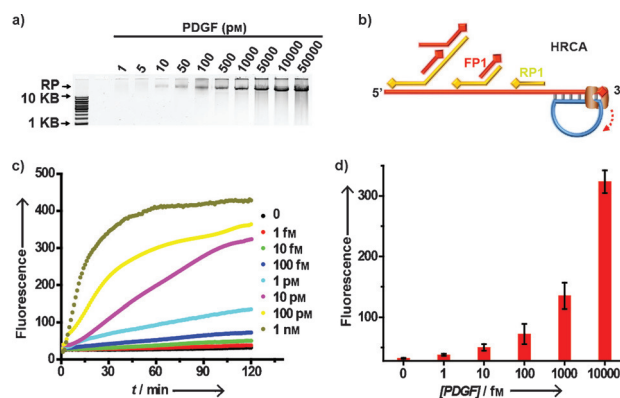


Figure 5. Detection of PDGF. a) Agarose gel analysis of RP in RCA reaction mixtures containing 1 μ M PP2, 1 μ M CT1, 1.5 μ M I-AP2, and increasing concentrations of PDGF. b) Working principle of hyper-branched RCA (HRCA). c) Real-time fluorescence monitoring of HRCA reaction with EvaGreen. d) Fluorescence readings at 120 min as a function of PDGF concentration.

To further improve detection sensitivity, we employed hyper-branched RCA (HRCA).^[15] In HRCA (illustrated in Figure 5b), DNA products generated from RCA using a forward primer (FP1) are further copied by ϕ 29DP using a second primer (reverse primer, RP1) into DNA products that can be further amplified using FP1. This process results in an exponential amplification.^[16] We adopted this strategy with the use of FP1 and RP1 as the cross-amplification primers. The DNA intercalating dye EvaGreen was used to achieve real-time monitoring of HRCA products. In the presence of PDGF, fluorescence intensity increased gradually with reaction time, indicating that PDGF can indeed initiate HRCA (Figure 5c). Using this method, we can detect PDGF at a concentration as low as 1 fM (Figure 5d). Remarkably, HRCA offers a detection sensitivity that is four orders of magnitude better than that of regular RCA (10 pM). The PDGF aptamer has a dissociation constant K_d of about 0.1 nM^[14] and the previously reported structure-switching fluorescent aptamer biosensor was only able to achieve a detection limit of about 2 nM.^[17a] Therefore, our integrated strategy offers a dramatically improved detection limit. To the best of our knowledge, the 1 fM limit of detection represents the lowest detected concentration ever achieved with the PDGF aptamer.^[7b,17]

To extend the digestion-primed RCA approach beyond aptamer-based detection, we applied the same strategy for DNA detection (Supporting Information, Figures S5 and S6). The DNA probe, I-DP1, has a specific DNA sequence designed to recognize HCV-1 DNA, representing a portion of the complementary DNA sequence from the hepatitis C virus genome.^[18] Once again, the HRCA strategy was adopted, along with the use of EvaGreen for real-time detection of DNA amplicons. The fluorescence intensity increased in response to HCV-1 DNA in a time-dependent manner (Supporting Information, Figure S6a,b). The limit of detection, established by plotting fluorescence intensity obtained at 180 min vs. DNA concentration (Supporting Information, Figure S6c), was found to be 20 aM, corresponding to 600 copies of DNA in 50 μ L. Besides the outstanding detection limit, this method also exhibited excellent selectivity. No increase of fluorescence was observed when the system was tested with unintended DNA targets, such as HCV-M1 and HCV-M2 (containing 7 and 9 mismatched nucleotides, respectively; Supporting Information, Figure S6d).

In summary, we have demonstrated a versatile amplified biosensing strategy that uniquely integrates structure-switching aptamers for target recognition with both exonucleolytic trimming and DNA-dependent polymerization functions of ϕ 29DP. The biosensor features a two-duplex tripartite DNA assembly consisting of a circular DNA template, a pre-primer, and an aptamer sequence. The target-induced aptamer structure-switching event acts as the control element for the trimming event carried out by ϕ 29DP, which in turn controls the DNA amplification event executed also by ϕ 29DP. To our knowledge, the integrated recognition–digestion–amplification strategy has never been reported before. Furthermore, this approach can be adopted for detection of wide-ranging targets, including small molecules, proteins, and DNA. With incorporation of HRCA, our strategy is capable of delivering a limit of detection that is several orders of magnitude lower than the dissociation constant of the aptamer. Therefore, this approach can turn an aptamer with a relatively low affinity for its target into an ultra-sensitive biosensing system. With a wide variety of aptamers currently available and new aptamers that can be conveniently produced by in vitro selection, we envision that the described strategy will find diverse applications.

Keywords: aptamers · biosensors · DNA amplification · rolling circle amplification · structure switching

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