Diagnosing viruses by the rolling circle amplified synthesis of DNAzymes†

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Circular DNA is used as a template for the amplified detection of M13 phage ssDNA by a rolling circle amplification (RCA) process that synthesizes DNAzyme chains, thus enabling the colorimetric or chemiluminescent detection of the analyte.

The amplified detection of DNA continues to attract interest directed to the sensitive detection of DNA.[1,2] Different methods have been employed to amplify the sensing of DNA by the application of enzymes,[3,4] nanoparticles[5,6] and magnetic particles,[7] and electrical,[8,9] optical[10,11] and piezoelectric[12] signals have been used to probe the nucleic acid recognition events. The RCA process has been employed in various sensing schemes,[13] and has been used to generate templates for nanoparticle aggregation.[14] In the present study, we developed a DNA-based machine that activates a rolling circle amplification (RCA) process upon recognition of the target DNA, yielding chains composed of DNAzyme units. The DNAzyme units amplify the recognition events and allow the colorimetric or chemiluminescent readout of the sensing process. Using this technique, we detect the M13 phage DNA with a sensitivity limit of 1×10^{-14} M.

Recently, the assembly of DNA-based machines has attracted substantial research activities,[15] and DNA-based "tweezers",[16] "walkers",[17] and "motors"[18] have been reported. The use of a DNA/protein-based cutter system was recently reported as an autonomous machine for the replication of DNA and for the amplified optical analysis of DNA.[19] Circular DNA is often used as a template for rolling circle amplification (RCA), which yields single-stranded chains of repeated units of the circular template.[20] The DNAzyme used in our study is a G-quadruplex nucleic acid sequence that, upon binding of hemin, mimics the functions of peroxidase.[21] This DNAzyme was previously used by us as a biocatalytic label for different sensing processes.[22,23] The DNAzyme catalyzes the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), $ABTS^{2-}$, by H_2O_2 to the green-colored oxidized ABTS,[22] or stimulates (in the presence of H_2O_2 and luminol) the generation of chemiluminescence.[23] The concept of activating a DNAzyme-synthesizing machine by the RCA process is depicted in Fig. 1A. The circular DNA was constructed from 75 bases that included one segment, A, complementary to the primer **1**, and three segments B, C, and D, each complementary to the DNAzyme, where the segments are separated one from another by a sequence of 4 bases.

The cyclic DNA was prepared by the hybridization of the ends of linear **2** with the nucleic acid **1** followed by phosphorylation of the nucleic acid with kinase, and ligation of the two ends with ligase to form the circular DNA **2**. Fig. 1B depicts the rate of $ABTS^{2-}$ oxidation by the DNAzymes synthesized by the RCA process, using a fixed concentration of the primer, **1**, and variable time-intervals for the RCA reaction. The longer the RCA process, the more DNAzyme units are generated, leading to an enhancement of the oxidation of $ABTS^{2-}$. Control experiments reveal that the interaction of a non-ligated circular ssDNA with the primer **1** does not lead, in the presence of dNTPs/polymerase, to any replication of the DNAzyme chain (Fig. 1B, curve e). Other control experiments that were performed revealed that no oxidation of $ABTS^{2-}$ was observed upon performing the RCA of the nucleic acid chains in the presence of **1** and dNTPs/polymerase and in the absence of hemin. Also, no oxidation of $ABTS^{2-}$ was observed when **1** was allowed to interact with the circular DNA **2** in the absence of either the dNTPs or polymerase. These results imply that only **1** triggers the RCA reaction and the synthesis of the DNAzyme units (hemin intercalated in the G-quadruplex units), that catalyze the oxidation of $ABTS^{2-}$.

The synthesis of the DNAzyme chains by the RCA process was also followed by chemiluminescence. Fig. 1C shows the light intensities generated by the DNAzyme chains synthesized by the RCA process, using a fixed concentration of the primer **1**, and H_2O_2/luminol as the substrates that stimulate the light emission. The longer the RCA reaction, the greater the content of synthesized DNAzymes, leading to an enhancement in the resulting chemiluminescence.

The synthesis of the DNAzyme chains through the RCA process was further supported by electrophoresis and atomic force microscopy (AFM) studies. Fig. 2A depicts the electrophoretic results corresponding to the products formed by the RCA system at various reaction time-intervals. It is evident that as the RCA process is allowed to continue, products of higher molecular weights are generated, and nucleic acid products containing *ca.* 1500 bp are formed. Fig. 2B,C show the AFM images of the resulting DNA products. Numerous nucleic acids exhibiting lengths of 20 to 30 nm (60 to 100 bp, respectively) are observed, together with substantially longer DNA chains (Fig. 2B). Some very long (micrometre length) DNA chains were detected (Fig. 2C).

The RCA reaction for the synthesis of DNAzymes may be used as a versatile catalytic process for the amplified analysis of any DNA. Fig. 3A presents the method used to trigger the RCA process for analyzing the ssDNA M13 phage, **3**, which includes 7229 bases. A hairpin nucleic acid, **4**, consisting of a single-stranded loop complementary to the M13 phage ssDNA, is designed. The hairpin nucleic acid **4** opens upon hybridization with M13 phage DNA, and the opening of the hairpin stem yields a single-stranded tether acting as a primer that binds to a segment of the circular DNA **2**. The hybridization of the resulting tethered primer to the circular DNA **2** triggers, in the presence

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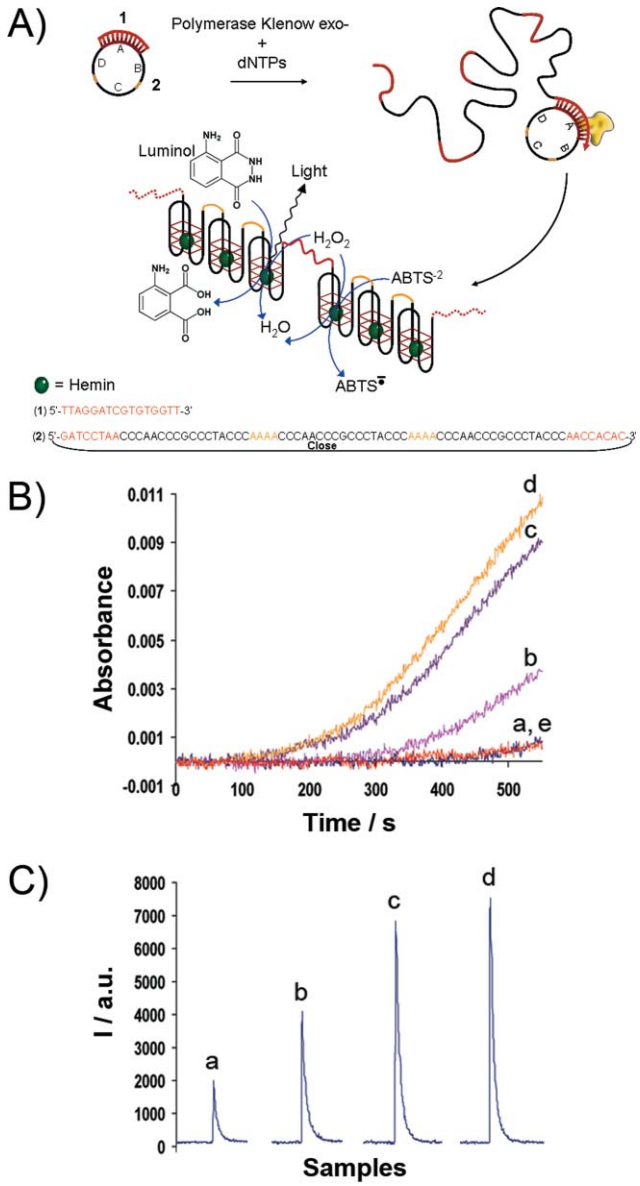

Fig. 1 A) Detection of DNA hybridization by RCA, leading to the synthesis of DNAzyme chains. B) Time-dependent absorbance changes of $ABTS^{2-}$ upon analyzing the nucleic acid **1** (2×10^{-8} M) by the RCA process that synthesizes the DNAzyme units, for different time-intervals: (a) 0 min, (b) 10 min, (c) 30 min, and (d) 60 min, (e) analysis of **1** with the open DNA **2** (not treated with kinase and ligase to form the closed circular DNA). In experiments (a) to (d) the circular DNA **2** (2×10^{-8} M) was present. C) Chemiluminescence intensities observed upon the light emission by luminol/H_2O_2 by the RCA process depicted in Fig. 1A, at different time-intervals and at the fixed concentrations of **1**, 2×10^{-8} M and **2**, 2×10^{-8} M : (a) 0 min, (b) 10 min, (c) 30 min, and (d) 60 min.

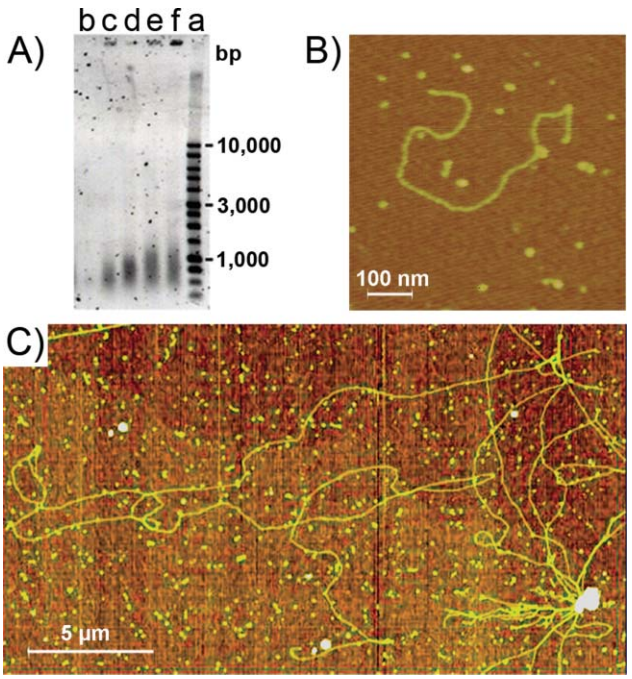

Fig. 2 A) Agarose-gel electrophoretic image corresponding to the time-dependent synthesis of the DNAzyme chains by the RCA process. Lane (a) 1 kb DNA ladder; (b) 0 min; (c) 10 min; (d) 30 min; (e) 60 min; (f) 90 min. B and C) AFM images of the DNA chains synthesized by the RCA process using **1** (2×10^{-8} M) as primer.

of dNTPs/polymerase, the RCA process, and the synthesis of the DNAzyme units. It should be noted that the hairpin structure **4** was designed in such a way that its stem hybridization affinities are stronger than the hybridization affinity with the circular DNA **2**. Consequently, **4** cannot be opened by **2**, yet the hybridization of **3** with the loop opens the stem structure. Fig. 3B shows the rate of $ABTS^{2-}$ oxidation by the DNAzyme synthesized by the RCA upon analyzing different concentrations of M13 phage ssDNA. As the concentration of M13 phage DNA increases, more of the primer units **4**/**2** are generated, and more RCA cycles are activated, leading to an increased content of the DNAzyme. A control experiment, where the foreign calf thymus DNA (1×10^{-8} M) is analyzed in the presence of the circular DNA **2**, according to the method shown in Fig. 3A, is shown in Fig. 3B, curve b. A minute rate of $ABTS^{2-}$ oxidation by the free hemin in the system is observed. This rate of $ABTS^{2-}$ oxidation may be considered as the background noise level of the system. Fig. 3C shows the light intensities observed upon analyzing different concentrations of M13 phage ssDNA according to the method shown in Fig. 3A, using chemiluminescence as the readout signal. As before, as the concentration of the M13 phage DNA rises, more DNAzyme units are synthesized, and the resulting light emission intensifies.

In conclusion, the present study has introduced the use of the RCA process for the synthesis of DNAzyme units that act as the catalytic units for the amplified analysis of DNA.[24] One amplification step involves the RCA synthesis of numerous DNAzyme units, as a result of a single recognition (hybridization) event. The second amplification step originates from the catalytic activities of the synthesized labels that lead to the colorimetric or chemiluminescent detection of DNA. In a recent study, the peroxidase-mimicking DNAzyme that was used in the present report was employed as a catalyst for the analysis of DNA in a PCR amplification process.[25] The sensitivity of the PCR amplification path that involves repeated thermal cycles is *ca.* 10^3-fold higher than the present RCA/DNAzyme process. This is mainly due to the exponential amplification features of the PCR process as compared to the RCA reaction. The fact, however, that the RCA/DNAzyme reaction process is isothermal, represents an important advantage of the system. The present study has used

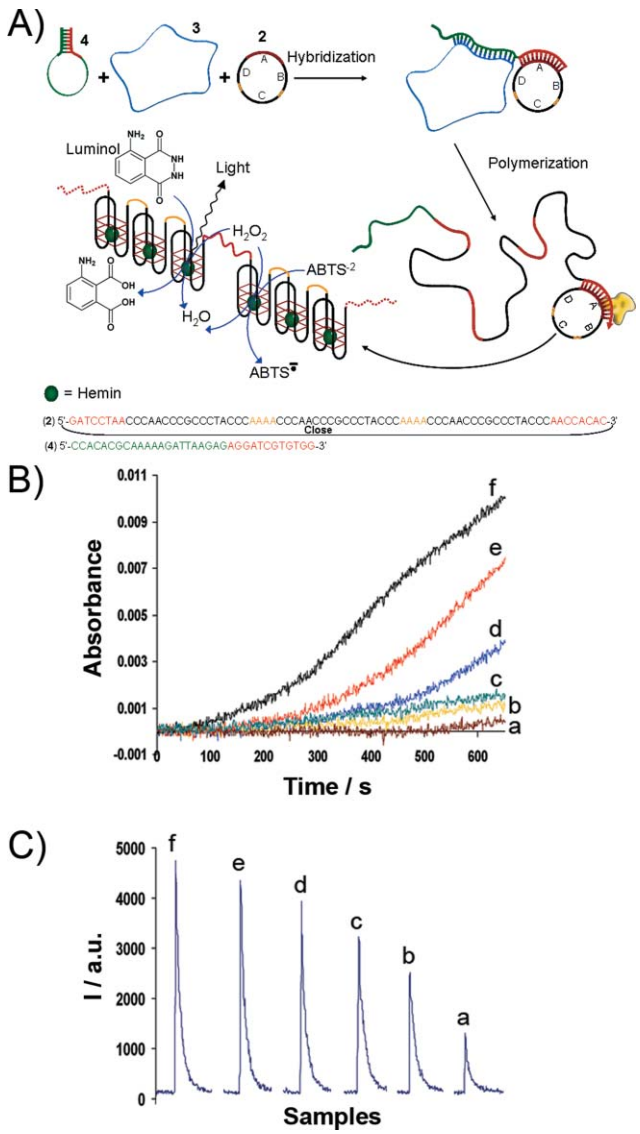

Fig. 3 A) Detection of M13 phage DNA by RCA, leading to the synthesis of DNAzyme chains. B) Absorbance changes upon the oxidation of $ABTS^{2-}$ by H_2O_2 in the DNA-based RCA process, and C) chemiluminescence intensities of light emission by luminol/H_2O_2 in the DNA-based RCA process. The following concentrations of M13 phage ssDNA **3** were used: (f) 1×10^{-9} M, (e) 1×10^{-11} M, (d) 1×10^{-12} M, (c) 1×10^{-14} M; and as controls: (b) 1×10^{-8} M foreign calf thymus ssDNA, and (a) absence of M13 phage DNA. In all systems fixed concentrations of the hairpin **4** (2×10^{-7} M) and the circular DNA **2** (2×10^{-8} M) were employed. Polymerase Klenow exo− (0.4 units μl^{-1}) and dNTPs (0.2 mM) were included in all of the systems. The RCA process was run for 60 minutes in all experiments.

the analysis of DNA as an example, but one might extend the concept to analyze other biorecognition events, such as immunocomplexes. In this case, primer-functionalized antibodies may act as triggers of the RCA synthesis of DNAzyme units that read out the immunocomplex formation.

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24 While preparing the manuscript we realized that Prof. Chengde Mao (Purdue University) is working on a similar project.
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