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Enzyme-Free Amplified Detection of DNA by an Autonomous Ligation DNAzyme Machinery

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Supporting Information

ABSTRACT: The Zn^{2+}-dependent ligation DNAzyme is implemented as a biocatalyst for the amplified detection of a target DNA by the autonomous replication of a nucleic acid reporter unit that is generated by the catalyzed ligation process. The reporter units enhance the formation of active DNAzyme units, thus leading to the isothermal autocatalytic formation of the reporter elements. The system was further developed and applied for the amplified detection of Tay-Sachs genetic disorder mutant, with a detection limit of 1.0×10^{-11} M. Besides providing a versatile paradigm for the amplified detection of DNA, the system reveals a new, enzyme-free, isothermal, autocatalytic mechanism that introduces means for effective programmed synthesis.

The self-replication of molecular or macromolecular units has attracted substantial research efforts, and the relevance of these systems to prebiotic chemical evolution and effective synthetic machineries has been discussed.[1] Various replication systems included nucleotide-based oligomers,[2] peptides,[3] molecular conjugates,[4] micelles,[5] and vesicles.[6] Amplification is an important aspect in bioanalytical science, and enzyme conjugates,[7] DNAzymes,[8] and nanoparticles[9] have been widely implemented as amplifying units for biorecognition events. Different enzyme-catalyzed machineries were suggested to amplify DNA detection, and these included the rolling circle amplified synthesis of DNAzyme units,[10] the cyclic nicking and polymerization of DNAzyme units on a DNA track,[11] and the Exo III-stimulated regeneration of the target analyte.[12] Recently, enzyme-free catalytic cycles for the amplified detection of DNA were developed, and these included the application of predesigned DNA constructs that trigger, upon sensing the target DNA, the autonomous catabolic cleavage that generates and recycles the analyte sequence or the autonomous synthesis of the catalytic DNAzyme label.[13] The self-replication of chemical elements may, in principle, provide a general method to amplify sensing events. Herein, we report on an enzyme-free, isothermal, autocatalytic DNA detection method that implements the E47 derived Zn^{2+}-dependent DNAzyme[14] as a biocatalyst. The Zn^{2+}-ligation DNAzyme catalyzes the formation of a new phosphodiester bond by the condensation of the 5′-hydroxyl of one oligonucleotide and a 3′-phosphorimidazolide on another oligonucleotide, with a catalytic rate of 0.07 min^{-1}.[14] It is demonstrated that the analyte DNA activates the ligation DNAzyme, and the resulting ligated product leads to the isothermal replication of the product through the subsequent formation of new ligating DNAzyme units. This is a unique example utilizing a ligation DNAzyme for amplified sensing. The method reveals a new concept that may substitute for PCR amplification and highlights the use of enzyme-free replication of a product for the amplified DNA sensing.

The operation of the ligation and replication machine is discussed with regards to, first, the formation of the ligation machinery. Figure 1A shows the principle of implementing the Zn^{2+}-ligating DNAzyme for the analysis of a target analyte. The DNAzyme/substrate structure is depicted in part (**a**). It includes the DNAzyme sequence (colored in blue and brown) tethered at its 5′ and 3′ ends to the specific substrate binding sequences (colored red and yellow, respectively) and the 5′-hydroxyl and 3′-phosphorimidazolide nucleic acids that are acting as the substrate components. In order to functionalize the ligation DNAzyme, the DNAzyme sequence (**a**) is split into two subunits i and ii, part (**b**), that cannot assemble to form an active DNAzyme. Subunit i is tethered to an auxiliary nucleic acid in the form of a hairpin (colored orange) that includes the analyte recognition domain, and a stem domain (colored blue) that blocks the DNAzyme subunits assembly and activity. In the presence of the analyte, the hairpin component opens, leading to the assembly of the active DNAzyme structure, part (**c**), that results in the ligation of the substrate subunits, part (**d**) (for the detailed construction procedure of the ligation DNAzyme subunits, see Figures S1 and S2, Supporting Information (SI)). Note that ligation of the substrate subunits leads to a product that severely inhibits the DNAzyme turnover or the further capturing of substrate subunits while releasing the ligated product.

To overcome this limitation, we developed an enzyme-free, isothermal, and autocatalytic detection method that can regenerate the DNAzyme structure and replicate the DNA analyte reporter by using the E47 derived Zn^{2+}-dependent DNAzyme as a biocatalyst. Figure 1B depicts the amplified analysis of the analyte DNA, (**1**), through the autonomous replication of a reporter unit for the analyte DNA by using the Zn^{2+}-dependent E47-type DNAzyme. The system consists of the hairpin (**2**) that includes in the loop the regions I, II, and III, which consists of the sequence complementary to the analyte (**1**). Domain IV in hairpin (**2**) is used for a strand-displacement reaction, and its function will be explained (vide infra). Domain V (gray) of hairpin (**2**) corresponds to one part of the Zn^{2+}-ligation DNAzyme. The stem region of hairpin (**2**) includes the domain VI (blue), and it is stabilized in a duplex structure. The system also includes DNAzyme subunit (**3**) that is composed of the domain VI′ (blue), which is complementary to domain VI of the hairpin (**2**). The protruding single-stranded region I (red) of hairpin (**2**) and domain III (yellow)

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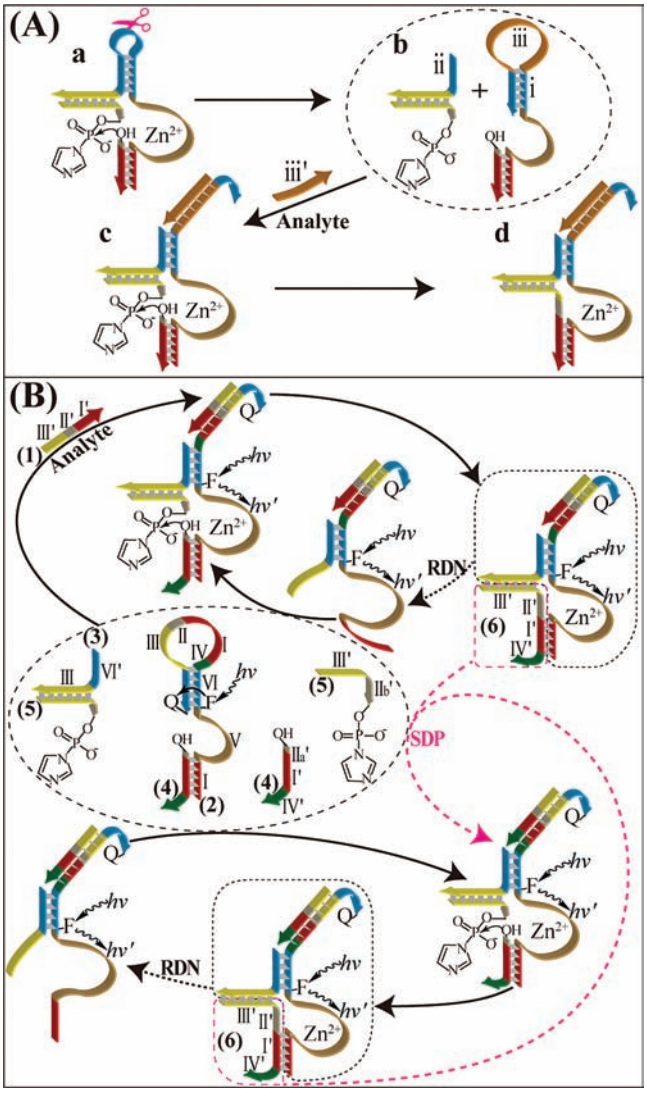

Figure 1. (A) General design of the Zn^{2+}-dependent DNAzyme subunits/substrates and their assembly in the presence of the DNA analyte into the catalytic ligation nanostructure. (B) Schematic autonomous replication of an analyte reporter unit by the Zn^{2+}-dependent ligation DNAzyme upon sensing the analyte and the autonomous assembly of the DNAzyme nanostructures. Throughout the paper, domains X and X′ in the respective analyte and DNAzyme or substrate subunits represent complementary base pair regions.

of (**3**) are used as recognition arms for the substrate subunits (**4**) and (**5**), respectively. In addition, the single-stranded nucleic acid substrate subunit (**4**) and the 3′-phosphorimidazolide-functionalized nucleic acid substrate subunit (**5**) consisting of the sequences I′ and III′, complementary to the respective protruding domains I and III of hairpin (**2**) and single-stranded nucleic acid (**3**), are included in the system. Note that the substrate subunits (**4**) and (**5**) also include additional domains IIa′, IIb′, and IV′, and their functions will be discussed later (vide infra). The hairpin (**2**) is functionalized in the stem region with an internally modified fluorophore, F (Cy5), and at the 3′-end with a quencher unit, Q (black hole quencher-2). In the closed hairpin structure, the fluorophore is quenched by Q. Furthermore, the stability of the duplex structure of the stem region of (**2**), in the absence of the analyte, prohibits the formation of the supramolecular nanostructure of the Zn^{2+}-ligation DNAzyme. In the presence of the analyte (**1**), the hairpin (**2**) opens, leading to the formation of the duplex structure between domains VI and VI′ of (**2**) and (**3**), respectively, resulting in the active loop sequence corresponding to the ligation DNAzyme. The binding of the nucleic acids (**4**) and (**5**) to the DNAzyme recognition domains of (**2**) and (**3**), I and III, and the association of Zn^{2+} ions to the loop region of the DNAzyme result in the active Zn^{2+}-ligation biocatalyst that ligates the substrate subunits (**4**) and (**5**) to form (**6**). Note that the domains IIa′ and IIb′ in the substrate subunits (**4**) and (**5**), respectively, are transformed to the domain II′ in the product (**6**) (for the detailed sequences of the nucleic acids comprising the system, see Table S1 (SI)). The product (**6**), however, includes the base sequence of the analyte (**1**) and a toe-hold region IV′ and thus acts as an activator unit, or amplifier, that opens hairpin (**2**) through the strand-displacement principle (SDP, indicated by purple dashed arrow), leading to the release of the ligated product (**6**) and to the concomitant opening of hairpin (**2**). This regenerates the DNAzyme nanostructures (RDN, indicated by black dashed arrow) that capture the free substrate subunits (**4**) and (**5**), and reactivates new DNAzyme units through the opening of hairpin (**2**). These two processes (SDP and RDN) enable the concomitant autonomous ligation and synthesis of (**6**). The activation of the DNAzyme machinery by the analyte results in the opening of hairpin (**2**), and the process is followed by the activation of the fluorescence of the fluorophore F. Several points related to the amplified detection of the analyte (**1**) by the system outlined in Figure 1B should be emphasized: (i) the ligated product (**6**) includes the sequence of the analyte (**1**), but it is further elongated by protruding oligonucleotide, domain IV′. Thus, the replicated product (**6**) is a reporter of the analyte. Evidently, the autonomous ligation and synthesis of (**6**) by using the ligation DNAzyme constructed by the reporter represents a replication process. (ii) The information encoded in the protruding chains is essential to enable the isothermal replication process, by providing the energetic driving force for the strand-displacement and the regeneration of the ligating DNAzyme.

Figure 2A shows the time-dependent fluorescence changes of the system upon analyzing different concentrations of the analyte (**1**). As the concentration of (**1**) increases, the fluorescence changes are intensified, consistent with the enhanced ligation of the substrate subunits (**4**) and (**5**), and the higher opening rate of hairpin (**2**). Nearly no fluorescence change is observed upon exclusion of the analyte (**1**) from the system. Control experiments revealed that these fluorescence changes do not occur upon exclusion of Zn^{2+} from the system or upon the substitution of the Zn^{2+} with Mg^{2+} ions (Figure 2B). These results indicate that the amplified time-dependent fluorescence changes originate from the Zn^{2+}-DNAzyme-stimulated ligation of (**4**) and (**5**) to form (**6**), a product that opens hairpin (**2**) and leads to the generation of fluorescence that provides the optical readout signal for the sensing of the analyte. It should be noted that the treatment of the oligonucleotides mixture shown in Figure 1B, in the absence of Zn^{2+} ions, leads, also, to a low intensity fluorescence, observed at a relatively high concentration of the target DNA (see Figure S3 (SI)). This is due to the opening of the hairpin (**2**) by the analyte (**1**). Figure 2B exemplifies the time-dependent fluorescence changes, upon analyzing the analyte (**1**), 10 nM, by the oligonucleotide mixture shown in Figure 1(B) in the presence of Zn^{2+} ions, curve (a), and the absence of Zn^{2+} ions, curve (b). Evidently, the fluorescence changes in the presence of Zn^{2+} are 100-fold intensified. The detection limit for analyzing (**1**) according to Figure 2A corresponded to 10 pM, whereas that for analyzing the target (**1**)

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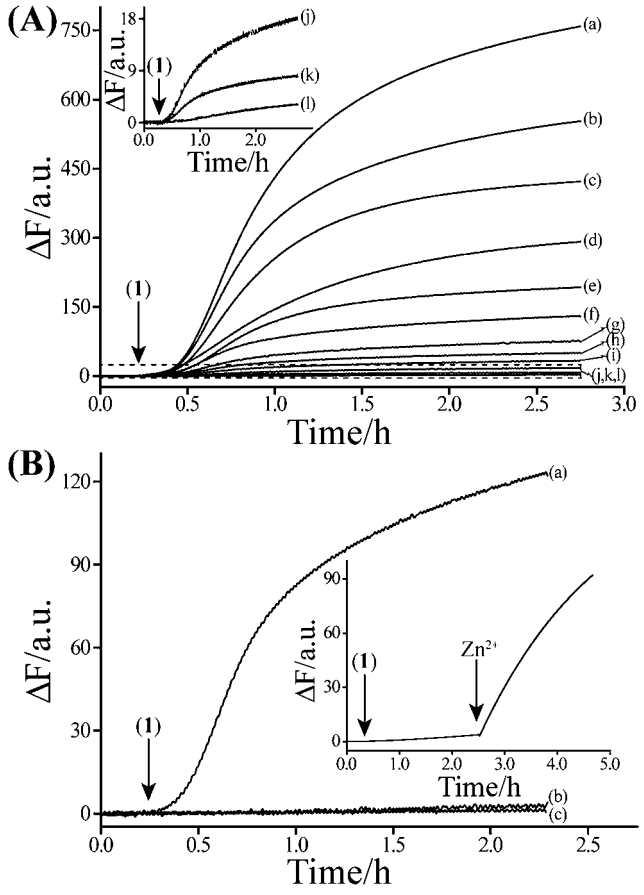

Figure 2. (A) Time-dependent fluorescence changes upon analyzing different concentrations of the analyte (**1**) according to Figure 1B: (a) 4.0×10^{-7} M, (b) 2.0×10^{-7} M, (c) 1.0×10^{-7} M, (d) 4.0×10^{-8} M, (e) 2.0×10^{-8} M, (f) 1.0×10^{-8} M, (g) 4.0×10^{-9} M, (h) 2.0×10^{-9} M, (i) 1.0×10^{-9} M, (j) 1.0×10^{-10} M, (k) 1.0×10^{-11} M, (l) 0 M. Arrow indicates the time of addition of the analyte. Inset: Enlargement of curves j–l. All experiments were performed in 10 mM HEPES buffer, pH = 7.0 that contained 300 mM NaCl and Zn^{2+}, 1 mM, (**2**) and (**3**), 0.5 μM each and (**4**) and (**5**), 0.8 μM each. (B) Time-dependent fluorescence changes upon the analysis of (**1**), 10 nM, according to Figure 1B: (a) in the presence of Zn^{2+}, 1 mM; (b) in the absence of Zn^{2+}; (c) only in the presence of Mg^{2+}, 1 mM. Inset: Fluorescence changes observed upon the stepwise analysis of (**1**); 10 nM of the analyte (**1**) are injected into the system in the absence of Zn^{2+}, followed by the introduction of Zn^{2+} ions, 1 mM. ΔF corresponds to the difference between the fluorescence intensity of the opened hairpin and the background fluorescence of the hairpin structure (**2**).

in the absence of Zn^{2+} ions corresponded to 10 nM (see Figure S3 (SI)). The amplified detection of the analyte by the DNAzyme ligation process is also evident from the fluorescence changes observed upon the stepwise detection of (**1**) (Figure 2B inset). Here, the oligonucleotide mixture is interacted with the analyte (**1**), 10 nM, in the absence of Zn^{2+} ions. A low value fluorescence change is observed because of the opening of (**2**) by (**1**). At the time marked with an arrow, Zn^{2+} is introduced into the system. This results in a sharp increase in the fluorescence of the system because of the activation of the autonomous ligation process and the formation of (**6**), or the continuous formation of active DNAzyme units and the amplified opening of (**2**) (for further gel electrophoresis characterization of the ligation process, see Figure S4 (SI)). Furthermore, the analysis of the target (**1**) is selective (see Figure S5 (SI)). We find that one-mismatch in the analyte leads to a 5-fold lower fluorescence intensity, whereas two- and three-mismatches scarcely activate the DNA sensing machinery.

The amplified detection of the analyte (**1**) by the set of oligonucleotides shown in Figure 1B requires optimization of the mixture of oligonucleotides. Specifically, the stem region of hairpin (**2**) must be sufficiently energetically stabilized to eliminate the activation of the ligation machinery in the absence of the analyte (high background signal), yet allowing the

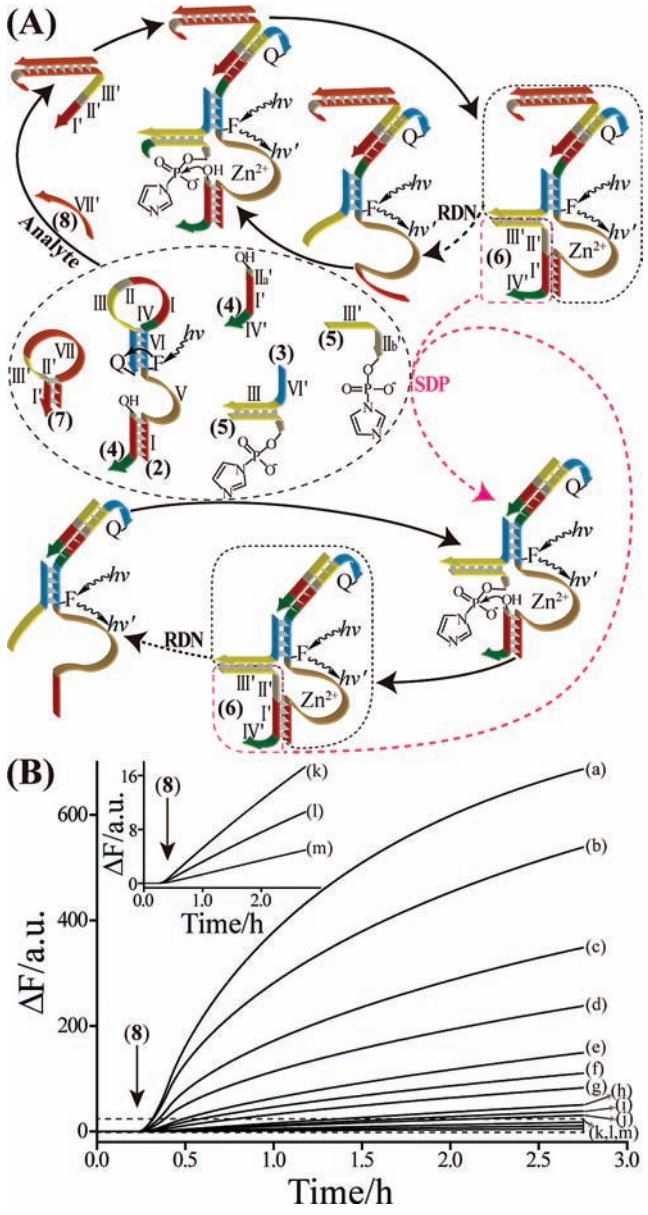

Figure 3. (A) Schematic analysis of a versatile DNA target using the Zn^{2+}-ligation DNAzyme as amplifying module and the hairpin (**7**) as sensor unit. The opening of the hairpin (**7**) by the target (**8**) triggers-on the autonomous replication of the reporter units and the assembly of active DNAzyme units. (B) Time-dependent fluorescence changes upon analyzing different concentrations of the analyte (**8**): (a) 1.0×10^{-6} M, (b) 4.0×10^{-7} M, (c) 2.0×10^{-7} M, (d) 1.0×10^{-7} M, (e) 4.0×10^{-8} M, (f) 2.0×10^{-8} M, (g) 1.0×10^{-8} M, (h) 4.0×10^{-9} M, (i) 2.0×10^{-9} M, (j) 1.0×10^{-9} M, (k) 1.0×10^{-10} M, (l) 1.0×10^{-11} M, (m) 0 M. Arrow indicates the time of addition of the analyte. Inset: Enlargement of curves k–m. All experiments were performed in 10 mM HEPES buffer, pH = 7.0 that contained 300 mM NaCl and Zn^{2+}, 1 mM, (**2**), (**3**), and (**7**), 0.5 μM each, and (**4**) and (**5**), 0.8 μM each.

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opening of the hairpin structure by the analyte, (**1**). Similarly, the protruding nucleic acids associated with (**4**) and (**5**) should be sufficiently long to favor the strand displacement of the ligated product (**6**), but they should eliminate cross-hybridization with other nucleic acids in the system, which may perturb the ligation machinery. These limitations seem as a serious disadvantage of the system as a versatile bioanalytical platform for sensing DNA, as it might imply that optimization of the system is required for any target. This apparent difficulty was resolved, however, by implementing the present system shown in Figure 1B as a common amplification module and conjugating an additional hairpin structure that senses the target DNA and activates the amplifying DNA ligation machinery (Figure 3A). The probe hairpin, (**7**), is designed to recognize the respective target, (**8**). The hairpin (**7**) includes in its stem region, in a caged configuration, the sequence comprising the domains I′, II′, and III′ that cannot activate the ligation machinery. Opening of the hairpin (**7**) by the target (**8**) results in the hybrid (**7**)/(**8**) that includes a duplex domain and the single-strand tethered sequences composed of I′, II′, and III′ that activate the autonomous DNA ligation machinery through two similar reaction processes (SDP and RDN). In the present study, we implemented the analytical platform consisting of the hairpin sensing module and the ligation amplification module (Figure 3A) to analyze one of the DNA mutants associated with the Tay-Sachs (TS) genetic disorder[15] (for the respective sequences, see Table S1 (SI)). The disorder leading to Tay-Sachs disease is caused by a deficiency of the enzyme hexosaminidase, which degrades GM2 ganglioside to GM3.[16] Figure 3B depicts the time-dependent fluorescence changes upon analyzing different concentrations of the TS mutant by the system outlined in Figure 3A. As the concentration of the TS mutant increases, the fluorescence changes are intensified. The method enabled the detection of the mutant with a detection limit that corresponded to 10 pM. For comparison, the nonamplified opening of hairpin (**7**) enabled the detection of the mutant with a detection limit corresponding to 1 nM (see Figure S6 (SI)).

In conclusion, the present study has introduced an enzyme-free, isothermal, autocatalytic amplified detection of DNA using the Zn^{2+}-ligation DNAzyme as a versatile amplifying unit. The ligated product acted as a reporter for the analyte, and the reporter-induced DNAzyme ligation process represents an autocatalytic replication mechanism. This concomitant autonomous ligation process leads to the regeneration of the DNAzyme, the enhanced formation of new active DNAzyme structures and the increased ligation of substrate subunits. Besides the analytical implications of the system, we demonstrate an enzyme-free reaction that provides a simple model for an evolutionary process, where a set of oligonucleotides can be selected and amplified to a target product from a mixture of nucleic acids through the independent ligation process.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

DNA sequences, experiment procedures and methods, the detailed construction of the Zn^{2+}-ligation DNAzyme subunits, gel electrophoresis results for Figure 2A, and the nonamplified detection results of Figures 1B and 3A, the selectivity for analyte (**1**) are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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