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Rolling Circle Amplification: Applications in Nanotechnology and Biodetection with Functional Nucleic Acids

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***R**olling circle amplification (RCA) is an isothermal, enzymatic process mediated by certain DNA polymerases in which long single-stranded (ss) DNA molecules are synthesized on a short circular ssDNA template by using a single DNA primer. A method traditionally used for ultrasensitive DNA detection in areas of genomics and diagnostics, RCA has been used more recently to generate large-scale DNA templates for the creation of periodic nanoassemblies. Various RCA strategies have also been developed for the production of repetitive sequences of DNA aptamers and DNAzymes as detection platforms for small molecules and proteins. In this way, RCA is rapidly becoming a highly versatile DNA amplification tool with wide-ranging applications in genomics, proteomics, diagnosis, biosensing, drug discovery, and nanotechnology.*

1. Introduction

In the mid-1990s, it was discovered that some special DNA polymerases have the ability to continuously lengthen a short DNA strand (primer) annealed to a small circular ssDNA template[1a–g] (typically containing 13–240 nucleotides (nt)) in a process now commonly known as rolling circle amplification (RCA; Figure 1 A).[1h,i] In a typical RCA process, DNA polymerase replicates the circular template hundreds to thousands of times. Therefore, the end products of an RCA reaction are extremely long ssDNA molecules (usually hundreds of thousands of nucleotides and between hundreds of nanometers and microns in length; Figure 1 B) with repetitive sequence units that are complementary to the circular DNA template.

Shortly after this discovery, RCA gained considerable attention as a novel DNA amplification tool and was explored as an important technique for ultrasensitive DNA, RNA, and protein detection in diagnostic genomics and proteomics.[2] In particular, RCA has been widely applied in DNA analysis with so-called padlock probes.[2a–c,3] The method uses a linear DNA probe in which both ends are juxtaposed by the specific hybridization to a target DNA (or RNA) sequence. The two ends of the DNA probe are joined by a DNA ligase, and the resulting DNA ring serves as the template for an RCA reaction with a suitable DNA polymerase (e.g., Phi29 DNA polymerase[4]) and dNTPs (dATP, dCTP, dTTP, and dGTP; dNTP = deoxynucleotide triphosphate). The RCA product can be visualized by confocal microscopy after hybridization with complementary DNA oligonucleotides that are labeled with a fluorophore.[5] Alternatively, the RCA process can be monitored in a real-time fashion by using molecular beacons,[6] molecular zippers,[7] or fluorogenic dyes.[8] RCA has also been used as a signal-amplification tool in microarray-based sandwich immunoassays for proteomic applications.[2e,9] With the coupling of the RCA primer to the detection antibody, antigen–antibody binding results in the immobilization of the primer on the microarray surface. The primer is then used to initiate an RCA reaction, which results in a long ssDNA molecule that can be visualized directly by hybridization with a fluorescent DNA probe.

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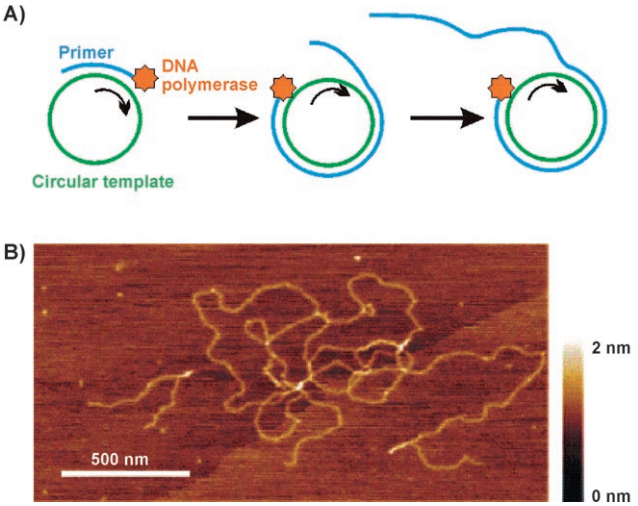
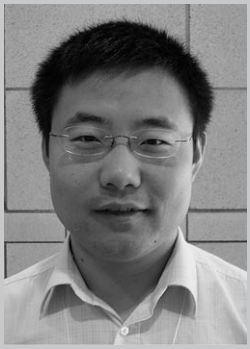


Figure 1. A) Schematic illustration of the RCA process. A short DNA primer (blue curve) first hybridizes with a circular ssDNA template (green circle). A DNA polymerase (orange octagon) then adds nucleotides continuously to the primer by copying the circular template. It is assumed that the ssDNA unwinds itself spontaneously from the circular DNA template during polymerase replication (instead of being displaced by the polymerase) as a result of the geometric constraints associated with the formation of a small circular double-stranded DNA duplex.[1i,g,2b,f] The length of newly synthesized chains is mainly limited by the finite processivity of the polymerase: Highly processive DNA polymerases (e.g., Phi29 DNA polymerase) remain bound to the circular DNA template longer (and therefore produce longer RCA products) than less processive polymerases.[2f] B) A typical atomic force microscope (AFM) image shows that the lengths of ssDNA molecules produced by RCA can range from hundreds of nanometers to microns. The image was reproduced from Ref. [10] with permission.

As a method for molecular amplification, RCA has several attractive features: 1) One advantage over the polymerase chain reaction (PCR)—the most commonly used DNA-amplification method—is the isothermal nature of Phi29 DNA polymerase, which catalyzes DNA polymerization at a constant temperature (at 30 °C or even room temperature). Therefore, neither a thermally stable DNA polymerase nor sophisticated instrumentation is required. 2) RCA-based assays offer both high sensitivity and high specificity. As a method for signal amplification (through either linear amplification or exponential growth;[3b,11] for an explanation of exponential growth, see the Supporting Information), RCA typically provides an approximately 1000-fold (for linear amplification) to 10000-fold increase in the intensity of the signal. Furthermore, single-molecule counting is possible, as the signal (thousands of fluorophores) is localized on a single molecule (or as a single spot on the solid substrate).[3b,12] An RCA-based assay is highly specific, because an RCA reaction can only be initiated after specific hybridization between a primer and the matching circular DNA template. Moreover, in the case of padlock-probe assays,[3] a specific ligase-mediated reaction is required to generate the circular template. The high specificity of the RCA-based assay makes the method particularly useful for the analysis of single-nucleotide polymorphisms (SNPs).[2a–c,3,13] 3) RCA can be adapted readily to numerous detection platforms (such as microarrays) and is suitable for parallel or high-throughput analysis.[2e,3b,9a–d] Indeed, extensive studies on the use of RCA as an amplification tool, particularly for nucleic acid diagnostics, have been conducted in the last decade, with great success. Several excellent



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reviews provide a general overview of fundamental aspects of RCA, padlock probes, and RCA-based protein microarrays.[2]

Some exciting new developments in the use of RCA have appeared during the past several years in the areas of nanotechnology and bioassays with functional nucleic acids. From the standpoint of materials science, the repetitive DNA sequence units within RCA products make these special DNA molecules coveted templates upon which periodic nanoassemblies can be produced.[10,14,15] Even more attractive is the fact that one can readily "tune" the properties of the circular template simply by manipulating its length and sequence information for a given RCA reaction. The large size of RCA products is advantageous for bridging nanoassemblies and for large-scale (micro- or macroscale) material fabrication.[16] Furthermore, RCA is capable of amplifying structural DNA motifs, such as nanojunctions[17] and DNAzymes.[18,19] When functional nucleic acids, such as DNA aptamers and DNAzymes,[20] are incorporated, the application of RCA-based assays can be extended from the detection of nucleic acids to the sensing of diverse target analytes, such as proteins[8,21] and small metabolites.[22] Finally, the use of RCA to produce enzymatic DNA products can result in further improvement of the detection sensitivity.[18,19] Herein, we focus on our discussion on the use of RCA for the construction of nanoassemblies, the synthesis of well-defined nanostructures, and biosensing with functional nucleic acids.

2. RCA in Nanotechnology

2.1. Templates for Periodic Assembly

In an RCA reaction, DNA polymerase continuously adds nucleotides to the primer to make a new DNA chain by copying the same circular DNA template round after round. This process leads to the formation of long ssDNA products containing tandemly repeated DNA-sequence units. Their repetitive structure makes RCA products excellent templates for one-dimensional periodic nanoassemblies (Figure 2A).

Research groups led by Mao and Simmel demonstrated the use of RCA products as templates for the assembly of gold nanoparticles (AuNPs) modified with complementary DNA oligonucleotides (Figure 2B).[10,14] We recently tested the feasibility of performing an RCA reaction directly on a nanoparticle surface and found that such a reaction can produce a three-dimensional scaffold onto which nanospecies tethered to a complementary oligonucleotide could be assembled periodically (Figure 2C,D).[15] Willner and coworkers demonstrated the use of RCA products to template the formation of complex protein nanostructures. The circular DNA template used in the RCA reaction in their study contained the antisense sequence for two different aptamers (for the recognition of thrombin and lysozyme, respectively; Figure 3).[23] They showed that the long RCA products can be used to assemble thrombin and/or lysozyme through aptamer–protein binding. The complexity of the assembly can be increased further by introducing AuNPs by chemical conjugation.[23]

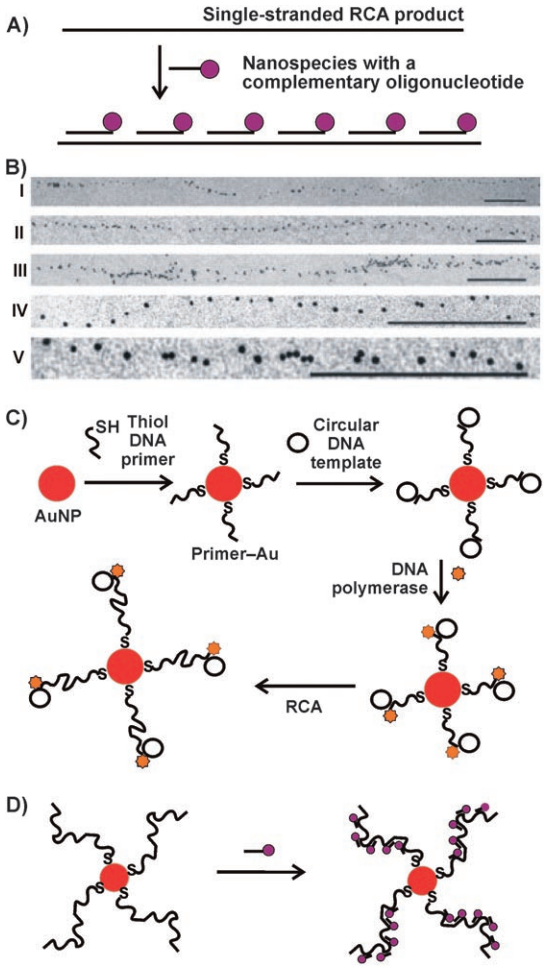

Figure 2. A) Schematic illustration of 1D nanoassembly on ssDNA templates prepared by an RCA reaction. B) Representative transmission electron microscopy (TEM) images of 1D AuNP arrays formed on an RCA product. IV and V are magnified views of parts of the arrays in I and II. Scale bars: 200 nm. The images were reproduced from Ref. [14] with permission. C) Preparation of a DNA–AuNP scaffold through an RCA reaction at an AuNP core.[15] Thiol-modified DNA primers attached to AuNPs are extended by DNA polymerase by using a circular DNA template. D) A structure composed of multiple RCA products on the same AuNP can act as a unique scaffold for larger periodic nanoassemblies. Parts (C, D) were adapted from Ref. [15].

There are several attractive features associated with the use of RCA products as templates for nanoassemblies: 1) As the length of the repeating units in an RCA product is identical to that of the matching circular template, one can readily tune the distance between two assembled nanospecies simply by adjusting the length of the circular template (although there are some practical limitations to the sizes available; see Ref. [1a]). 2) The sequence of the repeating units in an RCA product is encoded in (or complementary to) the circular template. Therefore, this strategy provides great versatility in the design of nanoconstructs. 3) Owing to the large size of RCA products, the formation of nanoassemblies can occur over larger scales (micro- or macroscale). This feature may prove particularly useful for the construction of future microscopic (or macroscopic) devices.[16] However, a

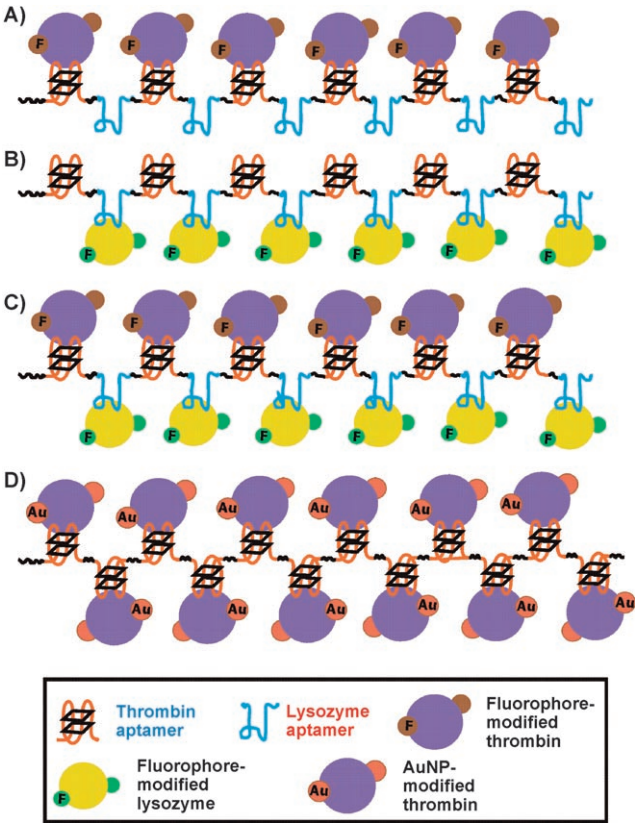

Figure 3. Schematic representation of protein and AuNP nanostructures produced by using RCA products as templates (adapted from Ref. [23]). The RCA products in (A–C) are ssDNA molecules containing repeating aptamer sequences for thrombin and lysozyme recognition. The RCA product in (D) contains only thrombin-binding aptamer sequences. The structures in (A–C) are RCA-product-templated assemblies of fluorophore-modified thrombin, fluorophore-modified lysozyme, and both, respectively. The structure in (D) is a RCA-product-templated AuNP-modified thrombin superstructure.

major problem is the nonspecific cross-linking that can occur between different RCA product molecules when the assembled nanospecies contain multiple oligonucleotides.[10,14] A solution to this problem is the use of monofunctionalized DNA nanospecies.[24] Another conceivable disadvantage of using RCA to generate nanoassembly templates is that the variability of binding sites for nanospecies immobilization is rather limited, given the fact that RCA only produces concatemers of identical sequence units (although Willner and co-workers demonstrated the simultaneous assembly of two kinds of nanospecies).[23] This issue might be addressed by constructing a scaffold that comprises a number of RCA products with different sequences.

2.2. Nanostructure Synthesis

The RCA process can also be used to produce interesting DNA nanostructures. Importantly, one can precisely define the sequence of the product by encoding the complementary sequence in the circular template. In early examples, Kool and co-workers demonstrated the use of RCA for the preparation of artificial human telomeres by using a circular template with a complementary hexamer sequence.[25] These synthetic telomeres are of great importance for the study of in vivo systems.[25]

Because of the high processivity of Phi29 DNA polymerase and its ability to displace double-stranded (ds) DNA,[4] RCA is effective even when the circular template presents challenging topological constraints in the form of secondary structures.[26] Thus, one can generate DNA products with various secondary structures (such as duplexes, hairpins, and junctions) that are important ingredients in functional nucleic acids and DNA nanotechnology.[27] Yan and co-workers recently demonstrated the successful amplification of a DNA four-way junction by using RCA and thus introduced a novel strategy for the preparation of branched DNA junctions on a large scale (Figure 4).[17a] An important method used in this study was the enzymatic digestion of RCA products by restriction enzymes (e.g., PstI) to yield monomeric DNA nanojunctions.[17a] We also found that by carefully tuning the reaction conditions (such as the reaction time) for the enzymatic digestion, one is able to define the length of the products of enzyme cleavage (that is, as monomers, dimers, trimers, etc.).[12a,15] These products can be separated and isolated by standard gel electrophoresis. Yan and co-workers showed that more complex, multi-crossover DNA nanostructures can also be amplified by

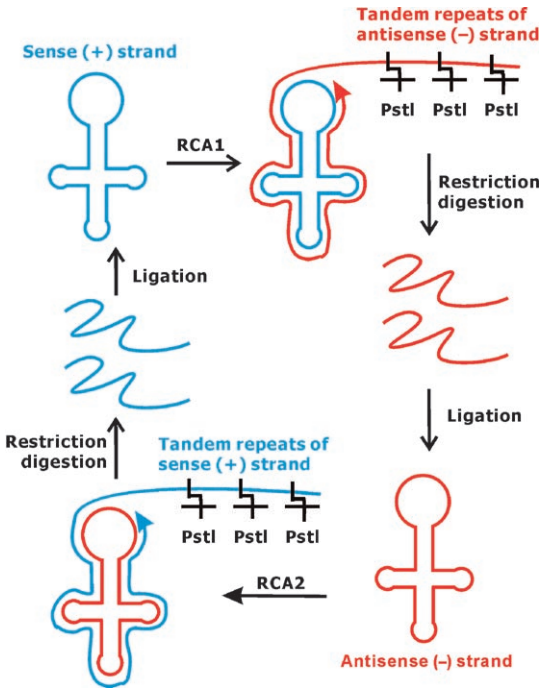

Figure 4. Amplification of a DNA four-way nanojunction by RCA (adapted from Ref. [17a]).[17] A ligated DNA four-way junction (blue; referred to as the sense (+) strand) serves as a template for an RCA reaction. The RCA product with tandem repeats that is complementary to the circular template (referred to as the antisense (–) strand) is cleaved by a restriction enzyme (PstI) into monomeric units (red). A subsequent RCA reaction on the ligated antisense (–) circular template, followed by digestion with the same restriction enzyme, produces a large number of sense strands.

RCA. Their study demonstrated a possible way to clone viral DNA in cells.[17b] The synthesis of DNAzymes with highly conserved secondary structures has also been demonstrated (see Section 3).[18,19]

These initial successes indicate clearly that RCA is emerging as a powerful method for the production of DNA strands with a desired length, sequence, and structure. These DNA molecules, which are not readily accessible by other methods, can be manipulated further by commonly used, powerful enzymatic reactions, such as digestion by restriction enzymes and ligation, depending on the specific application in nano- or biotechnology.[27]

3. RCA in Bioassays with Functional Nucleic Acids

The utility of RCA as a method for signal amplification was initially demonstrated with great success in nucleic-acid diagnostics.[3] In a representative example, circular DNA templates, termed "padlock probes", targeted the nucleic-acid sequence (often called the primer) of interest through hybridization, and this single binding event was then amplified several thousand times by the RCA process.[3] This type of assay has been applied successfully to the in situ genotyping of DNA sequences and SNP analysis. Moreover, sandwich immunoassays for the detection of proteins and other antigens on the basis of antibody–antigen interactions have since been developed.[2e,9] In a typical chip-based sandwich immunoassay, a target analyte is captured by a specific antibody that is tethered to a chip. A second antibody then binds to the captured analyte. A third, universal antibody to which the RCA primer is attached is conjugated subsequently to the second antibody, which is then subjected to RCA amplification.[2e] Since the first demonstration of this process in 2000,[9c] antibody chips amplified by RCA have been applied increasingly to multiplexed protein profiling in disease diagnostics and drug screening. However, the use of these assays might be limited by the inherent drawbacks of antibodies, including challenges in their production and their relatively poor stability.[2e,9] Furthermore, extra chemical conjugation steps are required to localize the RCA primer on the antibody.[9]

When RCA is combined with the use of functional nucleic acids (aptamers and aptazymes),[20] a significantly broader range of target structures can be detected than in standard RCA-based assays. DNA (or RNA) aptamers,[28] the nucleic-acid equivalents of antibodies, play important roles in biodetection, drug discovery, and nanotechnology.[20,27d] Aptamers, which can be obtained by simple yet powerful in vitro selection, are nucleic-acid sequences that bind specifically to their targets.[28] Since the development of in vitro selection strategies, aptamers have quickly become recognized as a promising class of molecules whose utility rivals that of antibodies in diagnostics.[20] Indeed, aptamers have certain advantages over antibodies:[20c] 1) Aptamers are created by in vitro selection, whereas animal cells are required for the production of antibodies. Furthermore, the conditions for in vitro selection can be manipulated readily to enable the production of aptamers with desirable properties. 2) Once identified, aptamers can be synthesized readily by chemical methods with excellent accuracy and reproducibility. Moreover, modifications (for example, in the form of fluorophores) can be introduced readily into the aptamer strands during chemical synthesis. 3) Aptamers are more stable towards denaturation (induced by temperature, for example). These attractive features make aptamers important alternatives in diagnostic assays dominated previously by antibodies.

The combination of aptamer binding and DNA amplification was first demonstrated in proximity-dependent assays for protein detection. Landegren and co-workers developed PDGF-sensing assays (PDGF = platelet-derived growth factor) in which the simultaneous binding of two aptamers promoted the ligation of these two aptamers, and the ligated DNA molecule was then amplified by PCR.[29] Later on, two research groups used RCA to replace PCR in such a proximity ligation system.[30]

On the basis of the isolation of two thrombin-binding DNA aptamers, each of which recognizes thrombin at a different location, King and co-workers came up with the following strategy for thrombin detection: They first converted one of the aptamers into the circular form, but left the other aptamer in the linear form. They also modified the sequences of both aptamers in such a way that the last six nucleotides at the end of the linear aptamer can form a short (thus extremely weak) duplex with the circular aptamer. In the absence of thrombin, the duplex was too weak to form; however, when thrombin was present, both aptamers engaged the same thrombin molecule for binding, which led to the formation of the DNA duplex and triggered the RCA process (the linear aptamer acted as the primer, and the circular aptamer as the template; see Figure 5A).[8] The RCA process was monitored in a real-time fashion by using fluorescent dyes. More recently, Ellington and co-workers developed an RCA-based sensing system for PDGF detection through the design of a DNA aptamer that can undergo a target-induced conformational change[31] to position the two ends of the DNA aptamer next to each other for a ligation reaction (Figure 5B).[21] The ligated DNA ring can then serve as the template for the subsequent RCA reaction. The same research group also demonstrated the use of RCA for the detection of a small molecule, ATP.[22] The functional nucleic acid used in this study was an aptazyme whose catalytic activity is modulated by ATP as an effector molecule. In the presence of ATP, this aptazyme functions like a DNA ligase and creates the circular template for the subsequent RCA reaction (Figure 6).

These assays can be performed homogeneously in solution or heterogeneously on microarray-based platforms.[22] The RCA process can be monitored in real time by molecular beacons or fluorescent probes.[8,21,22] The marriage of functional nucleic acids with RCA offers several advantages: 1) Whereas traditional RCA-based assays are mainly used for the detection of nucleic acids, the incorporation of aptamers enables the design of RCA-based assays for a much broader range of molecular targets (proteins, metabolites, small molecules, metal ions, and even live cells). In principle, it is possible to create an aptamer for any specific target by in vitro selection.[28] 2) The use of an aptamer avoids some steps

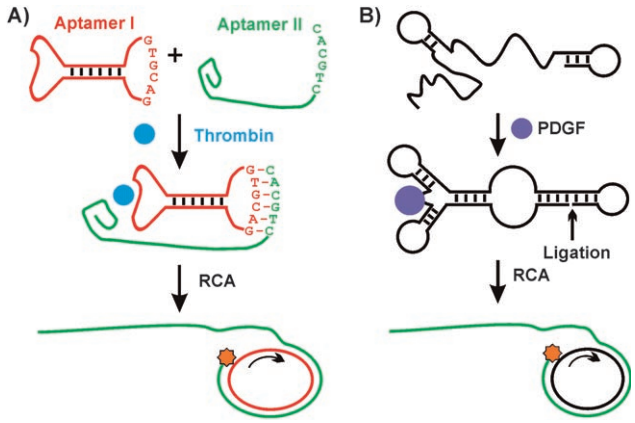

Figure 5. A) Proximity-dependent thrombin-detection assay with an aptamer as the recognition element and RCA as an amplification method (adapted from Ref. [8]). The simultaneous binding of a thrombin molecule by a linear aptamer (which also serves as the RCA primer) and a circular aptamer (which serves as the RCA template) facilitates an RCA reaction. B) Design of an assay for PDGF detection by using a structure-switching aptamer and RCA (adapted from Ref. [21a]). The binding of PDGF to the aptamer causes a conformational change in the aptamer and thus promotes the formation of a ligation junction. Following ligation by a DNA ligase, the circularized aptamer serves as the circular template for RCA. In both assays, a single protein–aptamer binding event initiates the RCA reaction, which amplifies the signal by a factor of several thousand.

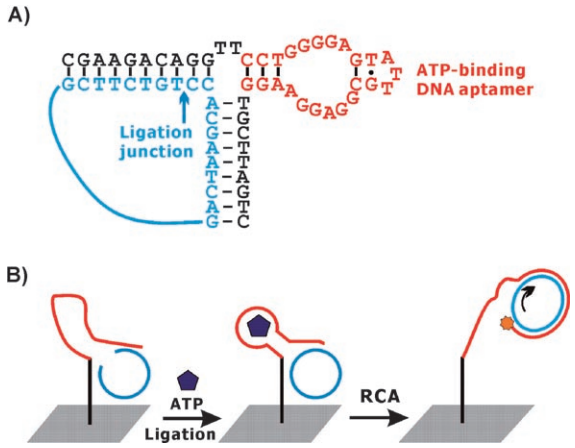

Figure 6. A) Sequence design of an aptazyme that catalyzes a DNA ligation reaction upon ATP binding (adapted from Ref. [22]). Black: DNA ligase, red: ATP-binding aptamer domain, blue: RCA padlock probe. B) Schematic representation of the aptazyme-mediated RCA reaction in a microarray format. First, the aptazyme is attached to a glass slide through a streptavidin–biotin interaction. The aptazyme is activated by ATP and ligates the padlock probe for the subsequent RCA reaction. The RCA product can be visualized by using fluorescent probes.

required for antibody-based RCA immunoassays, such as the chemical conjugation of the RCA primer with the antibody. 3) As mentioned previously, DNA aptamers are more stable and cheaper to synthesize than antibodies.[20c,32] Moreover, chemical functionalities, such as fluorophores, biotin, or thiols, can be introduced readily into aptamer sequences at precise locations, so that RCA assays based on functional nucleic acids can be adapted in a straightforward manner for a large variety of detection platforms.[20c,32]

Perhaps even more importantly, high sensitivities are observed in these assays, as a single biomolecular recognition event is amplified thousands of times by the RCA process. Recently, the research groups of Willner and Mao showed that the sensitivity can be improved further if DNAzymes (DNA sequences that are capable of catalyzing chemical reactions[33]) are produced as RCA products (Figure 7).[18,19]

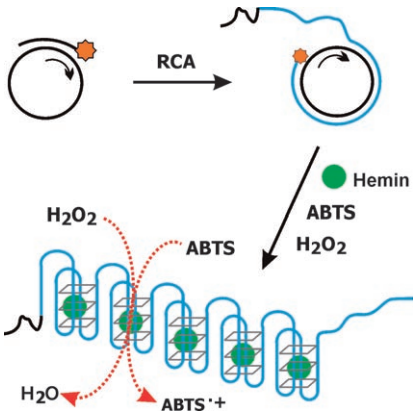

Figure 7. RCA process for the production of multiple copies of DNAzymes that mimic the functions of peroxidase and catalyze the oxidization of ABTS (adapted from Ref. [18]). The signal is amplified twice, first by RCA and then by the DNAzyme; thus, ultrasensitive detection results.

In their studies, RCA produced thousands of DNAzymes, which, upon the binding of hemin, mimic the functions of peroxidase. The DNAzyme catalyzes the oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to $ABTS^{\bullet+}$ (or $ABTS^{2-}$ to ABTS) by H_2O_2; the reaction can be monitored by measuring optical absorption.[34] As this enzymatic reaction has multiple turnovers (each DNA enzyme can generate several products), the signal is amplified at two different levels, which results in a much higher sensitivity. This assay was applied successfully for DNA detection.[18,19]

4. Summary and Outlook

Recent advances in RCA have revealed that this method for the production of functional nanostructures and templates for nanoassembly has great potential for the construction of nanodevices in the future. The assembly takes advantage of specific DNA hybridization (or aptamer–target recognition[35]), which ensures the precise localization of assembled nanospecies. The repetitive structure of RCA products makes them perfect templates for periodic nanoassembly. Significantly, RCA products can be produced with a desired sequence by encoding the complementary sequence in the circular template. The distance between assembled nanospecies can also be controlled readily by adjusting the length of the circular template. The large size (or high molecular

weight) of RCA products offers another attractive feature: They may serve as bridges to connect the nanoassembly to larger, independently prepared structures, or as a component of hierarchical assemblies at the micro- or macroscopic scale. This characteristic is particularly useful for the construction of practical micro- or macrodevices.

The RCA product, with a suitably designed sequence, may itself fold into well-defined nanostructures. By using over 200 short oligonucleotide "staple strands", Rothemund was able to fold long ssDNA molecules (7 kb) into various nanostructures with precisely defined shapes and patterns.[36] It is conceivable that long RCA products with carefully designed sequences could fold in a similar manner, even without the help of short staple strands. This hypothesis is based on the fact that these DNA molecules contain tandemly repeated units, and that the sequences required for the formation of well-defined shapes and patterns through specific intra- or intermolecular DNA hybridization can be produced by encoding the sequence information into the circular templates. Thus, RCA may find more applications in the field of DNA nanotechnology.[27b–e]

The RCA process can also produce a variety of functional nanostructures, such as nanojunctions, aptamers, and DNAzymes, which can be used for nanotechnology and biosensing. The use of functional nucleic acids, such as aptamers and aptazymes, in RCA-based assays can greatly extend the scope of RCA in terms of detectable analytes and the production of DNAzymes. Such processes are also considerably more sensitive than traditional RCA-based assays. As, in principle, any desired aptamer can be obtained by in vitro selection, RCA-based assays with aptamers as recognition elements could be used for the detection of almost any conceivable molecular target (nucleic acids, proteins, metabolites, metal ions, and even live cells). Moreover, as some of the drawbacks of antibodies can be overcome with aptamers, aptamer-based sandwich assays with RCA as an amplification tool show great potential for future immunoassays.

Although great progress has been made in this area, the study and application of RCA in nanotechnology and biodetection, particularly in relation to functional nucleic acids, are still in their infancy. For example, there are many natural or synthesized DNA (and RNA) aptamers and enzymes available for a large variety of applications,[20,27d,37] but only a few DNA aptamers and DNA enzymes have been used in RCA-based assays. Moreover, although the concept of using RCA assays based on functional nucleic acids for diagnostics has been demonstrated, their widespread use, particularly in a commercialized format, has not been fully realized. Thus, great opportunities exist for the exploration of functional nucleic acids in the development of future RCA-based diagnostic platforms, particularly in areas in which aptamers have advantages over antibodies. We believe that the incorporation of these functional nucleic-acid elements will popularize RCA as a powerful tool for diverse applications, including biosensing, diagnostics, drug discovery, and nanotechnology.

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[1] a) The circular DNA template used in the RCA process can be synthesized enzymatically or chemically through the intramolecular ligation of phosphate and hydroxy end groups. In the enzymatic synthesis (see Ref. [15] for a typical protocol), the two reactive ends of a linear DNA precursor are joined by an enzyme (e.g., T4 DNA ligase). The enzymatic synthesis proceeds efficiently (>90% yield) for relatively large DNA substrates, but may not be suitable for making small circular DNA (<30 nucleotides (nt), and particularly those <10 nt), presumably as a result of inadequate saturation of the enzyme binding sites and/or the strain induced upon the ring closure of short oligonucleotides (see Refs. [1b–d]). This shortcoming might be an obstacle to the use of the RCA method in some cases, as enzymatic synthesis is the most commonly used strategy for the creation of the circular DNA template in RCA-based diagnostic assays. However, the chemical (or nonenzymatic) cyclization of DNA oligonucleotides can also be used to produce circular DNA molecules in reasonably good yield (up to 85%) for both small (<14 nt; see Refs. [1e,f]) and large circular templates (>15 nt; see Ref. [1b]). For example, chemical ligation was used to produce a circular DNA molecule with 13 nt: the smallest circular DNA molecule studied to date that can be used effectively in an RCA process (see Ref. [1g]). b) E. Rubin, S. Rumney IV, S. Wang, E. T. Kool, *Nucleic Acids Res.* **1995**, *23*, 3547–3553; c) G. Kaufmann, T. Klein, U. Z. Littauer, *FEBS Lett.* **1974**, *46*, 271–275; d) T. J. Snopek, A. Sugino, K. L. Agarwal, N. R. Cozzarelli, *Biochem. Biophys. Res. Commun.* **1976**, *68*, 417–424; e) E. de Vroom, H. J. G. Broxterman, L. A. J. M. Sliedregt, G. A. van der Marel, J. H. van Boom, *Nucleic Acids Res.* **1988**, *16*, 4607–4620; f) M. L. Capobianco, A. Carcuro, L. Tondelli, A. Garbesi, G. M. Bonora, *Nucleic Acids Res.* **1990**, *18*, 2661–2669; g) M. Frieden, E. Pedroso, E. T. Kool, *Angew. Chem.* **1999**, *111*, 3870–3874; *Angew. Chem. Int. Ed.* **1999**, *38*, 3654–3657; h) A. Fire, S. Xu, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 4641–4645; i) D. Liu, S. L. Daubendiek, M. A. Zillman, K. Ryan, E. T. Kool, *J. Am. Chem. Soc.* **1996**, *118*, 1587–1594.

[2] For reviews, see: a) M. Nilsson, F. Dahl, C. Larsson, M. Gullberg, J. Stenberg, *Trends Biotechnol.* **2006**, *24*, 83–88; b) M. Nilsson, *Histochem. Cell Biol.* **2006**, *126*, 159–164; c) D. Zhang, J. Wu, F. Ye, T. Feng, I. Lee, B. Yin, *Clin. Chim. Acta* **2006**, *363*, 61–70; d) X. Qian, R. V. Lloyd, *Diagn. Mol. Pathol.* **2003**, *12*, 1–13; e) S. F. Kingsmore, D. D. Patel, *Curr. Opin. Biotechnol.* **2003**, *14*, 74–81; f) V. V. Demidov, *Expert Rev. Mol. Diagn.* **2002**, *2*, 89–95; g) S. C. Andras, J. B. Power, E. C. Cocking, M. R. Davey, *Mol. Biotechnol.* **2001**, *19*, 29–44; h) B. Schweitzer, S. Kingsmore, *Curr. Opin. Biotechnol.* **2001**, *12*, 21–27; i) M. M. Shi, *Clin. Chem.* **2001**, *47*, 164–172; j) N. O. Fischer, T. M. Tarasow, J. B.-H. Tok, *Anal. Biochem.* **2008**, *373*, 121–128.

[3] a) M. Nilsson, J. Banér, M. Mendel-Hartvig, F. Dahl, D. O. Antson, M. Gullberg, U. Landegren, *Hum. Mutat.* **2002**, *19*, 410–415; b) P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas, D. C. Ward, *Nat. Genet.* **1998**, *19*, 225–232; c) D. Y. Zhang, M. Brandwein, T. C. Hsuih, H. Li, *Gene* **1998**, *211*, 277–285; d) X. Qi, S. Bakht, K. M. Devos, M. D. Gale, A. Osbourn, *Nucleic Acids Res.* **2001**, *29*, 116e; e) A. T. Christian, M. S. Pattee, C. M. Attix, B. E. Reed, K. J. Sorensen, J. D. Tucker, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 14238–14243; f) C. Larsson, J. Koch, A. Nygren, G. Janssen, A. K. Raap, U. Landegren, M. Nilsson, *Nat. Methods* **2004**, *1*, 227–232; g) J. Banér, M. Nilsson,

M. Mendel-Hartvig, U. Landegren, *Nucleic Acids Res.* **1998**, *26*, 5073 – 5078; h) J. Pickering, A. Bamford, V. Godbole, J. Briggs, G. Scozzafava, P. Roe, C. Wheeler, F. Ghouze, S. Cuss, *Nucleic Acids Res.* **2002**, *30*, 60e; i) I. Smolina, C. Lee, M. Frank-Kamenetskii, *Appl. Environ. Microbiol.* **2007**, *73*, 2324 – 2328; j) I. V. Smolina, H. Kuhn, C. Lee, M. D. Frank-Kamenetskii, *Bioorg. Med. Chem.* **2008**, *16*, 84 – 93; k) J. S. Lohmann, M. Stougaard, J. Koch, *BMC Mol. Biol.* **2007**, *8*, 103 – 110.

[4] a) L. Blanco, A. Bernad, J. M. Lázaro, G. Martín, C. Garmendia, M. Salas, *J. Biol. Chem.* **1989**, *264*, 8935 – 8940; b) J. A. Esteban, M. Salas, L. Blanco, *J. Biol. Chem.* **1993**, *268*, 2719 – 2726.

[5] G. A. Blab, T. Schmidt, M. Nilsson, *Anal. Chem.* **2004**, *76*, 495 – 498.

[6] a) M. Nilsson, M. Gullberg, F. Dahl, K. Szuhai, A. K. Raap, *Nucleic Acids Res.* **2002**, *30*, 66e; b) I. V. Smolina, V. V. Demidov, C. R. Cantor, N. E. Broude, *Anal. Biochem.* **2004**, *335*, 326 – 329.

[7] J. Yi, W. Zhang, D. Y. Zhang, *Nucleic Acids Res.* **2006**, *34*, e81.

[8] D. A. Di Giusto, W. A. Wlassoff, J. J. Gooding, B. A. Messerle, G. C. King, *Nucleic Acids Res.* **2005**, *33*, e64.

[9] a) B. Schweitzer, S. Roberts, B. Grimwade, W. Shao, M. Wang, Q. Fu, Q. Shu, I. Laroche, Z. Zhou, V. T. Tchernev, J. Christiansen, M. Velleca, S. F. Kingsmore, *Nat. Biotechnol.* **2002**, *20*, 359 – 365; b) W. Shao, Z. Zhou, I. Laroche, H. Lu, Q. Zong, D. D. Patel, S. Kingsmore, S. P. Piccoli, *J. Biomed. Biotechnol.* **2003**, *5*, 299 – 307; c) B. Schweitzer, S. Wiltshire, J. Lambert, S. O'Malley, K. Kukanskis, Z. Zhu, S. F. Kingsmore, P. M. Lizardi, D. C. Ward, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 10113 – 10119; d) H. Zhou, K. Bouwman, M. Schotanus, C. Verweij, J. A. Marrero, D. Dillon, J. Costa, P. Lizardi, B. B. Haab, *Genome Biol.* **2004**, *5*, R28; e) Y. Y. Huang, H. Y. Hsu, C. J. Huang, *Biosens. Bioelectron.* **2007**, *22*, 980 – 985.

[10] S. Beyer, P. Nickels, F. C. Simmel, *Nano Lett.* **2005**, *5*, 719 – 722.

[11] a) F. Dahl, J. Baner, M. Gullberg, M. Mendel-Hartvig, U. Landegren, M. Nilsson, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4548 – 4553; b) D. Y. Zhang, W. Zhang, X. Li, Y. Konomi, *Gene* **2001**, *274*, 209 – 216; c) F. B. Dean, J. R. Nelson, T. L. Giesler, R. S. Lasken, *Genome Res.* **2001**, *11*, 1095 – 1099.

[12] a) M. M. Ali, S. Su, C. D. M. Filipe, R. Pelton, Y. Li, *Chem. Commun.* **2007**, *43*, 4459 – 4461; b) H. Y. Hsu, Y. Y. Huang, *Biosens. Bioelectron.* **2004**, *20*, 123 – 126.

[13] a) X. B. Zhong, P. M. Lizardi, X. H. Huang, P. L. Bray-Ward, D. C. Ward, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3940 – 3945; b) Y. Chen, M. R. Shortreed, M. Olivier, L. M. Smith, *Anal. Chem.* **2005**, *77*, 2400 – 2405; c) B. Nie, M. R. Shortreed, L. M. Smith, *Anal. Chem.* **2005**, *77*, 6594 – 6600; d) Y. Chen, M. R. Shortreed, D. Peelen, M. Lu, L. M. Smith, *J. Am. Chem. Soc.* **2004**, *126*, 3016 – 3017; e) H. Kuhn, Y. Hu, M. D. Frank-Kamenetskii, V. V. Demidov, *Biochemistry* **2003**, *42*, 4985 – 4992; f) J. Li, W. Zhong, *Anal. Chem.* **2007**, *79*, 9030 – 9038.

[14] Z. Deng, Y. Tian, S. H. Lee, A. E. Ribbe, C. Mao, *Angew. Chem.* **2005**, *117*, 3648 – 3651; *Angew. Chem. Int. Ed.* **2005**, *44*, 3582 – 3585.

[15] W. Zhao, Y. Gao, S. A. Kandadai, M. A. Brook, Y. Li, *Angew. Chem.* **2006**, *118*, 2469 – 2473; *Angew. Chem. Int. Ed.* **2006**, *45*, 2409 – 2413.

[16] W. Zhao, Y. Gao, M. A. Brook, Y. Li, *Chem. Commun.* **2006**, 3582 – 3584.

[17] a) C. Lin, M. Xie, J. J. Chen, Y. Liu, H. Yan, *Angew. Chem.* **2006**, *118*, 7699 – 7701; *Angew. Chem. Int. Ed.* **2006**, *45*, 7537 – 7539; b) C. Lin, X. Wang, Y. Liu, N. C. Seeman, H. Yan, *J. Am. Chem. Soc.* **2007**, *129*, 14475 – 14481.

[18] Z. Cheglakov, Y. Weizmann, B. Basnar, I. Willner, *Org. Biomol. Chem.* **2007**, *5*, 223 – 225.

[19] Y. Tian, Y. He, C. Mao, *ChemBioChem* **2006**, *7*, 1862 – 1864.

[20] a) N. K. Navani, Y. Li, *Curr. Opin. Chem. Biol.* **2006**, *10*, 272 – 281; b) J. J. Gooding, G. C. King, *J. Mater. Chem.* **2005**, *15*, 4876 – 4880; c) S. D. Jayasena, *Clin. Chem.* **1999**, *45*, 1628 – 1650.

[21] a) L. Yang, C. W. Fung, E. J. Cho, A. D. Ellington, *Anal. Chem.* **2007**, *79*, 3320 – 3329; b) L. Zhou, L.-J. Ou, X. Chu, G.-L. Shen, R.-Q. Yu, *Anal. Chem.* **2007**, *79*, 7492 – 7500.

[22] E. J. Cho, L. Yang, M. Levy, A. D. Ellington, *J. Am. Chem. Soc.* **2005**, *127*, 2022 – 2023.

[23] Z. Cheglakov, Y. Weizmann, A. B. Braunschweig, O. I. Wilner, I. Willner, *Angew. Chem.* **2008**, *120*, 132 – 136; *Angew. Chem. Int. Ed.* **2008**, *47*, 126 – 130.

[24] a) D. Zanchet, C. M. Micheel, W. J. Parak, D. Gerion, A. P. Alivisatos, *Nano Lett.* **2001**, *1*, 32 – 35; b) X. Xu, N. L. Rosi, Y. Wang, F. Huo, C. A. Mirkin, *J. Am. Chem. Soc.* **2006**, *128*, 9286 – 9287.

[25] a) U. M. Lindstrom, R. A. Chandrasekaran, L. Orbai, S. A. Helquist, G. P. Miller, E. Oroudjev, H. G. Hansma, E. T. Kool, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15953 – 15958; b) J. S. Hartig, S. Fernandez-Lopez, E. T. Kool, *ChemBioChem* **2005**, *6*, 1458 – 1462.

[26] H. Kuhn, V. V. Demidov, M. D. Frank-Kamenetskii, *Nucleic Acids Res.* **2002**, *30*, 574 – 580.

[27] a) G. M. Blackburn, M. J. Gait in *Nucleic Acids in Chemistry and Biology*, Oxford University Press, Oxford, **1990**; b) N. C. Seeman, *Nature* **2003**, *421*, 427 – 431; c) N. C. Seeman, *Chem. Biol.* **2003**, *10*, 1151 – 1159; d) Y. Lu, J. Liu, *Curr. Opin. Biotechnol.* **2006**, *17*, 580 – 588; e) F. C. Simmel, W. U. Dittmer, *Small* **2005**, *1*, 284 – 299; f) F. C. Simmel, *Nanomedicine* **2007**, *2*, 817 – 830.

[28] a) C. Tuerk, L. Gold, *Science* **1990**, *249*, 505 – 510; b) A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818 – 822; c) W. C. Winkler, R. R. Breaker, *Annu. Rev. Microbiol.* **2005**, *59*, 487 – 517.

[29] S. Fredriksson, M. Gullberg, J. Jarvius, C. Olsson, K. Pietras, S. M. Gustafsdottir, A. Ostman, U. Landegren, *Nat. Biotechnol.* **2002**, *20*, 473 – 477.

[30] a) O. Söderberg, M. Gullberg, M. Jarvius, K. Ridderstrale, K. J. Leuchowius, J. Jarvius, K. Wester, P. Hydbring, F. Bahram, L. G. Larsson, U. Landegren, *Nat. Methods* **2006**, *3*, 995 – 1000; b) J. Jarvius, J. Melin, J. Goransson, J. Stenberg, S. Fredriksson, C. Gonzalez-Rey, S. Bertilsson, M. Nilsson, *Nat. Methods* **2006**, *3*, 725 – 727.

[31] a) R. Nutiu, Y. Li, *J. Am. Chem. Soc.* **2003**, *125*, 4771 – 4778; b) X. Fang, A. Sen, M. Vicens, W. Tan, *ChemBioChem* **2003**, *4*, 829 – 834.

[32] S. Klussmann, *The Aptamer Handbook: Functional Oligonucleotides and Their Applications*, Wiley-VCH, Weinheim, **2006**.

[33] a) J. C. Achenbach, W. Chiuman, R. P. Cruz, Y. Li, *Curr. Pharm. Biotechnol.* **2004**, *5*, 321 – 336; b) Y. Li, R. R. Breaker, *Curr. Opin. Struct. Biol.* **1999**, *9*, 315 – 323.

[34] a) Y. Xiao, V. Pavlov, T. Niazov, A. Dishon, M. Kotler, I. Willner, *J. Am. Chem. Soc.* **2004**, *126*, 7430 – 7431; b) T. Niazov, V. Pavlov, Y. Xiao, R. Gill, I. Willner, *Nano Lett.* **2004**, *4*, 1683 – 1687.

[35] a) C. Lin, E. Katilius, Y. Liu, J. Zhang, H. Yan, *Angew. Chem.* **2006**, *118*, 5422 – 5427; *Angew. Chem. Int. Ed.* **2006**, *45*, 5296 – 5301; b) Y. Liu, C. Lin, H. Li, H. Yan, *Angew. Chem.* **2005**, *117*, 4407 – 4412; *Angew. Chem. Int. Ed.* **2005**, *44*, 4333 – 4338.

[36] P. W. Rothemund, *Nature* **2006**, *440*, 297 – 302.

[37] a) E. Katz, I. Willner, *Angew. Chem.* **2004**, *116*, 6166 – 6235; *Angew. Chem. Int. Ed.* **2004**, *43*, 6042 – 6108; b) N. L. Rosi, C. A. Mirkin, *Chem. Rev.* **2005**, *105*, 1547 – 1562.