Biosensing

DOI: 10.1002/anie.200805966

Colorimetric Sensing by Using Allosteric-DNAzyme-Coupled Rolling Circle Amplification and a Peptide Nucleic Acid–Organic Dye Probe**

*M. Monsur Ali and Yingfu Li**

DNA aptamers and DNAzymes have recently received considerable attention in chemical-biology research.[1] These two classes of synthetic DNA molecules, which can be isolated from random-sequence DNA pools by in vitro selection,[2] are regarded as attractive alternatives to antibodies and enzymes. Particular advantages are the greater chemical stability of DNA and its straightforward preparation by automated synthesis. A large number of DNA aptamers have been produced for the recognition of targets ranging from small molecules (such as adenosine triphosphate (ATP)) to proteins (such as thrombin) and complex molecular assemblies (such as cells).[3] Likewise, many DNAzymes have been synthesized for the catalysis of diverse chemical reactions, such as the cleavage and ligation of DNA and RNA.[4] More recently, the concept of allosteric ribozymes[5] has been adapted to the design of allosteric DNAzymes, in which a DNA aptamer is connected to a DNAzyme in such a way that the DNAzyme can only be activated by the ligand that binds to the aptamer.[6] Allosteric DNAzymes are interesting as biosensing tools, because the molecular-recognition event between an aptamer and its specific ligand can be translated into the activity of a DNAzyme for signal generation and amplification.

Rolling circle amplification (RCA) is a simple enzymatic process that can be used to generate very long single-stranded DNA (ssDNA) molecules with tandem repeats.[7] This process is carried out with a short DNA primer and a circular template under isothermal conditions by special DNA polymerases, such as ϕ29 DNA polymerase, with strand-displacement abilities. RCA has traditionally been used for the sensitive detection of DNA.[8] In recent years, however, RCA has been extended to the detection of other targets, such as proteins and small molecules, through the use of DNA aptamers and allosteric DNAzymes.[9] For example, the research groups of Willner and Mao recently used the RCA technique to generate repetitive units of a reporter DNAzyme for the highly sensitive detection of DNA.[9e,f] Ellington and co-workers created a ligand-dependent ligase DNAzyme that can generate a circular DNA template to initiate an RCA process as a way to detect small-molecule targets[9d] and proteins.[9b] However, the use of RCA and functional DNA for the sensing of non-nucleic-acid targets in a colorimetric format has yet to be demonstrated and was the focus of the current study. We believe such assays will expand the practical utility of functional nucleic acids significantly.

Our strategy is illustrated in Figure 1. Three key designs are implemented: an RNA-cleaving allosteric DNAzyme, RCA, and a colorimetric reporting mechanism based on a peptide nucleic acid (PNA) and an organic dye. In the presence of the intended target, the allosteric DNAzyme

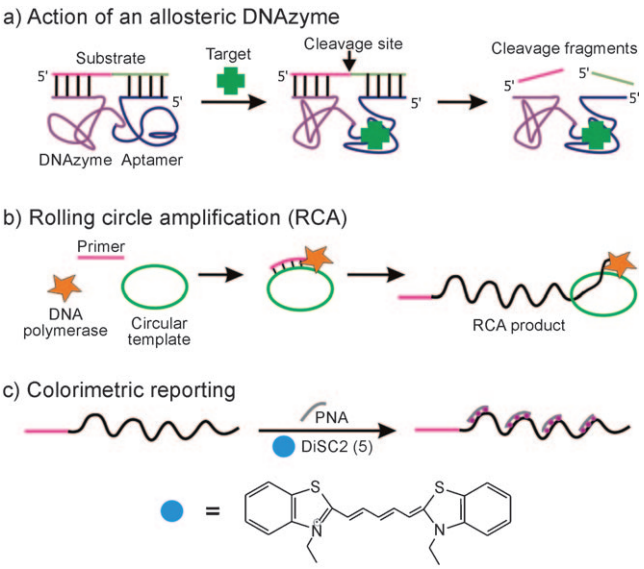

Figure 1. Schematic representation of the colorimetric detection of a specific target by using a) an RNA-cleaving allosteric DNAzyme, b) RCA, and c) PNA and DiSC2(5). When the target is present, the allosteric DNAzyme cleaves its substrate and generates a DNA primer to initiate RCA. The resulting long ssDNA forms a duplex with a complementary PNA. DiSC2(5) binds the PNA/DNA duplex and changes its color from blue to purple.

cleaves a special RNA-containing substrate and releases a DNA molecule that can be used by ϕ29 DNA polymerase as the primer to initiate an RCA reaction for the generation of a long ssDNA molecule. The RCA products are then detected colorimetrically upon hybridization with a complementary PNA in the presence of DiSC2(5) (3,3′-diethylthiadicarbocyanine). PNA molecules are known to form highly stable duplex structures with complementary DNA sequences.[10] It has also been reported that DiSC2(5) changes color from blue to purple upon binding to a DNA/PNA duplex. This

[*] Dr. M. M. Ali, Prof. Dr. Y. Li
Department of Biochemistry and Biomedical Sciences
and Department of Chemistry, McMaster University
1200 Main Street West, Hamilton, ON L8N 3Z5 (Canada)
Fax: (+1) 905-522-9033
E-mail: liying@mcmaster.ca

[**] This research was supported by research grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and SENTINEL Bioactive Paper Network. Y.L. holds a Canada Research Chair.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805966.

phenomenon has been used for colorimetric DNA detection.[11] As we demonstrate herein, by employing RCA, PNA, and DiSC2(5), we can translate the binding event between an allosteric RNA-cleaving DNAzyme and its cognate target into a colorimetric signal visible to the naked eye.

First, we examined the colorimetric characteristics of the DiSC2(5)–PNA probe in the presence of the RCA product. We synthesized a circular ssDNA template, named "RCA-T" (Figure 2a), by using a previously reported protocol.[12] RCA-T and the matching primer, "RCA-P", were used to perform

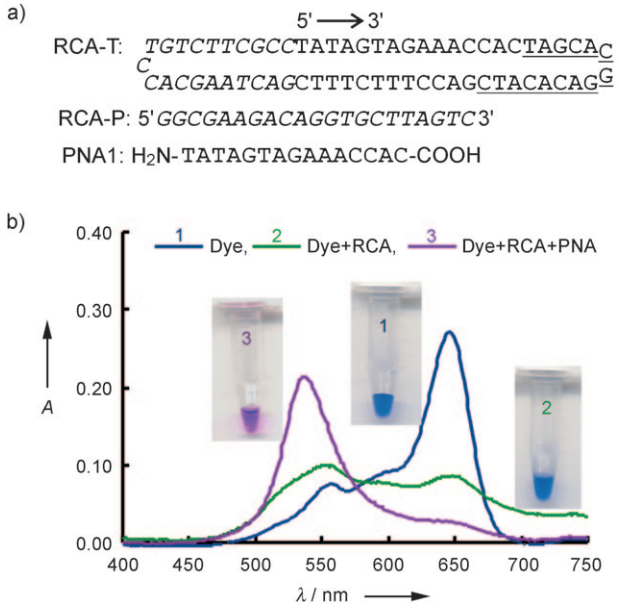

Figure 2. a) DNA sequences used for the test RCA reaction. b) Color and absorption spectra of DiSC2(5) in the hybridization buffer alone (1), with the RCA product (2), and in a mixture of the RCA product and the PNA probe (3).

the RCA reaction as described previously.[13] The details of this reaction and subsequent color-development procedures are provided in the Supporting Information. We found that DiSC2(5) retained the blue color in the hybridization buffer (50 mM Tris-HCl (Tris = 2-amino-2-hydroxymethylpropane-1,3-diol), pH 7.5, and 100 mM NaCl; Figure 2b, tube 1). When DiSC2(5) was mixed with the RCA product, no significant color change was observed (tube 2). However, when treated with the RCA product in the presence of the complementary PNA (named "PNA1"; its sequence is given in Figure 2a), the dye turned purple (tube 3). The dye slowly aggregated in the hybridization buffer with and without the RCA product; in contrast, no aggregation occurred in the presence of both the RCA product and the PNA. The dye gradually turned purple in the solution of the PNA alone, as in the solution of the PNA–DNA duplex. However, as previously reported,[11] upon heating and cooling, the purple color appeared faster (in less than 1 min) with the PNA–DNA duplex than with the PNA alone (at least 5 min was required).

We also analyzed the absorption spectra of solutions 1–3 (Figure 2b). The maximal absorbance of the dye in the hybridization buffer occurred at 646 nm. With the RCA product and PNA, the maximal absorbance was shifted to 537 nm. In comparison, the mixture of the dye and RCA product produced a broadened peak between 500 and 680 nm. These spectra are similar to those observed by others for PNA molecules hybridized to short DNA oligonucleotides.[11] In short, these results demonstrate that DiSC2(5)–PNA can indeed be used as colorimetric probe to visualize long ssDNA produced by RCA.

We next examined RCA carried out with "pH6-ET4" (Figure 3a), an ATP-sensing allosteric DNAzyme that we had designed previously[6d] by combining an RNA-cleaving and

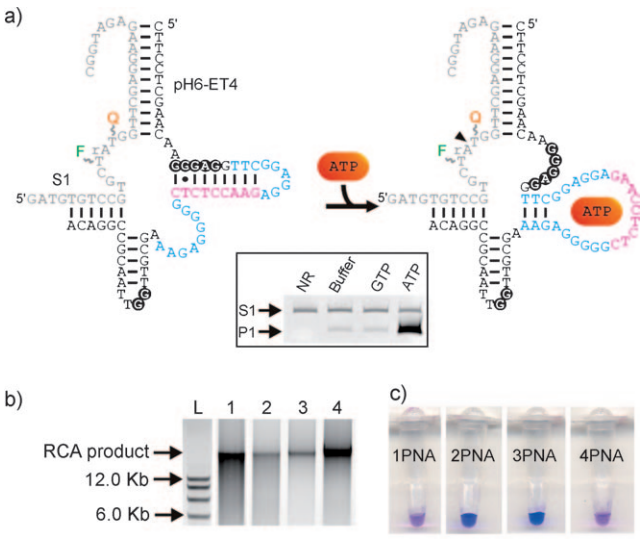

Figure 3. a) Conformations of "pH6-ET4", an ATP-dependent allosteric DNAzyme, and a PAGE gel showing the cleavage reaction in the reaction buffer alone (lane 2) and in the presence of GTP (lane 3) or ATP (lane 4). NR: no reaction. b) Agarose gel with RCA products: RCA with authentic P1 (lane 1); DNAzyme/S1 incubated in the cleavage buffer alone (lane 2) and in the presence GTP (lane 3) or ATP (lane 4). Lane L: DNA ladder. c) Color observed when samples 1–4 were mixed with PNA/DiSC2(5). P1 bears a 2′,3′-cyclic phosphate, which needs to be removed prior to RCA. T4 polynucleotide kinase (PNK) cleaves the 2′,3′-cyclic phosphate.[16]

fluorescence-signaling DNAzyme named "pH6DZ1"[14] with an ATP-binding DNA aptamer.[15] The DNAzyme pH6-ET4 can adopt two different conformations: In the absence of ATP, it adopts an inactive structure in which several catalytically important nucleotides form a short duplex with part of the aptamer sequence. In the presence of ATP, however, it switches to the active conformation, because the aptamer domain folds into its binding structure and frees the catalytic core of the DNAzyme, which then cleaves the RNA linkage ("rA", adenine ribonucleotide) embedded in a substrate (S1) otherwise composed of DNA. The cleavage event generates two separate DNA molecules, one of which (i.e. the cleaved fragment 5′ to the cleavage side, denoted "P1") is designated as the primer to initiate RCA. Key to this design is the placement of a masked primer that can only be retrieved for RCA upon the target-induced cleavage of S1 by the DNAzyme. Thus, the recognition of the target by the aptamer is translated into an RCA process.

Because of the fluorophore and quencher attached to the two T residues flanking the cleavage site in pH6-ET4 (F and Q in Figure 3a), the DNAzyme could also report target binding through the generation of a fluorescence signal. The activity of pH6-ET4 was confirmed to be dependent on the presence of ATP, as revealed by PAGE (polyacrylamide gel electrophoresis; Figure 3a): A much larger quantity of P1 (which is fluorescent and can be detected by fluorimaging) was produced in the mixture containing 1 mM ATP than in the mixture containing no ATP or 1 mM guanosine triphosphate (GTP, which has no affinity for the DNA aptamer).

P1 was designed to be complementary to part of the circular template RCA-T (underlined nucleotides in Figure 2a). Four separate RCA reactions were performed, and the RCA product was analyzed by agarose gel electrophoresis (Figure 3b). When RCA-T was incubated with precleaved P1 (a positive control), a significant amount of the RCA product (which was bigger than the 12000 base-pair marker in the DNA ladder in lane L) was observed (Figure 3b, lane 1). As expected, a similar amount of the RCA product was observed when RCA-T was incubated with the reaction mixture pH6-ET4/S1/ATP (Figure 3b, lane 4). In contrast, only a small amount of the RCA product was produced when ATP was omitted (Figure 3b, lane 2) or replaced with GTP (lane 3).

Each reaction mixture described above was combined with PNA1 (5 μM) and the DiSC2(5) dye (50 μM). The resulting solutions were heated at 90°C for 2 min and then cooled to room temperature to facilitate hybridization between the PNA and the RCA product and thus color development (Figure 3c). Both sample 2 (2PNA) and sample 3 (3PNA) retained the blue color of the dye; in contrast, sample 1 (1PNA) and sample 4 (4PNA) produced the expected purple color.

We also performed RCA reactions with the pH6-ET4/S1 cleavage products incubated with ATP (0, 50, 100, 250, 500, and 1000 μM). These reactions were conducted in the presence of a trace amount of [α-^{32}P]dGTP so that we could correlate the amount of the RCA product, measured on the basis of radioactivity (Figure 4a), with the development of the purple color (Figure 4b). As expected, more of the RCA product and a more intense purple color were observed when more ATP was used. For comparison, a fluorescent gel image of the cleavage reaction at varying concentrations of ATP is also provided in the Supporting Information as Figure S1: The cleavage product was observed when 100 μM ATP was used, in agreement with the colorimetric results in Figure 4.

Finally, we quantified the color response (CR) of the colorimetric samples (Figure 4c) by comparing absorbance according to a reported protocol[17] (see the Supporting Information for details). As mentioned earlier, a long incubation of DiSC2 with PNA alone can produce a purple color. To alleviate this problem, we measured the absorbance of the dye with the RCA product/PNA duplex in the presence of succinyl-β-cyclodextrin (Succ-β-CyD).[18] This reagent has been shown to interrupt the binding of the dye to PNA alone without interfering with its ability to bind to the RCA product/PNA duplex.[18] Therefore, the ratio of the absorbance of the dye at 535 nm to that at 647 nm can be related directly to the amount of RCA product produced at different concentrations of ATP. Indeed, the CR profile of the RCA reaction mixtures at tested concentrations of ATP (Figure 4c) reflects the increasing intensity of the purple color of relevant solutions (Figure 4b).

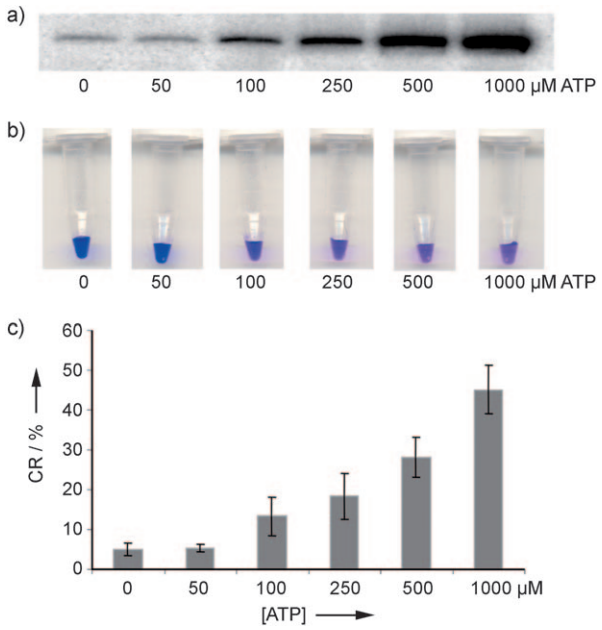

Figure 4. a) Analysis by 10% denaturing polyacrylamide gel electrophoresis of the radioactive RCA products following incubation of the pH6-ET4/S1 cleavage mixture with RCA-T in the presence of ATP (0, 50, 100, 250, 500, and 1000 μM). b) Color of the samples in (a) in the presence of 0.5% Succ-β-CyD. c) Color response (CR) of the samples as determined on the basis of absorbance (see the Supporting Information).

In summary, we have demonstrated that the action of an allosteric RNA-cleaving DNAzyme can be linked to RCA for the production of long ssDNA molecules to enable colorimetric sensing through the hybridization of a complementary PNA sequence in the presence of a duplex-binding dye, such as DiSC2(5). We believe that this approach is suitable as a general strategy for the discovery of colorimetric biosensors for the detection of a target analyte for which an allosteric RNA-cleaving DNAzyme can be designed or created.

Received: December 8, 2008
Published online: April 9, 2009

Keywords: biosensors · colorimetry · DNAzymes · peptide nucleic acids · rolling circle amplification

[1] a) N. K. Navani, Y. Li, *Curr. Opin. Chem. Biol.* **2006**, *10*, 272–281; b) J. F. Lee, G. M. Stovall, A. D. Ellington, *Curr. Opin. Chem. Biol.* **2006**, *10*, 282–289.

[2] a) A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818–822; b) C. Tuerk, L. Gold, *Science* **1990**, *249*, 505–510.

[3] a) M. Famulok, G. Mayer, M. Blind, *Acc. Chem. Res.* **2000**, *33*, 591–599; b) D. S. Wilson, J. W. Szostak, *Annu. Rev. Biochem.* **1999**, *68*, 611–647; c) D. Shangguan, Y. Li, Z. Tang, Z. C. Cao, H. W. Chen, P. Mallikaratchy, K. Sefah, C. J. Yang, W. Tan, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11838–11843.

[4] a) J. C. Achenbach, W. Chiuman, R. P. Cruz, Y. Li, *Curr. Pharm. Biotechnol.* **2004**, *5*, 321 – 336; b) S. K. Silverman, *Nucleic Acids Res.* **2005**, *33*, 6151 – 6163.

[5] a) G. A. Soukup, R. R. Breaker, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 3584 – 3589; b) R. R. Breaker, *Curr. Opin. Biotechnol.* **2002**, *13*, 31 – 39.

[6] a) M. Levy, A. D. Ellington, *Chem. Biol.* **2002**, *9*, 417 – 426; b) S. H. Mei, Z. Liu, J. D. Brennan, Y. Li, *J. Am. Chem. Soc.* **2003**, *125*, 412 – 420; c) J. C. Achenbach, R. Nutiu, Y. Li, *Anal. Chim. Acta* **2005**, *534*, 41 – 51; d) Y. Shen, W. Chiuman, J. D. Brennan, Y. Li, *ChemBioChem* **2006**, *7*, 1343 – 1348; e) W. Chiuman, Y. Li, *PLoS One* **2007**, *2*, e1224.

[7] a) A. Fire, S. Xu, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 4641 – 4645; b) D. Liu, S. L. Daubendiek, M. A. Zillman, K. Ryan, E. T. Kool, *J. Am. Chem. Soc.* **1996**, *118*, 1587 – 1594.

[8] a) P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas, D. C. Ward, *Nat. Genet.* **1998**, *19*, 225 – 232; b) C. Larsson, J. Koch, A. Nygren, G. Janssen, A. K. Raap, U. Landegren, M. Nilsson, *Nat. Methods* **2004**, *1*, 227 – 232.

[9] a) D. A. Di Giusto, W. A. Wlassoff, J. J. Gooding, B. A. Messerle, G. C. King, *Nucleic Acids Res.* **2005**, *33*, e64; b) L. Yang, C. W. Fung, E. J. Cho, A. D. Ellington, *Anal. Chem.* **2007**, *79*, 3320 – 3329; c) L. Zhou, L.-J. Ou, X. Chu, G.-L. Shen, R.-Q. Yu, *Anal. Chem.* **2007**, *79*, 7492 – 7500; d) E. J. Cho, L. Yang, M. Levy, A. D. Ellington, *J. Am. Chem. Soc.* **2005**, *127*, 2022 – 2023; e) Z. Cheglakov, Y. Weizmann, B. Basnar, I. Willner, *Org. Biomol. Chem.* **2007**, *5*, 223 – 225; f) Y. Tian, Y. He, C. Mao, *ChemBioChem* **2006**, *7*, 1862 – 1864; g) W. Zhao, M. M. Ali, M. A. Brook, Y. Li, *Angew. Chem.* **2008**, *120*, 6428 – 6436; *Angew. Chem. Int. Ed.* **2008**, *47*, 6330 – 6337.

[10] a) M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden, P. E. Nielsen, *Nature* **1993**, *365*, 566 – 568; b) O. Buchardt, M. Egholm, R. H. Berg, P. E. Nielsen, *Trends Biotechnol.* **1993**, *11*, 384 – 386.

[11] a) S. A. Kushon, J. P. Jordan, J. L. Seifert, H. Nielsen, P. E. Nielsen, B. A. Armitage, *J. Am. Chem. Soc.* **2001**, *123*, 10805 – 10813; b) M. Komiyama, S. Ye, X. Liang, Y. Yamamoto, T. Tomita, J.-M. Zhou, H. Aburatani, *J. Am. Chem. Soc.* **2003**, *125*, 3758 – 3762; c) I. Dilek, M. Madrid, R. Singh, C. P. Urrea, B. A. Armitage, *J. Am. Chem. Soc.* **2005**, *127*, 3339 – 3345; d) L. M. Wilhelmsson, B. Norden, K. Mukherjee, M. T. Dulay, R. N. Zare, *Nucleic Acids Res.* **2002**, *30*, e3.

[12] L. P. Billen, Y. Li, *Bioorg. Chem.* **2004**, *32*, 582 – 598.

[13] a) 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; b) W. Zhao, Y. Gao, M. A. Brook, Y. Li, *Chem. Commun.* **2006**, 3582 – 3584; c) M. M. Ali, S. Su, C. D. Filipe, R. Pelton, Y. Li, *Chem. Commun.* **2007**, 4459 – 4461.

[14] a) Z. Liu, S. H. Mei, J. D. Brennan, Y. Li, *J. Am. Chem. Soc.* **2003**, *125*, 7539 – 7545; b) Y. Shen, J. D. Brennan, Y. Li, *Biochemistry* **2005**, *44*, 12066 – 12076.

[15] a) D. E. Huizenga, J. W. Szostak, *Biochemistry* **1995**, *34*, 656 – 665; b) R. Nutiu, Y. Li, *Angew. Chem.* **2005**, *117*, 1085 – 1089; *Angew. Chem. Int. Ed.* **2005**, *44*, 1061 – 1065.

[16] S. A. Kandadai, W. Chiuman, Y. Li, *Chem. Commun.* **2006**, 2359 – 2361.

[17] A. Reichert, J. O. Nagy, W. Spevak, D. Charych, *J. Am. Chem. Soc.* **1995**, *117*, 829 – 830.

[18] T. Tedeschi, S. Sforza, S. Ye, R. Corradini, A. Dossena, M. Komiyama, R. Marchelli, *J. Biochem. Biophys. Methods* **2007**, *70*, 735 – 741.

