Bioanalysis

DOI: 10.1002/anie.201307418

Mismatches Improve the Performance of Strand-Displacement Nucleic Acid Circuits**

*Yu Sherry Jiang, Sanchita Bhadra, Bingling Li, and Andrew D. Ellington**

Abstract: *Catalytic hairpin assembly (CHA) has previously proven useful as a transduction and amplification method for nucleic acid detection. However, the two hairpin substrates in a CHA circuit can potentially react non-specifically even in the absence of a single-stranded catalyst, and this non-specific background degrades the signal-to-noise ratio. The introduction of mismatched base pairs that impede uncatalyzed strand exchange reactions led to a significant decrease of the background signal, while only partially damping the signal in the presence of a catalyst. Various types and lengths of mismatches were assayed by fluorimetry, and in many instances, our MismatCHA designs yielded 100-fold increased signal-to-background ratios compared to a ratio of 4:1 with the perfectly matched substrates. These observations could be of general utility for the design of non-enzymatic nucleic acid circuits.*

Nucleic acid circuits that are based on toehold-mediated strand exchange reactions have yielded interesting approaches to computation, nanotechnology, and diagnostics.[1] An example of a common amplification reaction, which is known as the catalytic hairpin assembly (CHA), is shown in Figure 1. Originally developed by Pierce, Yin, and co-workers,[1c] this circuit has subsequently been adapted to a variety of applications, including acting as a monitor of isothermal amplification reactions, both end-point[2] and real-time.[3]

Unfortunately, CHA circuits have also been shown to execute non-specifically, even in the absence of particular inputs.[4] This background leakage is characterized by an initial burst of signal, which is followed by a steady-state, non-catalyzed rate of circuit execution. In our previous work, the rate constant of the steady-state leakage of a typical CHA circuit was approximately $200\,M^{-1}s^{-1}$, while the corresponding catalytic rate at a catalyst concentration of 5 nM was $4000\,M^{-1}s^{-1}$.[4a] Although the 20-fold enhancement of the rate that was observed in the presence of the catalyst allowed for robust signal detection, any accompanying background leakage can potentially make quantitation of lower input concentrations more difficult. For example, we have found that although CHA circuits can be designed for a variety of sequence targets and applications, the signal-to-noise ratio for these circuits (that is, the catalyzed reaction relative to the uncatalyzed reaction) seldom exceeds a ratio of greater than 100:1.

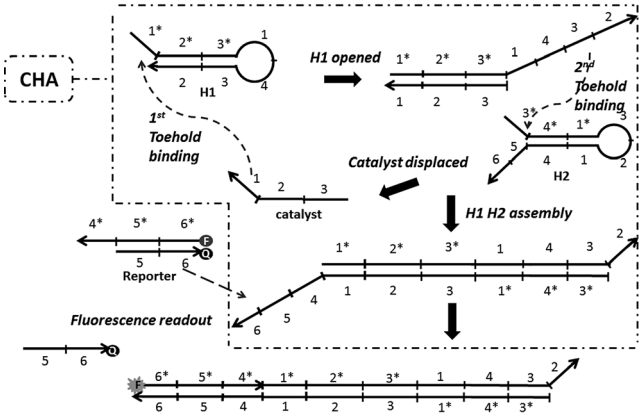

Figure 1. Catalytic hairpin assembly reaction with fluorescence readout. Briefly, one short linear oligonucleotide ("catalyst") will react with H1 through toehold binding and then initiate a branch migration reaction. The partially-opened H1 can interact with a toehold on H2 and similarly initiate a branch migration reaction. At the end of the second branch migration, the catalyst will be completely displaced from H1 and will be available for additional reaction cycles. Numbers in the Figure stand for different sequence domains; each domain includes eight bases.

The background leakage can be attributed to a number of factors, including the purity of the DNA samples[5] and the misfolding of nucleic acids into alternative conformers. Underlying many of these mechanisms, however, is the uncatalyzed binding of an otherwise occluded toehold to its hybridization partner, the subsequent initiation of strand exchange, and the continued propagation of the hairpin assembly reaction. For example, when the kinetically trapped hairpin substrates in CHA "breathe", they inadvertently reveal binding sites that can then initiate CHA even in the absence of a catalyst strand.

To reduce the prevalence of uncatalyzed strand exchange, we hypothesized that it might be possible to block either the revealed, inadvertent binding reaction and/or its continuation as a strand exchange reaction. In turn, the simplest way to introduce a block was to introduce mismatched nucleotides into the regions that are thought to breathe and/or into adjacent positions that might be involved in strand exchange. As the ends of helices are more likely to breathe than internal base pairs,[6] we chose to introduce mismatches into these portions of the hairpin substrates. A CHA circuit (Circuit A)

[*] Y. Jiang, Dr. S. Bhadra, B. Li, A. D. Ellington
Institute for Cellular and Molecular Biology
Center for Systems and Synthetic Biology
Department of Chemistry and Biochemistry
University of Texas at Austin
2500 Speedway, Austin, TX (USA)
E-mail: andy.ellington@mail.utexas.edu

[**] This work was supported by the Bill and Melinda Gates Foundation (OPP1028808), the Defense Advanced Research Projects Agency (HR0011-12-2-0001 and 5-35830), and the National Institute of Health TR01 (5 R01 AI092839).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201307418.

similar to those that were previously used by us and others[4a] was designed, except that domains 1 and 1* were shortened from ten to eight nucleotides, a length that we found could still act as an efficient toehold. Furthermore, mismatches were introduced at the 3′-end of domain 2 in H2 (CircA-H2D2M2, where CircA refers to the overall circuit, H2 refers to the hairpin substrate, D2 refers to the domain, and M2 refers to the type of mutation, that is, single, double, etc.; see also Table 1) to reduce its ability to hybridize to the complementary domain 2* in H1. To probe the potential contribution of different mismatches to background suppression, two consecutive mismatches were introduced at each of four sites (Figure 2 and Table 1).

The resultant "MismatCHA" circuits were assayed by monitoring the release of a fluorescent oligonucleotide from a quencher ("Reporter" in Figure 1; for the sequence, see the Supporting Information, Table S6). For example, CircA-H2D2M2 was paired with H1, and the development of a fluorescent signal was monitored as a function of time (Figure 3). When compared with the perfectly paired wild-type Circuit A (CircA-H1 paired with CircA-H2), the introduction of a double mismatch into domain 2 (CircA-H2D2M2; Table 1 and Figure 3a) led to a significant diminution of background signal development in the absence of catalyst. In contrast, when mismatches were introduced into domains 3 and 1 (CircA-H2D3M2, CircA-H1D1M2; Table 1), there was little effect on the performance of the circuit (Figure 3b and d), and the double mismatch in domain 4 (CircA-H1D4M2; Table 1) severely compromised the rate of the catalytic reaction (Figure 3c). We also studied a single mismatch on domain 4 and found that both the catalytic rate and the background leakage were reduced to one fifth of the values that were obtained for the wild-type constructs (Figure S1, Table S1).

Table 1: Wild-type sequence and mismatched sequences.[a]

Name	Sequence
CircA-H1	AGAGGCAT CAATGGGA ATGGGATC ATGCCTCT AACC-TAGC GATCCCAT TCCCATTG
CircA-H2	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCATTG ATGCCTCT AACCTAGC CCTTGTCA TAGAG-CAC
CircA-H2D2M2	ATGGGATC GCTAGGTT AGAGGCAT GATCCCAT TCCCA-Tac ATGCCTCT AACCTAGC CCTTGTCA TAGAGCAC
CircA-H2D3 M2	ATGGGATC GCTAGGTT AGAGGCAT atTCCCAT TCCCATTG ATGCCTCT AACCTAGC CCTTGTCA TAGAG-CAC
CircA-H1D4M2	AGAGGCAT CAATGGGA ATGGGATC ATGCCTCT AACC-TAcg GATCCCAT TCCCATTG
CircA-H1D1M2	AGAGGCAT CAATGGGA ATGGGATC taGCCTCT AACC-TAGC GATCCCAT TCCCATTG

[a] Mismatches in lowercase letters.

Whereas the rate of the reaction with CircA-H2D2M2 was slightly compromised (Figure 4), it nonetheless had a roughly 23-fold improved signal-to-noise ratio relative to the wild-type reaction. Although these initial results represent only a single set of designs, they can be rationalized by making two assumptions: First, breathing is more significant at the termini of the helices than at positions adjacent to loops. This implies that there was more opportunity for the suppression of the background signal for constructs CircA-H2D2M2 and CircA-H1D4M2 than for CircA-H2D3M2 and CircA-H1D1M2, as for CircA-H2D2M2 and CircA-H1D4M2, the intensity of the background signal should decrease because of breathing at the terminus of the hairpin helix, whereas CircA-H2D3M2 and CircA-H1D1M2 should reduce background interactions owing to breathing adjacent to the loop in hairpins. Second, in the case of CircA-H1D4M2, the double mismatch may effectively prevent not only the uncatalyzed reaction because of breathing, but also the initiation of the second strand exchange reaction that occurs following the opening of H1 (Figure 1). In contrast, the double mismatch in domain 2 would not interfere with the initiation, but only with the propagation of the second strand exchange reaction.

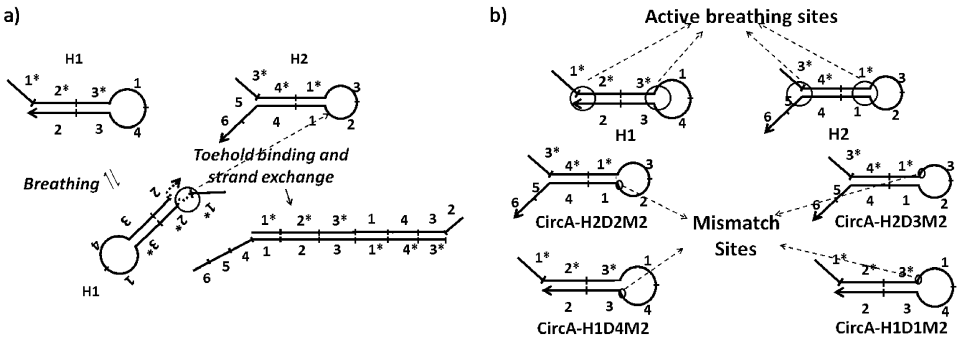

Figure 2. Possible pathways for leakage and positions of potential active breathing sites relative to the introduced mismatches: a) When the left end of the stem of H1 breathes, the 3′-end of domain 2 will be transiently exposed, revealing a partial toehold that is complementary to domain 2 of H2. This transient toehold exposure would permit H1 and H2 to react in the absence of catalyst. b) The four mismatch positions correspond to the revealed interactions that could initiate strand displacement reactions between H1 and H2. For example, mismatches at the 3′-end of domain 2 of H2 would disrupt binding and/or strand exchange with domain 2* of H1; similarly, mismatches at the 5′-end of domain 1 of H1 would disrupt binding and/or strand exchange with domain 1* of H2.

Building on the observation that mismatches in the H2 domain 2 (adjacent to the loop) can lead to better signal-to-noise characteristics, we generated a series of Circuit A variants by changing the number, position, and identity of the introduced mismatches (Figure 5; see also Table S2). A variety of single mismatches between H1 and H2 were tested (Figure 5a and Figure S2a). The mismatches were positioned at either the 3′-end of domain 2 (C:A, C:C, and C:T)or at the penultimate residue adjacent to the 3′-end of domain 2 (A:A). All mis-

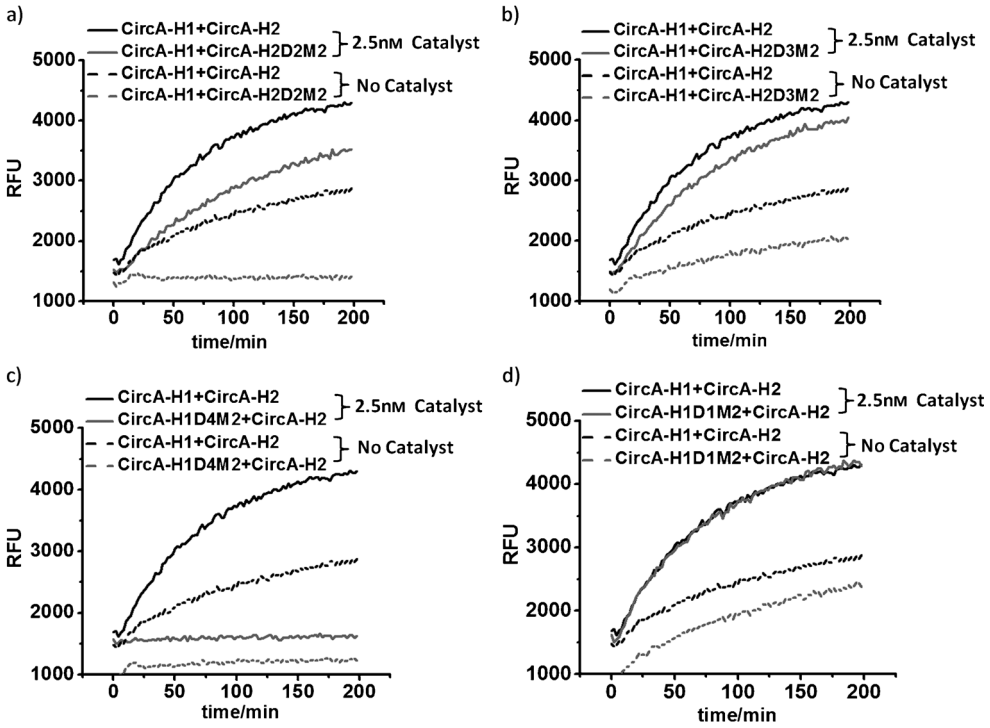

Figure 3. Signal generation with four different mismatches. a) Wild-type circuit and CircA-H2D2M2 circuit. b) Wild-type circuit and CircA-H2D3M2 circuit; c) Wild-type circuit and CircA-H1D4M2 circuit. d) Wild-type circuit and CircA-H1D1M2 circuit. The wild-type data are the same for each comparison; they are offset for clarity. RFU = relative fluorescence unit.

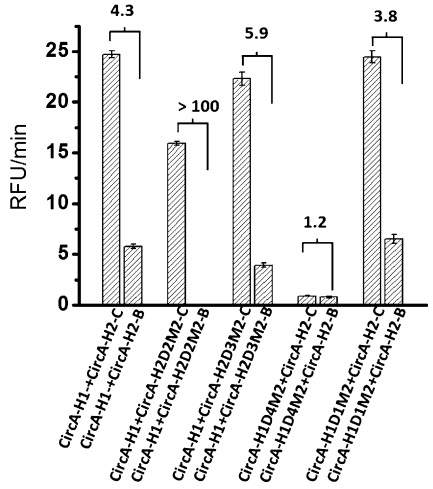

Figure 4. Signal-to-background ratios for four different mismatches. C denotes a catalyst concentration of 2.5 nM, B denotes an experiment conducted without catalyst. Signal-to-noise ratios were calculated from the linear portion of each fluorescence curve (such as those seen in Figure 3); each set of CHA reactions was repeated at least three times. The numbers at the tops of the columns represent the ratio of the catalyzed rate to background leakage.

matches improved the signal-to-background ratio, and the C:C mismatch gave a signal-to-background ratio of greater than 100:1. The performance of the C:C mismatch may be due to the fact that it is one of the strongest mismatches,[7] or may be due to the increase in the length of the H2 stem, which arises from a fortuitous pairing with an opposing guanosine in the loop. In Figure 5b and Figure S2b, we compared various multiple mismatches between H1 and H2. Four different double mismatches were assayed, either adjacent to one another (AC:CA, AC:AA) or separated by potentially paired residues (ATC:CAA, TTTC:CAAT; paired residues underlined). The double mismatches generally displayed higher signal-to-background ratios than the single mismatches, and three of the four ratios were greater than 100:1. Three mismatches (AAC:CAA) also yielded a high signal-to-background ratio. Both the double and triple mismatches decreased the catalytic rate of the CHA circuit, while generally improving the signal-to-background ratio.

To ensure that the circuits with mismatches were executing similarly to better known CHA circuits without mismatches, we also examined the strand exchange reactions by native gel electrophoresis of the reaction between CircA-H2 and CircA-H2D2M2 (Figure S3). Products were observed to be of the same sizes, irrespective of the presence or absence of mismatches. Consistent with the fluorescence assays, there is more background product from the perfectly paired CircA-H2 relative to the double mismatched CircA-H2D2M2 (roughly twice as much at end point).

To demonstrate that mismatches on domain 2 can generally improve signal-to-noise ratios, we used NUPACK,[8] a nucleic acid design software package, to generate a second circuit (Circuit B) with the same domain organization as in Figure 1, but with completely different sequences for the hairpin substrates. Again, we assayed circuit variants that contained mismatches at four positions (Table S3). Once more we found that only CircB-H2D2M2 (Figures S4 and S5) improved the signal-to-background ratio to over 100:1. To demonstrate the generality of these results, we also introduced different mismatches into Circuit B. Whereas wild-type Circuit B had a signal-to-background ratio of 4.5:1 (Figure S6c), a single mismatched H2 (CircB-H2D2M1, A:A; Figures S6a and C, Table S5) increased the signal-to-background ratio to 12.6:1, and a double mismatch (CircB-H2D2M2, CA:AC; Figure S6b and c, Table S5) increased the signal-to-background ratio to over 100:1.

Overall, MismatCHA designs substantially decreased the amounts of uncatalyzed background reactions in CHA

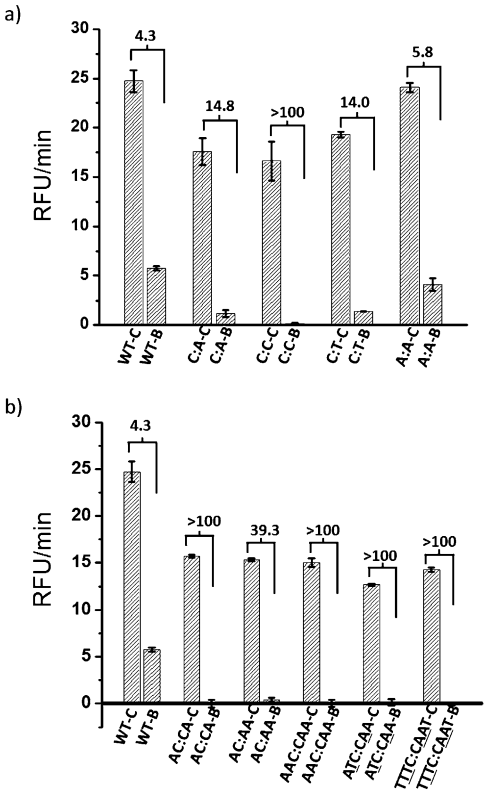

Figure 5. Signal generation in Circuit A with nine different domain 2 mismatches. Calculated signal-to-background ratios for a) single mismatches in CircA-H2 (named according to mismatches; see also Table S2); and b) double and triple mismatches. Each CHA reaction was carried out with H2 (50 nM; either wild-type or mismatched), CircA-H1 (50 nM), and CircA-reporter (50 nM). C denotes a catalyst concentration of 2.5 nM, B denotes an experiment conducted without catalyst.

amplification reactions. For a typical CHA circuit, introducing mismatches at the 3′-end of domain 2 generally gave higher signal-to-background ratios, with multiple mismatches almost always yielding much larger signal-to-background ratios while only modestly decreasing rates. As MismatCHA circuits can increase the signal-to-background ratio from single digits to over 100:1, they should prove useful for the sequence-specific signal transduction with amplicons that arise from isothermal amplification reactions.[9] In essence, CHA can now be used with isothermal amplification reactions in the same way a TaqMan probe is used for the polymerase chain reaction (PCR). The rules developed herein should now greatly extend the utility of CHA probes for many different amplicon sequences and for different types of isothermal amplification reactions.

Furthermore, these results continue to highlight the utility of non-canonical pairings in DNA nanotechnology. We have previously shown that defects in DNA nanostructures can potentially be detected using DNA circuits that are sensitive to mismatches[4a] (although in this instance, the presence of a mismatch in the structure led to a lower rate of reaction, rather than to a higher signal-to-background ratio as observed here with MismatCHA). Mismatches have also been shown to be specifically incorporated into DNA nanostructures, with regular DNA crystals being formed in part from non-Watson–Crick pairings[10] and even into larger structures, such as oligonucleotides hybridized to the surfaces of gold nanoparticles.[11] As the rules for the use of mismatches are further elucidated, the wealth of rationally designed DNA circuits and structures will continue to expand.

Received: August 22, 2013
Revised: November 25, 2013
Published online: January 8, 2014

Keywords: bioanalysis · DNA structures · hairpin loops · mismatch · nanobiotechnology

[1] a) D. Y. Zhang, E. Winfree, *J. Am. Chem. Soc.* **2009**, *131*, 17303–17314; b) C. Ma, W. Wang, Z. Li, L. Cao, Q. Wang, *Anal. Biochem.* **2012**, *429*, 99–102; c) P. Yin, H. M. T. Choi, C. R. Calvert, N. A. Pierce, *Nature* **2008**, *451*, 318–U314; d) D. Y. Zhang, A. J. Turberfield, B. Yurke, E. Winfree, *Science* **2007**, *318*, 1121–1125; e) H. Zhang, F. Li, B. Dever, X. Li, X. C. Le, *Chem. Rev.* **2013**, *113*, 2812–2841; f) J. Liu, Z. Cao, Y. Lu, *Chem. Rev.* **2009**, *109*, 1948–1998.

[2] B. Li, X. Chen, A. D. Ellington, *Anal. Chem.* **2012**, *84*, 8371–8377.

[3] Y. Jiang, B. Li, J. N. Milligan, S. Bhadra, A. D. Ellington, *J. Am. Chem. Soc.* **2013**, *135*, 7430–7433.

[4] a) B. Li, Y. Jiang, X. Chen, A. D. Ellington, *J. Am. Chem. Soc.* **2012**, *134*, 13918–13921; b) J. Huang, X. Su, Z. Li, *Anal. Chem.* **2012**, *84*, 5939–5943; c) J. Ren, J. Wang, L. Han, E. Wang, J. Wang, *Chem. Commun.* **2011**, *47*, 10563–10565.

[5] X. Chen, N. Briggs, J. R. McLain, A. D. Ellington, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 5386–5391.

[6] a) J. SantaLucia, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 1460–1465; b) J. SantaLucia, D. Hicks, *Annu. Rev. Cell Dev. Biol.* **2004**, *20*, 415–440.

[7] a) S. Kwok, S. Y. Chang, J. J. Sninsky, A. Wang, *Genome Res.* **1994**, *3*, S39–S47; b) S. Kwok, D. E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, J. J. Sninsky, *Nucleic Acids Res.* **1990**, *18*, 999–1005.

[8] a) J. N. Zadeh, C. D. Steenberg, J. S. Bois, B. R. Wolfe, M. B. Pierce, A. R. Khan, R. M. Dirks, N. A. Pierce, *J. Comput. Chem.* **2011**, *32*, 170–173; b) R. M. Dirks, J. S. Bois, J. M. Schaeffer, E. Winfree, N. A. Pierce, *Siam Rev.* **2007**, *49*, 65–88; c) R. M. Dirks, N. A. Pierce, *J. Comput. Chem.* **2003**, *24*, 1664–1677; d) R. M. Dirks, N. A. Pierce, *J. Comput. Chem.* **2004**, *25*, 1295–1304.

[9] a) J. Compton, *Nature* **1991**, *350*, 91–92; b) G. T. Walker, M. S. Fraiser, J. L. Schram, M. C. Little, J. G. Nadeau, D. P. Malinowski, *Nucleic Acids Res.* **1992**, *20*, 1691–1696; c) T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, *Nucleic Acids Res.* **2000**, *28*, e63.

[10] P. J. Paukstelis, J. Nowakowski, J. J. Birktoft, N. C. Seeman, *Chem. Biol.* **2004**, *11*, 1119–1126.

[11] H. D. Hill, S. J. Hurst, C. A. Mirkin, *Nano Lett.* **2009**, *9*, 317–321.

