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Published on Web 05/09/2003

Deoxyribozyme-Based Half-Adder

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Abstract: We have constructed a solution-phase array of three deoxyribozyme-based logic gates that behaves as a half-adder. Two deoxyribozymes mimic i_1ANDNOTi_2 and i_2ANDNOTi_1 gates that cleave a fluorogenic substrate, reporting through an increase in fluorescence emission at 570 nm. The third deoxyribozyme mimics an i_1ANDi_2 gate and cleaves the other fluorogenic substrate, reporting through an increase in fluorescence emission at 520 nm. Together, this system represents the first example of a decision-making enzymatic network with two inputs and two outputs. Similar systems could be applied to control autonomous therapeutic and diagnostic devices.

Introduction

A key requirement for digital computing is the ability to use logic functions to perform arithmetic operations.[1] The logic circuits that perform addition within central processing units of computers are called adders. A unit that adds two binary digits is called the *half-adder*. The half-adder by itself has only the ability to add two single binary digits (bits), but it is a key building block for a full adder, which in turn can add three bits and can be cascaded to yield serial adders for adding larger (multibit) integers. Consequently, the ability to construct a circuit that behaves as a half-adder is the crucial first test for any new computation medium. Accordingly, several new computation paradigms using molecular-scale logic gates[2] have been tested through the construction of half-adders. In the most striking prior example of solution-phase proof-of-concept molecular scale arithmetic, De Silva's group demonstrated how two fluorescent sensors can function as a half-adder with ions as inputs and the change in fluorescence as output.[3] In another published solution-phase approach, building a full adder from fluorophores using tools of multiphoton spectroscopy and fluorescence resonance energy transfer has been considered.[4]

We have recently reported in this journal a molecular-scale computation medium[5] based on a complete set of catalytic nucleic acid-based logic gates, thus fulfilling the early suggestion of Ellington and colleagues[6] that nucleic acid catalysts[7] constructed through modular design[8] can provide a novel form of DNA-based computation, unrelated to the Adleman−Lipton paradigm.[9] Our work has recently culminated in the construction of the first silicomimetic automaton capable of playing a type of tic-tac-toe game against a human opponent and never losing.[5b] We now report a strategy for constructing an array of three optimized deoxyribozyme[10]-based logic gates, which senses two inputs and makes a decision whether to produce either one of the two outputs. This array represents the first example of an artificial decision-making enzymatic network that mimics the half-adder and is the first example of artificial multiinput, multioutput decision-making networks with molecules as both inputs and outputs. While it is highly unlikely that such systems will ever be capable of high-speed computing competitive with silicon-based devices, these systems are fully biocompatible, and they may find applications as the control components of autonomous diagnostic and therapeutic agents. Our approach could also be used to control RNA-based enzymes, and these could be expressed in cells. This can lead to in vivo computation networks[11] and cells with engineered properties. To cite Leonard Adleman, who arguably pioneered biocomputing approaches using DNA:[9] "...molecular computers can be considered in a broader context...They may provide a much-needed means for controlling chemical/biological systems in the same way that electronic computers have provided a means for controlling electrical/mechanical systems."

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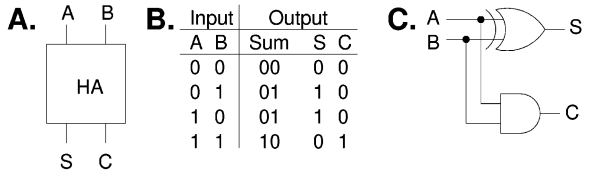

Figure 1. (A) The symbol for a half-adder. (B) The truth table for the sum digit S and the carry C. (C) The implementation for S with an exclusive OR (XOR) gate and for C with an AND gate.

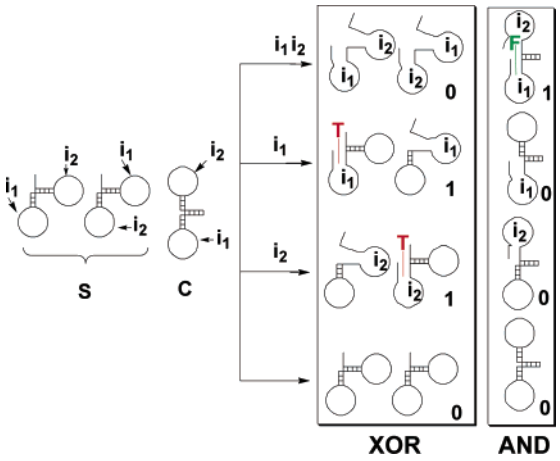

Figure 2. A schematic representation of technology mapping of the half-adder to deoxyribozyme-based logic gate technology. The sum digit is produced by an XOR system of two ANDNOT (∧¬) gates, and the carry digit is produced by an AND gate. Active enzymes have an intact catalytic core and a free substrate-recognition region and are presented here complexed with red and green substrates.

Results and Discussion

Design. A molecular half-adder system must analyze the presence of two input molecules and come up with two different output molecules in accordance with the following set of rules (cf., truth table, Figure 1): (1) the absence of both input molecules leaves the system as is, and neither of the outputs is produced, (2) the presence of any one (and only one) of the input molecules activates only the first output (the sum bit output), while (3) the presence of both inputs activates only the second output (the carry bit output). Thus, the sum bit output can be produced by the action of an exclusive OR (XOR) gate, whereas the carry bit output requires an AND gate. Both gates share the same inputs, while having different outputs.

Our complete basic set of nucleic-acid-based logic gates,[5a] with oligonucleotides as both inputs and outputs, was designed with the idea in mind that such gates can be arranged in solution to produce arbitrary Boolean calculations. The gates were constructed through modular design, combining deoxyribozymes with stem-loop[12] controlling elements. Because an XOR gate in our system is actually built from two deoxyribozyme ANDNOT (∧¬) gates, the half-adder needed a total of three deoxyribozymes. These three deoxyribozymes analyze two common inputs. The two deoxyribozyme ANDNOT gates behave together as an XOR gate to produce one output, and the remaining doxyribozyme AND gate produces the other output. This design is shown in Figure 2. Practically, to engineer a half-adder with deoxyribozymes, we had to expand our existing design capability to systems with multiple outputs. We also had to optimize deoxyribozyme structures to show satisfactory digital behavior in mixtures as well.

We defined the two inputs as two arbitrarily chosen oligonucleotides i_1 and i_2, (Figure 3) which will allosterically activate the deoxyribozymes. The two outputs are also oligonucleotides: the carry product $\mathbf{P_C}$ and the sum product $\mathbf{P_S}$. To produce two different oligonucleotide products, we must have two different deoxyribozyme/substrate couples. Furthermore, to facilitate visualization of the two outputs simultaneously, we need to optimize a two-color fluorogenic detection system for deoxyribozyme activity.[13] For this purpose, we chose to label the 5′ ends of the two deoxyribozyme substrates with two fluorophores with sufficiently separated fluorescent spectra to avoid any cross-talk in the two detection channels: fluorescein (**F**, $\lambda_{exc} = 480$, $\lambda_{em} = 520$ nm) for a "green channel" and tetramethylrhodamine (TAMRA, **T**, $\lambda_{exc} = 530$ nm, $\lambda_{em} = 570$ nm) for a "red channel".[5] On the 3′ ends of each substrate, we decided to attach "dark quenchers", that is, energy acceptors with no fluorescent emission: Black Hole 1 (BH_1) for the fluorescein-containing substrate $\mathbf{S_F}$, and Black Hole 2 (BH_2) for the TAMRA-containing substrate $\mathbf{S_T}$. We first established, by prolonged incubations of mismatched substrate−enzyme pairs, that there is exclusive cleavage of one substrate by a matched deoxyribozyme, that is, that there will be no cross-talk between the two detection channels. These experiments also established that prolonged incubation leads to up to a 20-fold increase in fluorescence in both substrates. Thus, the black-hole quenchers are significantly more efficient than the previously used fluorescence energy transfer quencher rhodamine.[5a]

Optimization of Individual Gates. Next, we examined the construction of three gates that will give the required behavior when arranged in a circuit. Two ANDNOT (∧¬) gates arranged in an XOR[14] circuit have one promoting and one inhibitory loop each ($i_1 \wedge \neg i_2$, which is active in the presence of i_1 but inactive in the presence of i_2; and $i_2 \wedge \neg i_1$, which is active in the presence of i_2 but inactive in the presence of i_1), as described earlier,[5a] and they cleave the substrate $\mathbf{S_T}$ to produce the output $\mathbf{P_S}$. We proceeded to optimize these two gates in accordance with the following three criteria: (1) unambiguous computation within 30 min in the presence of Mg^{2+} ions; (2) at least a 10-fold difference in relative increases in fluorescence in the samples with output **1** versus samples with output **0**; and (3) a similar increase in fluorescence for each component at the same concentrations.

Previously used ANDNOT gates[2] did not satisfy these conditions with new inputs, and we undertook a systematic effort to optimize system behavior. After changing structures of gates and testing eight different designs for each gate, we arrived at the structures shown in Figure 4 for the two components of the XOR system. In the $i_1 \wedge \neg i_2$ gate, we increased the overlap of the inhibitory stem with the catalytic core and shortened the substrate-recognition region. For the $i_2 \wedge \neg i_1$ gate, we had to

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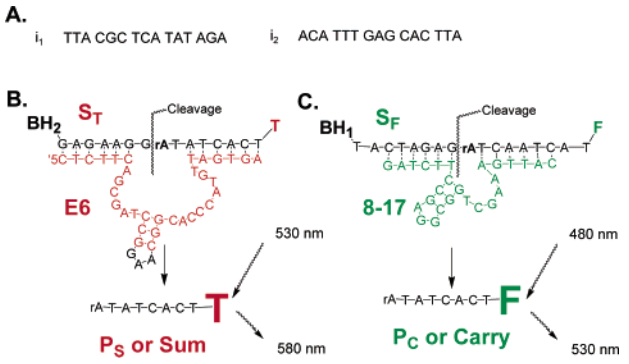

Figure 3. (A) Inputs i_1 and i_2. (B) Cleavage of the sum substrate S_T by the deoxyribozyme core E6[10a] provides the sum output P_S and an increase in TAMRA (**T**) fluorescence. (C) Cleavage of the carry substrate S_F by the deoxyribozyme core 8-17[10b] provides the carry output P_C and an increase in fluorescein (**F**) fluorescence.

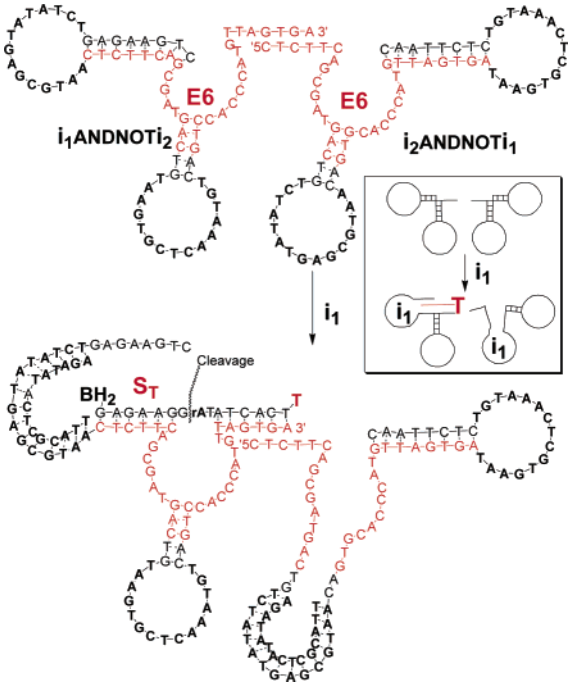

Figure 4. Deoxyribozyme E6 (red) incorporated into $i_1 \wedge \neg i_2$ and $i_2 \wedge \neg i_1$ is activated in the $i_1 \wedge \neg i_2$ gate upon addition of i_1. The insert schematically represents the state of the system upon addition of a single input i_1. The presence of both inputs would inhibit both gates.

change the position of the stem-loop at the 3′ end of deoxyribozyme to obtain a reaction rate similar to that of $i_1 \wedge \neg i_2$. The need for these adjustments is evidence of incompletely modular behavior and points to the importance of building databases of oligonucleotides with identical behavior as allosteric activators. Together in solution, at concentrations of 250 nM each, these two gates behaved as a perfect XOR system for a period of up to 6 h. In all experiments, we used a 10-fold excess of substrate, and each ANDNOT gate underwent almost six turnovers in the period of 6 h. While one important characteristic of our gates is their biocompatible nature, we have also been able to increase the rate of computation more than 10-fold by adding other bivalent ions, such as Zn^{2+} or Pb^{2+}.[10] For instance, by adding Zn^{2+} ions to the reaction mixture, we achieved computation times of less than 1 min.

The AND[15] ($i_1 \wedge i_2$) gate cleaves the $\mathbf{S_F}$ substrate to produce the output $\mathbf{P_C}$ and should be active only in the presence of both

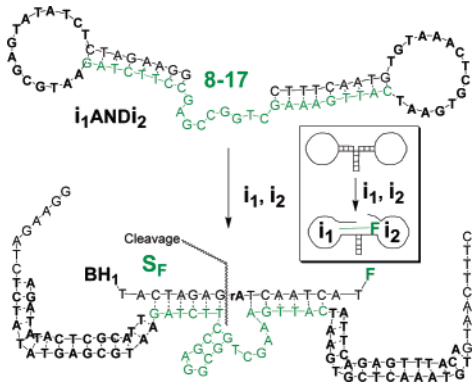

Figure 5. Deoxyribozyme E 8-17 (green) incorporated into $i_1 \wedge i_2$ is activated upon addition of both inputs. The insert schematically represents the active state.

inputs. Following the first two criteria used for XOR systems, we shortened the substrate-recognition region and significantly extended the inhibitory stem into the catalytic core at both ends of the deoxyribozyme. After the optimization process and testing seven different designs, the final structure of the new AND gate showed nearly perfect digital behavior and a reasonable rate of fluorescence increase. The structure in Figure 5 showed no detectable change in fluorescence in the presence of only one input after 6 h and could have its activity unambiguously detected within 30 min in the presence of both inputs. We note that this AND gate, at a concentration of 250 nM, had a lower turnover number than the ANDNOT gates. Specifically, with a 10-fold excess of substrate, each activated AND gate underwent slightly above one turnover in 6 h. Again, we were able to increase the rate of computation by using Zn^{2+} ions.

Half-Adder Function. We proceeded to construct a mixture of three gates, which was to behave as a half-adder (Figure 6). Interestingly, simply mixing up three gates at concentrations initially used to optimize individual components led to unexpectedly imperfect digital behavior, indicating that at higher total concentrations of oligonucleotides the gates influence each other, beyond what would be expected from simple additive behavior. This is an interesting observation that probably points to a limitation in the technology, that is, that there is a limitation of the maximal number of gates operating in solution. However, we combined in solution the three optimized individual deoxyribozymes at a total concentration of 300 nM and followed the real-time concentration changes in the presence of Mg^{2+} or Zn^{2+} cations. The presence of both oligonucleotides activated the AND gate, and this was detected through an increase in fluorescence in the green channel caused by the cleavage of $\mathbf{S_F}$, whereas the XOR system was inactive, as shown by the lack of increase in the red channel (Figure 6). Conversely, the presence of exactly one input left the AND gate inactive, with the green channel nearly constant over time, and activated the XOR system, as demonstrated through the increase in fluorescence measured in the red channel (caused by the cleavage of $\mathbf{S_T}$). The absence of both input oligonucleotides left both channels inactive. Therefore, this computation element, defined by the three deoxyribozymes operating in parallel, adds one single bit (defined by input oligonucleotide i_1) to another single bit (defined by input oligonucleotide i_2) to produce two output bits. Importantly, while addition itself was unambiguously performed in 30 min, satisfactory digital behavior continued even after prolonged incubation times (>5 h).

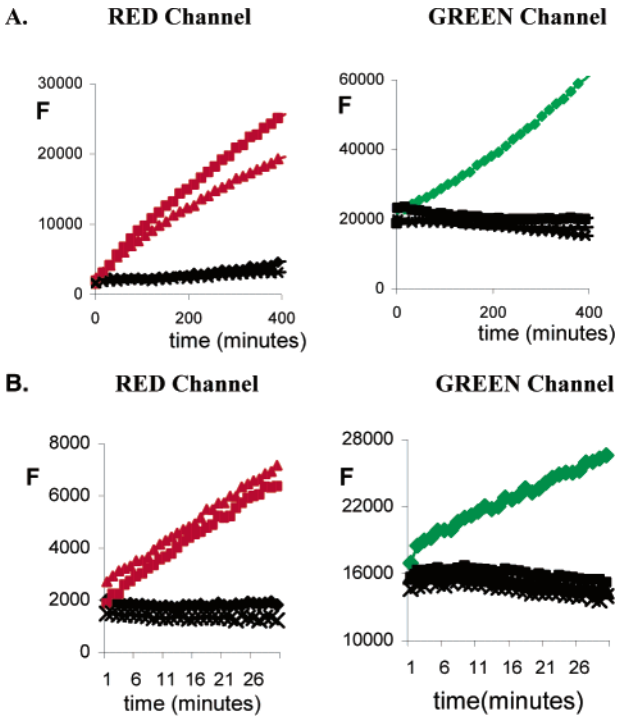

Figure 6. Half-adder behavior of the network. (A) Experiments with Mg^{2+}: Cleavage of the sum substrate by the deoxyribozyme core E6[10] provides the sum output in the red channel and an increase in TAMRA fluorescence. Cleavage of the carry substrate by the deoxyribozyme core 8-17[10b] provides the carry output in the green channel and an increase in fluorescein fluorescence. (B) Same experiments with Zn^{2+}. ◆, both inputs present; ■, only i_1 present; ▲, only i_2 present; ×, no inputs.

Conclusions

We have achieved the following progress in the area of decision-making molecular networks: (1) we have demonstrated the parallel operation, without cross-talk, of two separate systems of deoxyribozyme-based logic gates in solution; (2) we have established that multicolor detection is a viable way of observing outputs in these systems; (3) we have demonstrated that previously reported individual gates can be optimized to achieve fully digital behavior for specific outputs and inputs; (4) we have constructed the first common engineering element used in basic arithmetic operations, the half-adder, which is an important step toward the construction of a full adder; and (5) using this engineering element in solution, we have computed the sum and carry bits of 0+0, 0+1, 1+0, and 1+1. Importantly, although this is not the first solution-phase half-adder[3] ever reported, it is, to the best of our knowledge, the first example of an enzymatic half-adder with molecules as both inputs and outputs.

Our targeted applications in drug delivery do not require reversibility and do not require us to move beyond feed-forward circuits. However, our gates are fully reversible if attached to solid surfaces; the removal of the input oligonucleotides and the addition of new substrates resets the gates to their initial state. On the other hand, the move from feed-forward circuits to recurrent ones in solution would require the introduction of additional technologies to replenish the substrate, for example, the development of corresponding stem-loop-controlled ligase-based gates. Fully reversible circuits would also require open systems, which could be accomplished in cells or with microfluidic setups.

There is one significant corollary of our results, beyond the demonstration of the versatility and power of deoxyribozyme gates as the building blocks for solution-phase computation. These controlling elements for the solution-phase devices can now make one decision if only one of the two types of molecules is present, but they will make an entirely different decision when both of these molecules are sensed. We are not aware of any other fully artificial, solution-phase, molecular-scale system in which this kind of achievement has been possible, that is, in which enzymatic reactions can be triggered or inhibited under the precise control of such complex conditions. Extending these principles to disease markers will be an important step in the construction of autonomous diagnostic devices on the molecular scale. This alone justifies pursuing our next goal in this research: building the full adder, a three inputs−two outputs system.

Materials and Methods

Materials. All gates, inputs, and the fluorogenic substrate $\mathbf{S_F}$ were custom-made by Integrated DNA Technologies Inc. (Coralville, IA). Gates and substrate were used HPLC purified, while inputs were used crude. Fluorogenic substrates $\mathbf{S_F}$ were custom-made and purified by TriLink Biotechnologies (San Diego, CA) and used as received. All experiments were performed in 50 mM HEPES buffer (pH = 7.4) with 1 M NaCl, and DNA/RNAse purified water was used for all experiments.

Instrumental. Initial characterization of fluorescent spectra was performed on a Hitachi Instruments Inc. (San Jose, CA) F-2000 fluorescence spectrophotometer with Hamamatsu Xenon Lamp, while later characterizations were performed on a Perkin-Elmer LS-55 luminometer. All assays were performed using a Wallac Victor2 1420 Multilabel Counter (Perkin-Elmer Instruments, Shelton, CT) in 384-well plates (Wallac 384 black plates), using appropriate filters (λ_{em} = 530 ± 10 nm, λ_{exc} = 480 ± 10 nm for fluorescein−green channel and λ_{em} = 570 ± 10 nm, λ_{exc} = 530 ± 10 nm for TAMRA−red channel). The appropriate program for the plate reader was designed to allow simultaneous acquisition of fluorescence from both fluorophores.

Experiments. For the initial testing, individual gates were dissolved in reaction buffer at 1 μM, inputs and substrates at 10 μM concentrations. Three solutions were mixed together at equal volumes (15 μL each) in wells of a 384-well plate, and the reaction was initiated by addition of further aliquot of 40 mM Mg^{2+} or 1 mM Zn^{2+} solution. The reaction was followed directly from wells in real time. For the demonstration of half-adder behavior, gates were diluted to individual concentrations of 100 nM.

Acknowledgment. This work is dedicated to Prof. Vojin Mihaldzic on the occasion of his 70th birthday. Support from NASA (NAS2-02039 to M.N.S.), NIH (NIBIB, RO1 EB000675-1 to M.N.S.), NSF (EIA-0218262 to M.N.S. and D.S.), Microsoft Research (D.S.), and Hewlett-Packard (D.S.) is gratefully acknowledged. M.N.S. is supported by the Searle Scholars Program. We are grateful to Benjamin L. Andrews and Clint Morgan for careful reading of this manuscript.

Supporting Information Available: Fluorescence versus time curves for individual gates used in this paper (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0296632