Deoxyribozyme-Based Three-Input Logic Gates and Construction of a Molecular Full Adder†

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ABSTRACT: We have developed an array of seven deoxyribozyme-based molecular logic gates that behaves as a full adder in a single solution, with three oligonucleotides as inputs and two independent fluorogenic cleavage reactions as carry and sum outputs. The sum output consisted of four new deoxyribozyme-based logic gates: an ANDAND gate and three ANDNOTANDNOT gates. These gates required the design of a generic three-input deoxyribozyme-based logic gate that can use any three-way combination of activating or inactivating inputs. This generic gate design utilizes an additional inverting element that hybridizes to convert YES logic into NOT logic and vice versa. The system represents the first solution-phase, single test tube, enzymatic full adder and shows the complexity of control over molecular scale events that can be achieved with deoxyribozyme-based logic gates. Similar systems could be applied to control autonomous therapeutic and diagnostic devices.

We are interested in engineering biological molecules and pathways (*1*) for use in autonomous therapeutic and diagnostic devices (*2*). Such devices require the ability to process precisely and autonomously multiple signals (inputs) from the environment and generate appropriate responses (outputs) for each possible combination of multiple inputs. Nucleic acid catalysts allosterically regulated by oligonucleotides (*3*−*10*) can provide the basis for such an approach because specificity for each signal is obtained by selective binding of two nucleic acid sequences. Along these lines we have recently developed a solution-phase deoxyribozyme-based computation medium, which uses stem−loop controlled deoxyribozymes as molecular logic gates (*11*), oligonucleotides as inputs, and either cleaved (*11*−*13*) or ligated (*14*) oligonucleotides as outputs. Through this system we have developed a molecular automaton capable of playing a type of tic-tac-toe game against a human opponent (*13*), a molecular half-adder able to add two bits of oligonucleotide data (*12*), and established Boolean control over aptamers for the binding and release of analytes and enzymes (*15*). In these studies, gates could have up to two activating inputs and one inhibiting input (i_1ANDi_2ANDNOTi_3 in Figure 1). The complexity of the system was developed by increasing the number of gates in solution (*13*) and increasing both the number (*12*) and type (*15*) of output channels. Here we have combined these previous advances with an innovation at the level of deoxyribozyme control to construct a hitherto unavailable logical circuit: the full adder.

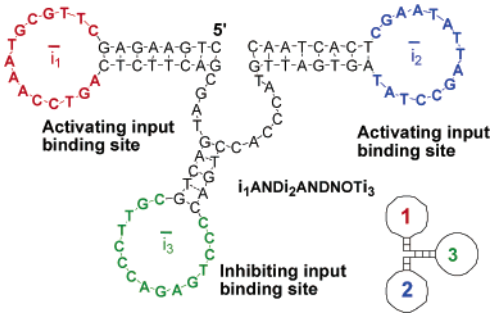

FIGURE 1: The elementary unit of molecular scale computation is a logic gate, such as the i_1ANDi_2ANDNOTi_3 gate shown here, which is activated by two input oligonucleotides and deactivated by a third input oligonucleotide. All inputs bind to the loop regions.

The full adder is a key digital logic element used in computer engineering as it is used in cascades to perform arithmetic operations. Creation of a full adder is a traditional challenge for any novel computational paradigm. The function of this element is to take three binary digits (called bits, or in our case input oligonucleotides) and add them together to produce two binary outputs, known as the sum and the carry digit (Figure 2). The inclusion of a third binary digit is significant [compared to a half-adder (*12*), which is only able to add two binary digits], because it enables cascading elements for multidigit addition: a downstream adder can take two independent binary digits and receive a third from a carry digit of an upstream adder.

Several other approaches using molecular scale logic gates (*16*) have been tested for the construction of adder elements, but none have resulted in the construction of a complete solution-phase full adder. In the first published example of solution-phase proof-of-concept molecular scale arithmetic, two fluorescent sensors were used as a half-adder with ions

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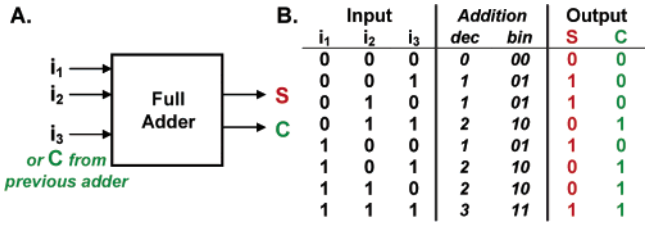

Input			Addition		Output	
i_1	i_2	i_3	dec	bin	S	C
0	0	0	0	00	0	0
0	0	1	1	01	1	0
0	1	0	1	01	1	0
0	1	1	2	10	0	1
1	0	0	1	01	1	0
1	0	1	2	10	0	1
1	1	0	2	10	0	1
1	1	1	3	11	1	1

FIGURE 2: (A) Full adder analyzes three inputs and produces two outputs: sum (S) and carry (C). (B) Truth table (set of rules for the conversion of inputs to outputs) of a full-adder engineering element.

as inputs and change in fluorescence as output (*17*). Another solution-phase approach considered building a full adder from fluorophores using multiphoton spectroscopy and fluorescence energy transfer (*18*). A third approach created a system based on hybridization-triggered photodamage of nucleic acids to demonstrate that individual solutions can behave as separate carry and sum digits (*19*).

Here we report the development of a generic three-input deoxyribozyme logic gate design that through the hybridization of controlling oligonucleotides enables processing of any three-way combination of activating or inactivating inputs. This enabled us to construct a solution-phase molecular full adder where all calculations are performed autonomously in a single tube. Our full adder has the potential to be used in cascades performing arithmetic operations and in the control of solution-phase devices.

MATERIALS AND METHODS

Materials. All gates, inputs, and the fluorogenic substrates S_F were custom-made by Integrated DNA Technologies Inc. (Coralville, IA). Gates and substrate were used PAGE purified, while inputs were used desalted. Fluorogenic substrate S_T was 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.

Instruments. Initial characterizations of fluorescent spectra were performed on a Hitachi Instruments Inc. (San Jose, CA) F-2000 fluorescence spectrophotometer with a Hamamatsu xenon lamp, while later characterizations were performed on a PerkinElmer LS-55 luminometer. Subsequent assays were performed using the Envision 2101 multilabel reader (PerkinElmer Instruments, Shelton, CT) in 384-well plates (Wallac 384 black plates), using 5% excitation light, 50% gain, and appropriate filters ($\lambda_{ex} = 485 \pm 14$ nm, $\lambda_{em} = 535 \pm 25$ nm for fluorescein, green channel, and $\lambda_{ex} = 531 \pm 60$ nm, $\lambda_{em} = 595 \pm 60$ nm for TAMRA, red channel). The plate reader was programed to allow simultaneous acquisition of fluorescence from both fluorophores.

Experiments. For initial testing, individual gates were dissolved in reaction buffer at 1 μM concentration, with 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 40 mM Mg^{2+} or 1 mM Zn^{2+} solution. The reaction was followed directly from wells in real time. For final demonstrations of full-adder behavior (Figure 7), gates were diluted to individual concentrations of 50 nM, pre-

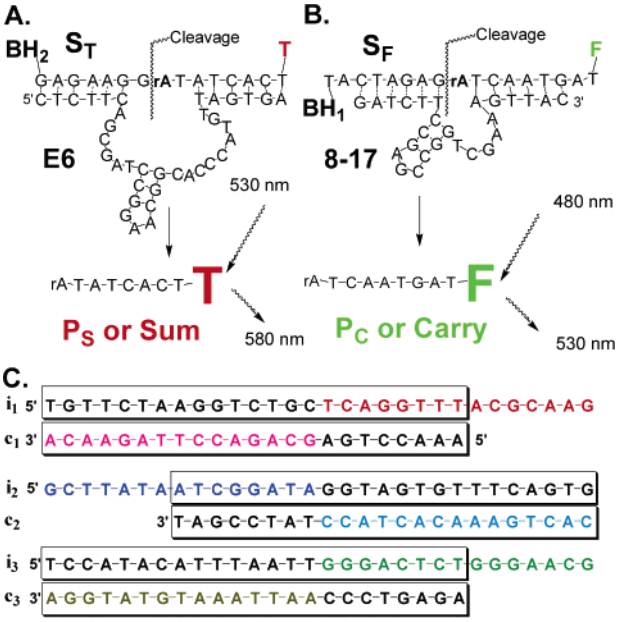

FIGURE 3: (A) Cleavage of substrate S_T by the deoxyribozyme core E6 (*21*) provides the sum output and an increase in TAMRA fluorescence. (B) Cleavage of substrate S_F by the deoxyribozyme core 8−17 (*22*) provides the carry output and an increase in fluorescein (F) fluorescence. (C) The three inputs (i_1, i_2, and i_3) and the corresponding inverting oligonucleotides (c_1, c_2, and c_3) aligned according to their complementary regions. Colored nucleotides interact with stem−loops in the gates. Boxed regions are mutually complementary.

complexed with 200 nM oligonucleotides c_1, c_2, and c_3 in reaction buffer containing 1 mM Zn^{2+} solution, and mixed with 1.25 μM S_T and 1 μM S_F. Individual wells of a 384-well plate were filled with 50 μL of mixture, and the reaction was activated by the addition of 1 μM appropriate inputs. The increase in fluorescence was measured every 15 min for 3 h.

RESULTS

Full-Adder Logic and Design. A molecular full adder must analyze the presence of three input molecules and produce two different output molecules according to the following rules (Figure 2): (a) The presence of any one (and only one) of the input molecules activates only the "sum" (S) output; (b) the presence of any two inputs activates only the "carry" (C) output; (c) the presence of all three inputs activates both outputs; and (d) the absence of all three input molecules leaves the system as is, and neither output is produced. The simplest full-adder construction uses seven molecular gates, since there are seven combinations of inputs leading to at least one of the outputs being activated. Because the carry output is activated in the presence of two or more inputs, it can be achieved by the use of three AND gates, which have been previously described (*11*−*13*). The sum digit required four gates not previously available: one logic gate active in the presence of all three inputs and three deoxyribozyme gates active only in the presence of one input (but not any two inputs or all three inputs). Development of these gates is described below.

Inputs and Outputs. Three oligonucleotides (i_1, i_2, and i_3) were arbitrarily chosen as inputs for the full adder. The resultant inputs (Figure 3C) were 30 bases long and contained

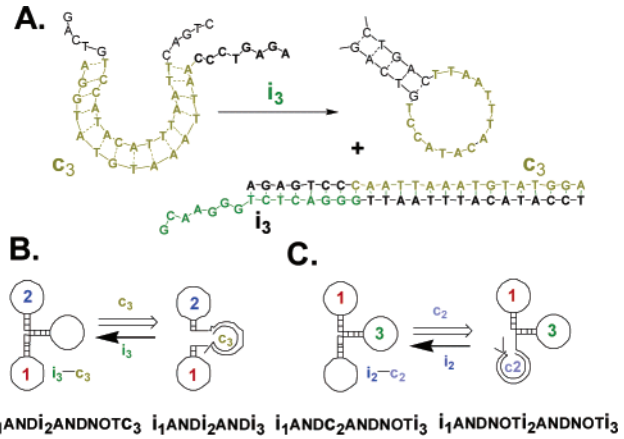

FIGURE 4: (A) A proxy input or inverter c_3 is used to open the stem, while the actual input, i_3, is used to remove c_3 from its complex with the stem−loop. This accomplishes a negation function for the i_3, and if c_3 was an inhibitory proxy input, i_3 will function as an activating input. (B) Inverter c_3 turns $i_1ANDi_2ANDNOTc_3$ into $i_1ANDi_2ANDi_3$ gate. (C) Inverter c_2 turns $i_1ANDc_2ANDNOTi_3$ into $i_1ANDNOTi_2ANDNOTi_3$ gate.

15-mer stretches that hybridized to loops involved in allosteric control of the deoxyribozyme logic gates. Inputs were designed such that they did not contain strong internal secondary structures, determined through available folding programs (*20*), and did not have more than a 45% GC content. The two outputs were identical to those designed for the half-adder (*12*). Briefly, the sum product (Figure 3A) was "red channel" TAMRA- (tetramethylrhodamine-) labeled oligonucleotides P_S, which is produced by the cleavage of substrate S_T by the deoxyribozyme E6 (*21*). The carry product (Figure 3B) was "green channel" fluorescein-labeled oligonucleotide P_C, produced by the cleavage of substrate S_F by deoxyribozyme 8−17 (*22*). Visualization of the two outputs simultaneously was achieved through a two-color fluorogenic detection system for deoxyribozyme activity (*12*), enabled by labeling the 3′ end of S_F with black hole 1 (BH_1) and S_T with black hole 2 (BH_2).

Generic Three-Input Gate Design. To create the new three-input logic gates required for full-adder construction, we defined three inverting oligonucleotides (c_1, c_2, and c_3) with stretches of 23 bases complementary to the inputs. These were inspired by the approach that Yurke and colleagues used to reset the early models of molecular motors (*23*) and were in keeping with the use of a modular design that avoids the use of special case oligonucleotides. When the inverting oligonucleotides c_1, c_2, or c_3 are precomplexed to complementary stem−loops on deoxyribozyme-based logic gates, input oligonucleotides i_1, i_2, and i_3 can strip the "inverters" from binding to the gates, reversing their action (Figure 4A). Thus hybridizing c_1, c_2, or c_3 with 15-mer complementary loops in our previous ANDANDNOT gates (*11*, *13*) (Figure 1) would negate the original effect, turning YES loops into NOT loops and vice versa.

ANDAND Gate. Construction of an ANDAND gate was achieved by negatively regulating the single stem−loop module for negative allosteric control of the ANDANDNOT gate (Figure 4A,B), because double negation leads to affirmation (NOT ○ NOT = YES). Gate $i_1ANDi_2ANDNOTc_3$, active in the presence of inputs i_1 and i_2 (Figure 4B), was precomplexed (and inhibited) by proxy input or inverter c_3,

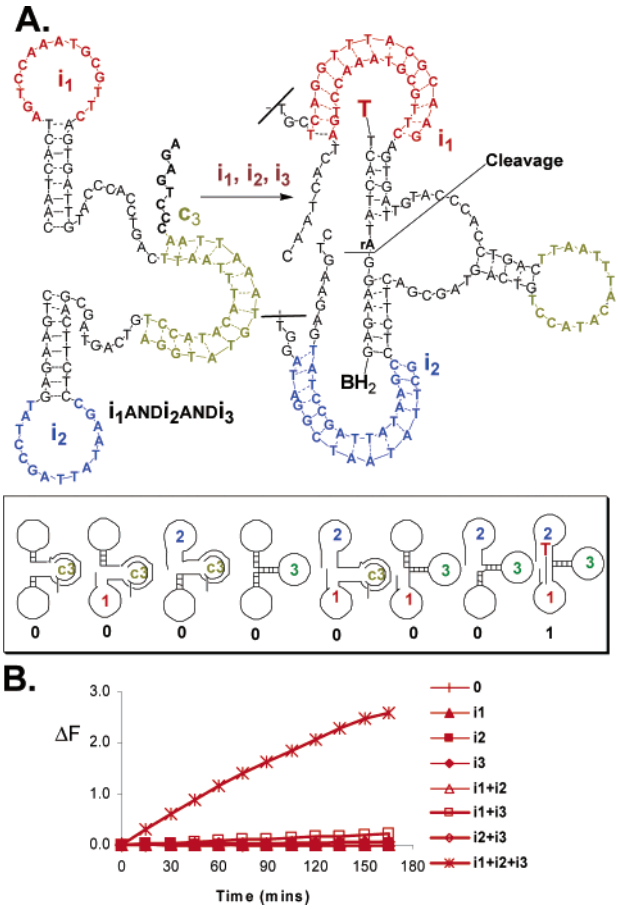

FIGURE 5: (A) Top: $i_1ANDi_2ANDi_3$ gate-like behavior of i_1ANDi_2-ANDNOTc_3 gate precomplexed with c_3 oligonucleotide. Addition of inputs i_1 and i_2 opens inhibitory stems in the stem−loops attached to the substrate recognition regions, while the addition of i_3 removes the inhibitory oligonucleotide c_3. Thus, the addition of all three inputs is necessary to switch the gate to the active form, which cleaves the fluorogenic substrate with TAMRA (T) and black hole 2 (BH_2). Bottom: Schematic representation of the gate in its eight states, of which only one is active. (B) Fluorescence activity (in millions) of the gate upon addition of each input combination. Gate $i_1ANDi_2ANDNOTc_3$ (100 nM) was combined with 400 nM oligonucleotide c_3 in reaction buffer containing 1.25 μM substrate S_T. Appropriate inputs were supplied at 2 μM, and the TAMRA (red channel) fluorescence was monitored every 15 min for 3 h.

which we designed to be complementary to a 23-mer region in the actual input i_3. In order for this gate to be active, not only i_1 and i_2 need to be present in solution but also i_3 to remove c_3 from its inhibitory complex. Because i_3 was used as a direct input in *other* gates, we designed the 30-mer input with a 15-mer region used as a direct input (green) and a 24-mer region used to remove c_3 from its complements (boxed) (Figure 3B). After some optimization of the stem lengths and the ratio of gate to inverter oligonucleotides, we found that the $i_1ANDi_2ANDNOTc_3$ (Figure 5A) gate behaved exactly as predicted in the presence of c_3, that is, as i_1ANDi_2-$ANDi_3$. Addition of all three inputs activated the system for fluorogenic cleavage (Figure 5B), while the omission of any of the inputs inhibited this increase. The $i_1ANDi_2ANDi_3$ gate is an eight-state system with only one active state, when all three inputs are present. Thus, for the first time we report a three-input deoxyribozyme-based logic gate with three positive inputs.

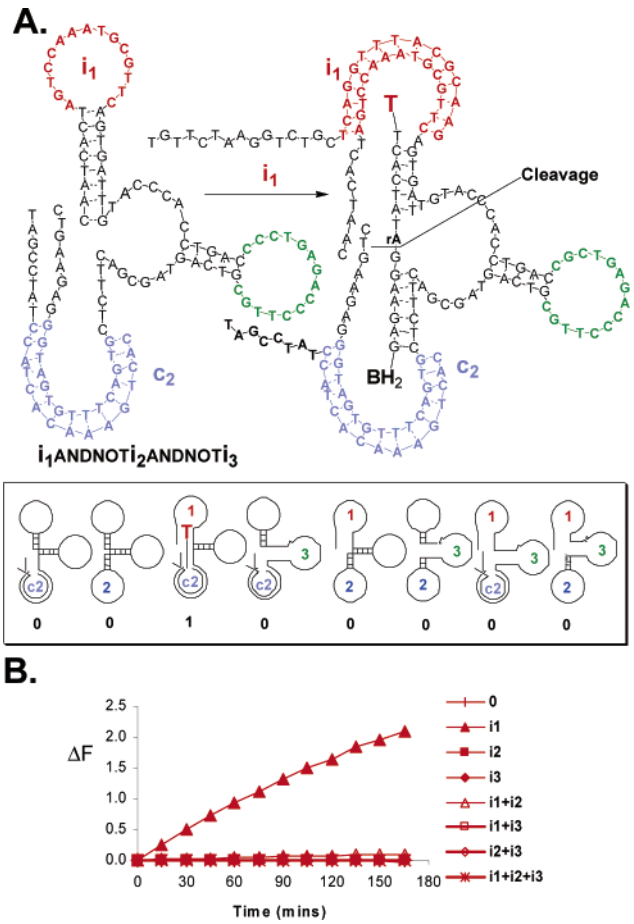

FIGURE 6: (A) Top: i_1ANDNOTi_2ANDNOTi_3 gate-like behavior of the i_1ANDc_2ANDNOTi_3 gate precomplexed with the c_2 oligonucleotide. The addition of input i_1 opens inhibitory stems in the stem−loops attached to the substrate recognition regions, while the addition of i_2 removes the activating oligonucleotide c_2. Thus, only the addition of i_1 will switch the gate into the active form, in which it cleaves the fluorogenic substrate with TAMRA (T) and black hole 2 (BH_2). Addition of any of the two remaining inputs will turn the gate off. Bottom: Schematic representation of the gate in its eight states, of which only one is active. (B) Fluorescence activity (in millions) of the gate upon addition of each input combination. Gate i_1ANDc_2ANDNOTi_3 (100 nM) was combined with 400 nM oligonucleotide c_2 in reaction buffer containing 1.25 μM substrate S_T. Appropriate inputs were supplied at 2 μM, and the TAMRA (red channel) fluorescence was monitored every 15 min for 3 h.

ANDNOTANDNOT Gates. The same principle was extended to accomplish i_1ANDNOTi_2ANDNOTi_3 behavior (Figure 4C). In this case we negated a positive allosteric input to construct i_1ANDc_2ANDNOTi_3, behaving as i_1ANDNOTi_2ANDNOTi_3 (Figure 6A). Oligonucleotide c_2 was precomplexed with the gate such that addition of input i_2 deactivated the gate. This protocol produced a gate with two inputs acting as inhibitors (i_2 and i_3) and only one input (i_1) being sufficient (and necessary) to switch the gate to the active state. Thus we created a three-input gate with one positive and two negative inputs. Again, complete digital behavior of all eight states of this gate was observed (Figure 6B).

The remaining two gates for computation of the sum digit were similarly constructed. Due to the idiosyncrasies of secondary structures, we could not apply our modular design straightforwardly, and simply rotate the inputs, but we had to go through trial-and-error loop rotation, which eventually led us to the gates i_3ANDc_1ANDNOTi_2 as i_3ANDNOTi_1ANDNOTi_2 and, finally, i_2ANDc_1ANDNOTi_3 as i_2ANDNOTi_1ANDNOTi_3. Again, complete digital behavior of all eight states of these gates was observed and is shown in the Supporting Information.

The Molecular Full Adder. The three AND gate components of the carry digit were constructed as previously described for a single gate in our half-adder work (*12*). Full structures and testing of all states in individual gates are given in the Supporting Information. A molecular system displaying full-adder behavior was obtained by combining all 10 components (seven gates and three inverters) of the system together into a single tube (Figure 7A). The best digital behavior of the system was observed at lower concentrations of gates (50 nM), which confirmed our earlier observation that a current limitation of the technology is defined by the total concentration of an increasing number of gates and the lowest concentration at which individual gates can be observed. At higher concentrations, some of the gates were interacting with each other, which led to the display of imperfect digital behavior and lower activity. Using every possible combination of inputs, the full adder produced a correct response with a readable output within 15 min or less (Figure 7B). Importantly for future biological applications, almost perfect digital behavior was still observed even after several hours.

DISCUSSION

Here we report the construction of a solution-phase array of seven optimized deoxyribozyme-based logic gates that senses three inputs and determines whether to produce one, both, or none of two outputs. This array represents the first example of an artificial decision-making enzymatic network that mimics an electrical engineering element called a full adder and has the potential to be cascaded into downstream recognition elements.

Development of the full adder required molecular scale gates capable of producing conjunctive outputs based on three inputs of arbitrary polarity, without resorting to serial connections between gates. Such gates were not previously available. Instead, they were created by the use of controlling oligonucleotides precomplexed to our three-input ANDANDNOT gates. Gates with excellent digital behavior were then constructed that have positive allosteric effects in the presence of all three inputs (an ANDAND logic gate) and one positive and two negative allosteric effectors (ANDNOTANDNOT gates). The remaining three-input gate not required for this work is NOTi_1ANDNOTi_2ANDNOTi_3 and is a technical extension of this general principle, indicating that the use of inverters provides the ability to create any three-input gate with arbitrary polarity of the inputs.

Two design issues were identified during our research. First, at higher gate concentrations (total concentrations >1 μM), the gates influenced each other, and therefore much lower concentrations were required to attain perfect full-adder behavior. It is likely that a judicious choice of oligonucleotides would allow the use of higher concentrations of gates, although probably at the expense of generality. We are currently working to generate in silico a set of oligonucleotides that would give us the ability to use the gates at a very high total concentration. This would allow us to perform more complex Boolean calculations with a larger number

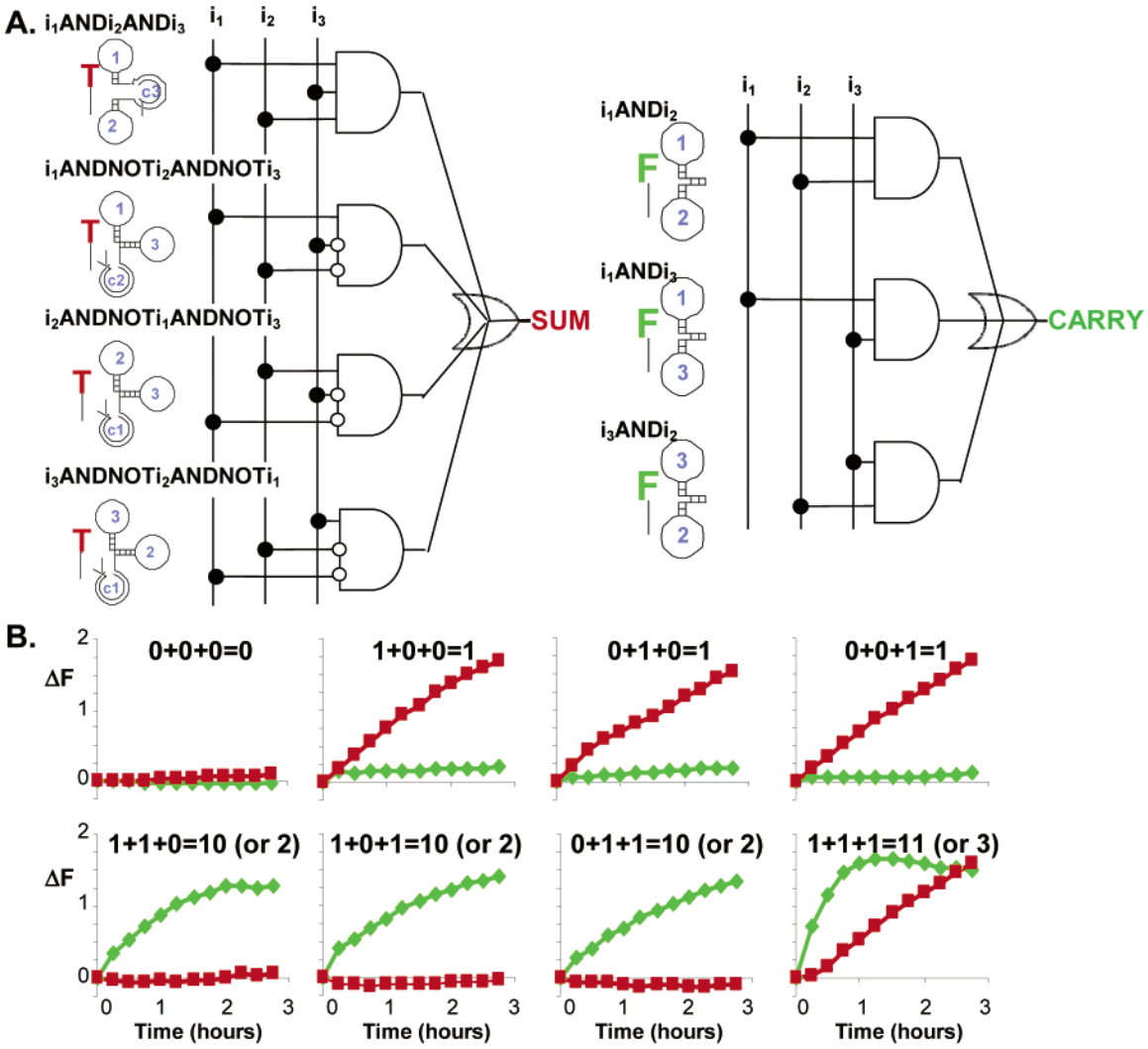

FIGURE 7: (A) Full-adder implementation in deoxyribozyme-based logic. In a single tube, the sum digit is created by the combination of a single ANDAND gate and three ANDNOTANDNOT gates, and the carry digit is created by the combination of three AND gates. (B) Ability of the deoxyribozyme-based full adder to add single bits. Gates (50 nM) were combined with 200 nM oligonucleotides c_1, c_2, and c_3 in reaction buffer containing 1.25 μM substrate S_T and 1 μM substrate S_F. Appropriate inputs were supplied at 1 μM. Red lines indicate the sum digit TAMRA fluorescence change over time (ΔF), and green lines indicate the carry digit fluorescein fluorescence; fluorescence units are in millions. Perfect digital behavior was observed for each possible arithmetical permutation.

of inputs and outputs. Nevertheless, the unexpected behavior of gates in complex mixtures might be of interest as a tool to study and understand the emergent behavior of complex networks that could not be explained as a sum of their individual components. As a second issue, we noticed that, despite the remarkable successes of modular design, we were not able routinely to rotate stem−loops to design all possible combinations of ANDNOTANDNOT gates. This apparent breakdown in the modular design will also likely be eliminated with the availability of improved oligonucleotide libraries that are specifically matched to the substrates and core enzymes.

The use of external controlling elements to alter gate behavior is reminiscent of some classical in vivo regulatory pathways. The combination of both our in cis stem−loop regulation and in trans controlling elements paves the way for the creation of, one hopes, even more complex artificial networks, which could mimic natural systems through the use of multifunctional regulatory components. The development of complex artificial biochemical networks capable of performing basic computational procedures is a key component of our long-term aim of developing autonomous therapeutic molecular devices.

SUPPORTING INFORMATION AVAILABLE

Sequences and individual testing of all gates used in the construction of full adder. This material is available free of charge via the Internet at http://pubs.acs.org.

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