Communication

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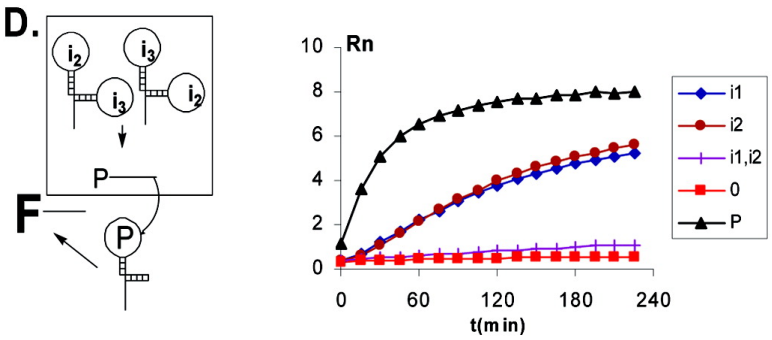

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Deoxyribozyme-Based Ligase Logic Gates and Their Initial Circuits

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Molecular scale logic gates[1] based on nucleic acid catalysts have been proposed as key components of autonomous therapeutic molecular-scale devices.[2] We envisage these devices as ensembles of molecules that analyze multiple disease markers in vivo and, using Boolean computation, determine whether to release a therapeutic agent. Previously we reported modularly[3] designed phosphodiesterase[4] logic gates,[2a] which analyze a series of oligonucleotide inputs and provide catalytically cleaved oligonucleotides as outputs. We used these cleavase gates to construct molecular circuits and automata.[5] The next step in this field is to expand available logic units to enzymes that can form larger oligonucleotides, that is, ligases.[6] The ligases would be useful because (1) longer outputs could be cascaded into downstream gates, allowing the construction of serial circuits, and (2) ligases could feed new substrate to phosphodiesterases, allowing us to reset circuits. We now report the first complete set of ligase logic gates,[7] which analyze two oligonucleotide inputs and produce a ligated oligonucleotide output. We have also constructed the first serial molecular logic gate circuits using gate-to-gate communication through molecular transfer, wherein output from the ligating gates is sensed by phosphodiesterase gates in a cascade[8] with ultimate fluorogenic output.

For a ligating module we used ligase **E47** (Figure 1) reported by Szostack's group,[6a] which requires chemically activated phosphoimidazole groups (**PIM**) at the 3′ end of a substrate $\mathbf{S_2}$ (Figure 1). This enzyme is Zn^{2+} (or Cu^{+})-dependent, with k_{cat} of ~2 h^{-1}, with the rate-limiting step being the product **P** release (as would be expected on the basis of base-pairing considerations). Whereas initial studies with this ligase used gel electrophoresis to visualize product formation, we performed the ligation reaction in the presence of a detector, the phosphodiesterase $_{P}$YES**P**[2a,4] gate. In the presence of the ligated product **P**, the detector gate is activated to fluorogenically cleave the substrate $\mathbf{S_F}$. We introduced a single point mutation in the downstream gate (boxed G−G mismatch in Figure 1) to eliminate the activation of the $_{P}$YES**P** gate by the substrates S_1 and S_2 in the absence of the ligase. In the absence of this modification, we did not see any difference between reaction and no-ligase controls.

Because the catalytic unit of **E47** has a general "hammerhead-like" structure, we hypothesized that stem−loop modules could be used to introduce allosteric control by oligonucleotides, as we have done previously with phosphodiesterase gates.[2a] Accordingly, we constructed a complete set of ligase logic gates, consisting of NOT and AND elements. We created the $_{L}$NOTi$_1$ gate—negatively regulated by a specific input, oligonucleotide i_1—by replacing a nonessential stem−loop with a molecular beacon stem−loop (Figure 2). In the absence of an input oligonucleotide complementary to the loop, the $_{L}$NOTi$_1$ gate catalytic core is undisturbed and the ligase is constitutively active. However, if i_1 is added to the reaction mixture, it binds to the loop opening the stem and distorts the catalytic core, inhibiting the ligation reaction. The activity of this gate is visualized (Figure 3A) by the creation of a molecular circuit $_{L}$NOTi$_1$ → $_{P}$YES**P** in which one molecular logic gate ($_{L}$NOTi$_1$) transfers its product (**P**) to another logic gate ($_{P}$YES**P**), which is then activated to cleave a fluorogenic substrate. The successful coupling of two gates provides us with more than just a convenient fluorogenic readout. To the best of our knowledge, this is the first artificial example of nonphotonic gate-to-gate communication of molecular scale in solution, with an upstream gate signaling its state to a reporting downstream gate. This result also offers a possible solution to a long-standing problem in solution-phase molecular computation, that is, construction of multicomponent circuits. Importantly, the omission of ligase (Figure 3A), or unactivated substrate $\mathbf{S_2}$ (not shown), fully suppresses the increase of fluorescence.

Our next step was to achieve positive regulation of ligases and YES gatelike behavior. To this end, we added a stem−loop to the 5′ end of the enzyme such that the stem inhibits the docking of 5′ substrate $\mathbf{S_1}$ and constructed $_{L}$YESi$_2$ (Figure 2, right side structure, only the blue 5′ stem−loop added). Again, the molecular circuit $_{L}$YESi$_2$ → $_{P}$YES**P** was used to visualize the activity of the upstream gate. In this circuit (or cascade), the input i_1, complementary to the loop, opens the inhibitory stem thereby allowing substrate $\mathbf{S_1}$

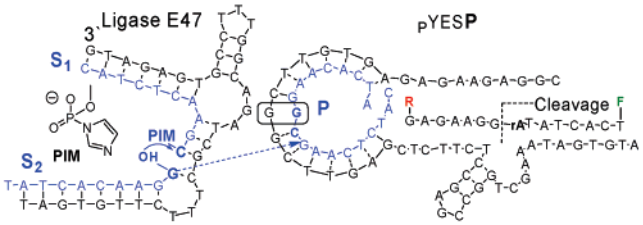

Figure 1. Ligase E47 combines two substrates ($\mathbf{S_1}$ and $\mathbf{S_2}$, activated by **PIM**), into the longer oligonucleotide product, **P**. The product of active ligases activates the downstream detector gate ($_{L}$E47 → $_{P}$YES**P**, where $_{P}$ stands for phosphodiesterase), which cleaves the fluorogenic substrate.

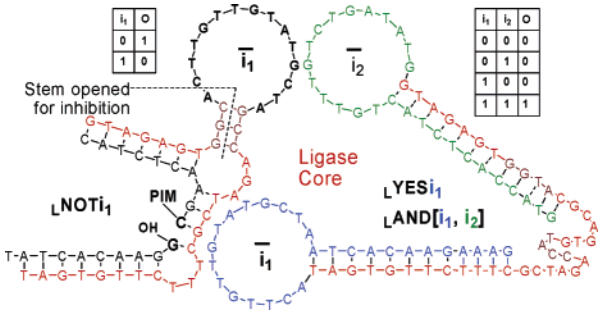

Figure 2. Ligase-based gates: $_{L}$NOTi$_1$, $_{L}$YESi$_2$ (blue loop only attached to red core), and $_{L}$AND[i_1, i_2] (both blue and green loops attached to red core, brown exchangeable stem−loop); $_{L}$ stands for ligase. Truth tables for NOT and AND gates shown.

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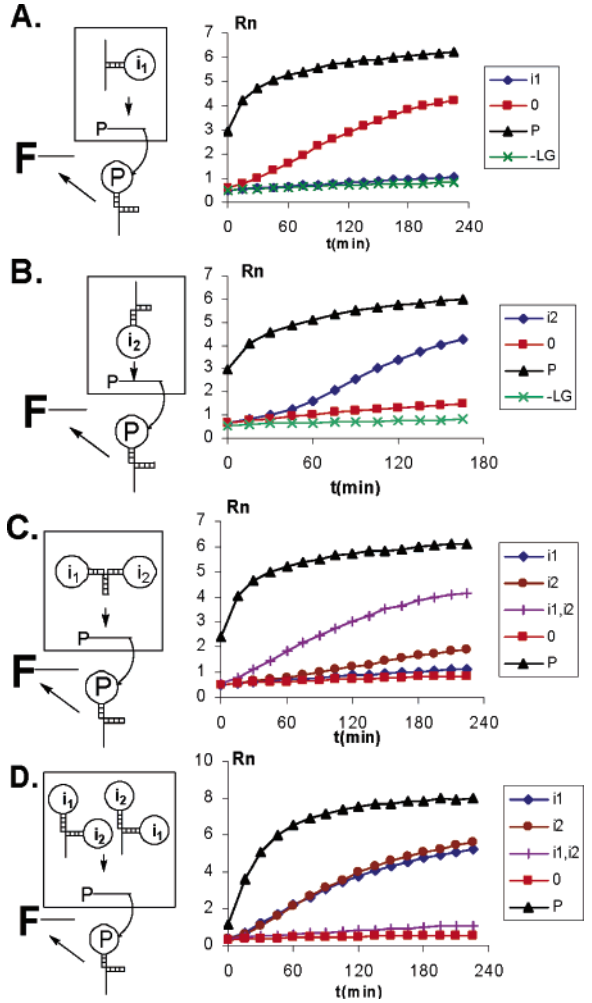

Figure 3. (A) Activity of circuit $_{L}$NOTi$_1$ → $_{P}$YES**P** (schematic representation shown on the left side) observed as time-dependent fluorescence change (Rn value is ratio of λem$_{520}$ and λem$_{580}$ with λexc$_{480}$). (B) Activity of the circuit $_{L}$YESi$_2$ → $_{P}$YES**P**. (C) Activity of circuit ($_{L}$AND[i$_1$,i$_2$]) → $_{P}$YES**P**. (D) Activity of circuit ($_{L}$ANDNOT[i$_2$, i$_3$] OR $_{L}$ANDNOT[i$_3$,i$_2$]) → $_{P}$YES**P**. Legend for all: 0, no inputs added; P, positive control; −LG, no logic gate negative control.

to bind to the ligase. This docking event triggers the formation of longer oligonucleotide product (**P**), which is then transferred to the detector gate, triggering fluorogenic cleavage of $\mathbf{S_F}$, as shown in Figure 3B.

The attachment of two stem−loops, one responsive to i$_1$ at the $\mathbf{S_1}$ recognition region and another, complementary to i$_2$, at the $\mathbf{S_2}$ recognition site, led to $_{L}$AND[i$_1$,i$_2$] gatelike behavior (Figure 2 right side structure, with both 5′ blue and 3′ green stem−loops). Again, activity of this molecular logic element was observed via the fluorogenic cleavage in the circuit $_{L}$AND[i$_1$,i$_2$] → $_{P}$YES**P**. Figure 3C shows the real-time fluorescence changes of the solution containing this cascade in the presence of both i$_1$ and i$_2$, in the presence of either i$_1$ or i$_2$, and in the absence of i$_1$ and i$_2$, together with positive and negative controls (+**P**, −gate). An important difference emerges from the comparison of $_{L}$YES, $_{L}$NOT, and $_{L}$-AND ligase gates with their phosphodiesterase gate analogues ($_{P}$-YES, $_{P}$NOT, and $_{P}$AND). The three ligase gates have comparable activities in circuits, whereas the phosphodiesterase $_{P}$AND gate is almost 1 order of magnitude slower than the corresponding $_{P}$YES logic element.[2] This difference in behavior is likely the result of the change in the rate-determining step from the productive substrate binding in $_{P}$AND gates to the product release in all ligase gates.

Finally, we designed a cascade of two $_{L}$ANDNOT gates in an implicit OR arrangement, feeding the phosphodiesterase detector gate to yield an XOR circuit: ($_{L}$ANDNOT[i$_1$, i$_2$] OR $_{L}$ANDNOT[i$_2$, i$_1$]) → $_{P}$YES**P** (Figure 3D). The two $_{L}$ANDNOT gates have opposite behavior: the first gate is active in the presence of i$_1$ and absence of i$_2$, while the second is active in the presence of i$_2$ and absence of i$_1$. The fluorogenic cleavage catalyzed by the downstream phosphodiesterase gate occurs in the presence of *either* i$_1$ or i$_2$ but occurs only to a negligible degree in the presence or absence of *both* inputs. To achieve an overlap in the intensity of active states we had to decrease the concentration of the faster of the two ligase gates.

In conclusion, we demonstrated that: (1) ligases can be controlled through the same principles of allosteric control (stem−loop attachment) as phosphodiesterases, (2) construction of a full basic set of molecular logic gates can be achieved using ligase units, (3) the upstream (donor) ligase-based logic gates can be cascaded into downstream (acceptor) phosphodiesterase gates, and (4) the activity of upstream gates can be followed by the fluorogenic cleavage performed by downstream gates. In light of our recent advances in the ability to control the binding of small molecules to aptamers,[9] these results bring us one step closer to our eventual goal of autonomous therapeutic ensembles of molecules in the form of feed-forward cascades starting from sensors and ending in the activity (or not) of drug delivery elements.

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Supporting Information Available: Experimental conditions and sequences of gates used to form cascades. This material is available free of charge via the Internet at http://pubs.acs.org.

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