Biocomputing

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Concatenated Logic Circuits Based on a Three-Way DNA Junction: A Keypad-Lock Security System with Visible Readout and an Automatic Reset Function**

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Abstract: *Concatenated logic circuits operating as a biocomputing keypad-lock security system with an automatic reset function have been successfully constructed on the basis of toehold-mediated strand displacement and three-way-DNA-junction architecture. In comparison with previously reported keypad locks, the distinctive advantage of the proposed security system is that it can be reset and cycled spontaneously a large number of times without an external stimulus, thus making practical applications possible. By the use of a split-G-quadruplex DNAzyme as the signal reporter, the output of the keypad lock can be recognized readily by the naked eye. The "lock" is opened only when the inputs are introduced in an exact order. This requirement provides defense against illegal invasion to protect information at the molecular scale.*

Molecular logic gates are molecular-scale computers that perform Boolean operations to carry out binary computational functions.[1] As a smart logic device, the keypad lock provides a new approach for protecting information against illegal invasion at the molecular level and has raised ever-increasing interest in recent years.[2] What distinguishes the molecular keypad lock from a simple logic gate is the fact that its output signals are dependent not only on the proper combination of inputs but also on the order in which these inputs are introduced.[3] In other words, one needs to know the exact password that opens this lock. Several research groups have successfully employed DNA, enzymes or antibodies as the building blocks to construct molecular keypad-lock systems with elegant design strategies.[4] Despite burgeoning developments, most keypad locks have no system-reset function[2d,4e–g] or require an external stimulus (for example, EDTA,[5a] a gas,[5b] DNA,[5c] or an enzyme[5d,e]) to activate the reset function, which limits their practical application. Therefore, it would be highly desirable to develop a keypad-lock security system with an automatic reset function.

Toehold-mediated DNA strand displacement has proven to be a powerful tool for receiving and transferring DNA input information without enzymes.[6] This reaction is a three-step process that includes toehold binding, branch migration, and strand dissociation.[7] The toehold sequestering principle, according to which the toehold domains of later gates are inaccessible and will not be triggered until the preceding gates in the circuit have been executed, has been successfully exploited to construct DNA-based logic gates,[1a,4b,8a,b] signaling cascades,[8c–g] and even neural networks.[8h,i] The remaining challenge is to design concatenated logic gates that can operate as a keypad-lock security system to protect information at the molecular level.

Peroxidase-mimicking G-quadruplex DNAzyme is an ideal signal reporter to monitor logic operations.[4d,8a,9] Upon binding to hemin, the active G-quadruplex/hemin complex exhibits superior peroxidase-like activity and is able to catalyze the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) by H_2O_2 to generate a colorimetric signal.[10] In contrast to indirect fluorescence and electrochemical signals, the G-quadruplex DNAzyme provides a direct and visible readout which can be readily distinguished by the naked eye.[11] Several research groups have designed a strategy whereby the G-quadruplex is split into two fragments that lack catalytic activity, but upon template-assisted formation of intact G-quadruplex DNAzyme from the split fragments, the peroxidation activity is restored.[12] It would be of great interest to integrate such a split-G-quadruplex strategy into molecular logic design for the construction of intelligent and visible keypad locks. In this study, we developed concatenated logic circuits operating as a biocomputing keypad-lock security system with an automatic reset function on the basis of toehold-mediated strand displacement and three-way-DNA-junction architecture. The output of the keypad lock can be directly recognized by the naked eye.

The mode of operation of the concatenated DNA-based logic circuits is illustrated in Scheme 1. A biotin-modified single-stranded initiator I was anchored on streptavidin-coated magnetic beads (MBs) through the interaction between biotin and streptavidin. Immobilized DNA I was used as the initial state. Three hairpin structures were selected as inputs (inputs A, B, and C). The two G-rich segments of the 3:1 split G-quadruplex sequence were distributed at the 5′ and 3′ ends of those hairpins. First, DNA strand I uses the red segment as a toehold to bind the red domain of input A, thus initiating a branch migration (toehold-mediated strand displacement) to open hairpin input A with the formation of an intermediate I·A in which the yellow segment of input A is open (Scheme 1, reaction 1). Then, the yellow domain of input B can hybridize to the newly exposed toehold (yellow segment) of input A, thus again initiating a branch migration

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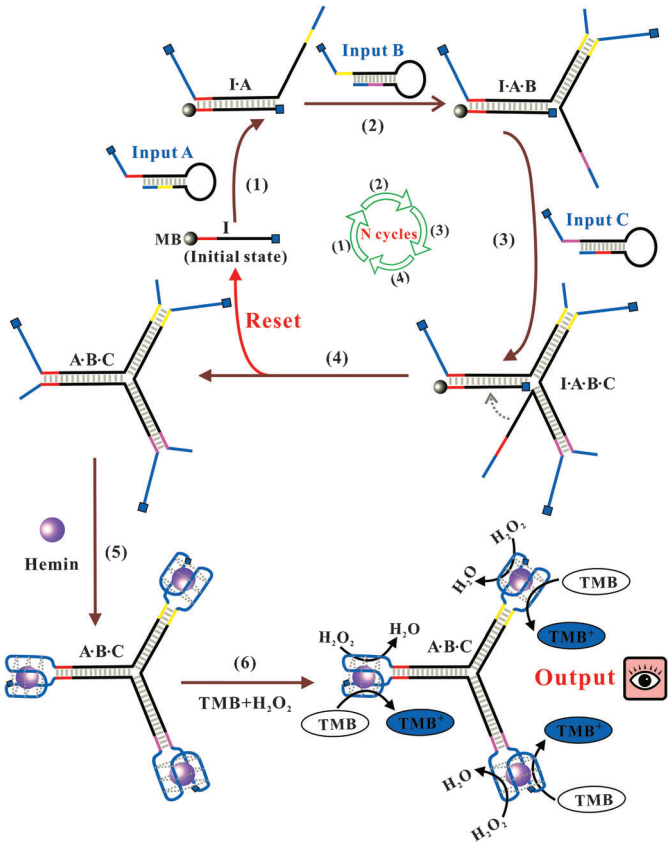


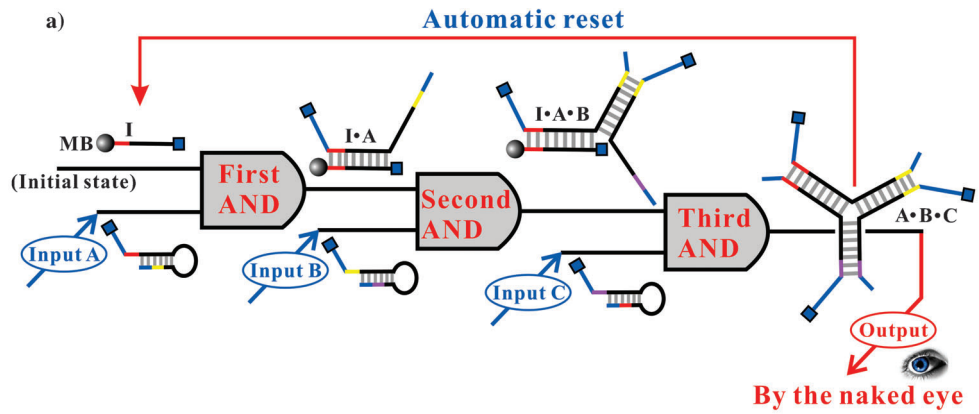
Scheme 1. Schematic illustration of the design principle of the concatenated logic circuits operating as a visible keypad-lock security system with an automatic reset function. The reaction is based on toehold-mediated strand displacement and three-way-DNA-junction architecture. Toeholds and toehold-binding regions are indicated in the same color (red, yellow, and purple). The fragments of the split G-quadruplex are marked in blue. DNA strands are drawn as lines with squares at the 5′ ends.

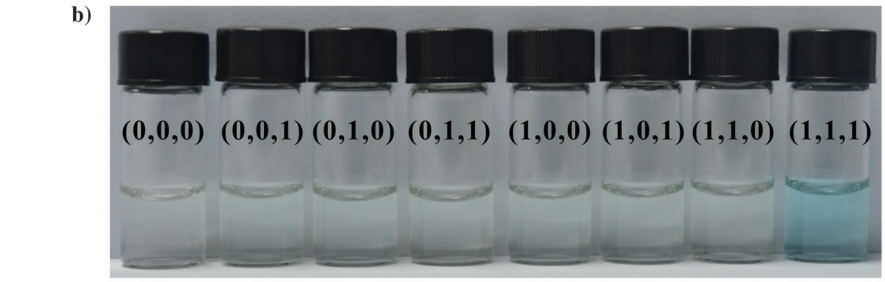
to open the hairpin of input B and form an I·A·B hybrid in which the purple segment of input B is no longer occluded (Scheme 1, reaction 2). Analogously, the purple domain of input C can then bind to the newly accessible toehold (purple segment) of input B to initiate strand displacement that opens the hairpin of input C and generates the I·A·B·C complex (Scheme 1, reaction 3). As this complex is inherently unstable, a spontaneous dissociation step occurs in which input C displaces I from the complex, thus freeing I to catalyze the logic operation of additional inputs (Scheme 1, reaction 4). Rinsing of the MBs after each hybridization reaction can remove unwanted hairpin inputs. Inputs can only hybridize in the right order with the previous input hairpin DNA through the toehold-mediated strand-displacement reaction. After magnetic separation, the three-way DNA junction (A·B·C product) will be left in solution. Such a three-way-junction structure can draw the 3:1 split G-quadruplex fragments together and induce the formation of intact G-quadruplex probes at each end of the three arms. Upon incubation with hemin, catalytic hemin/G-quadruplex peroxidase-mimicking DNAzymes should be formed (Scheme 1, reaction 5). The active DNAzymes can effectively catalyze the H_2O_2-mediated oxidation of TMB to generate a colored output signal, which can be readily distinguished by the naked eye (Scheme 1, reaction 6).

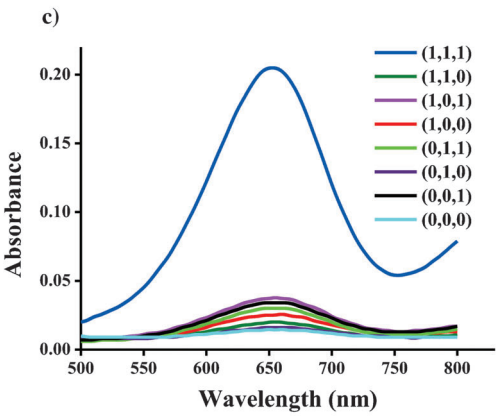
The formation of the three-way-DNA-junction structure through toehold-mediated strand-displacement reactions was confirmed by native polyacrylamide gel electrophoresis (PAGE; see Figure S1 in the Supporting Information). Fundamental logic gates are the basic building blocks for the construction of superior biocomputing security systems. Watson–Crick interactions and the toehold sequestering ability of hairpin DNA make it possible to fabricate a three-way DNA junction in a cascaded manner. The three-way junction can be regarded as a network composed of three concatenated AND logic gates. The DNA circuit involves stepwise treatment with different hairpins defined as input signals. The hairpin inputs (A, B, and C) are considered as 1 when they are present and 0 if they are absent. The output signal of the concatenated logic system is the color change of the solution (see Figure S2 for the effect of the concentration of hairpin strands on the color intensity of the proposed logic system). The colorless solution without the three-way DNA junction is defined as 0, and the blue solution containing the three-way DNA junction is defined as 1. The AND logic gate is represented by the situation in which the output is 1 ("On" state) only if all three inputs are present (1,1,1), whereas for other input combinations, the output reads 0 ("Off" state).

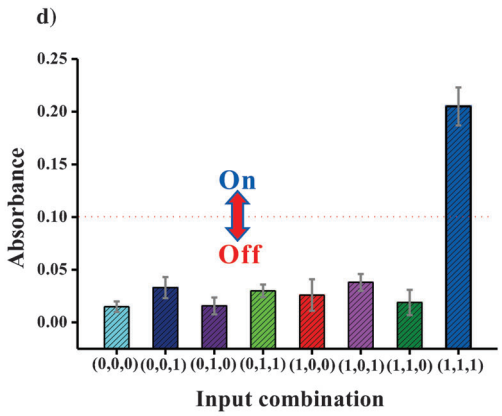
Figure 1 a shows the operation circuit of the concatenated AND logic gates. The output signal of one gate serves as an input signal for a downstream gate. The input of initiator I for the first AND gate was always 1. The I strand hybridizes with input A to form an intermediate I·A that acts as the output of the first AND gate. I·A generated by the first AND gate and input B activate the second AND gate, which yields a complex I·A·B as the output. Subsequently, input C and the complex I·A·B trigger the computation of the third AND gate to generate a product A·B·C as the output. The output A·B·C (three-way DNA junction) is released into solution if and only if all three inputs are present. In the presence of hemin, G-quadruplex DNAzymes are formed on the three-way DNA junction and catalyze the H_2O_2-mediated oxidation of TMB to generate a color change. Figure 1 b presents typical photographic images of the concatenated AND logic gates taken during the analysis of different input states. The solution of the system turned blue only for the (1,1,1) input state. No significant color change was observed for other input combinations. The corresponding absorption spectra obtained for the eight different combinations of the three inputs are shown in Figure 1 c. Accordingly, the system state (1,1,1) yielded a high absorbance value at $\lambda = 650$ nm. Figure 1 d presents the output of the concatenated gates in the form of an absorbance bar presentation. The high and weak absorbance values were considered as 1 ("On") and 0 ("Off"), respectively, with a threshold value of 0.1. The biocomputing responses obtained from the DNA circuit correspond to the truth table expected for the sequence of the concatenated AND logic gates (Figure 1 e).

The most important feature of the keypad-lock security system is the dependence of the output signal not only on the proper combination of the inputs but also on the order in









e)

Inputs			Output
Input A	Input B	Input C	Blue solution
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

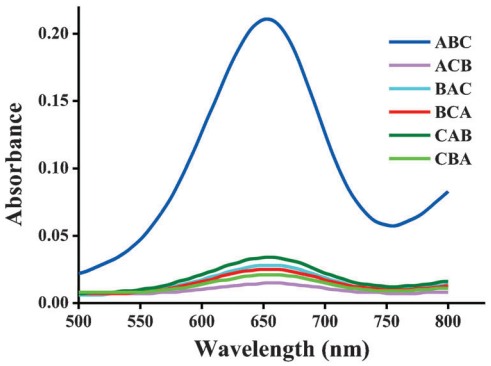
Figure 1. a) Representation of the DNA circuit as a network of three concatenated AND logic gates. b) Photographs of the concatenated AND logic gates during analysis of the eight different combinations of the three input signals. c) Corresponding absorbance features of the concatenated AND logic gates. d) Bar presentation of the output of the concatenated AND logic gates, as derived from the absorbance at $\lambda = 650$ nm. The threshold absorbance value of 0.1 is shown by the dotted line. Error bars represent the standard deviation of three independent experiments. e) Truth table for the network composed of three concatenated AND logic gates.

which these inputs are introduced. In other words, one needs to know the exact password that opens this lock. Thus, we varied the order of the three input signals in six different permutations (ABC, ACB, BAC, BCA, CAB, and CBA). As expected, only one correct order of the input signals (ABC) resulted in the appearance of the colorimetric signal (blue solution or high absorbance value at $\lambda = 650$ nm; Figure 2a,b). This color change was considered as the true output signal 1 ("On" state), corresponding to the correct application of the ABC password to open the keypad lock. All other wrong orders of the input signals (ACB, BAC, BCA, CAB, and CBA) did not generate the active G-quadruplex DNAzyme in solution, thus resulting in no signal production. The absence of a signal was considered as the false output signal 0 ("Off" state), which corresponds to the denial of unauthorized access and failure to open the keypad lock. Figure 2c shows a bar presentation of the output of the keypad lock. Only the ABC input can trigger the system to adopt the "On" state, with a threshold value of 0.1. A truth table of the keypad lock is given in Figure 2d.

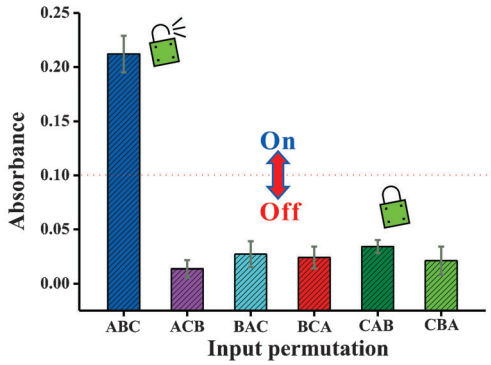
The reset capability is a key factor for keypad-lock operation from the viewpoint of practical application. However, many previously described keypad locks could perform the function only once owing to the accumulation of chemical waste. If an input password is wrong, the lock will never be

a)

b)



c)



d)

Input order			Output
Input 1	Input 2	Input 3	Blue solution
A	B	C	1
A	C	B	0
B	A	C	0
B	C	A	0
C	A	B	0
C	B	A	0

Figure 2. a) Photographs of the keypad lock in the presence of the three input signals (A, B, and C) added in different orders. b) Corresponding absorbance features of the keypad lock. c) Bar presentation of the output of the keypad lock, as derived from the absorbance at $\lambda = 650$ nm. The threshold absorbance value of 0.1 is shown by the dotted line. Error bars represent the standard deviation of three independent experiments. d) Truth table for the keypad-lock security system upon variation of the order of the A, B, and C input signals.

opened. If an input password is correct, the lock will be opened and cannot be closed again. This mode of operation limits the further development of the molecular keypad-lock system. In our case, the developed keypad lock can be readily reset from both "On" and "Off" states. In our current design strategy, after completion of the toehold-mediated strand-displacement reaction to generate the three-way-DNA-junction product (corresponding to the "On" state with the ABC password), the keypad-lock security system can be automatically reset to the initial state (Scheme 1 and Figure 1a, red arrows). This reset function of the biocomputing system occurs spontaneously without an external stimulus (for example, the introduction of EDTA, a gas, DNA, or an enzyme[5] to drive the reset function). The keypad lock with the wrong order of addition of the input signals (ACB, BAC, BCA, CAB, and CBA) stays in the "Off" state, which leads to the isolation of complex I·A by magnetic separation. For I to be released from I·A to reset the system, inputs B and C should be added in turn. Since the keypad lock is attached to a solid substrate (magnetic bead), it can be conveniently separated from the introduced chemical inputs by simple magnetic separation, thus eliminating chemical accumulation. Therefore, no waste is left in the security system after it is reset. After resetting of the keypad lock, the "On" or "Off" state could be reached again when the system was triggered by the inputs according to the order ABC or the other five permutations (Figure 3). The operation of the keypad lock with the reset function can be directly visualized in Figure S3 of the Supporting Information (see Figure S4 for the corresponding absorbance spectra for the reset operations). In previously reported keypad-lock systems, the signal of the "On" state appeared to significantly decrease after resetting.[4a,5c] Owing to the unique automatic reset function of the keypad lock in this study, the constructed biocomputing security system can be reset and cycled a large number of times without loss of the logic response. This behavior would be advantageous for practical applications of such keypad locks as well as the development of molecular security devices. Importantly, the keypad-lock system also exhibited excellent sequence specificity (see Figure S5), being able to differentiate a single-base mismatch in the toehold domain of the input DNA (see sequences in Table S1 of the Supporting Information).

In conclusion, we have successfully constructed the first example of concatenated logic circuits capable of operating as a biocomputing keypad-lock security system on the basis of toehold-mediated strand displacement and three-way-DNA-junction architecture. In comparison with previously reported keypad locks, the distinctive advantage of our proposed biocomputing security system is that it can be reset and cycled automatically a large number of times without an external stimulus, such as EDAT, DNA, a gas, or an enzyme, thus making practical applications possible. By the use of a split-G-quadruplex DNAzyme as the signal reporter, the output of the keypad lock can be readily recognized by the naked eye. The "lock" is opened only when the inputs are introduced in an exact order; this requirement offers defense against illegal invasion and the protection of information at the molecular scale. Importantly, owing to the design modularity and flexibility of DNA and its toehold sequestering ability in strand-displacement reactions, the present system may offer a promising platform for the future development of more powerful keypad-lock networks with enhanced complexity. Furthermore, in combination with aptamers or nanomaterials, the logic system based on three-way-DNA-junction architecture could be applied to biosensors, environmental monitoring, disease diagnosis, and drug delivery.

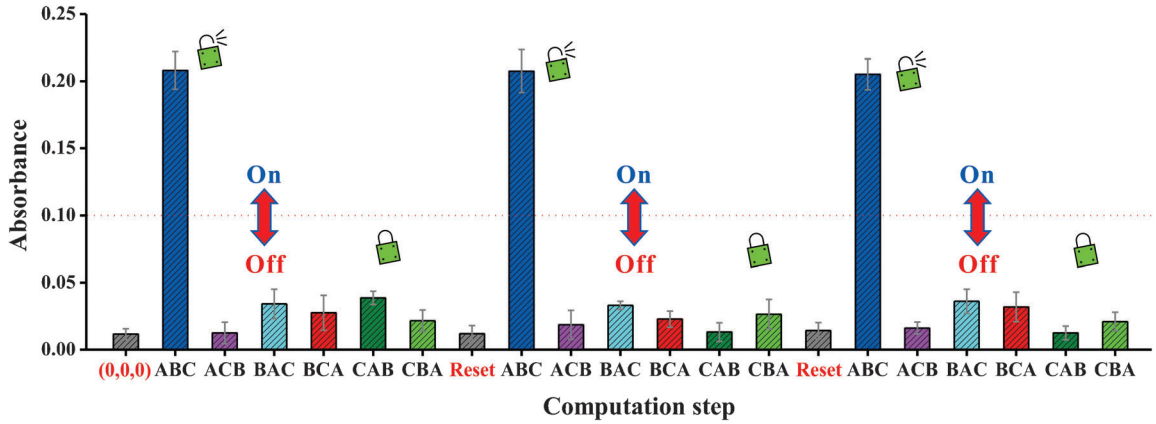


Figure 3. Computation and reset cycles of the keypad lock as triggered by different input permutations. The dotted line shows the threshold absorbance value. Error bars represent the standard deviation of three independent experiments.

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