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DNA Strand-Displacement Timer Circuits

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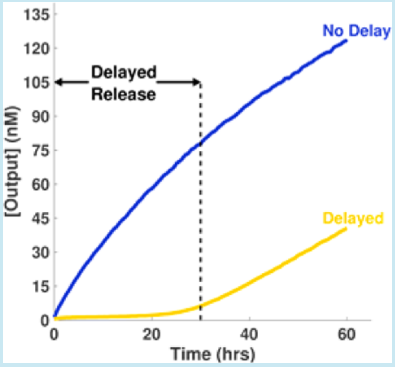
Supporting Information

ABSTRACT: Chemical circuits can coordinate elaborate sequences of events in cells and tissues, from the self-assembly of biological complexes to the sequence of embryonic development. However, autonomously directing the timing of events in synthetic systems using chemical signals remains challenging. Here we demonstrate that a simple synthetic DNA strand-displacement circuit can release target sequences of DNA into solution at a constant rate after a tunable delay that can range from hours to days. The rates of DNA release can be tuned to the order of 1−100 nM per day. Multiple timer circuits can release different DNA strands at different rates and times in the same solution. This circuit can thus facilitate precise coordination of chemical events *in vitro* without external stimulation.



While gene networks in cells can orchestrate intricate processes by modulating gene expression to release a series of target molecules at specified times,[1,2] synthetic *in vitro* biochemical protocols commonly involve manual steps performed by an experimenter, in which reagents are added, filtered, heated or otherwise altered. Artificial mechanisms to automate the temporal release of trigger molecules would make it possible to direct sequential events without the need for external stimulation. Further, the timed release of molecules using such a process could act as a trigger to control acellular self-assembly processes,[3−5] multistep reaction cascades,[6] or to time the release of signaling molecules or other reagents in cell culture.[7−9]

In this paper, we build a chemical timer circuit that releases a target sequence of DNA at a constant rate from DNA complexes after a tunable delay period. In contrast to previously designed synthetic *in vitro* transcriptional timer circuits,[10] our timer is controlled solely by DNA strand-displacement processes, which have previously been used to perform diverse information processing tasks including Boolean logic,[11−15] signal amplification,[16−19] neural network computation[20] and oscillatory signal generation.[21] The timer circuit is designed such that the strand that is released can be coupled to many of these systems in their present form, suggesting that timer circuits will make it possible to activate elaborate information processing tasks at specified times. Further, a design based on strand displacement reactions alone should allow the circuit to operate in a variety of buffers and at a variety of temperatures without redesign.[17]

The timer circuit works by suppressing the release of a single-stranded DNA molecule for a delay period, after which the molecule is allowed to increase in concentration at a constant rate. We show how to design a timer circuit within an abstract chemical reaction network, and then describe an implementation of the abstract network using a simple set of DNA molecules that interact through strand-displacement reactions. Next, we investigate the range of delay periods and release rates that are possible using our circuit, and finally demonstrate that multiple timer circuits can operate within the same solution.

The timer circuit consists of two simultaneous abstract chemical processes: *production* (eq 1) and *delay* (eq 2)

$$\varnothing_{\text{source}} \xrightarrow{k_{\text{prod}}} \mathbf{O} \tag{1}$$

$$\mathbf{O} + \mathbf{D} \xrightarrow{k_{\text{delay}}} \varnothing_{\text{waste}} \tag{2}$$

In the production process, the output **O** is released by a zero-order reaction at a constant rate k_{prod}. In the delay reaction, **O** is rapidly converted into inert waste when it reacts with a delay species **D**. If $k_{\text{delay}}[\mathbf{O}][\mathbf{D}] \gg k_{\text{prod}}$, **O** cannot accumulate until all of **D** has been depleted (Figure 1).

We call the time during which **O** cannot accumulate the delay time, t_{delay}, which is the time needed to produce enough **O** to consume all of the **D** that is initially present:

$$t_{\text{delay}} = \frac{[\mathbf{D}]_0}{k_{\text{prod}}} \tag{3}$$

This time can be easily tuned by changing the initial concentration of **D**.

During the delay period, [**O**] remains very small because any molecules of **O** that are produced are rapidly removed. After **D**

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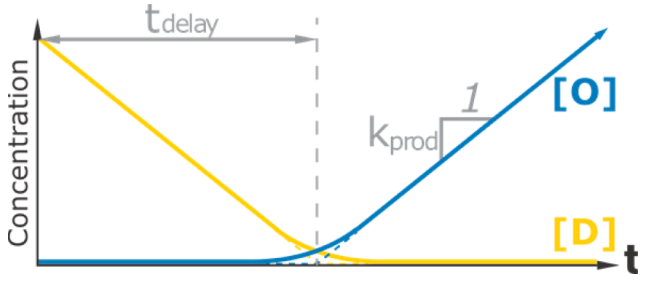

Figure 1. Schematic for the operation of a timer circuit. The output species (blue) is constantly produced at rate k_{prod}, but is rapidly consumed by the delay species (yellow). This rapid consumption prevents the accumulation of output until time t_{delay}, when the delay species is depleted.

is depleted, however, [**O**] increases linearly with time. The approximate concentration of free **O** is therefore

$$[\mathbf{O}] \approx \begin{cases} k_{prod}/(k_{delay}[\mathbf{D}]) \approx 0, & \text{if } t < t_{delay} \\ k_{prod}(t - t_{delay}), & \text{otherwise} \end{cases} \quad (4)$$

where [**O**] and [**D**] are functions of time.

To construct a timer circuit that controls the release of a DNA strand, we built a set of DNA strand-displacement reactions that emulate the abstract reactions in eqs 1 and 2 (Figure 2).[22,23] The domain level structure of our complexes follows a DNA architecture previously used for Boolean logic circuit evaluation.[15] Within this implementation, strand **O** is initially partially bound within a complex. The production process frees **O** from this complex, making **O** available in its full single-stranded form (Figure 2a). The delay process likewise sequesters **O** in a waste complex in which the toehold domain of **O** is covered (Figure 2b). Because an exposed toehold domain is generally required to initiate downstream strand-displacement reactions, the delay circuit will control when **O** is available in a functional form.

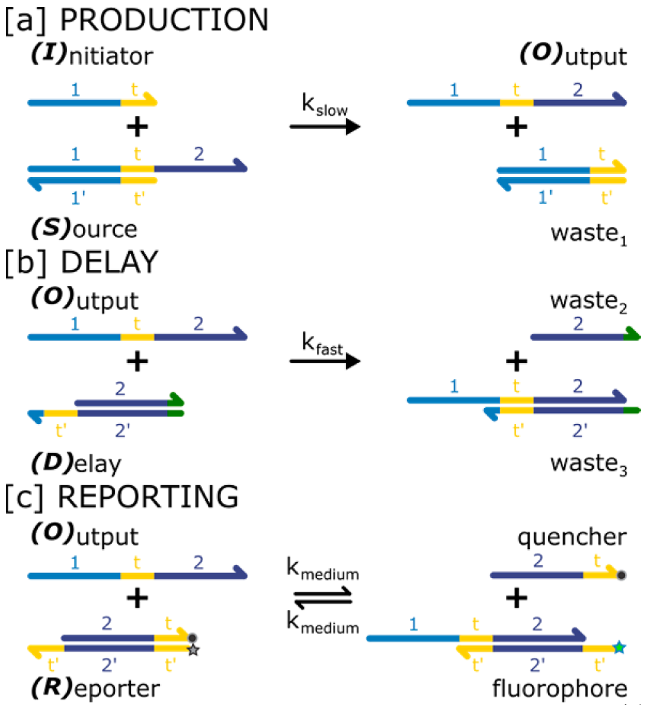

Figure 2. Strand-displacement reactions for a timer circuit. (a) PRODUCTION: Output is slowly released from source in the presence of initiator. (b) DELAY: Output is rapidly consumed by the delay complex. (c) REPORTING: Free output binds reversibly to a reporter complex, separating quencher and fluorophore modifiers. FAM and TexasRed paired with appropriate quenchers were used to report on two different output sequences (SI 0).

Production. The production reaction releases an output molecule **O** when a source complex **S** and an initiator strand **I** react (Figure 2a). This strand-displacement process is facilitated by the spontaneous pairing and unpairing of the bases on the ends of the source complex, *i.e.*, fraying. During the short time periods when these bases are frayed, **I** can bind and compete with **O** until one or the other is displaced.

Reactions initiated by fraying alone have an exceptionally small reaction rate constant, on the order of 0.5 M^{-1} s^{-1}, which we denote by k_{0bp}.[24,25] On the order of days, very little **S** and **I** react, allowing us to assume [**S**] and [**I**] remain effectively constant when considering shorter time scales. To release an appreciable concentration of **O**, we use a large amount of **S** and **I** in a reaction. The rate at which **O** is released into solution can therefore be approximated as a constant we term k_{prod}:

$$\frac{d[\mathbf{O}]}{dt} = k_{0bp}[\mathbf{S}][\mathbf{I}] \approx k_{prod} \quad (5)$$

Eq 5 shows that k_{prod} can be easily tuned by changing the initial concentrations of **S** and **I**. For simplicity, and to maximize the time during which the approximation of constant concentrations is reasonable, we keep their initial concentrations equal, *i.e.*, $[\mathbf{S}]_0 = [\mathbf{I}]_0$. At longer time scales, the approximation of a constant k_{prod} is violated and [**O**] increases according to second order reaction kinetics.

Delay. To keep [**O**] low while the delay species is present, the delay reaction must sequester **O** at a rate much faster than k_{prod}. The delay complex **D** (Figure 2b), which has a 7 base pair (7 bp) single-stranded toehold domain that binds to **O** and colocalizes it with **D**, acts as a concentration thresholding device.[14] Reactions mediated by 7 bp toeholds proceed at approximately 6 orders of magnitude faster than reactions without a mediating toehold.[24,25] A 2 bp clamp (green in Figure 2b) inhibits some undesired interactions between **S** and **D** while ensuring that the reaction between **O** and **D** remains strongly forward-biased. Clamps with only 1 bp may not reliably prevent interactions at that end and clamps with greater numbers of bases (*e.g.*, 5 bp) are expected to strongly decrease the sequestering ability of the thresholding device due to reaction reversibility.[11,17]

To monitor the reaction's progress, we also include a reporter complex modified with a fluorophore and an associated quencher to track the concentration of free **O** over time. This complex reacts reversibly with the output strand on a time scale much faster than the production reaction, but slower than the delay process, and produces fluorescence as a function of [**O**] at a given time (Figure 2c). The concentration of **O** is related to the fluorescence levels using a calibration curve (SI 1).[26,27] To build a timer circuit, the source complex **S** and the delay complex **D** are initially combined and the timer is triggered upon the addition of the initiator strand **I**.

To demonstrate that the individual reactions performed as desired and determine how the production rate varied with initial concentrations of **S** and **I**, we tested each reaction in isolation at 25 °C (Figure 3a,b, SI 2). To test the production reaction, we varied $[\mathbf{S}]_0 = [\mathbf{I}]_0$ from 0.25 μM to 2 μM (Figure

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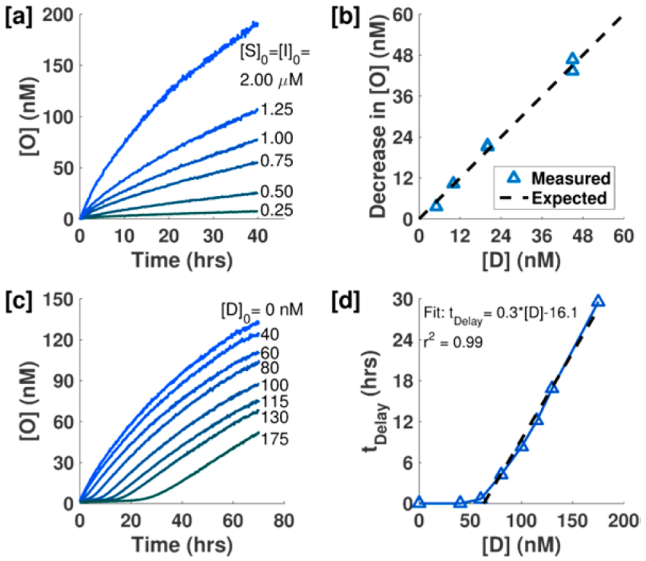

Figure 3. Production, Delay and Timer circuit reactions. (a) Production kinetics in the absence of **D**. Release rate is dependent upon initial [**S**] and [**I**]. (b) [**O**] decreases proportionally to added [**D**]. (c) Delay time was tuned by changing the initial concentration of the delay species **D**. $[\mathbf{S}]_0 = [\mathbf{I}]_0 = 1\ \mu$M. (d) Plot of delay time *vs* initial concentration of **D**, showing an approximately linear relationship after an initial offset.

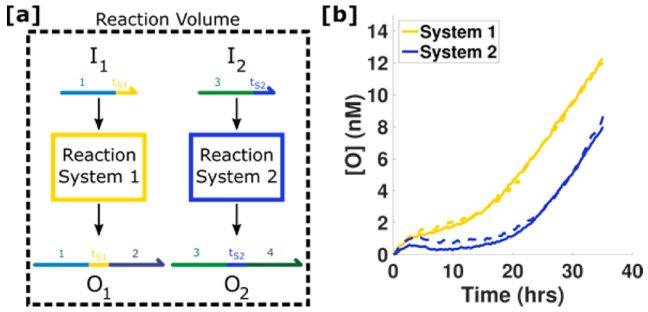

Figure 4. Multiplexed timers. (a) Schematic of multiple timer circuits operating within the same solution, releasing independent output strands at different times. (b) Two timers release output with the same rate but at different times (9 and 17 h). Here, $[\mathbf{S}]_0 = [\mathbf{I}]_0 = 0.5\ \mu$M and $\{[\mathbf{D}]_{Sys1}, [\mathbf{D}]_{Sys2}\} = \{46\text{ nM}, 63\text{ nM}\}$. Dashed lines indicate the same reactions except with each system in isolation.

3a) and determined the average k_{0bp} to be $0.49 \pm 0.13\ M^{-1}\ s^{-1}$ (SI Table S2) which is in good agreement with previous estimates.[24,25] We calculated, using $[\mathbf{S}]_0$ and $[\mathbf{I}]_0$, that k_{prod} varied from 0.15 to 4 nM/hour over the range of concentrations tested (SI Table S2). When the delay reaction was tested in isolation, the delay complex sequestered free **O**, resulting in a sudden decrease in output detected by the reporter (SI Figure S7). The decrease in [**O**] matched the concentration of **D** added to the reaction solution (Figure 3b).

To characterize the delay time before S begins accumulating as a function of $[\mathbf{D}]_0$ (eqs 3 and 4), we varied $[\mathbf{D}]_0$ while keeping the production rate (determined by $[\mathbf{S}]_0$ and $[\mathbf{I}]_0$) constant. **O** remained low for a delay period that increased with $[\mathbf{D}]_0$ (Figure 3c). For each trial, we used linear least-squares fitting to identify the portion of the production regime with the steepest slope (SI 3) and used the slope and y-intercept of this fit to calculate the delay time. This method allowed us to measure the delay time without being affected by the ramp up in release that occurs because small amounts of **D** are still present when release noticeably begins. We observed that the delay time varied linearly with respect to the initial concentration of **D** (Figure 3d). We also tested two other timer circuits with lower initial concentrations of **S**, **I** and **D**, and obtained systems with similar delay times but slower rates of output release (SI 3).

Because the delay circuit is based on DNA strand-displacement events involving a particular DNA sequence, it is possible to create multiple circuits that use different sequences and can trigger the delayed release of two different DNA strands (Figure 4). To characterize the operation of two timer circuits in a single solution, we prepared a second timer circuit and reporter complex with different sequences and fluorophore/quencher than our original system. The second system had the same qualitative behavior, and it was possible to programmatically tune both delay times and release rates. Differences in rates and delay times were observed between the two systems, possibly due to differences in toehold sequence that affect reaction rates (SI 4–5). The two systems were able to operate together in the same solution with virtually identical kinetics to those observed when the systems were operated apart (Figure 4).

The timer circuit developed here successfully releases target strands of DNA into solution at a constant rate after a delay period. The sequence, delay time and production rate were easily tuned without needing to redesign the release system. We demonstrated delay times on the order of hours to days and production rates from a few nM/day to a hundred nM/day, which for volumes of 100 μL are approximately 0.1–10 nmol/day.

While the release of **O** observed (Figure 3, SI Figures S8–S10) qualitatively followed our simple model described in eqs 1–5, there were differences between experiments and reaction curves predicted by the model. Notably we found that the release rate of **O** decreased faster and to a greater degree than the predicted reaction curves, suggesting the existence of uncharacterized reactions (*i.e.*, leak reactions) between **S** and **D** or the reporter. On the basis of the experiments, we hypothesize that DNA synthesis errors (*e.g.*, base-mismatches, truncations, additions or deletions) in the bottom strand of the **S** complex led to leak reactions and pathways that explained most of the deviation from the simple model. By accounting for these and other more minor leak reactions, a model was developed that provided a close fit with the experimental observations (SI 6), indicating that models can be used to program the rate and timing of output release.

By designing a circuit in which the output DNA sequence is an aptamer,[3,28] *i.e.*, sequences of DNA that bind specifically to non-DNA species such as proteins[29] and other small molecules,[30] the circuit described here could also be used to control the dynamics of a wide range of other chemical systems beyond DNA strand-displacement.

Timer devices that can be programmed to release a particular species with a prespecified delay could be important for designing cascades for therapeutics or for self-assembly[5,32] in which different species are activated at different times. The constant low-rate of production we have shown could be used to design therapeutic hydrogels with novel, linear release profiles or within a reliable pulsed delivery system by combining it with a threshold amplifier system such as those used for signal restoration in molecular logic circuits.[14,31]

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■ ASSOCIATED CONTENT

Supporting Information

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Materials and Methods; Reporter calibration and data processing; Production and Delay reaction characterization; Timer experiments; Multiplexing experiments; Modeling and Simulations (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Legewie, S., Dienst, D., Wilde, A., Herzel, H., and Axmann, I. M. (2008) Small RNAs establish delays and temporal thresholds in gene expression. *Biophys. J. 95*, 3232−3238.

(2) Zaslaver, A., Mayo, A. E., Rosenberg, R., and Bashkin, P. (2004) Just-in-time transcription program in metabolic pathways. *Nat. Genet. 36*, 486−491.

(3) Dirks, R. M., and Pierce, N. A. (2004) Triggered amplification by hybridization chain reaction. *Proc. Natl. Acad. Sci. U. S. A. 101* (43), 15275−15278.

(4) Yin, P., Choi, H. M., Calvert, C. R., and Pierce, N. A. (2008) Programming biomolecular self-assembly pathways. *Nature 451* (7176), 318−322.

(5) Zhang, D. Y., Hariadi, R. F., Choi, H. M., and Winfree, E. (2013) Integrating DNA strand-displacement circuitry with DNA tile self-assembly. *Nat. Commun.*, DOI: 10.1038/ncomms2965.

(6) Fu, J., Yang, Y. R., Johnson-Buck, A., Liu, M., Liu, Y., Walter, N. G., Woodbury, N. W., and Yan, H. (2014) Multi-enzyme complexes on DNA scaffolds capable of substrate channeling with an artificial swinging arm. *Nat. Nanotechnol. 9.7*, 531−536.

(7) Caldorera-Moore, M., and Peppas, N. A. (2009) Micro-and nanotechnologies for intelligent and responsive biomaterial based medical systems. *Adv. Drug Delivery Rev. 61.15*, 1391−1401.

(8) Santini, J. T., Jr, Richards, A. C., Scheidt, R., Cima, M. J., and Langer, R. (2000) Microchips as controlled drug-delivery devices. *Angew. Chem., Int. Ed. 39* (14), 2396−2407.

(9) Staples, M., Daniel, K., Cima, M. J., and Langer, R. (2006) Application of micro- and nanoelectromechanical devices to drug delivery. *Pharm. Res. 23* (5), 847−863.

(10) Kapsner, K., and Simmel, F. C. (2015) Partitioning Variability of a Compartmentalized In Vitro Transcriptional Thresholding Circuit. *ACS Synth. Biol. 4*, 1136−43.

(11) Seelig, G., Soloveichik, D., Zhang, D. Y., and Winfree, E. (2006) Enzyme-free nucleic acid logic circuits. *Science 314.5805*, 1585−1588.

(12) Takahashi, K., Yaegashi, S., Kameda, A., and Hagiya, M. (2006) Chain reaction systems based on loop dissociation of DNA. *DNA computing. 3892*, 347−358.

(13) Zhang, C., Yang, J., and Xu, J. (2009) Circular DNA logic gates with strand displacement. *Langmuir 26.3*, 1416−1419.

(14) Qian, L., and Winfree, E. (2011) Scaling up digital circuit computation with DNA strand displacement cascades. *Science 332.6034*, 1196−1201.

(15) Genot, A. J., Bath, J., and Turberfield, A. J. (2011) Reversible logic circuits made of DNA. *J. Am. Chem. Soc. 133.50*, 20080−20083.

(16) Zhang, D. Y., and Seelig, G. (2011) DNA-based fixed gain amplifiers and linear classifier circuits. *DNA Computing and Molecular Programming 6518*, 176−186.

(17) Zhang, D. Y., Turberfield, A. J., Yurke, B., and Winfree, E. (2007) Engineering entropy-driven reactions and networks catalyzed by DNA. *Science 318* (5853), 1121−1125.

(18) Chen, X., Briggs, N., McLain, J. R., and Ellington, A. D. (2013) Stacking nonenzymatic circuits for high signal gain. *Proc. Natl. Acad. Sci. U. S. A. 110* (14), 5386−5391.

(19) Seelig, G., Yurke, B., and Winfree, E. (2006) Catalyzed relaxation of a metastable fuel. *J. Am. Chem. Soc. 128.37*, 12211−12220.

(20) Qian, L., Winfree, E., and Bruck, J. (2011) Neural network computation with DNA strand displacement cascades. *Nature 475.7356*, 368−372.

(21) Srinivas, N. (2015) *Programming Chemical Kinetics: Engineering Dynamic Reaction Networks with DNA Strand Displacement*. Ph.D. dissertation, California Institute of Technology.

(22) Soloveichik, D., Seelig, G., and Winfree, E. (2010) DNA as a universal substrate for chemical kinetics. *Proc. Natl. Acad. Sci. U. S. A. 107.12*, 5393−5398.

(23) Chen, Y. J., Dalchau, N., Srinivas, N., Phillips, A., Cardelli, L., Soloveichik, D., and Seelig, G. (2013) Programmable chemical controllers made from DNA. *Nat. Nanotechnol. 8.10*, 755−762.

(24) Zhang, D. Y., and Winfree, E. (2009) Control of DNA strand displacement kinetics using toehold exchange. *J. Am. Chem. Soc. 131.47*, 17303−17314.

(25) Srinivas, N., Ouldridge, T. E., Šulc, P., Schaeffer, J. M., Yurke, B., Louis, A. A., Doye, J. P. K., and Winfree, E. (2013) On the biophysics and kinetics of toehold-mediated DNA strand displacement. *Nucleic Acids Res. 41.22*, 10641−10658.

(26) Qian, L., and Winfree, E. (2014) Parallel and scalable computation and spatial dynamics with DNA-based chemical reaction networks on a surface. *DNA Computing and Molecular Programming 8727*, 114−131.

(27) Wang, J. S., and Zhang, D. Y. (2015) Simulation-guided DNA probe design for consistently ultraspecific hybridization. *Nat. Chem.*, DOI: 10.1038/nchem.2266.

(28) Zhang, Z., Chen, N., Li, S., Battig, M. R., and Wang, Y. (2012) Programmable hydrogels for controlled cell catch and release using hybridized aptamers and complementary sequences. *J. Am. Chem. Soc. 134.38*, 15716−15719.

(29) Gatto, B., Palumbo, M., and Sissi, C. (2009) Nucleic acid aptamers based on the G-quadruplex structure: therapeutic and diagnostic potential. *Curr. Med. Chem. 16.10*, 1248−1265.

(30) Huizenga, D. E., and Szostak, J. W. (1995) A DNA aptamer that binds adenosine and ATP. *Biochemistry 34.2*, 656−665.

(31) Scalise, D., and Schulman, R. (2014) Designing modular reaction-diffusion programs for complex pattern formation. *Technology 2.01*, 55−66.

(32) Yao, D., Song, T., Sun, X., Xiao, S., Huang, F., and Liang, H. (2015) Integrating DNA-Strand-Displacement Circuitry with Self-Assembly of Spherical Nucleic Acids. *J. Am. Chem. Soc. 137*, 14107.

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