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Communication

Functional Constitutional Dynamic Networks Revealing Evolutionary Reproduction/Variation/Selection Principles

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ABSTRACT: Within the broad research efforts to engineer chemical pathways to yield high-throughput evolutionary synthesis of genes and their screening for dictated functionalities, we introduce the evolution of nucleic-acid-based constitutional dynamic networks (CDNs) that follow reproduction/variation/selection principles. These fundamental principles are demonstrated by assembling a library of nucleic-acid strands and hairpins as functional modules for evolving networks. Primary T_1-initiated selection of components from the library assembles a parent CDN X, where the evolved constituents exhibit catalytic properties to cleave the hairpins in the library. Cleavage of the hairpins yields fragments, which reproduces T_1 to replicate CDN X, whereas the other fragments T_2 and T_3 select other components to evolve two other CDNs, Y and Z (variation). By applying appropriate counter triggers, we demonstrate the guided selection of networks from the evolved CDNs. By integrating additional hairpin substrates into the system, CDN-dictated emergent catalytic transformations are accomplished. The study provides pathways to construct evolutionary dynamic networks revealing enhanced gated and cascaded functions.

Genome engineering by evolutionary pathways, e.g., CRISPR-Cas9,[1] PCR-based mutagenesis,[2] or compartmentalized self-replication,[3] is attracting growing interest as a means to yield high-throughput evolutionary synthesis of genes and their screening for improved functions, e.g., synthesis of novel proteins or ribozymes.[4] These evolution concepts require the development of amplified synthetic pathways of mutated gene libraries (diversity), selection of desired strands, ability to reproduce the selected gene(s), and utilization of the selected strand(s) for targeted functionalities.[5] Beyond the development of genetically engineered machineries, the design of evolutionary gene regulating networks is challenging.[6] In fact, constitutional regulating networks play central roles in living systems.[7] For example, the expansion of genes and their adaptive functions lead to the emergence and divergence of genetic networks.[8] Recently, we introduced DNA-based constitutional dynamic networks (CDNs) as functional modules mimicking the functions of native networks. The simplest CDN includes four equilibrated constituents, AA′, AB′, BA′, and BB′.[9] The triggered stabilization of one of the constituents, e.g., AA′, reconfigures the network, where AA′ and BB′ are upregulated while AB′ and BA′ are downregulated. Different triggers were used to reversibly reconfigure CDNs, e.g., the formation/dissociation of G-quadruplexes[9] or triplexes,[10] strand displacement,[11] or light.[12] These mechanisms were applied to construct DNA-based CDNs of enhanced complexities.[11,13−15] A key question in systems biology relates, however, to the evolutionary pathways leading to the emergence of complex networks in the living systems. Thus, one of the challenges in systems chemistry is an attempt to provide biomimetic pathways for evolving networks and specifically to demonstrate the emergence of networks from a component library, revealing intrinsic reproduction/variation/selection functions (fundamental principles of chemical evolutions[16]). In this context, we argued that nucleic acids could be an ideal versatile material to construct evolutionary networks mimicking natural systems: (i) The base sequences of nucleic acids provide an infinite set of supramolecular structures (library), e.g., duplexes, triplexes, or hairpins. (ii) Nucleic acids comprising the library may reveal recognition properties (aptamers[17]) or catalytic functions (ribozymes[18] or DNAzymes[19]). (iii) The collection of nucleic acids may lead to intermolecular transformations, e.g., hairpin-opening or strand displacement, to yield DNA structures of enhanced complexity.[20] (Note that we use terms and materials developed recently[21] and applied in DNA nanotechnology[22] and assume their coincidental existence in a nucleic-acid library.) Indeed, in a preliminary report,[23] we demonstrated the emergence of DNA-based CDNs by the rewiring mechanism. In the present study, we introduce a nucleic-acid library for the evolution of networks following reproduction/variation/selection principles. Beyond demonstrating the combined features of evolution, we highlight that evolutionary pathways allow the emergence of CDN-guided catalytic functions.

Figure 1 outlines the principle to construct a library leading to the reproduction, variation, and selection of CDNs. The library consists of a set of components A−F′ and hairpins H_1−H_3. Its interaction with initiator T_1 generates a parent CDN X including the T_1-stabilized constituents AA′, AB′, BA′, and BB′. Cleavage of H_1 by BA′ yields fragment T_1, which is

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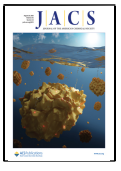

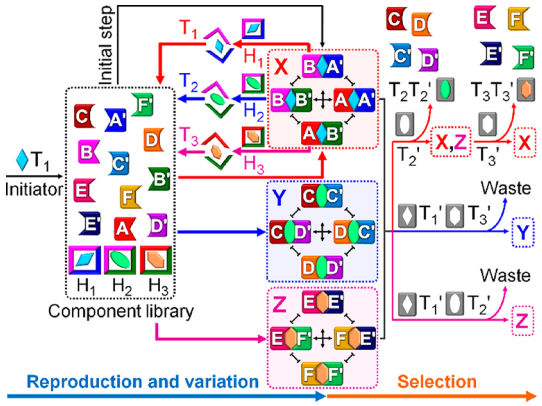

Figure 1. Schematic of triggered reproduction, variation, and selection of CDNs from a component library.

identical to initiator T_1 generating the parent CDN X and, thus, reactivates the selection of A, A′, B, and B′ to replicate CDN X. Cleavage of H_2 by BB′ yields fragment T_2, which interacts with C, C′, D, and D′ to generate CDN Y consisting of T_2-stabilized CC′, CD′, DC′, and DD′. Similarly, cleavage of H_3 by AB′ yields fragment T_3 to assemble CDN Z composed of the T_3-stabilized EE′, EF′, FE′, and FF′. Thus, the primary interaction of the library with initiator T_1 leads to the reproduction of CDN X and the concomitant diversification of the system by the emergence of two additional CDNs, Y and Z. Furthermore, subjecting the evolved CDN mixture to the counter triggers T_2'/T_3' displaces the stabilizing strands from the constituents comprising CDNs Y and Z (forming T_2T_2' and T_3T_3', respectively), resulting in their depletion and the survival of CDN X. Similarly, treatment of the CDN mixture with T_1'/T_3' or T_1'/T_2' leads to the selection of CDN Y or Z. It should be noted that Figure 1 outlines schematically the structural and functional requisites to follow the reproduction/variation/selection principles. For the specific constraints associated with the nucleic-acid structures that follow this figure, see Figure 2 and the accompanying discussion.

Figure 2 depicts the schematic structures of the nucleic-acid library used to assemble the evolutionary reproduction/variation/selection CDN systems. The library includes nucleic acids revealing the following features: (i) triggered assembly of the constituents in different CDNs through T_i-stabilized T-A·T triplexes. (ii) Each of the assembled constituents includes a Mg^{2+}-ion-dependent DNAzyme that provides a probe to quantitatively report on the concentrations of the constituents. By following the DNAzyme-catalyzed cleavage of the respective fluorophore/quencher (F_i/Q_i)-modified substrates, inset I, and using appropriate calibration curves corresponding to the fluorescence changes generated by different concentrations of intact constituents, the concentrations of the CDN constituents can be assessed. (iii) Beside the DNAzyme reporters, the constituents in CDN X include additional Mg^{2+}-ion-dependent DNAzyme activators (marked with a magenta frame) to cleave hairpins H_1−H_3 in the library, to stimulate the reproduction/diversification events. (iv) Each trigger T_i includes a toehold tether that allows its displacement by the counter trigger T_i' to yield T_iT_i', inset II, leading to the depletion of the respective constituents for the selection event. It should be noted that, upon designing the nucleic-acid library composed of components A−F′ and hairpins H_1−H_3, we ensured a lack of the undesirable cross-interactions between the different structures. In addition, we programmed the "arm" sequences of the Mg^{2+}-ion-dependent DNAzyme units to specifically cleave the respective substrates or hairpins.[24] Also, each of the triggers T_i is designed to selectively bind to the components by controlling the sequences and stabilities of the resulting T-A·T triplexes.[25] The specificity of the strand displacement processes was controlled by the base sequences of the respective duplexes and their relative energetic stabilization.[20c,26]

Figure 3A shows the catalytic activities of the CDN constituents before (curves i) and after subjecting the library

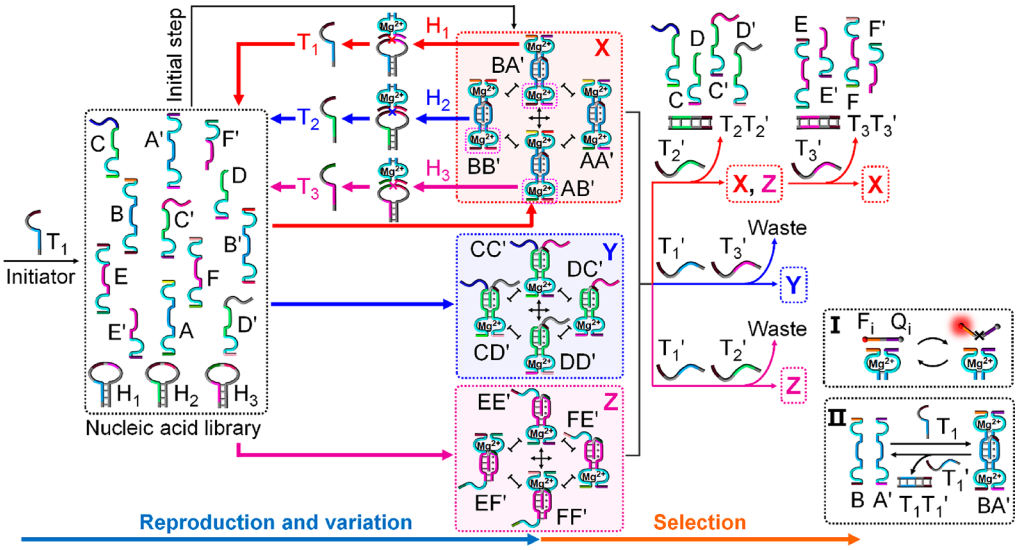

Figure 2. Schematic composition of a nucleic-acid library for the triggered formation of a parent DNAzyme-functionalized CDN X, including the functional information to reproduce itself and to guide the emergence of two other CDNs, Y and Z (reproduction/variation features). The resulting CDN mixture undergoes the triggered selection of target CDNs (selection feature). Insets: (I) Integrated Mg^{2+}-ion-dependent DNAzyme reporters provide catalytic paths to quantify the contents of the constituents. (II) Schematic of triggered formation/dissociation of CDN constituents (taking constituent BA′ as an example).

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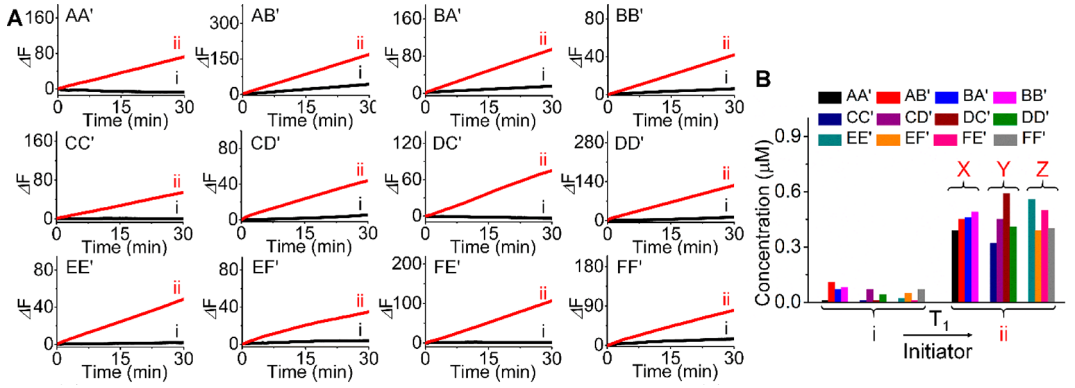

Figure 3. (A) Time-dependent fluorescence changes generated by the DNAzyme reporters and (B) quantified concentrations of the constituents: (i) after the incubation of the library for 24 h, in the absence of initiator T_1; (ii) after subjecting the library to T_1 and allowing it to incubate for 24 h.

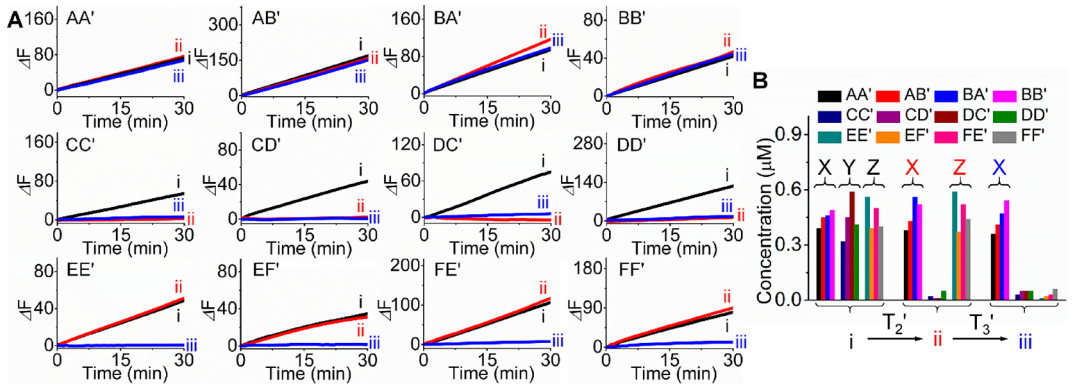

Figure 4. (A) Catalytic activities of the DNAzyme reporters and (B) quantified contents of the constituents in (i) the evolved CDNs X, Y, and Z; (ii) T_2'-selected CDNs Y and Z from the CDN mixture; (iii) T_3'-selected CDN X from the surviving CDNs X and Z.

to initiator T_1 for 24 h (curves ii). No constituents are present in the absence of T_1, yet interaction of the library with T_1 leads to the emergence of all constituents in CDNs X, Y, and Z. Using appropriate calibration curves, Figures S1–S3, we quantified the composition of the system, Table S1 and Figure 3B. The concentrations of the constituents in CDN X are 0.4–0.5 μM. To reach such concentrations, the concentration of T_1 has to be ca. 1.8 μM. The initiator T_1 concentration was, however, only 0.4 μM. Thus, there must be a source to generate T_1, consistent with the reproduction principle where BA′ in CDN X cleaves H_1 to generate fragment T_1 for replicating CDN X. These results demonstrate that T_1 is replicated ca. 4.5 times during this process. Control experiments reveal that exclusion of H_2/H_3 from the library evolves CDN X only (Figure S4), and exclusion of $H_1/H_2/H_3$ prohibits the reproduction of all CDNs (Figure S5). These results demonstrate the T_1-initiated replication of CDN X and CDN X-induced reproduction of two other CDNs (variation). Furthermore, we probed the kinetics of the emergence of the CDNs by following the time-dependent concentration changes of AA′ (of CDN X), CC′ (of CDN Y), and EE′ (of CDN Z), Figure S6. The emergence process, using initiator T_1 at 0.4 μM, reached saturation after ca. 12 h. This process is, however, affected by the concentration of T_1. Decreasing it to 0.2 μM decelerates the reproduction of CDN X, reaching the same saturation after ca. 24 h, Figure S7. In view of the complexity of the number of constituents and the limited number of fluorophore/quencher pairs, we adopted a sequential set of sample measurements to characterize the contents of the constituents in CDNs. For a detailed procedure, see Supporting Information and Figure S8.

The selection principle within the evolved CDNs, X, Y, and Z, is demonstrated in Figures 4, S9, and S10. Figure 4A shows the catalytic activities of the CDN constituents before (curves i) and after subjecting the CDN mixture to the counter trigger T_2' (curves ii), and subsequently to T_3' (curves iii). After subjecting the CDN mixture to T_2', the fluorescence signals of CDN Y are almost depleted, while those of CDNs X and Z are unchanged. Applying T_3' on the surviving CDNs X and Z depletes the fluorescence signals of CDN Z, while those of CDN X are still unchanged. By using the calibration curves (Figures S1–S3), these signals were translated into the constituent concentrations, Table S1 and Figure 4B. These results demonstrate the triggered sequential selection of the evolved CDNs, where CDNs X and Z are selected by T_2', followed by T_3'-guided selection of CDN X from the surviving CDNs X and Z. Similarly, treatment of the CDN mixture with T_1'/T_3' or T_1'/T_2' selects CDN Y or Z, Figures S9 and S10 and Table S1. The results demonstrate the trigger-guided selection of any desired CDN from the diversified CDNs. It should be noted that under the experimental conditions, the evolved CDNs consumed the respective components and hairpins comprising the library. Nonetheless, further reproduction of the selected CDNs is possible by readding a supply of the respective components and hairpins.

In the next step, we applied the evolutionary networks for the guided control over emerging catalytic functions of the system, Figures 5 and S11. Toward this goal, we introduced

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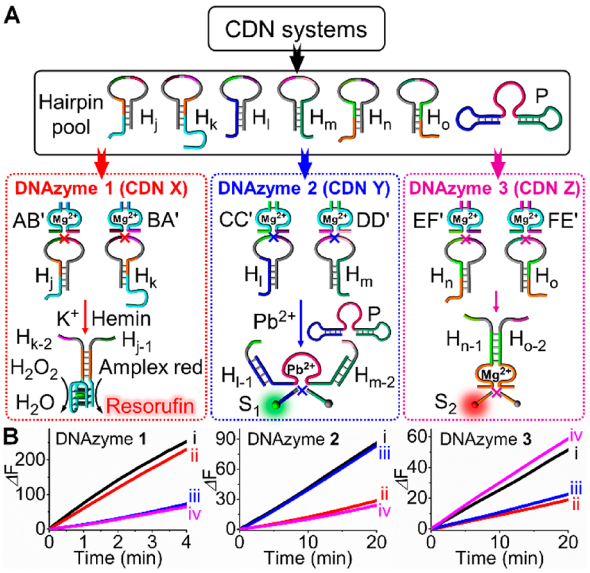

Figure 5. (A) Emerging catalytic functions dictated by the evolutionary CDNs. Upon subjecting the diversified and selected CDNs to the hairpin pool, each CDN selects and cleaves the respective hairpins to generate a catalytic DNAzyme. (B) Catalytic activities of emerging DNAzymes **1**, **2**, and **3** in (i) the evolved CDNs X, Y, and Z; (ii) T_2'/T_3'-selected CDN X; (iii) T_1'/T_3'-selected CDN Y; (iv) T_1'/T_2'-selected CDN Z.

into the library yielding the reproduction/variation/selection processes six additional hairpins, H_j–H_o, as substrates for the DNAzyme reporters in the constituents, and a bis-hairpin P. AB′ and BA′ in CDN X select and cleave H_j and H_k, respectively. The resulting fragmented strands H_{j-1} and H_{k-2} self-assemble, in the presence of hemin, into a K^+-ion-stabilized hemin/G-quadruplex, DNAzyme **1**, catalyzing the oxidation of Amplex Red to fluorescent Resorufin by H_2O_2. CC′ and DD′ in CDN Y cleave H_l and H_m to yield fragments H_{l-1} and H_{m-2}. They interact with P to self-assemble into a Pb^{2+}-ion-dependent DNAzyme, DNAzyme **2**, cleaving fluorophore/quencher-functionalized substrate S_1 to yield the fluorescence output signal. In addition, cleavage of H_n and H_o by EF′ and FE′ in CDN Z yields fragments H_{n-1} and H_{o-2}, assembling into a Mg^{2+}-ion-dependent DNAzyme, DNAzyme **3**, which cleaves fluorophore/quencher-modified substrate S_2 to provide the readout signal. That is, while all DNAzymes should be active in the diversified CDN mixture, the selection of targeted CDNs by T_1', T_2', and/or T_3' is anticipated to select the specific catalytic functions guided by the surviving networks. Figure 5B, curves (i), show the output signals of all DNAzymes in the diversified CDNs X, Y, and Z. The T_2'/T_3'-induced selection of CDN X from the CDN mixture selects DNAzyme **1**, while it switches-off DNAzymes **2** and **3**, curves (ii). Similarly, the selection of CDN Y or Z by using T_1'/T_3' or T_1'/T_2' results in the survival of DNAzyme **2**, curves (iii), or DNAzyme **3**, curves (iv). For further support of the formation of the structures of DNAzymes **1**–**3** by gel electrophoresis experiments, see Figures S12–S14 and the accompanying discussion.

In conclusion, the study has introduced the amplified emergent evolution of dynamic gene networks revealing reproduction/variation/selection principles and the ability to guide functionalities of the evolved networks. These principles were demonstrated by introducing a functional nucleic-acid library for the formation and replication of an initial network, diversification of networks guided by the initially evolved network, and selective selection of any desired networks from the evolved mixture. The success to duplicate these principles by the nucleic-acid library originates from the rich "tool-box" of information-encoded nucleic acids, e.g., programmed stabilities of constituents, and integration of cleavable hairpins and catalytic nucleic acids to dictate the reproduction/variation processes. Needless to state, the present library leading to the emergence of three networks included a limited number of components and hairpins, yet increasing the number of components and hairpins in the library is anticipated to yield diversified networks of higher complexity. In addition, the flexibility of reversible switching motives of nucleic acids may lead to versatile means to design evolutionary network platforms. Furthermore, the diversified and selective emerging functions dictated by the evolved networks were demonstrated, accompanied by the generation of "unused" strands. These waste strands may be used with other strands in the library as inputs for the operation of catalytic cascaded "gates", leading to evolutionary networks of enhanced complexities.[27]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c05669.

Materials, measurements, methods, calibration curves, kinetic profiles of the production/variation events, gel electrophoresis results, and additional results (PDF)

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Notes

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