Supporting Information

Controlling the Catalytic Functions of DNAzymes within Constitutional Dynamic Networks of DNA Nanostructures

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Experimental Section

Materials and Instrumentation

2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid sodium salt (HEPES), $ABTS^{2-}$, sodium chloride, magnesium chloride, potassium chloride, and 18-Crown-6 were purchased from Sigma-Aldrich. DNA oligonucleotides were purchased from Integrated DNA Technologies Inc. (Coralville, IA). Hydrogen peroxide solution was purchased from Fluka. Hemin was purchased from Porphyrin Products (Logan, UT). Tris–borate–EDTA (TBE) buffer solution was purchased from Biological Industries Israel BEIT HAEMEK LTD. (Kibutz Beit-Haemek, Israel). "SYBR Gold nucleic acid gel stain" was purchased from Invitrogen. Ultrapure water from NANOpure Diamond (Barnstead) source was used in all of the experiments.

Fluorescence spectra were recorded with a Cary Eclipse Fluorometer (Varian Inc.). Absorbance spectra were recorded with a Shimadzu UV-2401PC UV/Vis spectrophotometer. The gels were run on a Hoefer SE 600 electrophoresis unit.

The oligonucleic acid sequences used in the study include:

(1) 5'-CTGCTCAGCGATCTTACT*TTTCTTTTTT*AATGACGG*TTTTTTCTTT*AG-3'

(2) 5'-*AAAAAAGAAA*TCTAAGCACCCATGTTACTCT-3'

(3) 5'-GATATCAGCGATCTTACT*TTTCTTTTTT*ACCAGGAG*TTTTTTCTTT*AG-3'

(4) 5'-GAAAAGTAAGCACCCATGTTCGTCA-3'

(5) 5'-AAAAACCGTCAACAGCTCTC-3'

(6) 5'-AAAAACTCCTGCTTCCACAC-3'

(7) 5'-CY5-AGAGTAT**rA**GGAGCAG-BHQ2-3'

(8) 5'-ROX-TGACGAT**rA**GGAGCAG-BHQ2-3'

(9) 5'-FAM-AGAGTAT**rA**GGATATC-BHQ1-3'

(10) 5'-CY5.5-TGACGAT**rA**GGATATC-IBRQ-3'

(11) 5'-GAGAGCTGTTGACGGTTTTT-3'

(12) 5'-GTGTGGAAGCAGGAGTTTTT-3'

(13) 5'-GATATCAGCGATCTTAC*TTTTCTTTTTT*ACCAGGAG*TTTTTTCTTT*AG
CACACACACACACACACACACA-3'

(14) 5'-CACAGAC*AAAAAAGAAA*TCTAAGCACCCATGTTACTCT-3'

(15) 5'-TGGGTTATTGCCACCCATGT-3'

(16) 5'-AGCGATGCAATATGGGTAGGGCGGG-3'

(17) 5'-TAATGCCACCCATGTTCGTCA-3'

(18) 5'-CTGCTCAGCGATGCAATAT-3'

The ribonucleobase cleavage site, rA, in the substrates of the different Mg^{2+}-dependent DNAzymes is indicated in bold, the respective Mg^{2+} DNAzyme sequences are underlined, and the triplex domains associated with the different structures are presented in italic.

Methods and Systems

Preparation of CDNs:

The CDN shown in Figure 2, which includes the constituents AA', AB', BA' and BB', was prepared by the initial assembly of the A/C and B/D duplexes. A, 5 μM, was hybridized with C, 5 μM, and B, 5 μM, was hybridized with D, 5 μM, in a HEPES buffer solution, 10 mM, that included $MgCl_2$, 20 mM, pH = 7.2. Hybridization was carried out by the initial annealing of the respective mixtures at 95 ℃ followed by cooling to 25 ℃ at the rate of 0.47 ℃ $minute^{-1}$, and allowed to equilibrate for 2 hours at 25 ℃. Subsequently, a mixture of A/C, B/D, A' and B', 2 μM each, in HEPES buffer 10 mM, pH = 7.2, that included $MgCl_2$, 20 mM, was annealed

at 35 °C, cooled down to 25 °C at a rate of 0.25 °C minute^{-1}, and allowed to equilibrate for 2 hours at 25 °C, to yield the mixture of the AA', AB', BA' and BB' constituents of the CDN.

The CDN shown in Figure 7 was prepared by mixing the components W, W', X and X', 3 μM each, in a 10 mM HEPES buffer solution, pH = 7.2, that included NaCl, 20 mM, and $MgCl_2$, 20 mM, with the substrate (**8**), 6 μM. The mixture was annealed at 95 °C and cooled down to 25 °C, and allowed to equilibrate for 30 minutes at 25 °C, to yield the CDN in state "M".

Measurements

The excitation of FAM, ROX, Cy5 and Cy5.5 were performed at 496, 588, 648 and 685 nm, respectively. The emission of FAM, ROX, Cy5 and Cy5.5 were recorded at 516, 608, 668 and 706 nm, respectively. The activity of the hemin/G-quadruplex DNAzyme was followed spectroscopically by the DNAzyme-catalyzed oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), $ABTS^{2-}$, to the colored product $ABTS^{\bullet-}$, λ = 414 nm.

Probing the catalytic functions of CDNs in state "F", and stimuli-triggered re-equilibrated states "G" and "H", Figures 2 and 5; and states "M" and "N", Figure 7.

The equilibrated mixture of AA', AB', BA' and BB', 60 μL, was treated with the substrates (**7**), (**8**), (**9**) and (**10**), 3 μL 100 μM each, to yield state "F". Subsequently, the time-dependent fluorescence changes driven by the cleavage of the different catalytic constituents were followed. Also, the mixture of AA', AB', BA' and BB', was subjected to the effectors C' or D', 0.72 μL 100 μM each, and allowed to equilibrate at 25 °C for a time-interval of 12 hours, and then treated with the substrates (**7**), (**8**), (**9**) and (**10**), to yield the states "G" or "H", respectively. Subsequently, the time-dependent fluorescence changes generated upon the cleavage of the respective substrates by the catalytic constituents were monitored. It should be noted that similar rates of cleavage of the substrates (time-dependent fluorescence changes) were

observed upon the treatment of state "F" with the effectors C' or D', and subsequent monitoring of the fluorescence changes generated by the different catalytic constituents provided the CDN readout. For the reverse transition of states "G" or "H" to state "F": the CDNs "G" or "H", lacking the substrates (**7**), (**8**), (**9**) and (**10**), were treated with the counter effector C or D, 0.86 μL 100 μM each, allowed to equilibrate at 25 °C for a time-interval of 12 hours, and finally treated with the substrates (**7**), (**8**), (**9**) and (**10**), respectively, subsequently the time-dependent fluorescence changes of the different catalytic constituents were characterized. The results imply that the cleavage of the respective substrates is slow as compared to the equilibration time of the different CDNs. Using the appropriate calibration curves corresponding to the rates of cleavage of the different substrates by different concentrations of the intact constituents (see detailed description in Figure S2), the contents of the constituents in the different CDNs were evaluated.

Similarly, the equilibrated CDN shown in Figure 7, state "M", consisting of WW', WX', XW' and XX' was incubated with hemin, 150 nM, for 20 minutes. Subsequently, the catalytic functions of WW' and of XX' were probed by following the time-dependent absorbance changes (as a result of the catalyzed oxidation of $ABTS^{2-}$ by H_2O_2 to $ABTS^{\bullet-}$), and the time-dependent fluorescence changes generated by the catalyzed cleavage of (**8**), by the constituents WW' and XX', respectively. Afterwards, the CDN in state "M" was treated with KCl, 20 mM, to yield state "N". After an equilibration time-interval of 20 minutes, the catalytic functions of the constituents WW' and XX' were evaluated by probing the time-dependent absorbance changes and time-dependent fluorescence changes, generated by the respective constituents. The CDN in state "N" was reconfigured into the CDN in state "M" by the addition of 18-crown-6-ether, 25 mM. The contents of the different constituents in CDNs "M" and "N" were evaluated by using appropriate calibration curves, see Figure S8.

Quantitative evaluation of the contents of the constituents of the CDNs described in Figure 2 and Figure 5 by gel electrophoresis.

Native PAGE gel electrophoresis experiments were performed using an acrylamide gel (12%, acrylamide/bis acrylamide 19:1), gel thickness, 1 mm. Mixtures were separated upon applying a 100 V potential, 5 °C (to eliminate the dissociation of duplexes). The separation was conducted for a time-interval of 16 hours.

The mixtures of the constituents corresponding to the CDN were loaded as duplicates in the gel and the intact individual constituents AA', AB', BA', BB', AA'-T, AB'-C, BA'-T and BB'-D at identical concentration, 1.0 μM, were loaded as references in predefined lanes (Note that modified B, B_m, (**13**) instead of (**3**), and modified A', A'_m, (**14**) instead of (**2**) were used, see Figure S4). The separated constituents were stained with SYBR Gold and the intensities of the different bands in CDN were quantitatively analyzed by the ImageJ software by comparing the intensities of the respective separated bands to the intensities developed by the known concentration of the individual reference constituents.

The experiment shown in Figure 6 was repeated three times to allow reliable quantitative evaluation of the content (and experimental error bars) of the constituents in the CDNs. Table 3 summarizes the contents of the constituents of the CDNs.

Quantitative evaluation of the contents of the constituents in CDN "M" and "N" by gel electrophoresis.

Native PAGE gel electrophoresis experiments were performed using an acrylamide gel (acrylamide (12%)/bis acrylamide 19:1), gel thickness, 1 mm. Mixtures were separated upon applying a 30 V potential, 5 °C (to eliminate the dissociation of duplexes). The separation was conducted for a time-interval of 20 hours.

The mixtures of the constituents corresponding to the CDN "M" and "N" were loaded as duplicates in the gel and the intact individual constituents WW', WX', XW' and XX' at identical concentration, 3 μM, were loaded as references in predefined lanes. The separated constituents were stained with SYBR Gold and the intensities of the different bands in CDN "M" and "N" were quantitatively analyzed by the ImageJ software by comparing the intensities of the respective separated bands to the intensities developed by the known concentration of the individual reference constituents. Figure S9 shows the typical gel electrophoresis results in one experiment: In lanes (1) to (4) the separating of the intact reference structures, WW', WX', XW' and XX', are provided. In lanes (5) and (6), the separated mixture of constituents corresponding to CDN "M" (no added K^+-ions), as a duplicate, is presented. In lanes (7) and (8), a duplicate of the separated constituents of the mixture corresponding to CDN "N" (after the addition of K^+-ions) is presented.

The experiment shown in Figure S9 was repeated three times to allow reliable quantitative evaluation of the content (and experimental error bars) of the constituents in the CDNs "M" and "N". Table 4 summarizes the contents of the constituents in systems "M" and "N".

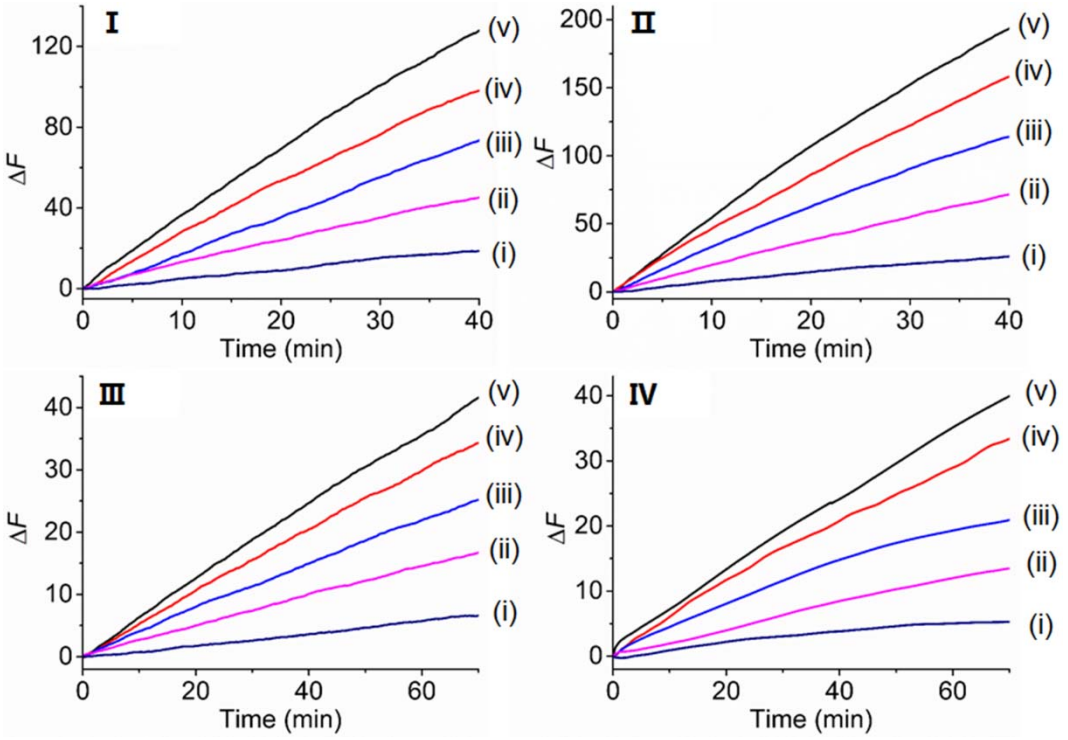

Figure S1. Time-dependent fluorescence changes generated upon cleavage of the respective substrates by variable concentrations of the different Mg^{2+}-dependent DNAzymes associated with the individual structural constituents depicted in Figure 2. Panel I – constituent AA'; Panel II – constituent AB'; Panel III – constituent BA'; Panel IV – BB'. For all systems the concentrations of the DNAzymes correspond to: (i) 0.17 μM, (ii) 0.33 μM, (iii) 0.5 μM, (iv) 0.67 μM, and (v) 0.83 (μM). It should be noted that the intact structures AA'-T and AB'-C show similar kinetic features to those displayed in Panel I and Panel II (for AA' and AB', respectively). Similarly, the constituents BA'-T and BB'-D, cf. Figure 5(A) reveal catalytic activities similar to those shown for BA' and BB', respectively, and Panel III and Panel IV, respectively.

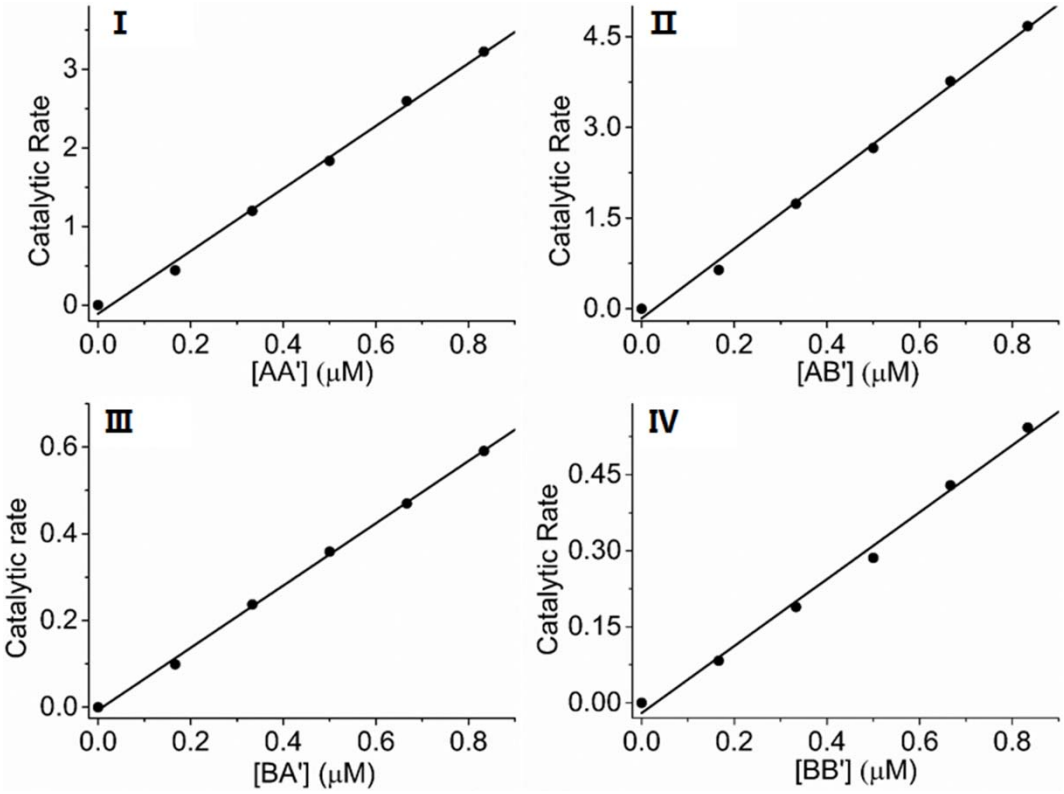

Figure S2. Calibration curves corresponding to rates of the catalytic activities of the different constituents of the CDNs shown in Figure 2 and Figure 5(A) as a function of their concentrations. Calibration curves are derived from the data shown in Figure S1. Panel I – constituent AA' (or AA'-T), Panel II – constituent AB' (or AB'-C), Panel III – constituent BA' (or BA'-T), Panel IV – constituent BB' (or BB'-D).

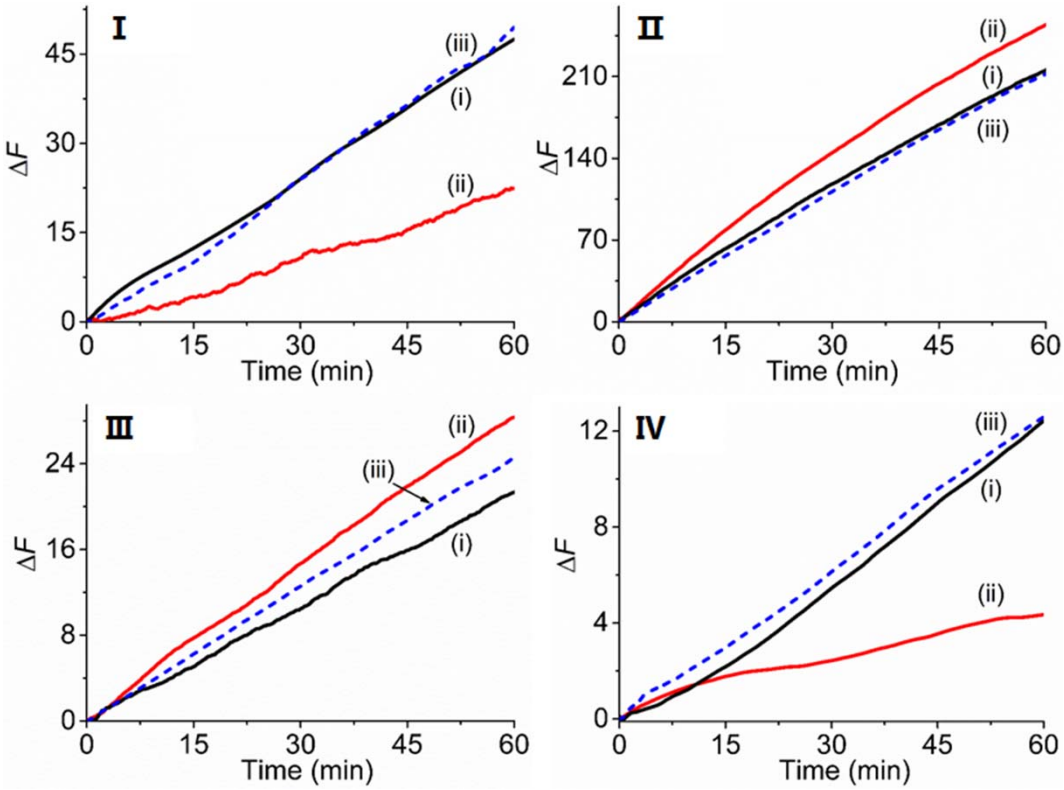

Figure S3. Kinetic features of the catalytic nucleic acids associated with the constituents of the CDNs shown in Figure 5(A). Results show the time-dependent fluorescence changes of observed upon the cleavage of the fluorophore/quencher-functionalized substrates of the respective Mg^{2+}-dependent DNAzymes. Panel I: AA' (fluorescence of CY5); Panel II: AB' (fluorescence of ROX); Panel III: BA' (fluorescence of FAM) and Panel IV: BB' (fluorescence of CY5.5). Kinetic features of the constituents of the CDNs: curves (i) the CDN "F"; curves (ii) the CDN "H" after subjecting the CDN "F" to the effector D'; curves (iii) the CDN "F" after subjecting the CDN "H" to the counter effector D.

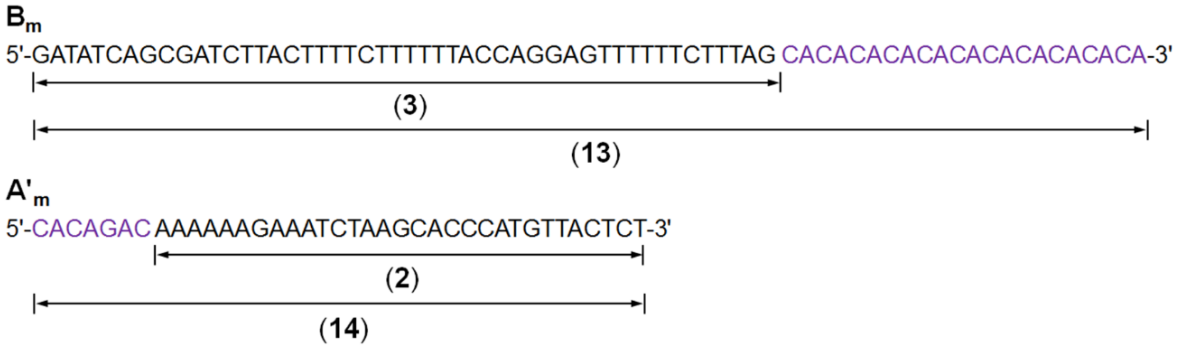

Figure S4. Modified nucleic acid sequences used for improved electrophoretic separation of the constituents in the different CDNs. Component B, (**3**), was elongated with the "innocent" tether marked in purple to yield the modified strand, B_m, (**13**), and component A', (**2**), was tethered to the "innocent" domain, marked in purple, to yield the modified strand, A'_m, (**14**).

To clarify the electrophoresis separation of the CDN systems shown in Figure 2 and Figure 5(A) being depicted as a continuous single gel, we cut the lanes corresponding to the separated constituents of CDN "F" and "G", and compare the results to the characteristic bands associated with the individual constituents, Figure S5. Lane (7) in Figure S5 shows the separated constituents of the CDN in state "F". Lanes (1) to (4) in Figure S5 show the bands corresponding to the individual constituents AA', AB', BA' and BB', respectively. Figure S5 lane (8) depicts the separated constituents associated with CDN "G", while lanes (3) to (6) show the bands corresponding to the individual constituents BA', BB', AA'-T and AB'-C, respectively. We realize that the transition of CDN "F" to CDN "G" results in the depletion of the bands corresponding to BA' and AB'-C and intensified bands corresponding to AA'-T and BB', respectively. The rectangles shown in lane (8) represent the "invisible" bands of BA' and of AB'-C that were detectable and quantitatively analyzed by the imaging software.

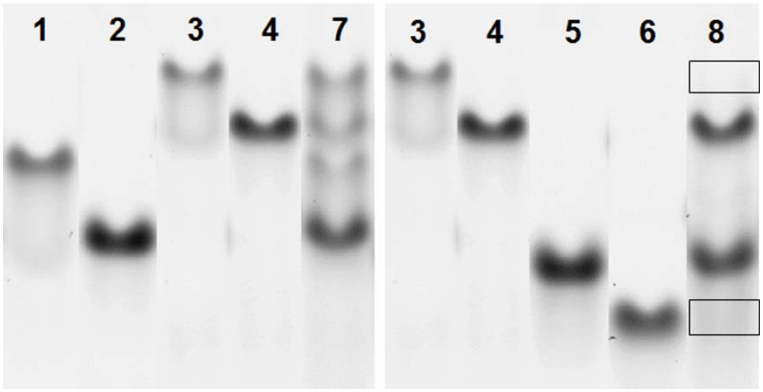

Figure S5. Native PAGE gel electrophoresis separation of the constituents upon transforming CDN "F" into CDN "G".

Similarly, we cut out the lanes corresponding to the separated constituents of CDN "F" and CDN "H", and compare the results to the characteristic bands associated with the individual constituents, Figure S6. Lane (7) in Figure S6 shows the separated constituents of the CDN in state "F". Lanes (1) to (4) in Figure S6 show the bands corresponding to the individual constituents AA', AB', BA' and BB', respectively. Figure S6 lane (11) depicts the separated constituents associated with CDN "H", while lanes (1), (2), (9) and (10) show the bands corresponding to the individual constituents AA', AB', BA'-T and BB'-D, respectively. We realize that the transition of CDN "F" to CDN "H" results in the depletion of the bands corresponding to AA' and BB'-D and intensified bands corresponding to BA'-T and AB', respectively. The rectangles shown in lane (11) represent the weak bands of AA' and of BB'-D that were detectable and quantitatively analyzed by the imaging software.

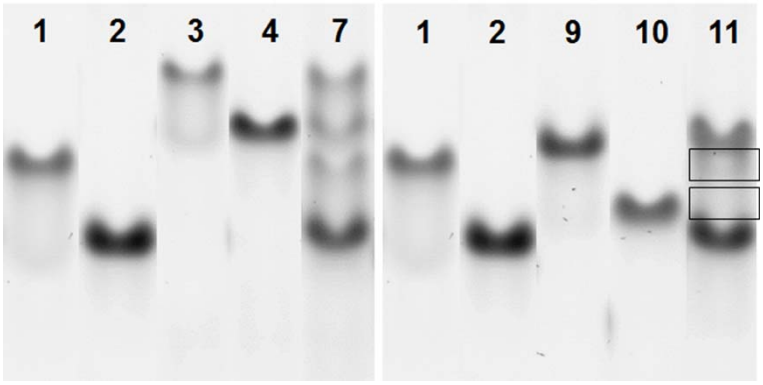

Figure S6. Native PAGE gel electrophoresis separation of the constituents upon transforming CDN "F" into CDN "H".

Kinetic results demonstrating that the DNAzyme activities of the constituents in CDNs "F" and "G" are unaffected by the modification of the constituents with "innocent" tethers. Figure S7 depicts the time-dependent fluorescence change induced by the Mg^{2+}-ion dependent DNAzymes associated with: (I) The AA' constituent, curve (a), and AA'$_m$ constituent, curve (b), of the CDN "F"; The AA' constituent, curve (c), and AA'$_m$ constituent, curve (d), of the CDN "G" (generated after treatment of CDN "F" with the effector C'). (II) The BB' constituent, curve (a), and B_mB' constituent, curve (b), of the CDN "F"; The BB' constituent, curve (c), and B_mB' constituent, curve (d), of the CDN "G" (generated after treatment of CDN "F" with the effector C').

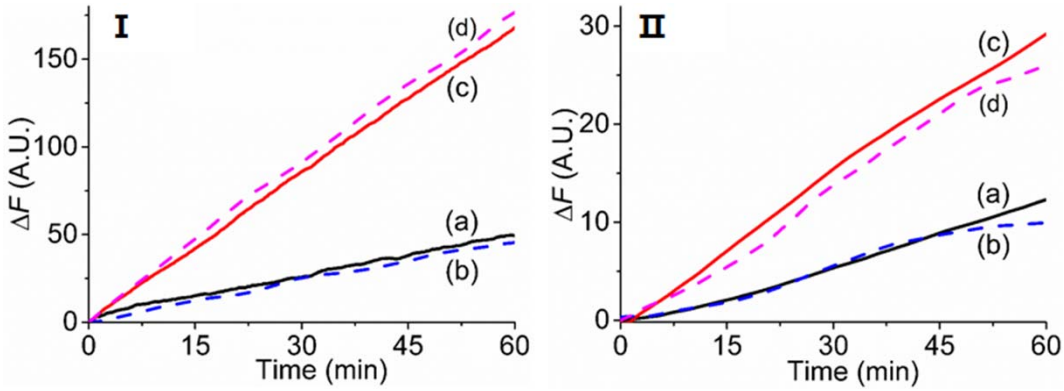

Figure S7. Time-dependent fluorescence changes generated from the cleavage of the substrates by the Mg^{2+}-dependent DNAzymes associated with: Panel I: The AA' constituent, curve (a), and AA'$_m$ constituent, curve (b), associated with CDN "F", and with the AA' constituent, curve (c), and AA'$_m$ constituent, curve (d), associated with the CDN "G". Panel II: The BB' constituent, curve (a), and B_mB' constituent, curve (b), associated with the CDN "F", and with the BB' constituent, curve (c), and B_mB' constituent, curve (d), associated with the CDN "G".

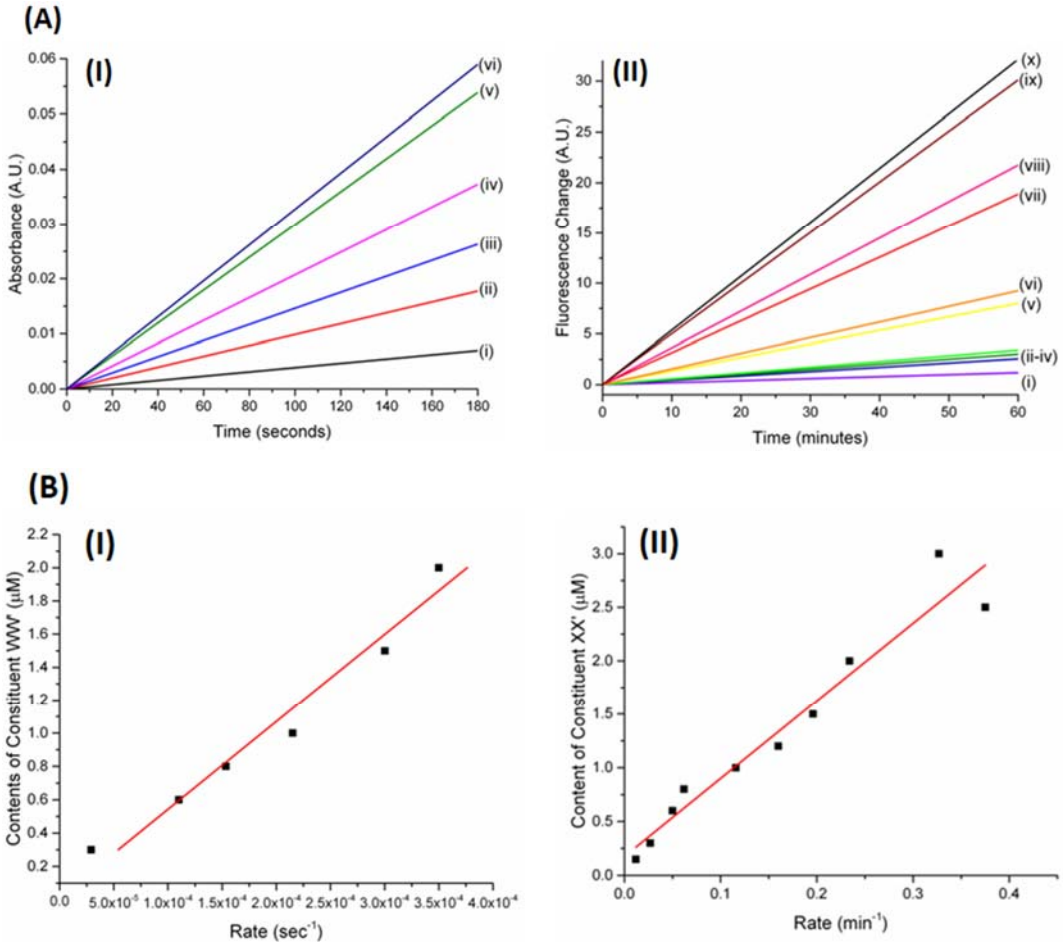

Figure S8. (A) Panel (I): Time-dependent absorbance changes corresponding to the intact WW', hemin-G-quadruplex constituent structure, that catalyzes the oxidation of $ABTS^{2-}$ to $ABTS^{\bullet-}$ by H_2O_2, as a function of the concentration of the DNAzyme catalyst: (i) 300 nM, (ii) 600 nM, (iii) 800 nM, (iv) 1 μM, (v) 1.5 μM and (vi) 2 μM. Panel (II): Time-dependent fluorescence changes corresponding to the intact XX' catalyzed cleavage of the substrate **(8)**, in the presence of variable concentrations of XX': (i) 150 nM, (ii) 300 nM, (iii) 600 nM, (iv) 800 nM, (v) 1 μM, (vi) 1.2 μM, (vii) 1.5 μM, (viii) 2 μM, (ix) 2.5 μM and (x) 3 μM. (B) Curves corresponding to the rates of the catalytic activities of (I) the constituent WW' and (II) the constituent XX', as a function of the concentrations of WW' and XX', respectively.

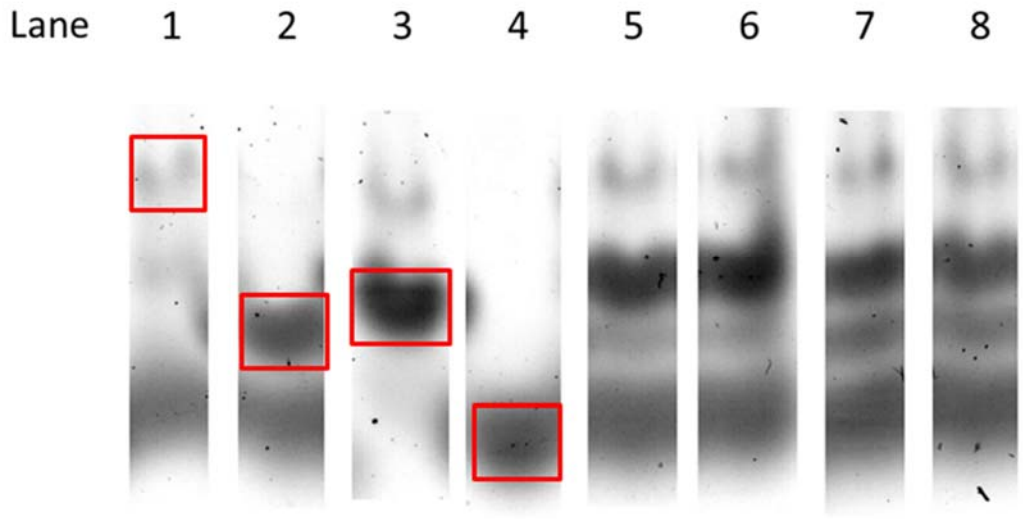

Figure S9. SYBR Gold stained gel of the electrophoretic-separated constituents of the CDN presented in Figure 7. Lane (1): WW'. Lane (2): XX'. Lane (3): WX'. Lane (4): XW'. Lane (5) and lane (6) duplicate separation of the CDN in state "M". Lane (7) and lane (8) duplicate separation of the CDN in state "N".

Effect of the concentration of the effector C' on the effectives of reconfiguration of CDN "F" into CDN "G".

The results presented in the paper use a concentration of the effector C' that corresponds to 1 μM. Realizing that the concentrations of A and C added to the mixture of constituents comprising CDN "F" are 0.83 μM, the concentration of added effector C' is sufficient to eliminate all the C strand associated with the different component A associated with the constituents of CDN "F". At lower concentrations of effector C', e.g. 0.33 μM, the incomplete elimination of the strand C from the components A is anticipated, and thus a lower conversion yield of CDN "F" to CDN "G" is expected. Indeed, Figure S10 reveals that lowering the concentration of the effector C' to 0.33 μM, yields an equilibrated "F"/"G" CDN systems where the concentrations of AA', AB', BA', and BB' correspond to 0.39 μM, 0.47 μM, 0.37 μM and 0.48 μM, respectively, whereas the concentrations of the equilibrated constituents formed upon subjecting CDN "F" to C', 1 μM, correspond to 0.72 μM, 0.14 μM, 0.15 μM and 0.75 μM, respectively. Evidently, as the concentration of C' is higher, the yield of the transforming CDN "F" to CDN "G" increases.

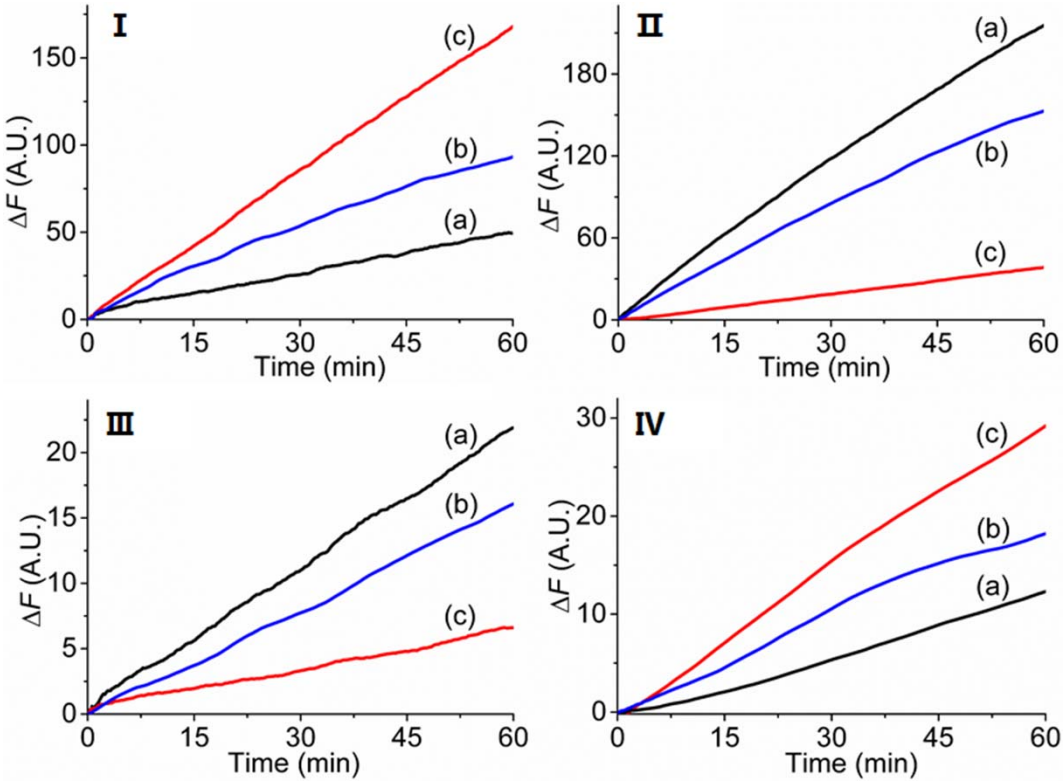

Figure S10. Time-dependent fluorescence changes generated from the cleavage of the substrates by the Mg^{2+}-dependent DNAzymes associated with: Panel I: AA' constituent. Panel II: AB' constituent. Panel III: BA' constituent. Panel IV: BB' constituent. Kinetic features of the constituents of the CDNs: (a) the CDN "F"; (b) the CDN after subjecting the CDN "F" to the effector C' (0.33 μM); (c) the CDN after subjecting the CDN "F" to the effector C' (1 μM).

Time-intervals for the effector-induced equilibration of CDN "F" into CDN "G".

The time-interval used to equilibrate the CDN "F" to the new CDN "G" upon subjecting the system to the effector C' is a crucial parameter to consider, in order to generate the optimal equilibrated system of the constituents associated with CDN "F" and CDN "G". As the time-interval of equilibration is prolonged, the "real" equilibrated composition of the constituents in CDN "G" is anticipated. Indeed, Figure S11 reveals that after subjecting CDN "F" to the effector C' for a time-interval of four hours, the equilibrated CDN "G" reached ca. 80% of the final fully equilibrated CDN "G" reached after twelve hours. Accordingly, to ensure for all systems fully equilibrated states, we applied a time-interval of twelve hours to examine the respective CDNs transitions.

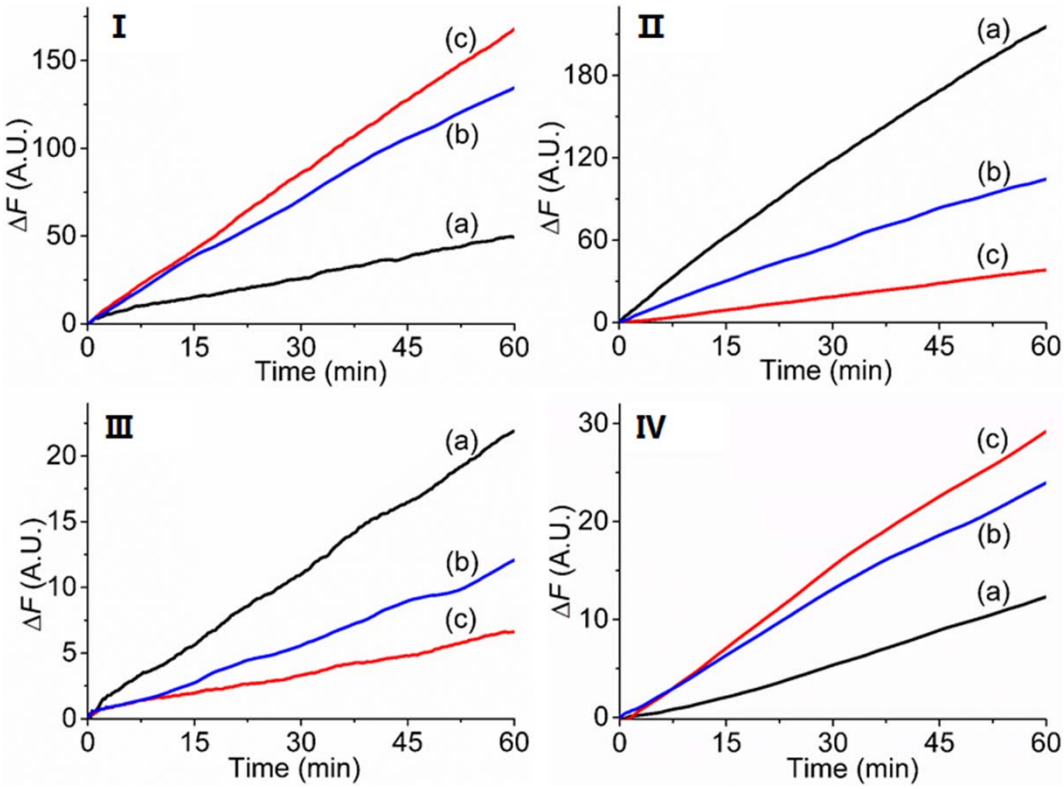

Figure S11. Time-dependent fluorescence changes generated from the cleavage of the substrates by the Mg^{2+}-dependent DNAzymes associated with: Panel I: AA' constituent. Panel II: AB' constituent. Panel III: BA' constituent. Panel IV: BB' constituent. Kinetic features of the constituents of the CDNs: (a) the CDN "F"; (b) the CDN after subjecting the CDN "F" to the effector C', 1 μM, and incubation for 4 hours; (c) the CDN after subjecting the CDN "F" to the effector C', 1 μM, and incubation for 12 hours.