Supporting Information for

Pathway Complexity in Fuel-Driven DNA Nanostructures with Autonomous Reconfiguration of Multiple Dynamic Steady States

Jie Deng [a-d], and Andreas Walther[a-d]*

[a] A[3]BMS Lab, Institute for Macromolecular Chemistry, University of Freiburg, Stefan-Meier-Straße 31, 79104 Freiburg, Germany

[b] DFG Cluster of Excellence "Living, Adaptive and Energy-Autonomous Materials Systems" (*livMatS*), 79110 Freiburg, Germany

[c] Freiburg Materials Research Center, University of Freiburg, Stefan-Meier-Straße 21, 79104 Freiburg, Germany

[d] Freiburg Center for Interactive Materials and Bioinspired Technologies, University of Freiburg, Georges-Köhler-Allee 105, 79110 Freiburg, Germany

andreas.walther@makro.uni-freiburg.de

Materials and methods

HPLC purified oligonucleotides were supplied by Integrated DNA Technologies Inc.. T4 DNA Ligase (HC, 20 Weiss Units (WU)/µL, recombinant *E. coli* strain) and BamHI (HC, 80 units/µL, *Bacillus amyloliquefaciens* H) were supplied by Promega. ATP solution (10 mM in 1 mM Tris-HCl pH 7.5) was purchased from Invitrogen. Agarose low EEO was supplied by PanReac AppliChem. Gene Ruler 1k bp and 50 bp DNA ladders (ready to use) were ordered from Thermo Scientific. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, biology grade) was supplied by CALBIOCHEM. Sodium chloride (NaCl, 99%) was ordered from ABCR. Tris (hydroxymethyl)aminomethane hydrochloride pH 8.0 (Tris-HCl), trizma base, sodium pyrophosphate dibasic (BioUltra, ≥ 99.0 %), and AMP (≥ 99.0 %) were ordered from Sigma-Aldrich. Acetic acid glacial (ACS, Reag. Ph. Eur. Analytical reagents) was supplied by VWR Chemicals. RotiR-GelStain (1,1'3,3',5,5'6,6'-Octamethyl-2,2'-spiro(2,3-dihydro-1H-Benzimidazol) was supplied by Carl Roth.

Buffer compositions:

T4 DNA Ligase Storage Buffer (Promega): 10 mM Tris-HCl (pH 7.4 at 25 °C), 50 mM KCl, 1 mM Dithiothreitol (DTT), 0.1 mM EDTA, 50% glycerol.

BamHI Storage Buffer (Promega): 10 mM Tris-HCl (pH 7.4), 300 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/mL BSA, 50% glycerol.

Restriction Enzyme 10x Buffer E (Promega): 60 mM Tris-HCl (pH 7.5), 1 M NaCl, 60 mM $MgCl_2$, 10 mM DTT.

Annealing Buffer: 10 mM TRIS-HCl (pH 8.0), 50 mM NaCl.

TAE Buffer: 40 mM TRIS, 20 mM acetic ccid, 1 mM EDTA.

Quenching Buffer: 200 mM EDTA, 10 mM TRIS-HCl (pH 8.0), 50 mM NaCl.

Supplementary Table 1: Oligonucleotide sequences. Sequences in red indicate the sticky ends for ligation.

	ID	Oligonucleotide sequence	#nt	Purification
M1	D_1	5'-/5Phos/**C**CCTTACGGTGAATGGAATTCTCATGCGAATAGAGGAT-3'	38	HPLC
	D_2	5'-/5Phos/**G**ATCCTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
M2	D_3	5'-/5Phos/**GATC**CCTTACGGTGAATGGAATTCTCATGCGAATAGAG-3'	38	HPLC
	D_2	5'-/5Phos/**GATC**CTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
C1	D_4	5'-/5Phos/**TC**CCTTACGGTGAATGGAATTCTCATGCGAATAGAGGA-3'	38	HPLC
	D_2	5'-/5Phos/**GA**TCCTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
C2	D_5	5'-/5Phos/**ATC**CCTTACGGTGAATGGAATTCTCATGCGAATAGAGG-3'	38	HPLC
	D_2	5'-/5Phos/**GAT**CCTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
M3	D_6	5'-/5Phos/**C**CCTTACGGTGAATGGAATTCTCATGCGAATAGAG-3'	35	HPLC
	D_2	5'-/5Phos/**GATC**CTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
M4	D_7	5'-/5Phos/**GATC**CCTTACGGTGAATGGAATTCTCATGCGAATAGAGGAT-3'	41	HPLC
	D_2	5'-/5Phos/**G**ATCCTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
M5	D_2	5'-/5Phos/**GATC**CTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
	D_9	5'-CCTTACGGTGAATGGAATTCTCATGCGAATAGAG-3'	34	HPLC
M6	D_2	5'-/5Phos/**GAT**CCTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	38	HPLC
	D_{10}	5'-CCTTACGGTGAATGGAATTCTCATGCGAATAGAGG-3'	35	HPLC

M7	D_{11}	5'-/5Phos/**ATC**CCTTACGGTGAATGGAATTCTCATGCGAATAGAG-3'	37	HPLC
	D_{12}	5'-CTCTATTCGCATGAGAATTCCATTCACCGTAAGG-3'	34	HPLC

Oligonucleotides:

All DNA strands were HPLC purified and were used as received, to which certain amounts of annealing buffer were added to make stock solutions and the concentrations were calculated via UV-VIS spectroscopy. The double-stranded (ds) DNA tiles in this study were annealed from two complementary single-stranded (ss) DNA with the same stoichiometry at room temperature overnight. The annealed stock solutions were stored at -20 °C for further use. In more details, M1 was annealed from D_1 and D_2. M2 was annealed from D_3 and D_2. C1 was annealed from D_4 and D_2. C2 was annealed from D_5 and D_2. M3 was annealed from D_6 and D_2. M4 was annealed from D_7 and D_2. M5 was annealed from D_2 and D_9. M6 was annealed from D_2 and D_{10}. M7 was annealed from D_{11} and D_{12}.

Definition of Activity Units of both Enzymes

***Definition of the Weiss Unit to describe the activity of T4 DNA ligase (Promega)*:** 0.01 Weiss Unit [WU] of T4 DNA Ligase is the amount of enzyme required to catalyze the ligation of greater than 95% of 1 µg of λ/HindIII fragments at 16 °C in 20 minutes.

***Unit definition to describe the activity of BamHI (Promega)*:** One Unit [U] is defined as the amount of enzyme required to completely digest 1 µg of lambda DNA in one hour at 37 °C in 50 µL assay buffer containing acetylated BSA added to a final concentration of 0.1 g/L.

Transient polymerization of M1 and M2 for lag time investigation

The experiment was performed at 25 °C in 1× *Promega* Buffer E with 0.05 mM M1 (or M2), 0.46 WU/µL T4 DNA ligase, 0.1 mg/mL bovine serum albumin (BSA), 10 units/µL BamHI, and 0.6 mM ATP. At different time intervals, 6 µL aliquots of the reaction solution were collected and quenched by 8 µL of quenching buffer. The aliquots were then analyzed by 2wt.% agarose gel electrophoresis (AGE) at 90 V for 2 h run time. The results were recorded by a transilluminator (Analytik Jena, Germany).

Control experiments of transient polymerization of tiles with 2 nt and 3 nt overhangs

The experiments were performed the same as above except 0.05 mM C1 (or C2) was used for the transient polymerization. 6 µL aliquots of the reaction solution were collected were collected and quenched by 8 µL of quenching buffer after different time intervals for the first 5 h to check the kinetics of the transient polymerizations. Afterwards, the collected samples were analyzed by AGE (see above). Results in Figure S3.

Pathway-controlled transient polymerization for transient multi-states

The experiments were performed at 25 °C in 1× *Promega* Buffer E with 0.025 mM M3, 0.025 mM M4, 0.46 WU/µL T4 DNA ligase, 0.1 mg/mL BSA, 10 units/µL BamHI, and 0.3, 0.6, or 0.9 mM ATP. At different time intervals, 6 µL aliquots of the reaction solution were collected and quenched by 8 µL of quenching buffer. The collected samples were analyzed by AGE (see above).

Disruption of dynamic steady-states for programmable transient multi-states

The experiments were performed at 25 °C in 1× *Promega* Buffer E with 0.05 mM dsDNA tiles (M3, M4, and M2 in different ratios; 1:1:0, 5:5:2, and 1:1:1), 0.46 WU/μL T4 DNA ligase, 0.1 mg/mL BSA, 10 units/μL BamHI, and 0.6 mM ATP. At different time intervals, 6 μL aliquots of the reaction solution were collected and quenched by 8 μL of quenching buffer. The collected samples were analyzed by AGE (see above).

Enzyme activities with the presence of waste products and by aging

T4 DNA ligase activity: the experiments were conducted at 25 °C in 1× *Promega* Buffer E with 0.05 mM M5, 0.46 WU/μL T4 DNA ligase, 0.1 mg/mL BSA, 0 or 0.3 mM of both pyrophosphate (PPi) and AMP, and 0.4 mM ATP. At different time intervals, 1 μL aliquots of the reaction solution were collected and quenched by 4 μL of quenching buffer. The collected samples were analyzed by 4wt.% AGE at 75 V for 3 h run time. Results in Figure S4.

The aging experiments were performed the same as above but without waste products and the enzymes were preincubated at 25 °C in 1× *Promega* Buffer E with 0.1 mg/mL BSA for 12 h, 1 day, 2 days, 3 days, 4 days, and 5 days, followed by the additions of M5 and ATP.

BamHI activity: the experiments were conducted at 25 °C in 1× *Promega* Buffer E with 0.02 mM dsDNA tiles (ligated from M6 and M7, and purified by spin filtration), 10 units/μL BamHI, 0.1 mg/mL BSA, and 0 or 0.3 mM of both PPi and AMP. At different time intervals, 1 μL aliquots of the reaction solution were collected and quenched by 4 μL of quenching buffer. The collected samples were analyzed by 4wt.% AGE at 75 V for 3 h run time. Results in Figure S4.

The aging experiments were performed the same as above but without waste products and the enzymes were preincubated at 25 °C in 1× *Promega* Buffer E with 0.1 mg/mL BSA for 12 h, 1 day, 2 days, 3 days, 4 days, and 5 days, followed by the addition of dsDNA for cleavage.

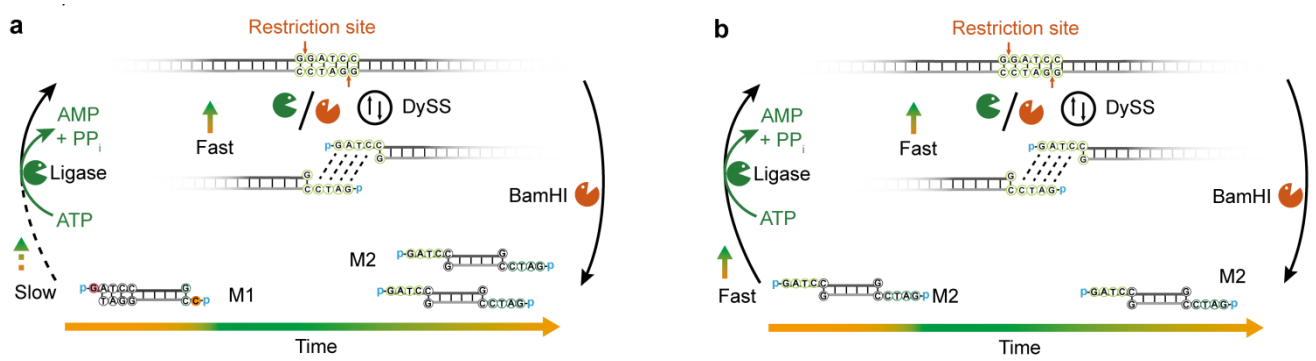

Figure S1. Evolution of sticky ends during the fuel-driven operation of the ERN. (**a**) Schematic illustration of transient polymerization of 1 nt sticky-end dsDNA tiles. Reconfiguration of the 1 nt ends into 4 nt ends occurs. (**b**) Schematic illustration of transient polymerization of 4 nt sticky-end dsDNA tiles. No reconfiguration of the sticky ends occurs.

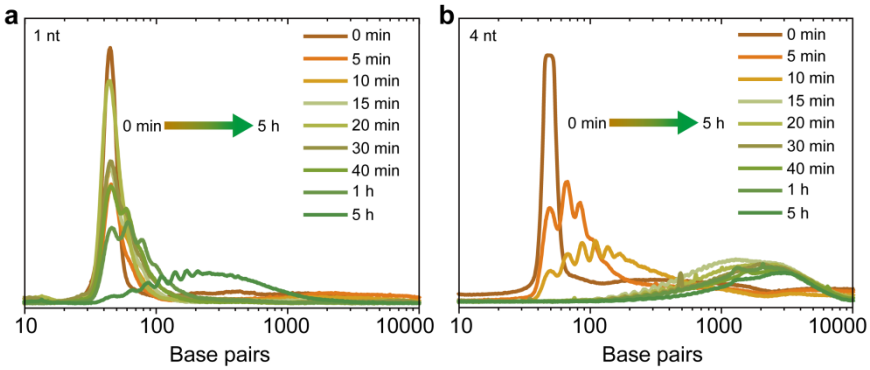

Figure S2. Length of the sticky ends controls the lag time in ATP-driven, dynamic covalent DNA assembly. (**a-b**) Grayscale profiles from

AGE for the first 5 h of transient DySS polymerization of (**a**) M1 and (**b**) M2. The results further show the kinetics of the band shifts for both polymerizations. The polymerization of M1 is not obvious at the first hour, and the elongation of the DNA polymer is very slow. Only after 5 h, there is obvious gel band shift. However, the chains for the M2 system rapidly grow to ca. 800 bp within 10 min and reach a DySS plateau at ca. 9000 bp after 15 min.

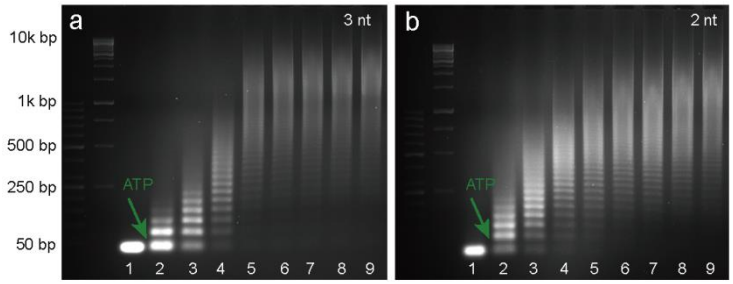

Figure S3. Lower lag times and less pronounced lag phases for 2 nt (C1) and 3 nt (C3) sticky ends on dsDNA monomers. AGE analysis for the transient DySS polymerization of (**a**) 3 nt and (**b**) 2 nt-overhang dsDNA tiles. Standard dsDNA tiles in the enzymatic reaction network have an overhang of 4 nucleotide (nt) for ligation (5'-GATC/GATC-3'), which has 10 pairs of hydrogen bonds. When the overhang is shortened to 3 nt (5'-ATC/GAT-3') and 2 nt (5'-TC/GA-3'), that have 7 and 5 pairs of hydrogen bonds, respectively, there is no obvious lag time as compared to the building blocks having an overhang of 1 nt (5'-C/G-3') with 3 pairs of hydrogen bonds. (Figure 2 in the main MS). Thus 1 nt overhangs were used throughout this study for significant lag time and pathway control. Conditions: 25 °C, 1× *Promega Buffer E*, 0.05 mM dsDNA (C1 or C2), 0.46 WU/µL T4 DNA ligase, 10 units/µL BamHI, and 0.6 mM ATP. Lane assignment: 1: 0 min, 2: 5 min, 3: 10 min, 4: 15 min, 5: 20 min, 6: 30 min, 7: 40 min, 8: 1 h, 9: 5 h.

Supplementary Note1:

We performed control experiments to check for the longevity and activity of the enzymes.

Background. According to literature, PPi could inhibit the activities of ligase and BamHI while AMP could mainly show inhibition on ligase.[1-3] The inhibition of ligase by PPi can be greatly eliminated by adding pyrophosphatase,[2] which hydrolyzes pyrophosphate to phosphates. For BamHI, the inhibition is not very significant when the PPi concentration is less than 1 mM (a concentration that cannot be reached in our experiments). For instance, at 0.6 mM PPi, the endonuclease activity could decrease by ca. 20 %.[3] Here, we checked the inhibition of both enzymes by the waste products (PPi and AMP).

(1) Enzyme activity in presence of waste products. We performed additional experiments to check the enzyme activity in the presence of waste products (AMP and PPi) for an average product concentration of 0.3 mM AMP and 0.3 mM PPi (Figure S4a,b). Briefly, the addition of 0.3 mM AMP and 0.3 mM PPi can decrease ligase activity by ca. 32 %, while it only decreases BamHI activity by ca. 4 %. Hence, the waste products show a bit stronger inhibition to ligase compared to that for BamHI. It needs to be noted however that the control experiments by directly exposing the enzymes to high concentration of wastes actually do not reflect the real situation of our system, in which the wastes are actually slowly accumulated and the poisoning of the enzymes should be much weaker and slower.

(2) Aging effects on enzyme activity. Additionally, we aged both enzymes in the buffer for different periods of time and then checked their activities. The results are displayed in Figure S4c,d. As the lifetimes of most of our dynamic DNA nanostructures were set within 3-4 days, we aged both enzymes for a maximum of 5 days. After aging for 3-4 days, both enzymes show some decreased activity, but only to a limited extent. For instance, T4 DNA ligase loses 35 % activity over a time frame of 4 days, while BamHI loses 39 % activity. Both enzymes are hence still highly active in the system. Additionally, we previously showed that both enzymes are still active even after 12 days, because we observed a linear relationship of the system lifetime with the ATP concentration, which indicates both enzymatic reactions are quite constant in the system.[4] In the present study we also observe similar linear relationship between lifetime and ATP concentration. It is important to note that aging in

free buffer corresponds to a *worst case scenario*, because there are no DNA structures present in solution that may stabilize both enzymes in their bound forms. Importantly, any change in the enzymatic activity, as long as no complete loss is observed, does not have any influence on the significance and realization of the concept for this study, that is to show pathway complexity by kinetic selection, molecular recognition, and species reconfiguration.

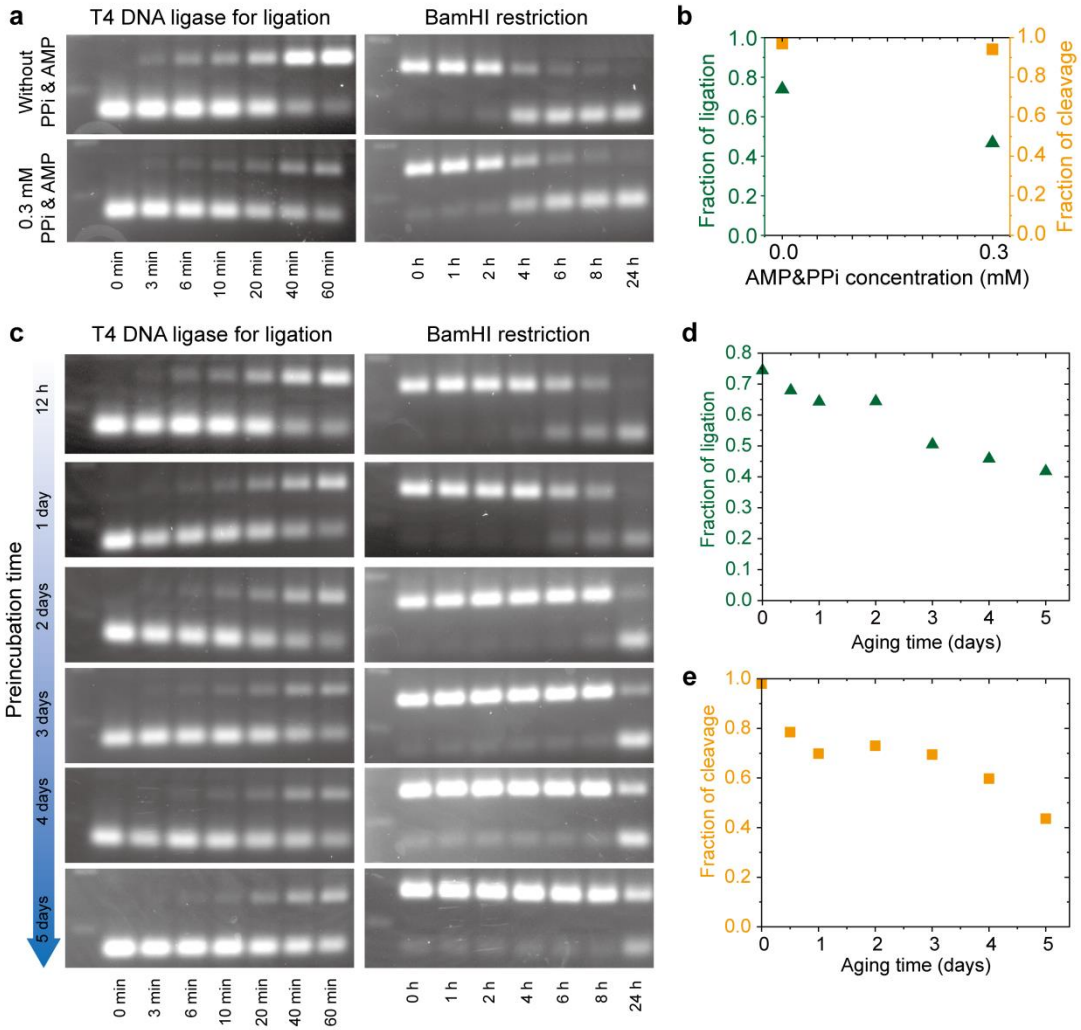

Figure S4. Enzyme activities in presence of waste products and aging effects. (**a**) AGE analysis for DNA ligation and cleavage without and with 0.3 mM AMP and 0.3 mM PPi. (**b**) Fraction of ligated DNA tiles after 60 min ligation and fraction of cleaved DNA tiles after 24 h cleavage without and with AMP and PPi. (**c**) AGE analysis of DNA ligation and cleavage by the enzymes that were preincubated in the buffer for different periods of time (in absence of any DNA). (**d**) Fraction of ligated DNA tiles after 60 min ligation by the enzymes after being preincubated for different time. (**e**) Fraction of cleaved DNA tiles after 24 h cleavage by the enzymes after being preincubated for different time. Conditions: (**a**) Ligation: 25 °C, 1× *Promega Buffer E*, 0.05 mM M5, 0.46 WU/μL T4 DNA ligase, 0 or 0.3 mM of both PPi and AMP, and 0.4 mM ATP. Restriction: 25 °C, 1× *Promega Buffer E*, 0.02 mM dsDNA from ligation of M6 and M7, 10 units/μL BamHI, and 0 or 0.3 mM of both PPi and AMP. (**c**) Ligation: 25 °C, 1× *Promega Buffer E*, 0.05 mM M5, 0.46 WU/μL T4 DNA ligase and 0.4 mM ATP. Restriction: 25 °C, 1× *Promega Buffer E*, 0.02 mM dsDNA from ligation of M6 and M7, 10 units/μL BamHI. The enzymes were preincubated in the buffer for 12 h, 1 day, 2 days, 3 days, 4 days, and 5 days before the ligation and cleavage experiments.

Reference

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