Programmable Dynamic Steady States in ATP-Driven Non-Equilibrium DNA Systems

Laura Heinen, Andreas Walther

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Inspired by the dynamics of the dissipative self-assembly of microtubules, chemically fueled synthetic systems with transient lifetimes are emerging for non-equilibrium materials design. However, realizing programmable or even adaptive structural dynamics has proven challenging because it requires synchronization of energy uptake and dissipation events within true steady states, which remains difficult to orthogonally control in supramolecular systems. Here, we demonstrate full synchronization of both events by ATP-fueled activation and dynamization of covalent DNA bonds via an enzymatic reaction network of concurrent ligation and cleavage. Critically, the average bond ratio and the frequency of bond exchange are imprinted into the energy dissipation kinetics of the network and tunable through its constituents. We introduce temporally and structurally programmable dynamics by polymerization of transient, dynamic covalent DNA polymers with adaptive steady-state properties in dependence of ATP fuel and enzyme concentrations. This approach enables generic access to non-equilibrium soft matter systems with adaptive and programmable dynamics.
Programmable Dynamic Steady States in ATP-Driven Non-Equilibrium DNA Systems

Authors
Laura Heinen\textsuperscript{1,2,3}, Andreas Walther\textsuperscript{1,2,3,4*}

Affiliations
\textsuperscript{1}Institute for Macromolecular Chemistry, University of Freiburg, Stefan-Meier-Straße 31, 79104 Freiburg, Germany.

\textsuperscript{2}Freiburg Materials Research Center (FMF), University of Freiburg, Stefan-Meier-Straße 21, 79104 Freiburg, Germany.

\textsuperscript{3}Freiburg Center for Interactive Materials and Bioinspired Technologies (FIT), University of Freiburg, Georges-Köhler-Allee 105, 79110 Freiburg, Germany.

\textsuperscript{4}Freiburg Institute for Advanced Studies (FRIAS), University of Freiburg, Albertstraße 19, 79104 Freiburg, Germany.

Corresponding Author
Email: andreas.walther@makro.uni-freiburg.de
Phone: +49 761-203-96895
Institute for Macromolecular Chemistry
Stefan-Meier-Strasse 31
Albert-Ludwigs-University Freiburg
D-79104 Freiburg
Germany
Abstract

Inspired by the dynamics of the dissipative self-assembly of microtubules, chemically fueled synthetic systems with transient lifetimes are emerging for non-equilibrium materials design. However, realizing programmable or even adaptive structural dynamics has proven challenging because it requires synchronization of energy uptake and dissipation events within true steady states, which remains difficult to orthogonally control in supramolecular systems. Here, we demonstrate full synchronization of both events by ATP-fueled activation and dynamization of covalent DNA bonds via an enzymatic reaction network of concurrent ligation and cleavage. Critically, the average bond ratio and the frequency of bond exchange are imprinted into the energy dissipation kinetics of the network and tunable through its constituents. We introduce temporally and structurally programmable dynamics by polymerization of transient, dynamic covalent DNA polymers with adaptive steady-state properties in dependence of ATP fuel and enzyme concentrations. This approach enables generic access to non-equilibrium soft matter systems with adaptive and programmable dynamics.
Introduction

Biological systems operate out-of-equilibrium under constant influx of energy and matter, and are orchestrated via signaling and reaction networks. For example, microtubules and actin filaments polymerize dynamically by consumption of chemical fuels, and persist in a fueled dynamic steady state (DySS) with unusual dynamics (e.g. instabilities) needed for rapid spatiotemporal reorganization in the cytoskeleton. Mimicking such biological dissipative structures with tunable structural dynamics in their steady states remains a profound challenge in the emergent pursuit for artificial, non-equilibrium molecular systems, but at the same time represents one of the most critical aspects for the design of next generation autonomous, active matter-type, functional material systems with truly adaptive or even life-like properties.

Research on chemically fueled systems has so far majorly focused on supramolecular structures, in which monomeric building blocks are embedded into a kinetically controlled reaction network and therein temporarily activated for self-assembly. However, energy-driven structural dynamics in such systems - with simultaneous formation, collapse and exchange of the structural units - is enabled only when chemical activation and deactivation occur concurrently, and, critically, synchronize appropriately with the kinetics of structure formation and destruction. Fiber dynamics were first and solely reported for Me$_2$SO$_4$-fueled supramolecular self-assemblies of carboxylate gelator molecules using transient esterification in alkaline hydrolytic environments, while it was not reported for other supramolecular fibrils of partly very similar structure. Structural dynamics are even harder to realize in fuel-dissipating environments with a modulated self-assembly trigger (e.g. pH or ATP), because deactivation of the fueling signal occurs for kinetic reasons preferentially outside the structure. For instance, although being highly valuable for designing autonomous systems with lifetimes, recent examples of ATP- or pH-triggered transient self-assemblies, that use enzymes to mediate the signals, lack energy-driven dynamics in their transient states, and those are also highly unlikely to occur.

Beyond such ATP-responsive self-assemblies with transient signal dissipation, ATP-fueled supramolecular peptide fibrils were reported by direct enzymatic phosphorylation of peptide residues and concurrent removal of it. In a dialysis reactor with continuous waste removal and fuel supply steady states were successfully sustained, however, structural dynamics remain elusive as the fibrils undergo unfavorable higher level aggregation.

Herein, we step away from supramolecular structures, and introduce the first example of a chemically fueled dissociative dynamic covalent bond system. Critically, this strategy enables facile access to adaptive and programmable structural DySSs by mechanistically synchronizing the energy events (uptake/dissipation) with structural transitions (bond formation/cleavage). In more detail, we present the ATP-fueled activation and dynamization of covalent phosphodiester DNA bonds via an enzymatic reaction network of concurrently acting ATP-dependent DNA ligase and counteracting endonuclease, which modulate jointly the average steady-state bond ratio and bond exchange frequencies. Bridging the fields of DNA nanotechnology and polymer science, we transduce this concept to non-equilibrium dynamic covalent and transient DNA chain growth with programmable DySS properties. The ATP fuel level in the system primarily programs the lifetime, whereas the kinetic balance between the ligation and the restriction reaction, as encoded by the concentrations and ratios of the enzymes, dictates the average steady-state chain length and the exchange frequencies between the polymer chains. Our approach introduces a generic dynamic covalent bond as a new concept into non-equilibrium DNA nanoscience. Moreover, we suggest chemically fueled dissipative dynamic covalent bonds as a generic concept for the nascent field of dissipative non-equilibrium systems design, that allows for engineering functional active matter with adaptive and autonomously programmable DySS behavior.
**Results**

### ATP-Fueled Dynamization of Covalent DNA Bonds

Our concept enabling this first example of a chemically fueled dynamic covalent bond with direct implications for higher level structure dynamics builds on the ATP-fueled enzymatic activation and dynamization of a DNA phosphodiester bond in presence of antagonistic enzymes joining and cutting this linkage. We apply this concept directly to the transient dynamic chain growth polymerization of α,ω-telechelic DNA monomer strands, $M_1$ (Fig. 1A). $M_1$ is a rigid duplex of 34 base pairs (bp) with a self-complementary single-stranded DNA (ssDNA) 4 nucleotide (nt) overhang at each side. These ssDNA ends carry the molecular recognition information to self-extend, but are too short to stably connect $M_1$ into elongated chains, as the 4 bp hybridization has a low melting temperature, $T_m \approx 0^\circ$C (Fig. S1). However, joining of two ends can be achieved by T4 DNA ligase, which catalyzes the phosphodiester bond formation between adjacent 5'-phosphate and 3'-OH groups in a DNA duplex under consumption of one ATP molecule (Fig. S1). Coupling of two $M_1$ requires two ligation steps and consumes two molecules of ATP. The $M_1$ ends are designed in a way that successful ligation creates the recognition site (GGATCC, orange box, Fig. 1A) for an antagonistic restriction enzyme, BamHI. BamHI cuts the dsDNA strands by hydrolytic cleavage of the phosphodiester bond at the position where the $M_1$ strands were just ligated. Cleavage is thus conditional on prior ligation and the phosphodiester bond formation is fully reversible. The ligation transfers chemical energy from ATP into a covalent phosphodiester bond in the DNA backbone, while the restriction enzyme dissipates this energy by breaking these bonds hydrolytically. The simultaneous action of both enzymes creates a dynamized phosphodiester bond under biocatalytic control. The kinetic boundary condition for formation of a transient polymer state is that ligation is faster than cleavage. The overall lifetime is given by the availability and consumption of chemical fuel and the concentrations of the enzymes, whereas the enzyme concentrations modulate the reaction frequencies needed to program the dynamics of the transient DySS.

The reaction network embedding the ATP-fueled dynamic phosphodiester bond fulfills the relevant features for formation of a dissipative non-equilibrium system: (i) Structure formation is coupled to an energy-fueled activation (ATP-dependent ligation). (ii) The deactivation dissipates energy (cleavage of a covalent bond, $\Delta G = -5.3$ kcal/mol$^{38}$). (iii) Activation and deactivation are chemically independent, selective and kinetically tunable reactions. (iv) The structure is completely reversible on a molecular level. Consequently, this ATP-fueled dynamization of a phosphodiester bond constitutes a general strategy to establish dissipative DNA-based structures and energy-driven active materials.

Critically, the chemical fuel acts only as an energy-providing source (a co-factor) to form the bond and connect DNA strands of choice (Fig. S1), but is not integrated into the structures as a terminal group. This is decisive to program larger molecular architectures, and opens considerable flexibility for rational design of functionalities and connectivity patterns.

Moreover, the present dissipative system fully synchronizes energetic and structural events, which provides the key advantage to mechanistically embed structural dynamics in the DySS. It enables deterministic access to material properties such as tunable exchange frequencies important for self-repair and adaptation in fueled DySSs.
Fig. 1. ATP-fueled dynamization of phosphodiester bonds by simultaneous action of two antagonistic DNA enzymes for transient dynamic covalent polymerization of DNA strands with a tunable lifetime and adjustable steady-state dynamics. (A) Short telechelic DNA monomers, M₁, with 4 nt self-complementary ssDNA ends are covalently joined via T4 DNA ligase-catalyzed phosphodiester formation under consumption of two ATP fuel molecules. This ligation forms the recognition site (highlighted by the orange box) of the endonuclease BamHI, which counteracts ligation by catalyzing the cleavage (restriction path as red line) of the just formed phosphodiester bonds. Simultaneous ligation and cutting at this site creates a dynamic covalent bond until the ATP runs out. (B) Transient growth of dynamically polymerizing DNA chains in a closed system is achieved by a faster ligation than restriction reaction (v_act >> v_deact). The lifetime is coupled to the ATP fuel and can be tuned together with the DySS properties of the dynamic covalent DNA chains under biocatalytic control.

Transient DySS DNA Polymerization System with ATP-Dependent Lifetimes

Dynamic covalent polymerization of DNA chains requires four main components (Fig. 1B): The DNA monomer M₁, T4 DNA ligase, BamHI restriction enzyme and ATP as chemical fuel in a suitable buffer. Without ATP the system is inactive. After addition of ATP, DNA chains grow immediately and evolve into a DySS with continuous joining and cutting. Once the ATP level becomes subcritical, cutting events overpower ligation and the DNA chains degrade back to the initial state. Each of the four system components and the reaction temperature control the dynamic polymerization and program its DySS properties as detailed below by systematic kinetic studies.

The basic reaction conditions were derived from extensive screening of the individual reactions as summarized in Supporting Note A (Figs. S2-S5). The DNA concentration [M₁] = 0.05 mM was set as fixed parameter in all kinetic experiments. All experiments contain at least equimolar ATP (related to the number of possible ligation sites, i.e. [ATP] ≥ 2•[M₁]) to avoid limitations in chain length from low conversion in the fueled step growth-like polymerization (Fig. S3, S8). From the individual kinetics of the enzyme-dependent DNA chain growth and degradation experiments (Fig. S2 and S5), we found 41.25 WU (Weiss Unit, Supplementary Note A) of T4 DNA ligase and 900 U of BamHI as suitable enzyme ratio fulfilling the kinetic requirement of a faster ligation than cleavage. This enzyme ratio is constant for all further dynamic polymerizations, unless when studying the influence of the enzyme concentrations.

Considering the importance of the chemical fuel in a dissipative system, we first discuss its influence on the ensemble system behavior of the transient DySS polymerization of dynamic covalent DNA chain growth (25°C). Experimentally, we analyze the time-dependent behavior from kinetic aliquots via agarose gel electrophoresis (GE; Fig. 2A). GE allows resolving the chain length distribution of the dynamically polymerizing M₁-based DNA chains accurately, in particular with regard to smaller oligomers.
Close inspection of a system fueled e.g. with 0.4 mM ATP reveals the monomer band (M1, 38 bp) at the bottom of lane 3 (t = 0; Fig. 2A, ii). Injection of ATP initiates chain growth rapidly and the system enters the DySS (lanes 5-9), where continuous exchange (ligation/cutting) occurs. After three days, the chain length declines. Analysis of the gray scale profiles of each lane allows quantification of the distributions and displays a shift to higher molecular weights at initial stages and back to M₁ when the system runs out of fuel (Fig. 2B). Those equal mass-weighted chain length distributions that can be calibrated using DNA ladders to derive mass-weighted average chain lengths, $\bar{b}_p$ (Supplementary Note B, Fig. S6).

**Fig. 2C** illustrates the corresponding transient polymerization profiles using the calculated $\bar{b}_p$ for increasing ATP concentrations. Evidently, the lifetimes of the continuously ATP-dissipating DySS polymers extend from less than one day to ca. 10 days with increasing fuel levels. Importantly, both enzymes remain fully operational even for such extended durations (Fig. S4). The lifetimes are defined to the point where $\bar{b}_p$ declines from the DySS plateau value. They show a linear correlation with the ATP concentration, underscoring an excellent control over the temporal programmability of the transient DySS of the DNA chains (Fig. 2C, D). Despite the different lifetimes, all systems evolve into the same plateau in the DySSs with $\bar{b}_p$s of ca. 1000 bp, which equals an average degree of polymerization $\bar{P}_w$ of ca. 26, programmed by the balance between ligation and cleavage. Given the high persistence length of ds-DNA (ca. 50 nm at 0.1 N NaCl), this corresponds to the formation of long semiflexible fibrils with a diameter of 2.0 nm and a mass-average length, $\bar{t}_w$, of ca. 3.5 µm, being similar to a range of mostly non-cooperatively assembling supramolecular fibrils.

**Fig. 2.** ATP-fueled transient DySS polymerization of dynamic covalent DNA chains with a tunable lifetime. (A) Time-dependent GE shows transient lifetimes programmed by ATP fuel concentration (0.1 to 1.0 mM ATP, left to right). Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 10 min, 5: 1 h, 6: 9 h, 7: 24 h, 8-16: 2d-10d (daily interval). (B) Gray scale profiles extracted from GE at 0.4 mM ATP (panel (ii)) quantify the transient, reversible shifts of molecular weight (top: growth, bottom: decline), which is used to calculate the mass-weighted average chain length ($\bar{b}_p$) for each kinetic aliquot. (C) The development of $\bar{b}_p$ over time reveals increasing lifetimes of the transient DNA polymerization with a constant steady-state chain length of around 1000 bp under the given enzymatic conditions when increasing the ATP concentration from 0.1 mM to 1.0 mM. Lines are guides to the eye. (D) The lifetime scales linearly with the amount of supplied ATP. Conditions: 0.05 mM M₁, 41.25 WU T4 DNA ligase, 900 U BamHI and varying amounts of ATP at 25°C in the enzyme reaction buffer.
Biocatalytic and Thermal Programming of Structural and Dynamic Steady-State Properties of the DNA Polymers

We hypothesized that the variation of the enzyme ratio could manipulate the DySS bond and the ensemble system behavior under biocatalytic control. To this end, we changed the T4 DNA ligase concentration ([T4]) while keeping the restriction enzyme concentration constant ([BamHI] = 900 U; Fig. 3A). The increase of [T4] from 11 to 110 WU has two main effects. First, it results in a faster build-up of the DySS (initial growth phase), and, second, it leads to longer DNA chains with an increase of $\bar{bp}_w$ from ca. 900 to 1200 bp. Both effects can be explained by a shift of the kinetic balance towards the ligation side by its selective acceleration. Likewise, cleavage can be favored when increasing [BamHI] (113 to 900 U, Fig. 3B), while [T4] stays unchanged (41.25 WU). More frequent cleavage events shorten $\bar{bp}_w$ and the lifetime of the DySS drastically. The transient DySS polymers degrade in the range of days faster for high concentrations of BamHI.

More intriguingly, the intermolecular bond exchange dynamics in the DySS polymerization system can be accelerated by a symmetric increase (here up to 8x) of both enzymes at a fixed ratio [T4]/[BamHI] = 5 WU/113 U. This leads to narrower time profiles of the DySS polymerization with both faster chain growth and degradation, and consequently to shorter lifetimes. Higher enzyme activities on both sides of the antagonistic reaction network mean faster conversion of ATP, and, more importantly, higher exchange frequencies of the dynamic covalent bond. The possibility to adjust the exchange frequencies within the DySS is instrumental regarding self-renewal/self-healing and adaptivity, and a unique advantage of this chemically fueled system with synchronized energetic and structural events.

The DySS polymerization can also be tuned by changing the temperature, which is particularly important to understand at near physiological conditions (37°C), as we operate a highly biocompatible system. Whereas BamHI shows higher activity at 37°C, the optimum temperature for the T4 DNA ligase is a trade-off between its activity and the hybridization probability of two 4 nt overhangs. Lower temperatures stabilize the complementary overhangs and thus facilitate ligation. This effect can be observed in the ATP-dependent DySS polymerization systems at 16°C (top) and 37°C (bottom: Fig. 3D). At 16°C, the chains evolve into a DySS with a $\bar{bp}_w \approx 1200$ bp ($\bar{l}_w \approx 4.1 \mu$m), hence, almost twice as high compared to 37°C ($\bar{bp}_w \approx 600$ bp; $\bar{l}_w \approx 2.0 \mu$m). The lower temperature (16°C) favors the ligation, whereas the higher temperature (37°C) shifts the reaction balance to the restriction side. The second important point is the difference in the DySS lifetimes at a given ATP concentration (Fig. 3E). Due to reduced enzymatic reaction rates at low temperatures and thus slower ATP conversion, the lifetimes of the DySS at 16°C exceed those at 37°C (e.g. by more than several days at 1.0 mM ATP), as less energy is dissipated per time. At 16°C and [ATP] $\geq$ 0.6 mM the DySS lifetime even exceeds the chosen experimental timeframe of 10 days. Since both enzymes are stable over time, this effect is clearly rooted in the slower conversion of ATP.

Critically, refueling experiments with a second addition of ATP after completion of one polymerization cycle underscore that ageing of the enzymes plays no significant role within the investigated timeframe (Fig. 3 C, D; Fig. S4, S7). The second cycle looks almost identical to the first one with respect to lifetime and average chain length (see also fluorescence experiments in Fig. S10 with 4 consecutive activation cycles). Control experiments without ATP fail to initiate the second cycle, and thereby confirm ATP clearly as the chemical driver of the DySS polymerization system. Overall, the ability to program DySS lifetimes up to weeks with high ATP concentrations following a linear dependence, to operate the system at different temperatures, and to reactivate several cycles confirms a very robust and long-living system with little problems concerning product inhibition (waste; AMP + PPi) or enzyme stability.
Fig. 3. Programming the transient ATP-fueled DySS polymerization of DNA chains by changing the dynamics of ligation and cleavage under biocatalytic and thermal control. The starting configuration of the systems comprises 0.05 mM M1 (38 bp), 41.25 WU T4 DNA ligase, 900 U BamHI, 0.1 mM ATP in the enzyme reaction buffer at 16°C, 25°C, 37°C. Each of these parameters is systematically varied to tune the dynamics of the transiently evolving chains: Increase of (A) T4 DNA ligase, (B) BamHI or (C) of both enzymes symmetrically, shifts the kinetic balance of the competing reactions either to the ligation or the restriction side, leading to different DySSs and lifetimes (0.1 mM ATP, 25°C). (D) Dynamics and ATP-dependent lifetimes can be further controlled by temperature: top: 16°C, dynamics slow down; bottom: 37°C, dynamics speed up. (E) Comparison of the temperature-dependent temporal development of the average chain length $\overline{bp_w}$ for selected ATP concentrations: top: 1.0 mM, bottom: 0.1 mM. (F) Time-dependent GE showing reactivation of transient chain growth by addition of ATP (both cycles fueled with 0.1 mM ATP, 37°C). (G) The corresponding plots of $\overline{bp_w}$ over time demonstrate identical dynamic system behavior for the second cycle. Control experiments elucidate ATP as the driving force for successful reinitiation. The lines in all graphs are drawn as a guide to the eye.

Molecular Dynamics and Adaptation within the DySSs

Finally, we investigate more closely the detailed dynamics of the system to evidence and understand the intermolecular exchange and the adaptation within true DySSs. We start with a simplified system of two DNA duplex dimers of different length, Dl (100 bp) and Ds (72 bp), with an internal restriction site to demonstrate the molecular exchange of DNA fragments (Fig. 4A). Upon ATP-fueled enzymatic dynamization, the duplexes are continuously cleaved and recombine randomly,
and thereby generate a new transient hybrid species D* of intermediate length (84 bp; Fig. 4A,B). Gray scale analysis highlights the transient occurrence of the hybrid species D* in the DySS between 1 h and 6 h (D* in green), before everything is eventually cleaved into the monomeric fragments M_L and M_S (Fig. 4C). This confirms unambiguously intermolecular exchange between the DNA dimers, and provides avenues to program transient functionality.

More importantly, on a polymer level, intermolecular exchange and bond shuffling occurs constantly between dynamized DNA polymers. To visualize and understand this time-dependent process, we dynamized a mixture of two fluorescently labeled DNA polymers with an excess of ATP. Using multicolor GE imaging, Fig. 4D,F illustrate how short fluorescein-labeled (P_Fl) and long Cy5-labeled DNA polymers (P_Cy5) undergo sequence randomization into a statistically mixed composition upon evolution of the DySS. At the beginning, individual fluorescent P_Fl and P_Cy5 oligomers are distinguishable by different migration behavior and colors in the composite GE image due to the influence of the attached fluorophores. However, upon bond shuffling in the DySS, the two initially separated red (P_Cy5) and green (P_Fl) static chain length distributions merge into a single mixed one of orange color, which adopts the DySS properties given by the specific enzymatic conditions. The disappearance of the oligomeric migration shift between the individual P_Fl and P_Cy5 bands and the convergence into one band can be convincingly visualized via gray scale analysis of the individual fluorophore channels and the composite image at t = 0 h (static mixture) and at t = 48 h (DySS; Fig. 4F).

All experiments presented so far indicate that the [T4]/[BamHI] ratio controls dynamically the degree of steady-state ligation and the molecular exchange frequencies within the DySS. This should make the DySS systems highly adaptive to changes in the enzymatic environment. To allow for an in-situ readout of the adaptive behavior, we used a DNA duplex F (42 bp) equipped with the Cy3/Cy5 FRET pair close to the internal restriction site. The FRET duplex F reports its DySS and the average steady-state bond strength of the ensemble by FRET-induced emission of the Cy5 acceptor dye, while the cleaved fragments F_Cy3 and F_Cy5 lack FRET (Fig. 4G). Spectral changes upon dynamization of the F_Cy3 and F_Cy5 fragments were evaluated by the FRET ratio (Cy5/Cy3 = I_674 nm/I_571 nm), which can be converted into a relative percentage of ligation, importantly, with greater precision and higher temporal resolution than in GE (details in Fig. S9). Fig. 4H demonstrates the evolution into programmable DySSs by variation of the enzyme ratio [T4]/[BamHI] at 25°C starting with the fully cleaved fragments. Increasing [T4] (1.14 WU to 18.33 WU) at a constant [BamHI] = 100 U (left panel) allows to reach the DySS faster and the extent of DySS ligation increases from ca. 63% to 83%. The stable plateau of the FRET ratio in the DySS confirms the development of true steady states with constant rates of ligation and cleavage. The degree of DySS ligation decreases drastically for higher [BamHI], as displayed in the right panel with a decrease down to ca. 34% at [BamHI] = 400 U, while [T4] = 2.29 WU is constant. Due to the rapid ATP conversion at this very high cleavage activity, the transient nature of the fueled system is visible with a final decay into the fully cleaved state.
Fig. 4. Adaptive DySSs and molecular exchange in the dynamic covalent DNA bond system. (A-C) Intermolecular exchange between two different dimer duplexes Ds (72 bp) and Dr (100 bp) upon enzymatic dynamization of the dynamic covalent restriction site. (A) DNA species formed during the transient ATP-fueled dynamization. (B) GE of ATP-fueled dimer exchange kinetics (37°C) shows the transient occurrence of a hybrid species D* (84 bp) and provides evidence for molecular reshuffling of the fragments. (C) Gray scale profiles highlight D* in green. Conditions: 0.5 µM Ds, 0.5 µM Dr, 37°C. (D-F) Dynamic sequence shuffling between fluorescently labeled DNA chains proves intermolecular subunit exchange also on the polymer level. (D) Two homopolymers, short fluorescein-tagged Pfl (green) and long Cy5-tagged Pcy5 (red), were mixed together and turned into a random copolymer upon DySS activation. (E) The shuffling process and evolution into a DySS polymer is followed by selective multicolor GE. The multicolor GE is a composite image of the fluorescein (green) and the Cy5 (red) channel. The fluorescent oligomers
show different migration distances and can be distinguished from each other (compare first two lanes of pure homopolymers P_E and P_Cy5). A randomized DySS sequence appears in orange color and by homogenization of the band migration. (F) Gray scale analysis of the individual fluorophore channels and the composite reveal the different composition of the static (0 h) and the dynamic (48 h) polymer “mix” (framed sections in GE). Convergence of the initially separated bands into one DySS band, c.f. the heptamer fraction (No. 7), demonstrates successful sequence shuffling and subunit exchange. Conditions: 5.0 µM M_E in P_E, 2.5 µM M_Cy5 in P_Cy5, 37°C. (G-I) Adaptive DySSs monitored by FRET duplex activation (Fig. S9, S10 for details). (G) The dynamic covalent bond was equipped with the Cy3/Cy5 FRET pair to report the DySS ligation level via the FRET ratio I_{max,Cy5(acceptor)/I_{max,Cy3(donor)}. The FRET ratio can be translated into a fraction of ligation, which is effectively an ensemble average steady-state bond strength. (H) Formation of different DySSs in dependence of the enzyme ratio [T4]/[BamHI] at 25°C: Variation of the T4 DNA ligase (left, [BamHI] = 100 U = const.) and BamHI (right, [T4] = 2.29 WU = const). (I) In-situ adaptation of the DySS in a transient ATP-fueled FRET duplex activation by sequential addition of individual enzymes. Conditions: 1 µM F_Cy3, 1 µM F_Cy5, 25°C, λ_exc = 505 nm.

Critically, the DySS ligation level adapts promptly to manipulations of the enzyme ratio as visualized by in-situ monitoring of the DySS and stepwise addition of the individual enzymes, T4 DNA ligase or BamHI (Fig. 4I). Starting from 1.14 WU T4 DNA ligase and 100 U BamHI, the FRET duplex system is activated by 30 µM ATP and evolves into its first DySS with a dynamic ligation plateau of ca. 64%. Another addition of T4 DNA ligase (+9.16 WU) shifts the DySS balance stronger towards the ligation side and increases the dynamic ligation ratio up to ca. 77%, while subsequent injections of BamHI (100 U) promote the cleavage and reduce the DySS plateau stepwise to ca. 31%. After each disturbance of the enzymatic balance, the system needs time for adaptation to form a new stable DySS. However, further manipulations of the DySS can be carried out until the system runs out of fuel (here ca. 15 h). Additional ATP-dependent lifetimes and refueling experiments monitored by FRET are in Fig. S10. Overall, this repeated adaptation to different DySSs with full reversibility of the dynamically cleaved bond underscore the robustness and integrity of the system.

**Discussion**

In this work, we bridge the gap between stable, robust covalent structure formation and the programmable dynamics of kinetically controlled molecular exchange in non-equilibrium systems by introduction of a fuel-driven dynamic covalent bond system. In contrast to classic, sensitized equilibrium-type dynamic covalent bonds, this dissipative system needs energy for making – and not for breaking – the covalent bond. This provides unprecedented controllability and inherent access to more complex, highly adaptive and autonomous steady-state behavior. The key properties of such a chemically fueled dynamic covalent bond are isothermally controlled DySSs with programmable and adaptive fractions of the bound state (bond ratio), tunable exchange frequencies, and transient lifetimes of the ensemble on a systems level. Critically, the chemical fuel is only an energy-providing co-factor and only serves to power the bond formation between two functional partners, and does not represent one of those. This provides the flexibility in molecular design, which is needed to access covalent connectivity patterns on larger length scales, of different topology and of emergent functionalities.

We investigated the structural implications of this by implementation of an ATP-fueled enzymatically activated and dynamized DNA phosphodiester bond, which was used for the transient polymerization of short dsDNA monomers into DySS polymers and thus micrometer-long semi-flexible fibrils. The integrated dissipative, dynamic covalent bond continuously consumes chemical energy by conversion of ATP, and the dynamics can be controlled by the kinetics of the enzymatic reaction network of ligation and cleavage. The availability of ATP controls mainly the lifetime of the dynamic polymers, while the absolute enzyme concentrations and the kinetic balance of ligation and cleavage regulate the average chain length and the exchange dynamics of the DySS.
The system is completely reversible and can be reactivated by addition of fresh ATP, with little effect of waste products on reactivation.

Strikingly, this system features simultaneous programmability on a temporal, structural and steady-state dynamics level in non-equilibrium molecular systems. A decisive advantage of the chemically fueled dynamic covalent bond is the fact that energetic events are merged with structural transitions, and modulate concurrently the intermolecular dynamics of the ensemble, which is not possible for chemically fueled supramolecular system approaches. Additionally, for the ATP-driven dynamic covalent DNA bond, the facile programmability of DNA systems and the availability of a large range of restriction enzymes will allow to proceed quickly towards rational design of the behavior in non-equilibrium systems, including different lifecycles, multicomponent systems, and for spatiotemporal organizations of functions in general. The next steps on a materials level will be to translate this emergent behavior into programmable non-equilibrium structure/property relationships. The integration of this ATP-fueled dynamic covalent bond into DNA hybrid soft matter systems is highly appealing, e.g. for active DNA hydrogels with programmable and adaptive stress relaxation behavior to study fundamental cell behavior, or for fueled self-healing via preorchestrated reshuffling of dynamic crosslinks. We believe that chemically fueled dynamic covalent bond systems are an avenue for robust and deterministic dissipative non-equilibrium materials systems and we are excited about finding further suitable coupling reactions that allow for this behavior in other material classes.

Materials and Methods

Hybridization of the DNA building blocks. The DNA monomer $M_1$ was obtained by mixing the complementary DNA strands $M_a$ and $M_b$ (each from 1 mM stock in the annealing buffer) in a stoichiometric ratio. The mixture (0.5 mM) was annealed in a thermocycler by heating to 95°C for two minutes and then cooling down to 20°C with a controlled temperature rate of 0.01°C/s. The fluorescently labeled DNA duplex strands $F$, $D_S$, $D_L$, $M_{Fl}$ and $M_{Cy5}$ were hybridized stoichiometrically in the 1x reaction buffer E from their single stranded constituents a and b by incubation at 37°C for 1 h to give a final storage solution of 25 µM dsDNA. Hybridized DNA stock solutions were stored at -20°C.

Transient dynamic DNA polymerization system. Enzymatic reactions of the dynamic chain growth were typically assembled in a total reaction volume of 90 µL as follows: Sterile water, DNA $M_1$, 10x buffer E, BSA, T4 DNA ligase and the BamHI restriction enzyme were added sequentially in a PCR tube. The solution was mixed gently by pipetting up and down and centrifuged shortly before addition of the ATP to initiate the reaction system. The enzymatic reaction was incubated in a thermoshaker at 250 rpm. Incubation temperatures (16°C, 25°C, 37°C) and the concentrations of the enzymes and the ATP (0.1 mM – 1.0 mM) varied depending on the experiment and are stated at the corresponding figures. The concentrations of all other components (0.05 mM DNA, 1x buffer E, 0.1 g/L BSA) were kept constant in the reaction mixture throughout all kinetic assays.

Time-dependent aliquots (6 µL) were withdrawn from the reaction tube and immediately quenched in the quenching buffer containing EDTA and subsequent freezing in liquid nitrogen. Time intervals were adapted to the kinetics of the experiments to follow the reaction progress appropriately. Kinetic aliquots were analyzed by electrophoretic mobility shift assays. Gel electrophoresis (GE) was carried out in 2 wt% agarose gels in TAE buffer applying 90 V = const., 300 mA, 90 min using in-cast staining with Roti®-GelStain.

Supplementary Materials

Supplementary material for this article is available at http://advances.sciencemag.org.
Materials and Methods

Experimental Protocols

Supplementary Note A: Development of the Conditions for the Dynamic Reaction Network by Characterization of the Individual Enzyme Reactions

Supplementary Note B: Routine of Gel Electrophoresis Analysis: From the Agarose Gel to an Average Chain Length

Supplementary Note C: ATP-Fueled Transient, Dynamic Steady-State DNA Polymerization System

Supplementary Note D: Dynamic Steady States and Molecular Exchange in ATP-fueled Dissociative Dynamic Covalent DNA Systems

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**Data and materials availability:** The data that support the plots within this paper and other finding of this study are available from the corresponding author upon reasonable request.
Supplementary Materials for

Programmable Dynamic Steady States in ATP-Driven Non-Equilibrium DNA Systems

Laura Heinen\textsuperscript{1,2,3}, Andreas Walther\textsuperscript{1,2,3,4}.*

\textsuperscript{1}Institute for Macromolecular Chemistry, University of Freiburg, Stefan-Meier-Straße 31, 79104 Freiburg, Germany.
\textsuperscript{2}Freiburg Materials Research Center (FMF), University of Freiburg, Stefan-Meier-Straße 21, 79104 Freiburg, Germany.
\textsuperscript{3}Freiburg Center for Interactive Materials and Bioinspired Technologies (FIT), University of Freiburg, Georges-Köhler-Allee 105, 79110 Freiburg, Germany.
\textsuperscript{4}Freiburg Institute for Advanced Studies (FRIAS), University of Freiburg, Albertstraße 19, 79104 Freiburg, Germany.

Correspondence to: andreas.walther@makro.uni-freiburg.de

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Selected Recurring Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ssDNA        | single-stranded DNA |
| dsDNA        | double-stranded DNA |
| GE           | gel electrophoresis |
| nt           | nucleotide |
| bp           | base pair |
| kbp          | kilo base pair |
| DySS         | dynamic steady state |
| FRET         | Förster resonance energy transfer |

1. Materials and Methods

Instrumentation:

TSC Thermoshaker (Analytik Jena), Thermocycler Tpersonal (Analytik Jena), U:Genius³ Gel electrophoresis documentation system (Syngene), UVsolo touch Gel electrophoresis documentation system (Analytik Jena), gel electrophoresis chambers (biostep), power source 250 V (VWR), Enduro power supply 300 V (Labnet International, Inc.), Large blue LED transilluminator (470 nm, IORodeo), Home-built RGB-LED-Transilluminator (Red: 620-630 nm, Green: 515-530 nm, Blue: 465-475 nm), Band pass filters: 545BP40-50x50 (Laser Components), 690BP40-50x50 (Laser Components), 605BP50-50x50 (Analytik Jena). Image J 1.51k, ScanDrop® UV-VIS spectrometer (Analytik Jena), QE Pro spectrometer with fluorescence measurement set-up (Ocean Optics), fiber coupled LED light source 505 nm (LLS series, Ocean Optics), CUV-QPOD Temperature Controlled Cuvette Holder qpod 2e™ (Ocean Optics).

Reagents:

The following chemicals, reagents and enzymes were used. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, biology grade, CALBIOCHEM), sodium chloride (NaCl, 99%, ABCR), tris (hydroxymethyl)aminomethane hydrochloride pH 8.8 and pH 8.0 (Tris-HCl, Trizma buffer substance pH 8.8 and pH 8.0, Sigma-Aldrich), Trizma base (Sigma-Aldrich), Acetic acid glacial (ACS, Reag. Ph. Eur. analytical reagents, VWR Chemicals), Gene Ruler 1kb DNA ladder (ready-to-use, Thermo Scientific, fragments [bp]: 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000), Gene Ruler 50bp DNA ladder (ready-to-use, Thermo Scientific, fragments [bp]: 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000), TrackIT™ Ultra Low Range DNA ladder (ready-to-use, Invitrogen, fragments [bp]: 10, 20, 35, 50, 75, 100, 150, 200, 300), Roti®-GelStain (1,1',3',5',5',6'-Octamethyl-2,2'-spiro(2,3-dihydro-1H-Benzimidazol), Carl Roth), SYBR™ Gold Nucleic Acid Gel Stain (10000x Concentrate in DMSO, Invitrogen), T4 DNA Ligase in Storage Buffer (20 WU/µL (HC) and 3 WU/µL , source: recombinant E. coli strain, Promega), BamHI in Storage Buffer (80 U/µL (HC) and 10 U/µL, source: Bacillus amyloliquefaciens H, Promega), 10x Buffer E (Promega), ATP solution (10 mM in 1 mM Tris-HCl pH 7.5, Invitrogen), ATP Solution Tris-buffered (100 mM, pH 7.3-7.5, Thermo Scientific), Agarose low EEO (Agarose Standard, PanReac AppliChem), Acetylated Bovine Serum Albumine (BSA, 10 g/L, Promega), Glycerol (Ultrapure, Invitrogen).
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 MgCl₂, 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 MgCl₂, 10 mM DTT.

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

*TAE Buffer:* 40 mM TRIS, 20 mM Acetic Acid, 1 mM EDTA.

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

Oligonucleotides:

Oligonucleotides were purchased from Integrated DNA Technologies Inc. (USA, Belgium) in HPLC purity. DNA stock solutions were prepared by resuspending the lyophilized oligonucleotides in annealing buffer, unless for the fluorescently labeled strands which were directly dissolved in the 1x reaction buffer E. The FRET duplex strands $F_a$ and $F_b$ were synthesized by biomers.net GmbH (Germany). Sterile ultrapure water with conductivity less than 0.055 mS/cm was used throughout all experiments. Table S1 presents an overview of the used DNA strands. Note the phosphorylation at the 5’-end.

**Table S1: Oligonucleotide sequences**

| ID   | oligonucleotide sequence                                      | #nt |
|------|----------------------------------------------------------------|-----|
| Mₐ   | 5’-/5Phos/GATC CT TAT TCG CAT GAG AAT TCC ATT CAC GTG AAG G-3’ | 38  |
| Mₕ   | 5’-/5Phos/GATC CCT TAC GTG GAA TGG AAT TCT CAT GCG AAT AGA G-3’| 38  |
| Dₛₐ  | 5’-CTCTATTCGAGATTTCCATTCACCGTAAAGGATCCTC/i6-TAMN/ATTGCATGAGAAATTCACCGTAAAGG-3’ | 72  |
| Dₛₕ  | 5’-CTTACCGGTAAGTTCTATGCGAATAGAGGATCCTC/i6-TAMN/ACGGTGAAATGAAATTTCATGCGAATAGAGG-3’ | 72  |
| Dₐ   | 5’-CTCTATTCGAGAGCTCTGAATAGGAATTTCATGCGAATAGAGGATCCTC/iF loopT/ATTGCATGAGAAATTCACCGTAAAGGCTGCGCTGCTGCGA-3’ | 100 |
| Dₐ   | 5’-CTCTATTCGAGAGCTCTGAATAGGAATTTCATGCGAATAGAGGATCCTC/iF loopT/ATTGCATGAGAAATTCACCGTAAAGGCTGCGCTGCTGCGA-3’ | 100 |
| Fₐ   | 5’-CATTTCCATTCACCGTAAAGGATCCTC/dT-Cy3/ATTGCATGAGAAATTTCATGCGAATAGAGGATCCTC/iF loopT/ATTGCATGAGAAATTCACCGTAAAGGCTGCGCTGCTGCGA-3’ | 42  |
| Fₕ   | 5’-AATTTCCATGCGAATAGAGGATCCTC/dT-Cy5/ACGGTGAAATGGAATAGG-3’ | 42  |
| Mₐ-Cy₅| 5’-/5Phos/GATCCTCTTATTCGAGAGCTCTGAATAGGAATTTCATGCGAATAGAGGATCCTC/iF loopT/ATTGCATGAGAAATTCACCGTAAAGGCTGCGCTGCTGCGA-3’ | 38  |
| Mₕ-Cy₅| 5’-/5Phos/GATCCTCTTATTCGAGAGCTCTGAATAGGAATTTCATGCGAATAGAGGATCCTC/iF loopT/ATTGCATGAGAAATTCACCGTAAAGGCTGCGCTGCTGCGA-3’ | 38  |
2. Experimental Protocols

**Ligation kinetics of the DNA chain growth as a function of T4 DNA ligase concentration.** The ligation assay was carried out as described above for the transient dynamic system at 25 °C, but omitting the BamHI restriction enzyme. The amount of T4 DNA ligase varied from 5.5 WU to 110 WU with 1.0 mM ATP in the reaction mixture. See Fig. S2.

**Ligation kinetics of the DNA chain growth as a function of ATP concentration.** The ATP-dependent ligation assay was carried out as described above for the transient dynamic system at 25 °C, but omitting the BamHI restriction enzyme. T4 DNA ligase activity was set constant to 41.25 WU, while the ATP concentration varied from 0.001 mM to 1.0 mM in the reaction mixture. See Fig. S3.

**Long-term ligation kinetics of the DNA chain growth.** The long-term ligation assay was carried out as described above for the transient dynamic system at 25 °C, but omitting the BamHI restriction enzyme. T4 DNA ligase activity was set to 41.25 WU with 1.0 mM ATP in the reaction mixture. Kinetic aliquots were taken over a period of 9 days to check for maximum DNA chain length development and conversion. See Fig. S4.

**Activity assay of T4 DNA ligase over time to check for potential ageing effects.** T4 DNA ligase (41.25 WU, 0.46 WU/µL) was incubated for a period of 10 days under the standard reaction conditions of the ligation reaction ($V_{tot} = 90$ µL, 1x buffer E, 0.1 g/L BSA) at 25 °C and 250 rpm. Ligation efficiency of this T4 DNA ligase stock was tested every 24 h by taking a small aliquot (8 µL), which was then mixed with fresh DNA M₁ (0.05 mM) and fresh ATP (1.0 mM). The mixture was reacted for 30 min (25 °C, 250 rpm) and subsequently quenched in quenching buffer by EDTA. The time-dependent ligation reactions were analyzed by GE as described above for the transient dynamic DNA chain growth reaction. See Fig. S4.

**Restriction kinetics of the DNA chain cleavage reaction as a function of BamHI concentration.** First, the DNA substrate of the enzymatic digestion assay of BamHI was prepared by ligation of the monomer fragment, M₁, to maximum conversion into long DNA chains. The ligation reaction was typically assembled in a total volume of 50 µL by sequential addition of water, hybridized DNA (M₁, 0.1 mM), 10x buffer E (1x dilution), BSA (0.1 g/L) and T4 DNA ligase (50 WU, 1 WU/µL). The solution was mixed gently by pipetting up and down and centrifuged shortly before addition of the ATP (1 mM) to initiate the ligation reaction. The ligation reaction was incubated in a thermoshaker at 25 °C and 250 rpm for 24 h and subsequently the T4 DNA ligase was heat-deactivated at 70 °C for 10 min. Several substrate batches of the ligated DNA chains were combined and carefully mixed to ensure the same starting material for every restriction assay. The kinetic assays of the cleavage reaction were assembled in a total volume of 90 µL by sequential addition of water, ligated DNA substrate (equivalent to 0.05 mM M₁), 10x buffer E (1x dilution) and BSA (0.1 g/L). The solution was mixed gently by pipetting up and down and centrifuged shortly before addition of the BamHI restriction enzyme to start the cleavage reaction. The concentration of the BamHI restriction enzyme varied from 450 U (5 U/µL) to 1800 U (20 U/µL). The enzymatic restriction reaction was incubated in a thermoshaker at 25 °C and 250 rpm and kinetic aliquots (6 µL) were taken at predetermined time...
Refueling experiments of the transient dynamic DNA polymerization reaction system. The transient dynamic system was conducted as described above using 41.25 WU T4 DNA ligase (0.46 WU/µL), 900 U BamHI restriction enzyme (10 U/µL) and 0.1 mM ATP as fuel. After completion of the first lifecycle with recovery of the original monomer sequence, the system was refueled by injection of either (i) ATP, (ii) T4 DNA ligase, (iii) ATP and T4 DNA ligase or (iv) no reagents added. The concentrations of the second injections were adjusted to match the conditions of the first cycle, corresponding to the reduced reaction volume due to withdrawn kinetic aliquots. Refueling of a second lifecycle is only successful upon addition of ATP. The refueling experiments were performed at 25 °C and 37 °C. See Fig. S7 and Fig. 3F,G in the main manuscript.

Dynamic dimer exchange between different DNA duplexes monitored by GE. Two differently sized DNA duplexes D_L (fluorescein-labeled, 100 bp) and D_S (TAMRA-labeled, 72 bp), each having the BamHI restriction site positioned in the center of the sequence, were assembled in an equimolar ratio (1:1) in a PCR reaction tube by mixing water, 10x buffer E (1x in reaction), duplex D_L (0.5 µM) and D_S (0.5 µM), BSA (0.1 g/L), ATP (2 µM), T4 DNA ligase (9.16 WU) and varying amounts of BamHI restriction enzyme (50 U, 100 U and 200 U) to give a final reaction volume of 200 µL. The solution was mixed gently by pipetting up and down and centrifuged shortly before being incubated in a thermoshaker at 37 °C at 300 rpm.

Time-dependent aliquots (20 µL) were withdrawn and immediately quenched in quenching buffer and subsequent freezing in liquid nitrogen. Kinetic aliquots were analyzed by GE in 4 wt% agarose gels in TAE buffer applying 75 V = const., 300 mA, 4 h and then poststained with SYBR gold for imaging. See Error! Reference source not found. Fig. 4A-C in the main manuscript.

Dynamic sequence shuffling of two different DNA homopolymers into one statistic copolymer and visualization by selective fluorophore GE imaging. First, two batches of DNA homopolymer chains with different degree of polymerization and different dye labels were prepared. Short, fluorescein-labeled DNA chains were obtained by ligation of M_F (10 µM) with 0.46 WU/µL T4 DNA ligase, 0.1 g/L BSA in 1x buffer E and substoichiometric ATP (3 µM) for 40 min at 25 °C to intentionally limit conversion to low molecular weights (V_\text{tot} = 150 µL). The T4 DNA ligase/DNA polymer solution was heat-deactivated (10 min, 70 °C) and spin filtered (3 kDa) with 1x buffer E to deactivate T4 DNA ligase and remove unreacted ATP.

Likewise, long, Cy5-labeled DNA chains were prepared by ligation of M_CY5 (10 µM) with 0.46 WU/µL T4 DNA ligase, 0.1 g/L BSA in 1x buffer E and excess ATP (1 mM) for 39 h at 25 °C to achieve maximum conversion (V_\text{tot} = 150 µL). The T4 DNA ligase/DNA polymer solution was heat-deactivated (10 min, 70 °C) and spin filtered (100 kDa) with 1x buffer E to deactivate T4 DNA ligase and remove unreacted ATP, as well as small DNA oligomers.

The dynamic sequence shuffling experiment was carried out in a total volume of 55 µL containing both DNA homopolymers (5 µM of short, fluorescein-labeled DNA chains, P_F, and 2.5 µM of long, Cy5-labeled DNA chains, P_CY5; molar concentrations relative to the monomer content) with 2 mM ATP, 20.6 U BamHI and 5.67 WU T4 DNA ligase, 0.1 g/L BSA in 1x buffer E at 37 °C.
Time-dependent aliquots (6 µL) were withdrawn from the reaction tube and immediately quenched in quenching buffer containing EDTA and subsequent freezing in liquid nitrogen. Kinetic aliquots were analyzed by GE in 2 wt% agarose gels in TAE buffer, 90 V = const., 300 mA, 2.5 h. The gel was analyzed by selective fluorophore imaging. The fluorescein-labeled DNA was imaged with excitation using a blue LED (ca. 465-475 nm) and a band-pass filter centered at 545 nm (BP 40 nm), while excitation with a red LED (ca. 620-630 nm) and a band-pass filter at 690 nm (40 nm) was used for the Cy5-labeled DNA. The DNA molecular weight ladders were visualized using the blue LED and a band-pass filter at 605 nm (50 nm) after post-staining with SYBR gold.

The gel images were stacked and processed in ImageJ, including correction of background, brightness, contrast and coloring, to obtain a multi-color composite image of both dyes, and further analyzed by gray scale profiling. See Fig. 4F in the main manuscript.

**Enzymatic cleavage of the FRET duplex (F) with BamHI.** In a typical cleavage reaction, the hybridized FRET duplex F (25 µM) was incubated with BSA (0.1 g/L) and the restriction enzyme BamHI (8 U/µL) in 1x buffer E at 37 °C, 300 rpm for 48 h. Full cleavage was verified by GE (60 min, 90 V = const., 300 mA, 4 wt% agarose stained with Roti-GelStain), and the sample was characterized by fluorescence spectroscopy (1 µM F, λexc = 505 nm, 1 s integration time, 25 °C). After heat-deactivation of the BamHI (10 min, 70 °C) the cleaved FRET duplex was used as starting material for the DySS FRET experiments. See Fig. S9.

**Enzymatic religation of the cleaved FRET duplex fragments with T4 DNA ligase.** T4 DNA ligase (2 U/µL) and ATP (1 mM) were added to the heat-deactivated, cleaved FRET duplex solution (25 µM) and incubated at 25 °C, 300 rpm for 48 h. Subsequently, T4 DNA ligase was heat-deactivated (10 min, 70 °C). Full religation was verified by GE (60 min, 90 V = const., 300 mA, 4 wt% agarose stained with Roti-GelStain). The sample was characterized by fluorescence spectroscopy (1 µM cleaved F, λexc = 505 nm, 1 s integration time, 25 °C). See Fig. S9.

**Cleavage kinetics of the FRET duplex (F) with 100 U BamHI at 25 °C as monitored by FRET in fluorescence spectroscopy.** The enzymatic cleavage reaction was assembled in a total volume of 200 µL by sequential addition of water, FRET duplex F (1 µM), 10x buffer E (1x dilution), BSA (0.2 g/L) and mixed gently by pipetting up and down. The overall glycerol content was typically kept at 5 vol%. The solution was transferred into a quartz glass cuvette (d = 3 mm) and equilibrated at 25 °C in the holder of the fluorescence spectrometer. The reaction was started by addition of BamHI (100 U, 0.5 U/µL). Cleavage of the FRET duplex was monitored by time-dependent emission spectra (λexc = 505 nm, 1 s integration time, 25 °C). See Fig. S9.

**Religation kinetics of the cleaved FRET duplex fragments with 4.58 WU T4 DNA ligase at 25 °C as monitored by FRET in fluorescence spectroscopy.** The enzymatic ligation reaction was assembled in a total volume of 200 µL by sequential addition of water, the cleaved fragments of the FRET duplex F (1 µM), 10x buffer E (1x dilution), BSA (0.2 g/L), and mixed gently by pipetting up and down. The overall glycerol content was typically kept at 5 vol%. The solution was transferred into a quartz glass cuvette (d = 3 mm) and equilibrated at 25 °C in the holder of the fluorescence spectrometer. The reaction was started by addition of T4 DNA ligase (4.58 WU, 0.023 WU/µL) and ATP (4 µM). Religation of the FRET duplex was monitored by time-dependent emission spectra (λexc = 505 nm, 1 s integration time, 25 °C). See Fig. S9.
**Enzymatic control over the DySSs of the ATP-fueled dynamic covalent DNA bond of the FRET duplex as monitored by FRET in fluorescence spectroscopy.** The DySS jump experiments presented in Figure 4H of the main manuscript were assembled in a total volume of 200 µL by sequential addition of water, the cleaved fragments of the FRET duplex F (1 µM), 10x buffer E (1x dilution), BSA (0.2 g/L), and mixed gently by pipetting up and down. The overall glycerol content was typically kept at 5 vol%. The solution was transferred into a quartz glass cuvette (d = 3 mm) and equilibrated at 25 °C in the holder of the fluorescence spectrometer. The enzymes were added after 5 min in varying ratios: (Series A) BamHI = 100 U + varying T4 DNA ligase, (Series B) T4 DNA ligase = 2.29 WU + varying BamHI. After 5 min of equilibration, the reaction was started by addition of ATP (4 µM). Temporal evolution of the DySS of the FRET signal was monitored by time-dependent emission spectra (λexc = 505 nm, 1 s integration time, 25 °C). See Fig. 4H in the main manuscript.

**In-situ adaptation of different DySSs of the dynamic covalent DNA bond in the FRET duplex by variation of the enzyme ratio as monitored by FRET in fluorescence spectroscopy.** The DySS adaptation experiment of the dynamically ligated FRET duplex was assembled in a total volume of 200 µL by sequential addition of water, the cleaved fragments of the FRET duplex F (1 µM), 10x buffer E (1x dilution), BSA (0.2 g/L) and mixed gently by pipetting up and down. The overall glycerol content was typically kept at 5 vol%. The solution was transferred into a quartz glass cuvette (d = 3 mm) and equilibrated at 25 °C in the holder of the fluorescence spectrometer. Both enzymes, BamHI (100 U) and T4 DNA ligase (1.14WU) were added after 5 min of incubation. After another 5 min of equilibration, the reaction was started by addition of ATP (30 µM). The extent of dynamic ligation (visible as FRET pair formation) was controlled by addition of the respective enzymes – either T4 DNA ligase or BamHI – to switch between different DySSs: (1st addition) 9.16 WU T4 DNA ligase, (2nd add.) 100 U BamHI, (3rd add.) 100 U BamHI, (4th add.) 4.58 WU T4 DNA ligase, (5th add.) 9.16 WU T4 DNA ligase. Temporal evolution of the DySSs of the dynamic FRET bond was monitored by time-dependent emission spectra (λexc = 505 nm, 1 s integration time, 25 °C). See Fig. 4I in the main manuscript.

**Lifetime control of the transient, dynamic FRET duplex ligation in dependence of ATP.** The transient, dynamic ligation of the FRET duplex (= dynamic covalent bond formation) with an ATP-dependent lifetime was assembled in a total volume of 200 µL by sequential addition of water, the cleaved fragments of the FRET duplex F (1 µM), 10x buffer E (1x dilution), BSA (0.2 g/L), and mixed gently by pipetting up and down. The overall glycerol content was typically kept at 5 vol%. The solution was transferred into a quartz glass cuvette (d = 3 mm) and equilibrated at 25 °C in the holder of the fluorescence spectrometer. The enzymes, BamHI (100 U) and T4 DNA ligase (18.33 WU), were added after 5 min. After another 5 min of equilibration, the reaction was started by addition of varying amounts of ATP (0.5 µM – 20 µM). Temporal evolution of the DySS DNA bond was monitored by the transient FRET signal in time-dependent emission spectra (λexc = 505 nm, 1 s integration time, 25 °C). See Fig. S10.

**Refueling of dynamic FRET duplex ligation by multiple additions of ATP.** Multiple repetitions of the transient, dynamic ligation of the FRET duplex (= dynamic covalent bond formation) were carried out in a total volume of 200 µL. The reaction solution was assembled by sequential addition of water, the cleaved fragments of the FRET duplex F (1 µM), 10x buffer E (1x dilution), BSA (0.2 g/L) and mixed gently by pipetting up and down. The overall glycerol content was typically kept at 5 vol%. After 5 min of equilibration, the reaction was started by addition of ATP (0.5 µM – 20 µM). Temporal evolution of the DySS DNA bond was monitored by the transient FRET signal in time-dependent emission spectra (λexc = 505 nm, 1 s integration time, 25 °C). See Fig. S10.
kept at 5 vol%. The solution was transferred into a quartz glass cuvette (d = 3 mm) and equilibrated at 25 °C in the holder of the fluorescence spectrometer. The enzymes, BamHI (100 U) and T4 DNA ligase (4.58 WU), were added after 5 min. After another 5 min of equilibration, the reaction was started by addition of ATP (2 μM). After completion of one such transient dynamic ligation cycle, the reaction was refueled by addition of ATP (2 μM) several times. Temporal evolution of the dissociative DySS bond was monitored by the transient FRET signal in time-dependent emission spectra (λ<sub>exc</sub> = 505 nm, 1 s integration time, 25 °C). See Fig. S10.
3. Supplementary Note A. Development of the Conditions for the Dynamic Reaction Network by Characterization of the Individual Enzyme Reactions

Both the T4 DNA ligase and the BamHI restriction enzyme-catalyzed reactions were characterized independently of each other with the DNA monomer M₁ and its ligated polymer as substrates to establish suitable reaction conditions for the ATP-fueled dynamic polymerization system. We analyze different reaction parameters, involving the influence of enzyme, DNA and ATP concentration, reaction time and temperature, and enzyme stability, and discuss their effect on a step-growth-like polymerization in the following. Methodological details on GE analysis are provided in Supplementary Note B.

3.1. Hybridization Efficiency and Melting Behavior of the Self-Complementary Ends of Monomer Strand M₁

The transient DySS polymerization is conditional on the fact that the monomers may only polymerize in dependence of energy input via a chemically fueled reaction. Therefore, the self-complementary ends, which serve as telechelic end groups of the DNA monomer strands, M₁, are intentionally kept short to prevent uncontrolled self-hybridization and elongation into polymer chains (Fig. S1 A, B). The NUPACK(39) prediction of the melting profile of the 4 bp DNA hybridization (GATC) underpins that the self-complementary ends are largely unpaired under our reaction conditions (16 – 37 °C).

Fig. S1 C describes the ATP-dependent ligation reaction in general terms (A) and with mechanistic details of the enzyme-catalyzed phosphodiester bond formation (B). With regard to later stoichiometry and energy considerations, it is important to note that complete joining of the ssDNA overhangs of two DNA monomers consumes two molecules of ATP because of nick sealing in both strands of the duplex. Note that the fuel is not incorporated into the final structure, but only mediates bond formation as energy source. This provides important flexibility in the design of partners to be joined.
Fig. S1: Hybridization of the self-complementary ends of the DNA monomer strands M₁ in dependence of temperature and ligation reaction catalyzed by T4 DNA ligase. (A) Scheme of the self-hybridization of M₁. M₁ consists of a 34 bp duplex body with a single-stranded 4 nt overhang at each side. These ends are self-complementary, but too short to hybridize permanently with each other. (B) The predicted melting profile of this 4 bp hybridization shows that the bases are largely unpaired over the whole temperature range from 0 °C to 45 °C (conditions similar to a typical polymerization system: 0.05 mM DNA, 100 mM Na⁺ and 6 mM Mg²⁺). (C) Ligation reaction of two DNA monomer strands M₁ as seen in our step growth reaction of DNA chains. Covalent coupling of two monomer strands consumes two molecules of ATP as the formation of a phosphodiester bond in each single strand is catalyzed by an ATP-dependent T4 DNA ligase reaction.

3.2. 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.
3.3. **Ligation Kinetics of the DNA Chain Growth in Dependence of T4 DNA Ligase**

The kinetics of the T4 DNA ligase-catalyzed polymerization of the M₁ monomer strands (0.05 mM) were analyzed at 25 °C. Variation of the enzyme concentration from 5.5 WU to 110 WU increases the turnover in the system, which leads to faster built-up of the polymer chains until a constant plateau is reached (Fig. S2H). All ligation experiments were carried out with an excess of 1 mM ATP to exclude any conversion-related limitations of this step-growth polymerization.
Fig. S2: Ligation kinetics of the DNA chain growth as a function of T4 DNA ligase concentration. (A-F) GE images of kinetic assays with increasing T4 DNA ligase concentration from 5.5 WU to 110 WU. (G) Evolution of the average DNA chain length $\bar{bp}$ with time (see Supplementary Note B for details). (H) Magnification of the initial growth phase shows acceleration with increasing T4 DNA ligase concentration. Conditions: 25 °C, 1x buffer E, 0.1 g/L BSA, 0.05 mM M1, 1.0 mM ATP and varying T4 DNA ligase concentration. Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 5 min, 5: 10 min, 6: 15 min, 7: 20 min, 8: 30 min, 9: 45 min, 10: 60 min, 11: 2 h, 12: 3 h, 13: 4 h, 14: 6 h, 15: 8 h, 16: 24 h.
3.4. Ligation Kinetics of the DNA Chain Growth in Dependence of ATP

Due to the step-growth character of the ATP-dependent T4 DNA ligase-catalyzed polymerization of the DNA monomer Mₙ, the amount of supplied ATP can be used to purposely limit the conversion, and, thus, the degree of polymerization (= average chain length) of the Mₙ-based DNA polymers. A substoichiometric amount of ATP compared to the theoretical number of ligation sites (= 2x monomer concentration) reduces the average chain length of the DNA polymer chains drastically (Fig. S3A-D). High molecular weights can only be reached at high conversions, which require at least equimolar amounts of ATP (Fig. S3E,F).
Fig. S3: Ligation kinetics of the DNA chain growth as a function of ATP concentration. (A-F) GE images of kinetic assays with increasing ATP concentration from 0.001 mM to 1.0 mM. (G) Evolution of the average DNA chain length $bp_w$ with time. Conditions: 25 °C, 1x buffer E, 0.1 g/L BSA, 0.05 mM M1, 41.25 WU T4 DNA ligase and varying concentrations of ATP. Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 5 min, 5: 10 min, 6: 15 min, 7: 20 min, 8: 30 min, 9: 45 min, 10: 60 min, 11: 2 h, 12: 3 h, 13: 4h, 14: 6 h, 15: 8 h, 16: 24 h.
3.5. Long-Term Development of T4 DNA Ligase-Catalyzed DNA Chain Growth and Time-Dependent Activity Assay of the T4 DNA Ligase

The maximum molecular weight attainable for the M₁-based DNA polymers is given by the extent of the step growth reaction. A T4 DNA ligase-catalyzed polymerization reaction (standard conditions: 25 °C, 0.05 mM M₁, 41.25 WU T4 DNA ligase and excess 1 mM ATP) was carried out over a continued period of nine days to monitor how quickly maximum conversion is obtained. Fig. S4 A, B displays a rapid growth of the DNA chains, which already levels off after the first hours and stays constant thereafter at around $\bar{L}_{p_w} = 1200$ bp. This excludes reduction of the average chain lengths due to time-limited conversion. However, given the complex nature of the system, the maximum chain lengths of the polymerization system may also be affected by slight batch-to-batch variations in reactant quality (e.g. enzyme activity, DNA purity and end group functionalization). To ensure comparability and reproducibility, all measurements within one experimental series of the kinetic studies were carried out using the same batches of reactants (DNA, enzymes, ATP).

The stability of the T4 DNA ligase over prolonged periods of time is of utmost importance for enabling the ATP-fueled transient DySS polymerization system, as it ensures the continuous energy input into the system via conversion of ATP and because it maintains the dynamic non-equilibrium steady-state character of the dynamic covalent system with continuous joining of phosphodiester bonds. The time-dependent T4 DNA ligase activity was assayed by short test ligation reactions (30 min reaction time). To this end, the DNA monomer fragment M₁ was incubated with T4 DNA ligase in a reaction tube under the standard reaction conditions at 25 °C (without ATP). Aliquots were taken on a daily basis and supplemented with fresh ATP to test for ligation efficiency. Fig. S4 C, D demonstrates that the obtained average chain length $\bar{L}_{p_w}$ of the polymer chains stays fairly constant over the investigated experimental time frame of 12 days. Minor decreases of enzyme activity do not interfere with the overall integrity of the DySS polymerization system.
**Fig. S4: Time-dependent T4 DNA ligase catalyzed ligation reaction.** Long-term kinetic analysis of the T4 DNA ligase-catalyzed ligation of the DNA chain growth. 

(A) GE image of the time-dependent ligation assay of M1. 

(B) Evolution of the average DNA chain length $\overline{bp_n}$ with time. Assay conditions: 25 °C, 1x buffer E, 0.1 g/L BSA, 0.05 mM M1, 41.25 WU T4 DNA ligase and 1.0 mM ATP. Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 10 min, 5: 1 h, 6: 6 h, 7: 9 h, 8: 24 h, 9: 2 d, 10: 3 d, 11: 4 d, 12: 5 d, 13: 6 d, 14: 7 d, 15: 8 d, 16: 9 d. 

(C) Ageing of T4 DNA ligase. GE image of samples obtained after injecting ATP into T4 DNA ligase/M1 solutions that were aged for different time frames as indicated. The polymerization time was set constant to 30 min. 

(D) Evolution of the average DNA chain length $\overline{bp_n}$ with time. The line is a linear fit to the data and indicates a slight loss of activity. Ligation reaction conditions: 25 °C, 1x buffer E, 0.1 g/L BSA, 0.05 mM M1, 0.46 WU/µL T4 DNA ligase, 1.0 mM ATP and incubation for 30 min. Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 1 d, 5: 2 d, 6: 3 d, 7: 4 d, 8: 5 d, 9: 6 d, 10: 7 d, 11: 8 d, 12: 9 d, 13: 10 d, 14: 50 bp, 15: 1 kbp.
3.6. Restriction Kinetics of the DNA Chain Degradation in Dependence of BamHI

An understanding of the time scales of the DNA cleavage in comparison to the ligation is critical to set up a suitable concentration ratio between the antagonistic enzymes, that fulfills the kinetic boundary condition for generating a transient system with faster activation than deactivation. We analyzed the restriction kinetics of BamHI, independently of the T4 DNA ligase, using previously ligated and heat-deactivated M1-based DNA polymers P1 as substrate. Increasing amounts of BamHI (450 U to 1800 U) strongly accelerate the digestion reaction to the original monomer M1. The speed of the digestions scales with the BamHI concentration. Full digestion of P1 takes up to four days for all BamHI concentrations and is hence much slower than the ligation reaction (see Fig. S2). This enables the kinetic condition necessary in a non-equilibrium DySS polymerization.

Fig. S5: Restriction kinetics of the DNA chain cleavage as a function of BamHI concentration. (A-D) GE images of kinetic assays of the cleavage of long M1-based DNA polymers, P1, with increasing BamHI activity from 450 U to 1800 U. (E) Evolution of the average DNA chain length $\langle P_n \rangle$ with time. Assay conditions: 25 °C, 1x buffer E, 0.1 g/L BSA, 0.05 mM P1 (polymer of M1), and varying concentrations of BamHI. Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 10 min, 5: 20 min, 6: 30 min, 7: 1 h, 8: 2 h, 9: 3 h, 10: 4 h, 11: 6 h, 12: 9 h, 13: 24 h, 14: 48 h, 15: 72 h, 16: 96 h.
4. Supplementary Note B. Routine of Gel Electrophoresis Analysis: From the Agarose Gel to an Average Chain Length

The enzyme-catalyzed step growth reaction of the DNA monomer, M₁, leads to a distribution of DNA chains with a broad range of molecular weights (38 bp to > 10000 bp). GE allows separation of DNA chains with molecular resolution down to a few base pairs (bp). Band resolution scales mainly with the agarose concentration (i.e. effective pore size of the gel), applied voltage and run time. GE conditions were optimized in TAE buffer to satisfy imaging of the broad chain length distribution. We chose an agarose concentration of 2 wt% (90 V, 90 min) to identify clearly the number of single repeat units of the oligomers (monomer, dimer, trimer, …) as illustrated in Fig. S6A.

![Fig. S6: Routine for analysis of GE data: From the agarose GE to an average DNA chain length \( \bar{bp}_{av} \). (A) Raw GE image of a time-dependent ligation experiment. The DNA base pair ladders (50 bp and 1 kbp) used for calibration are on the right-hand side. (B) Background-corrected GE image. (C) Extracted gray scale profiles of the base pair ladders. (D) Calibration curve to convert electrophoretic mobility into number of base pairs (\( \#bp \)) based on the band assignment of the DNA ladders. (E) Time-dependent gray scale profiles of the ligation assay as a function of \( \#bp \) aligned to the position of the 38 bp band of M1. (F) Evolution of the average DNA chain length (\( \bar{bp}_{av} \)) with time.](image)

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Band resolution gets poorer, i.e. molecular weight separation is less resolved, for longer DNA chains, which effectively cuts down the tail of the molecular weight distribution and reduces the measured average chain length and dispersity. DNA staining is based on Roti-GelStain, a benzimidazole dye binding to the minor groove of the DNA double helix. The binding scales with the length of the DNA duplex. Thus, the fluorescence signal obtained from the DNA staining is proportional to the DNA concentration and chain length (its mass), and the extracted gray scale profile from each lane of the agarose GE images corresponds to a mass-weighted chain length distribution.

DNA base pair ladders allow a calibration of the GE images and calculation of an average chain length as demonstrated below. The GE images of the kinetic electrophoretic mobility shift assays were routinely analyzed to obtain quantitative data on the development of the average chain length over time. The procedure is shown in Fig. S6 using the example of a time-dependent ligation reaction of the DNA chain growth. First, the raw GE image (Fig. S6A) is background corrected (Fig. S6B) using the sliding paraboloid algorithm to remove uneven illumination (Image J 1.51k). All GE images shown in the manuscript/SI are background-corrected. Afterwards gray scale profiles of each lane are extracted, giving intensity values as a function of migrated distance (Fig. S6C). The migrated distances $d_m$ are converted into electrophoretic mobilities $\mu_e$ according to Eq. 1 with drift velocity $v$, electric field strength $E$, time $t$, voltage $V$, distance between the electrodes $d_{el}$.

$$\mu_e = \frac{v}{E} = \frac{\frac{d_m}{t}}{\frac{V}{d_{el}}} \quad \text{Equation 1}$$

A 50 bp and 1 kbp DNA ladder are used for calibration of the gels by fitting electrophoretic mobility $\mu_e$ as a function of DNA chain length $x$ [bp] as described by Van Winkle et al. (40) in the following relationship (Eq. 2) with $\alpha$, $\beta$, and $\gamma$ as adjustable fit parameters (Fig. S6C, D).

$$\mu_e(x) = \frac{1}{\beta + \alpha \left(1-e^{-\frac{x}{\gamma}}\right)} \quad \text{Equation 2}$$

This fit function is used to recalculate the gray scale profiles as a function of base pair number. The band position of the monomer strand is normalized to its length of 38 bp (Fig. S6E). From this chain length distribution, the mass average chain length $\overline{bp_w}$ is obtained via applying Eq. 3 with $bp_i$ as the base pair number of fraction $i$ and $f_i$ as the intensity value of fluorescence intensity of the corresponding fraction $i$.

$$\overline{bp_w} = \frac{\sum_{i=1}^{n} f_i \cdot bp_i}{\sum_{i=1}^{n} f_i} \quad \text{Equation 3}$$

The average DNA chain length $\overline{bp_w}$ is plotted over time to estimate the temporal evolution of the enzymatic polymerization reaction (Fig. S6F).
5. Supplementary Note C. ATP-Fueled Transient, Dynamic Steady-State DNA Polymerization System

5.1. Refueling Experiments of the Transient DySS DNA Polymerization System

The aim of the refueling experiments is to clearly identify ATP as the driver of a chemically fueled dynamic polymerization reaction. To this end, a standard DySS polymerization reaction was initiated with 0.1 mM of ATP (Fig. S7). After completion of the first cycle, the system was supplemented with fresh reactants: A) ATP, B) T4 DNA ligase, C) ATP and T4 DNA ligase, and D) no reagent (negative control). A second transient polymerization cycle is only observed for the experiments that include further addition of ATP. Critically, the addition of fresh T4 (B) does not reinitiate the polymerization, proving that no loss of T4 DNA ligase enzyme activity causes the transient character of the polymerization. A similar behavior is obtained for the same refueling and control experiments at 37 °C, underscoring the robustness at higher temperature (Manuscript Fig. 3). Refueling experiments monitored by FRET are presented in Fig. S10.

![Fig. S7: Refueling experiments of the transient DySS DNA polymerization system at 25 °C. After completion of the first lifetime cycle (96 h), the systems are injected with (A) ATP (0.1 mM), (B) T4 DNA ligase (0.46 WU/µL), (C) ATP (0.1 mM) and T4 DNA ligase (0.46 WU/µL), (D) no reagents added. The concentrations of the reactants added during the second injection are equivalent to those applied in the first lifetime cycle. The GE images of the kinetic assays show successful re-initiation of the transient DySS DNA polymerization only in conjunction with ATP (A, C). Kinetic assay conditions: 25 °C, 1x buffer E, 0.1 g/L BSA, 0.05 mM MgCl₂, 41.25 WU T4 DNA ligase (0.46 WU/µL), 900 U BamHI (10 U/µL) and 0.1 mM ATP. Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 1 h, 5: 6 h, 6: 24 h, 7: 48 h, 8: 72 h, 9: 96 h, 10: 97 h, 11: 102 h, 12: 120 h, 13: 144 h, 14: 168 h, 15: 192 h.](image-url)
5.2. Transient DySS DNA Polymerization in Dependence of the DNA Concentration

The concentration of the DNA monomer $M_1$ impacts drastically the temporal evolution of the transient enzyme-catalyzed DySS polymerization with respect to the timescales needed to enter and leave the DySS and regarding the achievable average chain length of the dynamic DNA polymers. At a constant enzyme concentration ratio (41.25 WU T4 DNA ligase and 900 U BamHI) and ATP concentration (0.1 mM), higher monomer concentrations ($M_1$) slow down the absolute time scales of formation and degradation of the polymers as the substrate to enzyme ratio is higher, which delays high conversions needed for high molecular weights in step growth reactions and for complete digestion. Further, the different stoichiometry between DNA monomer $M_1$ and ATP can alter the degree of polymerization due to limitations of the chemical fuel for maximum conversion. This needs to be considered for the concentration of 0.1 mM DNA.

![Images of kinetic assays with increasing DNA concentration](image)

**Fig. S8:** Average chain length in the transient DySS DNA polymerization system in dependence of the concentration of the DNA monomer $M_1$. (A-D) GE images of kinetic assays with increasing DNA concentration ($M_1$) from 0.025 mM to 0.1 mM. (E) Evolution of the average DNA chain length $L^*$ with time. Kinetic assay conditions: 25 °C, 1x buffer E, 0.1 g/L BSA, 41.25 WU T4 DNA ligase, 900 U BamHI, 0.1 mM ATP and varying concentrations of $M_1$. Lane assignment: 1: 50 bp ladder, 2: 1 kbp ladder, 3: 0 min, 4: 10 min, 5: 20 min, 6: 30 min, 7: 1 h, 8: 2 h, 9: 4 h, 10: 6 h, 11: 9 h, 12: 12 h, 13: 24 h, 14: 48 h, 15: 72 h, 16: 96 h.
6. Supplementary Note D. Dynamic Steady States and Molecular Exchange in ATP-fueled Dissociative Dynamic Covalent DNA Systems

6.1. Characterization of the Different States of the FRET Duplex F

The FRET duplex F (42 bp) carries the FRET pair Cy3/Cy5 as fluorescent tags around the BamHI restriction site which is located at the center of the sequence. The FRET pair serves as a reporter for the extent of the ensemble average steady-state covalent bonding in the dynamized phosphodiester bond. The dyes are attached via amino-C6 linkers to thymines in the complementary strands 10 bp apart from each other. The fluorescent tags show no adverse effects on the enzymatic cleavage and religation as shown by the clean reactions (restriction, religation) in GE (Fig. S9A).

Fig. S9: Characterization of the FRET duplex F and its cleaved and religated DNA fragments as used for in-situ modulation of the DySS. (A) The FRET duplex F with the fluorescent tags Cy3 and Cy5 next to the restriction/ligation site can be cleaved by BamHI and religated by T4 DNA ligase completely without any residual traces as seen by the single DNA bands in GE. Lane assignment: 1: original FRET duplex, 2: cleaved state, 3: religated state. (B) Corresponding fluorescence spectra normalized to the Cy3 donor emission peak at 571 nm. The religated FRET duplexes show 1/3 of the original FRET emission at 674 nm (Cy5 acceptor) due to statistic recombination of the cleaved DNA duplex fragments F_{Cy3} and F_{Cy5} to F_{Cy3}F_{Cy3}, F_{Cy5}F_{Cy5} and F_{Cy3}F_{Cy5} (1:1:1). Critically, only F_{Cy3}F_{Cy5} induces FRET. (C) Schematic representation of the different FRET duplex bonding states. (D) Fluorescence spectra during cleavage of the original FRET duplex F with 100 U BamHI, and (E) evaluation of the Cy3 donor emission at 571 nm (green dot), the Cy5 acceptor emission at 674 nm (red triangle) and the FRET ratio (674 nm/571 nm, black dot) for different time points.
squares) over time. (F) Religation kinetics of the cleaved FRET duplex fragments (F_{Cy3}, F_{Cy5}) with 4.58 WU and 4 µM ATP as represented by the time-dependent fluorescence signals of the Cy3 donor at 571 nm (green dot), the Cy5 acceptor at 674 nm (red triangle) and the FRET ratio (674 nm/571 nm, black squares). All fluorescence spectra were recorded in 1x buffer E with 1 µM F, 0.2 g/L BSA, λ_{exc} = 505 nm, 1 s integration time, 25 °C.

The original FRET duplex F hybridized from strand F_a and F_b shows a maximum FRET emission at 674 nm (= 100 %, black line, Fig. S9B), because all Cy3 donors are positioned next to their Cy5 acceptor FRET partners. After complete enzymatic cleavage with BamHI, both dyes are fully separated into two duplex fragments (F_{Cy3} and F_{Cy5}, both 21 bp in length) with no FRET being observed (= 0 %, red line, Fig. S9B). Upon religation of these cleaved duplex fragments by T4 DNA ligase the maximum FRET emission does not and cannot recover to the original value. This is due to a statistic recombination of the palindromic (self-complementary) ends of the cleaved parts. Religation of the cleaved DNA duplex fragments F_{Cy3} and F_{Cy5} leads to F_{Cy3}F_{Cy3}, F_{Cy5}F_{Cy5} and F_{Cy3}F_{Cy5} in a 1:1:1 ratio. Critically, only F_{Cy3}F_{Cy5} shows FRET. The possible reaction products upon religation are illustrated in Fig. S9C. Hence, the FRET efficiency is reduced to 1/3 (= 33 %, blue line) of the original state. Ultimately, the fully cleaved and religated state represent the limiting cases of the dynamic DNA bond formation, and, thus, the accessible and tunable range of the fluorescence spectra changes due to FRET in the DySS experiments presented in the manuscript (Figure 4) and Section 6.2.

Fig. S9D-F show the spectral changes during cleavage of F and of the religation of F_{Cy3} and F_{Cy5}. Enzymatic cleavage of the FRET duplex reduces the FRET-induced emission of the Cy5 acceptor at 674 nm and strengthens the Cy3 donor emission at 571 nm (Fig. S9D,E). The opposite behavior is observed for the ligation reaction on the cleaved DNA by reformation of the FRET pair (however to a lesser extent due to statistical intermixing). In the following, the FRET ratio (Cy5/Cy3 = I_{674 nm}/I_{571 nm}) is used for the time-resolved evaluation of the dynamic bonding state of the FRET duplex and converted to a relative percentage of ligation using the accessible FRET signal limits of the DySS. Ultimately, this relative degree of bonding translates into an ensemble average steady-state bond strength of the dynamic system. We observed that the presence of the DNA-bound enzymes slightly lowers the FRET efficiency between the Cy3/Cy5 pair.
6.2. ATP-Dependent Temporal Control of the Dynamic DNA Bond Visualized by Time-Dependent FRET

The average lifetime of the dynamic covalent DNA bond, as represented here by the dynamic transient ligation of the FRET duplex fragments, $F_{Cy3}$ and $F_{Cy5}$, can be controlled by the amount of ATP as chemical fuel. Increasing amounts of ATP elongate the DySS phase by several hours under the chosen conditions (Fig. S10A,B). Transient dynamic ligation can be reactivated multiple times when supplemented with new ATP (Fig. S10C).

Fig. S10. ATP-dependent temporal control of the dynamic DNA bond with transient DySS FRET duplex formation. Programming of the transient lifetime of the dynamic FRET duplex (1 µM cleaved F ($F_{Cy3}$ + $F_{Cy5}$)) is shown for an enzyme ratio of 18.33 WU T4 DNA ligase and 100 U BamHI with varying amounts of ATP as chemical fuel in 1x buffer E with 0.2 g/L BSA at 25 °C. (A) Evaluated lifetime and (B) corresponding time-dependent traces of the FRET ratio for ATP concentrations from 0.5 µM to 20 µM. Molar ratios of ATP to ligation sites > 1 suggest a linear dependency of the lifetime on the ATP concentration (dashed line). This is in line with the trend presented in Figure 2 (manuscript). (C) After completion of one full transient cycle, the transient DySS cycle can be reinitiated multiple times by addition of ATP. The refueling experiment shows four consecutive cycles each initiated with 2 µM ATP in a dynamic system of 100 U BamHI and 4.58 WU T4 DNA ligase with 1 µM cleaved F ($F_{Cy3}$ and $F_{Cy5}$), 0.2 g/L BSA in 1x buffer E at 25 °C.

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