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Dissertation

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Abstract

DNA nanotechnology has traditionally relied on the precise Watson–Crick–Franklin base-pairing interaction for programming synthetic DNA oligonucleotides to construct well-defined nanostructure and nanodevices. The development of toehold-mediated strand displacement reactions further expanded the field into DNA reaction networks for applications in detection, diagnostics, and computation. Nevertheless, DNA nanotechnology faces a challenge in scaling to larger materials and systems. Recently, synthetic long-chain single-stranded DNA polymers have been shown to undergo temperature-induced phase separation, yielding micron-sized DNA condensates that extend the scope of DNA-based materials and open possibilities for new applications. These condensates closely resemble biomolecular condensates found in cells, formed through liquid–liquid phase separation of proteins and nucleic acids. They provide valuable model systems for studying the properties and mechanisms of biomolecular condensates in controlled settings. Meanwhile, their inherent compatibility with DNA nanotechnology and synthetic biology make DNA condensates promising synthetic cell models for integrating structures and functions. Finally, DNA condensates introduce a new hierarchical level of DNA-based materials for constructing complex multiscale architectures and provide a versatile compartmentalization strategy for DNA reaction networks to address challenges in scalability and modularity.

At the intersection of soft matter, supramolecular chemistry, DNA nanotechnology, and synthetic biology, this thesis explores the application of DNA condensates across different research topics:

In the first part of the thesis, DNA condensates are investigated as a model system for biomolecular condensates. They reveal how macromolecular transport within condensates can deviate from classical Fickian diffusion under certain conditions. This study identifies a new transport mechanism, termed ballistic wave diffusion. It emerges from molecular-recognition-mediated transition of condensates from arrested to dynamic states. This mechanism offers a new perspective on macromolecular transport within biomolecular condensates, providing fundamental insights into their behavior in cellular environments.

Beyond serving as model systems for biomolecular condensates, DNA condensates also provide a versatile platform for constructing minimalistic synthetic cells. In the second part of this thesis, they are used to incorporate subcellular structures and transcription function. A co-phase-separation strategy is developed for functionalizing their core with unique single-stranded DNA sequences as recognition barcodes for binding complementary strands. These barcodes enable them to modularly integrate DNA tiles at defined concentrations, enabling the controlled formation of DNA nanotubes inside synthetic cells as artificial cytoskeletons. These cytoskeletons enhance the mechanical stability of synthetic cells and support the formation of a stable synthetic cell-cell interface via cRGD-integrin binding. In parallel, co-phase separation has been leveraged to introduce transcription promoter sequences into DNA

condensates, enabling the sequestration of transcription templates and enzymes. This endows the condensates with the ability to locally transcribe RNA nanostar sequences in their cores, which subsequently condense into diverse artificial condensate patterns as intracellular compartments.

This thesis also explores the application of DNA condensates as a compartmentalization strategy for DNA reaction networks. DNA barcodes inside DNA condensates are used to compartmentalize DNA reaction components. In contrast to the global diffusion and reaction observed in solution-based systems, confining DNA reactions within condensates physically isolates sequences from different networks, thereby preventing unwanted diffusion and crosstalk. This allows multiple reaction networks to be independently executed in a single system. Moreover, this approach simplifies design principles and allows the reuse of identical sequence designs for different reactions without interference, facilitating improved scalability and modularity in DNA reaction networks.

From a future perspective, the concepts and approaches developed in this thesis open up opportunities across various research directions. DNA condensates and ballistic wave diffusion could be harnessed to design a gating mechanism that controls the transport of large macromolecules through size-exclusion effects, representing responsive material systems. Their core barcodes could be engineered to integrate multiple functional modules and enable synergistic effects in advanced synthetic cell systems. In addition, DNA condensates can enrich dilute species through barcode recognition and facilitate localized reactions via proximity effects, with potential applications in detection and diagnostics. Of particular interest, DNA condensates may be further developed for compartmentalizing complex DNA reaction networks, such as multi-bit DNA Adder gate arrays, thereby enabling more sophisticated and scalable DNA computing systems capable of addressing challenging tasks.

Kurzzusammenfassung

Die DNA-Nanotechnologie stützte sich traditionell auf die präzise Watson-Crick-Franklin-Basenpaarungsinteraktion, um synthetische DNA-Oligonukleotide für den Aufbau genau definierter Nanostrukturen und Nanobaulemente zu programmieren. Die Entwicklung von „toehold-vermittelten“ Strangverdrängungsreaktionen erweiterte das Feld hin zu DNA-Reaktionsnetzwerken für Anwendungen in der Detektion, Diagnostik und im Computing. Dennoch steht die DNA-Nanotechnologie vor der Herausforderung, auf größere Materialien und Systeme zu skalieren. Kürzlich wurde gezeigt, dass synthetische langkettige einzelsträngige DNA-Polymere eine temperaturinduzierte Phasentrennung durchlaufen, was zu mikrometergroßen DNA-Kondensaten führt, die den Anwendungsbereich von DNA-basierten Materialien erweitern und Möglichkeiten für neue Anwendungen eröffnen. Diese Kondensate ähneln stark den in Zellen vorkommenden biomolekularen Kondensaten, die durch Flüssig-Flüssig-Phasentrennung von Proteinen und Nukleinsäuren gebildet werden. Sie stellen wertvolle Modellsysteme dar, um die Eigenschaften und Mechanismen von biomolekularen Kondensaten unter kontrollierten Bedingungen zu untersuchen. Gleichzeitig macht ihre inhärente Kompatibilität mit der DNA-Nanotechnologie und der synthetischen Biologie DNA-Kondensate zu vielversprechenden synthetischen Zellmodellen für die Integration von Strukturen und Funktionen. Schließlich führen DNA-Kondensate eine neue hierarchische Ebene von DNA-basierten Materialien für den Aufbau komplexer, mehrskaliger Architekturen ein und bieten eine vielseitige Kompartimentierungsstrategie für DNA-Reaktionsnetzwerke, um Herausforderungen in der Skalierbarkeit und Modularität zu bewältigen.

An der Schnittstelle von weicher Materie, supramolekularer Chemie, DNA-Nanotechnologie und synthetischer Biologie untersucht diese Dissertation die Anwendung von DNA-Kondensaten in verschiedenen Forschungsthemen:

Im ersten Teil der Arbeit werden DNA-Kondensate als Modellsystem für biomolekulare Kondensate untersucht. Sie zeigen auf, wie der makromolekulare Transport innerhalb von Kondensaten unter bestimmten Bedingungen von der klassischen Fickschen Diffusion abweichen kann. Diese Studie identifiziert einen neuen Transportmechanismus, der als ballistische Wellendiffusion (ballistic wave diffusion) bezeichnet wird. Er entsteht durch einen molekular-erkennungs-vermittelten Übergang von Kondensaten von einem arretierten in einen dynamischen Zustand. Dieser Mechanismus bietet eine neue Perspektive auf den makromolekularen Transport innerhalb biomolekularer Kondensate und liefert grundlegende Einblicke in ihr Verhalten in zellulären Umgebungen.

Über ihre Funktion als Modellsysteme für biomolekulare Kondensate hinaus bieten DNA-Kondensate auch eine vielseitige Plattform für den Aufbau minimalistischer synthetischer Zellen. Im zweiten Teil dieser Arbeit werden sie genutzt, um subzelluläre Strukturen und Transkriptionsfunktionen zu integrieren. Es wird eine Ko-Phasentrennungsstrategie entwickelt,

um ihren Kern mit einzigartigen einzelsträngigen DNA-Sequenzen als Erkennungs-Barcodes für die Bindung komplementärer Stränge zu funktionalisieren. Diese Barcodes ermöglichen die modulare Integration von DNA-Kacheln (DNA tiles) in definierten Konzentrationen, was die kontrollierte Bildung von DNA-Nanoröhrchen (DNA nanotubes) im Inneren synthetischer Zellen als künstliche Zytoskelette erlaubt. Diese Zytoskelette erhöhen die mechanische Stabilität der synthetischen Zellen und unterstützen die Bildung einer stabilen synthetischen Zell-Zell-Schnittstelle über cRGD-Integrin-Bindung. Parallel dazu wurde die Ko-Phasentrennung genutzt, um Transkriptions-Promotor-Sequenzen in DNA-Kondensate einzuführen, was die Sequestrierung von Transkriptionsvorlagen und Enzymen ermöglicht. Dies verleiht den Kondensaten die Fähigkeit, in ihren Kernen lokal RNA-Nanostern-Sequenzen zu transkribieren, die anschließend zu vielfältigen künstlichen Kondensatmustern als intrazelluläre Kompartimente kondensieren.

Diese Arbeit untersucht auch die Anwendung von DNA-Kondensaten als Kompartimentierungsstrategie für DNA-Reaktionsnetzwerke. DNA-Barcodes im Inneren von DNA-Kondensaten werden verwendet, um Komponenten von DNA-Reaktionen zu kompartimentieren. Im Gegensatz zur globalen Diffusion und Reaktion, die in lösungsbasierten Systemen beobachtet wird, isoliert die Beschränkung von DNA-Reaktionen innerhalb von Kondensaten Sequenzen aus verschiedenen Netzwerken physisch und verhindert so unerwünschte Diffusion und Übersprechen (Crosstalk). Dies ermöglicht die unabhängige Ausführung mehrerer Reaktionsnetzwerke in einem einzigen System. Darüber hinaus vereinfacht dieser Ansatz die Designprinzipien und erlaubt die Wiederverwendung identischer Sequenzdesigns für verschiedene Reaktionen ohne Interferenzen, was eine verbesserte Skalierbarkeit und Modularität in DNA-Reaktionsnetzwerken ermöglicht.

Aus zukünftiger Perspektive eröffnen die in dieser Arbeit entwickelten Konzepte und Ansätze Möglichkeiten für verschiedene Forschungsrichtungen. DNA-Kondensate und die ballistische Wellendiffusion könnten genutzt werden, um einen Gating-Mechanismus zu entwerfen, der den Transport großer Makromoleküle durch Größenausschluss-Effekte steuert und so responsive Materialsysteme darstellt. Ihre Kern-Barcodes könnten so konstruiert werden, dass sie mehrere funktionelle Module integrieren und synergistische Effekte in fortschrittlichen synthetischen Zellsystemen ermöglichen. Zusätzlich können DNA-Kondensate verdünnte Spezies durch Barcode-Erkennung anreichern und lokalisierte Reaktionen über Proximity-Effekte erleichtern, mit potenziellen Anwendungen in der Detektion und Diagnostik. Von besonderem Interesse ist die Weiterentwicklung von DNA-Kondensaten zur Kompartimentierung komplexer DNA-Reaktionsnetzwerke, wie zum Beispiel Multi-Bit-DNA-Addiergatter-Arrays, um dadurch anspruchsvollere und skalierbarere DNA-Computingsysteme zu ermöglichen, die in der Lage sind, herausfordernde Aufgaben zu bewältigen.

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Abbreviations

AFM: Atomic force microscopy	FTD: Frontotemporal dementia
ALS: Amyotrophic lateral sclerosis	FtsZ: A bacterial homologue of tubulin
ATP: Adenosine-5'-triphosphate	FUS: Fused in sarcoma
AuNRs: Gold nanorods	GC: Methoxylated glycine
BCARS: Broadband coherent anti-Stokes Raman scattering	GFP: Green fluorescent protein
BrA: Broccoli aptamer	Glu: Ethoxylated glutamic acid
BSA: Bovine serum albumin	GTP: Guanosine-5'-triphosphate
CLSM: Confocal laser scanning microscopy	HCR: Hairpin catalytic reaction
CM-dextran: Carboxymethyldextran	HIV-1: Type 1 human immunodeficiency virus
DBD: DNA-binding domain	HRP: Horseradish peroxidase
DD: Dimerization domain	IDP(s): Intrinsically disordered protein(s)
DFHO: 3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime	ITS -G: Penicillin-Streptomycin and insulin-Transferrin-Selenium
DNA: Deoxyribonucleic acid	KL(s): Kissing loop(s)
DNA-RNs: DNA reaction networks	LCD(s): Low complexity domain(s)
dNTP: Deoxynucleotide triphosphate	LCST: Lower critical solution temperature
DNT(s): DNA nanotube(s)	LDH: L-lactate dehydrogenase
dParB: Truncated partition protein B variant	LLPS: Liquid-liquid phase separation
DSD: DNA strand displacement	MBP: Maltose-binding protein
dsDNA: Double-stranded DNA	MD: Molecular dynamics
EDTA: Ethylenediaminetetraacetic acid disodium salt dihydrate	MGA: Malachite green aptamer
ELP(s): Elastin-like polypeptide(s)	mRNP(s): Messenger ribonucleoprotein(s)
EMSA: Electrophoretic mobility shift assay	NIR: Near-infrared
ERK: Extracellular signal-regulated kinases	nt: Nucleotide
FBS: Fetal bovine serum	OMe: Methoxy
FLIM: Fluorescence lifetime imaging	PAO1: <i>Pseudomonas aeruginosa</i>
FRAP: Fluorescence recovery after photobleaching	PBS: Phosphate-buffered saline
	PCL: Photo-cleavable linker

PDDA: Poly(diallyldimethylammonium chloride)

pEGA: Poly(ethylene glycol acrylate)

PN: Protonuclei

PNA: Peptide nucleic acid

Poly(A): Adenine-rich DNA polymers

Poly(C): Cytosine-rich DNA polymers

Poly(G): Guanine-rich DNA polymers

Poly(T): Thymine-rich DNA polymers

Poly(P): Polyphosphate

PS: Phase separation

RCA: Rolling circle amplification

RCM: Ring-closing metathesis

RLP: Resilin-like polypeptide

RNA: Ribonucleic acid

rNTP(s): Ribonucleotide triphosphate(s)

ROI: Region of interest

SC(s): Synthetic cell(s)

SDR(s): Strand displacement reaction(s)

SIM: Structured illumination microscopy

ss: Cystamine

ssDNA: Single-stranded DNA

ssRNA: Single-stranded RNA

T7 RNAP: T7 RNA polymerase

TCEP: Tris(2-carboxyethyl)phosphine

TCSPC: Time correlated single photon counting

TEV: Tobacco Etch Virus protease

Tris-HCl: Tris(hydroxymethyl)-aminomethane hydrochloride

UCST: Upper critical solution temperature

YFP: Yellow fluorescent protein

For amino acids:

cRGD: Cyclic Arginyl-Glycyl-Aspartic acid peptides

FF: Diphenylalanine

FG: Phenylalanine-glycine

RG: Arginine-glycine

RGG: Arginine-glycine-glycine

F: Phenylalanine

G: Glycine

K: Lysine

P: Proline

R: Arginine

S: Serine

V: Valine

W: Tryptophan

Y: Tyrosine

1. Introduction

Phase separation is a ubiquitous phenomenon that can be observed in everyday life. Long before its underlying mechanisms were scientifically understood, such processes were already exploited in food production. For example, proteins in milk or soy milk undergo phase separation when acidity increases or when coagulants such as magnesium chloride are added, which are key steps in producing cheese and tofu, practices that date back thousands of years. The scientific concept of phase separation, however, was established much later in the late 19th century with the rise of modern colloid and physical chemistry through pioneers such as Thomas Graham and Josiah Willard Gibbs. With the development of polymer science, initiated by Hermann Staudinger, Paul Flory and Maurice Huggins extended phase separation theory to polymer systems in the 1940s to explain their anomalous phase behavior.¹⁻³

Now, phase separation has been recognized to also occur within cells, serving as a key mechanism for generating membraneless organelles that organize cellular processes and functions.⁴⁻⁶ This discovery has sparked a new focus on the investigation and application of phase separation across biology, chemistry, and physics. In particular, an essential biological macromolecule, the DNA polymer, has been found to undergo phase separation, which not only has significant biological relevance but also introduces a new class of DNA-based materials for diverse applications.⁷ Beyond that, the phase separation behavior of DNA provides a new perspective on how compartmentalization may have emerged during the origin of life.

This introduction provides relevant backgrounds around phase separation and condensate materials. It begins with a review of the fundamental theory governing macromolecular phase separation, establishing the scientific basis for understanding condensate formation and connecting it to biomolecular condensates formed inside cells. Next, it presents the development of various synthetic condensate materials, where DNA condensates are highlighted for their programmability and compatibility with DNA nanotechnology and synthetic biology. Finally, as this thesis focuses on developing DNA condensates as synthetic cell models and versatile building blocks for DNA-based materials and systems, it discusses recent developments, challenges, and open knowledge gaps in synthetic cell research and DNA nanotechnology, proposing DNA condensates as potential solutions to these limitations.

1.1 Macromolecular Phase Separation: From Polymer Concepts to Biomolecular Condensates inside Cells

1.1.1 Flory–Huggins Theory and Phase Diagram of Polymer Solution

Macromolecules are chains of covalently bonded small molecules (i.e., monomers). Unlike small molecules, their polymeric nature leads to markedly different mixing behavior in solvents, resulting in various **phase separation** behavior. While small molecules can gain

substantial entropy when dissolved in a solvent, this entropic driving force is markedly reduced for macromolecules due to the chain connectivity. This distinctive behavior was first explored in the early development of polymer science in the 1940s, when Flory and Huggins formulated a theoretical framework incorporating both entropic and enthalpic contributions for ideal polymers composed of identical monomers in solution.¹⁻³ One of the most important concepts of their theory framework is the extension of the entropy of mixing expression from small molecules to polymer solutions and polymer blends in a lattice model, yielding

$$\Delta S_{\text{mix}} = -kN \left[\frac{\phi_A}{N_A} \ln \phi_A + \frac{\phi_B}{N_B} \ln \phi_B \right], \quad (\text{Eq. 1-1})$$

where ΔS_{mix} is the entropy of mixing of a binary system containing A and B molecules (which could be either small molecules or polymers), k is the Boltzmann constant, N is the total number of lattices available for molecules. ϕ_A and ϕ_B are the volume fractions of the A and B, respectively, with $\phi_A + \phi_B = 1$. N_A and N_B denote the number of lattice sites occupied per molecule of A and B. For the case of small molecule A mixed with solvent B, $N_A = N_B = 1$, which gives the classical expression for the entropy of mixing of small molecule

$$\Delta S_{\text{mix}} = -kN [\phi_A \ln \phi_A + \phi_B \ln \phi_B]. \quad (\text{Eq. 1-2})$$

Since both ϕ_A and ϕ_B are less than 1, the entropy of mixing is always positive, meaning entropy always favors the mixing of two different molecules. The contributions of molecules A and B to the entropic gain are proportional to their respective terms $\phi_A \ln \phi_A$ and $\phi_B \ln \phi_B$.

In contrast, for a polymer solution with polymer A and solvent B, $N_A \gg 1$ (polymer), and $N_B = 1$ (solvent), which yields

$$\Delta S_{\text{mix}} = -kN \left[\frac{\phi_A}{N_A} \ln \phi_A + \phi_B \ln \phi_B \right]. \quad (\text{Eq. 1-3})$$

As $N_A \gg 1$, the polymer's contribution to the entropic gain is greatly diminished, making the solvent's contribution the primary driver of mixing. Given this inherent loss of entropy, many polymer solutions display phase separation behaviors.

With a substantially weakened entropic gain in polymer solutions, molecular interactions between the polymer and solvent become critical in determining phase separation behavior. To describe the energy of mixing (ΔU_{mix}), the other key concept of Flory–Huggins theory is the derivation of the enthalpic contribution based on mean-field theory:

$$\Delta U_{\text{mix}} = \frac{zN}{2} \phi(1 - \phi)(2u_{AB} - u_{AA} - u_{BB}). \quad (\text{Eq. 1-4})$$

Here, z is the coordination number, representing the number of nearest neighboring molecules that interact, N is the total number of lattices available, and ϕ is the polymer volume fraction in the solution. The terms u_{AB} , u_{AA} , and u_{BB} denote the interaction energies between polymer and solvent (A–B), polymer and polymer (A–A), as well as solvent and solvent (B–B), respectively. From this, the **Flory–Huggins interaction parameter** (χ) is defined as

$$\chi \equiv \frac{z}{2} \frac{(2u_{AB} - u_{AA} - u_{BB})}{kT}, \quad (\text{Eq. 1-5})$$

which leads to

$$\Delta U_{\text{mix}} = kTN\chi\phi(1 - \phi), \quad (\text{Eq. 1-6})$$

where T denotes the temperature.

The Helmholtz free energy of mixing can be calculated as

$$\Delta F_{\text{mix}} = kTN \left[\frac{\phi}{N_A} \ln \phi + (1 - \phi) \ln(1 - \phi) + \chi\phi(1 - \phi) \right], \quad (\text{Eq. 1-7})$$

which describes thermodynamics of polymer solutions.

From Eq. 1-7, the change of free energy is a function of polymer volume fraction ϕ . Figure 1-1a shows a typical free energy of mixing for a simple symmetric system at a specific temperature. Since mixing requires the free energy change to be negative, the mixing and demixing behavior can be analyzed by examining the first and second derivatives of ΔF_{mix} at each temperature. The extrema, determined by $\frac{\partial \Delta F_{\text{mix}}}{\partial \phi} = 0$, and the inflection points, determined by $\frac{\partial^2 \Delta F_{\text{mix}}}{\partial \phi^2} = 0$, define two key points in the phase diagram at each temperature: the binodal and spinodal points (Figure 1-1b). Varying the temperature alters the shape of the ΔF_{mix} , thereby yielding a series of binodal and spinodal points that can be connected to form the binodal and spinodal curves.

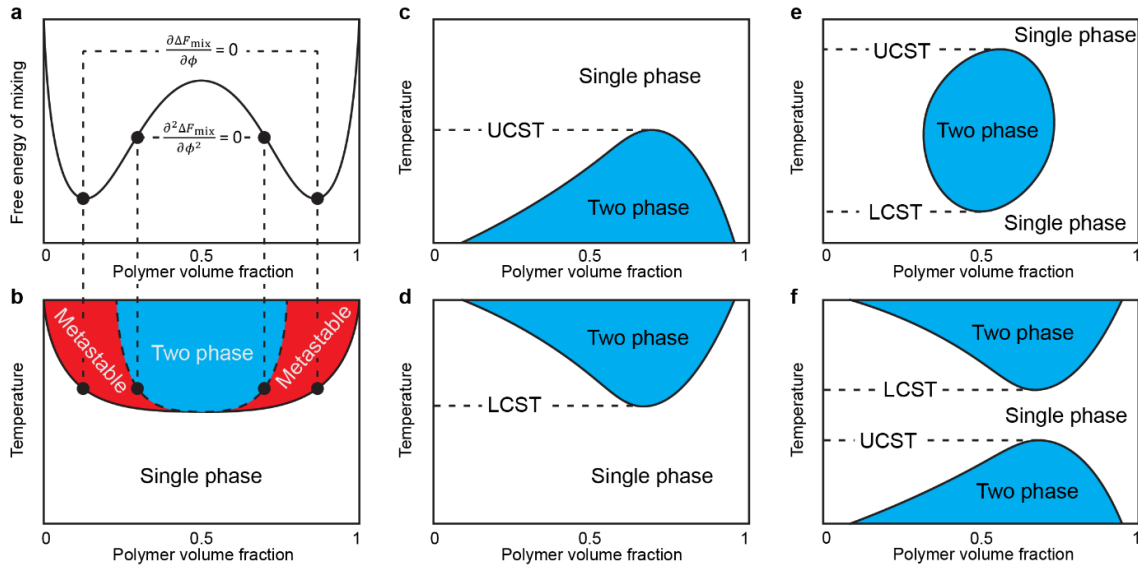


Figure 1-1. Free energy of mixing of polymer solution and various phase diagrams. **a**, Simplified free energy of mixing curve for a symmetric system at a specific temperature. **b**, Corresponding phase diagram obtained from the local minima and inflection points of the free energy of mixing function. **c**, Phase diagram of a system with UCST behavior. **d**, Phase diagram of a system with LCST behavior. **e**, Phase diagram of a system exhibiting both UCST and LCST behavior, where the two-phase region lies between the UCST and LCST, forming a closed-loop shape. **f**, Phase diagram of a system exhibiting both UCST and LCST behavior, where the two-phase region occurs either above the LCST or below the UCST, resulting in an hourglass shape.

Within the binodal region, the polymer solution is metastable and undergoes phase separation via a nucleation-and-growth mechanism, triggered by local concentration fluctuations. In the spinodal region, the solution is unstable and undergoes spontaneous spinodal decomposition, typically forming a bi-continuous structure initially, and breaking eventually into droplets to minimize interfacial area.

It is worth noting that the traditional Flory–Huggins theory assumes no volume change upon mixing. This assumption limits its applicability to explaining phase separation in polymer solutions only upon cooling, corresponding to an **upper critical solution temperature (UCST)**, and fails to account for phase separation of polymer solutions upon heating, i.e., a **lower critical solution temperature (LCST)**.⁸ The intriguing LCST behavior observed in some polymer solutions has led to reconsideration of free volume changes during mixing and the development of modified thermodynamic theory for polymer solutions.^{8–13}

Since the combinatorial ΔS_{mix} (Eq. 1-3) always favors the mixing of two phases, the phase separation behavior is ultimately governed by the enthalpy of mixing, and in particular, the interaction parameter χ . In the revised Flory–Huggins theory, χ is found not purely enthalpic. Polymer–solvent interactions also include a significant entropic contribution arising from free volume changes and ordering of solvent molecules around polymers. In practice, χ is often expressed using an empirical equation:

$$\chi(T) = A_\chi + \frac{B_\chi}{T}. \quad (\text{Eq. 1-8})$$

The temperature-independent term A_χ is referred to as “entropic part”, accounting for free volume change and ordering of solvent molecules, while B_χ/T represents the “enthalpic part”, describing whether the mixing process is endothermic or exothermic.^{8,14} For endothermic mixing ($B_\chi > 0$), χ decreases as temperature increases, favoring miscibility and leading to a UCST in the phase diagram (Figure 1-1c), as observed for poly(acrylic acid)/water solution.¹⁵ In contrast, for exothermic mixing with a negative entropy change from free volume effects ($B_\chi < 0$, and $A_\chi > 0$), χ decreases with decreasing temperature, resulting in a LCST in the phase diagram (Figure 1-1d), as observed for poly(N-isopropylacrylamide)/water solution.¹⁶ In more complicated systems, B_χ can change sign with temperature, resulting in both UCST and LCST behaviors that yield either a closed-loop shape (Figure 1-1e), e.g., for poly(vinyl alcohol)/water, or an hourglass shape (Figure 1-1f), e.g., for the polystyrene/acetone solution.^{17,18}

The development of the Flory–Huggins theory for polymer solution thermodynamics established a foundational framework in polymer physics for understanding polymer solutions and their phase separation. Beyond its original scope, this framework has found new relevance in biophysics, where it is adapted to study phase separation phenomena within living cells. In particular, the concepts of enthalpic and entropic contributions, interaction parameters, and critical solution temperatures provide valuable insights into the mechanism

of intracellular formation of biomolecular condensates that play essential roles in cellular organization and functions.^{19,20}

1.1.2 Phase Separation of Biomolecular Condensates inside Cells

Liquid–liquid phase separation (LLPS) of biological macromolecules, such as proteins and nucleic acids, has been recognized as a key mechanism in living cells for forming **biomolecular condensates** as membraneless organelles, which regulates physiochemical processes and functions (Figure 1-2a).^{4–6,21–25} The underlying mechanism of phase separation for biological macromolecules is far more complex than that for polymer solutions, as proteins and nucleic acids possess intricate structures and diverse interactions with solvents and among each other. Moreover, the cellular environment is also far from a simple plain solution. In a simplified framework, LLPS in cellular environments can be typically classified into two major categories: **segregative and associative** (Figure 1-2b).^{26,27} In segregative phase separation, macromolecules disfavor interactions with the solvent or coexisting molecules, leading to spontaneous solvent expulsion and the formation of macromolecule-rich droplets. In contrast, associative phase separation occurs when one or more types of macromolecules preferentially interact with each other, driving their assembly into a macromolecule-rich phase. Fundamentally, the distinction between the two arises from the balance between solute–solvent and solute–solute interactions, analogous to the role of the Flory–Huggins interaction parameter χ .

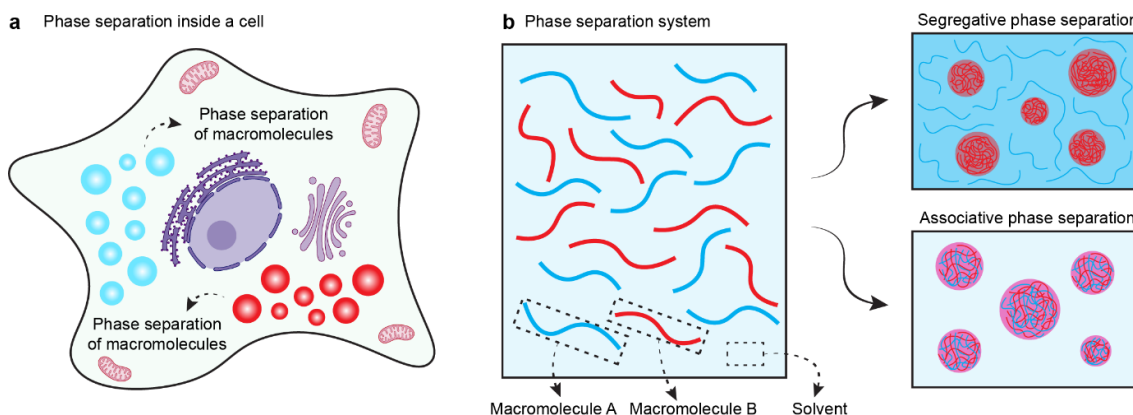


Figure 1-2. Phase separation in solution and in cells. **a**, Phase separation takes place inside living cells to form biomolecular condensates from proteins and nucleic acids. **b**, Two distinct mechanisms of phase separation for macromolecules A and B coexisting in the same solution: segregative phase separation, driven by repulsion between unlike molecules, and associative phase separation, driven by attraction between interacting molecules.

Proteins and nucleic acids often possess complex architectures and multiple interaction types. For example, proteins may contain intricate patterns of hydrophobic and hydrophilic domains, while nucleic acids can engage in specific interactions such as G-quadruplex formation or CAG repeat pairing.^{28–30} As a result, their phase separation is often a hybrid process, incorporating features of both segregative and associative phase separation. Thus, the **sticker–spacer model**,

originally inspired by associative polymers, was developed alongside the Flory–Huggins framework, to capture the interaction complexity of biological macromolecules.^{26,31,32} As a complementary framework to account for the reversibility of phase separation and dissolution in cellular systems, **phase separation coupled to percolation** has been recently proposed.²⁰ This model emphasizes that gelation and percolation play critical roles in phase separation process, in particular when strong interactions between associating macromolecules are present. By comparing phase separation temperature and percolation temperature, the model helps to explain the dynamic property of the formed condensates (liquid-like or solid-like) and phenomena such as condensate aging and their liquid-to-solid transitions.^{33–36}

Biomolecular condensates inside cells exhibit various functions. They can act as **reaction crucibles** and **organizational hubs** by: (1) concentrating key components required for biological reactions; (2) selectively enriching molecules to enhance activation or (3) modulate intracellular signaling levels; and (4) sequestering critical molecules to protect them from undesired reactions or degradation (Figure 1-3a–d).^{4,23,37} For example, P granules in *C. elegans* are spatially organized at the posterior of cells through controlled dissolution and condensation.²¹ Similarly, stress granules, formed from pools of untranslated messenger ribonucleoproteins (mRNPs), are thought to regulate cellular functions by concentrating key interaction partners.³⁸ In contrast, malfunction of condensates can have pathological consequences. This is typically related to the abnormal physical properties of the biomolecular condensates. For instance, the aging of protein fused in sarcoma (FUS) can drive a liquid-to-solid transition through aggregation, a process linked to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS).^{33,35} In general, the liquid nature of biomolecular condensates supports their normal regulatory functions, whereas mutations or aging can promote aberrant aggregation, leading to impaired cellular processes and disease.²³

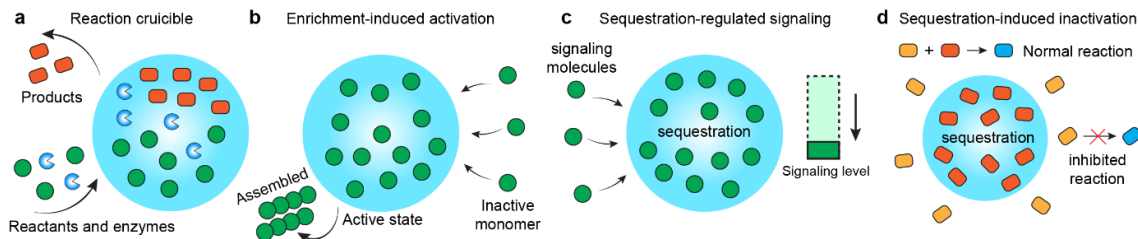


Figure 1-3. Roles and functions of biomolecular condensates in cellular processes. **a**, Biomolecular condensates act as reaction crucibles, enabling biochemical reactions to proceed inside. **b**, Biomolecular condensates enrich macromolecules beyond critical concentrations required for activation. **c**, Biomolecular condensates sequester macromolecules to reduce intracellular signaling levels. **d**, Biomolecular condensates sequester macromolecules to inhibit specific reactions.

However, a simple categorization of condensates as either liquid-like or solid-like may not fully capture their complexity. Viewed as macromolecules-enriched droplets, biomolecular condensates typically display **viscoelastic property** that are frequency-dependent.^{39–42} A recent study demonstrated that a series of biomolecular condensates, including PGL-3 protein found in *C. elegans* and four other proteins of the FUS family (FUS, EWSR1, DAZAP1, and

TAF15), can be regarded as aging viscoelastic Maxwell fluids, as probed by fluorescence recovery after photobleaching (FRAP) and microrheology experiments.³⁹ FRAP revealed a transition of condensates from highly dynamic to increasingly glassy states over time. Microrheology further indicated that condensates gradually shift from a liquid-like to a solid-like state. Beyond this aging behavior, condensates also exhibit frequency-dependent mechanical properties: at low shear rates, they behave as viscous liquids with storage modulus (G') smaller than loss modulus (G''), whereas at high shear rates, they become more solid-like, with G' greater than G'' . These unique mechanical characteristics enable cells to adaptively regulate their **mechanical properties** in response to environmental changes.³⁹ Fundamentally, this viscoelasticity arises from the relaxation dynamics of macromolecules within condensate droplet at different timescales, as described by Zimm, Rouse, and Reptation models.¹⁴

The recent focus on biomolecular condensates has greatly advanced the understanding of key roles of phase separation in cellular processes, including the regulation of biochemical reactions and the implications for certain diseases. However, much about their properties and mechanisms remains unknown, motivating the design of synthetic condensates as model systems to probe their properties and functions under controlled conditions.^{30,43} For instance, biomolecular condensates regulate cellular processes by enriching and organizing key macromolecules through molecular recognition interactions. Yet, the mechanisms governing the uptake and diffusion of macromolecules within condensates remain elusive. Furthermore, while aging is typically associated with a liquid-to-solid transition, it remains unclear whether pathways exist that allow condensates to undergo the reverse transition. These questions are systematically investigated in Chapter 2 using a model DNA condensate system, where an anomalous diffusion mechanism during macromolecular uptake is revealed and associated with the transition of condensates from a solid-like to a liquid-like state.

1.2 Synthetic Condensates from Different Materials

Phase separation and complex coacervation of polymers and polyelectrolytes have long been studied and applied in various contexts.^{44–47} Advances in understanding the properties and functions of cellular biomolecular condensates are now opening new opportunities to design synthetic condensates that are more relevant for biological applications.⁴⁸ Synthetic systems offer a controllable platform to dissect structure–property–function relationships of condensate materials. At the same time, the use of biological macromolecules as building blocks offer intrinsic bioactivity and molecular programmability, making them attractive for biomedical applications, despite potential risks of immunogenicity or toxicity. This section discusses various methods for designing and engineering synthetic condensates from different materials, starting with **peptide and protein condensates** in vitro and in vivo, valued for their biological relevance, and then turning to **deoxyribonucleic acid and ribonucleic acid (DNA and RNA) condensates**, whose high programmability and compatibility with DNA

nanotechnology make them ideal candidates for constructing material systems with structural and functional complexity.

1.2.1 Peptide-Based Condensates

Sequence-defined short peptides are among the simplest building blocks for creating synthetic condensates. A common strategy is to design peptides that mimic the **low complexity domains (LCDs)** of intrinsically disordered proteins (IDPs), classic protein motifs known to drive condensation.^{34,48–57} LCDs are typically enriched in a limited set of amino acids, including motifs rich in tyrosine (Y) flanked by glycine (G) or serine (S) residues (G/S–Y–G/S), phenylalanine–glycine (FG) repeats, and arginine–glycine-rich (e.g., RGG) domains.^{56,58} These sequences typically contain hydrophobic, π – π , cation– π , electrostatic, and hydrogen-bonding interactions, enabling multivalent interactions essential for phase separation. Another typical sequence used in synthetic peptide-based condensates is **elastin-like polypeptides (ELPs)**, composed of repeating VPGXG pentapeptides (where X can be any residue except proline), which is originally derived from the hydrophobic domain of tropoelastin. Notably, ELPs exhibit temperature-responsive phase separation behaviour.⁵⁹

Building on this knowledge, Leshem, Lampel, and coworkers designed a library of **minimalistic LLPS-promoting peptides** to study the sequence–property relationships of synthetic condensates.⁶⁰ Their design features an incorporation of three repeats of arginine–glycine (RG) domains, where glycine provides flexibility and arginine promotes electrostatic interactions with the terminal carboxylic group (Figure 1-4a). Considering that the arginine side chain can interact with side chains of aromatic amino acids, they further incorporated tryptophan (W) and tyrosine into the peptide sequence at a 1:1 ratio with arginine, with aromatic amino acids positioned at both sequence ends to enhance π – π stacking interactions. An additional WPGVG motif was included to mimic the ELPs repeat VPGXG to promote phase separation. The resulting peptide, WGRGRGRGWPGVGY, termed WGR-1, was confirmed to undergo LLPS at neutral pH in the presence of moderate NaCl concentration. Sequence modifications to WGR-1 revealed the respective contributions of the ELPs and RG domains to LLPS propensity (Figure 1-4b). Notably, substituting arginine with lysine (K) abolished phase separation, underscoring the essential role of π -interactions between arginine’s guanidinium group and aromatic side chains. Furthermore, sequence modification also modulated condensate polarity for enrichment of green fluorescent proteins and condensate dynamics (Figure 1-4c, d).

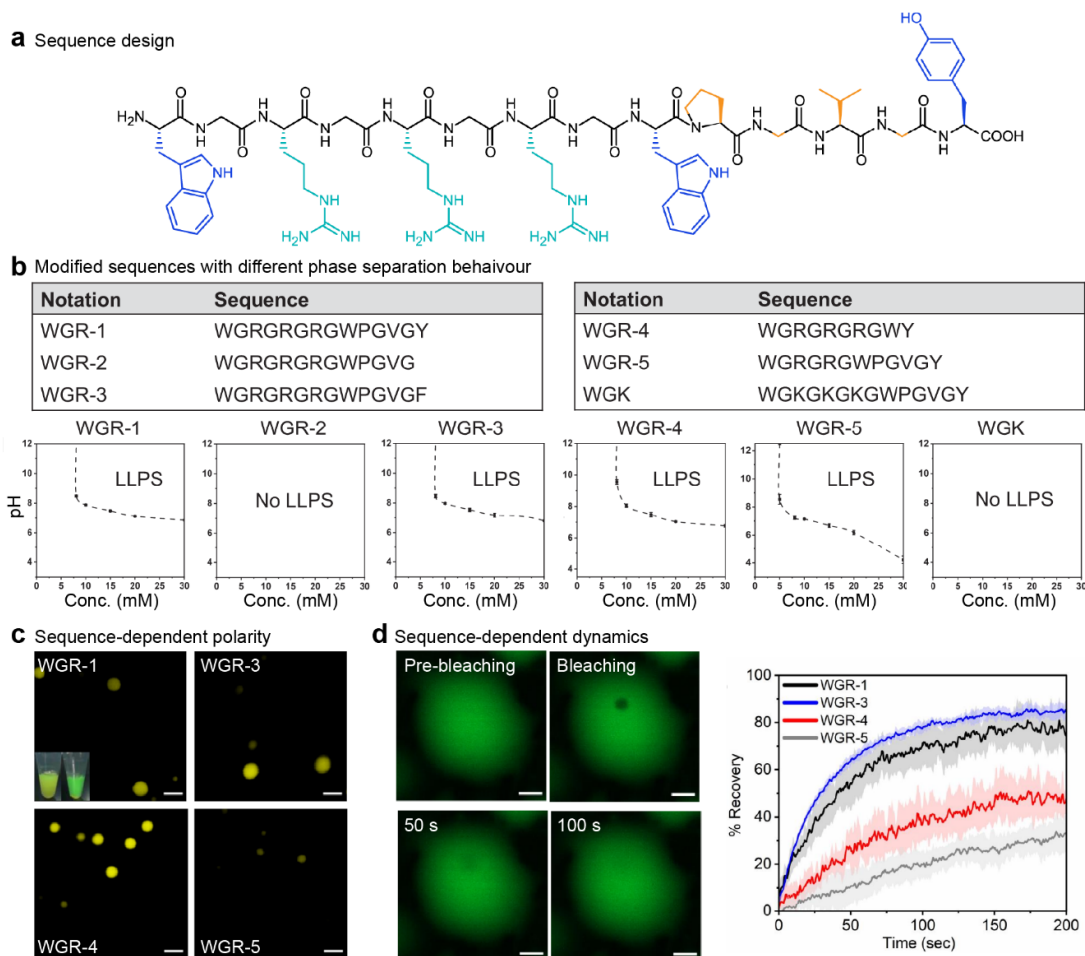


Figure 1-4. Synthetic condensates formed by minimalistic peptides. **a**, Sequence design of primary peptides for liquid-liquid phase separation (LLPS). **b**, Sequence modifications alter LLPS propensity. **c**, Condensates of different sequences show distinct enrichment of green fluorescent protein, reflecting sequence-dependent polarity. **d**, Fluorescence recovery after photobleaching (FRAP) reveals changes in condensate dynamics due to modified intermolecular interactions. Scale bars: 20 μm (**b**), 10 μm (**c**), 5 μm (**d**). Figures are adapted from ref. 60 with permission under the terms of the Creative Commons CC BY license. Copyright 2023, The Authors.

Molecular interactions of individual amino acids can also be harnessed to **de novo design** polypeptides that undergo phase separation. For example, Yu, Liu, and colleagues designed a synthetic peptide from scratch to promote interchain interactions, including π - π , cation- π , electrostatic, and hydrogen bonding.⁶¹ They primarily incorporated tyrosine, arginine, glycine, and aspartic acid (D) to promote intermolecular interactions, as well as hydrogen bond between water and serine, while remaining a net-zero charge to minimize electrostatic repulsion. Their design yielded the sequence RGYGSPDG, which undergoes LLPS under certain concentrations and salinity, exhibiting high dynamic properties that facilitate inter-condensate fusion (Figure 1-5a, b). Remarkably, using a synthetic biology approach, they expressed this synthetic peptide in *B. subtilis*, a model microorganism widely employed in the biosynthesis of nutraceuticals and fine chemicals. The peptide, fused to green fluorescent protein (GFP), formed fluorescent condensates within cells, as influenced by temperature and can be dissolved upon urea treatment (Figure 1-5c, d). Furthermore, these intracellular synthetic

condensates could be engineered with specific interaction motifs to function as synthetic crucibles, enabling the co-localization of client proteins via corresponding peptide tags and the regulation of protein translation.⁶¹

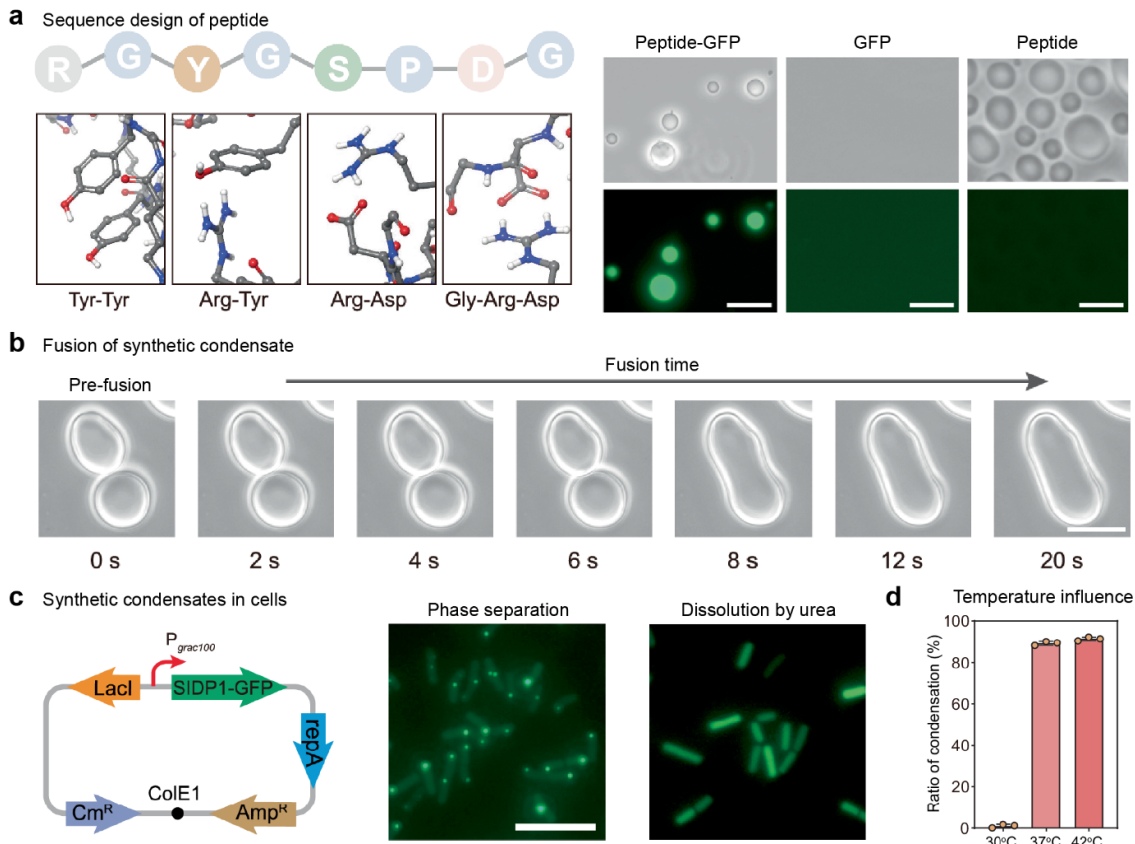


Figure 1-5. De novo engineering of synthetic condensates inside cells. **a**, Sequence design for the minimalistic peptide for LLPS. **b**, Fusion behavior of the synthetic condensates. **c**, GFP-fused peptides expressed in *B. subtilis* form condensates via LLPS, which can be dissolved upon urea treatment. **d**, Elevated temperature accelerates peptide expression, promoting condensate formation in *B. subtilis*. Scale bars: 10 μ m (**a–c**). Figures are adapted from ref. 61 with permission under the Creative Commons CC-BY-NC-ND license. Copyright 2024, The Authors.

Interestingly, even oligopeptides with minimal sequences and simple designs can undergo phase separation by balancing molecular flexibility and intermolecular interactions. **Diphenylalanine (FF)** is commonly used building block in supramolecular chemistry to form fibers and hydrogels due to strong hydrophobic interactions.^{62–64} Abbas, Spruijt, and coworkers demonstrated that introducing a flexible spacer, such as cystamine (ss), between two FF motifs, i.e., FFssFF, enhances their conformational flexibility and drives their LLPS into condensates. The molecular structure of FFssFF also exemplifies the sticker–spacer model (Figure 1-6a).⁶⁵ The FFssFF peptide exhibits **pH-responsive** phase behavior. At low pH, the ammonium group of phenylalanine is protonated, enhancing solvation and maintaining solubility. As pH increases, deprotonation weakens solvation, promoting phase separation into a dense phase. This system is also **redox-responsive**: reduction of the cystamine linker into

free thiols dissolves the condensates, whereas oxidation reforms FFssFF, which spontaneously phase separates (Figure 1-6b).

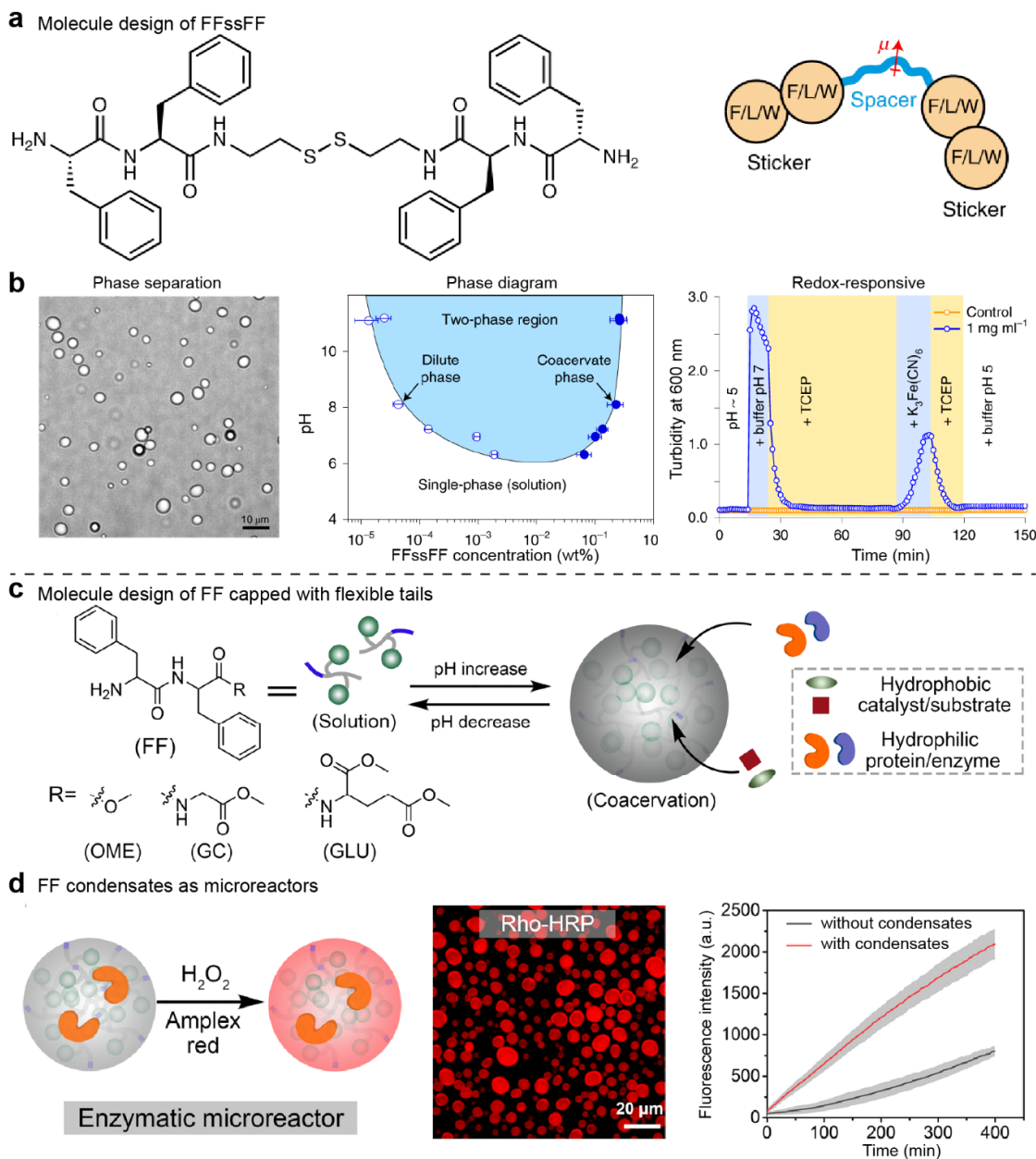


Figure 1-6. FF motifs as building blocks for synthetic condensates. **a**, Molecular design of FFssFF, comprising two L-phenylalanyl-L-phenylalanine dipeptide stickers (FF) linked at the C-termini by a cystamine (ss) spacer. This peptide can be described by the sticker–spacer model. **b**, FFssFF peptide undergoes LLPS upon pH increase. FFssFF condensates dissolve upon reduction with tris(2-carboxyethyl)phosphine (TCEP), while oxidation by $K_3Fe(CN)_6$ induces phase separation. **c**, FF motifs capped with flexible tails exhibit pH-responsive phase separation. **d**, FF-OMe condensates act as microreactors, encapsulating HRP to catalyze Amplex Red oxidation in the presence of H_2O_2 with enhanced reaction rates. Scale bars are provided in each image. Figures **a** and **b** are adapted from ref. 65 with permission. Copyright 2021, Springer Nature. Figures **c** and **d** are adapted from ref. 66 with permission under the terms of the Creative Commons CC BY license. Copyright 2024, The Authors.

Similarly, Cao, Landfester, da Silva, and coworkers demonstrated a minimal peptide system containing only a FF motif capped with flexible tails, such as methoxy (OMe), methoxylated glycine (GC), or ethoxylated glutamic acid (Glu), that could undergo pH-induced phase separation (Figure 1-6c).⁶⁶ Remarkably, the system could function as microreactors, enriching enzymes and substrates to enhance enzymatic reactions. For example, horseradish peroxidase (HRP) was enriched within the condensates with a partition coefficient of 96, resulting in an approximately twofold increase in reaction rate compared to the same reaction in solution (Figure 1-6d).

1.2.2 Protein-Based Condensates

Moving beyond the peptide-based condensates, condensates formed by natural proteins, such as FUS, histone H1, and prothymosin- α , have been studied extensively in vitro for revealing their properties and mechanisms.^{39,67} **Synthetic biology** allows further engineering of synthetic protein-based condensates inside cells, which hold greater relevance for cell biology and biomedical applications. A typical strategy is to fuse a condensate scaffold, such as synthetic IDPs, with functional binding domains that target specific proteins or nucleic acids.^{48,68} Once formed, the condensate can spontaneously recruit target molecules for local enrichment and reaction regulation. It is also possible to directly fuse synthetic IDPs to target proteins, however, this will restrict flexibility in post-translational regulation due to the covalent linkage.

As an example, Dai, You, and coworkers reported synthetic protein-based condensates with programmable dynamics to regulate diverse cellular processes.⁶⁹ They engineered fusion proteins by linking zwitterionic synthetic IDPs, which is a resilin-like polypeptide (RLP) with repeating units GRGDSPYS, to a truncated partition protein B variant (dParB) containing a DNA-binding domain (DBD) and a C-terminal dimerization domain (DD) (Figure 1-7a). The DBD is used for plasmid recruitment, while the DD increases interaction valence to drive phase separation. When expressed in *E. coli*, these fusion proteins formed condensates with controlled dynamics from highly fluid-like to arrested, tuned by altering the RLP sequence (Figure 1-7b, c). The distinct dynamic properties of synthetic protein condensates within cells give rise to various functions. For example, arrested condensates selectively sequester plasmids, blocking their access to replication machinery and causing plasmid loss in daughter cells (Figure 1-7d). In contrast, dynamic and permeable condensates can colocalize target DNA with transcriptional machinery to accelerate gene expression (Figure 1-7e). Moreover, the arrested condensates could also be applied to enrich target proteins and protect them from degradation (Figure 1-7f).

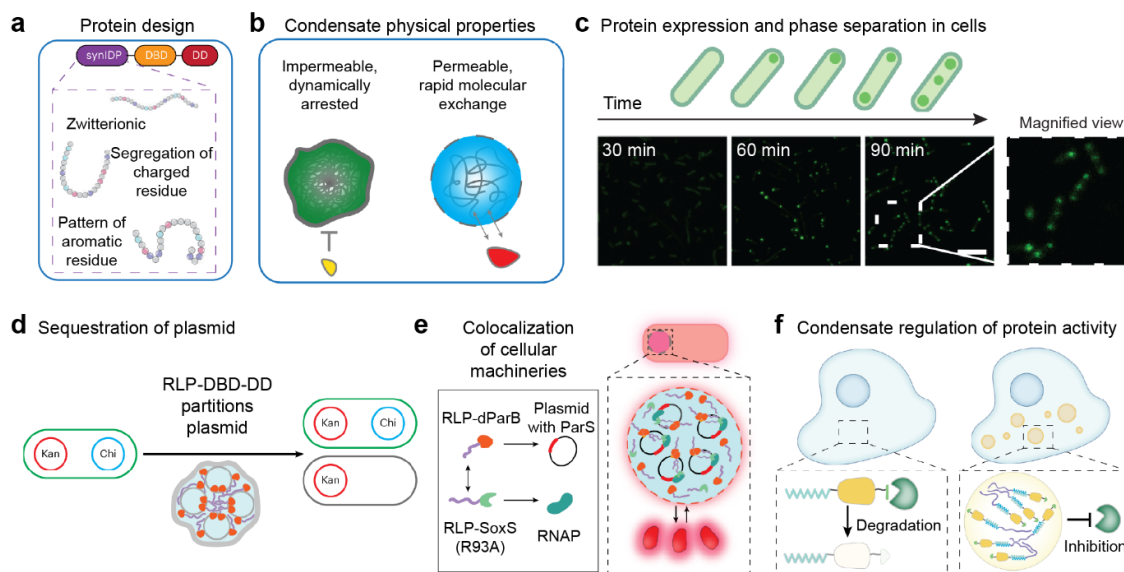


Figure 1-7. Synthetic protein condensates with tunable dynamics and various functions in cells. **a**, Design of synthetic protein condensate, including a synthetic intrinsically disordered protein domain (synIDP), a target-binding domain (DBD), and a dimerization domain (DD) to enhance valency for phase separation. **b**, Condensate dynamics regulate molecular exchange with the environment and therefore modulate cellular functions. **c**, Expression and phase separation of synthetic protein condensates inside *E. coli*. **d**, Arrested condensates can be used to sequester target plasmid to prevent its replication and inheritance. **e**, Dynamic co-condensates can be used to enrich specific plasmids and transcriptional machinery for enhancing transcription and expression of fluorescent proteins. **f**, Arrested condensate can be used to sequester specific proteins and prevent them from degradation. Scale bars: 7.5 μm (c). Figures are adapted from ref. 69 with permission. Copyright 2023, The Authors, under exclusive license to Springer Nature America, Inc.

Recent advances have also reported synthetic protein condensate systems that respond to light, small molecules, and enzymatic cues, enabling spatiotemporal control of condensate behavior inside cells.⁶⁸ Such systems are interesting toolkits for biologists to probe and manipulate cellular processes, and for chemists to engineer functional synthetic cells.

1.2.3 DNA- and RNA-Based Condensates

Nucleic acids are commonly associated with proteins to form co-condensates that regulate diverse cellular functions.^{70–73} Beyond these ribonucleoprotein assemblies, the inherent multivalency of nucleic acids by programmable base-pairing interactions can also drive the formation of pure RNA and DNA condensates in the absence of proteins. In comparison to peptide and protein condensates discussed earlier, the **programmable interactions** among nucleobases in DNA and RNA enable more straightforward design and fabrication of condensates with controlled properties. Furthermore, nucleic acid condensates can be seamlessly integrated with established DNA and RNA nanotechnology tools for constructing secondary structures and incorporating functional modules. These advantages are particularly relevant for building **synthetic cells**. This section focuses on strategies for engineering nucleic acid condensates through two mechanisms: beginning with **sticky-end interactions** mediated

by base pairing at the molecular level, and then turning to **LCST-type phase separation** of DNA/RNA polymers driven by their intrinsic polymeric characteristics at larger length scales.

CAG and GGGGCC repeats of RNA

In an early example, Jain and Vale demonstrated that RNA sequences containing tens of **CAG repeats** could undergo phase separation in solution, forming RNA condensates through multivalent C–G nucleobase interactions (Figure 1-8a, b).²⁹ These repeats, when expressed in cells, could enrich and phase separate into nuclear RNA foci (Figure 1-8c). Beyond base-pairing-driven multivalency, Hoogsteen interactions such as those in G-quadruplexes can also promote phase separation (Figure 1-8d), which are linked to amyotrophic lateral sclerosis and frontotemporal dementia (ALS/FTD).^{74,75} For instance, RNA polymers with **GGGGCC repeats** could form condensates and clusters both in solution and in the nucleus (Figure 1-8e, f). These results provide insightful perspective into mechanism of repeat expansion disorders.^{76,77}

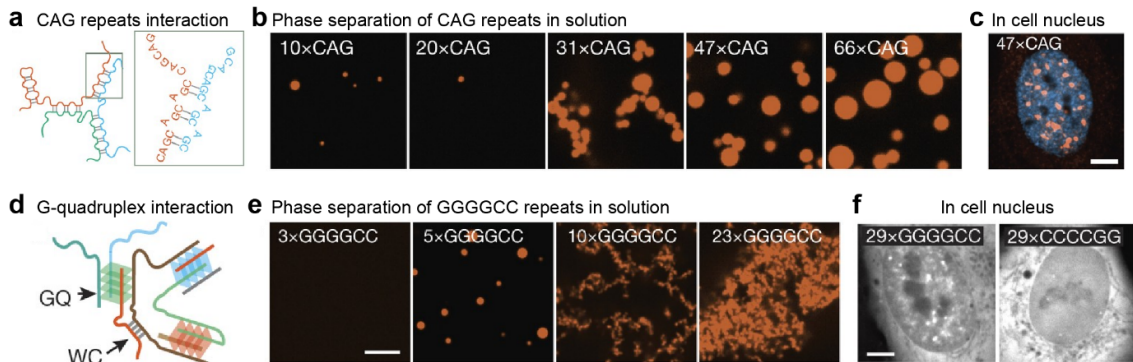


Figure 1-8. Phase separation of RNA sequences of CAG or GGGGCC repeats. **a**, Scheme showing multivalent interaction among CAG repeats in RNA. **b**, Fluorescence microscopy images showing the formation of condensates in solution by RNA sequence containing different numbers of CAG repeats. **c**, Fluorescence in situ hybridization image showing the condensation of expressed 47×CAG RNA in a cell nucleus. **d**, Scheme showing the G-quadruplex interaction among GGGGCC repeats in RNA. **e**, Fluorescence microscopy images showing the formation and clustering of condensates in solution by RNA sequencing containing different numbers of GGGGCC repeats. **f**, Microscopy images showing the condensation of expressed 29×GGGGCC RNA in a cell nucleus, whereas expressed 29×CCCCCG repeats remain soluble. Scale bars: 5 μm for all. Figures are adapted from ref. 29 with permission. Copyright 2017, Macmillan Publishers Limited, part of Springer Nature.

Sticky-end interactions for DNA nanostars

The strategy of exploiting multivalent interactions has been adapted in DNA nanotechnology to create DNA-based condensates. Building on the original **Y-shaped DNA** design, researchers have developed **branched DNA nanostar motifs** that can assemble via **sticky-end interactions** at their arms to form DNA condensates.^{78–82} This platform is highly versatile, as the tools and principles of DNA nanotechnology (such as base-pairing and toehold-mediated strand displacement reaction) can be leveraged to program condensate properties and precisely control their assembly and disassembly. Agarwal, Franco, and colleagues demonstrated that the **size** of DNA condensates can be tuned by adjusting the arm length of the DNA nanostars. At a given DNA nanostar concentration, longer arms generally produce larger condensates and promote a faster condensate growth rate (Figure 1-9a).⁸⁰

DNA condensates can be controlled in their **dynamic properties**: Sato, Takinoue, and colleagues found that the length of sticky-end eventually governs interaction strength and, consequently, the dynamic properties of DNA condensates.⁸¹ They characterized DNA condensates by droplet temperature and gel temperature. The droplet temperature is always higher, defining the melting of sticky-end interactions, while the gel temperature marks the transition point, below which the condensate becomes dynamically arrested. Between them, condensates remain liquid-like. Both temperatures increase with sticky-end length. Combining two DNA nanostar systems with orthogonal sticky ends could yield two distinct condensates in the same solution that do not fuse (Figure 1-9b).

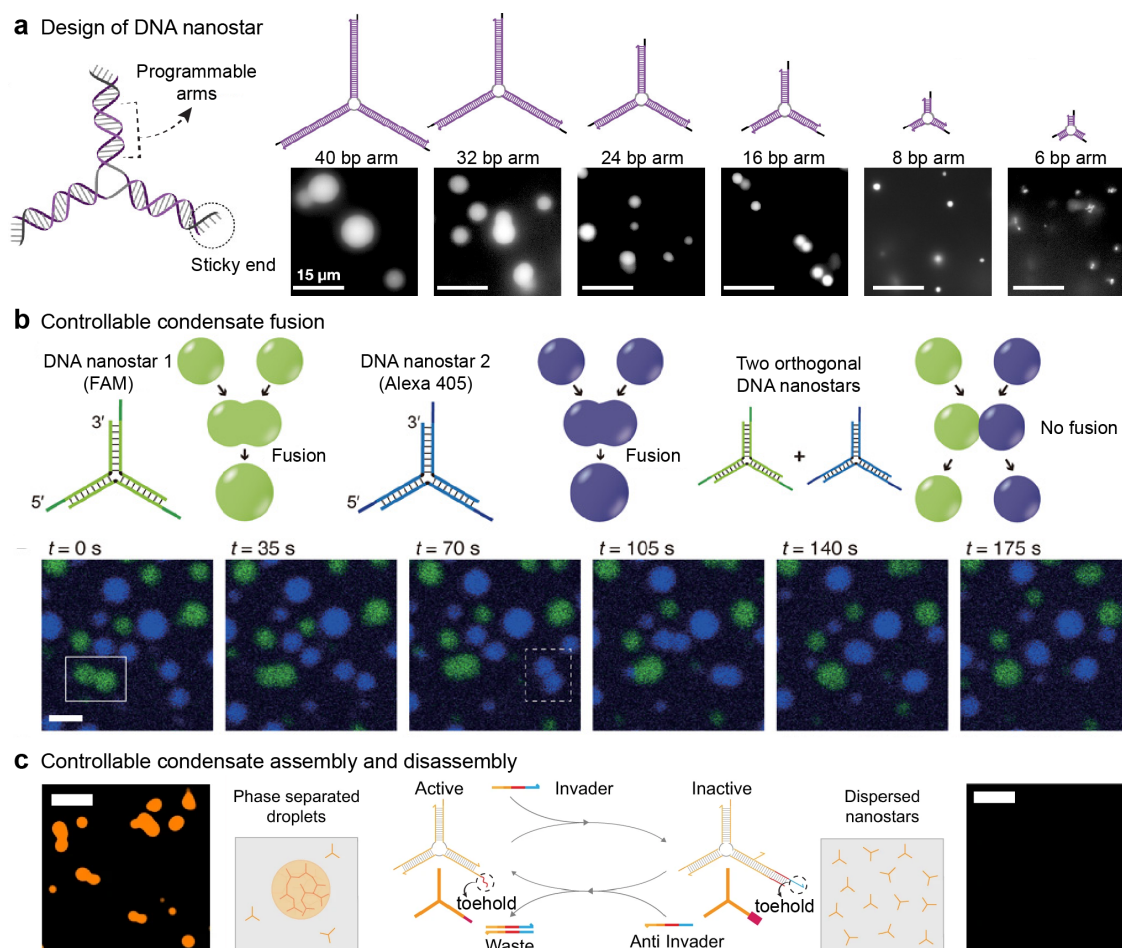


Figure 1-9. DNA condensates formed by DNA nanostars via sticky-end interactions. **a**, Design and microscopy images showing self-assembly and phase separation of DNA nanostars into condensates, with arm length determining condensate size. **b**, Formation of orthogonal DNA condensates in the same solution using two types of DNA nanostars with distinct sticky ends. **c**, Dissolution of DNA condensates by blocking sticky ends with an invader strand through toehold-mediated strand displacement reaction, and reassembly upon addition of an anti-invader strand. Scale bars: 15 μm (**a**), 30 μm (**b**), 10 μm (**c**). Figure **a** is adapted from ref. 80 with permission. Copyright 2022, American Chemical Society. Figure **b** is adapted from ref. 81 with permission under the terms of the Creative Commons CC BY-NC 4.0 license. Copyright 2020, The Authors. Figure **c** is adapted from ref. 82 with permission under the terms of the Creative Commons CC BY license. Copyright 2024, The Authors.

DNA condensates can be further programmed in their **formation and destruction**: Agarwal, Franco, and colleagues demonstrated that the assembly and disassembly of DNA condensates can be regulated through toehold-mediated strand displacement reactions (SDRs).⁸² The addition of an invader strand, which binds to the sticky ends of DNA nanostars and prevents intermolecular interactions, causes the condensates to dissolve into solution. Subsequent introduction of an anti-invader strand displaces the invader, freeing the sticky ends and enabling the nanostars to reassemble into condensates (Figure 1-9c). In terms of applications, such DNA condensates have also been shown to accelerate DNA strand displacement reactions and enable logic gate computations.⁸³

Sticky-end interactions for RNA nanostars

Similarly, as RNA also relies on base-pairing interactions, RNA condensates can be assembled from **RNA nanostars**. Stewart, Franco, and coworkers introduced both multi-stranded four-arm RNA nanostar and single-stranded three-arm RNA nanostar to phase separate into condensates (Figure 1-10a, b).⁸⁴

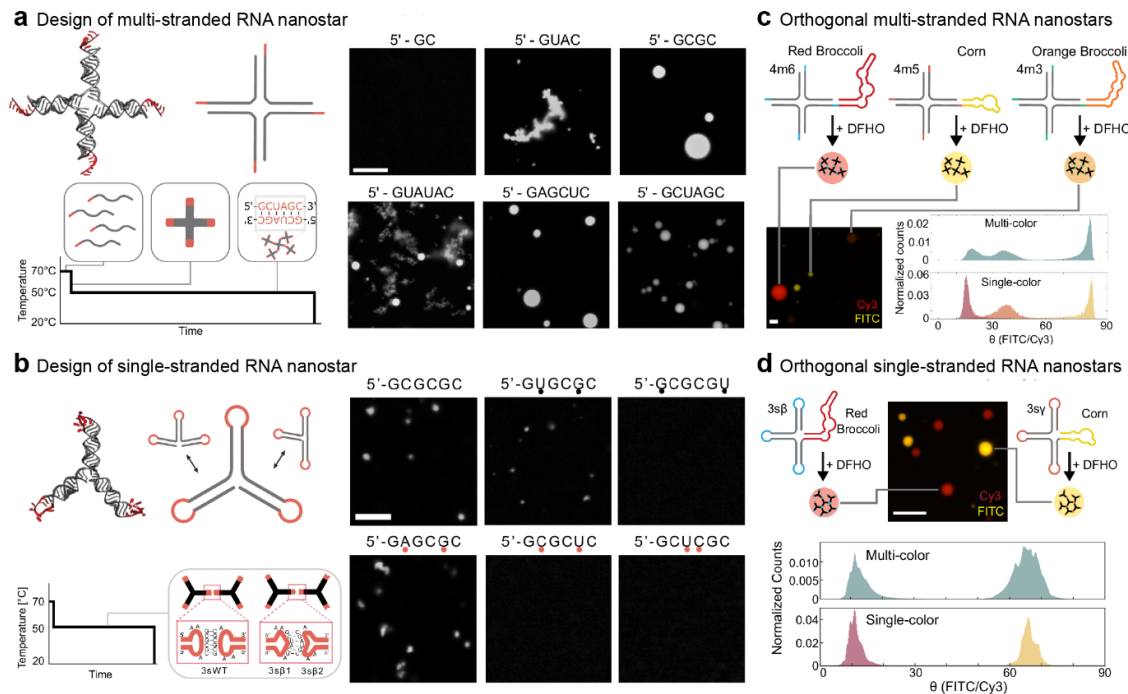


Figure 1-10. RNA condensates formed by RNA nanostars via sticky-end interactions. **a**, Design and microscopy images of multi-stranded RNA nanostars bearing sticky ends at each arm end. Such RNA nanostars undergo phase separation in solution, with the final structures determined by the strength of the sticky-end interactions. **b**, Design and microscopy images of single-stranded RNA nanostars and their phase separation, with condensate structure similarly governed by sticky-end interactions. **c**, Orthogonal multi-stranded RNA nanostars bearing distinct sticky ends and fluorescent light-up aptamers. These nanostars can form distinct condensate populations that bind the same fluorescent molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime (DFHO), to yield different fluorescence signals. **d**, Orthogonal single-stranded RNA nanostars with distinct sticky ends and fluorescent aptamers. Similarly, they can form distinct condensate populations with DFHO-based fluorescence. Scale bars: 40 μm (**a**), 10 μm (**b**), 20 μm (**c**, **d**). Figures are adapted from ref. 84 with permission under the terms of the Creative Commons CC BY license. Copyright 2024, The Authors.

In both designs, the CG content and sticky-end length influence binding strength and proper condensate formation in solution. An advantage of the RNA-based system is that it can directly incorporate **RNA aptamers** as overhangs in the RNA nanostars, which can selectively bind to specific molecules. As proof of concept, different aptamers including Red Broccoli, Corn, and Orange Broccoli, were incorporated into orthogonal RNA nanostar designs. These modified RNA nanostars retained their ability to form stable condensates in solution and bound the fluorescent light-up molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone-2-oxime (DFHO) to generate distinct fluorescence signals (Figure 1-10c, d).

Co-transcriptional condensation of RNA nanostars

RNA nanostars can also be generated in situ via transcription from DNA templates encoding their sequences, enabling **co-transcriptional condensation**. Fabrini, Di Michele, and coworkers demonstrated transcription of single-stranded RNA nanostars, each having distinct sticky ends and aptamers, by combining T7 RNA polymerase, double-stranded DNA (dsDNA) templates, and ribonucleotide triphosphates (rNTPs), and their subsequent phase separation in the solution and in water-in-oil emulsions (Figure 1-11a, b).⁸⁵

When two different RNA nanostars were co-transcribed inside the same liposome, the ratio of DNA templates determined the relative sizes of the resulting condensates, reflecting competition for the shared monomer pool (Figure 1-11c). They further designed RNA nanostars with short overhang strands, which can be modularly used for supramolecular binding to external aptamers such as yellow fluorescent protein (YFP)-binding aptamers, streptavidin-binding aptamers, and functional strands like biotinylated DNA, enabling selective recruitment of diverse proteins (Figure 1-11d).

Such co-transcriptional condensation system is further explored and integrated with DNA condensate system in Chapter 4, where DNA condensates are endowed with transcription function to enable localized transcription of RNA nanostars and their subsequent condensation into diverse architectures in a DNA-rich, nucleus-mimicking environment.

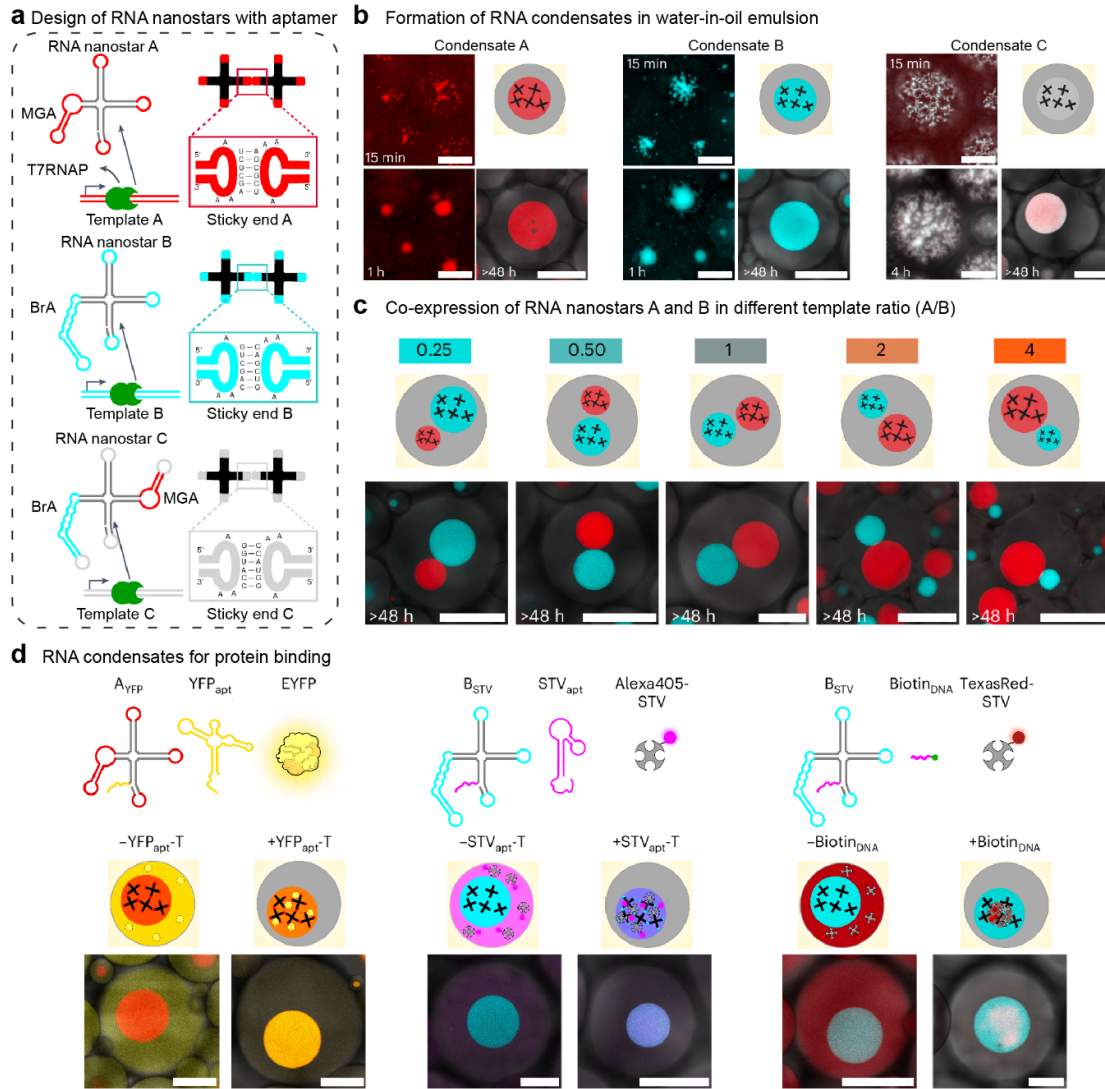


Figure 1-11. Co-transcriptional condensation of RNA nanostars in water-in-oil emulsions. **a**, Scheme of the design for three orthogonal RNA nanostars with distinct sticky ends and aptamers. RNA nanostar A contains a malachite green aptamer (MGA), RNA nanostar B contains a Broccoli aptamer (BrA), and RNA nanostar C contains both MGA and BrA, for binding different fluorescent molecules, respectively. **b**, Confocal laser scanning microscopy (CLSM) images showing co-transcriptional formation of RNA condensates in water-in-oil emulsions. **c**, CLSM images showing the co-expression of RNA nanostars A and B in the same water-in-oil emulsion. Altering the template ratio changes the relative sizes of the formed two condensates. **d**, RNA nanostars engineered with 5' overhangs for binding specific protein binding moieties, including yellow fluorescent protein (YFP)-binding aptamer, streptavidin-binding aptamer, and biotinylated DNA oligonucleotides. Their transcription in emulsions induces phase separation of RNA nanostars into condensates for recruitment of the target proteins. Scale bars: 50 μ m for all. Figures are adapted from ref. 85 with permission under the terms of the Creative Commons CC BY license. Copyright 2024, The Authors.

Phase separation of DNA polymers

Beyond base-pairing interactions, it has recently been found that long chain **DNA and RNA polymers** can undergo **phase separation** due to their polymeric characteristics, providing a complementary route to nucleic acid condensate formation. Merindol, Walther, and coworkers demonstrated that single-stranded adenine-rich and guanine-rich DNA polymers

(poly(A) and poly(G)), synthesized by template-guided rolling circle amplification (RCA), exhibit **LCST-type phase separation** behavior, with the cloud point temperature (T_{cp}) dependent on both polymer length and ionic strength (Figure 1-12a).⁷ In contrast, thymine-rich and cytosine-rich DNA polymers (poly(T) and poly(C)) remain soluble under the same conditions.

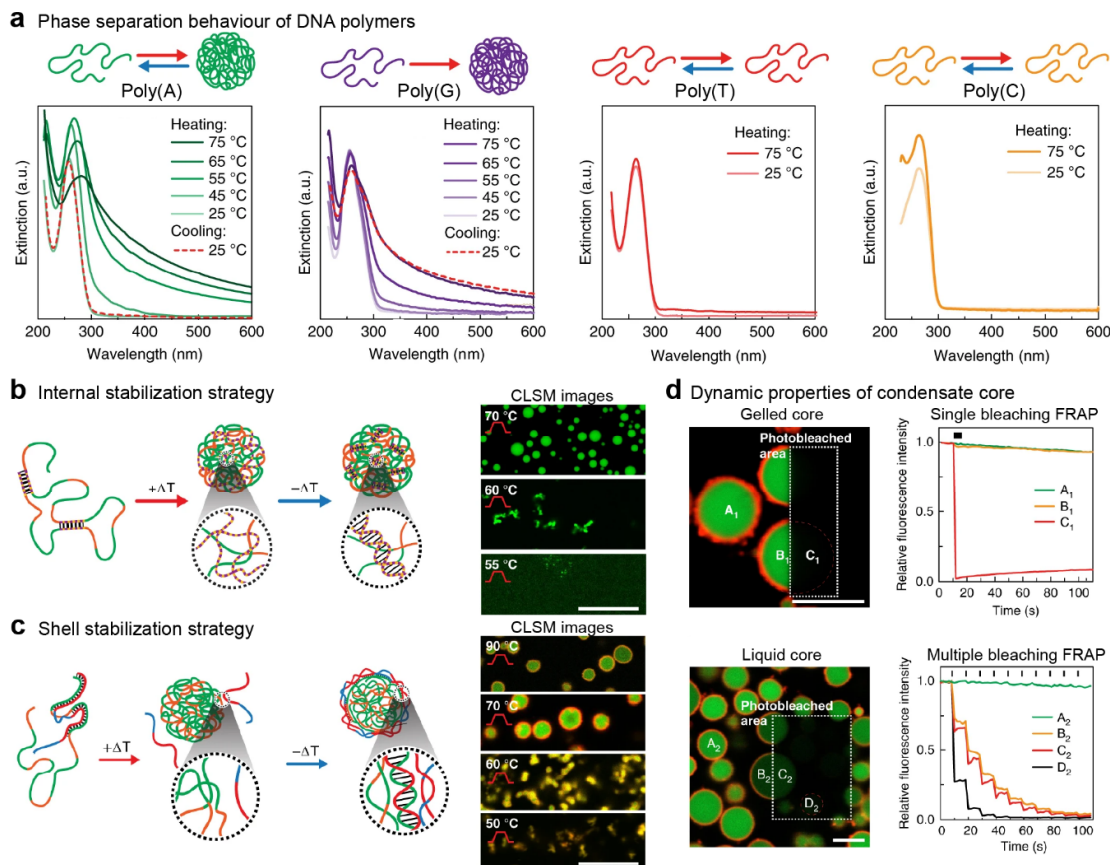


Figure 1-12. Formation of DNA condensates via phase separation of DNA polymers. **a**, DNA polymers exhibit sequence-dependent phase separation: adenine-rich and guanine-rich DNA polymers (poly(A) and poly (G)) undergo LCST-type phase separation, whereas thymine-rich and cytosine-rich DNA polymers (poly(T) and poly(C)) remain soluble, as shown by temperature-dependent light scattering experiments. **b**, DNA condensates formed by internal stabilization strategy, which incorporates palindromic domains in poly(A) for internal crosslinking. **c**, DNA condensates formed by shell stabilization strategy, which uses poly(T) to form a crosslinked shell on poly(A) condensates surface for stabilization. Both strategies require sufficient high temperature for proper phase separation. **d**, FRAP reveals solid-like property for DNA condensates formed by internal stabilization strategy, and liquid-like property for DNA condensates formed by shell stabilization strategy. Scale bars: 10 μ m (**b**, **c**), 5 μ m (**d**). Figures are adapted from ref. 7 with permission. Copyright 2018, The Authors.

Because the phase transition for poly(A) is reversible, two strategies were developed to stabilize poly(A)-based condensates at room temperature. In the internal stabilization approach, repetitive palindromic domains are incorporated into the poly(A), which crosslinks the condensate interior for stabilization against dissolution (Figure 1-12b). In the shell stabilization approach, a solution containing both poly(A) and poly(T) polymers is heated, causing the poly(A) to phase separate and form condensates. Upon cooling, the poly(T)

hybridizes at the poly(A) condensate surface, forming a stabilizing hydrogel shell (Figure 1-12c). In both cases, sufficient high temperature is necessary to melt the hybridization domains between DNA polymers in the original solution and allow proper phase separation. The applied stabilization strategies also determine the final dynamic states of the condensates. DNA condensates with internal stabilization exhibit solid-like behavior due to a crosslinked core, whereas core-shell condensates maintain a highly liquid-like interior (Figure 1-12d).

A key advantage of the DNA condensate system lies in the use of DNA polymers containing repeating recognition sequences that serve as **binding barcodes** for complementary oligonucleotides. The barcodes enable modular enrichment and functionalization of specific modules within the condensates by programmable binding interaction. However, such binding process lacks a robust mechanism to control local concentrations of enriched molecules, as the number of barcodes within condensates is inherently dictated by the dense phase of the phase diagram. This in fact restricts the use of barcodes for enriching multiple target molecules at designated concentrations for specific interactions and functions. To overcome this limitation, Chapter 3 introduces a co-phase-separation strategy, in which target barcode DNA polymers are mixed with dummy filler DNA polymers to induce co-phase separation. This approach employs filler polymers to effectively dilute the target barcode polymer within the condensate matrix, enabling precise tuning of internal barcode concentrations across several orders of magnitude, thereby achieving controlled enrichment of functional molecules for various applications.

Phase separation of RNA polymers

Similarly, RNA polymers have recently been reported by Banerjee, Wadsworth, and coworkers to undergo **LCST-type phase separation** (Figure 1-13a).⁸⁶ They observed that polyphosphate (poly(P)) also exhibits phase separation at elevated temperatures, and suggested that this behavior may be a general feature of nucleic acids. Molecular dynamics (MD) simulations further revealed increased Mg^{2+} binding to the oxygen atoms in polyphosphate, at higher temperatures (Figure 1-13b). To rationalize the (ir)reversibility of RNA phase separation under different conditions, they proposed a **phase separation coupled to percolation** hypothesis, with two characteristic temperatures: phase separation temperature (T_{ph}) and percolation temperature (T_{prc}) (Figure 1-13c).^{20,25} Phase separation takes place at temperatures above T_{ph} , whereas percolation and system-spanning network formation happen below T_{prc} . Therefore, when $T_{ph} > T_{prc}$, the phase separation will always be reversible, as percolation cannot happen at such high temperature during condensate formation. In contrast, when $T_{ph} < T_{prc}$, the system spanning network can form during the phase separation process within the condensates, which crosslinks and stabilizes the condensates to prevent dissolution.

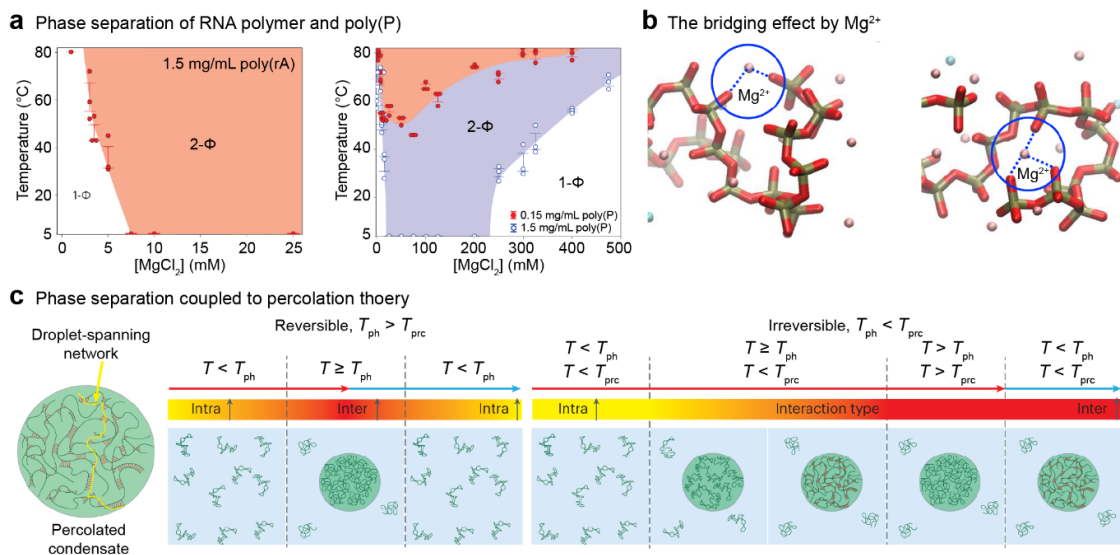


Figure 1-13. Formation of RNA condensates via phase separation of RNA polymers. **a**, RNA polymers exhibit LCST-type phase separation behavior. Poly(P) also undergoes LCST-type phase separation. **b**, Molecular dynamics (MD) simulation suggests Mg²⁺ binding to several oxygen atoms in the poly(P). **c**, Proposed phase separation coupled to percolation theory to explain reversible and irreversible phase separation of RNA polymers. Figures are adapted from ref. 86 with permission. Copyright 2023, The Authors, under exclusive license to Springer Nature Limited.

Among all synthetic condensate materials discussed in this section, DNA- and RNA-based condensates are particularly valuable for **synthetic biology** and **DNA nanotechnology**. From the perspective of synthetic biology, DNA and RNA condensates offer a powerful compartmentalization strategy, which can leverage their programmability and inherent compatibility with DNA/RNA nanoscience tools for building synthetic cells with desired properties and functions. This is exemplified in Chapter 3, where DNA condensates serve as synthetic cells to regulate the growth of artificial cytoskeletons, and in Chapter 4, where they are endowed with transcriptional functionality to act as nucleus mimics. In parallel, the **LCST-type phase separation** of DNA polymers will fuel the development of DNA nanotechnology by providing new interactions, which complements base-pairing, to program the assembly, dynamics, structures and functions of DNA-based materials. Chapter 5 further exploits the use of DNA condensates to compartmentalize DNA reaction networks for preventing reaction crosstalk between individual reaction networks.

The following sections review how condensate materials, particularly DNA condensates, have been harnessed in synthetic cell research and DNA nanotechnology as programmable and versatile compartments.

1.3 Synthetic Condensates as Minimalistic Synthetic Cells

A cell is the basic unit of life, which operates as a compartmentalized system sustained far from equilibrium.^{87,88} **Synthetic cell (SC)** research aims to create minimalistic cell-like entities with chemistry, biology, and engineering tools. A minimalistic SC should possess one or several **cell-like properties and functions**, such as metabolism, signal processing, enzymatic functions, motility, secondary structures, growth and division, and self-replicating.⁸⁹ It should become a functional entity with embodied intelligence to sense signals, process signals, and act correspondingly.^{90,91}

To construct a minimalistic SC from a **bottom-up chemistry approach**, the first requirement is establishing a **compartmentalized environment**.^{92,93} Amphiphilic molecules are typically used to create membrane-enclosed aqueous compartments, including liposomes, polymersomes, and colloidosomes.^{94–102} However, natural cells are far more crowded than a simple aqueous solution. The cytoplasm is densely packed with proteins and nucleic acids (Figure 1-14a).^{103–106} For example, the cytosol of *E. coli* was found to contain about 300–400 mg/mL of macromolecules.¹⁰⁷ This **macromolecular crowding** profoundly affects biochemical reaction kinetics and transport of macromolecules, which can be primarily attributed to the excluded volume effect (Figure 1-14b).^{108–113} Besides, the crowded environment can induce an entropy-driven depletion attraction between macromolecules, as described by Asakura–Oosawa model (Figure 1-14c).^{114–116} In addition to macromolecular crowding, the cytoplasm exhibits complex **viscoelastic behavior**, arising from the dynamic relaxation of macromolecules within the cell, that influences physicochemical processes.^{117–121} In this regard, synthetic condensates enriched in macromolecules represent compartments close to real cellular environments, featuring macromolecular crowding and viscoelastic property.

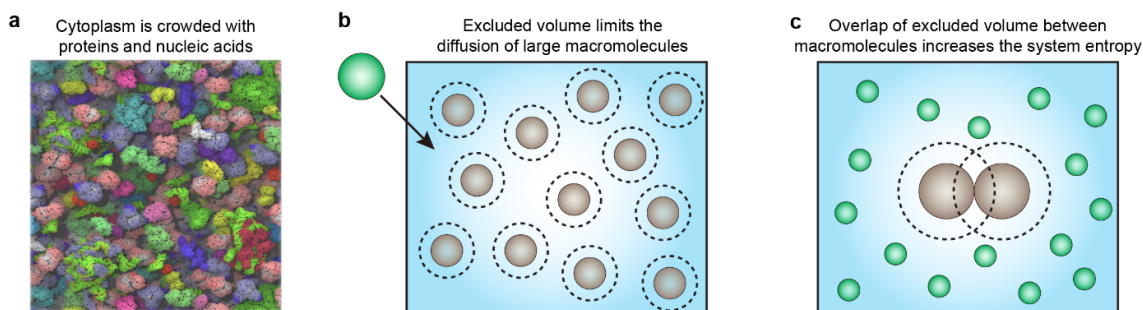


Figure 1-14. Macromolecular crowding inside cell and the excluded volume effect. **a**, Inside a cell, the high concentration of macromolecules creates a crowded environment full of proteins and nucleic acids, which influences the biochemical processes. **b**, Large macromolecules experience strong steric hindrance from neighboring molecules, leading to the excluded volume effect (indicated by dashed circles) and limited transport and diffusion. **c**, Depletion-induced attraction between large macromolecules, driven by entropic gain resulting from the overlap of excluded volumes. Figure **a** is adapted from ref. 105 with permission under the terms of the Creative Commons CC BY license. Copyright 2010, The Authors.

This section discusses **condensate-based synthetic cells (SCs)**, focusing on their various **cell-like functions**, including metabolism, motility, internal secondary structures, morphogenesis, information processing, and prototissue formation. In particular, **DNA condensates** are

highlighted for their unique ability to couple with DNA nanotechnology tools, enabling facile and versatile integration of structures and functions in synthetic cells.

1.3.1 Condensate-Based Synthetic Cells

For integrating **cell-like functions** into condensate-based SCs, Testa, Laurino, and coworkers demonstrated the use of bovine serum albumin (BSA) to form dense condensates, which enrich enzymes such as L-lactate dehydrogenase (LDH) and urease (Figure 1-15a).⁴³ The compartmentalization of enzymes within these protein condensates improve their enzymatic activity due to macromolecular crowding effect. Notably, when encapsulating urease in such condensates, these condensates can produce a pH gradient in presence of urea, which then leads to an **internal flow** inside condensates, possibly driven by Marangoni effect due to change in interfacial tension (Figure 1-15b).¹²² In the future, such internal flow might be utilized to enable **motility** or **division** in SCs.¹²³

For constructing internal **cell-like structures** within SCs, te Brinke, Huck, and coworkers further demonstrated a condensate system based on the condensation between RNA and the cationic supercharged polypeptide fused to GFP (GFP-K72) (Figure 1-15c).¹²⁴ Such condensates can be used to enrich FtsZ protein, which is a bacterial homologue of tubulin. In the presence of fuel guanosine-5'-triphosphate (GTP), FtsZ protein dynamically assembled into branched FtsZ filaments as **cytoskeleton structures** inside the condensates (Figure 1-15d). Over time, the FtsZ filaments could even deform the condensates, indicating the generated force during their assembly.

For reaching a SC with **structural and functional complexity**, Xu, Mann, and coworkers developed bacteriogenic synthetic cells by integrating bacterial cells into condensates.¹²⁵ Their approach is based on the co-capture and on-site processing of bacterial colonies (*E. coli* and *Pseudomonas aeruginosa* (PAO1 strain)) that are co-associated with individual poly(diallyldimethylammonium chloride) (PDDA)/adenosine-5'-triphosphate (ATP) condensates. When mixing these condensates with *E. coli* and PAO1, *E. coli* cells were spontaneously sequestered into the condensate core, whereas PAO1 were enriched at the condensate surface to form a shell (Figure 1-15e). Further addition of cell wall hydrolase (lysozyme) and antimicrobial peptide (melittin) led to the lysis of both cells. This gave rise to a condensate with a spatially segregated membrane of PAO1 lipids and with a core matrix consisting of incarcerated *E. coli* cytoplasmic components. The released components from bacterial cells endow the SCs with various active enzymes including alkaline phosphatase, protease, lipase, and β -galactosidase for different enzymatic functions. Furthermore, it supports glycolytic **enzyme cascade reactions**, thereby confirming the retention of sufficient enzymes and components inside SCs necessary for achieving cell-like functions.

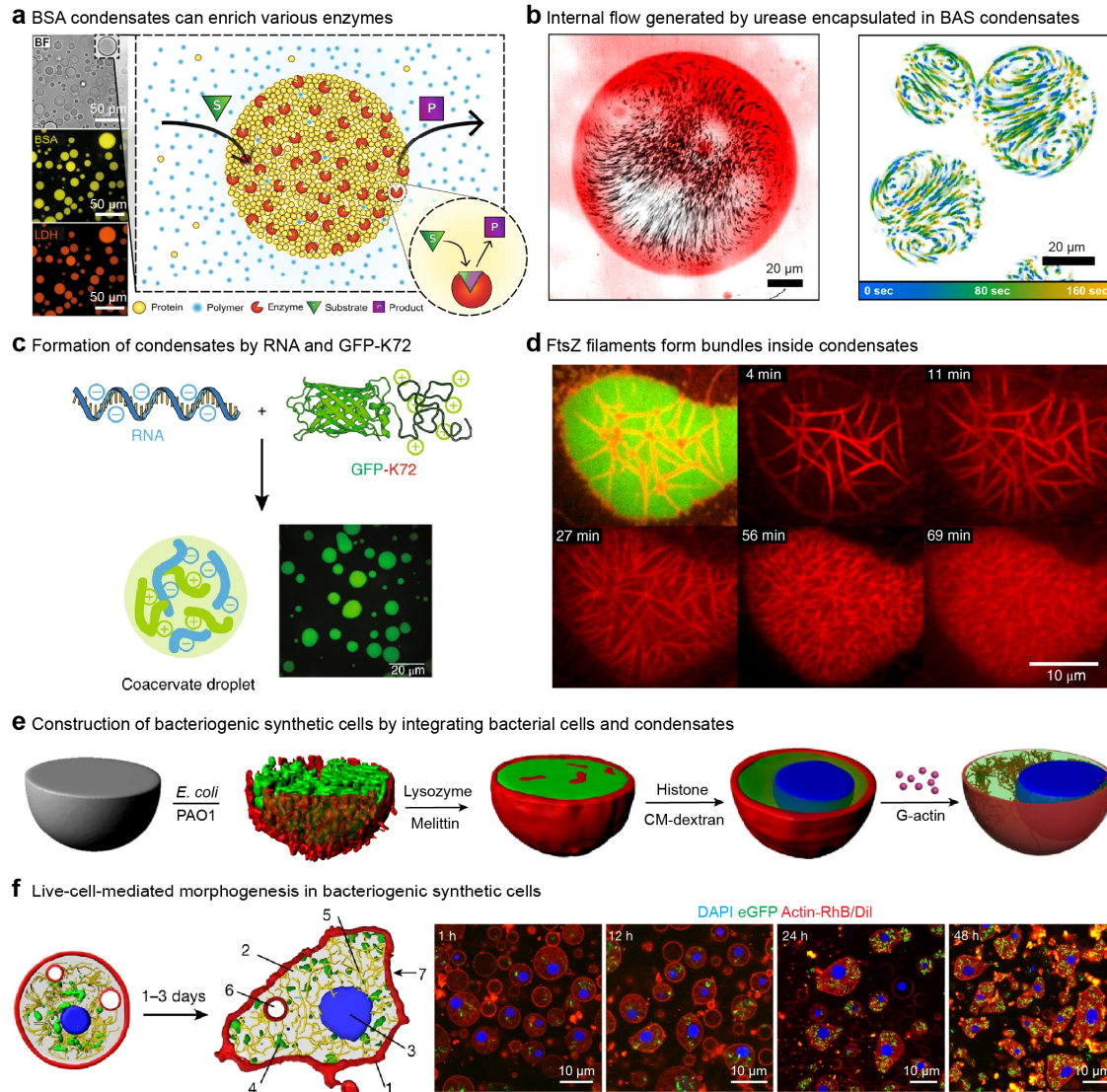


Figure 1-15. Synthetic cells based on condensate materials exhibit macromolecular crowding effect and can be engineered for cell-like structures and functions. **a**, BSA condensates can enrich enzymes for improved enzymatic reactions. **b**, BSA condensates enriched with urease can create pH gradient for generating internal flows. **c**, RNA and the cationic supercharged polypeptide fused to GFP (GFP-K72) form condensates when mixed together. **d**, These condensates can partition FtsZ proteins for bundling formation as artificial cytoskeletons. **e**, Construction of bacteriogenic SCs by partitioning *E. coli* and *Pseudomonas aeruginosa* (PAO1) bacterial cells into PDDA/ATP condensate droplets with their subsequent lysis catalyzed by lysozyme. These SCs can further recruit histone and carboxymethyldextran (CM-dextran) for condensation with bacterial DNA as a nuclear, and G-actin for growing proto-cytoskeletons. **f**, Bacteriogenic SCs entrapped with live *E. coli* eGFP cells and F-actin show progressive morphogenesis into amoeba-like living/synthetic constructs due to on-site *E. coli* activity. Individual components in the SC: (1) Outer membrane; (2) Molecularly crowded proto-cytoplasm; (3) Membrane-free DNA–histone proto-nucleus; (4) Surrogate mitochondria (*E. coli* cells); (5) Proto-cytoskeleton; (6) Membrane-bounded spherical water vacuoles; (7) Amoeba-like cell morphology. Scale bars are provided in each figure. Figures **a** and **b** are adapted from ref. 43 with permission under the terms of the Creative Commons CC BY license. Copyright 2021, The Authors. Figure **c** and **d** are adapted from ref. 124 with permission. Copyright 2018, The Authors. Figure **e** and **f** are adapted from ref. 125 with permission. Copyright 2022, The Authors.

This system can be further developed for achieving complex structures. Histone and carboxymethyldextran (CM-dextran) can be enriched in the SC core, where they condense

with bacterial DNA to form a nucleus-like mimic. Introducing G-actin promotes the formation of cytoskeletal structures within the SCs. Remarkably, these bacteriogenic synthetic cells can also encapsulate live *E. coli*, whose ongoing production of metabolic products modulates membrane interfacial tension, thereby inducing **morphogenesis** into non-spherical morphologies (Figure 1-15f).

However, the systems discussed above primarily rely on the partitioning of macromolecules into condensates to enrich functional modules. This enrichment approach has limited control over both the types of functional molecules and their local concentrations. To achieve a SC system with precisely controlled functional integration, it is necessary to establish specific enrichment interactions between condensates and target molecules. In this context, DNA base-pairing offers programmable interaction that can be used to direct the enrichment of functional molecules within SCs. Therefore, DNA condensates can serve as a promising platform for constructing SCs with enhanced controllability and programmability over properties and functions, as exemplified in the following section as well as in Chapter 3 and 4.

1.3.2 DNA Condensate-Based Synthetic Cells

Core-shell DNA condensates formed via DNA polymer phase separation have been recently applied as SC models.^{7,126–128} These DNA condensates are dynamic, featuring a permeable shell that enables efficient molecular exchange with the surrounding milieu for nutrient uptake and waste release. Interestingly, DNA nanotechnology approaches can be applied to engineer DNA condensates with diverse functions.⁹⁰ For example, DNA can serve as a structural scaffold for intracellular structure formation such as artificial cytoskeletons, assemble into nanopores and transmembrane motifs for macromolecular transport and signaling, and be programmed through base pairing to enable layered functionalization in DNA condensates.^{101,129–133}

As DNA condensates are formed through phase separation of synthetic single-stranded DNA (ssDNA) polymers. These polymers can be programmed to bear recognition sequences as **DNA barcodes**, for binding complementary ssDNA, which allows applying simple base-pairing interactions to integrate functional modules. Building on this principle, Samanta, Walther, and coworkers demonstrated the encapsulation of an artificial metalloenzyme inside DNA condensate-based synthetic cells.¹²⁶ In their design, DNA barcodes in the SCs were used to hybridize with biotinylated complementary strands. These biotins were further used to bind free streptavidin, which in turn captured biotinylated artificial metalloenzymes, thereby localizing catalytic activity within the SCs (Figure 1-16a, b). The obtained SCs possess **catalytic functions** to catalyze a ring-closing metathesis reaction, with the macromolecularly crowded environment enhancing reaction kinetics (Figure 1-16c). Localized product formation could further induce **growth** of the synthetic cells and membrane dynamization, which ultimately led to **fusion** between individual synthetic cells (Figure 1-16c). In a similar way, they also demonstrated the encapsulation of DNAzyme inside SCs, which allowed the SCs to recruit

substrates and catalyze their cleavage. The cleaved substrates acted as crosslinkers on the SC shell, promoting intercellular crosslinking and leading to the formation of **prototissue-like structures** (Figure 1-16d).¹²⁷

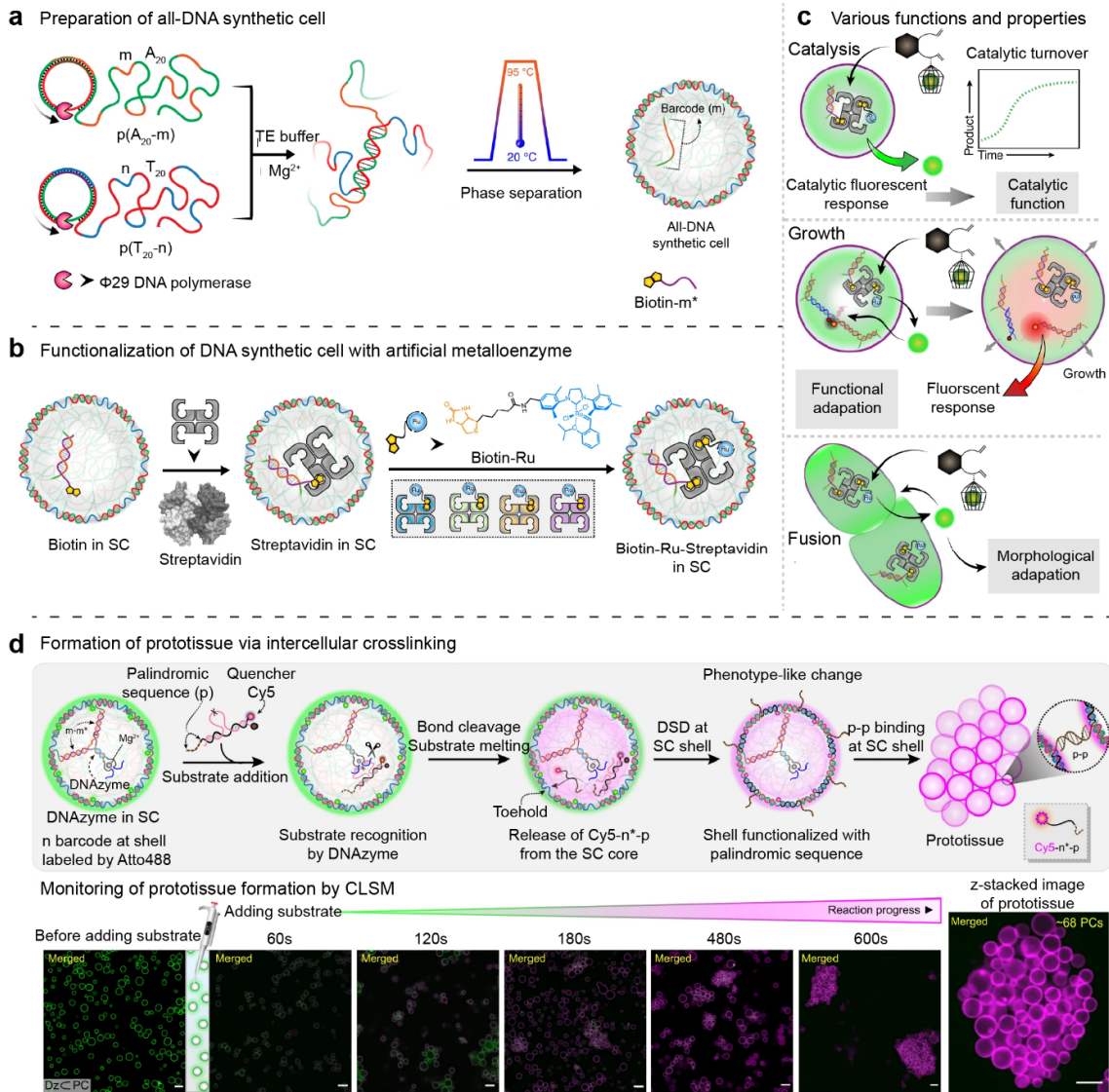


Figure 1-16. DNA condensates-based synthetic cells with cell-like functions. **a**, Scheme showing the synthesis of DNA polymers and their phase separation into core-shell SCs. **b**, DNA SC can encapsulate an artificial metalloenzyme via base-pairing and biotin-streptavidin interactions. **c**, DNA SC is capable of catalytic ring-closing metathesis for uncaging a fluorescent signal. The resulting product induces swelling and membrane dynamization, driving morphological transitions and fusion between SCs. **d**, Scheme and representative CLSM images showing DNA SCs encapsulating DNAzyme, which cleaves a fluorescent substrate. The resulting product serves as a crosslinker on the SC shell, driving intercellular crosslinking and assembly into prototissue-like structures. Scale bars: 5 μm (**d**). Figures **a–c** are adapted from ref. 126 with permission. Copyright 2020, The Authors, under exclusive license to Springer Nature Limited. Figure **d** is adapted from ref. 127 with permission under the terms of the Creative Commons CC BY license. Copyright 2022, The Authors.

In parallel to function integration, DNA condensate-based SCs were also explored to grow and organize **subcellular structures**. However, constructing secondary structures inside SCs is non-

trivial due to the challenge of manipulating monomers and self-assembly process inside the confined environment. Liu, Lupfer, Walther, and coworkers demonstrated the intracellular phase separation of an LCST-type polymer (poly(ethylene glycol acrylate); pEGA) inside them to generate subcellular compartments. These compartments establish a local hydrophobic environment, which facilitates a retro-Diels–Alder reaction with accelerated reaction rates.¹²⁸ Along this line of building secondary structures inside SCs, Chapter 3 further extends the DNA condensates to host self-assembling DNA tiles for the growth of artificial cytoskeletons, which enhances the mechanical property of the SCs.

1.4 DNA Nanotechnology and DNA Reaction Networks

DNA condensates represent a new class of mesoscale DNA materials, offering new opportunities for advancing DNA nanotechnology. Specifically, they can be used as colloids at mesoscale for assembling hierarchical DNA structures and materials. They can also be exploited as compartments to host DNA nanostructures, nanodevices, and DNA reaction networks. This section provides a brief overview of **DNA nanotechnology**, highlighting its key achievements as well as current limitations. Building on this foundation, this section will also shortly propose how DNA condensates can complement conventional DNA nanotechnology approaches to overcome existing challenges, enabling more complex and functional DNA materials and systems.

1.4.1 DNA Nanotechnology for Nanostructures

In the 1980s, Seeman first envisioned taking DNA out of its biological context and using synthetic DNA oligonucleotides to construct **self-assembled nanostructures** through predictable Watson–Crick–Franklin base pairing interactions.¹³⁴ This idea, particularly the use of **branched DNA with sticky ends** to assemble periodic crystalline materials, laid the fundamental foundation for the field of DNA nanotechnology.^{135,136} Subsequently, DNA tiles in form of double-crossover molecules were developed as more robust building blocks for assembling **DNA nanotubes (DNTs)**, which are now still widely applied in diverse applications (Figure 1-17a).^{137,138} A major breakthrough for creating arbitrary nanostructures came in 2006 with Rothemund’s development of **DNA origami**, in which hundreds of short DNA strands hybridize with a long scaffold strand (up to 7,000 nucleotides), guiding the folding of the scaffold DNA into defined shapes (Figure 1-17b).¹³⁹ Later, Wei, Yin, and coworkers introduced an alternative approach using distinct **DNA tiles** to program arbitrary assemblies. They designed tens of 42-base single-stranded DNA tiles forming a so-called “Brick-wall” structure. By selecting an appropriate subset of tiles from this common pool, arbitrary shapes can be generated (Figure 1-17c).¹⁴⁰ Conceptually, the “Brick-wall” can be imagined as a tile canvas and the finally formed shapes will be defined by the tiles selected. Finally, **three-dimensional DNA nanostructures** can also be assembled by folding a DNA sheet, achieved through

programming base-pairing interactions among specific subsets of staple strands within the scaffold (Figure 1-17d).¹⁴¹ These developments collectively demonstrate the remarkable programmability and versatility of DNA nanotechnology.

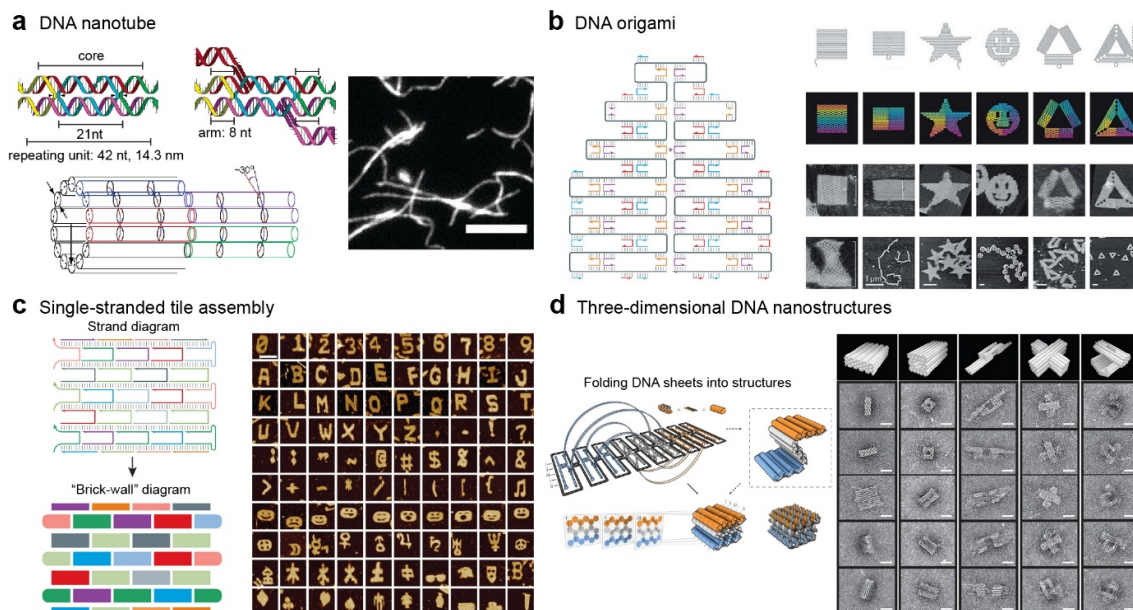


Figure 1-17. DNA nanotechnology for creating nanostructures. **a**, Scheme and representative fluorescence microscopy image of DNA nanotubes formed by double-crossover DNA tile motifs. **b**, Scheme and representative AFM images of various DNA origami shapes generated by folding a long DNA strand with hundreds of short staple strands. **c** Scheme and representative AFM images illustrating complex shapes formed by self-assembly of tens of distinct single-stranded DNA tiles. **d**, Scheme and representative transmission electron microscope (TEM) images of three-dimensional DNA nanostructures created by folding DNA sheets into target structures. Unless noted otherwise, scale bars: 5 μm (**a**), 100 nm (**b**, **c**), 20 nm (**d**). Figure **a** is adapted from ref. 138 with permission. Copyright 2004, American Chemical Society. Figure **b** is adapted from ref. 139 with permission. Copyright 2006, Springer Nature Limited. Figure **c** is adapted from ref. 140 with permission. Copyright 2012, Springer Nature Limited. Figure **d** is adapted from ref. 141 with permission. Copyright 2009, Macmillan Publishers Limited.

However, DNA structures and materials are typically confined to the nanoscale, which limits their broader applications in various fields. While some studies have explored the integration of DNA into soft materials, such as hydrogels and colloids, all-DNA hierarchical material systems have remained largely undeveloped.^{142–145} To construct **hierarchical DNA materials**, additional interactions orthogonal to base-pairing are required. In this context, DNA condensates formed by phase separation of DNA polymers offer a promising tool. Because phase separation and hybridization operate on different length scales and with distinct interaction strengths, they can be combined to create DNA materials with tunable structures and functions. As a start, Merindol, Walther, and coworkers showed in 2018, the hierarchical assembly of various DNA condensates.⁷ Moreover, DNA condensates also can serve as compartments to host DNA nanostructures, enabling precise control over their spatial organization and functions. This advantage is further explored in Chapter 3, where DNA condensates are combined with DNA nanotubes for generating fibril structures within mesoscale DNA condensates, thereby achieving structural complexity in all-DNA materials.

1.4.2 DNA Reaction Networks

Moving beyond DNA nanostructures, toehold-mediated strand displacement reaction (SDR) reactions enable the construction of **DNA reaction networks (DAN-RNs)** capable of performing diverse functions, including signal amplification, disease diagnostics, and molecular computation.^{146–154} The key principle is that a strand with a longer complementary domain gains more enthalpy when displacing a strand with a shorter complementary domain from its binding substrate (Figure 1-18a).¹⁵⁵ This difference in binding energy provides a programmable driving force to control reaction direction. For example, SDR can be used to program time-dependent release of DNA signals or to switch self-assembly processes on and off.¹⁵⁶ When coupled with enzymes, it can further enable dissipative self-assembly, where DNA structures and materials form and disassemble in a controlled way.^{157–162} Additionally, these reactions can be integrated with external stimuli, such as light and fuel, to achieve light-driven and fuel-driven DNA assemblies, opening possibilities for more sophisticated and responsive DNA-based materials and systems.^{163–169}

One of the most exciting applications of DAN-RNs is **DNA computing** using DNA logic gates. In an early study, Seelig, Winfree, and coworkers demonstrated the design of an **AND gate** composed of three DNA strands (Figure 1-18b).¹⁴⁶ These three DNA strands are hybridized such that one strand hybridizes to a substrate and exposes a toehold, while another strand binds the substrate with a loop. Upon receiving both input strands, the first input displaces the first bound strand, generating a new toehold domain. This newly exposed toehold is then recognized by the second input, which displaces the second bound strand, producing an output signal for downstream reactions. If only one or none of the inputs is present, the output is not generated, thereby fulfilling the logic of an AND gate. Remarkably, this system is enzyme-free, relying purely on DNA hybridization reactions, and exhibits high robustness. Beyond its conceptual significance, this approach can be extended to messenger ribonucleic acid (mRNA) detection and other sensing applications. This study marked the beginning of DNA logic gate-based computation, inspiring a wide range of subsequent developments in more advanced DNA computing systems.^{148,149,153}

However, in signal detection and diagnostic applications, such stoichiometry-dependent systems are limited, as they require equimolar amounts of input to proceed and therefore struggle to detect trace amounts of substances. To overcome this limitation, Yin, Pierce, and coworkers developed an autocatalytic DNA-RN capable of **signal amplification** (Figure 1-18c).¹⁷⁰ Their design employs a **hairpin catalytic reaction (HCR)**, comprising two DNA hairpin motifs and an initiator strand. The complementary domains are engineered such that the initiator first binds to the first hairpin, opening it and exposing a domain complementary to the second hairpin. Subsequent hybridization between the first and second hairpins releases the initiator back into solution, allowing it to further catalyze hairpin interactions for unopened DNA hairpins. As a result, even a trace amount of initiator can be recycled repeatedly, guiding the hybridization of all available hairpins over time and achieving robust

signal amplification. This strategy has been widely adapted in different applications for sensitive molecular detection and diagnostics.^{171–173}

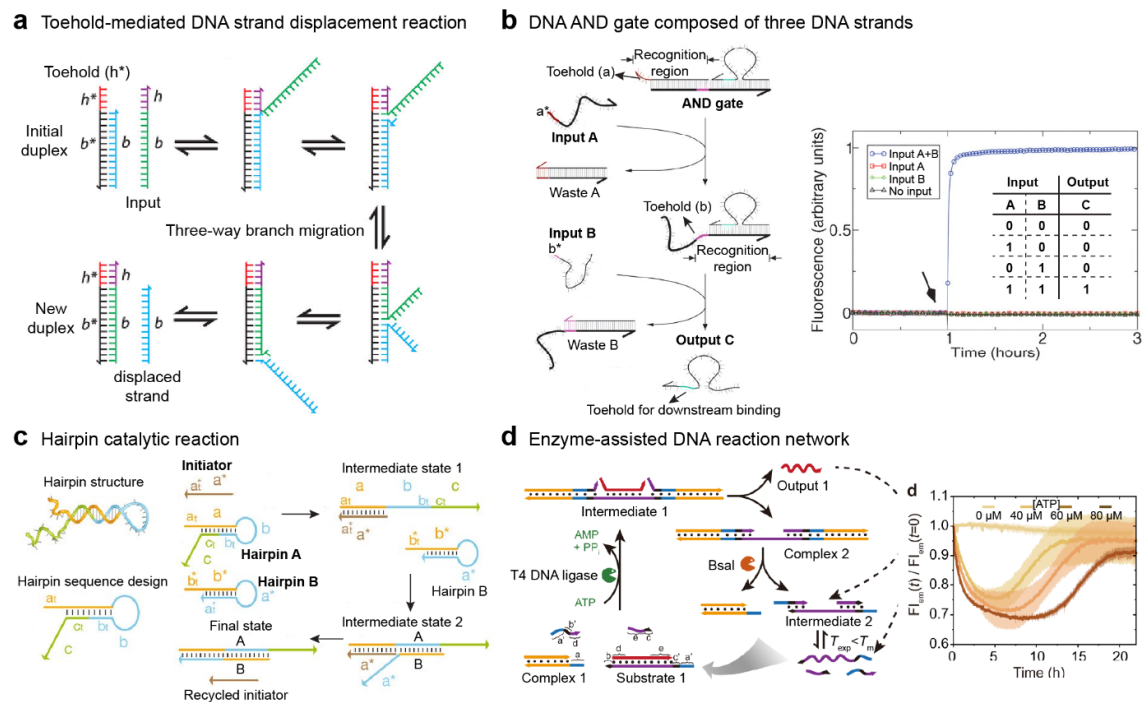


Figure 1-18. DNA reaction networks enabled by toehold-mediated strand displacement reactions. **a**, Scheme of the toehold-mediated strand displacement reaction via three-way branch migration. **b**, Representative DNA AND gate composed of three DNA strands, which can be triggered in presence of both inputs A and B. The right plot shows the response and corresponding truth table of the AND gate upon addition of different inputs. **c**, Scheme of hairpin catalytic reaction of two different DNA hairpin motifs and an initiator. **d**, Enzyme-assisted DNA reaction network with competitive ligation and cleavage reactions, enabling transient release of an output strand. Figures adapted from references (^{146,155,170,174}). Figure **a** is adapted from ref. 155 with permission. Copyright 2019, American Chemical Society. Figure **b** is adapted from ref. 146 with permission from AAAS. Figure **c** is adapted from ref. 170 with permission. Copyright 2008, Springer Nature Limited. Figure **d** is adapted from ref. 174 with permission under the terms of the Creative Commons CC BY license. Copyright 2021, The Authors.

By combining **DNA-RNs with enzymes**, **transient SDR** can be established. For example, Deng and Walther demonstrated a rationally designed system in which T4 DNA ligase and BsaI were combined in the same solution to perform competitive ligation and cleavage reactions (Figure 1-18d).¹⁷⁴ The activity of T4 DNA ligase is dependent on ATP, allowing the use of ATP to tune the balance between ligation and cleavage kinetics. By consuming ATP, T4 DNA ligase catalyzes the ligation of several designed DNA constructs including two Complex 1, a Substrate 1, an Input 1, and an Input 2 to form an Intermediate 1. The following SDR reaction ejects the originally bound Output 1 from Substrate 1 (now in form of the ligated Intermediate 1) to the solution. Once ATP is depleted, the cleavage reaction catalyzed by BsaI outcompetes the ligation reaction, resulting in the cleavage of Complex 2 back into Complex 1, Substrate 1 and Inputs. Therefore, such DNA-RN generates a transient DNA output. This transient signal can be further exploited to induce dissipative self-assembly, enabling the formation of transient patterns and structures, including colloidal assemblies, DNA nanotubes, and other dynamic architectures.^{166,174,175}

However, two major challenges have largely constrained the development of DNA-RNs: (1) they have been limited in scales and complexity for minimizing sequence interference and preventing unwanted reaction crosstalk;¹⁷⁶ (2) individual DNA-RNs cannot be simply integrated into a unified system without major sequence modification and redesigning. To run large-scale DNA-RNs, a recent approach divides DNA strands into subunits and operates them in separate reaction vessels.¹⁵³ Yet, this requires manual intervention in multiple steps and falls short of achieving fully autonomous operation. It is necessary to think about compartmentalization strategies for confining individual reaction networks in the same pool, so that strand transport across different reaction networks is regulated. There have been some attempts using DNA origami to fix DNA strands and guide their reaction through space, which enabled the reuse of same sequence design for different DNA-RNs.¹⁷⁷ However, a DNA origami can only fix limited number of DNA strands, which possesses little diffusivity. As a result, interactions are restricted to local neighborhoods, preventing fully dynamic, diffusion-driven reactions similar to those in a bulk solution.

Ideally, a compartmentalization system for DNA-RNs should meet three key criteria: (1) it must be permeable, enabling material exchange between the inside and outside for receiving inputs, releasing outputs, and communicating across different DNA-RNs; (2) it should selectively enrich desired DNA strands while preventing unwanted diffusion out of the compartment; and (3) it should be dynamic and allow free diffusion of DNA strands within the compartment for reactions to occur. In this regard, DNA condensate can serve as a suitable candidate, which is exploited in Chapter 5 to host and regulate DNA reaction networks for improved modularity and scalability.

1.5 Motivation and Aim

DNA condensates formed by phase separation of DNA polymers are versatile materials. This thesis aims to apply DNA condensates as: (1) **model systems for investigating fundamental properties and mechanisms of condensates**; (2) **synthetic cells for incorporating structural and functional elements**; (3) **reaction hubs for compartmentalizing DNA reaction networks**.

Inside cells, **biomolecular condensates** enrich essential macromolecules for organizing physicochemical processes. However, a mechanistic understanding of how macromolecules are transported into and within condensates remains limited. Because condensates exhibit distinct viscoelastic properties, concepts from polymer physics can be applied for providing mechanistic insights into these phenomena. This thesis aims to apply DNA condensates as in vitro models to study diffusion of macromolecules inside condensates. The overarching goal is to develop a quantitative framework for understanding **macromolecular transport** within biomolecular condensates.

In **synthetic cell** research, DNA condensate-based SC model still lacks structural and functional complexity. This thesis aims to leverage the inherent compatibility of DNA condensates with

DNA nanotechnology and synthetic biology to develop SCs with internal **artificial cytoskeletons** and **transcription functions**. Moreover, intracellular structures such as cytoskeletons form through self-assembly within the crowded, viscoelastic cytoplasm, a process that remains poorly understood compared to assembly in solution. This thesis aims to employ DNA condensates as a controllable platform that mimics macromolecular crowding and viscoelasticity, to study self-assembly and phase separation within them.

Of particular relevance to **DNA nanotechnology**, DNA condensates introduce a new level of hierarchy in DNA-based materials and systems. Conventional DNA nanotechnology, relying on base-pairing interactions, restricts the scale of all-DNA materials to relatively small dimensions. This thesis aims to incorporate DNA condensates into DNA nanotechnology to expand the design space, thereby enabling the construction of hierarchical DNA materials, such as core-shell DNA condensates with self-organizing artificial cytoskeletons. Beyond material design, DNA-RNs are traditionally limited by sequence interference, which constrains their scalability and modularity. To address this challenge, this thesis aims to employ DNA condensates as a **compartmentalization strategy** for DNA-RNs, enabling spatial control over DNA sequences and allowing multiple DNA-RNs with identical sequence designs to operate independently without interference. This strategy could lay the foundation for building large-scale DNA computing systems capable of executing advanced functions.

To summarize, this thesis aims to establish DNA condensates as a versatile platform that bridges fundamental sciences and functional applications and demonstrates the functionality of DNA condensates as a promising new material in DNA-based materials and systems.

1.6 Synopsis

With the relevant background, knowledge gaps, motivations, and aims established in Chapter 1, the following chapters will present the use of DNA condensates formed by DNA polymer phase separation in different research projects:

Chapter 2 employs DNA condensates as a **model system** to investigate **diffusion phenomena** within biomolecular condensates under controlled conditions (Figure 1-19a). Specifically, a new diffusion phenomenon is observed during the uptake of macromolecules into condensates driven by molecular recognition. This unprecedented diffusion mechanism is triggered by the addition of a short oligonucleotide, termed the “invader,” which selectively binds to complementary domains within the DNA condensate. During uptake, a sharp diffusion front forms, with a high local concentration of the invader accumulating at the front, propagating linearly with time. This behavior contrasts with the blurry concentration gradients and nonlinear propagation kinetics predicted by classical Fickian diffusion. We quantify, model, and extrapolate the multiscale, nonequilibrium characteristics of this diffusion mode, highlighting its distinction from conventional understandings of transport in condensates.

Finally, we discuss the generalization of these findings based on the physicochemical interplay between condensate dynamics, molecular recognition, and chemical kinetics.

Chapter 3 applies DNA condensates as viscoelastic **synthetic cells**, focusing on the construction of **internal secondary structures**. Leveraging the inherent compatibility of DNA condensates with DNA nanotechnology tools, DNA tiles are used as building blocks for growing DNA nanotubes as artificial cytoskeletons inside synthetic cells (Figure 1-19b). We first introduce a co-phase-separation approach to integrate multiple barcodes with controlled concentrations into the SCs, for enriching DNA tiles. We then study how artificial cytoskeletons grow in these confinements, and how the concentration of DNA tiles and the viscoelastic properties regulate the assembly process. Programmed barcode recognition between SCs and DNA tiles is further explored for achieving self-sorted cytoskeletons within SCs and self-sorted SCs containing distinct artificial cytoskeletons in SC communities. Finally, we characterize the mechanical enhancement by growing artificial cytoskeletons inside SCs. These SCs are exploited to build a SC-mammalian cell interface.

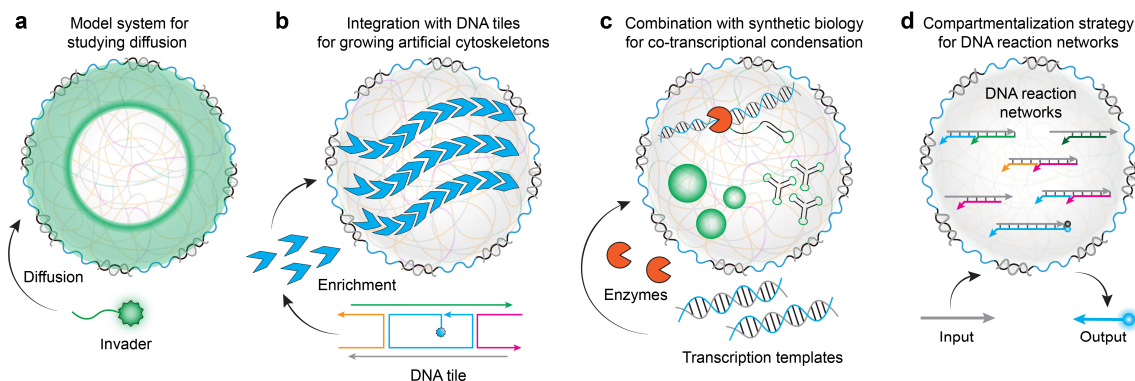


Figure 1-19. DNA condensates for diverse applications and functions. **a**, DNA condensates can serve as synthetic model system to study fundamental diffusion mechanism within condensates. **b**, DNA condensates can serve as synthetic cells and combined with DNA tiles for growing internal artificial cytoskeletons. **c**, DNA condensates can be integrated with synthetic biology for acquiring transcriptional functions, which enables co-transcriptional condensation of RNA nanostars inside. **d**, DNA condensates can compartmentalize DNA reaction networks for preventing sequence interference and crosstalk across different reaction networks.

Chapter 4 repurposes DNA condensates as **protonuclei (PN)** and couples them with synthetic biology tools to achieve **transcriptional functions** (Figure 1-19c). As the internal composition of the PN can be flexibly tuned based on the ssDNA polymer used through co-phase separation strategy, we incorporate T7 promoter sequences into the PN core to recruit transcription templates and facilitate localized *in-protonucleo* transcription. RNA nanostars are transcribed within these PN, leading to the formation of co-transcriptional RNA condensates with various morphologies. We demonstrate a range of synthetic nuclear architectures, including single condensates, multiple condensates, interfacial condensates formed through secondary nucleation, and biphasic condensates of orthogonal RNA nanostars, and investigate the controlling parameters including salinity, PN-RNA nanostars affinity, and competing PN-RNA nanostars interactions, respectively.

Chapter 5 explores DNA condensates as a **compartmentalization strategy** for hosting **DNA-RNs** (Figure 1-19d). We design DNA condensates containing different DNA barcodes for anchoring individual DNA-RNs. We further introduce input-selective Transducer module for reacting with specific input and releasing a unified output Messenger. Coupling between different Transducer modules with same DNA duplex Reporter allows us to use the same Reporter to detect different DNA input in distinct DNA condensates. We study such Input-Transducer-Reporter system both in solution and in condensate and demonstrate the advantage of compartmentalization in enabling parallel and orthogonal execution of interfering DNA-RNs.

Chapter 6 provides a comprehensive review of our current understanding of DNA condensates. It discusses the control of physical and chemical properties of DNA condensates and the strategies devised to engineer their compositions. It highlights the applications of DNA condensates across diverse fields, from serving as model systems in biophysical studies and probing self-assembly in crowded environments to advancing synthetic cell research, expanding the scope of DNA nanotechnology, and enabling biomedical applications. This review integrates insights and contributions from individual works with an outlook on future directions for harnessing DNA condensates in fundamental research and various applications.

The thesis concludes in Chapter 7 with a summary and conclusion of the main results, accompanied by perspectives on the future development of DNA condensate materials.

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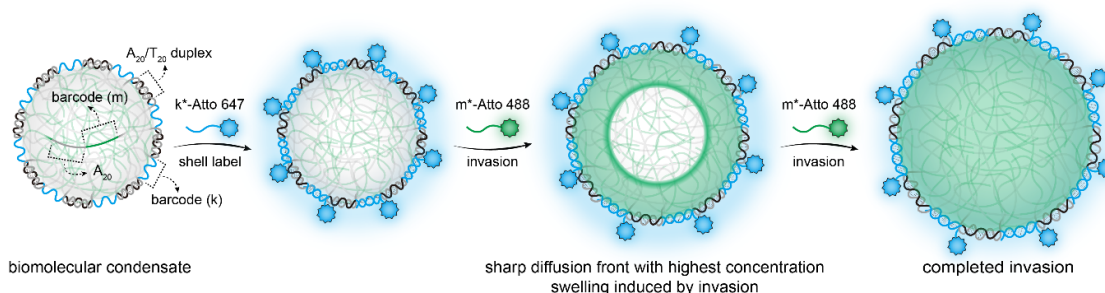
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2. Ballistic Diffusion Fronts in Biomolecular Condensates



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Author contributions: W.C. and A.W. conceived the project and designed the experiments. W.C. performed the majority of experiments and analyzed the data. W.C. and B.D. built the reaction-diffusion model, performed the simulations, and analyzed the results. P.G.A. performed the BCARS experiments and analyzed the data. S.V.B. performed oxDNA simulation and analyzed the data. W.L. helped with DNA synthesis. W.C., B.D., P.G.A., S.V.B., A.S., S.H.P., M.B., and A.W. discussed the data. W.C. and A.W. wrote the manuscript with input from all authors. A.W. supervised the project.

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Abstract: Biomolecular condensates in cells compartmentalize vital processes by enriching molecules through molecular recognition. However, it remains elusive how transport occurs in biomolecular condensates and how it relates to their dynamic and/or viscoelastic state. We show that the transport of molecules in DNA model condensates does not follow classical Fickian diffusion, which has a blurry front with a square root of time dependence. In contrast, we identify a new type of transport with an ultrasharp front that propagates linearly with time. Our data reveals that this ultrasharp ballistic diffusion front originates from molecular recognition and an arrested-to-dynamic transition in the condensate properties. This diffusion mechanism is the result of intertwining chemical kinetics and condensate dynamics on transport in biomolecular condensates. We believe that our understanding will help to better explain and tune dynamics and properties in synthetic condensate systems and for biological functions.

2.1 Introduction

Biomolecular condensates formed by liquid–liquid phase separation (LLPS) of nucleic acids^{1,2} and proteins^{3,4} in cells serve as signaling and reaction hubs^{5,6}, and gate accessibility, for instance to the nucleus⁷. The transfer of concepts from polymer science has greatly advanced the understanding of condensate formation, stabilization, and their physicochemical properties^{8–16}.

Despite this progress, a mechanistic understanding of how macromolecules are transported into and within condensates remains limited. Biomolecular condensates possess complex viscoelastic properties and are typically classified as liquid-like or solid-like^{17–19}. However, the functional significance of the solid-like state beyond regulating mechanical properties and arresting dynamics is poorly understood^{5,20}. In the crowded and heterogenous environment of cells and condensates with complex viscoelastic properties^{21–24} transport may deviate from classical diffusion, and be influenced by complex relaxation dynamics²⁵ or specific interactions^{26,27}, leading to anomalous diffusion behavior^{7,28}. To dissect this intricacies, minimalistic synthetic model systems offer powerful platforms to study behavior in well-controlled settings^{29,30}.

In this study, we report on the observation of a new diffusion behavior during the uptake of macromolecules into biomolecular condensates driven by molecular recognition. Our findings reveal this unprecedented diffusion mechanism upon the addition of a short oligonucleotide, termed “invader”, to DNA condensates composed of long single-stranded DNA (ssDNA). The invader selectively binds to complementary domains in the DNA condensate. During its uptake, a sharp diffusion front is formed with an accumulation of a high concentration of the invader at this front, which propagates with a linear time dependence. This contradicts the commonly observed fuzzy concentration gradients and nonlinear propagation kinetics described by Fickian diffusion, which widely applies to many diffusion processes in solutions. We quantify, model, and extrapolate the multiscale nonequilibrium nature of this new diffusion mode and

distinguish it from the current understanding of diffusion and transport in condensates. We discuss the generalization of the findings based on the physicochemical interplay between condensate dynamics, molecular recognition, and chemical kinetics.

2.2 Results and Discussion

2.2.1 Observation of Ballistic Diffusion Fronts

We prepared DNA condensates by temperature-induced coacervation of two long ssDNA copolymers, $p(A_{20}-m)_n$ and $p(T_{20}-k)_n$ in TE buffer at 50 mM $MgCl_2$ (n = repeating units; Extended Data Figure 2-1)³¹⁻³³. A_{20} and T_{20} are homorepeats of adenine- and thymine-containing nucleotides, whereas m and k are DNA barcode sequences that can bind complementary oligonucleotides (Extended Data Figure 2-1a). Briefly, upon heating a $p(A_{20}-m)_n/p(T_{20}-k)_n$ mixture, $p(A_{20}-m)_n$ undergoes selective temperature-induced LLPS and forms spherical condensates above its cloud point temperature (ca. 42 °C)³¹, whereas $p(T_{20}-k)_n$ remains in solution. During cooling, $p(T_{20}-k)_n$ localizes at the periphery of $p(A_{20}-m)_n$ condensates by A_{20}/T_{20} hybridization and forms a thin and permeable shell (Extended Data Figure 2-1c)^{32,33}. This process results in micrometer-sized core-shell condensates with addressable barcodes in the core (m) and shell (k). The shells are typically labeled at the k barcodes with fluorescent ssDNA (k^* -dye) for visualization (Extended Data Figure 2-1d)^{31,32}. The m barcodes in the core provide recognition sites for the diffusing molecules (invaders, m^*) and mimic molecular recognition in biological systems.

Surprisingly, the uptake of invaders (m^* -Atto488), that specifically bind to the m barcodes inside the condensate, results in a sharp, high-intensity diffusion front (Figure 2-1a, b). The front propagates linearly with time, ΔX (front displacement) $\sim t$ (time) (Figure 2-1c, d). This represents a ballistic behavior (constant velocity) of the macroscopic diffusion front. Significant swelling occurs in the invaded region to a final swelling ratio of about 4-fold in volume (Figure 2-1b, e). The high-intensity front arises from the spatial accumulation of the invaders and is stable in shape during propagation (Figure 2-1f and Extended Data Figure 2-2). This propagating steady state indicates balanced kinetic processes of invader binding and condensate swelling at the front interface. This behavior is fundamentally different from classical Fickian diffusion, which exhibits unavoidable concentration gradients and a power-law relationship of the front propagation with time, $\Delta X \sim t^{1/2}$ (Figure 2-1a).

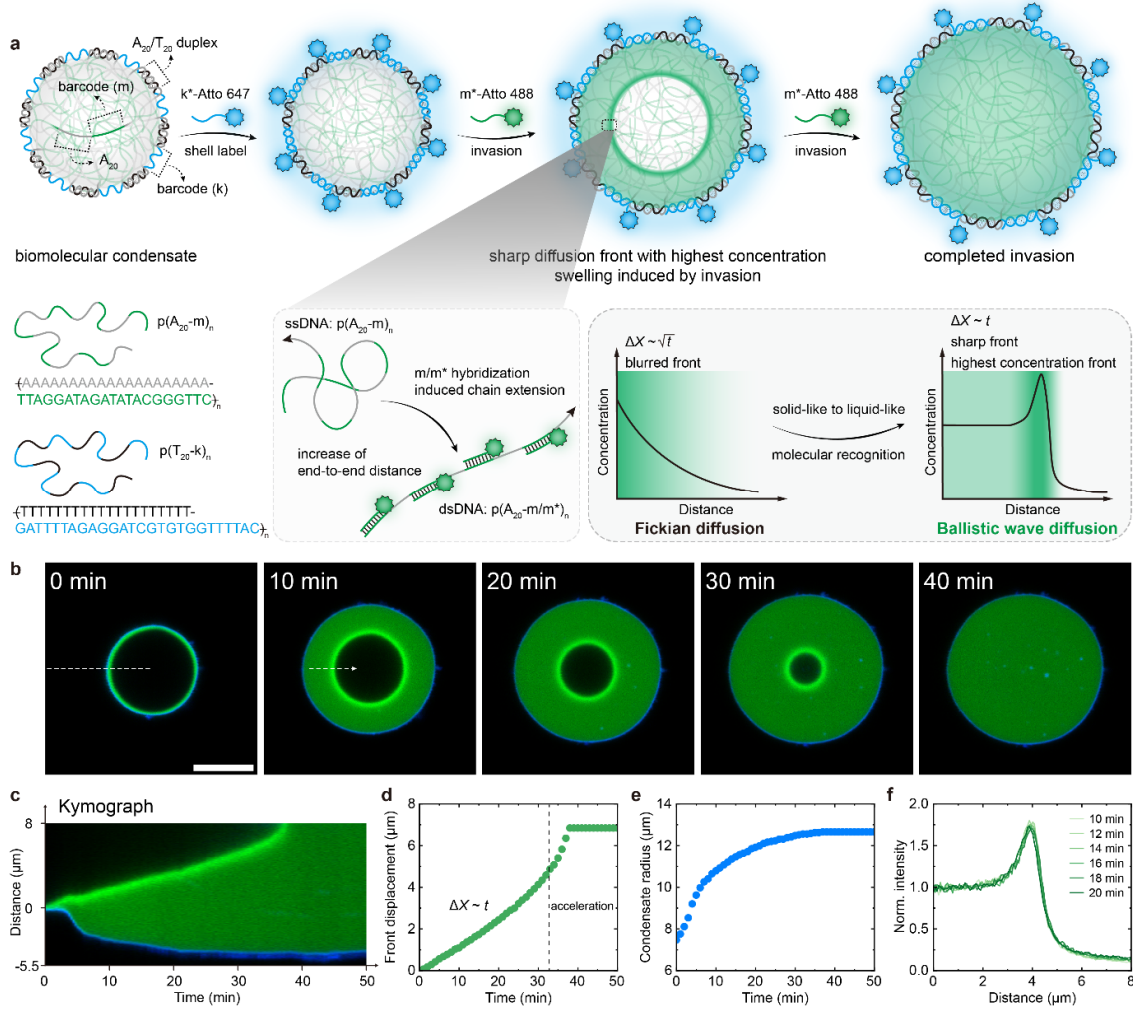


Figure 2-1. Not Fickian but ballistic wave diffusion with a high-intensity zone occurs during invasion of ssDNA invaders into DNA condensates. **a**, Scheme of ballistic wave diffusion in DNA condensates, whereby invaders m^* bind to m barcodes in the DNA condensates, inducing a structural and conformational transition from ssDNA to dsDNA. Differences between classical Fickian diffusion and ballistic wave diffusion identified in this study. **b**, Time-series CLSM images showing the invasion process of invaders (m^* -Atto488, 2 μ M; green) into a DNA condensate (shell labelled by k^* -Atto647; blue) at 50 mM of $MgCl_2$. The ballistic wave diffusion process has been consistently reproduced in 20 individual concentration-dependent experiments discussed below as well as in other experiments throughout this research. **c**, Kymograph (space–time plot) of the invasion process along the white dashed line in **b**. **d**, Front displacement during invasion is linear with time. Acceleration happens at the end stage (noted with the dashed line) because of the interference between diffusion fronts from all the radial directions. **e**, Radius of DNA condensate during invasion. **f**, Cross-sectional line profiles taken at various times along the white dashed arrow in **b**. The distances are rescaled to the maximum intensity of the front to highlight the self-similar shape during invasion. Scale bar, 10 μ m.

The sharp diffusion front is a nonequilibrium steady-state interface, where DNA chain extension occurs because m barcodes of the $p(A_{20}-m)_n$ ssDNA are converted to m/m^* dsDNA segments. Molecular dynamics simulations using oxDNA yield a 64 % increase in the radius of gyration from $p(A_{20}-m)_n$ to $p(A_{20}-m/m^*)_n$ (Extended Data Figure 2-3 and Supplementary Note 2-1)^{34–36}. An acceleration of the front propagation is visible in the final invasion stage, likely caused by the interference of individual diffusion fronts and the smaller surface area once the

fronts start to share the same non-invaded center. The process loosely resembles “Case II diffusion”^{37,38} that describes the diffusion of solvents with sharp fronts into specific thermoplastics. However, despite a phenomenological similarity, Case II diffusion in thermoplastics cannot account for a high-intensity front, and, critically, the origin and underlying physical principles are profoundly different. Condensates, unlike thermoplastics, are solvent-rich entities, and we elucidate that molecular recognition plays a key role, a specific property inherent to biomacromolecules and responsible for condensate formation in cells. We term this new molecular-recognition-enabled frontal diffusion mechanism with a high-intensity front as “ballistic wave diffusion,” which satisfies the condition of $\Delta X \sim t$.

2.2.2 Mechanistic Details of Ballistic Wave Diffusion

To gain mechanistic insights, we synthesized fluorescent $p(A_{20-m_{dye}})_n$ to study the dynamic properties of the $p(A_{20-m_{dye}})_n$ chains in the condensate before and after invasion. During invasion with m^* -Atto488, the dilution of the $p(A_{20-m_{Atto594}})_n$ condensate in the outer region is now evident (Figure 2-2b, magenta channel). The approximately 4-fold swelling causes a 4-fold drop in the intensity of the core material $p(A_{20-m_{Atto594}})_n$. The invader front localizes precisely at the boundary from the compact non-invaded to the swollen invaded condensate (Figure 2-2c). We correlate relative changes in the dynamic properties of the DNA condensates before and after invasion using fluorescence recovery after photobleaching (FRAP) and fluorescence lifetime imaging (FLIM)³⁹, which provide complementary insights into mesoscale diffusion behavior and nanoscale chain flexibility. FRAP shows drastic differences in recovery kinetics before and after invasion. Before invasion, the condensates are in an arrested state in which the bleached region does not recover even after 6000 s (Figure 2-2d–f). In contrast, after invasion, the condensates become dynamic with fluorescence recovery (Figure 2-2e, f). Quantification using the reciprocal of the half recovery time ($1/t_{1/2}$) reveals differences of at least 2 orders of magnitude (Figure 2-2g). FLIM and lifetime analysis show a significant increase from a lifetime of about 2.9 ns in the arrested/non-invaded region to about 3.4 ns in the dynamic/invaded region. The phasor plot shows two distributions (Figure 2-2h–j)^{40,41}.

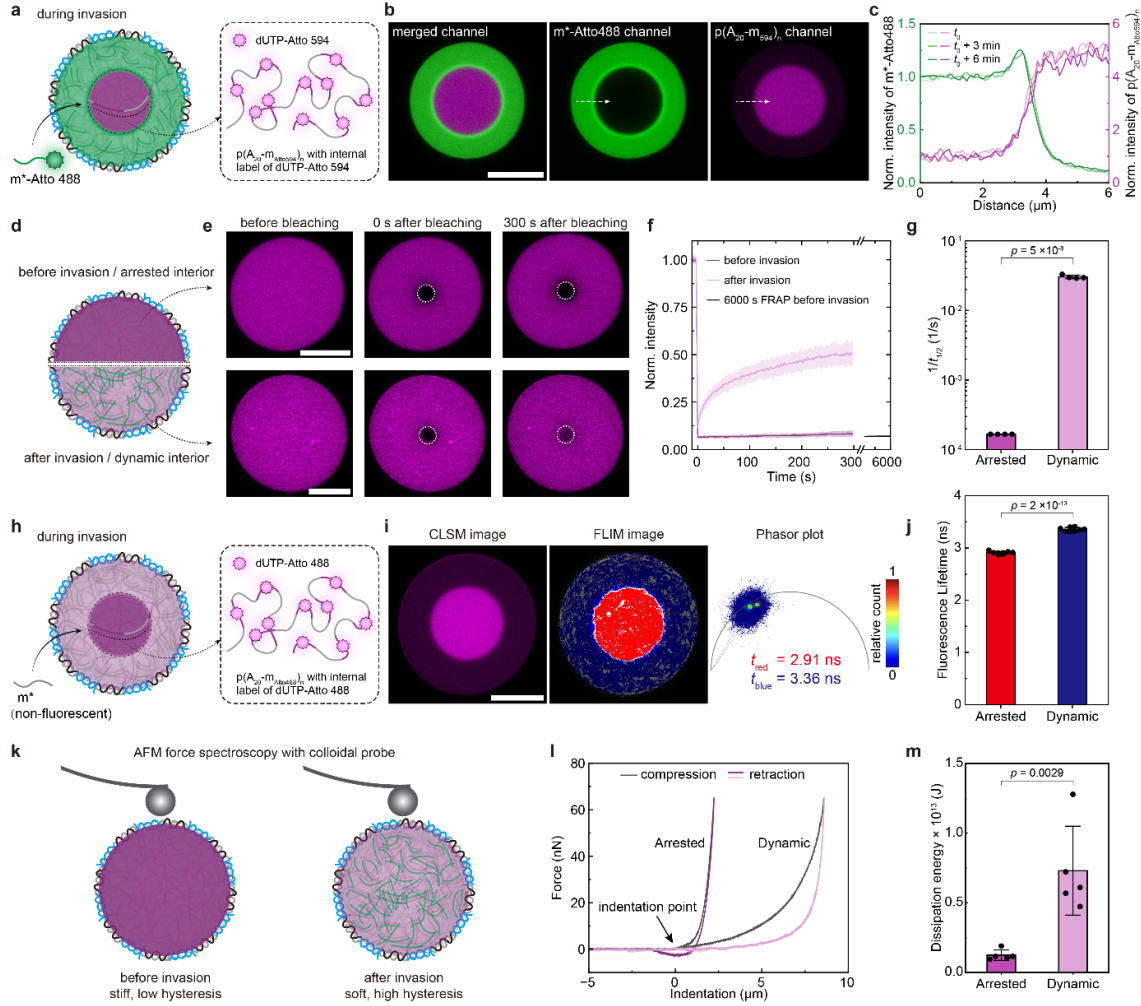


Figure 2-2. Changes in condensate dynamical and mechanical properties during ballistic wave diffusion. **a**, Scheme of a DNA condensate with in-chain fluorophores ($p(A_{20}-m_{Atto594})_n$; magenta) during the invasion of m^* -Atto488 (green). **b**, CLSM images of a DNA condensate during invasion by $1 \mu\text{M}$ of m^* -Atto488 at 50-mM MgCl_2 . **c**, Time-dependent cross-section profiles along the white dashed arrows in **b**, with the distance rescaled to the maximum intensity of the front. **d**, Scheme of a DNA condensate before (top) and after (bottom) invasion. Size increase is not shown for clarity. **e**, CLSM images showing FRAP experiments on DNA condensates before and after invasion. **f**, Recovery profiles of FRAP circles in **e** before and after invasion at 50-mM MgCl_2 . Unless specified, the curves represent the mean values, and the shaded areas indicate the s.d. Sample size (N) = 4 condensates measured for each condition. The black curve after the axis break shows a 6,000-s-long FRAP experiment on a non-invaded condensate at 20-mM MgCl_2 , proving its arrested state. **g**, Reciprocal of the half-recovery time ($1/t_{1/2}$) extracted from **f**. $1/6,000 \text{ s}^{-1}$ is taken as $1/t_{1/2}$ for the arrested condensates due to the absence of recovery in FRAP within 6,000 s (mean $\pm$ s.d., N = 4 condensates measured for both conditions). **h**, Scheme of a fluorescent DNA condensate ($p(A_{20}-m_{Atto488})_n$; magenta) during the invasion of non-fluorescent invaders m^* . **i**, CLSM and FLIM images during invasion by $3\text{-}\mu\text{M}$ m^* at 50-mM MgCl_2 , with the corresponding phasor plot depicting the lifetime distribution. **j**, Fluorescence lifetime of in-chain fluorophores in arrested/non-invaded and dynamic/invaded regions (mean $\pm$ s.d., N = 8 condensates). **k**, Scheme of the AFM indentation measurement of the condensates before and after invasion. **l**, Representative force spectroscopy for DNA condensates before and after invasion show distinct force–distance relationships and hysteresis behaviour during compression and retraction of the AFM cantilever. Five independent experiments were performed on the arrested and dynamic condensates (Supplementary Figure 2-1). **m**, Dissipation energy for condensates in different states, measured by quantifying the hysteresis area in the compression and retraction curves (mean $\pm$ s.d., N = 5 condensates measured for each condition). One-way analysis of variance test was used for the P values for **g**, **j** and **m**. Scale bars, $10 \mu\text{m}$.

Since our condensates are based on semi-dilute and entangled ssDNA polymer solutions that are viscoelastic, we used force spectroscopy via colloidal probe atomic force microscopy⁴² to study differences in mechanical properties for the condensates before and after invasion (Figure 2-2k, l, and Supplementary Figure 2-1). The force response during indentation increases more drastically for condensates before invasion, whereas after invasion, the condensates are significantly softer. This is a direct comparative measure for the elastic response. Additionally, a larger hysteresis is visible between the compression and retraction curves for condensates after invasion (Figure 2-2m and Supplementary Figure 2-1). Since hysteresis is a measure of energy dissipation, this indicates increasingly liquid-like properties for condensates after invasion. In contrast, the near-superimposing compression/retraction curves of the condensate before invasion are closer to an elastic material with little energy dissipation. These insights provide direct evidence for distinct mechanical properties of the two types of condensates, as regulated by ballistic wave diffusion.

Based on these data, the following picture emerges. The initial DNA condensates are in an arrested state, similar to some aged condensates in biology literature^{5,17,19,20,43}. This arrested state is due to the efficient arrest of $p(A_{20}-m)_n$ with divalent Mg^{2+} ions⁴⁴. During invasion, m/m^* hybridization occurs by molecular recognition, and invasion is restricted to the frontier zone. The high intensity of the front is due to the initial high concentration of m barcodes in the compact condensate. The m/m^* binding causes swelling, dilution of $p(A_{20}-m/m^*)_n$, and simultaneous liquefaction in this volume. The relaxation from the compact high-concentration front to the swollen and dynamic final state is a time-dependent process due to the polymeric nature of the condensate. The swelling is induced by chain stiffening and expansion due to partial dsDNA formation in partly hybridized $p(A_{20}-m/m^*)_n$, a solubilizing effect of m^* binding, and a minor increase in osmotic pressure. Simulations in Extended Data Figure 2-3 confirm the expansion, and further control experiments support this physical picture: (1) A non-binding ssDNA diffuses about two orders of magnitude faster in dynamic/invaded condensates than in arrested/non-invaded condensates (Extended Data Figure 2-4). Diffusion of small ssDNA is strongly suppressed in the arrested/non-invaded condensates. The measured diffusion coefficients are in line with those found in other condensates^{45,46}. (2) Invasion with a non-charged peptide nucleic acid (PNA) strand (m^*_{PNA} -Atto488) results in substantially less swelling, and the condensates remain in an arrested state after invasion (Extended Data Figure 2-5a–e). (3) Hybridization of $p(A_{20}-m)_n$ with DNA- m^* increases the solubility of $p(A_{20}-m)_n$, while PNA- m^* has an intermediate effect (Extended Data Figure 2-5f–h). Points (2) and (3) confirm that the negative charges of the DNA invader contribute to the swelling and regulation of the arrested-to-dynamic transition of the invaded condensates.

2.2.3 Reaction-Diffusion Model and Simulations

To rationalize and predict the behavior of ballistic wave diffusion, we built a model using reaction-diffusion equations (Extended Data Figure 2-6a and Supplementary Note 2-2). The model includes different diffusivities of the invader (m^*) in the arrested/non-invaded (D_0) and dynamic/invaded part (D_1), the binding reaction of m/m^* (k_{bind}), and the subsequent swelling (k_{swell}). The swelling is associated with a swelling factor (f_{swell}). Since we know that volumetric swelling is about 4-fold and the m barcode concentration ($[m]$) inside the condensates is about 1 mM (Supplementary Figure 2-2), we can assign realistic starting points to these simulations (Supplementary Table 2-2).

Let us hypothesize a ballistic wave diffusion process. When m^* diffuses into the condensate, it binds to an unoccupied m and forms a compact $p(A_{20}-m/m^*)_n$ that is part of the arrested interior ($m/m^* = M_{\text{arrested}}$). After binding, solubilization of the local segment occurs, leading to swelling and a transition from M_{arrested} to M_{swollen} . Binding can be considered diffusion-controlled and unbinding can be neglected because of the high binding strength of m/m^* ($T_m \approx 71.5^\circ\text{C}$, Supplementary Note 2-3). Once swelling has occurred, *i.e.*, $[M_{\text{swollen}}]$ exceeds a threshold, D_0 for m^* in this volume element increases to D_1 ($D_1 \gg D_0$). Therefore, subsequent m^* can diffuse rapidly through the swollen region but is drastically slowed down at the transition to the arrested region (Figure 2-3a).

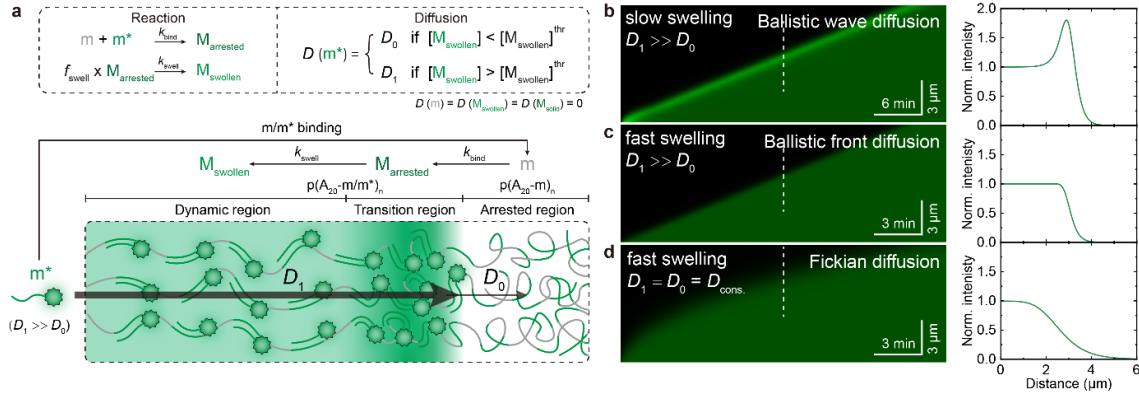


Figure 2-3. Modelling of ballistic wave diffusion using a reaction–diffusion model. **a**, Chemical reactions and diffusion rules in the ballistic wave diffusion model with a graphical explanation of the processes. **b–d**, Representative simulated kymographs (space–time plots) demonstrating ballistic wave diffusion (**b**), ballistic front diffusion (**c**) and Fickian diffusion (**d**) at different conditions. The right plots show individual front shapes by cross-sectional line profiles taken along the white dashed lines in the corresponding kymographs. Supplementary Table 2-3 lists the parameter sets.

This model can successfully reproduce ballistic wave diffusion with ballistic propagation kinetics ($\Delta X \sim t$) and a sharp and stably propagating diffusion front of high intensity with locally high m/m^* concentration (Figure 2-3b). It can also extrapolate behavior. For instance, when the swelling process is accelerated (k_{swell} is increased) but the difference between two diffusion coefficients is maintained, the high-intensity front (*i.e.*, the wave) disappears, but the front remains sharp and still propagates linearly with time (Figure 2-3c). We term such a state “ballistic front diffusion”. When the diffusion coefficient for m^* is assumed to be the

same inside the arrested/non-invaded and dynamic/invaded parts of the condensate, the transport follows Fickian diffusion with a blurry front and a front displacement proportional to $t^{1/2}$ (Figure 2-3d). A full phase diagram with varied D_0 and D_1 shows that the appearance of the ballistic front and wave diffusion originates from the difference between D_0 and D_1 (Extended Data Figure 2-6b and Supplementary Note 2-7). Additional orienting simulations in 2D and 3D are in line with the 1D simulation and show a slight acceleration at late stages as also observed in experiments (Figure 2-3c, d, Supplementary Figure 2-3 and Supplementary Note 2-4).

Simulations suggest that the sharpness and height of the front (I_p/I_0) should decrease with enhanced swelling kinetics (Figure 2-4a, b). At the molecular level, swelling kinetics depend on the length of the $p(A_{20-m})_n$ chains because swelling occurs through relaxation, reptation, and disentanglement of the polymer chains⁴⁷. Indeed, we find a less pronounced high-intensity wave for shorter $p(A_{20-m})_n$ (Figure 2-4c and Extended Data Figure 2-7).

Moreover, the simulations predict a transition from ballistic wave diffusion to ballistic front diffusion and even to classical Fickian diffusion for condensates with increasing swelling kinetics and when the differences in D_1 and D_0 of m^* are reduced (Figure 2-3b–d). This can be achieved experimentally by decreasing the ionic strength. Lower $[Mg^{2+}]$ increases the net charge density of the phosphodiester backbone of $p(A_{20-m})_n$ ^{48,49}. In addition to increasing the persistence length of $p(A_{20-m})_n$, the chain-bridging effect of Mg^{2+} is also reduced⁴⁴. This is reflected in a Mg^{2+} -dependent swelling of DNA condensates (Figure 2-4e). A gradual decrease of $[Mg^{2+}]$ from 50 to 15 mM leads to a change of the invasion process from ballistic wave diffusion to ballistic front diffusion and finally to Fickian diffusion (Figure 2-4d). FRAP experiments on non-invaded and invaded DNA condensates allow quantification of the internal dynamics (Figure 2-4f and Extended Data Figure 2-8). For $[Mg^{2+}] \geq 20$ mM, the condensates are arrested before invasion and show a dynamic character only after invasion. At this point, the swelling due to lower $[Mg^{2+}]$ before invasion does not yet overcompensate for the interaction between the $p(A_{20-m})_n$ chains. Further decrease of $[Mg^{2+}]$ yields a discontinuous and drastic property change. At the transition point to Fickian behavior ($[Mg^{2+}] = 17.5$ mM), the DNA condensates already exhibit a comparatively dynamic interior that still increases by one order of magnitude after invasion. Lower $[Mg^{2+}]$ (15 mM) then shows similar dynamics before and after invasion. This clearly underscores two points: First, the observation of sharp diffusion fronts requires a distinct transition from an arrested to a dynamic condensate during invasion. Second, the transition between the two regimes (and thus the appearance of a high-intensity wave) depends on preinvasion swelling, because this eliminates the need for a swelling process after the invader binds to its barcode.

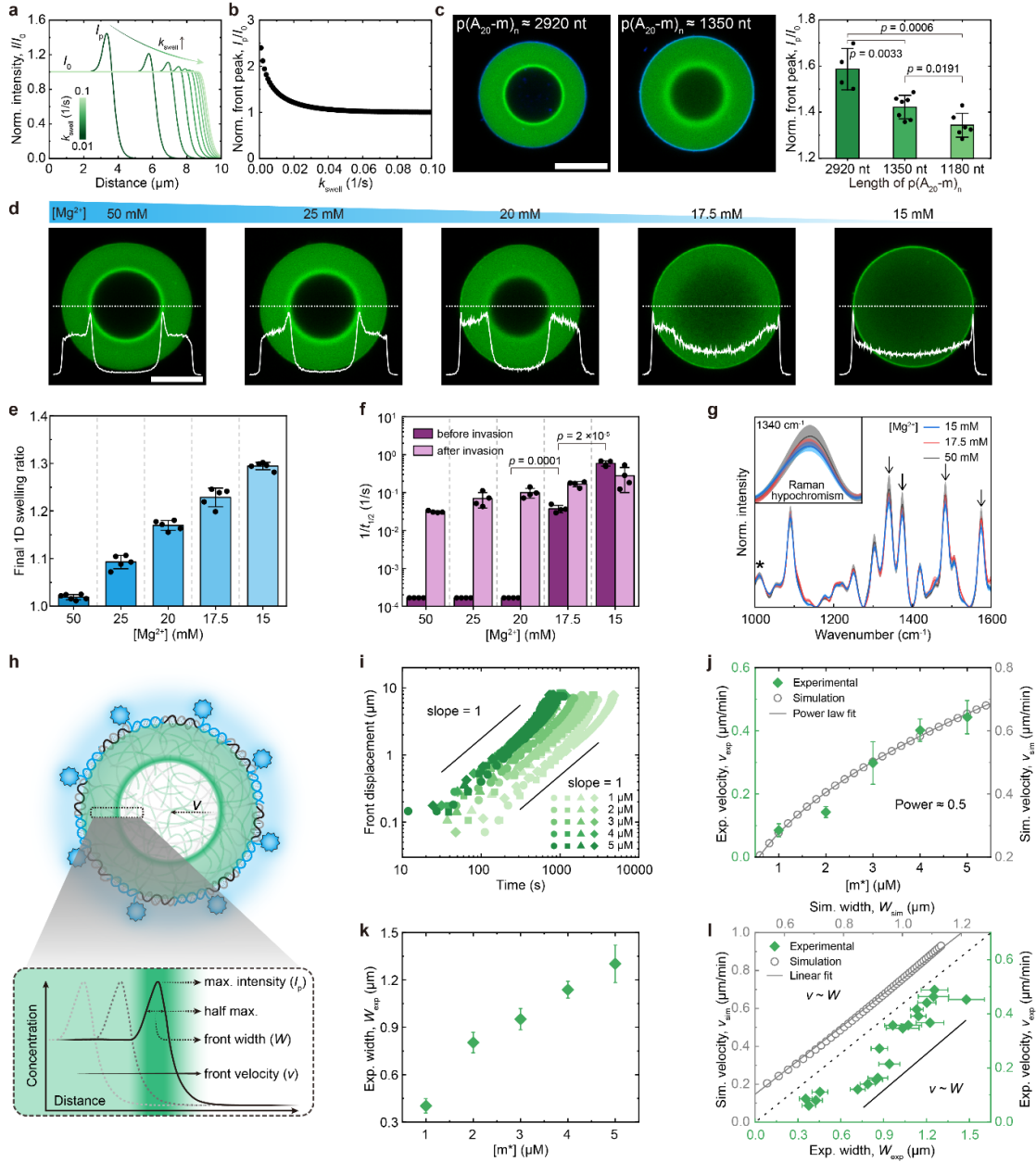


Figure 2-4. Tuning the diffusion mechanism and kinetics by combined simulations and experiments. **a**, Simulated intensity profiles (normalized by intensity at a fully swollen region, I_0) of ballistic wave diffusion show a decrease in the front height for increase swelling kinetics, k_{swell} . **b**, Simulated peak intensity (I_p) normalized by I_0 as a function of k_{swell} . **c**, CLSM images and quantification show a reduced front peak for shorter $p(A_{20}-m)_n$ chains (length in nucleotides (nt); mean $\pm$ s.d., $N = 4, 7$ and 6 condensates measured for 2,920, 1,350 and 1,180 nt). **d**, CLSM shows a transition from ballistic wave to Fickian diffusion when reducing $[Mg^{2+}]$. The white lines depict the intensity profiles. Experiments were repeated at least two times for each $[Mg^{2+}]$ with similar results. **e**, $[Mg^{2+}]$ -dependent swelling of non-invaded condensates (mean $\pm$ s.d., $N = 6$ condensates measured for 50-mM Mg^{2+} , and $N = 5$ condensates measured for others). **f**, $[Mg^{2+}]$ -dependent $1/t_{1/2}$ of DNA condensates from FRAP experiments before and after invasion (mean $\pm$ s.d., $N = 4$ condensates measured). **g**, $[Mg^{2+}]$ -dependent Raman spectroscopy of DNA condensates before invasion. Spectra are normalized to the peak at 1,014 cm^{-1} of the deoxyribose (*) (mean $\pm$ standard error, $N = 10, 9$ and 8 condensates measured for 50, 17.5 and 15 mM of Mg^{2+}). The black arrows indicate Raman hypochromism, with one magnification around 1,340 cm^{-1} . **h**, Scheme for the quantification of front velocity (v) and front width (W). **i**, Representative front displacement during ballistic wave diffusion at various invader concentrations. **j**, v as a function of invader concentration (mean $\pm$ s.d., $N = 4$ condensates measured). **k**, W as a function of invader concentration (mean $\pm$ s.d., $N = 4$ condensates measured). **l**, v plotted against W shows the linear correlation (mean $\pm$ s.d. of W in each experiment; W is measured six times for each invasion process at different times). The axis scales for the simulated and experimental results are scaled but show similar relationships. Supplementary Table 2-3 lists the parameter sets. One-way analysis of variance test was used for calculating the P values for **c** and **f**. Scale bars, 10 μm .

Broadband coherent anti-Stokes Raman scattering (BCARS) provides additional information on the chain conformation of $p(A_{20-m})_n$. Lower $[Mg^{2+}]$ leads to a marked decrease in Raman intensity for specific peaks in the 1300 to 1600 cm^{-1} range (Figure 2-4g), characteristic of nucleobases⁵⁰. This phenomenon, Raman hypochromism, suggests that the $p(A_{20-m})_n$ chains in the condensates adopt a more compact coil conformation with less base stacking due to geometric confinement at high $[Mg^{2+}]$. In contrast, lower $[Mg^{2+}]$ leads to a more extended, relaxed conformation due to an increase of effective charges along the backbone, resulting in swelling of the condensates, and a gain in free volume and dynamics, which facilitates the base stacking interactions known for adenine in ssDNA⁵¹⁻⁵³. Using a fluorescent reporter for Mg^{2+} , we find a slightly higher Mg^{2+} concentration inside the condensates than the solution prior invasion, whereas such differences vanish after the invasion process. This arises from the lower negative charge density inside the condensates after invader-induced swelling (Supplementary Figure 2-4).

Finally, we ask how well the model can capture and predict the tunability of the frontal velocity and the width of the high-intensity diffusion front. Our approach targets establishing generic scaling laws rather than a perfect fit that would require accurate assumptions for unknown kinetic parameters. Simulations predict a power-law dependence of the front velocity (v_{sim}) on the concentration of the invader ($v_{sim} \sim [m^*]^{0.5}$, Figure 2-4j and Extended Data Figure 2-6c, d). This dependence is reflected in experiments (Figure 2-4i,j). Similar good agreement is found for the dependence of the front velocity (v) with front width (W), which follows a linear correlation ($v \sim W$; Figure 2-4h, k, l and Extended Data Figure 2-6e). The model and the experimental agreement provide a clear physical picture of the dynamization and relaxation processes at the diffusion front. Higher $[m^*]$ can penetrate deeper into the arrested region at the steady-state diffusion front, resulting in a wider front interface and transition region. Accordingly, more compact $p(A_{20-m})_n$ chains bind m^* in a given volume and time element, leading to faster relaxation in a larger volume element, and, consequently, to a faster transition from compact $p(A_{20-m})_n$ to swollen $p(A_{20-m}/m^*)_n$.

2.3 Conclusions

We have identified a new diffusion mechanism in soft matter in general and in biomolecular condensates in particular, namely ballistic wave diffusion. Unlike Fickian diffusion, ballistic wave diffusion is characterized by an ultrasharp diffusion front that propagates with ballistic properties, *i.e.*, linearly with time. The invading molecules accumulate in a nonequilibrium high-intensity front, representing a transition interface that relaxes with time. Molecular recognition and a transition from arrested to dynamic and swollen condensates during invasion of a binding macromolecule are critical elements for ballistic wave diffusion. This diffusion mechanism could not have been previously identified in classical polymer coacervates, because the molecular recognition event between the invader and its 'receptor' plays a key role in the nonequilibrium process. To verify the general relevance of ballistic wave

diffusion, we also investigate the invasion of m^* into a membraneless sticker-spacer DNA condensate formed by $p(A_{20}-m-XL)_n$, as internally stabilized by palindromic XL domains. Indeed, ballistic wave diffusion also manifests there (Extended Data Figure 2-9). This is an important generalization as such membraneless sticker-spacer condensates are generic models for membraneless organelles formed by RNA or proteins in biology^{15,54,55}. In future, it will be relevant to understand how domain variations, such as extending or reducing the adenine repeats, and modulating thermodynamic binding interactions, by introducing mismatches in the invader sequence or changing its length, influence the diffusion behavior.

Although we show ballistic wave diffusion in purely synthetic DNA model condensates, molecular recognition (*e.g.*, based on protein/RNA binding or coiled-coil interactions) and different dynamic states are ubiquitous in biological coacervates^{2,15,17,20,24,28}. Interestingly, sharp diffusion fronts can be found in biology literature, *e.g.*, for condensates based on coiled-coil interactions²⁸, however, without noticing its importance or physical origin. Notably, our study explains these observations with non-Fickian diffusion concepts, bridging the gap between polymer physics and cellular processes. Although the implications of this observation for biological function and its generalization remain to be elucidated, the significance of ballistic wave diffusion lies in its ability to generate sharper signal fronts with clearer spatial concentration thresholds compared to traditional Fickian diffusion. Moreover, the ballistic wave diffusion mechanism highlights how invading molecules can – in real-time – modify the dynamic properties in condensates. This has important implications for the spatial and temporal sequestration of molecules within cellular condensates and for controlling transport processes at membranes, such as the nuclear pore complex⁷. Besides, it suggests an additional role of condensate dynamics with respect to signal selection and rejection, and for how biology might adaptively regulate these processes through environmental triggers that alter condensate dynamics. As a proof of concept, we encapsulate a ribonuclease⁵⁶ inside our condensates and found that arrested condensates prior invasion reject the substrate from diffusing into the condensate, and the reaction only occurs at the condensate surface. In contrast, dynamic condensates after invasion enable reactions in the entire interior (Extended Data Figure 2-10). This demonstrates a functional gating effect related to the condensate dynamics as regulated by ballistic wave diffusion.

Our study suggests that ballistic wave diffusion can be a potentially important pathway for cells to regulate the dynamics of condensates and prevent them from aging and aggregation, which guarantees their normal functions during cellular processes. This could potentially guide therapeutic solutions for neurodegenerative diseases, which typically involve the liquid-to-solid transition of relevant biomolecular condensates formed by various proteins and nucleic acids, such as hnRNPA1, TDP-43, and FUS^{19,57-59}. One may speculate that because fiber formation was observed to occur at the surface of such condensates⁶⁰, their frontal suppression could make for an effective therapeutic intervention. We anticipate that our study provides a new perspective to comprehend the intricate interplay of dynamics and chemical kinetics in biomolecular condensates and their relation to cellular function.

2.4 Experimental

2.4.1 Materials

Single-stranded DNA (ssDNA) were purchased from Biomers and Integrated DNA Technologies (IDT) (Supplementary Table 2-1). The peptide nucleic acid (PNA) was purchased from PANAGENE. Supplementary Data 2 summarizes all sequences. T4 DNA Ligase (2 U/ μ L), Exonuclease I (40 U/ μ L), Exonuclease III (200 U/ μ L), and Φ_{29} polymerase (10 U/ μ L) were purchased from Lucigen. Thermostable Inorganic Pyrophosphatase (2 U/ μ L), Inorganic pyrophosphatase (0.1 U/ μ L), MluCI (10 U/ μ L), Terminal Transferase (20 U/mL), and nuclease-free water were bought from New England BioLabs (NEB). Deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP) (100 mM), aminoallyl-dUTP-XX-ATTO-488 (1 mM), and aminoallyl-dUTP-XX-ATTO-594 (1 mM) were purchased from Jena Bioscience. Hexadecane, NaCl, $MgCl_2$, Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl, pH=8), Trizma base, acetic acid and Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), were purchased (as bioreagent grade if available) from Sigma-Aldrich. Agarose Low EEO was purchased from AppliChem. RNase Inhibitor (40 U/ μ L), RNase-free TE buffer (Invitrogen, 10 mM Tris and 1 mM EDTA, pH 8.0, 500 mL), SYBR gold, GeneRuler 50 bp DNA Ladder, GeneRuler 1 kb DNA Ladder, Mag-fluo-4 AM, and Duke Standards dry borosilicate glass microspheres ($18.2 \pm 1 \mu m$) were purchased from ThermoFisher Scientific. 384 well high-content imaging glass bottom microplates were purchased from Corning. AFM probes cantilevers (HQ:NSC36/tipless/No Al) with 3 different AFM cantilevers with spring constants between 0.6 N/m and 2.0 N/m were purchased from MikroMasch.

2.4.2 Instruments

Thermal heating ramps were performed on a TPersonal Thermocycler (Analytik Jena). Incubation with shaking was carried out on an Eppendorf ThermoMixer C. DNA concentrations were determined by a ScanDrop (Analytik Jena) and DS-11 Spectrophotometer (DeNovix). Confocal laser scanning microscopy (CLSM) was performed on a Leica Stellaris 5. Fluorescence lifetime imaging microscopy (FLIM) was performed on a Leica Stellaris 8. Fluorescence microscopy experiments for monitoring swelling kinetics of DNA condensates after diluting to lower $[Mg^{2+}]$ were performed on an EVOS M7000 (Invitrogen). Temperature-dependent UV-Vis measurements were performed on Scandrop (Analytik Jena) connecting with a temperature controller (JUMO DTRON 308). Gel electrophoresis was performed in a gel chamber from Biostep. Fluorescence measurements were performed by a plate reader (Tecan). Raman fingerprint measurements were performed by a home-built broadband coherent anti-Stokes Raman scattering (BCARS) microscope (details below). AFM colloid probes were fabricated by attaching glass beads to the tipless AFM cantilevers using a three-axis oil hydraulic micromanipulator (MMO-203 from Narishige) under a bright field optical microscope (Zeiss Axiovert). AFM measurements were performed using a JPK SPM NanoWizard III with a liquid sample cell on top of an inverted microscope stage (Zeiss Axiovert).

2.4.3 Methods

Synthesis of circular ssDNA templates and long ssDNA polymers

The synthesis of the circular DNA template and its corresponding ssDNA polymer is adapted from our previous report with slight modifications (Extended Data Figure 2-1)^{31,33}. The linear ssDNA template and the corresponding ligation strand were firstly mixed at concentration of 1 μ M in 100 μ L TE buffer containing 100 mM NaCl. The solution was heated to 85 $^{\circ}$ C for 5 min before cooling to 25 $^{\circ}$ C with a cooling rate of 0.01 $^{\circ}$ C/s for complete hybridization. Afterwards, 20 μ L of 10 $\times$ Ligase buffer (500 mM Tris-HCl, 100 mM MgCl₂, 50 mM dithiothreitol and 10 mM ATP (Lucigen)), 70 μ L of nuclease-free water and 10 μ L of T4 DNA Ligase (2 U/ μ L (Lucigen)) were introduced into the reaction mixture and gently mixed before leaving at room temperature for 3 h reaction. The reaction mixture was then heated to 70 $^{\circ}$ C for 20 min to deactivate the enzyme. Then, 10 μ L of Exonuclease I (40 U/ μ L (Lucigen)) and 10 μ L of Exonuclease III (200 U/ μ L (Lucigen)) were added into the reaction mixture for further overnight reaction at 37 $^{\circ}$ C to fully remove the ligation strands and any non-circularized templates in solution. Afterwards, the reaction mixture was heated to 80 $^{\circ}$ C for 40 min to deactivate the enzymes. To obtain the final circular ssDNA templates, the reaction mixture was washed by adding 400 μ L TE buffer and filtrated using Amicon ultra-centrifugal filters with a 10 kDa cut-off (Merck Millipore) three times. The concentrations of the collected circular ssDNA templates were measured by the DS-11 Spectrophotometer (DeNovix), and the templates were stored in TE buffer at -20 $^{\circ}$ C.

For the synthesis of the long ssDNA polymers, we used rolling circle amplification (RCA). 5 μ L of circular template (1 μ M in TE buffer) and 1 μ L of exonuclease resistant primer (10 μ M in TE buffer) were mixed with 76 μ L nuclease-free water, 10 μ L of commercial 10 $\times$ polymerase buffer (500 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 40 mM dithiothreitol, 100 mM MgCl₂ (Lucigen)), 2 μ L of Φ ₂₉ DNA polymerase (10 U/ μ L (Lucigen)), 20 μ L of inorganic pyrophosphatase (0.1 U/ μ L (NEB)) and 5 μ L of adjusted deoxyribose nucleoside 5'-triphosphate mix (100 mM, the mix contains pure dATP, dTTP, dCTP, and dGTP solutions mixed in corresponding proportions of the exact composition of the desired ssDNA polymer repeating units (Jena Bioscience)). Note that for the synthesis of ssDNA polymers with in-chain fluorophores of Atto488 and Atto594, *i.e.*, p(A₂₀-mAtto488)_n, p(T₂₀-kAtto488)_n, and p(A₂₀-mAtto594)_n, we replaced 2 mol% of the dTTP in the mix with either aminoallyl-dUTP-XX-ATTO-488 or aminoallyl-dUTP-XX-ATTO-594 for random insertion of the dye along the ssDNA chains during RCA. The reaction mixture was kept at 30 $^{\circ}$ C for 50 h before thermal cleavage at 95 $^{\circ}$ C for 15 min to shorten the ultrahigh molecular weight ssDNA³¹. The final products were purified by rinsing with 400 μ L TE buffer and filtration in Amicon Ultra-centrifugal filters with 30 kDa cut-off (Merck Millipore) three times. The concentrations of the collected final ssDNA polymers were measured using the DS-11 Spectrophotometer (DeNovix), and the DNA polymers (cleaved at 95 $^{\circ}$ C for 15 min) were stored in TE buffer at -20 $^{\circ}$ C.

Preparation of DNA condensates via liquid–liquid phase separation (LLPS)

The preparation of the DNA condensates is adapted from our previous reports with modifications for the formation of larger DNA condensates (Extended Data Figure 2-1)^{31,33}. For core-shell condensates, $p(A_{20-m})_n$ (0.5556 g/L) and $p(T_{20-k})_n$ (0.0694 g/L) were mixed in TE buffer without any salt at a final volume of 9 μ L. The solution mixture was then heated at 95 °C for 15 min (in addition to the first thermal cleavage during ssDNA synthesis mentioned above) for thermal cleavage to further reduce the chain length of the ssDNA polymers. Afterwards, 1 μ L of TE buffer containing 500 mM $MgCl_2$ was introduced into the reaction mixture. The solution containing finally 0.5 g/L $p(A_{20-m})_n$ and 0.0625 g/L $p(T_{20-k})_n$ with 50 mM $MgCl_2$ was heated to 95 °C for 20 min and subsequently cooled down to room temperature using 3 °C/s³¹. Finally, the 10 μ L solution containing the core-shell DNA condensates was diluted 5 times by adding 40 μ L TE buffer containing various amounts of $MgCl_2$ to adjust $[Mg^{2+}]$ from 15 to 50 mM. The obtained 50 μ L DNA condensates solution (as 5 times diluted) has 0.1 g/L $p(A_{20-m})_n$ and 0.0125 g/L $p(T_{20-k})_n$, corresponding to ca. 8 μ M m barcode and ca. 1 μ M k barcode, respectively, in total solution. The solution was then stored in a fridge at 4 °C for 1 week for equilibration. Before usage, the condensate solutions were always slightly vortexed for redispersion. For membraneless condensate formed by $p(A_{20-m-XL})_n$, the same method was used, except the $p(T_{20-k})_n$ was not added in solution. All heating ramps were performed in PCR tubes with negligible evaporation. All imaging was done by CLSM at room temperature after preparation.

The chain lengths of the $p(A_{20-m})_n$ and $p(T_{20-k})_n$ for the DNA condensates were quantified by gel electrophoresis using 2 wt% agarose gel under 5 V/cm for 150 min in TAE buffer containing 40 mM Tris-HCl, 20 mM acetic acid and 1 mM of EDTA (Extended Data Figure 2-1b). Post staining by SYBR gold was applied. Quantification of chain length distribution and average chain length of $p(A_{20-m})_n$ and $p(T_{20-k})_n$ uses a previously reported method^{61,62}.

Ballistic wave diffusion kinetics

19 μ L solution containing k^* -Atto647 (0 to 0.5 μ M), m^* -Atto488 (1 to 5 μ M), and desired concentration of $MgCl_2$ in TE buffer was prepared and homogeneously mixed before the final addition of 1 μ L DNA condensates stock solution (5 times diluted). The DNA condensates were thus 100 times diluted, equaling a content of 0.4 μ M m barcode in the solution. 20 μ L of the mixture was added into a 384 well plate with another 10 to 20 μ L hexadecane on top to prevent evaporation. Typically, DNA condensates with diameters around 15 ± 2 μ m were chosen for the observation and recording of the invasion process. The invasion process was followed by taking time-series CLSM images with identical settings. The displacement of the front was calculated in reference to the center of the condensate. Due to the change of the center plane of the DNA condensates along the z-direction during invasion-induced swelling, the z-focused cross-sectional plane in the CLSM was always slightly adjusted before each image acquisition to record the diffusion in the same two-dimensional plane.

Determination of the interior dynamics of DNA condensates by FRAP

To determine the dynamic properties of the DNA condensates before and after invasion, we used the ROI bleaching method (Figure 2-2d-g, Figure 2-4f, and Extended Data Figure 2-8). DNA condensates composed of $p(A_{20-mAtto594})_n$ were firstly imaged with low laser intensity 5 times (2 s/frame) as pre-bleaching images, before 3 times bleaching in a small circular region of interest (ROI) with diameter of 2 μm by 100 % laser intensity in the 561 nm channel. Then, 150 frames of post-bleaching images were recorded at a rate of 2 s/frame over 300 s. The intensities within the circular ROI (I_{ROI}), and intensities in a circular region of same size away from bleached region within the condensates (I_{ref}), in pre- and post-bleaching images were measured in ImageJ for performing double normalization in bleached regions by:

$$I_{Norm}(t) = \frac{I_{ROI}(t)}{I_{ROI}(t_0)} \times \frac{I_{ref}(t_0)}{I_{ref}(t)} \quad (1)$$

to quantify the recovery kinetics over time. Note that $I(t_0)$ represents the intensity measured in the first image before bleaching. The half recovery times ($t_{1/2}$) from individual experiments were extracted from their corresponding recovery kinetics.

Note that the FRAP protocol is different for the 6000 s FRAP experiment for the confirmation of arrested state of non-invaded DNA condensates at 20 mM $MgCl_2$ (Figure 2-2f). For this experiment, post-bleaching images were manually recorded every several minutes over 6000 s. Before each image acquisition, the focal plane was tuned to the bleached plane of the condensates to make sure the same ROI was monitored, avoiding interference caused by the movement of DNA condensate over the long experimental time. Due to the absence of recovery in this FRAP experiment over 6000 s, we take 6000 s as $t_{1/2}$ for all arrested DNA condensates.

Fluorescence lifetime imaging (FLIM) experiments

To determine the lifetime changes of the in-chain fluorophores along the $p(A_{20-m})_n$ chain during ballistic wave diffusion, we used DNA condensates with $p(A_{20-mAtto488})_n$ chains. We used non-fluorescent m^* as invader to avoid unnecessary interference between dyes. The reaction mixture contained 50 times diluted DNA condensates (0.8 μM m barcode) and 3 μM m^* in TE buffer with 50 mM $MgCl_2$.

FLIM images were recorded (Figure 2-2h-j) based on FALCON-modified time correlated single photon counting (TCSPC) method with the Leica Stellaris 8 FLIM module. The phasor plots were generated by transforming the lifetime determined at each pixel of the FLIM image into a 2D map according to the following equations⁴¹:

$$g_{i,j}(\omega) = \int_0^T I(t) \cdot \cos(n\omega t) dt / \int_0^t I(t) dt \quad (2)$$

$$s_{i,j}(\omega) = \int_0^T I(t) \cdot \sin(n\omega t) dt / \int_0^t I(t) dt \quad (3)$$

where $g_{i,j}(\omega)$ and $s_{i,j}(\omega)$ are the x and y coordinates of the phasor plot, n and ω are the harmonic frequency and the angular frequency of excitation, respectively, and T is the repeat

frequency of the acquisition. Major distributions of phasors were identified by cursors in the phasor plot, and the corresponding lifetimes were assigned into the grey scale images in red and blue colors.

Determination of diffusion coefficients of non-binding short ssDNA in DNA condensates by FRAP

The diffusion coefficients of invader (m^*) within the invaded and non-invaded condensates cannot be directly determined due to the specific binding interactions between m^*/m . Thus, we used a non-binding dummy strand of 20 nucleotides (nt) labeled by Cy5 (Cy5-20nt-dummy). To measure the diffusion coefficients of Cy5-20nt-dummy in DNA condensates before invasion, we prepared a solution containing 100 times diluted $p(A_{20}-m)_n$ DNA condensates and 10 μ M Cy5-20nt-dummy in TE buffer with 50 mM $MgCl_2$. For diffusion coefficients of Cy5-20nt-dummy in DNA condensates after invasion, we prepared a solution containing 100 times diluted $p(A_{20}-m/m^*)_n$ condensates, (complete m^* invasion was reached before the FRAP experiments; shell labeled with k^* -Atto488), and 10 μ M Cy5-20nt-dummy in TE buffer with 50 mM $MgCl_2$.

We used the point-bleaching method (Extended Data Figure 2-4): Several pre-bleaching images were firstly recorded, before bleaching by a 638 nm laser with 100 % intensity at a single pixel for 3 s. Then, post-bleaching images were recorded with maximum frame rates over 1 min. Cross-sectional line profiles crossing the bleached center in post-bleaching images were taken over time, giving Gaussian distributions of the fluorescence intensity. Fitting the line profiles with a Gaussian function yielded a squared standard deviation of Gaussian distribution σ^2 , which changed over time. The diffusion coefficients (D) were then extracted by plotting σ^2 over time with the slope being $2D^{63}$.

Diffusion of non-charged PNA invader into DNA condensates

The diffusion of PNA invaders (m^*_{PNA} -Atto488) was investigated by diluting 1 μ L of the stock solution of non-fluorescent DNA condensates (5 times diluted, in 50 mM $MgCl_2$) in 20 μ L solution containing 5 μ M m^*_{PNA} -Atto488 invader at 50 mM $MgCl_2$ in TE buffer. The diffusion process was recorded with CLSM (Extended Data Figure 2-5b). The DNA condensates were finally 100 times diluted (corresponding to 0.4 μ M m barcode), so that the ratio of Atto488- m^*_{PNA}/m was 12.5/1. FRAP experiments were performed on $p(A_{20}-m_{Atto594})_n$ chains of the fully invaded DNA condensates by PNA invader with the ROI bleaching methods described above (Extended Data Figure 2-5d, e).

Cloud point temperature (T_{cp}) measurement of DNA polymers

0.5 g/L $p(A_{20}-m)_n$ was prepared in 10 μ L TE buffer and heated at 95 $^{\circ}C$ for 15 min. The cleaved $p(A_{20}-m)_n$ was either mixed with stoichiometric amount m^* -Atto488 (PNA or DNA invader), or dissolved alone at a concentration of 0.04 g/L (3.2 μ M) in TE buffer with 50 mM $MgCl_2$. Temperature-dependent UV-Vis spectra were measured from 20 $^{\circ}C$ to 80 $^{\circ}C$ (1 $^{\circ}C$ /min) and the onset of the absorbance increase at 350 nm was used to determine the T_{cp} .

Enzymatic cleavage of $p(A_{20-m})_n$ DNA polymers inside the DNA condensates to reduce the chain length for modulation of the front peak during ballistic wave diffusion

Since the temperature-induced coacervation of $p(A_{20-m})_n$ is a molecular weight-dependent phenomenon, it is not possible to cleave the $p(A_{20-m})_n$ before condensate formation as this could lead to a preferred incorporation of long $p(A_{20-m})_n$, which is unwanted. Therefore, we developed a multistep protocol to cleave the $p(A_{20-m})_n$ chains after DNA condensates formation (Extended Data Figure 2-7). The process uses enzymatic cleavage with a restriction enzyme (MluCI) that recognizes restriction sites generated by hybridization between $p(A_{20-m})_n$ and a specifically designed cleaving strand (c^*). c^* contains a 10 nt sequence that can hybridize to $p(A_{20-m})_n$, generating restriction sites for the MluCI enzyme, and another 5 nt spacer sequence attached with fluorophores (FITC) at the 5' end as reporter for hybridization and subsequent melting events once cleaved. Briefly, c^* hybridizes to A_{20-m} , the MluCI cleaves A_{20-m}/c^* at its restriction site, and then the shorter c^* fragments melt away due to their low T_{ms} .

For this process, the condensates first need to be made dynamic at low $[Mg^{2+}]$ (for the enzyme processing, and also for homogenous mixing with c^* within the condensates) and later made arrested state again at high $[Mg^{2+}]$. To perform enzymatic cleavage of $p(A_{20-m})_n$ chains within DNA condensates, we firstly diluted 10 μ L of stock solution of non-fluorescent DNA condensates (5 times diluted, in 15 mM $MgCl_2$) into 20 μ L solution containing either 0.1 μ M or 0.2 μ M c^* (2.5 or 5 mol% relative to 4 μ M A_{20-m} repeats concentration in the total solution, respectively) at 15 mM $MgCl_2$ in TE buffer. After 3 h incubation at room temperature with gentle shaking (300 rpm) for complete binding of c^* into the DNA condensates, 2 μ L of the reaction mixture was then diluted in 10 μ L solution containing 0.5 U/ μ L MluCI restriction enzyme at 15 mM $MgCl_2$ in TE buffer for subsequent restriction overnight at room temperature. The final enzyme (U) to $p(A_{20-m})_n$ (μ g) ratio was 50 U/ μ g. MluCI recognizes a part of the hybridization domain between c^* and $p(A_{20-m})_n$ and cleaves the $p(A_{20-m}/c^*)_n$ chain at this position. After cleavage, both fragments ($c^{*'} and c^{*''}$) of the restricted c^* only have 5 base pairs with $p(A_{20-m})_n$, and thus dissociate due to their low T_{ms} . This was confirmed by the disappearance of the fluorescence of c^* (-FITC) in the condensates (Extended Data Figure 2-7b, c). The amount of cleaved sites, and hence the statistical shortening of the $p(A_{20-m})_n$ depends on the amount of c^* added. Afterwards, the $[Mg^{2+}]$ of the 10 μ L reaction mixture was further adjusted by adding 0.8 μ L TE buffer containing 500 mM $MgCl_2$ to get a final $[Mg^{2+}]$ of 50 mM. The total 10.8 μ L mixture was then stored for at least one week for equilibration.

To test the front shape of ballistic wave diffusion, the enzymatically cleaved DNA condensates, in equilibrium with $[Mg^{2+}]$, were further diluted into a solution containing 1 μ M m^* -Atto488, 0.25 μ M k^* -Atto647 in TE buffer with a total solution volume of 20 μ L at 50 mM $MgCl_2$ in a 384 well plate with 10 to 20 μ L hexadecane covering it to prevent evaporation. The DNA condensates were thus 100 times diluted with m barcode concentration at 0.4 μ M in the total solution. The invasion process was then recorded by CLSM (Figure 2-4c).

A direct quantification of $p(A_{20-m})_n$ chain length in cleaved DNA condensates by gel electrophoresis is not possible due to the existence of the $p(T_{20-k})_n$ in the system, which can associate with $p(A_{20-m})_n$ and interfere with the mobility of $p(A_{20-m})_n$ chains in gel electrophoresis. Thus, we prepared 20 μL reference solutions containing 0.05 g/L $p(A_{20-m})_n$ (heated previously at 95 °C totally for 30 min, same as the $p(A_{20-m})_n$ used for preparing DNA condensates) with 2.5, and 5 mol% of c^* in TE buffer at 15 mM MgCl_2 incubated at room temperature for 3 h. Subsequently, 5 μL of 10 U/ μL MluCI was added to reach the same enzyme (U) to $p(A_{20-m})_n$ (μg) ratio (50 U/ μg) for overnight cleavage at room temperature. These samples thus had the same c^* to A_{20-m} ratio and the same enzyme to $p(A_{20-m})_n$ ratio as in the condensates. The samples were then characterized by gel electrophoresis (2 wt% agarose gel, 5 V/cm, 150 min in TAE buffer containing 40 mM Tris-HCl, 20 mM acetic acid and 1 mM of EDTA, post staining by SYBR gold) to quantify the cleaved chain length in comparison with the chain length of the non-cleaved $p(A_{20-m})_n$ chains (Extended Data Figure 2-7d–f). The chain length distribution and average chain length of cleaved and non-cleaved $p(A_{20-m})_n$ were calculated from the gel electrophoresis result using a previously reported method^{61,62}.

Determination of the swelling ratio of DNA condensates in different salinity

DNA condensates composed of $p(A_{20-m\text{Atto594}})_n$ and $p(T_{20-k\text{Atto488}})_n$ were prepared at 50 mM MgCl_2 . 1 μL of stock solution of DNA condensates (5 times diluted, in 50 mM MgCl_2) were diluted to lower MgCl_2 concentrations, ranging from 15 mM to 50 mM in TE buffer with a total volume of 20 μL , yielding a m barcode concentration at 0.4 μM in the solution. The sizes of several condensates were recorded every 24 h for a total time of 120 h. The size of each condensate was then normalized to its initial size to determine the final swelling ratio in equilibrium after 120 h (Figure 2-4e).

Tuning the diffusion mechanism of invaders into DNA condensates

DNA condensates composed of $p(A_{20-m\text{Atto594}})_n$ and $p(T_{20-k})_n$ prepared at 50 mM MgCl_2 were diluted by 5 times to individual MgCl_2 concentrations (15 to 50 mM). Diluted DNA condensates were then stored as stock at 4 °C for at least 1 week to reach equilibrium. For experiments with $[\text{MgCl}_2] \geq 20$ mM, 19 μL solution containing $m^*\text{-Atto488}$ (1 μM) in the given MgCl_2 concentration in TE buffer was prepared, followed by the addition of 1 μL DNA condensates stock solution (5 times diluted). The 20 μL solution was then added into a 384 well plate with another 10 to 20 μL hexadecane added on top of the solution to prevent evaporation during experiments. The final DNA condensates were thus 100 times diluted with a m barcode concentration of 0.4 μM in the total solution. The diffusion of $m^*\text{-Atto488}$ into the DNA condensates was then recorded by CLSM (Figure 2-4d). For experiments with MgCl_2 below 20 mM, 1 μL DNA condensate stock solution was firstly diluted to 19 μL TE buffer containing the given MgCl_2 concentration and transferred into a 384 well plate. Then a small amount of concentrated $m^*\text{-Atto488}$ was added into the same well, so that the overall $m^*\text{-Atto488}$ concentration reached to 1 μM in the mixture. 10 to 20 μL hexadecane was added on top to prevent evaporation. DNA condensates, which were far away from the concentrated $m^*\text{-}$

Atto488 spot, were chosen for observation of the rapid diffusion process. This method is necessary to allow enough time for setting up imaging for the rapid diffusion process at low MgCl_2 . Direct dilution of DNA condensates into mixture of invaders resulted in excessively fast completion of the invasion process before transferring and observing under CLSM.

Broadband coherent anti-Stokes Raman scattering microscopy

Raman fingerprint measurements were conducted using a home-built broadband coherent anti-Stokes Raman scattering (BCARS) microscope, extensively described elsewhere⁶⁴. Briefly, pump/probe and Stokes pulses were generated in a dual-output subnanosecond laser source (CARS-SM-30, Leukos), spatially and temporally overlapped at the sample plane of an inverted microscope (Eclipse Ti-U, Nikon). These pulses were then tightly focused onto a sample using a 0.85 NA air objective (LCPlan N, Olympus). The samples were prepared by adding 1 μL condensates solution (5 times diluted, at individual MgCl_2 concentrations) into 19 μL TE buffer with same concentration of MgCl_2 . The 20 μL samples were placed within a sealed channel created by attaching double-sided tape strips between a glass coverslip and a glass slide and positioned with the cover slip facing the objective. The BCARS signal was filtered from the excitation pulses and focused onto the slit of a spectrograph (Shamrock 303i, Andor), which disperses the spectral components on a cooled CCD camera (Newport DU920P-BR-DD, Andor). A piezo stage (Nano-PDQ 375 HS, Mad City Labs) was employed to raster scan the samples. Data acquisition was controlled using LabView 2015 (National Instruments) software, and its processing was carried out using IgorPro (WaveMetrics). The Raman-like spectra were obtained through a modified Kramers-Kronig transform⁶⁵, and background phase removal was performed using a Savitzky-Golay filter with a 3rd order polynomial along a B-spline baseline.

DNAzyme-catalyzed RNA cleavage in DNA condensate

DNA condensates containing 80 % m barcode and 20 % q barcode (obtained by using a mixture of $p(\text{A}_{20}\text{-m})_n$ and $p(\text{A}_{20}\text{-q})_n$ during condensate formation) were used for DNAzyme⁵⁶ encapsulation by q/q* hybridization. 5 μL DNA condensates (1.6 μM q barcode, 6.4 μM m barcode) were mixed with 1 μL q*-DNAzyme (8 μM) and 2 μL m* (50 μM) into total 12 μL TE buffer containing 50 mM MgCl_2 for 1 h for complete DNAzyme encapsulation and invasion-induced arrested-to-dynamic transition of DNA condensates. For arrested DNA condensates loaded with DNAzyme, the same solution was prepared except m* was not added. A substrate solution was prepared by mixing 2 μL p-A₁₀₀₀ (2 μM , synthesized by Terminal deoxynucleotidyl Transferase (TdT)³¹), 2 μL p*-Substrate (400 nM), and 1 μL RNase Inhibitor (40 U/ μL (Thermo Scientific)) into total 8 μL TE buffer containing 50 mM MgCl_2 for 1 h for complete hybridization between p-A₁₀₀₀ and p*-Substrate. Then, the substrate solution was introduced into the solution containing arrested or dynamic DNAzyme-loaded DNA condensates. The reaction was monitored under CLSM (Extended Data Figure 2-10).

Atomic force microscopy measurements

To perform force spectroscopy on condensates, we added condensates (before or after invasion, in TE buffer containing 50 mM MgCl₂) into an AFM liquid cell on a glass slide on top of the stage of the inverted microscope. The AFM colloid probe (borosilicate glass bead diameter = 18.2 ± 1 μm , spring constant = 0.6 N/m) was then approached to the top of a condensate under the microscope. The force spectroscopy measurement was then conducted in contact mode. For each condensate type, we measured over 5 force spectroscopy curves in compression-retraction cycle, the hysteresis is quantified by integrating the area within compression-retraction curves.

Statistical analysis

All data points featuring statistical analysis are presented as mean $\pm$ standard deviation or mean $\pm$ standard error. The sample size (N) for each statistical analysis is reported in the relevant figure legend. FRAP, FLIM, and AFM measurements were performed on several condensates from 2 independent samples. Diffusion mechanism tuned by chain length and salinity were measured on several condensates from 2 independent samples. BCARS measurements were performed on several condensates from 3 independent samples. Titration and swelling experiments were carried out on several condensates from a series of samples at each condition. Diffusion kinetics were measured from 4 independent samples at each condition. P values are calculated by One-way ANOVA test. Statistical analysis was carried out using Origin and Microsoft Excel.

Code availability

The code for reaction-diffusion simulation and oxDNA MD simulation is available via GitHub at <https://github.com/WaltherLab/reaction-diffusion-in-biomolecular-condensates>.

2.4.4 Supplementary Tables

	Name	Sequence (5' → 3')	Purification	Modification	Supplier
Template and RCA	Tp(A ₂₀ -m)	/Phos/ATC TAT CCT AAT TTT TTT TTT TTT TTT TTT TGA ACC CGT AT	HPLC	5'-Phosphorylation	Biomers
	Tp(T ₂₀ -k)	/Phos/ATC CTC TAA AAT CAA AAA AAA AAA AAA AAA AAA AAA GTA AAA CCA CAC G	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -q)	/Phos/TAC CTC AAT GCT TTT TTT TTT TTT TTT TTT TTT TTT GGA GCA ACA A	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -m-XL)	/phos/TAT ATC TAT CCT AAT TTT TTT TTT TTT TTT TTT TTT TGC TAG CGA ACC CG	HPLC	5'-Phosphorylation	Biomers
	ligation-m	TTA GGA TAG ATA TAC GGG TTC	HPLC	None	Biomers
	ligation-k	TTT TAG AGG ATC GTG TGG TTT T	HPLC	None	Biomers
	Ligation-q	GCA TTG AGG TAT TGT TGC TCC CA	HPLC	None	Biomers
	ligation-m-XL	GGA TAG ATA TAC GGG TTC GCT	HPLC	None	Biomers
	primer-m	TTA GGA TAG ATA TAC GGG T*T*C	Desalting	Phosphorothioate d twice	IDT
	primer-k	TTT TAG AGG ATC GTG TGG TT*T*T	Desalting	Phosphorothioate d twice	IDT
	primer-q	GCA TTG AGG TAT TGT TGC TCC*C*A	Desalting	Phosphorothioate d twice	IDT
	primer-m-XL	GGA TAG ATA TAC GGG TTC G*C*T	Desalting	Phosphorothioate d twice	IDT
Polymer	p(A ₂₀ -m) _n	p(AAA AAA AAA AAA AAA AAA AAT TAG GAT AGA TAT ACG GGT TC) ₇₄	Desalting	74 repeating units on average	
	p(T ₂₀ -k) _n	p(TTT TTT TTT TTT TTT TTT TTG ATT TTA GAG GAT CGT GTG GTT TTAC) _{>90}	Desalting	over 90 repeating units on average	
Barcode*	m*-Atto488	/ATTO488/ TGA ACC CGT ATA TCT ATC CTA A	HPLC	5'-Atto488	Biomers
	n*-Atto647	/Atto647N/AAA ACC ACA CGA TCC TCT AAA A	HPLC	5'-Atto647N	Biomers
	m*	TGA ACC CGT ATA TCT ATC CTA A	HPLC	None	Biomers
DNAzyme	p	TAA TAC GAC TCA CTA TAG GGA T	HPLC	None	Biomers
	q*-DNAzyme	TGG GAG CAA CAA TAC CTC AAT GCA AAG AGT CAT GAG GCT AGC TAC AAC GAG GTT AGG TC	HPLC	None	Biomers
	p*-Substrate (RNA)	AUC CCU AUA GUG AGU CGU AUU A/BMN-Q1/G ACC UAA CCA UCA UGA/6FAM/	HPLC	Internal BMN-Q1 3'-6FAM	Biomers
Others	Cy5-20nt-dummy	/Cy5/TCG TCT ACT AGC TCC AAC CT	HPLC	5'-Cy5	IDT
	c*	/FITC/AAA AAA TCC TAA TTT	HPLC	5'-FITC	Biomers
	m* _{PNA} -Atto488	/ATTO488/ TGA ACC CGT ATA TCT ATC CTA A	HPLC	5'-Atto488	PANAGENE

Supplementary Table 2-1. Oligomers used, with their names, sequences, purification methods, modifications, and suppliers.

Chapter 2. Ballistic Diffusion Fronts in Biomolecular Condensates

Parameter	Value	Unit	From
k_{bind}	100	$\text{m}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$	Manual optimization
k_{swell}	Varied from 0.001 – 0.1	s^{-1}	Manual optimization
D_0	$5 \cdot 10^{-12}$	$\text{m}^2 \cdot \text{s}^{-1}$	Estimated from experiments (Extended Data Figure 2-4)
D_1	$5 \cdot 10^{-9}$	$\text{m}^2 \cdot \text{s}^{-1}$	Estimated from experiments (Extended Data Figure 2-4) + manual optimization
$D_{\text{cons.}}$	$5 \cdot 10^{-11}$	$\text{m}^2 \cdot \text{s}^{-1}$	Between D_0 and D_1 + manual optimization
$[\text{m}^*]_{\text{i}}$	Varied from 0.0002 – 0.1	mM	Input
$[\text{m}]_{\text{i}}$	1	mM	Estimated from experiments (Supplementary Figure 2-2)
$[\text{M}_{\text{swollen}}]_{\text{thres}}$	0.24	mM	Manual optimization
f_{swell}	4	None	Estimated from experiments (Figure 2-1e)

Supplementary Table 2-2. General parameters used in the reaction-diffusion simulations.

Figures	k_{bind} ($\text{m}^3 \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$)	k_{swell} (s^{-1})	D_0 ($\text{m}^2 \cdot \text{s}^{-1}$)	D_1 ($\text{m}^2 \cdot \text{s}^{-1}$)	$[\text{m}^*]_{\text{i}}$ (mM)	$[\text{m}]_{\text{i}}$ (mM)	$[\text{M}_{\text{swollen}}]_{\text{thres}}$ (mM)	f_{swell} (/)
Figure 2-3b	100	0.004	$5 \cdot 10^{-12}$	$5 \cdot 10^{-9}$	0.001	1	0.24	4
Figure 2-3c	100	0.1	$5 \cdot 10^{-12}$	$5 \cdot 10^{-9}$	0.001	1	0.24	4
Figure 2-3d	100	0.1	$5 \cdot 10^{-11}$	$5 \cdot 10^{-11}$	0.001	1	0.24	4
Figure 2-4a, b	100	0.001 - 0.1	$5 \cdot 10^{-12}$	$5 \cdot 10^{-9}$	0.001	1	0.24	4
Figure 2-4j, l	100	0.004	$5 \cdot 10^{-12}$	$5 \cdot 10^{-9}$	0.0002 - 0.01	1	0.24	4
Extended Data Figure 2-6b	100	0.004	10^{-13} - 10^{-8}	10^{-13} - 10^{-8}	0.001	1	0.24	4
Extended Data Figure 2-6c-e	100	0.004	$5 \cdot 10^{-12}$	$5 \cdot 10^{-9}$	0.001 - 0.1	1	0.24	4
Supplementary Figure 2-3	100	0.001	$5 \cdot 10^{-12}$	$5 \cdot 10^{-9}$	0.001	1	0.24	4

Supplementary Table 2-3. Specific parameter sets for reaction-diffusion simulation results in different figures.

2.4.5 Supplementary Notes

Supplementary Note 2-1. Molecular Dynamics (MD) simulations using oxDNA

Theoretical predictions for the change in rigidity of $p(A_{20-m})_n$ upon hybridization with invader strands m^* and corresponding expansion were calculated on the basis of the oxDNA model^{34,66,67}. End-to-end distances were calculated for (i) a ssDNA $p(A_{20-m})_n$ with $n = 25$ repetition units and (ii) its partly hybridized dsDNA $p(A_{20-m}/m^*)_n$ counterpart hybridized with 25 invader strands m^* . Molecular dynamics (MD) simulations were performed with the standalone version of oxDNA (v3.6.0) using oxDNA2 interactions with sequence-dependent parameters as provided in the standalone version. Accounting for sequence dependency is advisable to study the mechanics of our system because poly(A) has been found to be exceptionally straight and stiff⁶⁸. Results were analyzed with oxDNA analysis tools included in the standalone version, and with a custom-made ready-to-use tool that determines radii of gyration (R_g) from oxDNA data.

1. Simulation Setup

All simulations were performed within the canonical ensemble with temperature $T = 20$ °C. A Bussi-Donadio-Parrinello thermostat was used during the relaxation period and a Johnson thermostat during data collection, with a diffusion coefficient of 2.5 simulation unit (s.u.) and 103 Newtonian steps (steps during which the system evolves according to Newton's equations of motions, before a random subset of particle momenta is sampled from a Maxwell distribution for thermostating). The skin parameter for Verlet lists was set to 0.1. Interactions were always calculated for oxDNA interaction type DNA2 using sequence-dependent parameters provided by the oxDNA standalone version.

Since Mg^{2+} is not included in the oxDNA model, we adjusted ionic strength by setting $[Na^+] = 1$ M as recommended by Doye et al.⁶⁹ for establishing representative conditions for DNA systems operating at $[Mg^{2+}] = 12.5$ mM. Using this value is further justified by the fact that, while the related quantity of persistence length depends on salt concentrations, $[Na^+] = 1$ M fits very well $[Mg^{2+}] = 50$ mM in the case of a 1201 bp dsDNA⁴⁷.

1.1 Initialization

Initial configurations were generated using the tool `generate-sa.py` included in the standalone version of oxDNA with box lengths set to $L = 500$ s.u.. The initial configuration of the hybridized dsDNA was derived from the one of a hypothetical full dsDNA by manual deletion of T_{20} parts that would be complementary to the unhybridized A_{20} parts of the dsDNA.

1.2 Relaxation

To relax the initial configurations, we followed the Rovigatti protocol⁷⁰: As first stage, a short MC run (10^3 steps) with limited backbone forces was performed to account for the possibility of an unphysically stressed initial configuration. As second stage, an MD simulation with limited backbone forces (3.1×10^7 steps, $\Delta t = 10^{-3}$ s.u.) followed up. In the case of ssDNA, the

simulation box lengths had to be reduced to $L = 200$ s.u. after 10^6 steps, because the maximum number of particles per cell grew too large due to the pronounced contraction of ssDNA compared to the initial configuration. We added another MD simulation with unlimited backbone interactions (4×10^7 steps, $\Delta t = 10^{-3}$ s.u.) as third stage for relaxation until changes in both total and hydrogen-bond energies had reduced to thermal fluctuations.

1.3 Data collection

MD simulations were performed on a GPU for 2×10^9 steps with a step size of $\Delta t = 5 \times 10^{-3}$ s.u.. Configurations and energies were evaluated every 10^5 steps, giving a sample size of $N = 2 \times 10^4$ configurations for each model DNA. On an NVIDIA GeForce RTX 3060 Ti, this took about 2.8 days in the case of ssDNA and 3.3 days in the case of our hybridized dsDNA (wall time).

2. Data analysis

2.1 Analysis tool for gyration analysis

A command line tool was implemented in Python 3 to calculate radii of gyration, R_g , from oxDNA topology and trajectory files. It is available via GitHub repository (<https://github.com/WaltherLab/reaction-diffusion-in-biomolecular-condensates>) as `gyration.py` and can readily be used on any data from oxDNA simulations (provided in the “traditional” oxDNA format).

2.2 Calculations

The mean squared radius of gyration $\langle R_g^2 \rangle$ were calculated both in a sequence-averaged and a sequence-dependent way, thereby treating the analyzed nucleotides as monomeric units in a homopolymer or a heteropolymer, respectively. For a homopolymer with N monomeric units at positions r_i , the root mean square radius of gyration was calculated as

$$\langle R_g^2 \rangle^{1/2} = \sqrt{\left\langle \frac{1}{N} \sum_{i=1}^N (r_i - r)^2 \right\rangle},$$

where $\langle \rangle$ is the ensemble average and $r = \frac{1}{N} \sum_{i=1}^N r_i$ is the mean particle position. In this case, $\langle R_g^2 \rangle$ and mean square end-to-end distance ($\langle h^2 \rangle$) are related via $\langle R_g^2 \rangle = \langle h^2 \rangle / 6$ relationship⁷². Thus, the root mean square end-to-end distance ($\langle h^2 \rangle^{1/2}$) is calculated, too. For a heteropolymer, the root mean square radius of gyration was calculated as

$$\langle R_g^2 \rangle^{1/2} = \sqrt{\left\langle \frac{1}{M} \sum_{i=1}^N m_i s_i^2 \right\rangle},$$

where, for a certain nucleotide i , m_i is its mass, $M = \sum_{j=1}^N m_j$ the total mass of all nucleotides, and $s_i = \left| r_i - \frac{1}{M} \sum_{j=1}^N m_j r_j \right|$ is its distance from the common center of mass⁷³.

Supplementary Note 2-2. Numerical model for the simulation

The reaction-diffusion model implements two steps, i.e., binding (R1) and swelling (R2), and is based on the following stoichiometric and rate equations:



$$r_1 = k_{\text{bind}}[m^*][m] \quad (1)$$



$$r_2 = k_{\text{swell}}[M_{\text{arrested}}], \quad (2)$$

Where m^* is the invader strand diffusing from outside, m is the ssDNA barcode inside the condensate, M_{arrested} and M_{swollen} are the hybridized dsDNA forms of m/m^* in the arrested state and in the dynamic state, respectively. The f_{swell} stoichiometric coefficient in (R2) mimics the volume expansion. Molar concentrations are indicated by $[]$, k_{bind} and k_{swell} are reaction rate coefficients.

The spatiotemporal behavior of the condensate is described by following set of equations in 1D space with the spatial coordinates $x \in \{0, \dots, L\}$, where $x = 0$ corresponds to the condensate/outer medium interface and $x = L$ is the center of the condensate (Extended Data Figure 2-6a):

$$\partial_t[m^*] = -k_{\text{bind}}[m^*][m] + D_{m^*}\Delta[m^*] \quad (3)$$

$$\partial_t[m] = -k_{\text{bind}}[m^*][m] + D_m\Delta[m] \quad (4)$$

$$\partial_t[M_{\text{arrested}}] = k_{\text{bind}}[m^*][m] - f_{\text{swell}} k_{\text{swell}}[M_{\text{arrested}}] + D_{M_{\text{arrested}}}\Delta[M_{\text{arrested}}] \quad (5)$$

$$\partial_t[M_{\text{swollen}}] = k_{\text{swell}}[M_{\text{arrested}}] + D_{M_{\text{swollen}}}\Delta[M_{\text{swollen}}] \quad (6)$$

In our numerical model, for simplicity, m^* is considered to be the only diffusively transported component with the diffusion coefficient D_{m^*} , whereas $D_m = D_{M_{\text{arrested}}} = D_{M_{\text{swollen}}} = 0$.

At the definition of D_{m^*} we apply a deviation from the classical Fick's second law: D_{m^*} depends on the local state (arrested or swollen) of the condensate. The state is defined by comparing the local $[M_{\text{swollen}}]$ with a threshold value $[M_{\text{swollen}}]^{\text{thres}}$.

$$D_{m^*} = \begin{cases} D_0, & \text{if } [M_{\text{swollen}}] < [M_{\text{swollen}}]^{\text{thres}} & \text{arrested state} \\ D_1, & \text{if } [M_{\text{swollen}}] \geq [M_{\text{swollen}}]^{\text{thres}} & \text{dynamic state.} \end{cases} \quad (7)$$

In simple terms, this governs whether m^* diffuses quickly in already dynamic/invaded parts, or whether it diffuses slowly in arrested/non-invaded parts. Dirichlet boundary conditions are applied at the condensate surface, i.e., $[m^*]_{x=0} = [m^*]_i$, and no-flux boundary conditions at the inner side of the condensate, i.e., $\partial_x[m^*]_{x=L} = 0$. The initial conditions are $[m^*]_{t=0} = 0$, $[m]_{t=0} = [m]_i$, and $[M_{\text{arrested}}]_{t=0} = [M_{\text{swollen}}]_{t=0} = 0$ at $x = 1, \dots, L$. Here $[]$ is the space-

and time-dependent concentration in the condensate, ∂_t is the time derivative, and $\Delta = \frac{\partial^2}{\partial x^2}$ is the Laplace operator. The parameter values are listed in Supplementary Table 2-2 and Supplementary Table 2-3.

The partial differential equations were discretized with standard second order finite difference scheme, and the resulting systems were solved by the SUNDIALS CVODE solver using backward differentiation formula method⁷¹. In Figure 2-3b, the mesh size was 400, the grid spacing was $\Delta x = 0.05 \mu\text{m}$, the time step was $\Delta t = 0.001 \text{ s}$, the absolute and relative error tolerances were 10^{-10} and 10^{-12} , respectively. In Figure 2-4a, b the mesh size was 7000, the grid spacing was $\Delta x = 0.005 \mu\text{m}$, the time step was $\Delta t = 0.005 \text{ s}$, the absolute and relative error tolerances were 10^{-6} and 10^{-12} , respectively. In Figure 2-4j, l the mesh size was 75000, the grid spacing was $\Delta x = 0.006 \mu\text{m}$, the time step was $\Delta t = 0.01 \text{ s}$, the absolute and relative error tolerances were 10^{-5} and 10^{-8} , respectively. In Extended Data Figure 2-7c–e, the mesh size was 7000, the grid spacing was $\Delta x = 0.005 \mu\text{m}$, the time step was $\Delta t = 0.005 \text{ s}$, the absolute and relative error tolerances were 10^{-6} and 10^{-12} , respectively.

The front position $x_f(t)$ is determined as the x , where the $I_t(x) = [m^*]_t(x) + [M_{\text{arrested}}]_t(x) + [M_{\text{swollen}}]_t(x)$ curve has its maximum. It is in accordance with the experiments, where these species correspond to the detectable fluorescence. The front velocity (v) is determined by linear fitting of the $x_f(t)$ data:

$$x_f(t) = v_0 + vt, \quad (8)$$

where $v_0 = 0$. The width of the front (W) is the full width at half maximum (FWHM) of the $I_t(x)$ curve. The half maximum value is defined as $I_{t,\text{HM}} = \frac{I_t(x_f) + I_t(x_0)}{2}$, where $I_t(x_0)$ indicates the fluorescence at the equilibrium swollen region backwards the front (Figure 2-4h).

The simulation code is available at <https://github.com/duzsbrigitta/Reaction-Diffusion-in-Condensates>.

Supplementary Note 2-3. Melting temperature estimation

All melting temperatures (T_{ms}) were estimated by OligoAnalyzer Tool (IDT) with experimental conditions as input for oligonucleotide concentration and salinity.

The m barcode concentration inside DNA condensates was determined in Supplementary Figure 2-2 and used for the determination of important T_{ms} as follows: For T_{m} of m/m^* (71.5 °C), we applied 200 μM (note that there is swelling induced by m/m^* hybridization) as the oligonucleotide concentration, and 50 mM Mg^{2+} for salinity.

For melting points of $c^*/p(\text{A}_{20}\text{-}m)_n$ (40.2 – 44.4 °C, before cleavage), $c^*/p(\text{A}_{20}\text{-}m)_n$ (0 °C, after cleavage), and $c^{**}/p(\text{A}_{20}\text{-}m)_n$ (3.4 – 11.6 °C, after cleavage), we applied 10, 20, and 40 μM as the oligonucleotide concentration. Since the DNA condensates swell by ca. 2.2-fold in volume at 15 mM MgCl_2 (Figure 2-4e), the m barcode concentration in the condensates is roughly 400

μM . The applied concentrations correspond to experimentally used 2.5, 5, 10 mol% c^* relative to m-barcode concentration in the condensates. We apply 15 mM Mg^{2+} for salinity.

Supplementary Note 2-4. Finite element simulation with COMSOL

We used COMSOL Multiphysics 5.6 to model ballistic wave diffusion in different dimensions. We combined two modules in COMSOL, i.e., Chemistry and Transport of Diluted Species for simulations. We used the same input as our numerical model and the inputs are summarized in Supplementary Table 2-3. We used here Heaviside function for the change of diffusion coefficients at different regions for consistency. The geometry is a rectangle ($1 \times 20 \mu\text{m}^2$) for 1D simulation, a circle (radius = $20 \mu\text{m}$) for 2D simulation, and a sphere (radius = $20 \mu\text{m}$) for 3D simulation. For 1D simulation, we applied Dirichlet boundary conditions with $[m^*] = 1$ at the left side of the rectangle, while for 2D and 3D simulation we applied Dirichlet boundary conditions with $[m^*] = 1$ at the circumference of the circle and surface of the sphere to induce invasion process.

Supplementary Note 2-5. Determination of m barcode concentration inside DNA condensates

We used a titration method to measure the concentration of the m barcode in the $p(\text{A}_{20}\text{-m})_n$ DNA condensates (Supplementary Figure 2-2). Non-fluorescent DNA condensates at 50 mM MgCl_2 were 100 times diluted ($0.4 \mu\text{M}$ barcode in total solution) in a $20 \mu\text{L}$ solution containing different concentrations of $m^*\text{-Atto488}$ ranging from 50 to $400 \mu\text{M}$ at 50 mM MgCl_2 in TE buffer. We confirmed complete invasion and swelling of the $p(\text{A}_{20}\text{-m})_n$ DNA condensates before taking CLSM images for the quantification of fluorescence intensity inside (I_{in}) and outside (I_{out}) of the condensates. The corresponding intensity ratio ($R = I_{\text{in}}/I_{\text{out}}$) was plotted against the $m^*\text{-Atto488}$ concentration and fitted to extract the m barcode concentration inside dynamic/invaded DNA condensate. By knowing the swelling during invasion, it is possible to calculate the original m barcode concentration in the pristine condensates.

Supplementary Note 2-6. Determination of Mg^{2+} concentration inside and outside DNA condensates

We used the Mg^{2+} indicator Mag-fluo-4 AM to determine the Mg^{2+} concentrations inside and outside the condensates before and after invasion. First, we calibrated the Mg^{2+} indicator by preparing a series of $10 \mu\text{M}$ Mag-fluo-4 AM solutions in PBS buffer with varying Mg^{2+} concentrations, confirming that fluorescence intensity increases with the Mg^{2+} concentration. Next, we verified that the presence of $p(\text{A}_{20}\text{-m})_n$, $p(\text{T}_{20}\text{-k})_n$, and m^* does not significantly affect the fluorescence intensity of $10 \mu\text{M}$ Mag-fluo-4 AM at 2 mM Mg^{2+} , which is the relevant Mg^{2+} concentration range for our experiments (measured in dilution from an original condensate). For the condensate experiments, we prepared triplicate samples of condensates at 50 mM

Mg²⁺ before and after invasion. Each sample was centrifuged, and 1 μ L of both the supernatant and sediment (=condensate) was collected and diluted into 20 μ L of PBS buffer containing 10 μ M Mag-fluo-4 AM. The fluorescence intensities of these samples were measured with a plate reader. Excitation = 485 nm, emission = 525 nm, band width = 15 nm.

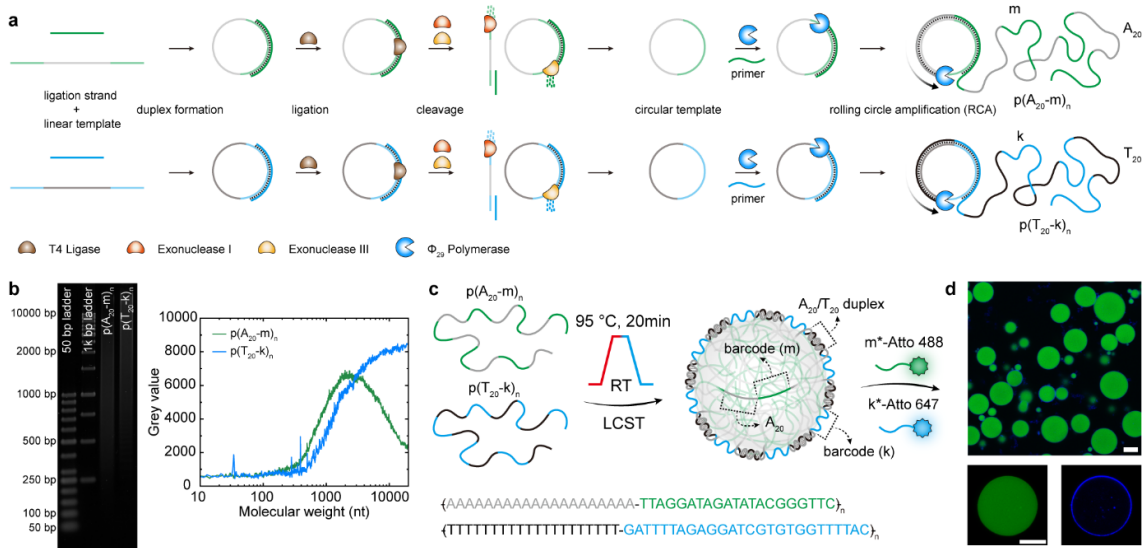
Supplementary Note 2-7. Parameter sweeps in simulations to derive the influence of differences in diffusion coefficients on the appearance of ballistic wave diffusion

Extended Data Figure 2-6b shows extended parameter sweep for different diffusion coefficients in the arrested/dense interior (D_0) versus the dynamic/swollen exterior (D_1). The data helps to demonstrate that the occurrence of the ballistic diffusion front, *i.e.*, (1) sharp diffusion front and (2) linear front propagation kinetics, follows a gradual transition from Fickian diffusion based on the differences between D_0 and D_1 (See from (v) to (i)). Once the difference exceeds two orders of magnitude, the front propagation follows linear kinetics in certain length (tens of micrometers) and time scales (hundreds of minutes). In the transition period, where D_0 and D_1 only have a small difference, at long time and length scales, there will be a transition from ballistic diffusion to Fickian diffusion. This aligns with case II diffusion for solvent penetration into plastics³⁸. Compare the two top lines in b (i) – (vi) – (viii) (and – (x) for very long-term simulations) versus (ii) – (vii) – (ix). Ultimately, the difference between D_0 and D_1 will determine the length and time scales of the ballistic diffusion front. In the context of biomolecular condensates, whose size are 1 ~ 10 μ m, short length and time scales dominate, which therefore locates them in the regime with ballistic diffusion front.

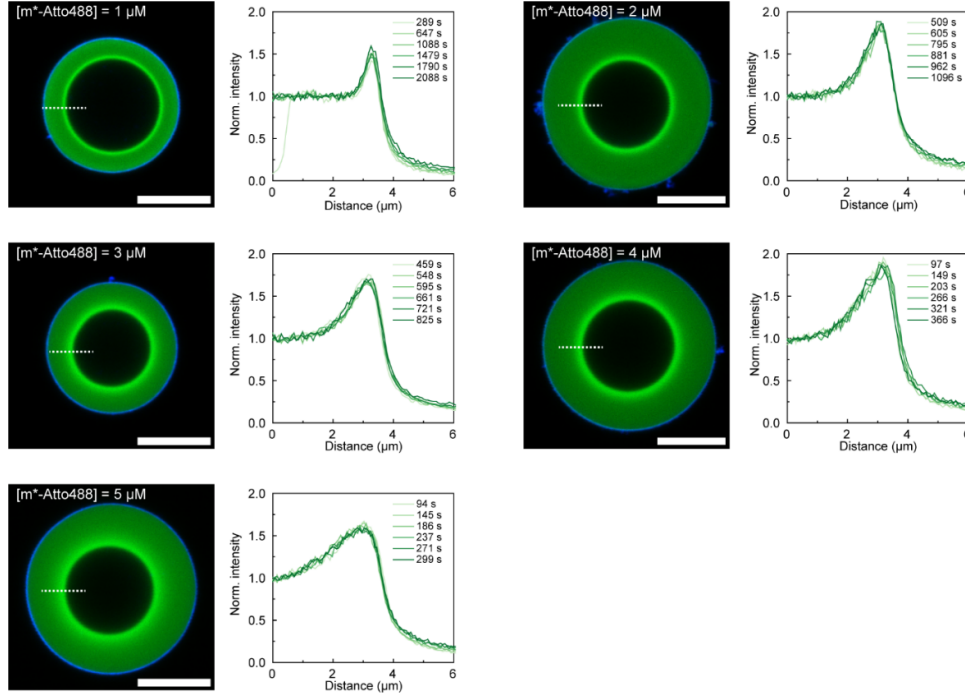
Besides, since our model uses fast binding kinetics of the invader m^* to the core $p(A_{20}-m)_n$ and a slow relaxation kinetics, there can even be a front accumulation in case of Fickian diffusion with a mild D_0 and D_1 difference (see beginning of (iii), (iv), and (v)). Critically, for all Fickian diffusion cases, the accumulation at the front vanishes over time, while the ballistic wave diffusion can maintain the front accumulation with a stable high intensity front controlled by balanced binding and relaxation kinetics.

To summarize, it is the difference between D_0 and D_1 that defines the ballistic wave diffusion in time and length scales, while the interplay between binding and relaxation kinetics determines the front accumulation.

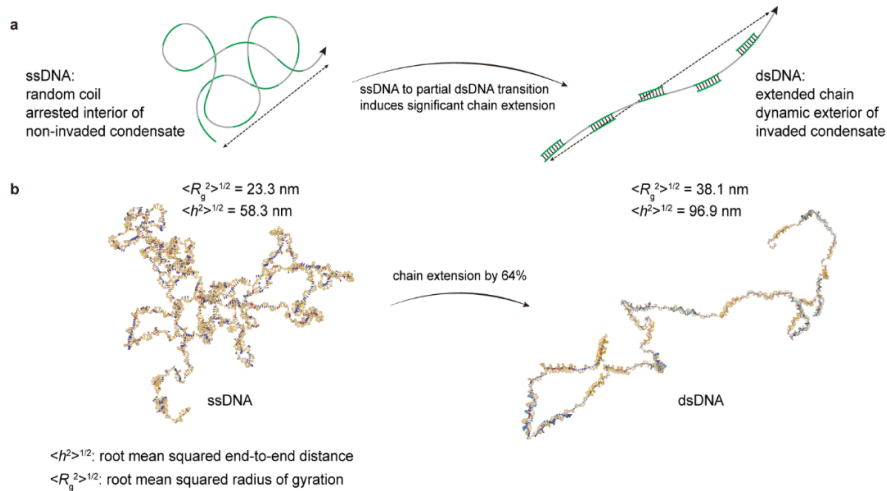
2.4.6 Extended Data Figures and Supplementary Figures



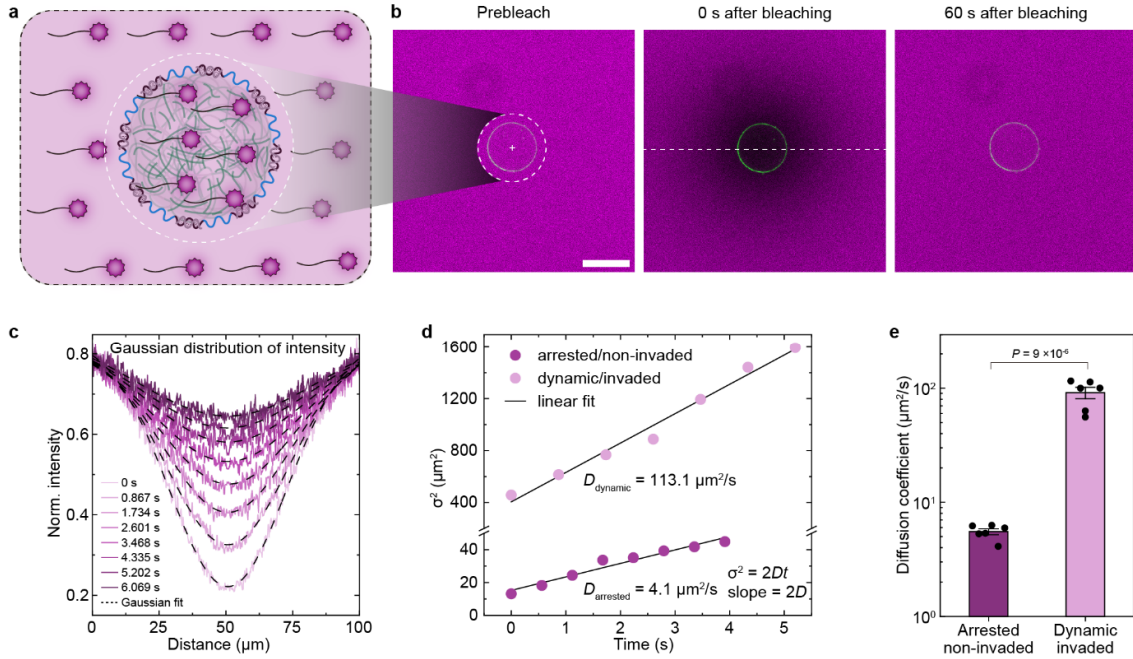
Extended Data Figure 2-1. Synthesis of ssDNA polymers and preparation of DNA condensates. **a**, Schematic representation for the enzymatic synthesis of circular template by T4 Ligase and subsequent removal of non-circular strands by Exonuclease I and Exonuclease III. The circular template is then used for the enzymatic synthesis of DNA polymers $p(A_{20}-m)_n$ and $p(T_{20}-k)_n$ by rolling circle amplification (RCA) using Φ_{29} DNA polymerase and a primer. **b**, Background-subtracted gel electrophoresis image of $p(A_{20}-m)_n$ and $p(T_{20}-k)_n$ after thermal cleavage at 95 °C for 30 min in total. Quantification of chain length of $p(A_{20}-m)_n$ and $p(T_{20}-k)_n$ is shown in the right plot. The molecular weight is reported as nucleotide number (nt), corresponding to double of the calibrated base pair number (bp). Average repeats of $p(A_{20}-m)_{24}$ can be calculated. $p(T_{20}-k)_n$ has a tailing to higher molecular weights ($n > 90$). This material is used for preparing the DNA condensates. **c**, Scheme for the preparation of DNA condensates by a heating ramp applied to a solution of 0.5 g/L of $p(A_{20}-m)_n$, and 0.0625 g/L $p(T_{20}-k)_n$ incubated in TE buffer containing 50 mM $MgCl_2$ at 95 °C for 20 min, followed by cooling to room temperature. The heating and cooling rate are both 3 °C/s. The formed condensates are composed of a $p(A_{20}-m)_n$ core and a $p(T_{20}-k)_n$ shell, that can be individually addressed by the complementary strands (m^* and k^*) conjugated with fluorophores. Details can be found in our preceding publications^{31, 33}. **d**, Representative CLSM image showing the resulting DNA condensates with the bottom images showing the respectively addressed $p(A_{20}-m)_n$ core by m^* -Atto488 (left, green channel) and $p(T_{20}-k)_n$ shell by k^* -Atto647 (right, blue channel). Note the thin blue shell. The scale bars are all 10 μ m.



Extended Data Figure 2-2. Stable diffusion fronts at individual concentrations of invader m^* . Representative CLSM images showing ballistic wave diffusion at different invader concentrations ($[m^*\text{-Atto488}]$) at 50 mM MgCl_2 . Time-dependent cross-sectional line profiles along the white dashed lines in each CLSM image are shown to the right of the corresponding CLSM images. The line profiles are rescaled on the distance axis to the maximum intensity of the front. As can be seen from the line profile analysis, the front is self-similar and always has a stable shape over time, indicating balanced kinetics at the front. 0.25 μM $k^*\text{-Atto647}$ was added for the labeling of the shell (blue channel). Scale bars for all are 10 μm .

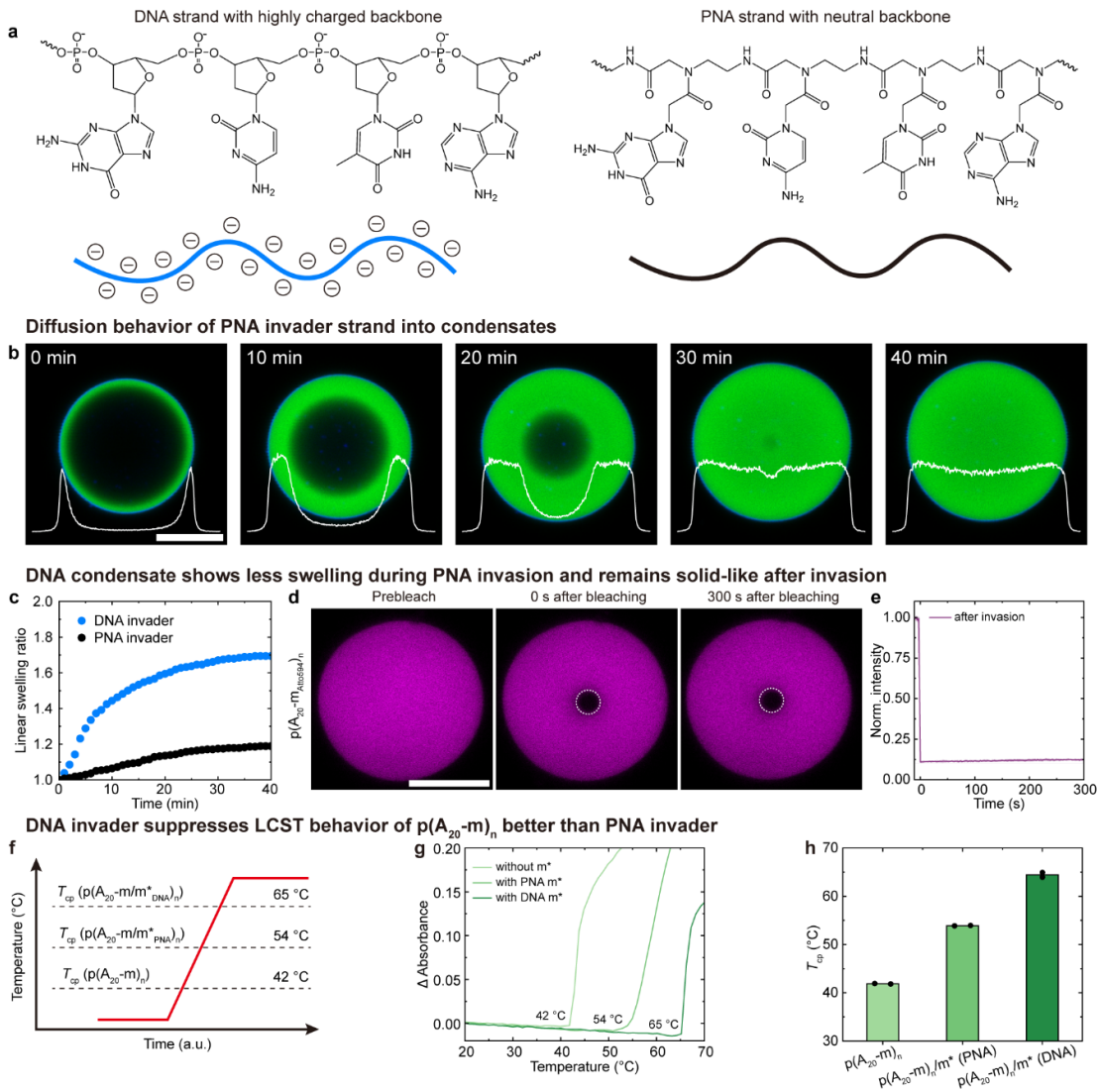


Extended Data Figure 2-3. Molecular Dynamics (MD) simulations of the DNA chain extension induced by ssDNA to partial dsDNA transition upon hybridization of the invader m^* to $p(\text{A}_{20}\text{-m})_n$, leading to $p(\text{A}_{20}\text{-m}/m^*)_n$. **a**, Schematic representation for the DNA chain extension after hybridization. **b**, Representative chain conformation of ssDNA and dsDNA obtained from oxDNA MD simulations. The root mean squared end-to-end distance ($\langle h^2 \rangle^{1/2}$) and root mean squared radius of gyration ($\langle R_g^2 \rangle^{1/2}$) for ssDNA ($p(\text{A}_{20}\text{-m})_n$) and dsDNA ($p(\text{A}_{20}\text{-m}/m^*)_n$) are calculated over a simulation sample size of $N = 20000$. The total length of the strand is 1025 nt.

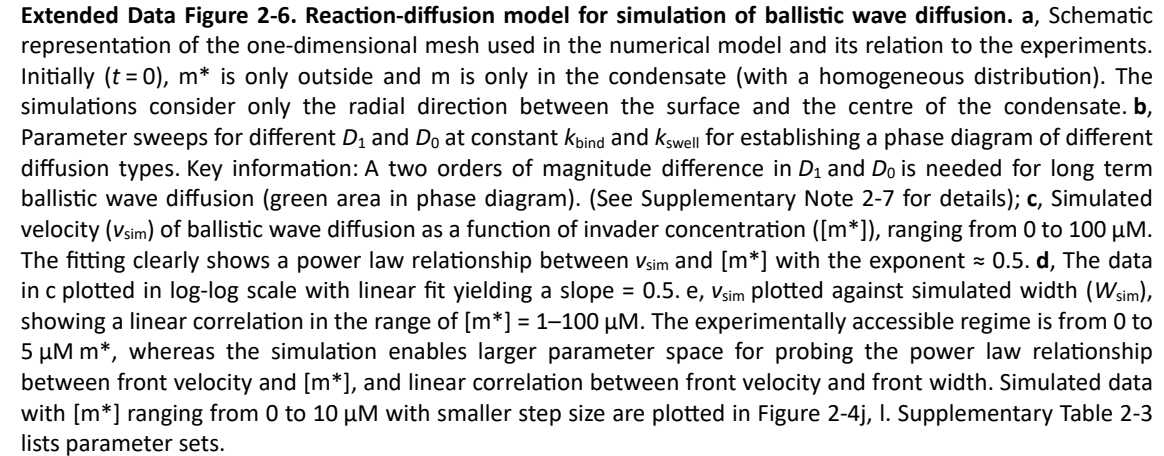


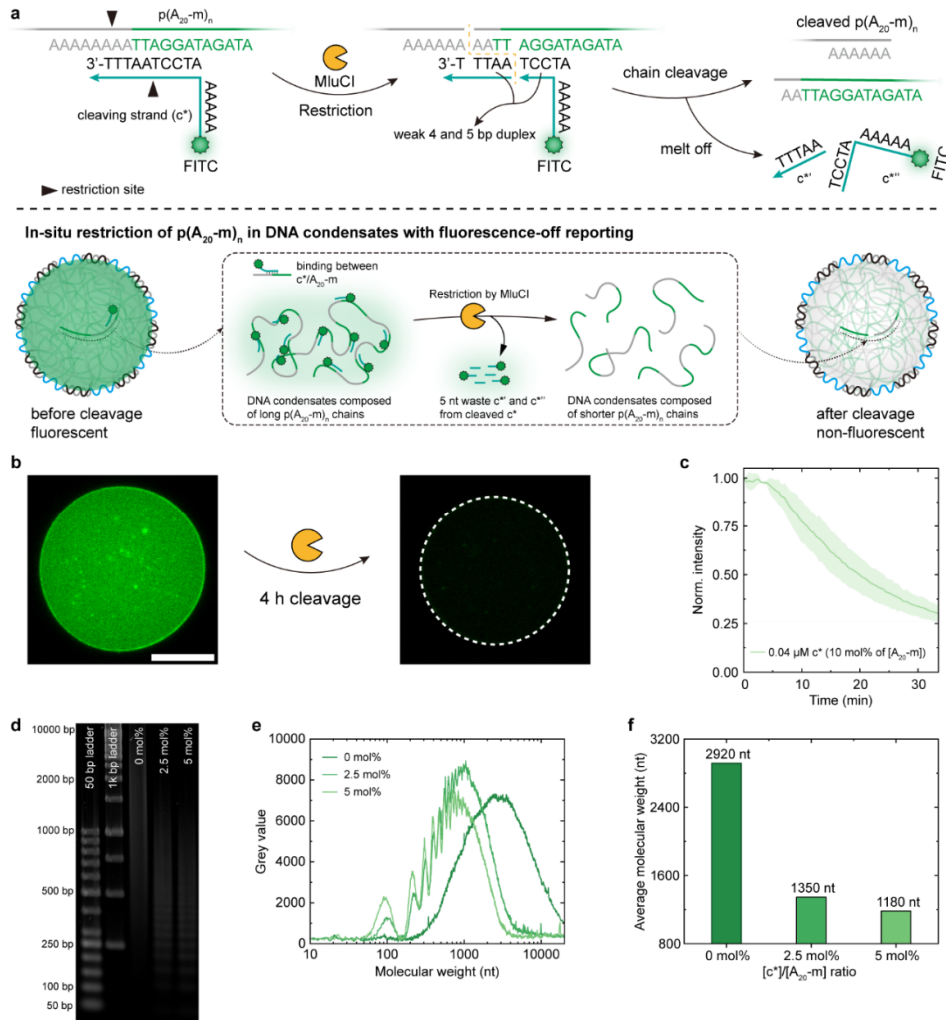
Extended Data Figure 2-4. Determination of the diffusion coefficients of short non-binding dummy ssDNA in arrested/non-invaded and dynamic/invaded DNA condensates. **a**, Scheme of a dynamic/invaded DNA condensate immersed in a solution containing Cy5-20nt-dummy. **b**, Representative CLSM images showing point bleaching method on freely diffusing Cy5-20nt-dummy inside a dynamic/invaded non-fluorescent $p(A_{20-m})_n$ condensate. The condensates were invaded by $1 \mu\text{M}$ non-fluorescent m^* to become dynamic and mixed with $10 \mu\text{M}$ Cy5-20nt-dummy (magenta channel). The shell of the condensates was labeled with a small amount of k^* -Atto488 ($0.5 \mu\text{M}$, green channel) for identification, as inside the white dashed circle. 3 s bleaching was applied in the centre pixel (indicated by the white cross in prebleach image) in the condensate before taking post-bleaching images. **c**, Representative cross-sectional line profiles along the white dashed lines in post-bleaching images in **b** for a dynamic/invaded condensate over time, giving Gaussian distribution of the fluorescence intensity, which represents Brownian motion of the bleached Cy5-20nt-dummy strands. The line profiles can be fitted with Gaussian fit to obtain their squared standard deviation (σ^2) at different time points. σ^2 changes over time during fluorescence recovery. The diffusion coefficients (D) of Cy5-20nt-dummy strands can be extracted by plotting σ^2 over time with the slope being $2D$ ⁶³. The same method was also applied on the arrested/non-invaded DNA condensates, respectively. **d**, Representative σ^2 for Cy5-20nt-dummy in arrested/non-invaded and dynamic/invaded DNA condensates, respectively. **e**, Comparison of diffusion coefficients of dummy strands in non-invaded and invaded DNA condensates (mean $\pm$ s.d., $N = 6$ condensates measured). One-way analysis of variance test was used for calculating P values for **e**. Scale bar is $20 \mu\text{m}$.

Chapter 2. Ballistic Diffusion Fronts in Biomolecular Condensates

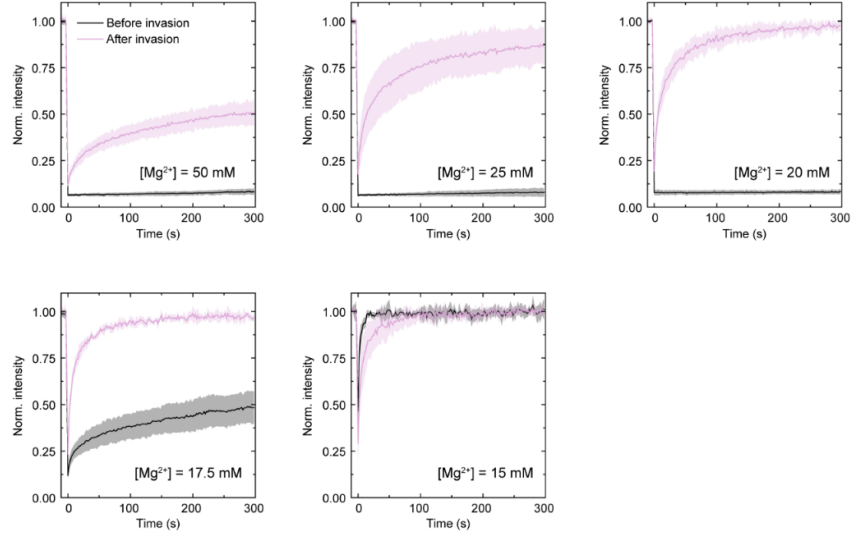


Extended Data Figure 2-5. Diffusion mechanism of non-charged PNA invader into DNA condensate. **a**, Structural comparison between a DNA strand and a PNA strand: A DNA strand has a phosphodiester backbone containing negative charges, while a PNA strand is composed of N-(2-aminoethyl)glycine backbone, which is non-charged. PNA binds efficiently to complementary DNA. **b**, Time series CLSM images of DNA condensate (m barcode concentration at $0.4 \mu\text{M}$ in total solution) during invasion by $5 \mu\text{M } m^*_{\text{PNA}}\text{-Atto488}$ PNA invader at 50 mM MgCl_2 (shell labeled with $0.25 \mu\text{M } k^*\text{-Atto647}$, blue channel). **c**, PNA only causes minor swelling during invasion. The swelling kinetics for the DNA invader is the same data as Figure 2-1e in main text. **d**, Representative CLSM images showing FRAP experiment on DNA condensates after invasion by $m^*_{\text{PNA}}\text{-Atto488}$ at 50 mM MgCl_2 . **e**, Corresponding intensity recovery kinetics of FRAP circles in **d** showing the arrested nature of the DNA condensates after invasion by $m^*_{\text{PNA}}\text{-Atto488}$ (mean $\pm$ s.d., $N = 4$ condensates measured). **f**, Scheme showing the increasing T_{cp} s when $p(A_{20}-m)_n$ is hybridized with PNA and DNA m^* invaders. **g**, Temperature-dependent change of absorbance at 350 nm of $p(A_{20}-m)_n$ with and without the existence of PNA or DNA m^* invaders. The onset of the increase in absorbance indicates the T_{cp} . **h**, Comparison of T_{cp} for $p(A_{20}-m)_n$ with and without hybridized PNA or DNA m^* invaders (mean, $N = 2$ condensates measured). Scale bars for all are $10 \mu\text{m}$. **Summary**: These experiments demonstrate the importance of introducing repulsive charges for regulating the dynamic properties of the invaded condensate. The invasion by a DNA invader brings more charges into the condensate, which generates stronger repulsive electrostatic force within and between DNA chains. Thus, it leads to higher swelling of the invaded condensate and a transition from arrested to dynamic interior. PNA is a poor solubilizer. The absence of a high intensity front in **b** is due to the persistence of the arrested nature of invaded condensates and a lack of swelling in the process.

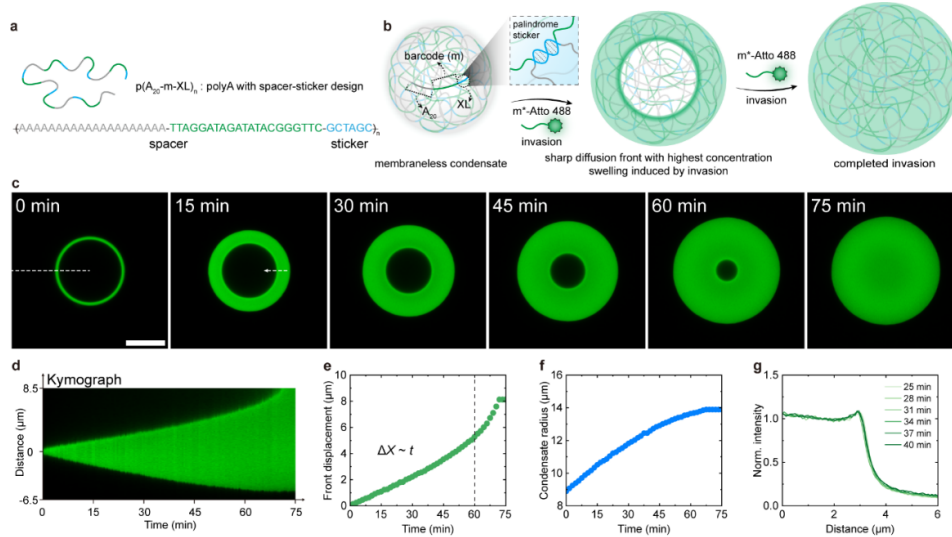




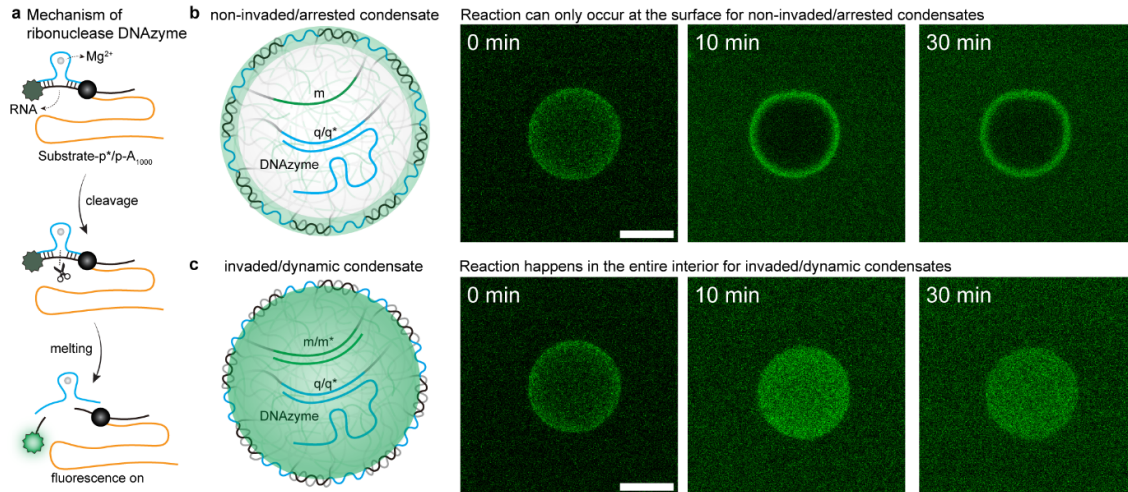
Extended Data Figure 2-7. Preparation of DNA condensates containing shorter $p(A_{20}-m)_n$ chains by in-condensate restriction. **Rationale:** Shortening of the $p(A_{20}-m)_n$ strands cannot be done before condensate preparation because excessively shortened strands might not integrate into the condensates due to the molecular weight dependence of the LCST behavior³¹. Hence, we applied restriction inside the condensate. **a**, Scheme for the design of the enzymatic restriction by MluCI on $p(A_{20}-m)_n$ chains with help of a cleaving strand (c^*) inside the condensates. c^* hybridizes to $p(A_{20}-m)_n$ and generates a recognition site for the restriction enzyme MluCI. The ratio of $[c^*]/[A_{20}-m]$ codes the extent of cleavage. c^* contains a spacer (A_5) with a fluorophore (FITC) at the 5' end. Before cleavage, c^* has 10 base pairs with $p(A_{20}-m)_n$ ($T_m \approx 40.2 - 44.4^\circ\text{C}$, Supplementary Note 2-3) and will thus bind tightly at experimental temperature (room temperature). After cleavage, both subparts ($c^{*'} and $c^{*''}$) of the c^* only have 5 base pairs with $p(A_{20}-m)_n$ ($T_m \approx 0^\circ\text{C}$ and $3.4 - 11.6^\circ\text{C}$, respectively, Supplementary Note 2-3) and will thus melt off. The fluorophore at the 5' end of the cleaving strand serves as a reporter for monitoring the restriction as fluorescence vanishes from the condensates into the surrounding. The finally cleaved DNA condensates will not contain any cleaved subparts of c^* due to their low T_m s, but only contain shorter $p(A_{20}-m)_n$ chains. **b**, Representative CLSM images showing the enzymatic restriction of DNA condensates loaded with sub-stoichiometric amount c^* (10 mol%). **c**, Restriction kinetics (light green) indicated by decrease of normalized fluorescence intensity within DNA condensate. Conditions: $0.04\ \mu\text{M } c^* = 10\ \text{mol}\%$ of $[A_{20}-m]$, $0.5\ \text{U}/\mu\text{L}$ MluCI enzyme, $15\ \text{mM } \text{MgCl}_2$, room temperature (mean $\pm$ s.d., $N = 2$ kinetic experiments). **d**, Gel electrophoresis of non-cleaved (with 0 mol% c^*) and cleaved $p(A_{20}-m)_n$ (with 2.5 and 5 mol% c^*). The gel electrophoresis image is background-subtracted. **e**, Quantification of molecular weight distribution by grey value profiles extracted from **d**. 50 bp and 1k bp ladders are used for the calibration of molecular weight as a function of migration distance. The molecular weight is reported as nucleotide number (nt), corresponding to double the calibrated base pair number (bp). **f**, Number average molecular weight of individual $p(A_{20}-m)_n$ chains calculated from the molecular weight distributions of individual lanes in **e**. Scale bar is $10\ \mu\text{m}$.$



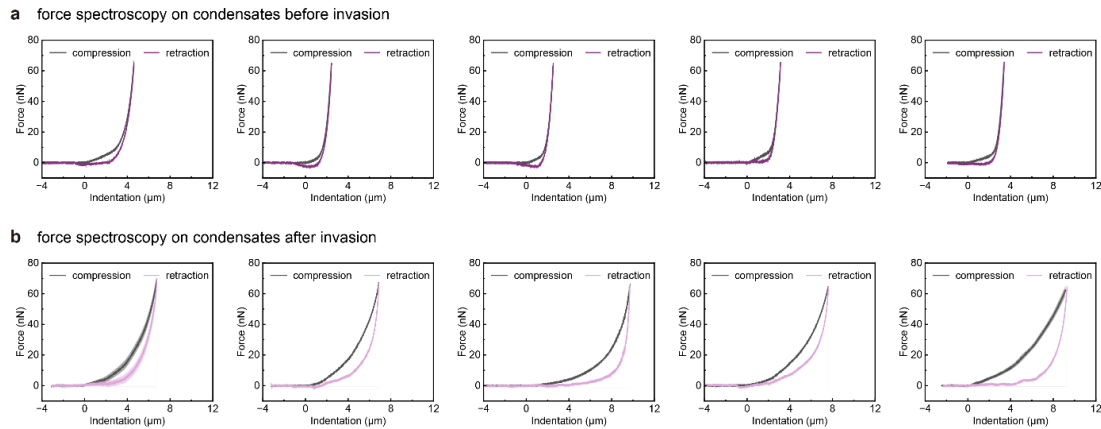
Extended Data Figure 2-8. FRAP of DNA condensates before and after invasion at different $[Mg^{2+}]$. FRAP experiments performed on DNA condensates of $p(A_{20}-m_{Atto594})_n$ before (black) and after (violet) invasion by $1 \mu M$ m^* -Atto488 at the $MgCl_2$ concentration mentioned inside the panels (mean $\pm$ s.d., $N = 4$ condensates measured for all conditions). The plots shown here are used for the determination of $1/t_{1/2}$ for Figure 2-4f in the main text.



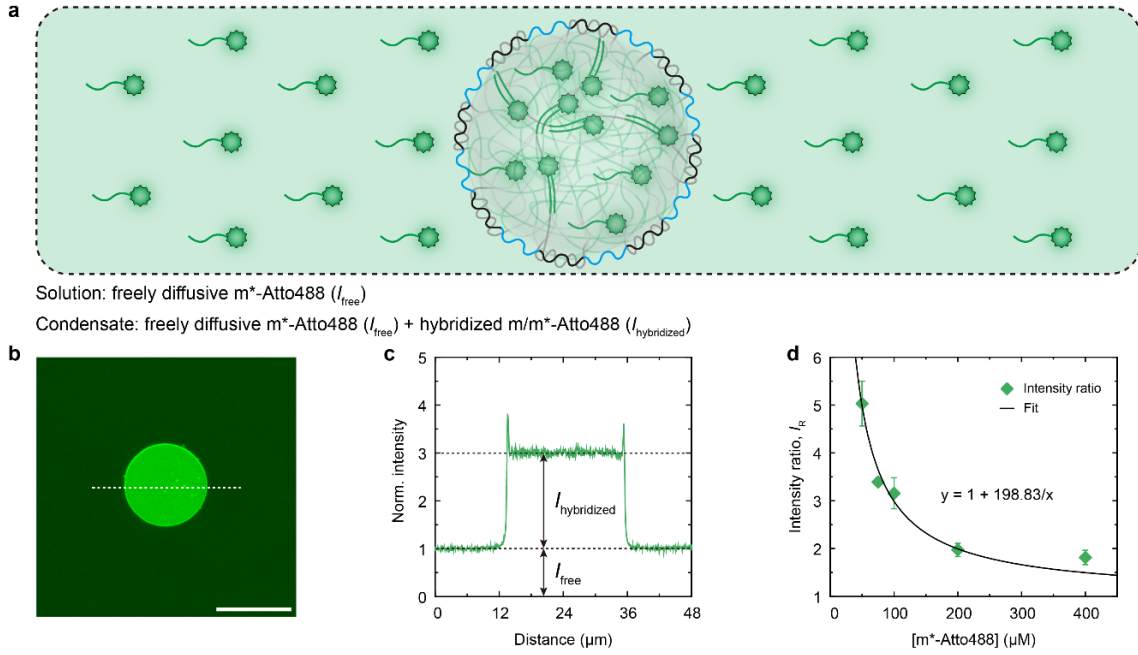
Extended Data Figure 2-9. Ballistic wave diffusion in membraneless DNA condensates with spacer-sticker model. **a**, $p(A_{20}-m-XL)_n$ sequence design of adenine-rich DNA polymer with palindromic domain (XL) mimicking spacer-sticker model. XL domains are self-complementary DNA sequences that serve as crosslinks. **b**, Schematic representation of the ballistic wave diffusion occurring during the invasion of m^* -Atto488 into such membraneless $p(A_{20}-m-XL)_n$ DNA condensates. **c**, Time-series CLSM images showing the invasion process of invaders (m^* -Atto488, 3 μM ; green; 50 mM $MgCl_2$). **d**, Kymograph of the invasion process along the white dashed line in **c**. **e**, Front displacement during invasion is linear with time. Acceleration happens at the end stage (noted with the dashed line) because of the interference between diffusion fronts from all radial directions. **f**, Radius of DNA condensate during invasion. **g**, Cross-sectional line profiles taken at various times along the white dashed arrow in **c**. The distances are rescaled to the maximum intensity of the wave to highlight the self-similar shape during invasion. Scale bar is 10 μm and applies to all micrographs in **c**.



Extended Data Figure 2-10. Catalytic cleavage by ribonuclease DNAzyme in DNA condensates is regulated by different dynamic states. **a**, Schematic representation for the catalytic cleavage process of p*-Substrate/p-A₁₀₀₀ by 10-23 ribonuclease DNAzyme tethered via additional q/q* domains. The DNAzyme is able to break RNA bonds once the substrate is hybridized with the blue recognition parts. DNA condensates containing 80 % m barcode and 20 % q barcode (obtained by using a mixture of p(A₂₀-m)_n and p(A₂₀-q)_n during condensate formation) were used for DNAzyme encapsulation by q/q* hybridization. **b**, Before invasion, DNA condensates are in arrested state. The catalytic cleavage of p*-Substrate at the DNAzyme encapsulated (by q/q* hybridization) in the arrested condensates only happens at the condensate surface. **c**, After invasion with m*, the DNA condensates are in a dynamic state. Now the catalytic cleavage of the DNAzyme can happen in the entirety of the condensate. The m* invader has served as a gate opener for the catalysis. Scale bars are 10 μm.

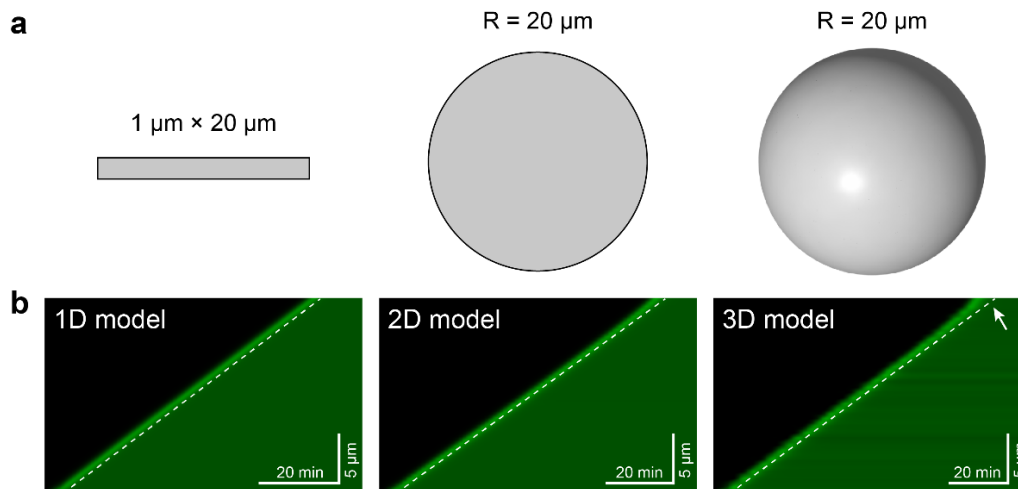


Supplementary Figure 2-1. Individual force spectroscopy on condensates before and after invasion. **a**, Individual force spectroscopy measurements on condensates *before invasion*, showing rather elastic behavior with high stiffness, and low hysteresis (mean ± s.d., $N = 5$ condensates measured). **b**, Individual force spectroscopy measurements on condensates *after invasion*, showing rather liquid-like behavior with low stiffness and high hysteresis (mean ± s.d., $N = 5$ condensates measured).

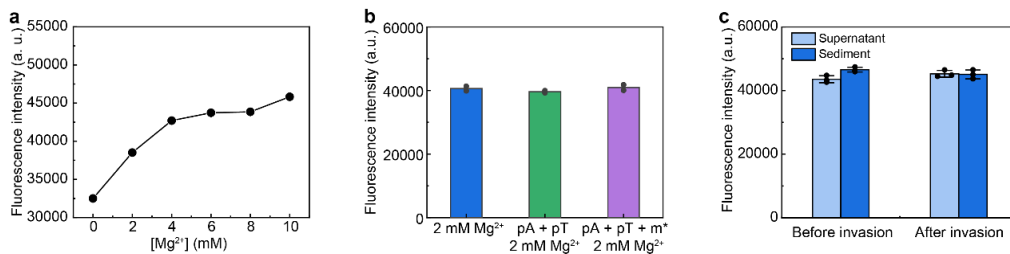


Supplementary Figure 2-2. Determination of the m barcode concentration in DNA condensate by titration. **a**, Schematic representation of the titration experiment for determining the m barcode concentration in DNA condensates (Supplementary Note 2-5). **b**, Representative CLSM image of a dynamic condensate in solution containing 100 μM m*-Atto488. **c**, Corresponding cross-sectional line profile along the dashed line in **b**. The intensity is normalized to the background solution intensity (I_{free}). Thus, normalized intensity = 1 means the intensity contributed from freely diffusive fluorophores, while the additional contributions above 1 within a DNA condensate stem from the hybridized m*-Atto488, $I_{\text{hybridized}}$. **d**, Intensity ratio (I_R) as a function of m*-Atto488 concentration (mean $\pm$ s.d., $N = 6$ condensates measured for 50 and 400 μM [m*-Atto488], and 4 condensates measured for other [m*-Atto488]). The scale bar is 20 μm .

Analysis and experiment method: DNA condensates were mixed with high concentrations m*-Atto488 (50–400 μM) with a total concentration of m barcodes in the solution at 0.4 μM (inside the condensates). During hybridization of m*-Atto488 into the condensates, the consumption of m*-Atto488 in the background solution can be neglected due to the high excess. Within the DNA condensate, all m barcodes are hybridized by m*-Atto488, and the condensate is in a dynamic and swollen state. The fluorescence intensity in the solution is only due to freely diffusing m*-Atto488, yielding I_{free} . In the condensate, there are both hybridized m*-Atto488 and freely diffusing m*-Atto488, giving $I_{\text{hybridized}} + I_{\text{free}}$. We define the intensity ratio (I_R) as the ratio between intensity inside the condensate and intensity in the background solution, which can be written as $I_R = (I_{\text{hybridized}} + I_{\text{free}})/I_{\text{hybridized}}$. We consider a linear correlation of intensity with dye concentration ($c \propto I$). Under this consideration, we can get $(c_{\text{hybridized}} + c_{\text{free}})/c_{\text{free}} = (I_{\text{hybridized}} + I_{\text{free}})/I_{\text{free}} = I_R$, which can be re-written to $c_{\text{hybridized}}/c_{\text{free}} + 1 = I_R$. c_{free} is the concentration of m*-Atto488 added in solution (known, independent variable), and I_R can be experimentally measured (dependent variable). Therefore, when performing a series of concentration-dependent experiments and measuring the corresponding I_R , it is possible to fit all data points with the equation $c_{\text{hybridized}}/c_{\text{free}} + 1 = I_R$. This yields the parameter $c_{\text{hybridized}}$, which is the concentration of hybridized m*-Atto488, and thus also the concentration of m barcode inside the condensates in the swollen state. The data points in **d** are fitted, and the hybridized concentration of m*-Atto488 is determined to be ca. 200 μM , reflecting the concentration of m barcode within the condensates after invasion and swelling. Based on a 4-fold swelling ratio in volume due to invasion (Figure 2-1e), the barcode concentration can be calculated to ca. 800 $\mu\text{M} \approx 1 \text{ mM}$ in the non-invaded pristine condensates.



Supplementary Figure 2-3. Acceleration of front propagation at the end stage for 2D and 3D simulations. **a**, Geometries used for simulation in different dimensions. **b**, Simulated kymographs show the front propagation behavior of ballistic wave diffusion in different dimensions. No acceleration of front propagation in 1D, negligible acceleration in 2D, and more pronounced acceleration in 3D. The acceleration at the end stage in 3D is indicated with a white arrow.



Supplementary Figure 2-4. Comparison of Mg²⁺ concentrations inside and outside condensates. **a**, Calibration curve showing the fluorescence intensity of 10 μ M Mg²⁺ indicator (Mag-fluo-4 AM) in PBS buffer at varying Mg²⁺ concentrations (mean $\pm$ s.d., $N = 2$ independent experiments for each [Mg²⁺]). **b**, Fluorescence intensity of 10 μ M Mg²⁺ indicator in PBS buffer with and without the presence of DNA polymers (pA and pT) and invader strand (m*) show no significant differences. Note that the concentrations of pA, pT, and m* were matched to those in the sediment samples before and after invasion in c (mean, $N = 2$ independent experiments for each condition). **c**, Fluorescence intensity of 10 μ M Mg²⁺ indicator in 20-fold diluted supernatant and sediment samples from condensates at 50 mM Mg²⁺ before and after invasion. (mean $\pm$ s.d., $N = 3$ independent experiments for each condition). See Supplementary Note 2-6 for experiment details.

2.5 References

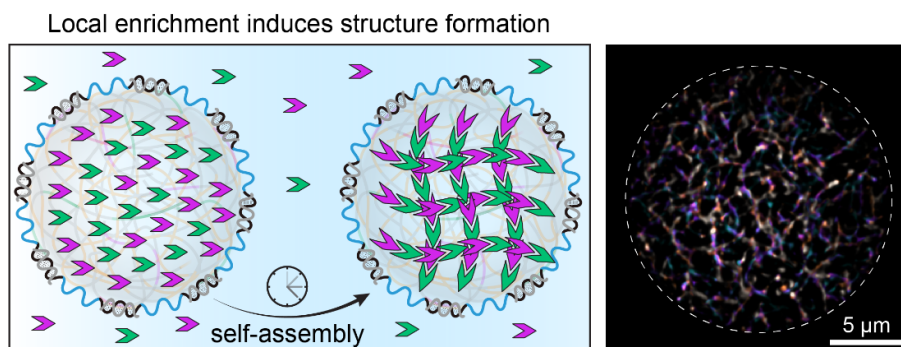
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3. Growing Functional Artificial Cytoskeletons in the Viscoelastic Confinement of DNA Synthetic Cells



Preliminary note: This chapter is based on the journal article published in *Nat. Chem. Eng.* **2**, 627–639 (2025). <https://doi.org/10.1038/s44286-025-00289-5>, by **Weixiang Chen**, Siyu Song, Avik Samanta, Soumya Sethi, Christoph Drees, Michael Kappl, Hans-Jürgen Butt & Andreas Walther*. Minor changes have been made concerning formatting style.

Author contributions: W.C. and A.W. conceived the project and designed the experiments. W.C. performed the majority of experiments and analyzed the data. A.S. helped with SC preparation. S.Song and S.Sethi synthesized the ssDNA-cRGD conjugate. S.Song performed SC-mammalian cell contact experiments. D.C. performed and evaluated the SIM measurements. M.K. and H.-J.B. helped with AFM measurement and analysis. W.C. and A.W. discussed the data. W.C. and A.W. wrote the manuscript with input from all authors. A.W. supervised the project.

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Abstract: Intracellular structures, like the cytoskeletons, form within a crowded cytoplasm with viscoelastic properties. While self-assembly in crowding is well studied, the effects of coupled viscoelastic environments remain elusive. Here, we engineer all-DNA synthetic cells (SCs) with tunable viscoelastic interiors to investigate this phenomenon. We introduce facile DNA barcode engineering to selectively enrich DNA tiles with adjustable concentrations into SCs to form artificial cytoskeletons coupled to their interior. Distinct mechanistic differences in assembly occur compared to solution or simple crowding. Furthermore, we develop light, molecular, and metabolic switches to direct structure formation and create self-sorted SC populations with distinct artificial cytoskeletons. These cytoskeletons strengthen SCs and support stable contacts with mammalian cells. Our SC system integrates molecular-scale DNA nanotube assembly with mesoscale DNA condensate structures, expanding further the DNA nanoscience toolbox and may serve as a versatile platform to study self-assembly under viscoelastic constraints and leverage sub-SC structures for challenging applications.

3.1 Introduction

Cells are compartmentalized dynamic systems that sustain out-of-equilibrium functions and structures by selective exchange of nutrients, signals, and wastes with the environment¹⁻⁴. The intracellular cytoplasm is a macromolecularly crowded interior with defined viscoelastic properties, which influences both catalysis and subcellular structure formation⁵⁻⁸. Fundamentally, viscoelasticity comes from the relaxation dynamics of macromolecules in solutions of different relaxation modes at different timescales, which is well described by Zimm, Rouse, and Reptation models for macromolecular solutions at different concentration regimes⁹. The viscoelastic properties are in particular relevant to the formation of subcellular structures because the length and time scales of their formation may interface with the dynamics of the viscoelastic cytoplasm matrix¹⁰⁻¹³. These effects are beyond simple crowding. Mimicking defined crowded and/or viscoelastic environments in synthetic model systems, such as synthetic cells (SCs), may provide insights into such cellular processes. Efforts have been dedicated to building SCs to tackle the challenge of mimicking cell-inspired structures and functions, and to better understand cellular processes¹⁴⁻¹⁹. SCs can be obtained as water enclosed compartments from liposomes²⁰⁻²³, polymersomes^{24,25}, or colloidosomes²⁶⁻²⁸, or from liquid-liquid phase separation (LLPS) and complex coacervation enriching polymers, proteins and nucleic acids²⁹⁻³³. The latter are suitable mimics of the intracellular environment as they reproduce features of the cytoplasm regarding macromolecular crowding and viscoelastic properties^{6,13,34}.

Construction of secondary structures inside SCs, such as cytoskeletons and membraneless organelles is non-trivial due to the challenge to manipulate monomers and self-assembly inside the confinement^{10,11,35-37}. Recent examples to build complex structures inside SCs include artificial cytoskeletons and droplet-like subcompartments^{22,38-41}. In the context of this work, DNA nanotubes (DNTs)⁴² have been organized as an artificial cytoskeleton in water-in-

oil emulsions³⁸ and in liposomes^{22,39,43}. Excluded volume effects by dextran or addition of Mg^{2+} have been used to induce bundling of DNTs^{39,43}.

The influence of macromolecular crowding on enzymatic catalysis and structure formation through excluded volume effects has been extensively studied^{19,44-47}. Molecular crowding in fact requires absence of interactions between a colloidal species and the used crowder to form the excluded volume layer. The Asakura–Oosawa model is commonly used to describe depletion attraction dominated by an entropic gain^{48,49}. However, in addition to being densely crowded, the cellular environment is also viscoelastic, as a result of macromolecular relaxation processes. In contrast to the well-studied effects of crowding, much less is known about how viscoelasticity—superimposed on macromolecular crowding—affects self-assembly and structure formation (Figure 3-1). In this case coupling may occur purely through physical constraints, akin to soft interactions, or can occur in regimes with strong coupling, for instance, by tethering the building blocks to a matrix. We focus on the second aspect because bonding of the self-assembling DNT tiles is essential for their enrichment in SCs and for studying their assembly. Only a few recent studies started to look at structure formation in macroscopic gels, hence in situations where soft coupling can be studied without the need for enriching building blocks as in case of SCs⁵⁰⁻⁵³. Bringing viscoelastically coupled self-assembly approaches to the field of SCs requires to develop SC models that allow manipulation of their viscoelastic properties and controlled enrichment of building blocks, which will benefit both the fundamental understanding and the improvement of the engineering principles of SCs towards structural and functional properties.

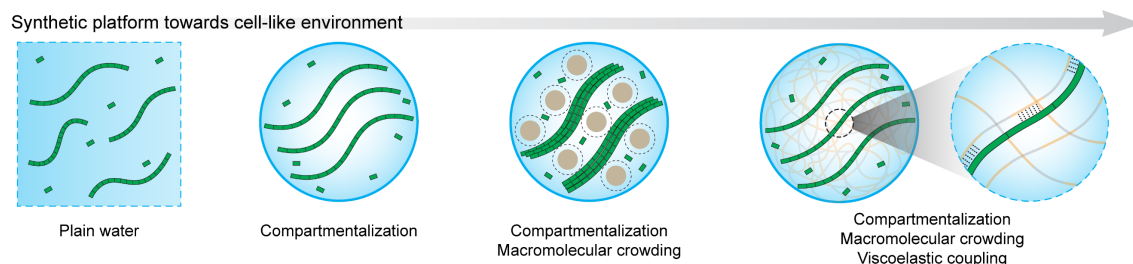


Figure 3-1. Self-assembly in different environments: From plain solution via crowded compartments to viscoelastic coupling to a matrix environment (also termed viscoelastic confinement).

Self-assembly of DNTs was originally studied in plain solution, which was later on extended to compartmentalized system with crowding agents. In contrast, we develop systems in a crowded and viscoelastic compartment by coupling the assembling DNT monomers to the viscoelastic compartment matrix with a supramolecular binding mechanism.

Here, we develop a crowded all-DNA SC system to study the formation of artificial cytoskeletons in viscoelastic confinement under strong coupling conditions, by combining DNA nanotechnology between DNA self-assembly at molecular scale to DNA polymer phase separation at mesoscale. The SC is characterized as a confined, semidilute polymer solution, whose viscoelasticity is intrinsically related to concentration and temperature. The SCs allow selective enrichment of cytoskeleton monomers that are bonded to the intra-SC matrix using short DNA barcode domains. The SCs are formed by a temperature-induced condensation

process and contain a liquid interior composed of long ssDNA stabilized by a permeable DNA hydrogel shell^{17,41,54,55}. We will first introduce a *co*-phase-separation approach to integrate multiple barcodes with controlled concentrations into the all-DNA SCs. We then demonstrate how artificial cytoskeletons grow in these confinements, and how the concentration of DNA tiles and the viscoelastic properties regulate the assembly process. We further showcase the versatility of this approach to control the selective enrichment of different cytoskeletal building blocks to achieve self-sorted cytoskeletons within SCs and self-sorted SCs containing distinct artificial cytoskeletons in SC communities. We demonstrate the functional benefit of forming artificial cytoskeletons to enhance the stiffness of SCs, as well as providing stability during SC-mammalian cell contact when building a mechano-interface to cells using RGD-peptide-integrin binding. Our molecular engineering approach provides versatile SC systems to fundamentally study self-assembly in viscoelastic confinement and guide the design of future SCs with emergent functions via embedded secondary structures.

3.2 Results and Discussion

3.2.1 Barcode Engineering inside SCs by Co-Phase Separation

The preparation of all-DNA core-shell SCs builds on our original discovery of the lower critical solution temperature (LCST) behavior of adenine-rich ssDNA polymers^{54,55}. Previously, we had shown that heating a mixture of adenine-rich and thymine-rich ssDNA polymers, *i.e.*, $p(A_{20}-m)_n$ and $p(T_{20}-k)_n$, induces condensation of $p(A_{20}-m)_n$ into spherical condensates. During cooling the $p(T_{20}-k)_n$ hybridizes on the periphery of the $p(A_{20}-m)_n$ droplets, trapping the core-shell SCs with homogenous distribution of $p(A_{20}-m)_n$ in the core and $p(T_{20}-k)_n$ at the shell^{19,54}. A_{20} and T_{20} are repeats of 20 nucleotides of adenine and thymine, whereas m and k are short ssDNA barcodes embedded in the repeating unit of the polymer chains (Figure 3-2a). The subscript 'n' denotes the number of repeats, which is around 70 for adenine-rich DNA polymers, and above 80 for thymine-rich DNA polymers (Supplementary Figure 3-1). The barcodes allow selective functionalization of the core and shell by complementary ssDNA.

The concentration of the m barcode ($[m]$) in the core is a fixed value determined by the thermodynamics of phase separation of $p(A_{20}-m)_n$. As we will show below, tuning the barcode concentration is required to precisely control the enrichment of building blocks. Hence, to finetune $[m]$ inside the core matrix of a SC, we introduce a new *co*-phase-separation method by mixing various adenine-rich ssDNA polymers with different barcode sequences. To this end, we synthesized two additional adenine-rich DNA polymers, namely $p(A_{20}-p)_n$ and $p(A_{20}-s)_n$ (Supplementary Figure 3-1). We first focus on binary mixtures of $p(A_{20}-p)_n$ and $p(A_{20}-m)_n$ in different ratios (Figure 3-2a). As the thermodynamic driving force for phase separation is encoded in the A_{20} repeats present in both ssDNA polymers, we expected similar temperature-dependent condensation of both $p(A_{20}-p)_n$ and $p(A_{20}-m)_n$, as well as *co*-condensation of them due to their chemical similarity.

To verify our concept, we prepared a series of SCs with different ratios of $p(A_{20-m})_n$ and $p(A_{20-p})_n$, while keeping their total concentration constant at 0.5 g/L. $p(A_{20-p})_n$ is introduced as a non-functional filler strand which constitutes the majority weight fraction (from 90% to 99.9%), while $p(A_{20-m})_n$ ranges from 10% to 0.1%. We labeled these SCs with Atto488-m* and Atto647-k* for microscopy and found morphologically similar SCs for all mixtures, independent of the used ratio of $p(A_{20-m})_n/p(A_{20-p})_n$ (Figure 3-2b). Quantification of the Atto488 fluorescence in the core shows a clear dependence of the fluorescence intensity on the weight fraction of $p(A_{20-m})_n$ used (Figure 3-2b, c). The intensity differences cross 3 orders of magnitude, confirming that the [m] can be controlled by simply adjusting the ratio of $p(A_{20-m})_n/p(A_{20-p})_n$. Mesoscopic demixing of $p(A_{20-m})_n$ and $p(A_{20-p})_n$ is absent. Based on titration measurements, the absolute [m] inside the SC core ranges from 100 nM for 0.025% of $p(A_{20-m})_n$ to 40000 nM for 10% of $p(A_{20-m})_n$ (Supplementary Figure 3-2 and Supplementary Note 3-1). The total concentration of all A_{20-x} ($x = m, p$) repeating units inside SCs is around 400 μ M (5 g/L) (Supplementary Note 3-1)⁵⁶.

3.2.2 Formation of Artificial Cytoskeletons inside SCs

We use DNA nanotubes formed by DNA tiles as cytoskeleton-mimic. Such DNTs are versatile and robust systems, and their growth has been demonstrated in solution as well as in water compartments^{38,39,42}. For the integration into all-DNA SCs, we first focus on identifying the correct concentration, and establishing conditions providing enrichment inside the SCs. We start by using a binary DNT-tile system^{38,42} composed of tile A and tile B (Supplementary Figure 3-3), which are mutually complementary but not self-complementary based on their sticky end interaction. For visualization and enrichment inside the SCs, we modified both tiles with fluorescent labels (Atto488 for tile A and Atto647 for tile B) and a dangling strand complementary to the m barcode inside the SCs (Figure 3-2d). The two DNA tiles were annealed separately so that no fibrils can form during annealing.

We then mixed individual SCs having different [m] in the core with 20 nM DNA tiles to enrich them inside the SCs and avoid undesired DNT formation in solution. Depending on the SCs used (different [m]), the DNA tiles can be over-stoichiometric, stoichiometric, or sub-stoichiometric with respect to the m barcode. A sufficient concentration of added DNA tiles is important to facilitate their enrichment inside SCs, particularly at low total barcode concentration (Supplementary Figure 3-4). Supplementary Table 3-1 lists details of the experimental settings. As expected, the tiles do not form any observable structure in solution due to their low concentration in solution. Rather, they accumulate in SCs via m/m* recognition to controlled concentrations programmed by the m content of the co-phase-segregated $p(A_{20-m})_n$ and $p(A_{20-p})_n$. This selective enrichment of the tile concentration is of critical importance, as it enables structure formation exclusively within the compartment. In contrast, when mixing SCs with DNA tiles without barcodes, a preferred partitioning of the tiles into the SCs does not occur (Supplementary Figure 3-5). This underscores that chemical

interactions are needed to sequester the DNT tiles into the SCs, allowing to study structure formation under strong coupling conditions to the matrix.

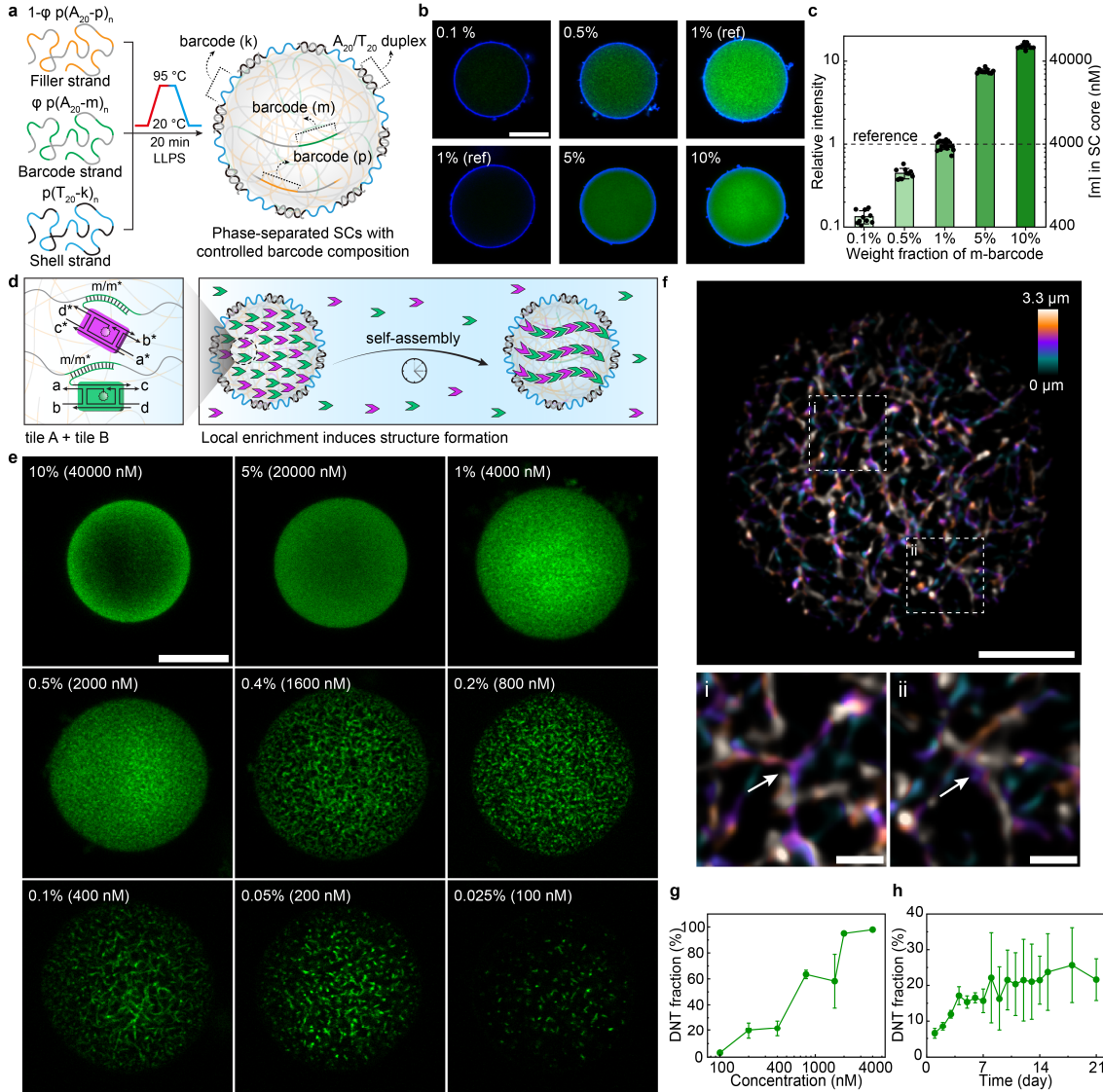


Figure 3-2. Preparation of core-shell all-DNA synthetic cells containing multiple barcodes with controlled local concentrations and formation of artificial cytoskeletons inside synthetic cells by controlling monomer enrichment. **a**, Schematic representation for the preparation of SCs containing two core barcodes (namely, m barcode and p barcode). **b**, Representative CLSM images of SCs containing different weight fraction of m barcode in the core ranging from 0.1% to 10%, labeled by Atto488-m* (green) in the core and Atto647-k* (blue) at the shell. Note that for each row of the CLSM images, laser settings are the same for direct comparison of internal fluorescence intensity to quantify relative m barcode concentrations. **c**, Quantification of m barcode concentrations, [m], inside the SC core by the measured fluorescence intensity inside SCs as a function of weight fraction of p(A₂₀-m)_n in the mixture of p(A₂₀-m)_n (from 0.1% to 10%) and p(A₂₀-p)_n (from 99.9% to 90%) used for preparation of SCs. **d**, Schematic representation for the enrichment of DNA tiles into SCs by barcode interactions and concentration-dependent formation of artificial cytoskeletons inside SCs. **e**, Representative CLSM images showing that artificial cytoskeletons start to form at proper concentration range and the corresponding morphology varies as a function of [m]. Experiment temperature = 20 °C. **f**, Structured illumination microscopy (SIM) on an artificial cytoskeleton formed in a SC with [m] = 400 nM. The color-coded z-projection image reveals branching as indicated by white arrows in the zoomed-in images (i and ii). **g**, Increasing DNT fraction within SCs with the increase of [m] inside SCs after 20 ± 1 days of DNT growth. **h**, Growth of DNT inside SCs with [m] = 400 nM, as quantified by the DNT fraction inside SCs. The DNT fraction is quantified by the fraction of pixel counts inside SC over a threshold intensity. Experiment temperature = 20 °C. The DNTs have two co-localized fluorescent labels (Atto488, Atto647). Only Atto488 (green) is shown for highest resolution and better contrast. Sample size (*n*) = 10 for **c**, *n* = 4 – 10 for **g**, and *n* = 6 – 14 for **h**. Error bars represent standard deviation. The scale bar is 10 μm for CLSM images, 5 μm for SIM image, and 2 μm for zoomed-in SIM images.

We compare DNT growth in the SCs after 20 ± 1 days under CLSM (Figure 3-2e, g). Three main regimes can be delineated (Extended Data Figure 3-1): (1) Absence of distinguishable DNTs at high $[m]$, (2) Proper DNT formation at intermediate $[m]$, and (3) formation of very short DNTs at low $[m]$. In SCs with 10% m barcode ($[m] = 40000$ nM), the tiles cannot fully address all barcodes due to their sub-stoichiometric amount (50%, see Supplementary Table 3-1). Therefore, a higher concentration at the rim of the SCs can be observed (Figure 3-2e). DNT formation is absent even after 20 days of incubation. Down to SCs containing 0.5% m barcode ($[m] = 2000$ nM), no clear fibrillar structures are formed. We suggest that the absence of fiber formation in this first regime is due to the excessive concentration and the freezing of dynamics required for rearrangement in the viscoelastic interior. The sticky end interactions of the tiles prevent reorientation to form well-defined structures. Increasingly arrested dynamics of the SC interior at higher $[m]$ can be confirmed by fluorescence recovery after photobleaching (FRAP) experiments (Supplementary Figure 3-6b–d). Upon further lowering of $[m]$ in the SCs to 0.4% (1600 nM), clear fibrils start to appear in a very dense state. When $[m]$ inside the SCs reaches 0.1% (400 nM), the formed artificial cytoskeleton becomes clearly discernible in CLSM. A Further reduction of $[m]$ leads to insufficient concentrations of the DNA tiles, which ultimately limits the growth of any artificial cytoskeleton to short fibers. This underscores the importance of precisely engineering the enrichment and local concentration of building blocks to ensure proper structure formation in crowded and viscoelastic environments. Details of the process also depend on the temperature of assembly. For instance, higher temperatures facilitate the formation of DNTs even at higher loading concentrations ($[m] = 10\%$) on account of higher dynamics (Supplementary Figure 3-7).

Superresolution imaging by structured illumination microscopy (SIM) provides further details and reveals a significantly higher degree of branching of the DNTs in SCs compared to DNTs formed in plain solution (z-section in Figure 3-2f; Supplementary Figure 3-3b). Time series CLSM reveals massive nucleation events at an early assembly stage, forming bright nuclei, which slowly evolve to fibrils after 2 to 3 weeks (Figure 3-2h, Extended Data Figure 3-2, and Supplementary Figure 3-8a). This observation suggests that fibril growth occurs by end-to-end joining and rearrangement of short DNT segments and not by classical addition of DNT tiles to seeds and growing DNTs in solution⁵⁷⁻⁵⁹. This is because nucleation into short segments has plenty of time to occur in small viscoelastic volumes, whereas the subsequent slow growth requires relaxation of the polymeric and viscoelastic ssDNA matrix and larger scale rearrangement to accommodate structure formation. The growth of DNTs is dominated by the tiles bonded via m/m^* hybridization inside SCs, whereas free tiles in solution only have a minor contribution (Supplementary Figure 3-9). The continuous structural evolution of the artificial cytoskeletons over time confirms that certain dynamics exist in the system. This is also verified by a two-day long FRAP experiment that shows recovery of the cytoskeleton (Supplementary Figure 3-6a). Crosslinking the entire SC matrix via the p barcodes of co-assembled $p(A_{20}-p)_n$ prevents assembly, as the SC matrix transforms from a semidilute polymer solution to a hydrogel network, and the SC's viscoelastic properties are largely altered

from more viscous to more elastic (Supplementary Figure 3-10). Importantly, clustering of DNTs into bundles cannot be observed as has been reported for simple crowders (dextran)³⁹. The absence of bundling is due to the fact that the tiles are all bonded to the $p(A_{20-m})_n$ core strands to prevent segregation (Figure 3-2d). In contrast, when non-binding DNA tiles assemble in a concentrated $p(A_{20-p})_n$ solution at 5 g/L, which nominally represents the SC interior, bundling of DNTs occurs. This is similar to the aforementioned simple crowders such as PEG or dextran³⁹ (Supplementary Figure 3-11).

Since the SCs can be considered as a semi-dilute polymer solution at the level of entanglement concentration (c_e) (5 g/L, average molecular weight around 900 kDa, $c_e \approx 2.8$ g/L, see Supplementary Note 3-2), its viscoelastic properties can be described by established semidilute polymer solution dynamics following combined Zimm, Rouse, and, above c_e , Reptation models⁹. For instance, zero shear viscosity increases with concentration as well as decreases with higher temperature on account of the Arrhenius relationship. On a molecular level this relates to polymer relaxation dynamics and possibly disentanglement of individual chains. Since matrix rearrangement is critical to accommodate growing DNTs, we hypothesized an impact of viscosity on DNT growth dynamics. We note that for non-crosslinked SCs, the assembly process is so slow that it couples to the low-frequency regime that probes rather the viscous properties of the SC matrix.

To understand the impact of viscosity at constant temperature, we designed SCs composed of three pA strands (5 g/L) and degraded one of them using selective enzyme degradation (Supplementary Figure 3-12). This yields SCs with only 3 g/L pA in the core, while [m] is the same as for the standard SCs. Afterwards, we incubated DNA tiles with SCs before (5 g/L) and after degradation (3 g/L). FRAP experiments confirm enhanced dynamics for SCs with 3 g/L as seen by ca. 40% increase in the reciprocal of half recovery time ($1/t_{1/2}$), thus confirming the impact of reduced concentration on dynamics (Supplementary Figure 3-12f, g). This leads to a marked increase in the DNT growth kinetics inside the SCs with 3 g/L, wherein DNTs are clearly visible within 3 days of assembly time (Supplementary Figure 3-12e, h, i) — approximately half the time required for standard SCs (7 days). This underscores the importance of matrix dynamics on the self-assembly timescale.

Temperature has a further influence on semidilute polymer solution dynamics and therefore impacts the corresponding viscoelasticity. We studied DNT growth in standard SCs with 5 g/L at 20, 25, 30, and 35 °C. In general, temperature has two effects: First, higher temperature enhances reptation dynamics and thereby contributes to faster stress relaxation and lower viscosity, as can be probed by FRAP (Supplementary Figure 3-13e, f). Second, higher temperature dynamizes DNA duplexes and lowers the equilibrium fraction of binding according to the melting temperature. Profound differences in the growth kinetics and final DNT density inside SCs occur (Supplementary Figure 3-13a–c). A temperature increase accelerates the DNT growth when going from 20 to 25 and 30 °C, however, DNT formation is fully absent at 35 °C (Supplementary Figure 3-13d). For instance, at day 3, DNTs are barely

visible at 20 °C, whereas they have clearly formed already at 25 °C and 30 °C. Additionally, the DNT density inside SCs starts to decrease at 30 °C. This can be explained as follows: Faster growth originates from lower viscosity at higher temperatures. However, once reaching 30 °C, increased reaction kinetics due to fast reptation are outbalanced by the weaker sticky end interactions between the DNT tiles responsible for their assembly (interaction only based on 5 nt) and furthermore by the lower DNT tile recruitment due to insufficient m/m* interactions to sequester DNTs into the SCs. At 35 °C, the weakened DNA interactions dominate the system and prevent assembly. Therefore, we determine 25 °C to be the optimum temperature, balancing quickest growth and high density of DNTs inside SCs.

Interestingly, the dynamic properties of the final DNT cytoskeleton inside SCs are different at different temperatures. When placing DNT-containing SCs grown at 25 °C to 20 °C and 30 °C, the DNT structure is very stable and shows almost no movements over 1 h at 20 °C, whereas considerable dynamics are visible at 30 °C. This enhanced movement relates to more dynamic interactions at the m/m* interface by which the cytoskeleton is bonded to the SC matrix.

3.2.3 Stimuli-Induced and Metabolic Cytoskeleton Formation in SCs

Next, to achieve controlled formation of artificial cytoskeletons inside SCs, we engineer control mechanisms to the DNT system, notably light and strand displacement triggers, as well as a metabolic induction. For that, we move to DNTs formed solely by one tile, and first designed a light-activated tile C (Supplementary Figure 3-14). Tile C has sticky ends that are self-complementary, inhibited by a hairpin at one of its sticky ends (Figure 3-3a), that contains a photo-cleavable linker (PCL) in its loop. The design utilizes an important principle of DNA duplex stability: A double-stranded DNA (dsDNA) in a hairpin structure is thermally more stable than the same dsDNA formed by two ssDNAs. We designed the hairpin to have 5 base pairs (bp) in its stem region with a melting temperature (T_m) around 60 °C. The same dsDNA formed by corresponding ssDNA is however unstable even down to 0 °C (Supplementary Figure 3-14). Hence, without UV illumination, the hairpin is stable and inhibits the sticky end interactions. Once the DNA tiles are exposed to UV-light, the PCL is cleaved and the resulting 5 bp dsDNA becomes thermally unstable and melts. This activates the DNA tiles for self-assembly into fibrils. To realize light-activated formation of artificial cytoskeletons inside the SCs, we charged SCs with 10 nM tile C, followed by illumination at 365 nm. As expected, artificial cytoskeletons gradually form over time (Figure 3-3b and Supplementary Figure 3-8c). In contrast, a control without illumination only shows small aggregates after 14 days, likely caused by ambient light pollution over long experimental time (Supplementary Figure 3-15).

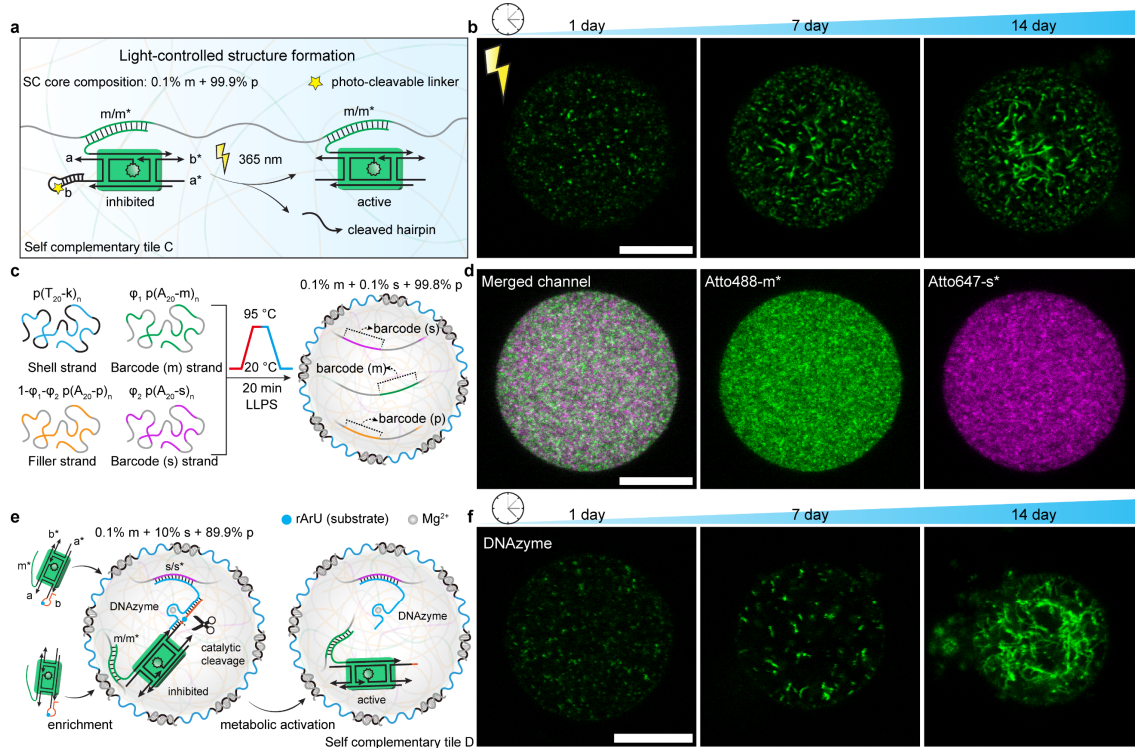


Figure 3-3. Light-triggered and DNAzyme catalyzed metabolic formation of artificial cytoskeletons inside synthetic cells. **a**, Schematic representation for the design of tile C and light-controlled growth of artificial cytoskeletons in SCs. **b**, Time series CLSM images showing formation of artificial cytoskeletons by tile C triggered by UV illumination inside a SC with $[m] = 400 \text{ nM}$. Experiment temperature = 20 $^{\circ}\text{C}$. **c**, Schematic representation for the preparation of SCs containing 3 core barcodes (m, p, s). **d**, Representative CLSM images showing a SC containing 99.8% p (400000 nM), 0.1% m (400 nM), and 0.1% s (400 nM) barcodes addressed by Atto488-m* and Atto647-s*. **e**, Schematic representation for the design of tile D and DNAzyme catalyzed metabolic formation of artificial cytoskeletons in SCs. **f**, Time series CLSM images showing formation of artificial cytoskeletons by tile D catalyzed by DNAzyme inside a SC with $[m] = 400 \text{ nM}$. Experiment temperature = 20 $^{\circ}\text{C}$. Scale bars are 10 μm .

For integration of multiple structural units and metabolic functions within a single SC, we considered to generalize our *co-phase separation* approach to a ternary system. For that, we designed and synthesized an additional adenine-rich ssDNA polymer with a new barcode, namely, $p(A_{20-s})_n$ (Supplementary Figure 3-1). Applying the same *co-phase-separation* strategy, we mixed $p(A_{20-p})_n$, $p(A_{20-m})_n$, $p(A_{20-s})_n$, and $p(T_{20-k})_n$ together, and prepared SCs via a temperature ramp (Figure 3-3c). The majority of the SC core is $p(A_{20-p})_n$ (99.8%), whereas 0.1% $p(A_{20-m})_n$ and 0.1% $p(A_{20-s})_n$ are homogeneously distributed at 400 nM each. $p(T_{20-k})_n$ stabilizes the shell. CLSM confirms the successful encapsulation and homogenous distribution of both m and s barcodes inside a single SC (Figure 3-3d).

We then designed a metabolic SC that can achieve metabolic structure formation by embedding a DNAzyme^{60,61} via the s barcodes. The DNAzyme is engineered to recognize the hairpin of a protected DNA tile D containing two RNA point mutation (rArU; Figure 3-3e, Supplementary Figure 3-16). Tile D has an affinity via the orthogonal m barcodes. Indeed, once the DNAzyme and tile D meet in the same SC, the DNAzyme cleaves the hairpin at the RNA mutation and enables artificial cytoskeleton formation inside SCs by melting off of the protective hairpin part (Figure 3-3f and Supplementary Figure 3-8d).

The ternary barcode SC system also enables the assembly of orthogonal cytoskeleton mimics within the same entity, similar as real cells can organize distinct fibrillar structures. As a proof of concept, we introduce a second one-tile DNT system based on tile E with a dangling strand that binds to the s barcode (Supplementary Figure 3-17). This tile is inhibited by an inhibitor strand at one of its sticky ends and can be activated by an activator strand via a DNA strand displacement (DSD) reaction to initiate artificial cytoskeleton growth (Figure 3-4a). After charging the SCs with both the light-activatable tile C and the DSD-switchable tile E, orthogonal triggers selectively control the formation of two different artificial cytoskeletons in the same compartment (Figure 3-4b and Supplementary Figure 3-8b). As expected, the sole formation of green cytoskeletons by tile C is visible after applying light stimulation. In contrast, only magenta cytoskeletons form when the appropriate activator strand is provided for the DSD reaction. Furthermore, the DSD-activated cytoskeletons can even be deconstructed by adding a deactivator strand, which displaces the activator strand and sets free the inhibitor strand to bind to the sticky ends for disassembly of the whole structure. Applying both stimuli at the same time results in formation of two orthogonal artificial cytoskeletons in the same SCs.

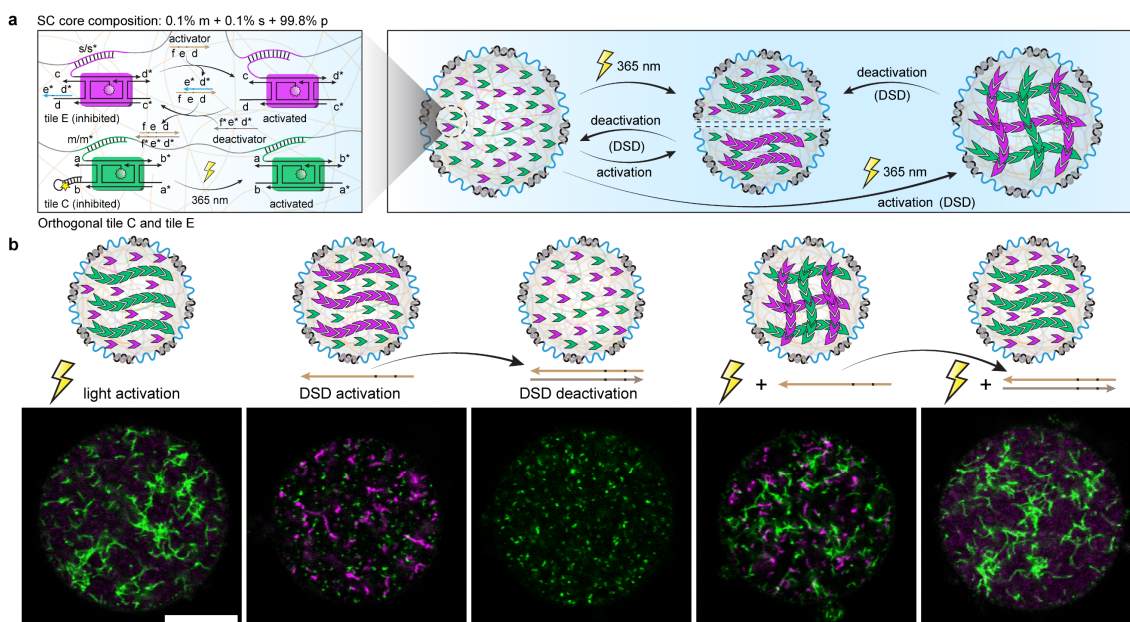


Figure 3-4. Formation of dual artificial cytoskeletons in synthetic cells enabled by multiple barcodes and orthogonal stimuli pathways. **a**, Schematic representation for the enrichment of tile C and tile E in SCs by individual barcodes (m and s), which can be orthogonally triggered by different stimuli to form and deconstruct artificial cytoskeletons. **b**, Schemes and representative CLSM images showing the formation and deconstruction of artificial cytoskeletons by light and DSD triggers inside SCs containing 400 nM m and 400 nM s barcodes. Green channel for Atto488 (tile C) and magenta channel for Atto647 (tile E). Experiment temperature = 20 °C. Scale bar is 10 μ m.

3.2.4 Programmable Selectivity in Diverse SC Communities

Finally, we hypothesized that the precise selectivity for enrichment in our SCs can be exploited on a system level to generate different populations of cytoskeleton-mimic-containing SCs in the same system by selective enrichment in a pool of different DNT building blocks. To this end, we first synthesized a series of $p(T_{20-k})_n$ with different in-chain fluorophores to label the SC shells (Supplementary Figure 3-1). Next we prepared four different SCs, where the differently labeled $p(T_{20-k})_n$ shells serve as identifiers for SCs containing different barcodes in the core for enrichment of different DNT systems: (i) cyan-shell SCs without any addressable barcode to enrich DNT tiles as control, (ii) green-shell SCs with the s barcode to enrich tile E for DSD activation, (iii) magenta-shell SCs with the m barcode to enrich tile C with the PCL, and (iv) yellow-shell SCs with the m and s barcodes together.

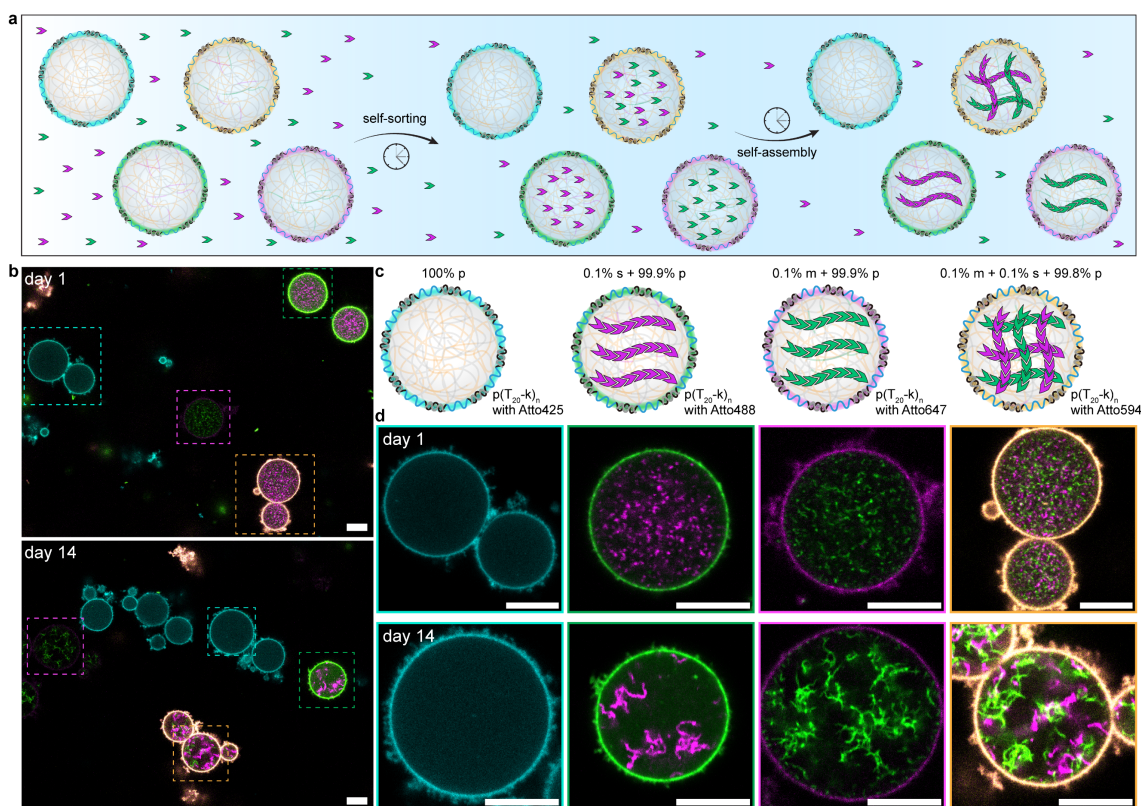


Figure 3-5. Selective uptake and growth of artificial cytoskeletons in differentiated synthetic cells from a species pool of cytoskeletal building blocks. **a**, Schematic representation of different DNA tiles self-sorting into various SCs inside a common pool based on programmed molecular recognition interaction and their subsequent self-assembly in defined compartments. **b**, Overview CLSM images showing selective uptake of DNA tiles and subsequent growth of artificial cytoskeletons (by tile C and tile E) in SCs containing different barcodes, which can be identified by their distinct shell labels. **c**, Schematic representation of SCs having different barcodes, with their shells labeled by $p(T_{20-k})_n$ with different in-chain fluorophores. **d**, Corresponding zoomed-in CLSM images of SCs in the dashed boxes in **b** for day 1 and day 14. CLSM image channel assignment: Atto425 (cyan channel), Atto488 (green channel), Atto 594 (yellow channel), Atto647 (magenta channel). Experiment temperature = 20 °C. Scale bars are all 10 μ m.

Subsequently, we mixed all different SCs and added tile C and tile E in the same solution (Figure 3-5a–d). Indeed, the SCs accumulate their corresponding tiles selectively, and, after triggering

with the appropriate stimuli, self-sorted populations occur. The cyan-shell SCs containing only the p barcode do not show enrichment of any tiles and thus no cytoskeleton. The SCs with s or m barcode enrich their corresponding tiles E or tile C, respectively, which grow into artificial cytoskeletons in the distinct SCs over time. The SCs with both m and s barcode enrich both tiles and form two different kinds of cytoskeletons inside the same compartment. This demonstrates ultimately the strength of the co-phase-separation approach for barcoding individual populations, and for reaching selectivity in a diverse community of synthetic cells.

3.2.5 Mechanical Properties of SCs and Durable SC-Cell Contacts

After establishing various control mechanisms for growing artificial cytoskeletons inside SCs, we further asked to what extent the DNT cytoskeleton indeed endow SCs with functional benefits regarding mechanical properties and stability. Studying the functional properties is an important aspect for transitioning the field of SCs from control over structure to functional molecular systems and ultimately applications. Similar to how cytoskeletons endow cells with mechanical strength¹⁰, the artificial cytoskeletons formed in our SCs also significantly enhance their elastic modulus by about two orders of magnitude compared to pristine SCs, as probed by atomic force microscopy (AFM) with a colloid probe⁶² (Figure 3-6a–c). Interestingly, the compression-retraction force cycle for the pristine SCs without DNT cytoskeletons displays a hysteresis that signifies viscous energy dissipation and liquid-like properties. In contrast, SCs with DNT cytoskeletons exhibit largely elastic behavior without hysteresis.

The enhanced elastic modulus reaches the level of real cells reported in literature⁶³, which opens possibilities for further exploring interactions between our SCs with real mammalian cells by building an interface to the mechanosensory system of cells. To this end, we synthesized a ssDNA-cRGD conjugate (r^* -cRGD) based on our previously reported method and immobilized it to the SC shell via the k barcode using Atto425-k^{*}-r/ r^* -cRGD binding⁶⁴. cRGD is a peptide motif that can be recognized and bound by integrin of cells⁶⁵. When incubating SCs with HeLa cells (green cell mask stain visualizes the plasma membrane), a marked deformation of SCs in contact with the cells can be observed, confirming a tight bonding as expected. This deformation is similar for both types of SCs regardless of whether they contain a cytoskeleton or not (Figure 3-6d, e). A slight tendency can be observed towards a bit more corrugated interface in absence of the cytoskeleton. However, while the cytoskeleton-containing SCs retain their content ($p(A_{20}-p)_n$), SCs without a cytoskeleton leak some of their interior. The leakage is significant and can be traced by a reduction of the yellow fluorescence in the interior that arises from the $p(A_{20}-p)_n$, labeled by Atto565-p^{*}, that constitutes the majority of the SC core (Figure 3-6d–g). This beneficial mechanical durability is because all artificial cytoskeletons grown within the SC are strongly bound to the core matrix, so that the mechanical property of these cytoskeletons can be tethered and coupled to the SC core. This represents a distinct functional benefit for building communicative mechano-SC-cell interfaces in the future.

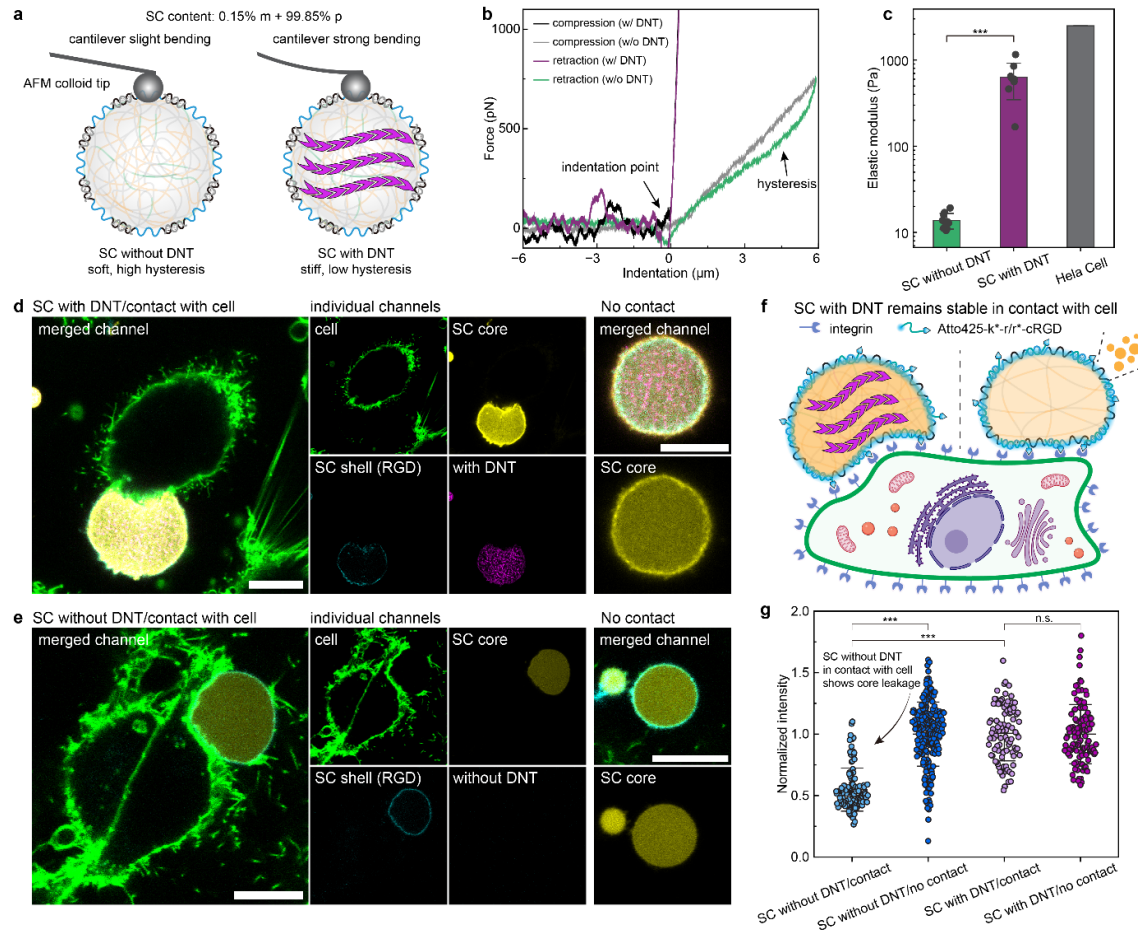


Figure 3-6. Artificial cytoskeleton enhances SC stiffness and stabilizes SC during SC-mammalian cell contact. **a**, Schematic representation for the SC stiffness probed by colloid probe AFM. **b**, Representative force-distance curves in a full compression-retraction cycle for SCs with and without DNT cytoskeleton. SCs with DNT cytoskeleton show elastic behavior without hysteresis, whereas SCs without DNTs show more viscous behavior with marked hysteresis in the compression/retraction cycle. **c**, Comparison of elastic moduli of SCs with and without a DNT cytoskeleton, and compared to HeLa cells reported in literature⁶³. **d**, Representative CLSM images in different channels for SC (with DNT)-mammalian cell contact and no contact control (HeLa cells; cell mask stain visualizes the plasma membrane). **e**, Representative CLSM images in different channels for SC (without DNT)-mammalian cell contact and no contact control. High cell viability confirmed in Supplementary Figure 3-18. **f**, Schematic representation for SCs with and without DNT in contact with cell. The SC without DNT becomes unstable and show leakage when in contact with cell. **g**, Quantification of leakage of SC with and without DNT in contact with cell, indicated by the normalized intensity of SC core. CLSM image channel assignment: Cell membrane (green channel), SC shell (cyan channel), SC core (yellow channel), DNT (magenta channel). $n = 8$ for SC with DNT and $n = 10$ for SC without DNT in **c**, $n > 90$ for **g**. Error bars represent standard deviation. DNTs were firstly grown in SCs at 20 °C. The SC-cell co-incubation were performed at 25 °C with 5 % CO₂. Scale bars are all 10 μ m.

3.3 Conclusions

We have introduced a versatile SC system that allows to study the assembly of subcellular-SC structures in viscoelastic environment, as exemplified for the formation of artificial cytoskeletons. We introduced *co*-phase-separation as a molecular engineering approach to

embed multiple orthogonal ssDNA barcodes inside the SCs to allow for selective enrichment of self-assembling species. In contrast to classical crowding conditions, such as in dextran solutions³⁹, the DNT building blocks and the arising DNTs remain tethered and hence coupled to the viscoelastic interior of the SCs. The growth of DNA tiles into extended fibers depends on the enriched tile concentration and the process is much slower than in plain solution due to the coupling with the DNA polymer dynamics. On a mechanistic level, in contrast to classical crowding, bundling of the DNTs does not occur. Additionally, the cytoskeleton growth rather proceeds by end-to-end joining of small fibrillar nuclei and not by tile addition commonly observed in solution⁵⁷⁻⁵⁹. We note that the current DNT assembly system used in our study only proceeds on a certain timescale of its own, and it can therefore also only couple to one particular timescale regime of the viscoelastic matrix (here in our case, the terminal flow regime for uncrosslinked SCs). Nonetheless, by tuning the viscoelastic property of the SC matrix via crosslinking, temperature, and concentration, we were able to modify the dynamics of the coupled SC matrix and influence the DNT assembly kinetics. In the future, it will be intriguing to explore how a more sophisticated assembly system, involving multiple kinetic processes across different timescales, would interact with various relaxation regimes of the viscoelastic environment and exhibit behaviors distinct from those observed in a simple solution.

With ternary barcode SC systems, different stimuli pathways, and orthogonal tiles, we further realized metabolic growth of cytoskeleton inside SCs, as well as controlled formation and deconstruction of multiple artificial cytoskeletons inside the same SC. This is a step forward to mimic real cells that can organize multiple cytoskeletons in response to different stimuli or even in a metabolic fashion³⁵. Moreover, we also showed at a system level how SCs can selectively enrich desired monomers from a common pool in a diverse SC community to form distinct structures. Finally, artificial cytoskeletons enhance the SC stiffness and lead to stability when in contact with real mammalian cells via a strong mechano-contact brought about by cRGD/integrin binding. To our knowledge, this also constitutes the first mechano-interface between cells and SCs.

In a wider perspective, in contrast to classical SCs made by liposomes and polymersomes with plain water compartments^{25,39}, or coacervates with certain level of crowding^{32,66,67}, our SC represents a step forward to mimic real cells featuring not only macromolecular crowding, but also viscoelastic environment, programmable uptake, and controlled local enrichment. These features are ubiquitous in biological and living systems, but have been rare in synthetic cells. Our SC can thus serve as a modular molecular systems engineering platform for studying self-assembly and structure formation under cell-like environments. In addition, our co-phase-separation approach endows SC systems with highly flexible programmable barcodes in their cytoplasm-mimetic matrix, which can be modularly utilized to design and functionalize SCs with higher degree of complexity and functionality. The selective enrichment behavior of our barcoded SCs in SC communities also opens simple pathways to establish communication, controlled targeting, and programmable signaling. It can for instance be applied in DNA-based

computing, circuits, and diagnostics. In addition, our SC-mammalian cell contact study also serves as a starting point for further exploration of SC interactions with biological systems. To summarize, we anticipate that this work provides a new perspective for understanding self-assembly processes in cell-mimicking environments, for the development of interactive SC protoecologies, and for future investigation and design of SC-mammalian cell interactions.

3.4 Experimental

3.4.1 Materials

Single-stranded DNA (ssDNA) were purchased from Biomers and Integrated DNA Technologies (IDT). Supplementary Table 3-2 and Supplementary Table 3-3 summarize all sequences used in this study. T4 DNA Ligase (2 U/ μ L), Exonuclease I (40 U/ μ L), Exonuclease III (200 U/ μ L), and Φ_{29} polymerase (10 U/ μ L) were purchased from Lucigen. Thermal stable inorganic pyrophosphatase (2 U/ μ L), Avall restriction enzyme (10 U/ μ L), and nuclease-free water were bought from New England BioLabs (NEB). Deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP) (100 mM), aminoallyl-dUTP-ATTO-425 (1 mM), aminoallyl-dUTP-XX-ATTO-488 (1 mM), aminoallyl-dUTP-XX-ATTO-594 (1 mM) and aminoallyl-dUTP-ATTO-647N (1 mM) were purchased from Jena Bioscience. Hexadecane, sodium chloride, magnesium chloride, Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl, pH=8), Trizma base, acetic acid and Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and Fetal bovine serum (FBS) were purchased (as bioreagent grade if available) from Sigma-Aldrich. Agarose Low EEO was purchased from AppliChem. RNase-free TE buffer (Invitrogen, 10 mM Tris and 1 mM EDTA, pH 8.0, 500 mL), RNase Inhibitor (40 U/ μ L), SYBR gold, GeneRuler 50 bp DNA Ladder, GeneRuler 1 kb DNA Ladder, and Duke Standards dry borosilicate glass microspheres ($18.2 \pm 1 \mu\text{m}$) were purchased from ThermoFisher Scientific. 96 well μ -plates were purchased from ibidi. 384 well high-content imaging glass bottom microplates were purchased from Corning. Tipless AFM probes cantilevers (HQ:NSC36/tipless/No Al) with 3 different AFM cantilevers with spring constants between 0.6 N/m and 2.0 N/m were purchased from MikroMasch. HeLa cell lines was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Cyclo[Arg-Gly-Asp-d-Phe-Lys(PEG-PEG-azide)] (cRGDfK-N₃; RGD-3759-PI) was purchased from Biosynth. Cell Eagle's Minimum Essential Medium (MEM), MEM-NEAA solution and phosphate-buffered saline (PBS) were purchased from CARL ROTH. Penicillin-Streptomycin and insulin-Transferrin-Selenium (ITS -G) were obtained from Gibco. CellMask™ green plasma membrane stain and Live/dead™ viability/cytotoxicity assay kit (green/deep red) were purchased from Invitrogen.

3.4.2 Instruments

All thermal annealing and heating ramps were performed on TPersonal Thermocycler (Analytik Jena) or Thermocycler Nexus GX2 (Eppendorf). Incubation was carried out on an

Eppendorf ThermoMixer C. DNA concentrations were determined by a ScanDrop (Analytik Jena) or a DS-11 Spectrophotometer (DeNovix). UV illumination was performed by BioLED Light Source Control Module (Mightex). Confocal laser scanning microscopy (CLSM) was performed on a Leica Stellaris 5. Structured illumination microscopy was performed on a ZEISS Elyra 7 with Lattice SIM². AFM colloid probes were fabricated by attaching glass beads to the tipless AFM cantilevers using a three-axis oil hydraulic micromanipulator (MMO-203 from Narishige) under a bright field optical microscope (Zeiss Axiotech). AFM measurements were performed using a JPK SPM NanoWizard III with a liquid sample cell on top of an inverted microscope stage (Zeiss Axiovert). The cells were cultured in MCO-170AIC CO₂ Incubator (PHC Holdings Corporation). Cell viability experiments were performed on an EVOS M7000 (Invitrogen). Gel electrophoresis was performed in a gel chamber from Biostep.

3.4.3 Methods

Synthesis of circular ssDNA templates and long ssDNA polymers

Synthesis of circular DNA template and its corresponding ssDNA polymer is adapted from our previous reports (Supplementary Figure 3-1)^{19,54}. The linear ssDNA template and the corresponding ligation strand were firstly mixed at concentration of 1 μ M in 100 μ L TE buffer containing 100 mM NaCl. The solution was heated to 85 $^{\circ}$ C (3 $^{\circ}$ C/s) for 5 min before cooling to 25 $^{\circ}$ C (0.01 $^{\circ}$ C/s) for complete hybridization. Afterwards, 20 μ L of 10 $\times$ Ligase buffer (500 mM Tris-HCl, 100 mM MgCl₂, 50 mM dithiothreitol and 10 mM ATP (Lucigen)), 70 μ L of nuclease-free water and 10 μ L of T4 DNA Ligase (2 U/ μ L (Lucigen)) were introduced into the reaction mixture and gently mixed before leaving at room temperature for 3 h. The reaction mixture was then heated to 70 $^{\circ}$ C for 20 min to deactivate the enzyme. Then, 10 μ L of Exonuclease I (40 U/ μ L (Lucigen)) and 10 μ L of Exonuclease III (200 U/ μ L (Lucigen)) were added into the reaction mixture to react overnight at 37 $^{\circ}$ C for degradation of the ligation strands and any non-circularized templates in solution. Afterwards, the reaction mixture was heated to 80 $^{\circ}$ C for 40 min to deactivate the enzymes. To obtain the final circular ssDNA templates, the reaction mixture was washed by adding 400 μ L TE buffer and filtrated using Amicon Ultra-centrifugal filters with a 10 kDa cut-off (Merck Millipore) for three times. The concentrations of the collected circular ssDNA templates were measured by the DS-11 Spectrophotometer (DeNovix), and the templates were stored in TE buffer at -20 $^{\circ}$ C.

For synthesis of long ssDNA polymers, we used rolling circle amplification (RCA). 5 μ L of circular template (1 μ M in TE buffer) and 1 μ L of exonuclease resistant primer (10 μ M in TE buffer) were mixed with 76 μ L nuclease-free water, 10 μ L of commercial 10 $\times$ polymerase buffer (500 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 40 mM dithiothreitol, 100 mM MgCl₂ (Lucigen)), 2 μ L of Φ ₂₉ DNA polymerase (10 U/ μ L (Lucigen)), 1 μ L of thermal stable inorganic pyrophosphatase (2 U/ μ L (NEB)) and 5 μ L of adjusted deoxyribose nucleoside 5'-triphosphate mix (100 mM, the mix contains pure dATP, dTTP, dCTP, and dGTP solutions mixed in corresponding proportions of the exact composition of the desired ssDNA polymer repeating

units (Jena Bioscience)). Note that for synthesis of ssDNA polymers with in-chain fluorophores, we further add 0.017 mM dUTP with desired fluorescent labels (aminoallyl-dUTP-ATTO-425, aminoallyl-dUTP-XX-ATTO-488, aminoallyl-dUTP-XX-ATTO-594, or aminoallyl-dUTP-ATTO-647N) for random insertion of the dye along the ssDNA chains during RCA. The reaction mixture was kept at 30 °C for 50 – 60 h before thermal cleavage at 95 °C for 15 min to shorten the ultrahigh molecular weight ssDNA. The final products were purified by rinsing with 400 μ L TE buffer and filtration in Amicon Ultra-centrifugal filters with 30 kDa cut-off (Merck Millipore) for three times. The concentrations of the collected final ssDNA polymers were measured using the DS-11 Spectrophotometer (DeNovix), and the DNA polymers (cleaved at 95 °C for 15 min) were stored in TE buffer at -20 °C.

Preparation of DNA synthetic cells via co-phase separation

Preparation of DNA SCs containing only p barcode is adapted from our previous reports with modifications for the formation of larger DNA SCs (Supplementary Figure 3-1)^{19,54}. $p(A_{20}-p)_n$ (0.5556 g/L) and $p(T_{20}-k)_n$ (0.0694 g/L) were mixed in TE buffer without any salt at a final volume of 9 μ L. The solution mixture was then heated at 95 °C for 15 min (in addition to the first thermal cleavage during ssDNA synthesis mentioned above) for thermal cleavage to further reduce the chain length of the ssDNA polymers. Afterwards, 1 μ L of TE buffer containing 500 mM $MgCl_2$ was introduced into the reaction mixture. The solution containing finally 0.5 g/L $p(A_{20}-p)_n$ and 0.0625 g/L $p(T_{20}-k)_n$ with 50 mM $MgCl_2$ was heated to 95 °C for 20 min (to induce $p(A_{20}-p)_n$ phase separation) and subsequently cooled down to room temperature, during which the $p(T_{20}-k)_n$ localized as shell on the periphery of the $p(A_{20}-m)_n$ core by A_{20}/T_{20} hybridization. The heating and cooling rates were both 3 °C/s. Finally, the 10 μ L solution containing the core-shell DNA SCs was diluted 5 times by adding 40 μ L TE buffer containing various amounts of $MgCl_2$ to reach desired salinity, e.g., 15 or 50 mM of $MgCl_2$. The obtained 50 μ L DNA SCs solution (as 5 times diluted) has 0.1 g/L $p(A_{20}-p)_n$ and 0.0125 g/L $p(T_{20}-k)_n$, corresponding to ca. 8 μ M p barcode and ca. 1 μ M k barcode, respectively, in total solution. The solution was then stored in a fridge at 4 °C for 1 week for equilibration. Before usage, the DNA SCs solution was always slightly vortexed to redisperse the DNA SCs homogenously in the solution.

To make SCs containing other barcodes (m, s, p, c) at different concentrations (from 400 to 40000 nM), certain fraction of the $p(A_{20}-p)_n$ was replaced correspondingly by $p(A_{20}-m)_n$, $p(A_{20}-s)_n$, and $p(A_{20}-c)_n$, while maintaining the total concentration of $p(A_{20}-x)_n$ ssDNA ($x = m, s, p, c$) constant at 0.5 g/L. The successful integration of the desired barcodes in the SCs was then confirmed by CLSM (Figure 3-2b and Figure 3-3d).

The DNA polymers (cleaved for 30 min at 95 °C) used to prepare the DNA SCs were quantified by gel electrophoresis performed by 2 wt% agarose gel under 7 V/cm for 150 min in TAE buffer containing 40 mM Tris-HCl, 20 mM acetic acid and 1 mM EDTA (Supplementary Figure 3-1). Post staining by SYBR gold was used to stain the DNA polymers. Quantification of chain length

distribution and average chain length of DNA polymers was derived from the gel electrophoresis result based on previously reported method^{68,69}.

Preparation of DNA tiles and DNA nanotubes in solution

DNA tiles design was adapted from previous reports with modifications⁴². The DNA tiles were prepared by heating the solution containing 5 μM of each of the needed strands (Supplementary Table 3-3) with 1 $\times$ TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and 12.5 mM MgCl_2 to 90 $^\circ\text{C}$ for 5 min and then cooling down to 20 $^\circ\text{C}$ (0.2 $^\circ\text{C}/\text{min}$) over a total time of ca. 6 h. The formed DNA tile solutions were stored at -20 $^\circ\text{C}$ for further experiments. To assemble DNTs of tile A and tile B in solution, 250 nM tile A and 250 nM tile B were mixed in 20 μL solution containing 1 $\times$ TAE buffer and 12.5 mM MgCl_2 . During assembly at different time, 1 μL reaction mixture was taken and diluted into 10 μL solution containing 1 $\times$ TAE buffer and 12.5 mM MgCl_2 for CLSM imaging (Supplementary Figure 3-3). To assemble DNAzyme-catalyzed DNTs of tile D in solution, 500 nM tile D, 5 μM DNAzyme, and 8 U/ μL RNase Inhibitor were mixed in 20 μL solution containing 1 $\times$ TAE buffer and 12.5 mM MgCl_2 . During assembly at different time, 2 μL reaction mixture was taken and diluted into 20 μL solution containing 1 $\times$ TAE buffer and 12.5 mM MgCl_2 for CLSM imaging.

Formation of artificial cytoskeletons in synthetic cells

For DNT formation by tile A and tile B in SCs with different m barcode concentrations (Figure 3-2), 1 μL of 5 $\times$ diluted SCs containing specific m barcode concentrations (at 15 mM MgCl_2), 2 μL of 100 nM tile A and 2 μL of 100 nM tile B were added to 15 μL TE buffer containing 15 mM MgCl_2 and gently mixed in a 200 μL transparent PCR tube. The solution mixture was then transferred to a 384 well plate, and covered with 20 μL of hexadecane to prevent evaporation. The sample was incubated at different temperatures (20 – 35 $^\circ\text{C}$) and imaged by CLSM over time.

For light-activated artificial cytoskeleton formation in SCs (Figure 3-3a, b), 1 μL of the 5 $\times$ diluted SCs (at 15 mM MgCl_2) containing 400 nM m barcode and 2 μL of 100 nM tile C with a photo-cleavable hairpin were added to 17 μL TE buffer containing 15 mM MgCl_2 and gently mixed in a 200 μL transparent PCR tube. The sample in the PCR tube was incubated at room temperature for roughly 30 min for tile enrichment and then illuminated with a 365 nm LED light for 5 min with an intensity of 3.5 mW, before transferring to a well of a 384 well plate, and subsequently covered with 20 μL of hexadecane to prevent evaporation. The sample was incubated at 20 $^\circ\text{C}$ and imaged by CLSM over time to track the temporal evolution of the formed artificial cytoskeleton inside SCs.

For DNAzyme-catalyzed artificial cytoskeleton formation in SCs (Figure 3-3e, f), 1 μL of the 5 $\times$ diluted SCs ([m] = 400 nM, [s] = 40000 nM, at 15 mM MgCl_2), 2 μL of 100 nM tile D, 2 μL of 40 U/ μL RNase Inhibitor, 0.9 μL of 800 nM DNAzyme were added to 14.1 μL TE buffer containing 15 mM MgCl_2 and gently mixed in a 200 μL transparent PCR tube. The solution mixture was

then transferred to a well of a 384 well plate, and covered with 20 μ L of hexadecane to prevent evaporation. The sample was incubated at 20 °C and imaged by CLSM over time.

For the formation of orthogonal artificial cytoskeletons inside the SCs (Figure 3-4), 1 μ L of the 5 $\times$ diluted SCs (at 15 mM $MgCl_2$) containing 400 nM m barcode and 400 nM s barcode, 2 μ L of 100 nM tile C and 2 μ L of 100 nM tile E were added to 15 μ L TE buffer containing 15 mM $MgCl_2$ and gently mixed in a 200 μ L transparent PCR tube. For the light trigger, we applied 365 nm LED light illumination for 5 min with intensity of 3.5 mW. For the DSD trigger, we used 50 nM activator (2.5 $\times$ over-stoichiometric to the inhibitor) to trigger the assembly of the tiles. The sample was incubated at 20 °C and imaged by CLSM over time to track the temporal evolution of the formed artificial cytoskeletons inside SCs. After 7 days of assembly, we added 100 nM deactivator (2 $\times$ over-stoichiometric to the activator) to deconstruct the formed artificial cytoskeletons by tile E.

For selective formation of distinct artificial cytoskeletons inside individual SCs having different barcodes within a SC community (Figure 3-5), 1 μ L of each 5 $\times$ diluted SCs containing different barcodes (blue-shell, magenta-shell, green-shell, and yellow-shell SCs, at 15 mM $MgCl_2$), 2 μ L of 100 nM tile C and 2 μ L of 100 nM tile E were added to 12 μ L TE buffer containing 15 mM $MgCl_2$ and gently mixed in a 200 μ L transparent PCR tube. For the light trigger, we applied 365 nm LED light illumination for 5 min with intensity of 3.5 mW. For the DSD trigger, we used 50 nM activator (2.5 $\times$ over-stoichiometric to the inhibitor) to trigger the assembly of the tiles. The sample was incubated at 20 °C and imaged by CLSM over time to track the temporal evolution of the formed artificial cytoskeletons inside different SCs.

Fluorescence recovery after photobleaching experiments

We performed FRAP experiment with a Leica Stellaris 5 on light-activated artificial cytoskeletons formed by tile A and tile B (0.1 %, 1% and 10%) inside the SCs after 20 days of growth to probe dynamic properties of the artificial cytoskeletons (Supplementary Figure 3-6). A SC containing artificial cytoskeletons labeled by Atto488 was firstly imaged with low laser intensity 5 times (2 s/frame) as pre-bleaching images, before 10 times bleaching by 100% laser intensity in a rectangular region of interest (ROI) covering half of the SC to completely bleach the artificial cytoskeletons inside. Then, post-bleaching images were manually recorded at different times over 48h.

We also performed FRAP on various SCs (crosslinked, uncrosslinked, cleaved, etc.) to probe the internal dynamics of the SCs (Supplementary Figure 3-10, Supplementary Figure 3-11, and Supplementary Figure 3-12). SCs labeled by Atto488-m* attached to the m barcode were firstly imaged with low laser intensity 5 times (1 s/frame) as pre-bleaching images, before 5 times bleaching by 100% laser intensity in a circular ROI with a diameter of 2 μ m inside the SC. Then, 120 frames of post-bleaching images were recorded at a rate of 1 s/frame over 120 s. For quantification, we measured intensities within the circular ROI (I_{ROI}), and intensities in a circular ROI away from bleached region within the SCs (I_{ref}), in pre- and post-bleaching images.

We applied double normalization by $I_{\text{Norm}}(t) = \frac{I_{\text{ROI}}(t)}{I_{\text{ROI}}(t_0)} \times \frac{I_{\text{ref}}(t_0)}{I_{\text{ref}}(t)}$, where $I(t_0)$ represents the intensity measured in the first image before bleaching and $I(t)$ is the intensity measured over time.

Reduction of DNA polymer concentration of SC matrix by enzymatic degradation

SCs containing 59.9% p barcode, 40% c barcode, and 0.1% m barcode were prepared with the methods described above. To perform enzymatic degradation, 2 μL of such SCs (5 $\times$ diluted, in TE buffer at 15 mM MgCl_2) was mixed with 0.4 μL of 50 μM c^* and 12.6 μL of TE buffer containing 15 mM MgCl_2 . After 1 h incubation for c/c^* hybridization, 1 μL of Avall enzyme (10 U/ μL) was added to enable sequence specific restriction at c/c^* for another 1 – 2 h. The sample was then used to mix with 2 μL 100 nM tile A and 2 μL 100 nM tile B for growing artificial cytoskeletons inside the SCs.

Structured illumination microscopy measurements

Structured illumination microscopy was conducted at an Elyra7 (ZEISS) using the proprietary ZEN black software for acquisition and data processing (Figure 3-2f). SCs containing 400 nM m barcode with formed DNT inside were imaged in Lattice SIM mode using the following parameters: Objective: Plan-Apochromat 63x/1.4 Oil DIC M27. Beam splitters (primary, secondary): LBF 405/488/561/642, SBS BP 490-560 + LP 640. Magnifying lens: 1.6x. Laser: 642 nm diode, output: 0.5% (ca. 400 μW). Exposure time: 100 ms/phase, 13 phases. SIM grating period: 32 μm . Z-Stack: Leap mode, Z-step 328.7 nm. Pixel size (xy): 62.6 nm. Raw data was processed using the 'SIM' processing function with the following parameters: Algorithm: SIM² 3D Leap. Iterations: 20. Regularization weight: 0.08. Sampling rates (input and output): 2 $\times$. Sectioning: 100. Intensity: Scale to raw.

Atomic force microscopy measurements

Colloid AFM probes were fabricated by attaching borosilicate glass beads ($18.2 \pm 1 \mu\text{m}$) to the tipless AFM cantilever end (spring constant 0.6 N/m) with the help of micromanipulator under a bright field microscope. To perform AFM measurement on SC, we added SCs (99.85% p barcode, 0.15% m barcode) with and without DNT (formed via tile A and tile B) into an AFM liquid cell on a glass slide on top of the stage of the inverted microscope. The AFM colloid probe was then approached to the top of a SC under microscope. The force-distance measurement was then conducted in contact mode. The force spectroscopy data was fitted with the following equation, where F is the force, E is modulus, ν is Poisson's ratio (we used 0.5 here), R is the effective radius calculated by harmonic mean of the radius of the SC (R_{SC}) and the radius of the colloidal probe (R_{probe}), i.e., $R = R_{\text{SC}} \times R_{\text{probe}} / (R_{\text{SC}} + R_{\text{probe}})$ and d is indentation distance:

$$F = \frac{4}{3} \times \frac{E}{1-\nu^2} \times \sqrt{R} \times d^{1.5} \quad (1)$$

according to Hertzian model (sphere-sphere geometry) to yield the modulus. (Figure 3-6a–c).

Synthetic cell-mammalian cell contact and cell viability assay

The HeLa cells were routinely cultured with complete MEM supplemented with 10% FBS (v/v), 1% MEM-NEAA and penicillin/streptomycin (v/v) in an incubator with 5% CO₂ at 37 °C. We used click chemistry to conjugate cRGDfK-N₃ to DBCO-r* for obtaining cRGD-r* based on our reported method⁶⁴. cRGD-r* was further annealed with Atto425-k*-r to form Atto425-k*-r/r*-cRGD complex by heating up to 90 °C for 3 min (3 °C/s), which was then slowly cooled down to 20 °C (0.02 °C/s). SCs with DNT inside were labeled on their shell by mixing SCs with Atto425-k*-r/r*-cRGD complex. The ratio between Atto425-k*-r/r*-cRGD and the k barcode in the SC shells was always kept at 80% to avoid free cRGD in solution.

For the SC-mammalian cell contact experiment, the HeLa cells were firstly incubated in a 96 well plate with complete MEM culture medium (10 % FBS) for 15 h for adhering to the well surface, then the complete medium was replaced with adapted MEM medium which contains 5 % FBS, 1× ITS-G, 100 µg/mL actin, and 12.5 mM MgCl₂. Then, we added SCs with cRGD on the shell to the prepared Hela cells in the well for co-incubation at 25 °C with 5 % CO₂ for 3 h. Before CLSM study, the cells were stained by cell mask green plasma membrane dye (500× diluted) for 10 min.

To verify cell viability during co-incubation with SCs, we prepared independent Hela cells with the same method described above. We then performed Live/Dead Viability/Cytotoxicity Assay with 2000× dilution factor for the Calcein, AM and SYTOX™ Deep Red Nucleic Acid Stain, according to the manufacturer's instructions, and imaged the cells under CLSM and EVOS microscope (Supplementary Figure 3-18).

Statistical analysis

All data points featuring statistical analysis are presented as mean ± standard deviation. The sample size (*n*) for each statistical analysis is reported in the relevant figure legend. *p* values are calculated by Anova test (single factor) with ****p* ≤ 0.001, ***p* ≤ 0.01, **p* ≤ 0.05. Statistical analysis was carried out using Origin 2023 and Microsoft Excel.

3.4.4 Supplementary Tables

	SC composition	Starting materials	[m] or [s] in SC	[m] or [s] in solution (100× diluted) ¹	[DNA tile] in solution	Stoichiometry ([DNA tile] / [m] in solution)
Fig. 1	10% m 90% p	0.05 g/L p(A ₂₀ -m) _n 0.45 g/L p(A ₂₀ -p) _n	40000 nM [m]	40 nM [m]	10 nM tile A 10 nM tile B	0.5
	5% m 95% p	0.025 g/L p(A ₂₀ -m) _n 0.475 g/L p(A ₂₀ -p) _n	20000 nM [m]	20 nM [m]	10 nM tile A 10 nM tile B	1
	1% m 99% p	0.005 g/L p(A ₂₀ -m) _n 0.495 g/L p(A ₂₀ -p) _n	4000 nM [m]	4 nM [m]	10 nM tile A 10 nM tile B	5
	0.5% m 99.5% p	0.0025 g/L p(A ₂₀ -m) _n 0.4975 g/L p(A ₂₀ -p) _n	2000 nM [m]	2 nM [m]	10 nM tile A 10 nM tile B	10
	0.4% m 99.6% p	0.002 g/L p(A ₂₀ -m) _n 0.498 g/L p(A ₂₀ -p) _n	1600 nM [m]	1.6 nM [m]	10 nM tile A 10 nM tile B	12.5
	0.2% m 99.8% p	0.001 g/L p(A ₂₀ -m) _n 0.499 g/L p(A ₂₀ -p) _n	800 nM [m]	0.8 nM [m]	10 nM tile A 10 nM tile B	25
	0.1% m 99.9% p	0.0005 g/L p(A ₂₀ -m) _n 0.4995 g/L p(A ₂₀ -p) _n	400 nM [m]	0.4 nM [m]	10 nM tile A 10 nM tile B	50
	0.05% m 99.95% p	0.00025 g/L p(A ₂₀ -m) _n 0.49975 g/L p(A ₂₀ -p) _n	200 nM [m]	0.2 nM [m]	10 nM tile A 10 nM tile B	100
	0.025% m 99.975% p	0.000125 g/L p(A ₂₀ -m) _n 0.499875 g/L p(A ₂₀ -p) _n	100 nM [m]	0.1 nM [m]	10 nM tile A 10 nM tile B	200
	0.1% m 99.9% p	0.0005 g/L p(A ₂₀ -m) _n 0.4995 g/L p(A ₂₀ -p) _n	400 nM [m]	0.4 nM [m]	10 nM tile C	25
Fig. 2	0.1% m 10% s 89.9% p	0.0005 g/L p(A ₂₀ -m) _n 0.05 g/L p(A ₂₀ -s) _n 0.4495 g/L p(A ₂₀ -p) _n	400 nM [m] 40000 nM [s]	0.4 nM [m] 40 nM [s]	10 nM tile D	25
	0.1% m 0.1% s 99.9% p	0.0005 g/L p(A ₂₀ -m) _n 0.0005 g/L p(A ₂₀ -s) _n 0.4995 g/L p(A ₂₀ -p) _n	400 nM [m] 400 nM [s]	0.4 nM [m] 0.4 nM [s]	10 nM tile C 10 nM tile E	25
Fig. 4 ²	100% p	0.5 g/L p(A ₂₀ -p) _n	0 nM	0 nM		
	0.1% m 99.9% p	0.0005 g/L p(A ₂₀ -m) _n 0.4995 g/L p(A ₂₀ -p) _n	400 nM [m]	0.4 nM [m]		
	0.1% s 99.9% p	0.0005 g/L p(A ₂₀ -s) _n 0.4995 g/L p(A ₂₀ -p) _n	400 nM [s]	0.4 nM [s]	10 nM tile C 10 nM tile E	12.5
	0.1% m 0.1% s 99.9% p	0.0005 g/L p(A ₂₀ -m) _n 0.0005 g/L p(A ₂₀ -s) _n 0.4995 g/L p(A ₂₀ -p) _n	400 nM [m] 4000 nM [s]	0.4 nM [m] 0.4 nM [s]		
Fig. 5	0.15% m 99.85% p	0.00075 g/L p(A ₂₀ -m) _n 0.49925 g/L p(A ₂₀ -p) _n	600 nM [m]	0.6 nM [m]	10 nM tile A 10 nM tile B	33.33

¹ SCs were always used in 100× dilution with respect to the starting materials.

² For experiment shown in Fig. 4 in main text, 100× diluted SCs of each type are mixed together, resulting in 0.8 nM m barcode and 0.8 nM s barcode in the solution, with 10 nM tile C and 10 nM tile D added to grow two distinct artificial cytoskeletons.

Supplementary Table 3-1. Details about composition of SCs with varied internal barcode concentrations, barcode concentration in solution, DNA tile concentration in solution, and stoichiometry between DNA tile and barcode in individual experiments.

Chapter 3. Growing Functional Artificial Cytoskeletons in the Viscoelastic DNA SCs

	Name	Sequence (5' → 3')	Purification	Modification	Supplier
Template and RCA	Tp(A ₂₀ -p)	/Phosphate/ATA GTG AGT CGT ATT ATT TTT TTT TTT TTT TTT TTT ATC CCT	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -m)	/Phosphate/ATC TAT CCT AAT TTT TTT TTT TTT TTT TTT TGA ACC CGT AT	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -s)	/Phosphate/CAC AAA CAT AGT TTT TTT TTT TTT TTT TTT TCA ACA AGA GT	HPLC	5'-Phosphorylation	Biomers
	Tp(T ₂₀ -k)	/Phosphate/ATC CTC TAA AAT CAA AAA AAA AAA AAA AAA AAA GTA AAA CCA CAC G	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -c)	/Phosphate/TCG TTC AGA TGT TTT TTT TTT TTT TTT TTT TTG GGG TCC TTA C	HPLC	5'-Phosphorylation	Biomers
	ligation-p	TAA TAC GAC TCA CTA TAG GGA T	HPLC	None	Biomers
	ligation-m	TTA GGA TAG ATA TAC GGG TTC	HPLC	None	Biomers
	ligation-s	CTA TGT TTG TGA CTC TTG TTG	HPLC	None	Biomers
	ligation-k	TTT TAG AGG ATC GTG TGG TTT T	HPLC	None	Biomers
	ligation-c	CAT CTG AAC GAG TAA GGA CCC CA	HPLC	None	Biomers
	primer-p	TAA TAC GAC TCA CTA TAG GG*A*T	Desalting	Phosphorothioated twice (*)	IDT
	primer-m	TTA GGA TAG ATA TAC GGG T*T*C	Desalting	Phosphorothioated twice (*)	IDT
	primer-s	CTA TGT TTG TGA CTC TTG T*T*G	Desalting	Phosphorothioated twice (*)	IDT
	primer-k	TTT TAG AGG ATC GTG TGG TT*T*T	Desalting	Phosphorothioated twice (*)	IDT
	primer-c	CAT CTG AAC GAG TAA GGA CCC*C*A	Desalting	Phosphorothioated twice (*)	IDT
Label, Crosslinker, Degradation, and RGD	Atto488-m*	/ATTO488/TGA ACC CGT ATA TCT ATC CTA A	HPLC	5'-Atto488	Biomers
	Atto647-s*	/ATTO647N/CAA CAA GAG TCA CAA ACA TAG	HPLC	5'-Atto647N	Biomers
	Atto565-p*	/ATTO565/ATC CCT ATA GTG AGT CGT ATT A	HPLC	5'-Atto565	Biomers
	Atto647-k*	/ATTO647N/AAA ACC ACA CGA TCC TCT AAA A	HPLC	5'-Atto647N	Biomers
	Atto425-k*-r	/ATTO425/AAA ACC ACA CGA TCC TCT AAA ACC AAT CCA ATC ACA GCA CGG AGG CAC GAC ACA AAA AAA AA	HPLC	5'-Atto425	Biomers
	p*-A5-p*	ATC CCT ATA GTG AGT CGT ATT A AAAAA ATC CCT ATA GTG AGT CGT ATT A	HPLC	None	Biomers
	c*	TGG GGT CCT TAC T	HPLC	None	Biomers
	Atto565-c*	/ATTO565/TGG GGT CCT TAC T	HPLC	5'-Atto565	Biomers
	DBCO-r*	/DBCO/TTT TTT TTT GTG TCG TGC CTC CGT GCT GTG	HPLC	5'-DBCO	Biomers

Supplementary Table 3-2. Oligomers for SCs and labels, with their names, sequences, purification methods, modifications, and suppliers.

Chapter 3. Growing Functional Artificial Cytoskeletons in the Viscoelastic DNA SCs

	Name	Sequence (5' → 3')	Purification	Modification	Supplier
Tile A	m ₁₃ *-A1	ATA TCT ATC CTA AAA ATA AAC TCA GTG GAC AGC CGT TCT GGA GCG TTG GAC GAA ACT	HPLC	None	Biomers
	A1	CTC AGT GGA CAG CCG TTC TGG AGC GTT GGA CGA AAC T	HPLC	None	Biomers
	A2	GTC TGG TAG AGC ACC ACT GAG GCA TT	HPLC	None	Biomers
	A3	/ATTO488/TTC CAG AAC GGC TGT GGC TAA ACA GTA ACC GAA GCA CCA ACG CT	HPLC	5'-Atto488	Biomers
	A4	TGA GGA GTT TCG TGG TCA TCG TAC CT	HPLC	None	Biomers
	A5	CGA TGA CCT GCT TCG GTT ACT GTT TAG CCT GCT CTA C	HPLC	None	Biomers
Tile B	m ₁₃ *-B1	ATA TCT ATC CTA AAA ATT AAC GTA TTG GAC ATT TCC GTA GAC CGA CTG GAC ATC TTC	HPLC	None	Biomers
	B1	CGT ATT GGA CAT TTC CGT AGA CCG ACT GGA CAT CTT C	HPLC	None	Biomers
	B2	CCT CAC CTT CAC ACC AAT ACG AGG TA	HPLC	None	Biomers
	B3	/ATTO647N/TTT CTA CGG AAA TGT GGC AGA ATC AAT CAT AAG ACA CCA GTC GG	HPLC	5'-Atto647N	Biomers
	B4	CAG ACG AAG ATG TGG TAG TGG AAT GC	HPLC	None	Biomers
	B5	CCA CTA CCT GTC TTA TGA TTG ATT CTG CCT GTG AAG G	HPLC	None	Biomers
Tile C (Light control)	m ₁₃ *-C1 (same as m ₁₃ *-A1)	ATA TCT ATC CTA AAA ATA AAC TCA GTG GAC AGC CGT TCT GGA GCG TTG GAC GAA ACT	HPLC	None	Biomers
	C2-PC Linker	CAG ACT TTT T/PC Linker BMN/G TCT GGT AGA GCA CCA CTG AGA GGT A	HPLC	Photocleavable Linker	Biomers
	C3 (same as A3)	/ATTO488/TTC CAG AAC GGC TGT GGC TAA ACA GTA ACC GAA GCA CCA ACG CT	HPLC	5'-Atto488	Biomers
	C4	CAG ACA GTT TCG TGG TCA TCG TAC CT	HPLC	None	Biomers
	C5 (same as A5)	CGA TGA CCT GCT TCG GTT ACT GTT TAG CCT GCT CTA C	HPLC	None	Biomers
Tile D (DNAzyme control)	m ₁₃ *-D1 (same as m ₁₃ *-A1)	ATA TCT ATC CTA AAA ATA AAC TCA GTG GAC AGC CGT TCT GGA GCG TTG GAC GAA ACT	HPLC	None	Biomers
	D2-substrate	TTG CAG ACT TTrA rUGT CTG GTA GAG CAC CAC TGA GAG GTA	HPLC	rA, rU	Biomers
	D3 (same as A3)	/ATTO488/TTC CAG AAC GGC TGT GGC TAA ACA GTA ACC GAA GCA CCA ACG CT	HPLC	5'-Atto488	Biomers
	D4 (same as C4)	CAG ACA GTT TCG TGG TCA TCG TAC CT	HPLC	None	Biomers
	D5 (same as A5)	CGA TGA CCT GCT TCG GTT ACT GTT TAG CCT GCT CTA C	HPLC	None	Biomers
	s*-DNAzyme	CAA CAA GAG TCA CAA ACA TAG CAG ACA GGC TAG CTA CAA CGA AAA GTC TGC AA	HPLC	None	Biomers
Tile E (DSD)	s ₁₉ *-E1	ACA AGA GTC ACA AAC ATA GAA AAA CGT ATT GGA CAT TTC CGT AGA CCG ACT GGA CAT CTT C	HPLC	None	Biomers
	E2	AGT TCA ACT GGT CCT TCA CAC CAA TAC GGC ATT	HPLC	None	Biomers
	E3 (same as B3)	/ATTO647N/TTT CTA CGG AAA TGT GGC AGA ATC AAT CAT AAG ACA CCA GTC GG	HPLC	5'-Atto647N	Biomers
	E4	ACC AGG AAG ATG TGG TAG TGG AAT GC	HPLC	None	Biomers
	E5 (same as B5)	CCA CTA CCT GTC TTA TGA TTG ATT CTG CCT GTG AAG G	HPLC	None	Biomers
	E2-inhibitor	CCA GTT GAA CTC AAG	HPLC	None	Biomers
	E2-activator	CCG GCT TGA GTT CAA CTG G	HPLC	None	Biomers
	E2-deactivator	CCA GTT GAA CTC AAG CCG G	HPLC	None	Biomers

Supplementary Table 3-3. Oligomers for all DNA tiles, with their names, sequences, purification methods, modifications, and suppliers.

3.4.5 Supplementary Notes

Supplementary Note 3-1. Estimation of total barcode concentration in SCs.

In Supplementary Figure 3-2, we determined the local concentration of total barcode inside SCs to be 850 μM in TE buffer containing 50 mM Mg^{2+} . Given a roughly 2.2 times volumetric swelling after lowering the salinity from 50 mM Mg^{2+} to 15 mM Mg^{2+} (Supplementary Figure 3-2e), the total barcode concentration inside the SCs is about 400 μM , which is relevant to the salinity used throughout this study and corresponds to 100% of barcode inside SCs.

The molecular weight of the repeating unit of $p(\text{A}_{20-x})_n$ ($x = p, m, \text{ or } s$) is in the range of 12600 – 13000 g/mol depending on the barcode. Therefore, 400 μM barcode in the SC core corresponds to a local concentration of 5.04 – 5.20 g/L.

For SCs made by mixture of adenine-rich DNA polymers having different barcodes, the concentration of a specific barcode inside the SCs scales with the fraction of this particular barcode used in the mixture. For instance, 10% of m barcode corresponds to 40 μM , 1% of m barcode corresponds to 4 μM , and 0.1% of m barcode corresponds to 400 nM, etc.

Supplementary Note 3-2. Calculation of overlap concentration (c^*) and entanglement concentration (c_e).

In our recent publication, we have used MD simulations to obtain the end-to-end distance and radius of gyration of a DNA polymer with 1025 nt (end-to-end distance, $R = 58.3$ nm, radius of gyration $R_g = 23.3$ nm)⁵⁶.

Assuming a good solvent scenario, $R \sim N^{0.588}$ and taking 3 different DNA polymer lengths, which are characteristic of our DNA polymer size distribution (see Supplementary Figure 3-1e, 3,000 nt as average molecular weight, 10,000 nt and 20,000 nt for superlong polymers), we can first calculate the end-to-end distance for three DNA polymer lengths:

$$R: 3,000 \text{ nt} = (3000/1025)^{0.588} \times 58.3 = 109.63 \text{ nm.}$$

$$10,000 \text{ nt} = (10000/1025)^{0.588} \times 58.3 = 222.52 \text{ nm.}$$

$$20,000 \text{ nt} = (20000/1025)^{0.588} \times 58.3 = 334.49 \text{ nm.}$$

The overlap concentration (c^*) can be estimated based on

$$c^* = \frac{3M}{N_A 4\pi R^3} \quad (2)$$

where M is the molecular weight and N_A is the Avogadro constant.

Each repeating unit has a molecular weight (on average) of 313 Da. Therefore, we get

$$c^*: 3,000 \text{ nt} = 0.28 \text{ g/L.}$$

$$10,000 \text{ nt} = 0.11 \text{ g/L.}$$

$$20,000 \text{ nt} = 0.066 \text{ g/L.}$$

(Note that the calculation of 10,000 nt and 20,000 nt values serve as a reference since our DNA polymers have a broad molecular weight distribution and the longest chain expand to over 20,000 nt, which will influence the SC matrix property.)

The calculated c^* for the average DNA polymer length (3,000 nt) is one order of magnitude below the concentration of our SCs (ca. 5 g/L). **Thus, the SC matrix is above the overlap concentration** and in the semi-dilute regime, where polymers overlap with each other⁹.

As the **entanglement concentration** (c_e) is typically 10 times the c^* , the calculated c^* has to be multiplied by 10 to reach c_e , and we have

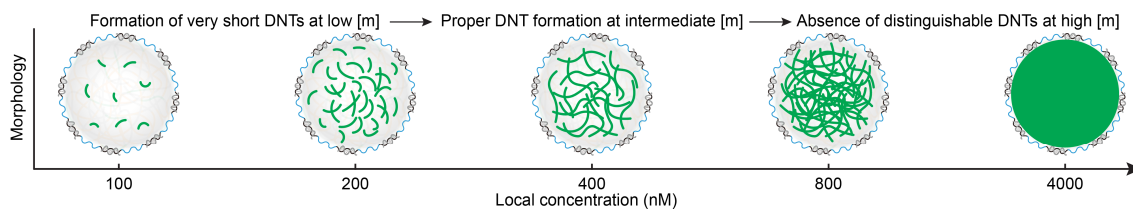
$$c_e: 3,000 \text{ nt} \approx 2.8 \text{ g/L.}$$

$$10,000 \text{ nt} \approx 1.1 \text{ g/L.}$$

$$20,000 \text{ nt} \approx 0.66 \text{ g/L.}$$

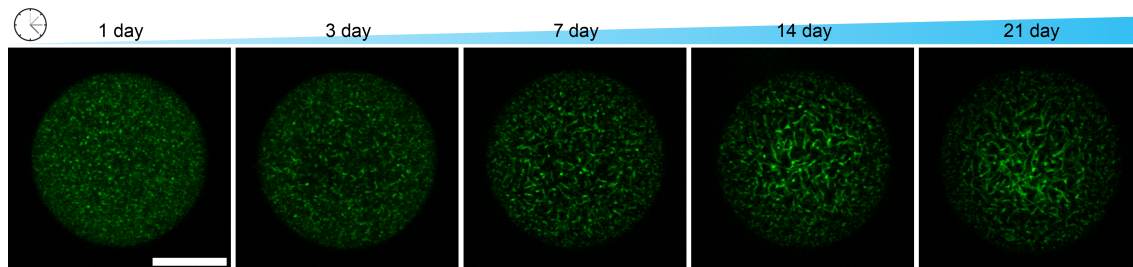
From this, we conclude that the SC matrix to be above, or with *conservative estimate* on the order of, c_e .

3.4.6 Extended Data Figures and Supplementary Figures



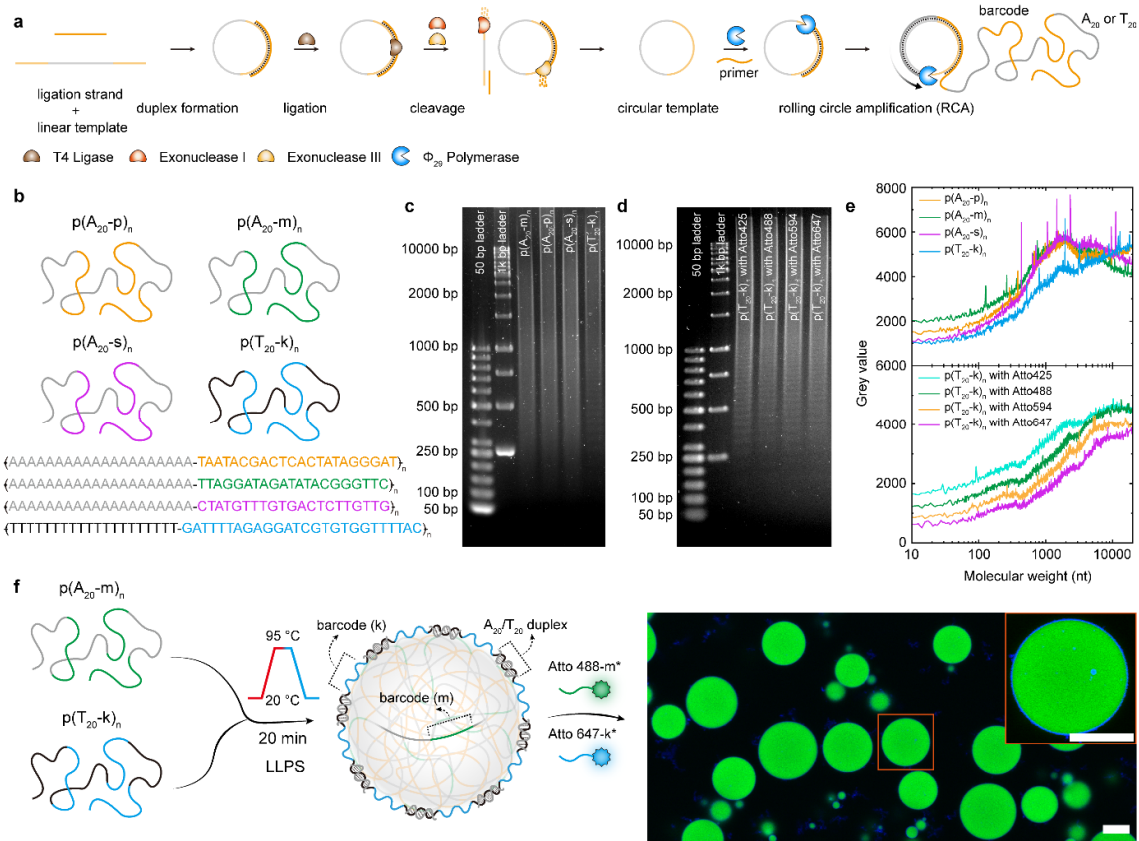
Extended Data Figure 3-1. Phase diagram of cytoskeleton morphologies inside SCs as a function of [m].

At low concentrations (<200 nM), DNA tiles assemble only into short fibers. When the local concentration increases to ~400 nM, DNTs form well-defined network structures inside SCs. At higher concentrations (>800 nM), individual DNTs become indistinguishable. The absence of DNT formation at high concentration is due to the excessive tile concentration and the freezing of dynamics required for rearrangement in the SC interior.

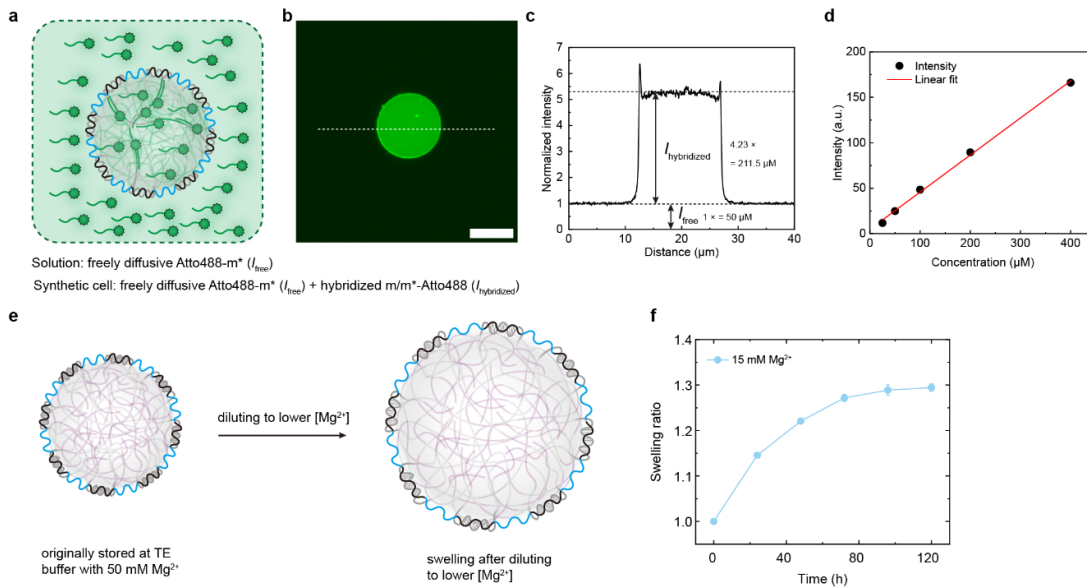


Extended Data Figure 3-2. Growth kinetics of artificial cytoskeletons inside SCs.

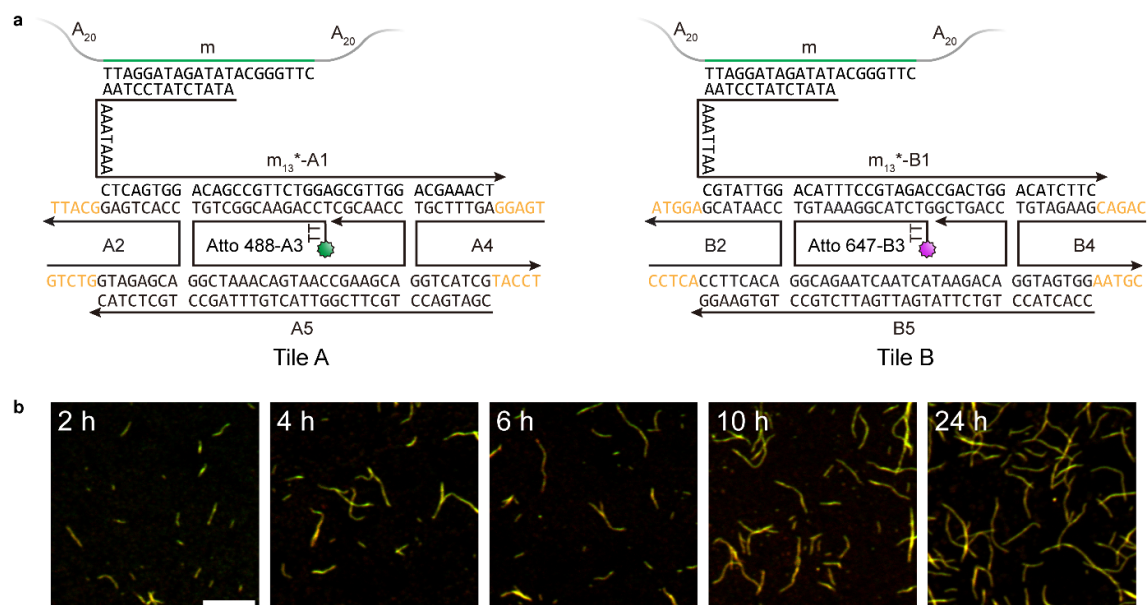
Time series CLSM images for the growth of artificial cytoskeletons inside a SC with $[m] = 0.1\%$ (400 nM) and $[p] = 99.9\%$ (400,000 nM). Experiment temperature = 20 °C. Scale bar, 10 μm .



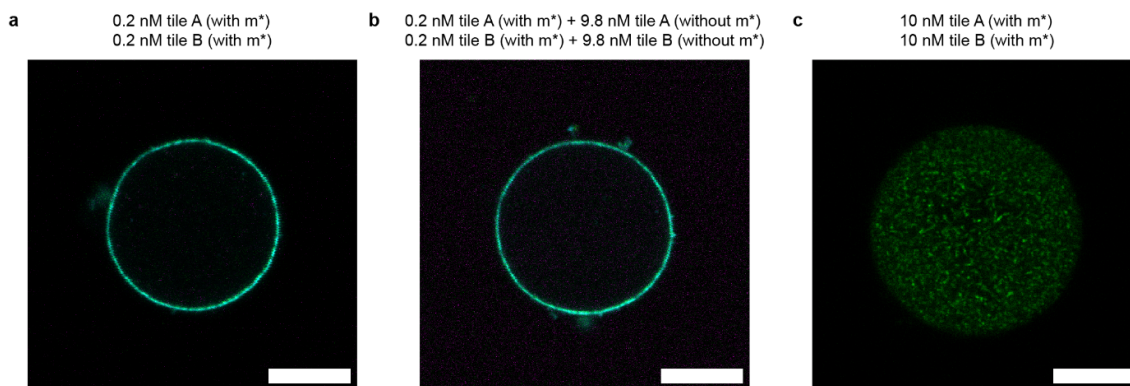
Supplementary Figure 3-1. Synthesis of ssDNA multiblock copolymers by rolling circle amplification (RCA) and formation of synthetic cells by liquid-liquid phase separation. **a**, Schematic representation for the synthesis of DNA polymers including preparation of circular template and RCA. **b**, DNA polymers synthesized and used in this work and their sequences. **c**, Representative gel electrophoresis of the generally used adenine-rich and thymine-rich DNA polymers, which are thermally heated at 95 °C for 30 min and used to make SCs. **d**, Gel electrophoresis of $p(T_{20}-k)_n$ containing different in-chain fluorophores, which are thermally heated at 95 °C for 30 min and used to make SCs. **e**, Quantification for the molecular weight distribution of DNA polymers shown in **c** and **d**. The molecular weight is reported as nucleotide number (nt), corresponding to double of the calibrated base pair number (bp). **f**, Scheme and representative CLSM image for the formation of SCs by LLPS of DNA polymers, resulting in core-shell SCs with addressable barcodes in the core and at the shell, which are labeled by Atto488-m* (green channel) and Atto647-k* (blue channel). The inset shows a zoomed-in SC corresponding to the orange box with visible shell. Scale bars are all 10 μ m.



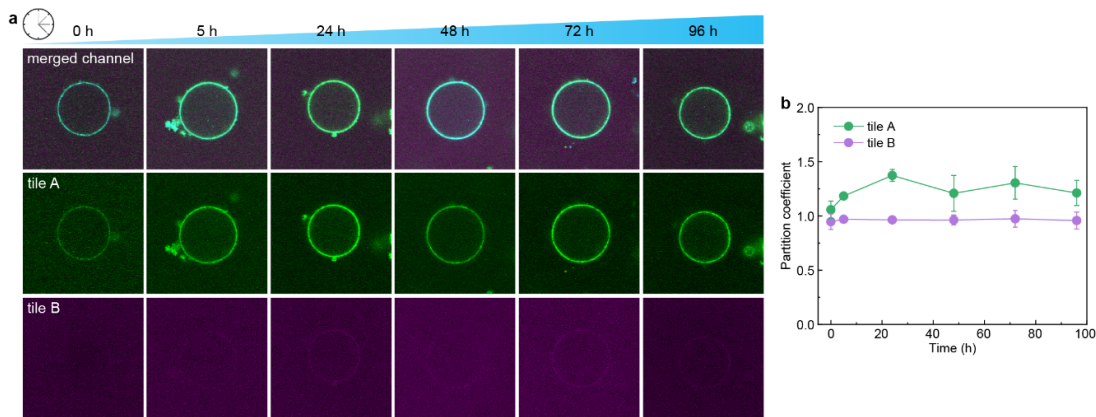
Supplementary Figure 3-2. Determination of total barcode concentration in DNA SC. **a**, Schematic representation of the titration experiment for determining the total barcode concentration in DNA SC. DNA SCs with 100% m barcode were mixed with high concentrations of Atto488-m* (50 μM) with a total concentration of m barcodes in the solution at 0.4 μM . Within the DNA SCs, all m barcodes are hybridized by Atto488-m*, and the SC is in a swollen state. The fluorescence intensity in the solution is only due to freely diffusing Atto488-m*, yielding I_{free} . In the SC, there are both hybridized Atto488-m* and freely diffusing Atto488-m*, giving $I_{\text{hybridized}} + I_{\text{free}}$. The intensity ratio (I_{R}) inside and outside the SC is then $(I_{\text{hybridized}} + I_{\text{free}})/I_{\text{hybridized}}$. **b**, Representative CLSM image of a SC in solution containing 50 μM Atto488-m*. **c**, Corresponding cross-sectional line profile along the dashed line in **b**. The intensity is normalized to the solution intensity, *i.e.*, divided by I_{free} . Thus, normalized intensity = 1 means the intensity contributed from freely diffusive Atto488-m*, while the additional contributions above 1 within a DNA SC stem from the hybridized Atto488-m*, $I_{\text{hybridized}}$. As the solution contains 50 μM Atto488-m*, given the intensity ratio in and outside SC, $(I_{\text{hybridized}} - I_{\text{free}})/I_{\text{free}} = 4.23$, the SC therefore contains $50 \mu\text{M} \times 4.23 = 211.5 \mu\text{M}$ (Note that a linear correlation between fluorescence intensity and fluorophore concentration is verified in **d**), reflecting the concentration of m barcode within the SCs after hybridization and swelling. Based on a roughly 4-fold swelling ratio in volume after m/m* hybridization, the barcode concentration can be calculated to be ca. 850 μM in the non-hybridized pristine SCs. **d**, Linear correlation between fluorescence intensity and Atto488-m* concentration. **e**, Schematic representation of the swelling of the SC induced by diluting the salinity (Mg^{2+}) in the solution. **f**, Swelling kinetics of the SCs after diluting to $[\text{Mg}^{2+}] = 15 \text{ mM}$. The SCs are in equilibrium swollen state without further swelling after ca. 120 h. $n = 5$ for **e**. Error bars represent standard deviation. Experiment temperature = 20 $^{\circ}\text{C}$. The scale bar is 10 μm .



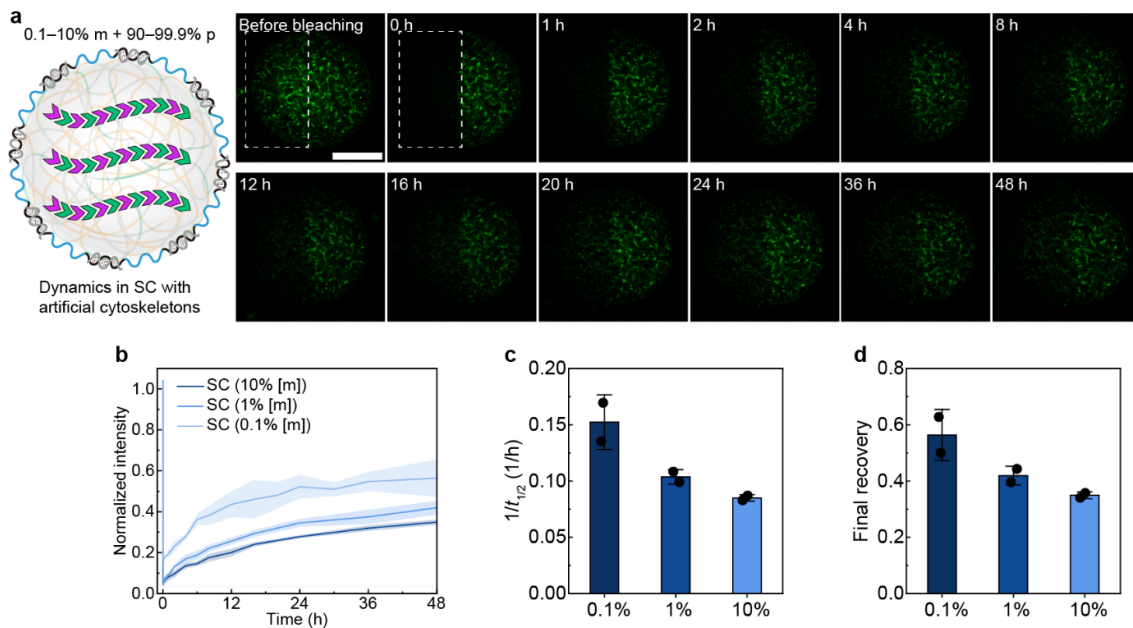
Supplementary Figure 3-3. Complementary tile A and tile B form DNA nanotubes (DNTs) with low degree of branching in plain solution. **a**, Design of the mutually complementary DNA tiles, tile A and tile B. Both contain a dangling strand partially complementary to a part of the m barcode of $p(A_{20}-m)_n$. Tile A contains Atto488 and tile B contains Atto647 as fluorescent labels. **b**, Time series CLSM images showing the formation of DNTs in solution over time. Experiment temperature = 20 °C. The scale bar is 5 μ m.



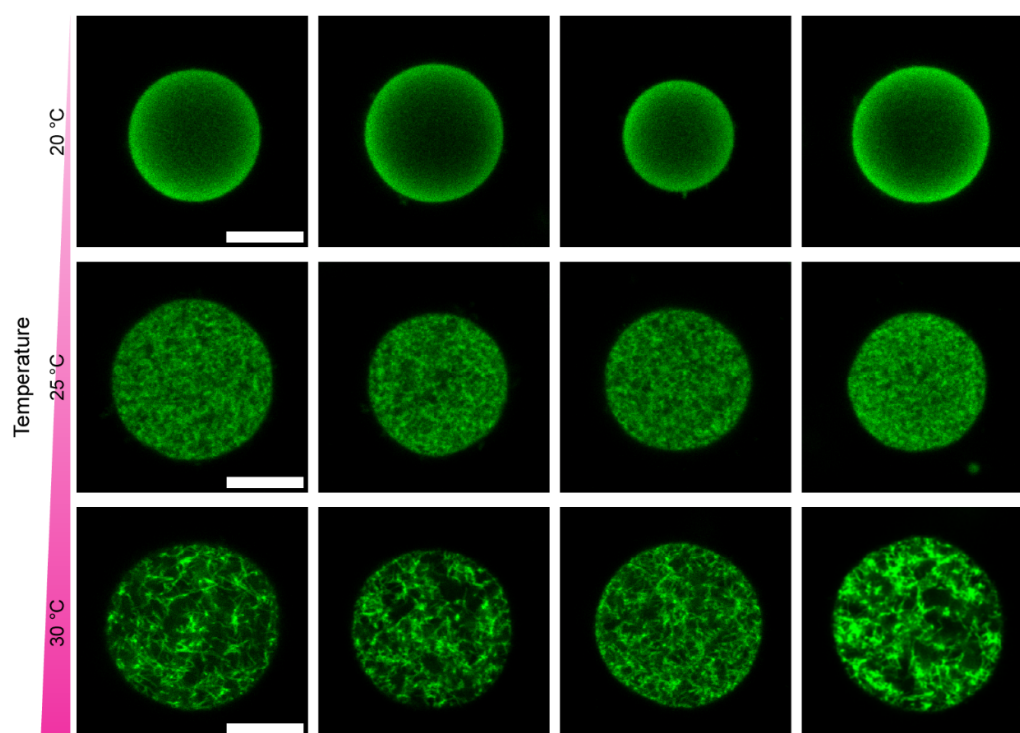
Supplementary Figure 3-4. Excess of DNA tiles allows enrichment of tiles inside the SCs through hybridization at the barcodes. **a**, Representative CLSM image of a SC incubated with 0.2 nM tile A (labeled by Atto488, green channel) and 0.2 nM tile B (labeled by Atto647, magenta channel), both containing a barcode m^* , shows no enrichment of tiles inside the SCs (100 h incubation time, 20 °C, SC shell is labeled by Atto390-k* (cyan channel)). **b**, Representative CLSM image of a SC incubated with 0.2 nM tile A (labeled by Atto488, green channel) and 0.2 nM tile B (labeled by Atto647, magenta channel), both with barcode m^* , as well as 9.8 nM tile A (labeled by Atto488, green channel) and 9.8 nM tile B (labeled by Atto647, magenta channel) without barcode m^* shows no enrichment of tiles inside the SCs (100 h incubation time, 20 °C, SC shell is labeled by Atto390-k* (cyan channel)). **c**, Representative CLSM image of a SC incubated with 10 nM tile A (labeled by Atto488, green channel) and 10 nM tile B (labeled by Atto647, magenta channel), both with barcode m^* , shows enrichment and initial assembly of DNA tiles inside the SC (100 h incubation time, 20 °C, SC shell is not labeled). Only green channel is shown in **c** for clarity. Scale bars are all 10 μ m.



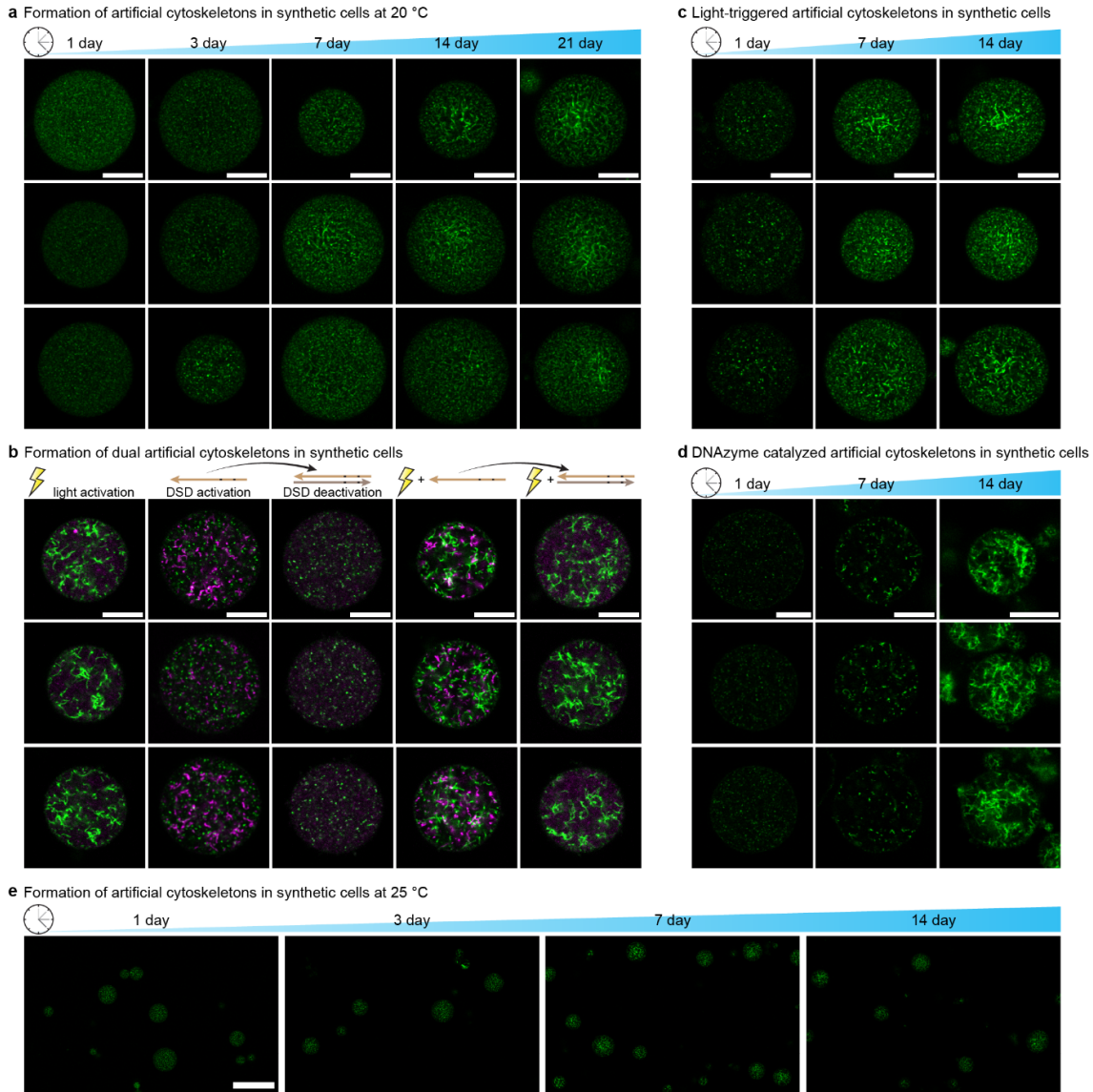
Supplementary Figure 3-5. No significant partitioning of tiles without barcodes into SCs. **a**, Representative CLSM images showing the distribution of tile A (Atto488, green channel) and tile B (Atto647N, magenta channel) across SCs (labeled at shell by Atto390-k*, cyan channel) and background solution over 96 h. Both tiles do not have a barcode interaction (m/m*) as otherwise used throughout this study. **b**, Partition coefficient of tile A and tile B as a function of time. The partition coefficient is quantified by the ratio between mean intensity inside the SCs and outside the SCs. The partition coefficients of both tiles are near 1, indicating a rather even distribution of tiles across SCs and solution. $n = 3 - 4$ for **b**. Error bars represent standard deviation. Experimental temperature = 20 °C. The scale bar is 10 μ m.



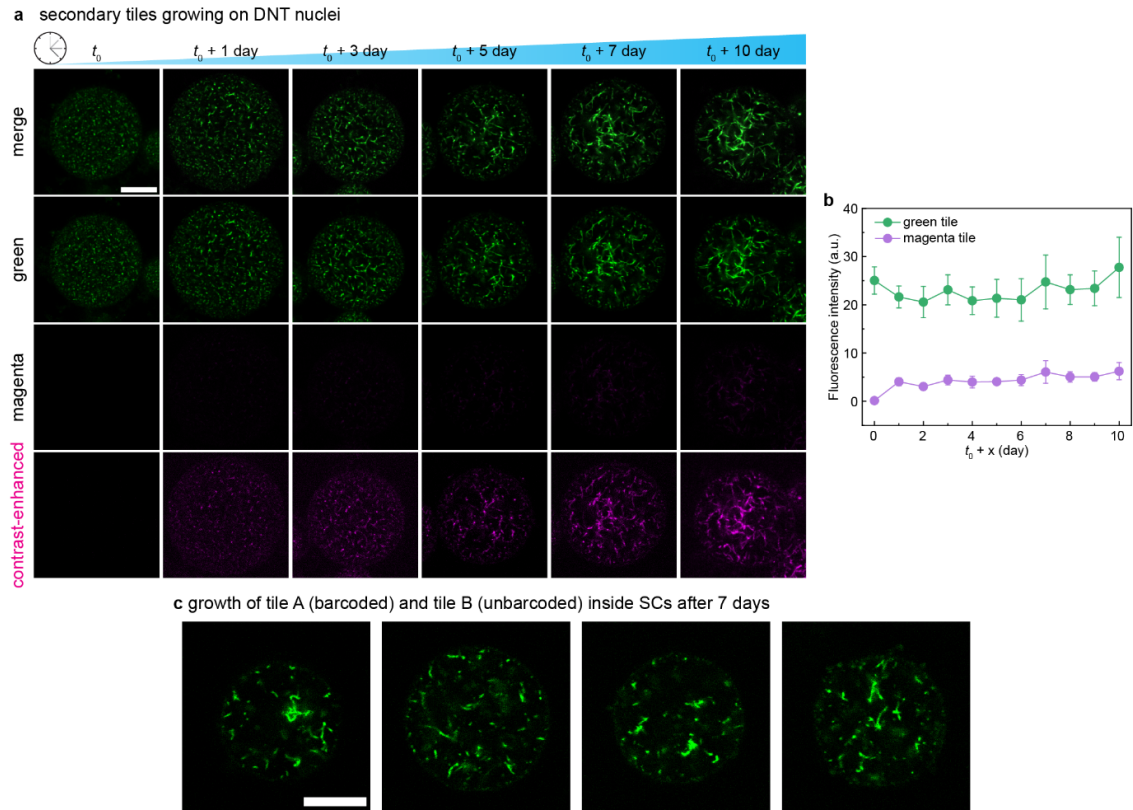
Supplementary Figure 3-6. Dynamics of DNTs inside SCs by fluorescence recovery after photobleaching (FRAP) measurements. **a**, Scheme for a SC with DNTs inside and representative time-dependent CLSM images of an SC with 0.1% DNTs inside, showing the FRAP (white dashed box) at different time points, revealing rather slow dynamics. **b**, Normalized intensity in the bleached area as a function of time for half-bleaching FRAP experiments performed on DNTs inside SCs after 14 days of assembly at different concentrations from 0.1% to 10% ([m] = 400 nM – 40000 nM inside SC). **c**, Half recovery time extracted from the FRAP data in **b** for DNTs inside SCs at different concentrations. **d**, Final recovery percentage extracted from the FRAP data in **b** for DNTs inside SCs at different concentrations. $n = 2$ for **b – d**. Recovery is faster and more complete for lower degree of loading of DNTs. Error regions and error bars represent standard deviation. Experiment temperature = 20 °C. The scale bar is 10 μ m.



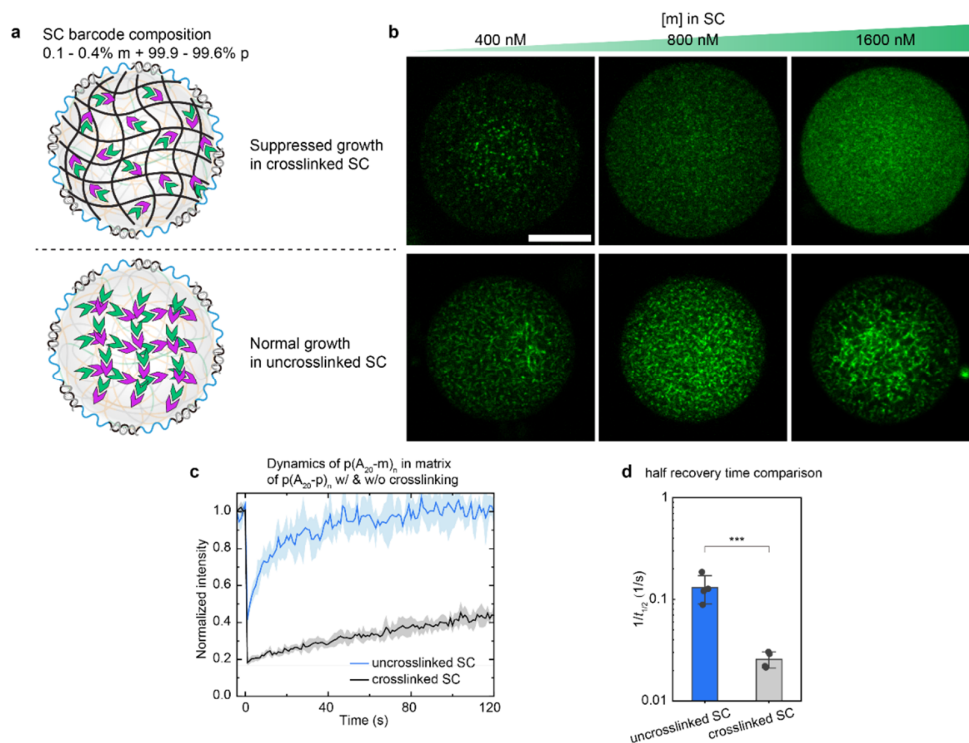
Supplementary Figure 3-7. Increase of temperature enables the formation of DNTs in SCs with high $[m]$. Representative CLSM images showing DNA tile assembly inside SCs ($[m] = 40,000 \text{ nM}$) after 2 days at different temperatures. At 20 °C, DNT formation is absent inside SCs. At 25 °C, there is a high density of DNA tiles inside, with a certain level of assembly into short DNTs. At 30 °C, the DNTs inside the SCs are discernible and yield a network structure. The assembly at higher temperature is possible due to enhanced dynamics of the SC matrix and weaker m/m^* interactions. Scale bars are all 10 μm .



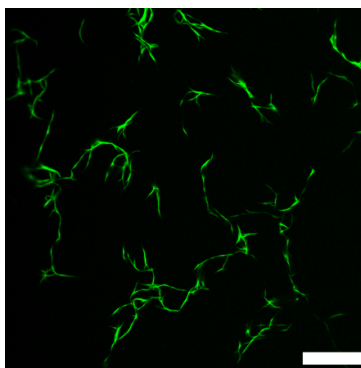
Supplementary Figure 3-8. Additional CLSM images showing the consistency in the formed structures of artificial cytoskeletons across individual SCs. **a**, CLSM images showing consistent structures for the formation of artificial cytoskeletons in SCs over time for Extended Data Figure 3-2. **b**, CLSM images showing consistent structures for the formation of dual artificial cytoskeletons in SCs enabled by light and DSD activation for Figure 3-4b. **c**, CLSM images showing consistent structures for the light-triggered artificial cytoskeletons in SCs for Figure 3-3b. **d**, CLSM images showing consistent structures for the DNAzyme catalyzed artificial cytoskeletons in SCs for Figure 3-3f. **e**, CLSM images showing several SCs with consistent structures of artificial cytoskeletons during their growth over time at 25 °C. Scale bars are 10 μm for **a–d** and 40 μm for **e**. We refer to the corresponding figures in the main text for details on the conditions.



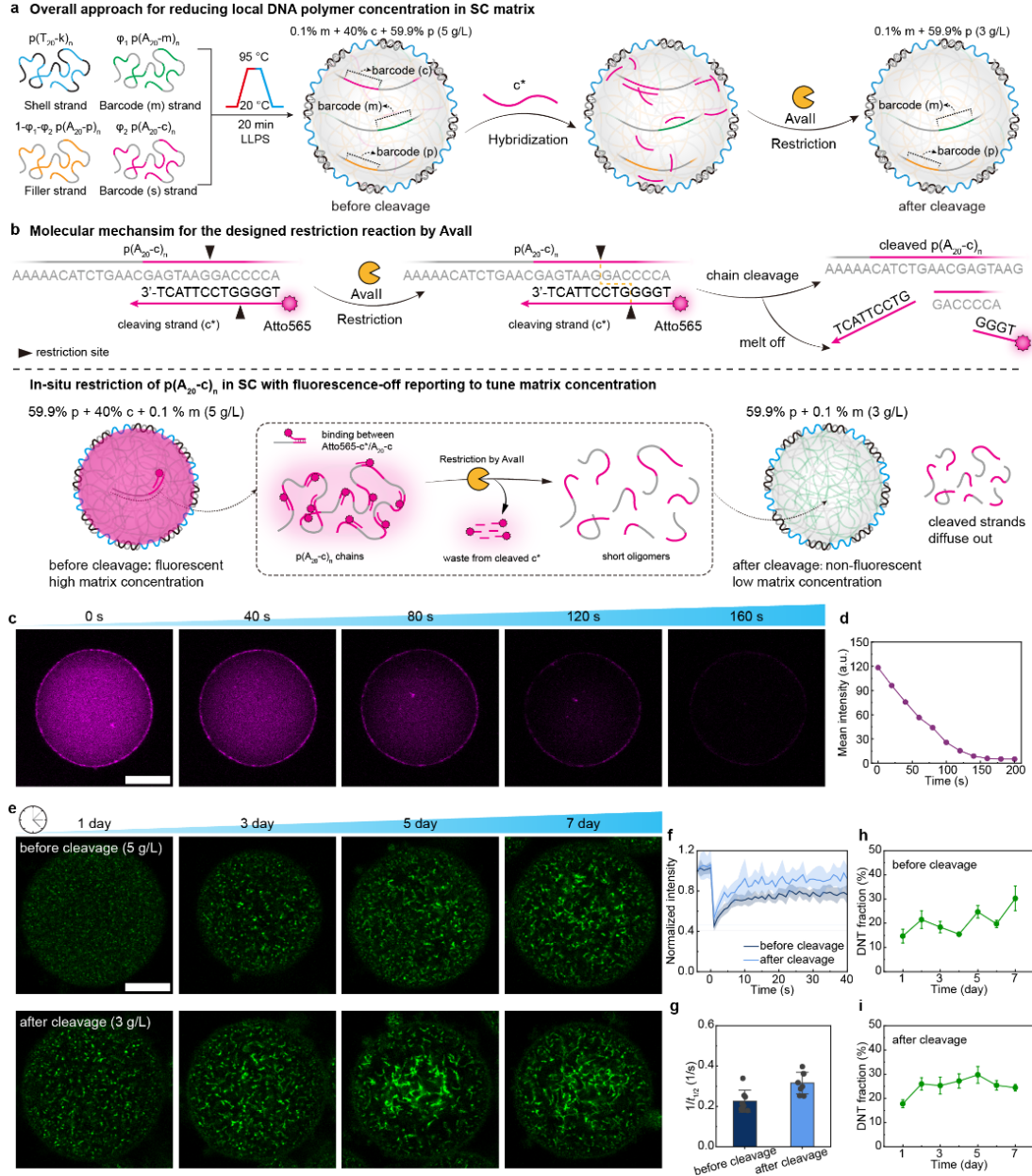
Supplementary Figure 3-9. Unbound DNA tiles in solution contribute only marginally to the growth of DNTs inside SCs. We firstly incubated SCs with green DNA tiles (10 nM tile A and 10 nM tile B) for 2 days, allowing them to grow into nuclei stage. After 2 days (t_0), we then added magenta tiles (10 nM tile A and 10 nM tile B) to see if they can co-assemble with the existing DNT nuclei. **a**, Representative CLSM images and contrast-enhanced CLSM images in the magenta channel in the fourth row visualize a marginal integration of the magenta tiles. **b**, Fluorescence intensity of green tile and magenta tile measured inside the SCs at different times. **c**, Further control: Representative CLSM images showing that the growth of barcoded tile A and unbarcoded/not immobilized tile B after 7 days results in ill-defined, short DNT structures, and a lack of mature network structure inside the SCs. $n = 6 - 10$ for **b**. Error bars represent standard deviation. Scale bars are all 10 μm . Experimental temperature = 20 $^{\circ}\text{C}$.



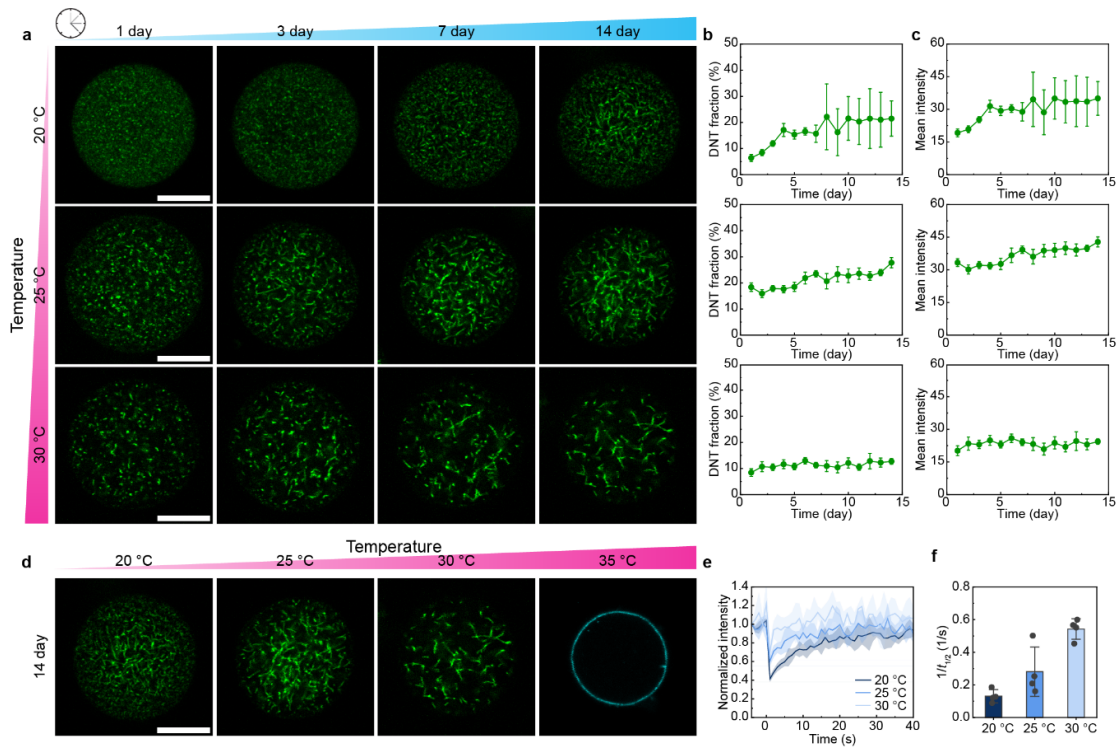
Supplementary Figure 3-10. Crosslinking prevents cytoskeleton assembly. **a**, Scheme for the DNT nucleation and growth behavior differences in crosslinked and uncrosslinked SCs. **b**, CLSM images depicting arrested and normal growth of DNTs in fully crosslinked $p(A_{20-p})_n/p^*-A_5-p^*$ and uncrosslinked SCs (21 days) for different $[m]$. **c**, FRAP on the $p(A_{20-m})_n$ (0.1%) chains labeled by Atto488- m^* in the SCs with crosslinked and uncrosslinked $p(A_{20-p})_n$ matrix (99.9%). The blue and black curves correspond to SCs in crosslinked and uncrosslinked states before the growth of artificial cytoskeletons. **d**, Reciprocal of half recovery time ($1/t_{1/2}$) extracted from **c**. The $1/t_{1/2}$ is a quantification for the internal dynamics of the SCs, which reflects their viscoelastic properties. For all CLSM images, the DNTs have two co-localized fluorescent labels (Atto488, Atto647). Only Atto488 (green) is shown for highest resolution. $n = 4$ for **c** and **d**. Error bars and error regions represent standard deviation. Experiment temperature = 20 °C. The scale bar is 10 μ m.



Supplementary Figure 3-11. Representative CLSM image showing bundling of DNTs in a concentrated $p(A_{20-p})_n$ solution. We added 250 nM tile A and 250 nM tile B without binding strands to a concentrated $p(A_{20-p})_n$ solution at 5 g/L in TE buffer with 15 mM $MgCl_2$, which nominally represents the SC interior. The DNA tiles do not bind to the p barcodes of $p(A_{20-p})_n$. In this case, the DNA polymer $p(A_{20-p})_n$ induces bundling of DNTs during their assembly. Such a structure is similar to the reported DNT bundling caused by crowding effects with simple crowders such as PEG or dextran. Experiment temperature = 20 °C. The scale bar is 20 μ m.

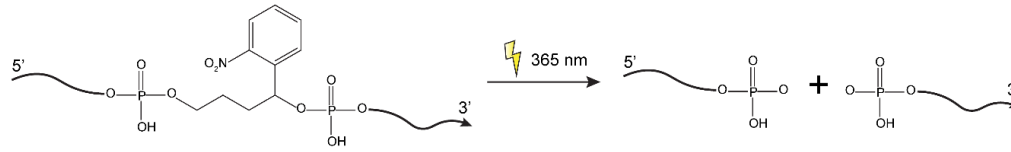


Supplementary Figure 3-12. Reduction of DNA polymer concentration of SC matrix accelerates the DNT assembly inside SCs. **a**, Scheme for the overall approach for reducing the local DNA concentration inside the SC matrix. First, we prepared SCs based on co-phase-segregation of three pAs with different barcodes (0.1% m, 59.9% p, and 40% c). This is followed by hybridization to c^* and enzymatic restriction by Avall at c/c^* dsDNA, leading to removal of the degradation products by diffusion **b**, Details of the enzymatic restriction by Avall on $p(A_{20}-c)_n$ chains with a cleaving strand (Atto565- c^*) inside the SC. Atto565- c^* hybridizes to $p(A_{20}-c)_n$ and generates a recognition site for the Avall. c^* has 13 base pairs with $p(A_{20}-c)_n$ ($T_m \approx 69.7$ °C, analyzed with IDT Oligo Analyzer) and binds tightly at experimental temperature (20 °C). After cleavage, $p(A_{20}-c)_n$ will become short oligomers with 43 nt, which diffuse out of the SC with the cleaved Atto565- c^* . The fluorophore at the 5' end of the c^* serves as a reporter for monitoring the restriction as fluorescence vanishes from the SC. The final SC will only contain $p(A_{20}-m)_n$ and $p(A_{20}-p)_n$ with local concentration of DNA polymers at 3 g/L. **c**, Representative CLSM images showing the enzymatic restriction of a SC containing 59.9% $p(A_{20}-p)_n$, 40% $p(A_{20}-c)_n$, and 0.1% $p(A_{20}-m)_n$, loaded with Atto565- c^* . **d**, Restriction kinetics indicated by decrease of fluorescence intensity within SC. **e**, Representative CLSM images showing the growth of DNA tiles inside the original SC with DNA matrix concentration at 5 g/L (top panel) and inside a SC after cleavage treatment with reduced DNA matrix concentration at 3 g/L (bottom panel). **f**, FRAP on the $p(A_{20}-m)_n$ (0.1%) chains labeled by Atto488-m* in the SCs before and after cleavage treatment. **g**, Reciprocal of half recovery time ($1/t_{1/2}$) extracted from **f**. The $1/t_{1/2}$ is a quantification for the internal dynamics of the SCs. **h**, Growth of DNTs inside SCs before cleavage treatment, as quantified by the DNT fraction inside SCs as a function of time. **i**, Growth of DNTs inside SCs after cleavage treatment, as quantified by the DNT fraction inside SCs as a function of time. $n = 6$ for **f** and **g**. $n = 8$ for **h** and **i**. Error bars and error regions represent standard deviation. Scale bars are all 10 μ m. Experimental temperature = 20 °C.

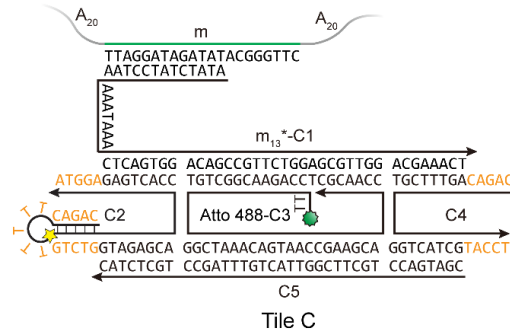


Supplementary Figure 3-13. Formation of artificial cytoskeletons inside SCs at different temperatures. **a**, Representative CLSM images showing the growth of DNTs inside SCs ([m] = 400 nM) at different temperatures over time. **b**, Growth of DNTs inside SCs at different temperatures, as quantified by the DNT fraction inside SCs as a function of time. **c**, Mean intensity of DNTs inside SCs at different temperatures as a function of time. **d**, Representative CLSM images comparing the growth of DNTs inside SCs after 14 days at different temperatures. **e**, FRAP on pristine p(A₂₀-m)_n/p(A₂₀-p)_n SCs (0.1% m; 99.9% p) with simple labeling by Atto488-m* reveals accelerated recovery at higher temperature on account of increased reptation dynamics. **f**, Reciprocal of half recovery time (1/t_{1/2}) extracted from (e). The 1/t_{1/2} is a quantification for the internal dynamics of the SCs, which reflects their viscoelastic properties. We note that the scaling law for concentration-viscosity relationship at entanglement regime for polymer solution does not hold due to the complexity of the studied polyelectrolyte solution complexed and interacting with bivalent counterions. Error bars and error regions represent standard deviation. Scale bars are all 10 μm.

a Cleavage mechanism of the nitrobenzene-based photocleavable linker in DNA strand



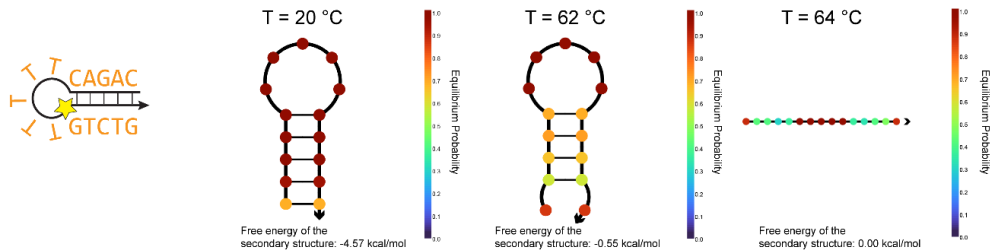
b Light-activated DNA tile C design



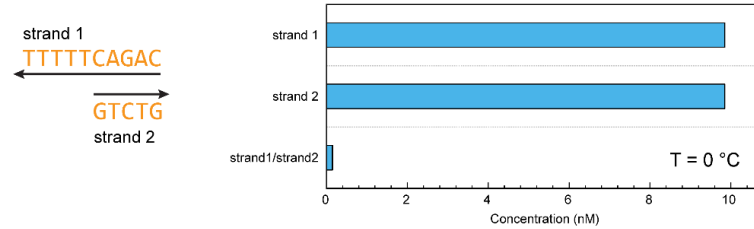
c Light-induced melting of duplex



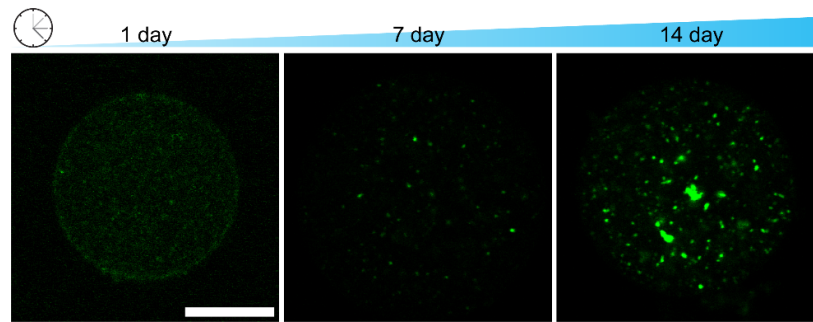
d Thermal stability analysis of the hairpin



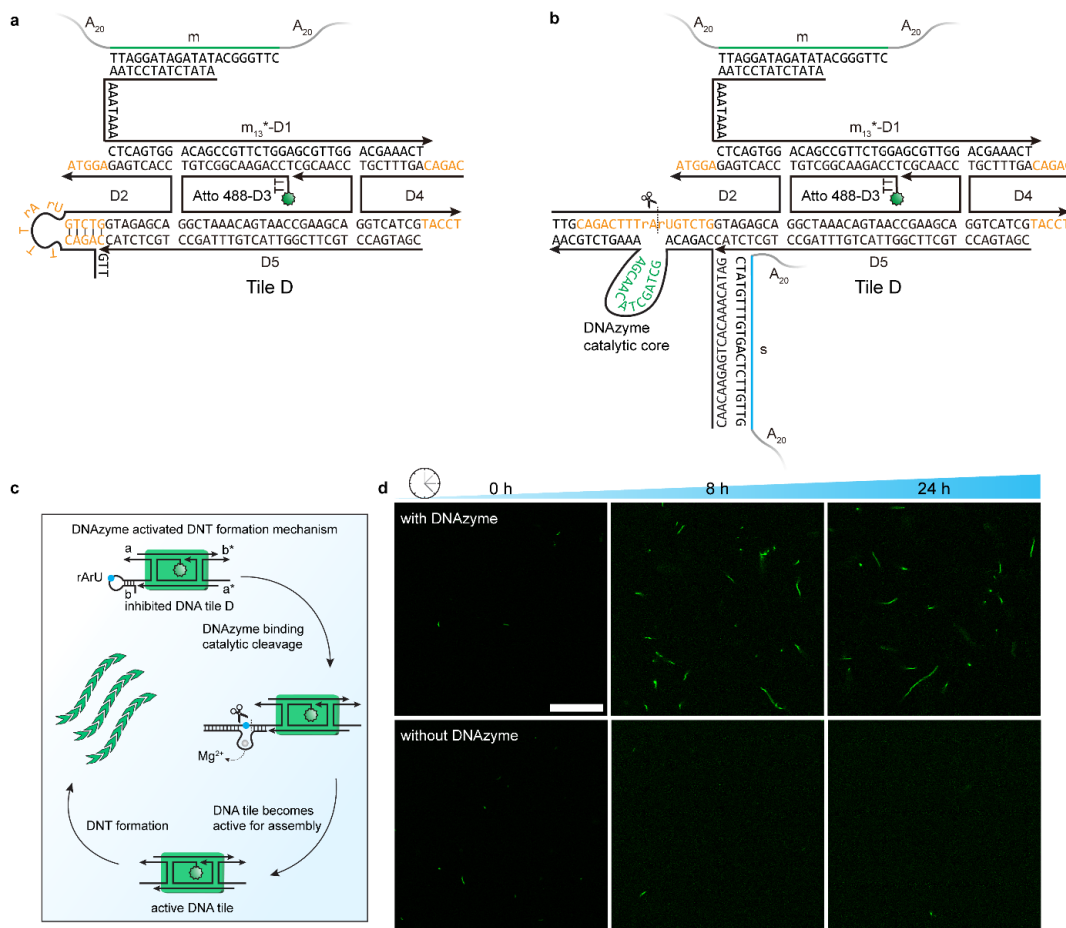
e Thermal stability analysis of the duplex



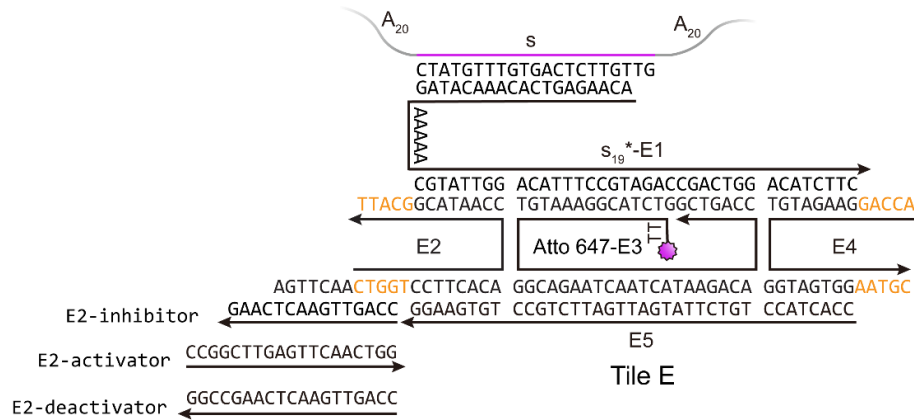
Supplementary Figure 3-14. Light-activated DNT assembly using photocaged DNA tile C. **a**, Cleavage mechanism of the photocleavable linker (nitrobenzene) used in this work. **b**, Design of the light-activated DNA tile, containing Atto488 and a dangling strand complementary to a part of the m barcode of $p(A_{20}-m)_n$. **c**, Schematic representation for the cleavage-induced melting of the duplex utilizing the significant difference in their melting temperatures (T_m). **d**, NUPACK analysis of thermal stability of the hairpin. **e**, NUPACK analysis of thermal stability of the duplex after the hairpin is cleaved. NUPACK condition for both analysis: 10 nM of strand, 50 mM Na⁺, 15 mM Mg²⁺.



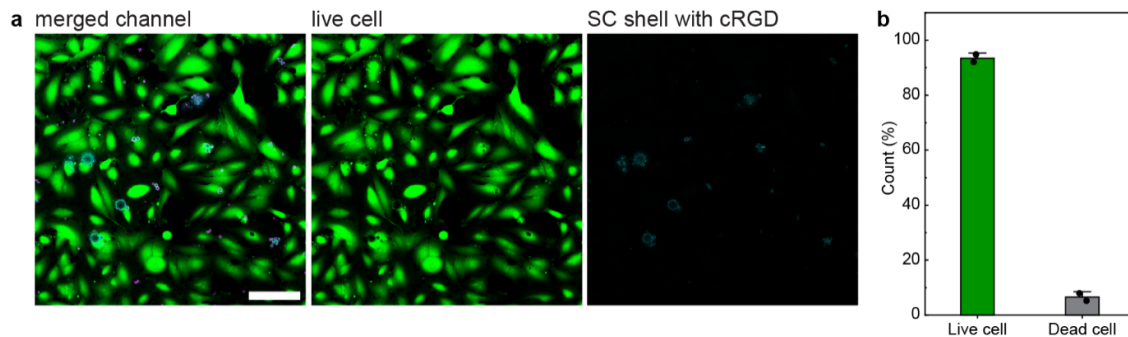
Supplementary Figure 3-15. Control for light-activated artificial cytoskeleton inside SC shows absence of assembly. Time-series CLSM images showing the absence of artificial cytoskeleton formation by tile C in a SC without UV illumination. Small aggregates appeared inside SC after 14 days, likely caused by ambient light pollution over long experimental time, which may have activated a small fraction of the initially inhibited tile C. Experimental temperature = 20 °C. The scale bar is 10 μ m.



Supplementary Figure 3-16. DNAzyme-catalyzed DNT assembly using DNA tile D in plain solution. **a**, Design of the DNAzyme-catalyzed DNA tile D containing a dangling strand complementary to a part of the m barcode of p(A₂₀-m)_n. **b**, Design of DNAzyme and how the DNAzyme can bind to the tile D on its sticky end for catalytic cleavage of the RNA substrate (rArU unit). **c**, Schematic representation for the cleavage and assembly of tile D, activated by DNAzyme. **d**, Time-series CLSM images showing the formation of DNT in solution with DNAzyme over time. The control experiment without DNAzyme shows the absence of DNT formed. Experimental temperature = 20 °C. The scale bar is 20 μ m.



Supplementary Figure 3-17. DSD-activated DNT assembly using DNA tile E. Design of the DSD-activated DNA tile E containing a dangling strand complementary to a part of the s barcode of p(A₂₀-s)_n. This tile has a E2-inhibitor strand, which binds to one of the sticky ends and avoid autonomous formation of artificial cytoskeletons. The assembly of tiles can be triggered by adding E2-activator strand, which displaces the E2-inhibitor to set free the sticky end. The formed artificial cytoskeletons can be further deconstructed by adding E2-deactivator. E2-deactivator will displace the E2-activator so that the E2-inhibitor is available again in solution and will bind to the sticky ends to deconstruct the formed structures.



Supplementary Figure 3-18. Cell viability after co-incubation with SCs. **a**, Representative CLSM images in different channels for cell viability assay of co-incubated cells and SCs with cRGD at shell. The live cells are shown in green channel, the SC shells with cRGD are shown in cyan channel, and the dead cells and SCs are shown in magenta channel. The dead cells are labeled by SYTOX™ Deep Red Nucleic Acid Stain, which also labels the SC on the shell. **b**, Live and dead cell percentage for over 1000 cell counts. Co-incubation of cells and SCs with cRGD at shell was performed in a 96 well plate at 25 °C with 5 % CO₂ for 3 h. The co-incubation condition used here is the same as the experiments for SC-mammalian cell contact. $n = 2$ for **b**. Error bars represent standard deviation. The scale bar is 100 μ m.

3.5 References

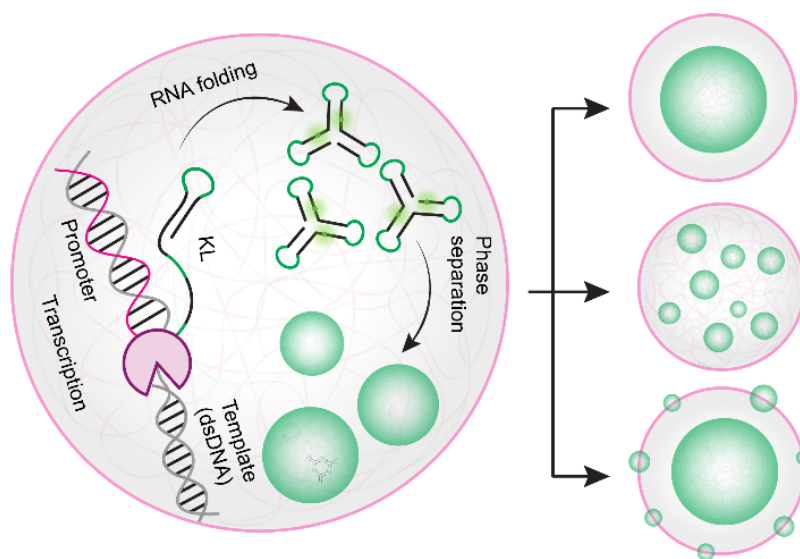
1. Fang, X., Kruse, K., Lu, T. & Wang, J. Nonequilibrium physics in biology. *Rev. Mod. Phys.* **91** (2019).
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4. Constructing Synthetic Nuclear Architectures via Transcriptional Condensates in a DNA Protonucleus



Preliminary note: This chapter is based on the journal article published in **Nat. Commun. 16, 8254 (2025)**. <https://doi.org/10.1038/s41467-025-63445-8>, by Miao Xie^{*,#}, Weixiang Chen[#], Maria Vonk-de Roy & Andreas Walther^{*}. ([#]Equal contribution). Minor changes have been made concerning formatting style.

Author contributions: M.X. and A.W. conceived the project. M.X. designed and performed experiments related to the system of transcriptional condensates in PN. W.C. developed the transcription system in PN. M.R. helped with the initial transcription experiments. M.X. prepared the draft manuscript. M.X., W.C., and A.W. reviewed and edited the manuscript. A.W. supervised the project. M.X. and W.C. contributed equally.

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Abstract: Nuclear biomolecular condensates are essential sub-compartments within the cell nucleus and play key roles in transcription and RNA processing. Bottom-up construction of nuclear architectures in synthetic settings is non-trivial but vital for understanding the mechanisms of condensates in real cellular systems. Here, we present a facile and versatile synthetic DNA protonucleus (PN) platform that facilitates localized transcription of branched RNA motifs with kissing loops (KLs) for subsequent condensation into complex condensate architectures. We identify salinity, monomer feeding, and KL-PN interactions as key parameters to control co-transcriptional condensation of these KLs into diverse artificial nuclear patterns, including single and multiple condensates, interface condensates, and biphasic condensates. Over time, KL transcripts co-condense with the PN matrix, with the final architecture determined by their interactions, which can be precisely modulated using a short DNA invader strand that outcompetes these interactions. Our findings deepen the understanding of RNA condensation in nuclear environments and provide strategies for designing functional nucleus-mimetic systems with precise architectural control.

4.1 Introduction

In eukaryotic cells, the nucleus provides a compartment for essential processes such as transcription, mRNA pre-splicing, and ribosome assembly¹. To ensure precise spatial and temporal regulation of these biochemical processes², membrane-less organelles such as nucleolus, Cajal bodies, and nuclear speckles form sub-compartments within the nucleus, which are biomolecular condensates that concentrate specific nucleic acids, enzymes, and metabolites³⁻⁶. Beyond regulating these crucial processes, unique nuclear patterns formed by biomolecular condensates vary across cell types, adapting to specific demands and functional cell states⁷. Importantly, dysfunctions in nuclear condensates have been implicated in diseases such as cancer, ribosomopathy, and neurodegeneration^{6,8,9}. Thus, understanding and reconstructing nuclear biomolecular condensates is not only essential for uncovering their mechanisms but also holds significant potential for therapeutic applications.

Despite considerable advances in studying natural biomolecular condensates and attempts to engineer transcriptional condensates within the nucleus^{8,10-12} based on specific or non-specific interactions of protein-protein, protein-nucleic acid, and RNA-RNA pairs^{2,13,14}, much still remains unknown about their formation mechanisms and the involved kinetic processes. Specifically, the mechanisms by which these condensates concentrate molecules, maintain structural integrity, regulate composition, and modulate internal biochemical activities remain elusive, largely due to the complexity of *in vivo* environments. In contrast, *in vitro* models of biomolecular condensates allow for precise control over composition in a simplified setting¹¹, enabling detailed mechanism assessment through experiments and computational modeling¹⁵. Here, studies presently however rely on plain solutions that are far from the conditions in a nucleus.

Transcriptional RNAs with specific sequences have been identified to play a key role in many biomolecular condensation processes¹⁵. However, achieving control in synthetic nuclear architectures and functions requires more advanced RNA designs capable of forming higher-order structures. In nature, the self-complementary kissing loop sequence in type 1 human immunodeficiency virus (HIV-1) virions has been identified as framework for systematically manipulating genomic dimerization¹⁶. Similar kissing loop interactions have been shown to facilitate condensation in bacterial riboswitches^{13,17}. Inspired by the sequence-dependent interaction of kissing loops, which enables specific pairing between internally folded RNAs^{18,19}, the groups of Takinoue²⁰, di Michele²¹, and Franco²² have recently introduced programmable condensates in solution formed by nanostar-like RNA motifs. The latter two groups have further shown that RNA nanostars with kissing loops at the end of each arm (KLS) could co-transcriptionally condense into condensates with controlled size, number, morphology, and composition either in solution or confined within water-in-oil emulsions^{21,22}. Through integration of RNA aptamers into KLS, such condensates can mimic natural membrane-less organelles capable of selective capture of client molecules with biofunctions²¹. However, it remains unexplored whether RNA condensates can form in crowded conditions and how they may interact with DNA-rich environments resembling the cellular nucleus, where intricate RNA-DNA interactions occur. How such DNA environments influence the organizational principles of such designer condensates is unknown.

We have recently introduced core-shell DNA coacervates, formed by single-stranded DNA (ssDNA) polymers, with a highly concentrated DNA-enriched core²³, that can flexibly recruit molecules and proteins for enzymatic functions^{24,25} and chemical reactions²⁶. These DNA coacervates closely resemble the crowded environment of the cellular nucleus, making them an ideal platform for constructing nucleus mimics²⁷. Therefore, we term them protonuclei (PN) in this study. As the internal composition of the PN can be flexibly tuned based on the ssDNA polymer selection, we incorporate T7 promoter sequences into the DNA core to recruit transcription templates and facilitate localized *in-protonucleo* transcription. We demonstrate that KL can be transcribed within these PN, leading to the formation of co-transcriptional KL condensates with various morphologies. We demonstrate a range of synthetic nuclear architectures, including single condensates, multiple condensates, interfacial condensates formed through secondary nucleation, and biphasic condensates of orthogonal KLS, all controlled by salinity, PN-KL affinity, and competing PN-KL interactions, respectively. Given the design flexibility of transcriptional KLS and the tunable condensate patterns in our crowded PN system, we believe this artificial nucleus platform will significantly advance the field of synthetic biology, in particular synthetic cells, providing a powerful toolkit for designing and constructing synthetic nuclear architectures with unprecedented control and precision.

4.2 Results and Discussion

4.2.1 Formation of Transcriptional KL Condensates inside PN

Figure 4-1 shows an overview of our entire approach. It consists of constructing a modular PN platform using DNA nanoscience approaches, followed by immobilization of short KL templates to initiate transcription therein. The transcribed KLs are designed to undergo phase separation by complementary interactions. By precisely controlling KL-PN interactions and environmental conditions, we study structure formation and response in detail through easily accessible pathways. In more detail, the DNA PN are derived from our previous work on DNA protocells^{23,24}, where we have identified that temperature ramps of mixtures of long poly(A₂₀-m)_n ssDNA and long poly(T₂₀-k)_n ssDNA form micron-sized core-shell coacervates with an adenine-rich ssDNA polymer (polyA) core and a thymine-rich ssDNA polymer (polyT) shell^{23-25,28}. This process features a selective liquid-liquid phase separation (LLPS) of polyA during heating, forming polyA droplets at high temperature, which are then stabilized by polyT with A₂₀/T₂₀ hybridization during cooling, forming a thin and crosslinked hydrogel shell. This ultimately furnishes a highly concentrated polyA core of around 10 g/L²⁹. The dynamic properties of the PN can be regulated from an arrested state to a liquid-like state by tuning the salinity. Additional ssDNA barcode sequences (o, p, k) can be modularly incorporated into the ssDNA polymers for integrating functionalities into the core and the shell (Figure 4-1).

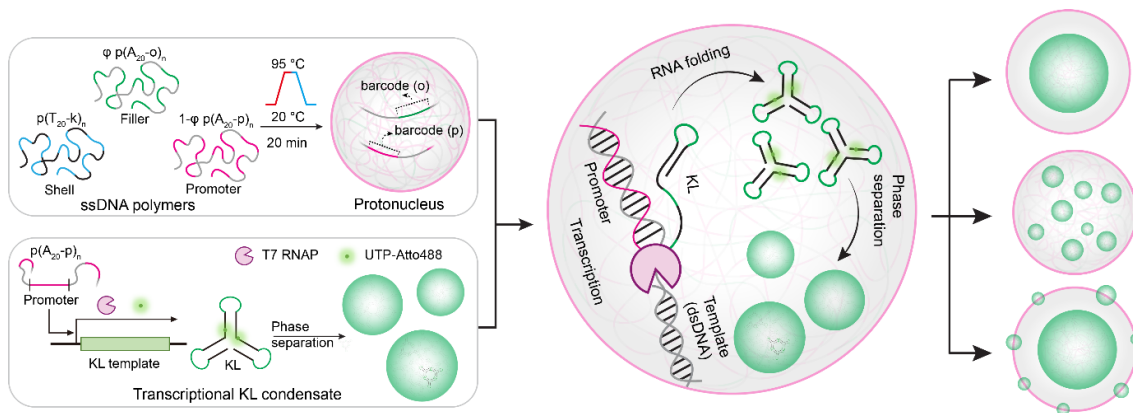


Figure 4-1. Transcriptional kissing loop (KL) condensates form different synthetic nuclear patterns in DNA protonuclei (PN). An adenine-rich ssDNA polymer (polyA) strands with barcode p (T7 RNA polymerase promoter sequence), polyA strands with dummy barcode (o), and a thymine-rich ssDNA polymer (polyT) with k barcode are used for LLPS process to form PN with an incorporated promoter region. The promoter barcodes inside the PN recruit DNA templates, T7 RNA Polymerase (T7 RNAP), and nucleotide triphosphate (NTP) monomers to induce a localized transcription and enrichment of KL sequences, forming distinct nuclear patterns via different nucleation and condensation processes.

We synthesized several ssDNA polymers using rolling circle amplification (details in Supplementary Table 4-1), including poly(A₂₀-p)_n, poly(A₂₀-o)_n, and poly(T₂₀-k)_n with n ranging roughly from 10 to 60 repeating units²³. The barcodes p, o, and k serve specific functions. The most critical part is the p barcode in poly(A₂₀-p)_n, which is the T7 RNA polymerase (T7 RNAP) promoter sequence that allows for the flexible integration of DNA templates (short genes) amenable to transcription of RNA in the PN through simple addition of the templates after

formation of the PN. Poly(A₂₀-o)_n serves to homogeneously dilute the p barcode and provides an addressable matrix barcode to tune properties and (as we will see below) adjust the affinity to the transcribed RNA, which regulates the subsequent growth of the transcriptional condensates. Following our established protocols^{23-25,28}, we prepared a set of core-shell PN by mixing poly(A₂₀-p)_n and poly(A₂₀-o)_n for the core, and poly(T₂₀-k)_n for the shell, using a temperature ramp in TE buffer at 50 mM Mg²⁺. Functionalization of the p and o barcodes with complementary dye-appended ssDNA confirms a homogeneous integration of both polyAs in the PN cores (confocal laser scanning microscopy (CLSM) images in Supplementary Figure 4-1). The PN can be conditioned to different Mg²⁺ concentration after preparation. We focus on a PN system where 10% of promoter sequences (poly(A₂₀-p)_n) are diluted with 90% of a matrix (poly(A₂₀-o)_n).

To verify transcription to occur inside the PN, we hybridized a transcription template T_x* (ssDNA template) containing p* for hybridization with the promoter sequence p and an active transcription region x* at stoichiometric ratio into the PN (sequences in Supplementary Table 4-2). x* codes for a simple RNA not amenable to undergo condensation. Subsequent addition of T7 RNAP and a nucleotide triphosphate (NTP) monomer mix containing 1% fluorescent monomer (UTP-Atto488) induces transcription with local formation of fluorescent RNA strands (Figure 4-2a, b).

To better quantify the transcription efficiency and kinetics inside the PN and compare it to free transcription in solution, we further designed a reporter (R) containing a fluorophore-quencher pair, which is a partially complementary double-stranded DNA (dsDNA; Rep/Rep' sequences in Supplementary Table 4-2; ' denotes a partially complementary sequence leaving a toehold on Rep). The transcribed Rep* from template T_{Rep}* will trigger a strand displacement reaction (SDR) with R by fully hybridizing with the Rep strand, generating a fluorescent signal, which can be monitored by fluorescence measurements using a plate reader. In more detail, we compared the transcription kinetics among PN with embedded promoter sequence of poly(A₂₀-p)_n, pure poly(A₂₀-p)_n ssDNA in solution, and short p ssDNA in solution—all at identical p concentration and under otherwise identical transcription conditions (Figure 4-2a, c). All systems show relatively similar kinetic profiles, with the free promoter in solution being the most active transcription system, and the PN showing a slightly lower activity compared to the free poly(A₂₀-p)_n in solution. The slightly lower activity can be understood considering constraints of the diffusion of T7 RNAP and NTPs into the PN, and RNA strands out of the PN.

After confirming successful transcription in the PN, we turn to KL-condensate formation by transcriptional control in the PN versus in solution. As a proof of concept, we first focus on a three-armed singled-stranded RNA (ssRNA) nanostar with a wildtype palindromic KL sequence²⁰⁻²² at the tip of each arm (KL1 in Figure 4-2d). We used a dsDNA template (T_{KL1}/T_{KL1}') because ssDNA templates (T_{KL1}) alone do not allow for efficient transcription and condensate formation on account of intramolecular folding of such ssDNA template (Supplementary Figure 4-2). T_{KL1} contains a p* ssDNA sequence for hybridization to poly(A₂₀-p)_n inside the PN to initiate transcription (sequences in Supplementary Table 4-2).

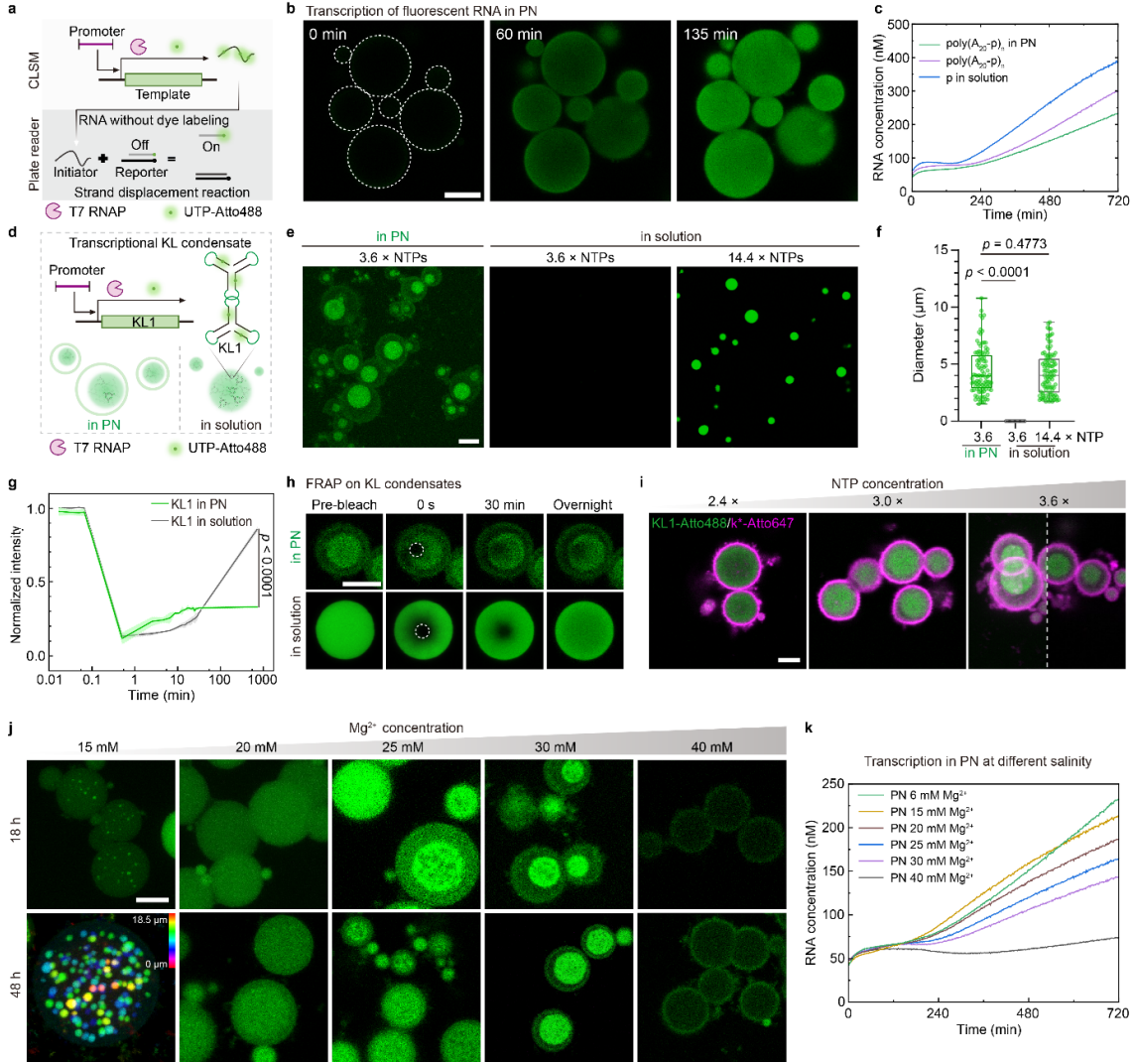


Figure 4-2. Transcriptional KL condensates in PN show different nuclear patterns. **a**, Scheme of transcription in PN characterized by CLSM and plate reader. UTP-Atto488 labels transcripts for CLSM. In plate reader assays, dsDNA reporters (R) with fluorophore-quencher pairs generate fluorescence via RNA-triggered strand displacement reaction (SDR). **b**, Representative CLSM showing localized transcription process of x^* (green, UTP-Atto488 labeled) inside PN ($[NTP] : [T_{x^*}] : [p] = 2500 : 1 : 1$). **c**, Transcription kinetics in PN with embedded poly(A_{20} -p) $_n$, in solution with poly(A_{20} -p) $_n$, or free promoter (p), monitored by SDR ($[NTP] : [R] : [T_{Rep^*}] : [p] = 2000 : 10 : 1 : 1$, 6 mM Mg^{2+} , mean of $N=2$). **d**, Scheme of transcriptional KL1 condensate formation in PN and solution; KL1 labeled by UTP-Atto488. **e**, CLSM images (maximum z-stack projections) showing transcriptional KL1 condensates in PN with poly(A_{20} -p) $_n$ ($[NTP] : [T_{KL1}] = 3.6$), and in solution with free p ($[NTP] : [T_{KL1}] = 3.6 : 1$ or $14.4 : 1$). **f**, Diameter distributions of KL1 condensates in PN and solution at varying $[NTP]$ ($N = 100$ condensates from 3 experiments). Box plot: median (central line), interquartile range (box), min-max (whiskers). **g**, **h**, FRAP on KL1 condensates in PN and solution at 30 °C. **g**, Normalized fluorescence recovery kinetics in the bleached areas (**h**), with intensity normalized to pre-bleach levels (mean $\pm$ SD, $N = 3$). **h**, Time-lapse CLSM images with dashed circles marking bleached areas. **i**, Representative CLSM images showing effect of $[NTP]$ on KL1 condensate formation in PN. The left half of the $3.6 \times [NTP]$ image is a z-stack projection. Green channel: KL1-Atto488; Magenta channel: k^* -Atto647/poly(T_{20} -k) $_n$. **j**, Representative CLSM showing effect of $[Mg^{2+}]$ on transcriptional KL1 condensate formation in PN (15 mM Mg^{2+} image is a color-coded z-depth projection). **k**, Effect of $[Mg^{2+}]$ on transcription kinetics in PN monitored via SDR (as in **c** with indicated $[Mg^{2+}]$, mean of $N = 2$). Two-sided t -test (**g**, **f**). Scale bars: 10 μm (**b**, **e**); 5 μm (**h**, **i**, **j**).

We compared differences in KL1 condensate formation at low $[NTP] = 3.6 \times [T_{KL1}]$ after 18 h transcription. $[NTP]$ is defined as the maximum amount of RNA transcripts that can be produced per template (see Method for details). The PN system clearly shows a single KL1 condensate in every PN with an average diameter of approximately $4 \mu m$ for PN with an average diameter of around $6.7 \mu m$ (Figure 4-2e, f). In striking contrast, no KL1 condensates can be found in solution due to the limited concentration of RNA transcripts (Figure 4-2e, f). Transcriptional KL1 condensates in solution start to appear with diameter of $\sim 4.5 \mu m$ at increased $[NTP]$ ($[NTP] = 14.4 \times$; Figure 4-2e, f). The size of the transcriptional KL1 condensate in solution increases with $[NTP]$ due to the increased amount of RNA transcripts (Supplementary Figure 4-3). This comparison demonstrates that the spatial transcription of the KL1 in PN leads to locally high concentrations sufficient for condensation, similar to the enrichment mechanism in natural nuclear condensates². In addition, as we will demonstrate below, the KL-PN affinity also plays an important role in the condensation process.

Interestingly, one single KL1 condensate forms in each PN, confirming sufficient dynamics within the PN to follow energy minimization constraints to yield a minimum surface area (Figure 4-2e and Supplementary Figure 4-4). We further performed fluorescence recovery after photobleaching (FRAP) experiments on the KL1 condensates in PN and in solution to study their dynamic properties. Strikingly, their fluorescence recovery kinetics differ substantially. Whereas KL1 condensates in solution show near full recovery overnight, KL1 condensates in PN only show limited recovery, highlighting much better diffusion dynamics of individual KL1 RNA in KL1 condensate in solution than in PN (Figure 4-2g, h). A complementary half-bleaching experiment shows a bright edge of transcriptional KL1 condensates in solution during recovery, indicating a dynamic exchange of soluble KL1 transcripts from the solution with the condensate phase (Supplementary Figure 4-5). In contrast, half-bleached KL1 condensates in PN show less recovery and lack the bright edge, likely due to their restricted dynamics in a DNA-crowded environment and interactions between PN matrix and the KL1 transcripts, as we will further discuss below.

Next, we discuss the effects of $[NTP]$ and $[Mg^{2+}]$ on transcriptional KL1 condensate formation inside the PN. KL1 transcription with $[NTP]$ varying from $2.4 \times$ to $3.6 \times$ show a morphological transition from peripheral localization of KL1 transcripts to reorganization and compaction into a single condensate (Figure 4-2i). The formation of peripheral KL1 transcripts at low $[NTP]$ shows that incoming NTPs are converted to RNA as they reach the embedded transcription templates in the outer PN parts. The lack of a centrally compacted condensate points to the fact that, at this low concentration of KL1 transcripts, phase segregation is at least not very pronounced. The remaining ring indicates an interaction between the PN matrix and the KL1 transcripts. At higher $[NTP]$, KL1 transcripts are homogeneously produced throughout the PN, and phase segregation drives the formation of the KL1 condensate.

$[Mg^{2+}]$ shows a profound impact on nucleation and condensate morphology. Multiple small condensates can be observed at $15 \text{ mM } Mg^{2+}$, whereas $[Mg^{2+}] > 20 \text{ mM}$ leads to the formation of a single condensate droplet. $20 \text{ mM } Mg^{2+}$ corresponds to a transition point. Interestingly, a

transition in the condensate formation process is visible. Whereas isolated nucleation events dominate at 15 mM Mg^{2+} , co-continuous phase separation is visible above 25 mM Mg^{2+} with a sponge-like structure. At 40 mM Mg^{2+} , condensate formation in PN is no longer visible (Figure 4-2j). Such distinct condensate formation in PN is associated with multiple influences of Mg^{2+} on the system: First, higher $[Mg^{2+}]$ leads to reduced dynamics in the crowded environment of PN, as previously studied by us in detail^{23,24}. Second, higher $[Mg^{2+}]$ also assists in tighter condensation of the KL condensates and potentially increases non-specific interactions between the KL condensates and the PN matrix²². Third, increasing $[Mg^{2+}]$ leads to a continuous decrease of the transcription kinetics as depicted in Figure 4-2k. Thus, multiple isolated nucleation events and binodal phase separation occur at 15 mM Mg^{2+} , driven by the high dynamics of the PN core and the high transcription kinetics. In contrast, spinodal or viscoelastic phase separation^{26,30,31} is favored at high Mg^{2+} concentrations, where the dynamics of the PN become more arrested (see also discussion for time-lapse data at Figure 4-3b-e). Further, charge screening increases the propensity for non-specific interactions between nucleic acids (RNA and DNA). The transcription is strongly suppressed at 40 mM Mg^{2+} with limited KL1 transcripts so that condensation of KL1 cannot take place (Figure 4-2j, k).

4.2.2 Co-Condensation of Transcriptional KL with the PN Matrix

To get a deeper understanding of the morphological development of single KL condensates in the PN at 30 mM Mg^{2+} , we monitored the whole process over 24 h through CLSM (Figure 4-3a, b). Two distinct stages occur. In the first 12 h, transcription takes place from the edge of the PN to their center due to the continuous consumption of NTPs as well as diffusive uptake of T7 RNAP and NTPs. The KL1 intensity gradient can be diminished if the PN are pre-equilibrated with T7 RNAP (for 2 h) prior to the addition of the NTPs (Supplementary Figure 4-6). The overall structure formation proceeds however in a very similar fashion. The entire structures reach maximum fluorescence intensities at 12 h (Figure 4-3b-d). Spongy structures of KL1 condensates during phase separation start to appear at ca. 8-10 h, whereas significant coarsening and compaction into single spherical condensates follows in the later 12-24 h (Figure 4-3b-e). The sponge-like, co-continuous structures of KL-PN co-condensates arises from spinodal or viscoelastic phase separation²⁶ of both polyA and KL transcripts in PN, that can appear in phase-segregating systems of high concentration of components and for situations of low dynamics. Different from the binodal phase separation via a nucleation and growth process for in-solution KL condensate formation at comparably high dilution³², KL transcripts transcribed inside PN continuously compact and co-phase-segregate with the PN matrix, ultimately forming interconnected networks. KL transcripts co-phase-segregating with the polyA matrix constitute slowly diffusing components with viscoelastic properties. Solvent molecules as the quickly diffusing component are expelled in the process, leading to interconnected network-like phase segregation that ultimately collapses into a spherical domain by interfacial energy minimization³³. Interestingly, we can observe a relatively slow and continuous increase in the PN dimensions, as facilitated by the relaxation of polyA/polyT

shell as a result of the increasing negative charge density inside the PN during localized RNA production and condensation (Figure 4-3c, e).

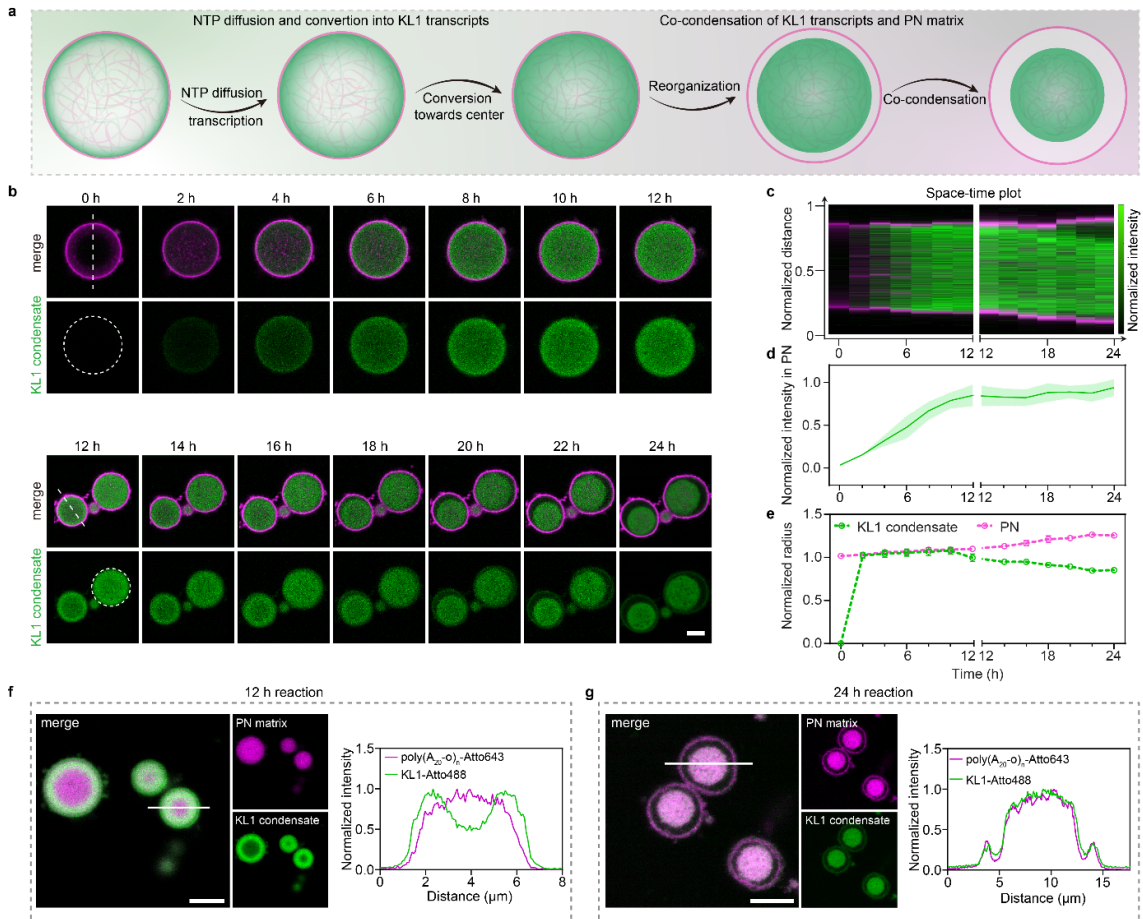


Figure 4-3. Mechanism of co-condensation of transcriptional KL1 with the PN matrix. **a**, Scheme for formation of single co-condensates of transcriptional KL1 and polyA in PN through peripheral initiation of transcription, reorganization, and co-condensation. **b**, Representative CLSM images of PN conducting KL1 transcription over 24 h ([NTP] : [T_{KL1}] = 3.6 : 1, 1 mol% UTP-Atto488). Note a slight slow-down of co-condensation kinetics of KL1 compared with results shown in Figure 4-2e, j after 18 h, due to the interruption of shaking during incubation for CLSM imaging. Green channel: KL1 condensate labeled by UTP-Atto488; Magenta channel: PN shell (poly(T₂₀-k)_n labeled by k*-Atto647). **c**, Space-time plot analysis corresponding to the two dashed lines in **b** over 24 h shows the KL1 transcription, condensation, and reorganization process. **d**, Fluorescence intensity in the KL1 condensate channel over 24 h, measured within the two white dashed circles in **b**. Intensity values were normalized to the 24 h intensity and expressed as mean $\pm$ SD from $N = 3$ PN measured. **e**, Normalized radius of KL1 condensate and PN as measured from **b** over 24 h. Data are presented as mean $\pm$ SD ($N = 3$ PN measurements). **f, g** Representative CLSM images of fluorescent PN (magenta) containing KL1 transcripts (green) at 12 h (**f**) and 24 h (**g**). The right plots correspond to line segment analysis of the white line in the CLSM images, showing fluorescence distribution for KL1-Atto488 and poly(A₂₀-o)-Atto643. The KL1 transcripts show a peripheral distribution in the PN matrix at 12 h (**f**), while colocalization and co-condensation between KL1 and PN matrix occur after 24 h (**g**). Scale bars: 5 μ m (**b, f, g**).

For further probing the universality of this single condensate formation phenomena for various KL structures, we adapted a KL1 condensate with an RNA light-up broccoli aptamer (BrA) as one of the arms, termed KL1-BrA (NUPACK-simulated structure shown in Supplementary Figure 4-7a). After 12-24 h transcription, single condensates are formed in

each PN. In contrast, KL1-BrA only forms irregular aggregates in solution. Here, the interaction between the PN matrix and the KL1-BrA condensate may facilitate better relaxation and stabilization of KL1-BrA condensate within the PN (Supplementary Figure 4-7). Taken together, KL condensation in solution and in-PN differ profoundly in both the kinetic formation process and the formed final structures at what could be considered closer to the thermodynamic equilibrium. The system can be easily tuned by adjusting the NTP and Mg^{2+} concentrations and is robust to changes in the KL components.

To study the behavior and aforementioned interactions of the PN DNA matrix with the KL1 RNA condensates, we covalently labeled poly(A₂₀-o)_n with Atto643 to prepare fluorescent PN and used these new PN to initiate localized KL1 transcription. As expected, the initial production and localization of KL1 transcripts occur at the periphery of the PN (Figure 4-3f). Unexpectedly, co-condensation of the PN matrix with the KL1 condensates occurs over time. These co-condensates deposit at the bottom of PN after 24 h in the imaging chamber, highlighting their high density and compactness (Figure 4-3g, Supplementary Figure 4-8). FRAP experiments reveal a better recovery for the KL1 components compared to the PN matrix, corresponding to higher dynamics for the KL1 condensate composed of small RNAs than the PN matrix composed of long ssDNA polymers (Supplementary Figure 4-9). This demonstrates the molecular level diffusivity of the RNA nanostars in this co-condensate structure.

4.2.3 Hybridization-Induced Disassembly of KL-PN Co-condensate

Overall, this co-condensation between PN and KL1 condensate comes unexpectedly because the KL1 condensate is not designed to have any specific interactions with the PN matrix. Indeed, a NUPACK simulation suggests no specific hybridization between the A₂₀-o repeats and the KL1 sequence (Supplementary Figure 4-10). Experimentally, we probed interactions between mature KL1 condensates and poly(A₂₀-o)_n inside PN by adding different quantities of o*-Atto647 (from 10% – 300%) that can bind to the majority phase of poly(A₂₀-o)_n in the PN. We hypothesized that the hybridization between o/o* may break non-specific KL1-PN interactions. To do so, we added different stoichiometric amounts of o*-Atto647 into solutions of PN containing already formed KL1-poly(A₂₀-o)_n co-condensates and investigated how the invasion by o*-Atto647 would alter the pre-existing KL1-poly(A₂₀-o)_n co-condensates. A gradual invasion of o*-Atto647 into the KL1-poly(A₂₀-o)_n co-condensates takes place as the amount of o*-Atto647 increases (Figure 4-4a, b). A continuous surface erosion of the co-condensates occurs because the o/o*-Atto647 hybridization reduces the affinity between KL condensate and PN interior by introducing stronger electrostatic and steric repulsion inside PN due to increased negative charge density and increasing persistence length of the formed dsDNA parts^{34,35} (Figure 4-4c–e). A sharp interface defined by a bright ring of o*-Atto647 with a locally high concentration appears.

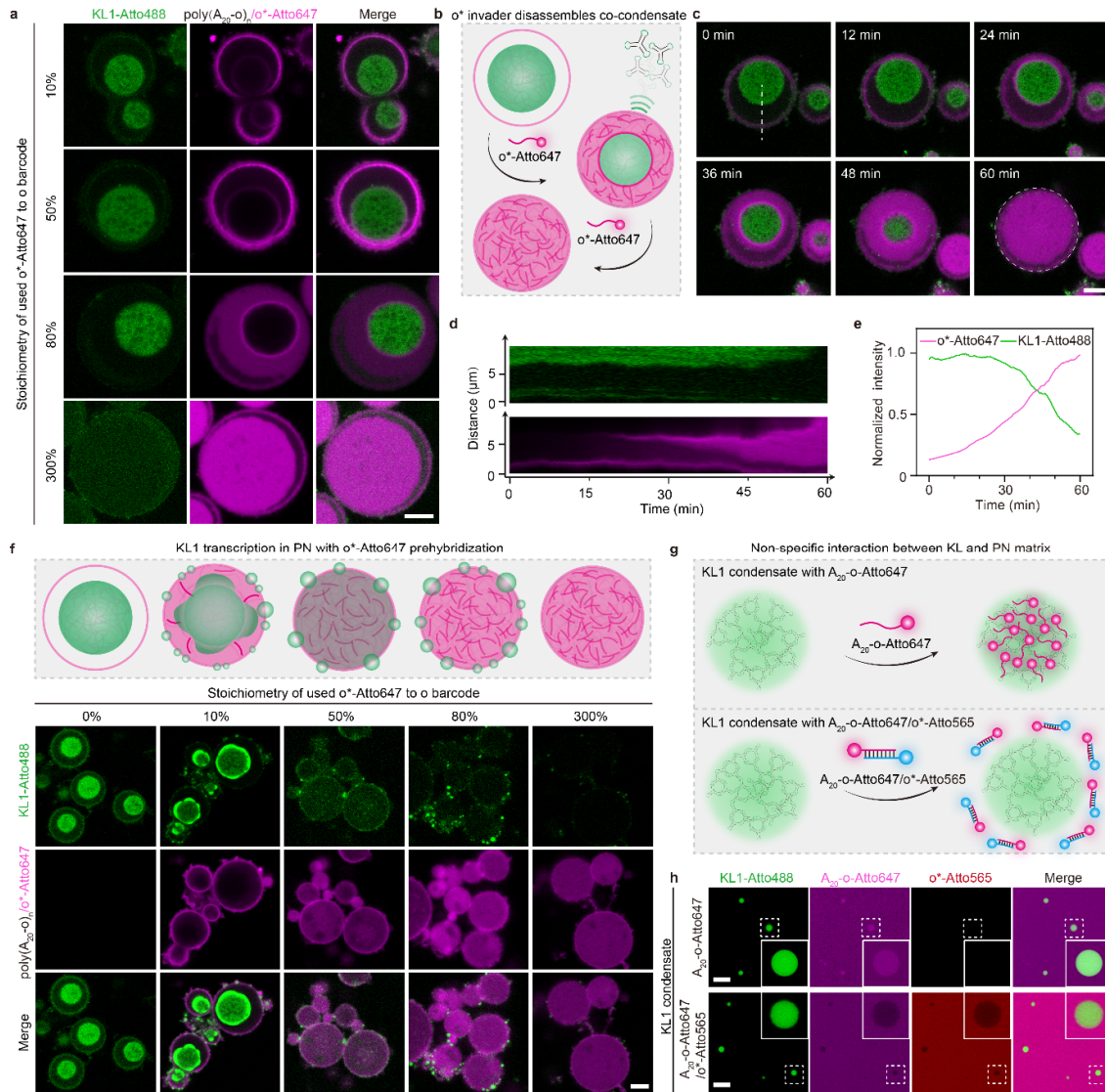


Figure 4-4. Hybridization of the polyA matrix of PN induces disassembly of KL1-PN co-condensate. **a**, Representative CLSM images of transcriptional KL1 condensates (Atto488, green channel) in PN (Atto647, magenta channel) 60 min after adding 10%, 50%, 80% and 300% o*-Atto647 as an invader strand. **b**, Schematic illustration of the o*-Atto647 invasion process. Hybridization of o*-Atto647 with poly(A₂₀-o)_n starts from the edge of the KL1-polyA co-condensate with a bright and sharp invading front to final dissolution of the whole co-condensate. **c**, Representative CLSM images showing the process of co-condensate (Atto488, green channel) dissolution by adding 300% o*-Atto647 (magenta channel) to hybridize to the poly(A₂₀-o)_n of the PN. **d**, Space-time plot analysis along the white dashed line in **c** shows the gradual dissolution of the condensate. **e**, Normalized fluorescence intensities of KL1 condensates (KL1-Atto488) and invader strand (o*-Atto647) measured in the white dashed circle in **c** during the invasion process. Data are presented as mean values from *N* = 2 PN measured. **f**, Scheme and representative CLSM images of KL1 transcription and condensation (Atto488, green channel) after 18 h in pre-hybridized PN with 0%, 10%, 50%, 80%, and 300% o*-Atto647 (magenta channel). **g**, Scheme shows the attractive interaction between KL1 condensate and ssDNA A₂₀-o-Atto647, and repulsive interaction between KL1 condensate and dsDNA A₂₀-o-Atto647/o*-Atto565. **h**, Representative CLSM images of pure transcriptional KL1 condensates (Atto488, green channel) prepared in solution, with the addition of (top) ssDNA A₂₀-o-Atto647 (magenta channel) for 1 h, showing preferential partitioning, or (bottom) dsDNA A₂₀-o-Atto647/o*-Atto565 (red channel) showing rejection. Three independent experiments show similar results for **a**, **f**, and **h**. Scale bars: 5 μm (**a**, **c**, **f**); 10 μm (**h**).

The poly(A₂₀-o)_n/o*-Atto647 thereafter occupies the space within the entire PN, whereas the KL1 transcripts are squeezed to the PN periphery and eventually dissolve into solution to equilibrate to their low concentration there. This process verifies that the interaction between KL1 and poly(A₂₀-o)_n PN matrix promotes the formation of the KL1-poly(A₂₀-o)_n co-condensate. Moreover, an invasion process of o*-Atto647 at 100 mM Mg²⁺ shows a slower disassembly of the co-condensate compared to 30 mM Mg²⁺ (Figure 4-4c), implying the critical role of Mg²⁺ in stabilizing interactions of KLs and KL-PN (Supplementary Figure 4-11).

Seeing such a profound impact, we then investigated KL1 transcription in PN with a poly(A₂₀-o)_n matrix pre-hybridized by different amounts of o*-Atto647 (from 0% – 300%) to provide weakened affinity between PN matrix and KL1 transcripts. In analogy with the above result, single KL1 condensates form in pristine PN (Figure 4-4f). When applying 10% o*-Atto647, the KL1 transcripts form single condensates with irregular secondary nucleation on its surface inside the PN, along with multiple tiny nuclei outside the PN shell (Figure 4-4f). The brighter green parts are condensates purely enriched with KL1 transcripts that remain inside the PN due to relatively sufficient affinity. The marked difference – heterogeneously structured RNA condensates in Figure 4-4f compared to the rather spherical and homogenous structures formed after invasion of pre-formed condensate in Figure 4-4a, c – can be attributed to their different formation processes. Increasing the content of pre-hybridized o*-Atto647 domains from 10% to 300% gradually prevents KL1 condensate formation inside the PN due to weakened PN-KL1 interaction, which likely becomes even repulsive at higher pre-hybridization degrees because the ssDNA to dsDNA transition leads to higher negative charge density and persistence length, allowing for stronger electrostatic and steric repulsion^{34,35}, respectively, inside the PN. As a result, the KL1 transcripts formed inside the PN do not yield condensates inside the PN, instead, multiple small transcriptional KL1 condensates form in the PN surroundings. These results highlight the importance of the interaction between the DNA matrix of the PN and the KL1 transcripts in both the formation and the maintenance of the condensates within the PN. Hence, modulating the DNA-RNA interaction is a way for regulating nucleus condensate architectures.

To directly study the affinity between polyA sequence of the PN matrix and KL1 condensate, we prepared transcriptional KL1 condensates in solution and added A₂₀-o-Atto647 ssDNA, and A₂₀-o/o*-Atto565 dsDNA. Pure KL1 condensates sequester A₂₀-o-Atto647 whereas A₂₀-o/o*-Atto565 is excluded (Figure 4-4g, h). Such marked differences among interactions between KL1-to-ssDNA versus KL1-to-dsDNA confirm some level of unspecific interaction between the KL1 transcript and the o region, which can be removed through hybridization into o/o*. Furthermore, electrostatic repulsion from increased negative charge density, and steric repulsion from higher persistence length after dsDNA formation also play important roles^{34,35}, as in analogy to re-entrant phenomena in living cells, where transcriptional condensate formation is promoted at low rates of RNA synthesis up to a point of charge imbalance, beyond which higher rates of RNA synthesis disfavors condensate formation^{11,36}.

4.2.4 Formation of Orthogonal Transcriptional KL Condensates in PN

Finally, we attempted to integrate orthogonal KL transcription systems into PN for constructing more complex structures to mimic multiple RNA condensates in the crowded environment of natural cell nuclei. We adapted two KL nanostars (KL1-R1 and KL2-R2) with orthogonal kissing loop sequences at the end of their arms, and distinct tail regions (R1 and R2) for specific labeling by R1*-Atto488 and R2*-Atto647, respectively (Figure 4-5a). We firstly confirmed the transcription and the formation of centrally located condensates for both KL1-R1 or KL2-R2 inside PN (Supplementary Figure 4-12a, b). Hence, both systems form similar condensate structure as the original KL1 system and the KL1-BrA system studied above.

Since $[Mg^{2+}]$ can control the condensate morphology (Figure 4-2j), we conducted co-transcription of both KLs in the same PN at 15 and 30 mM Mg^{2+} , respectively (Figure 4-5a). At 30 mM Mg^{2+} , KL1-R1 assembles to a large single condensate (~0.7-fold the diameter of the host) at the PN center, while KL2-R2 forms small condensates, budding at the PN shell, with diameters less than 0.2-fold of the host PN (Figure 4-5b–d). This suggests a preferred interaction between KL1-R1 and PN matrix, retaining the KL1-R1 condensate inside the PN, whereas KL2-R2 gets obviously expelled. KL1-R1 dominates the interaction with the PN matrix in this competitive system, whereas pure KL2-R2-PN would form a single central condensate (Supplementary Figure 4-12b).

To ensure that this bias in structural development does not arise solely from different transcription efficiency, we conducted three control experiments: First, we compared the in-solution transcription efficiency of KL1-R1 and KL2-R2, finding out comparable transcription efficiency of both KL2-R2 and KL1-R1 (Supplementary Figure 4-13). Second, we further screened whether biasing the in-PN transcription towards KL2-R2 by increasing its template concentration to a 3-fold excess over the KL1-R1 template would change the structure formation. However, even under such conditions, the co-condensate morphology does not invert, but KL1 condensates remain inside the PN and KL2 condensates form on the surface of the PN (Supplementary Figure 4-14). Taken together, these results provide mutually reinforcing evidence supporting that the interaction between different KL and PN dominates the formation of the co-condensates and not the pure transcription efficiency. Third, we also probed whether KL2-R2 transcribed in solution can be enriched into pristine PN to check for interactions. Here, distinct differences are visible when comparing the structures formed by in-PN transcription of KL2-R2 versus KL2-R2 transcripts recruited from solution (Supplementary Figure 4-12c). The in-PN transcription clearly induces phase segregation by KL2/KL2 interactions, whereas the latter rather points to some KL2-R2/PN interactions that enable a certain level of recruitment. Clear phase segregation via contraction of a spongy co-condensate phase is not visible for the latter. Overall, the last control also emphasizes the critical role of in-PN transcription to facilitate co-condensate formation.

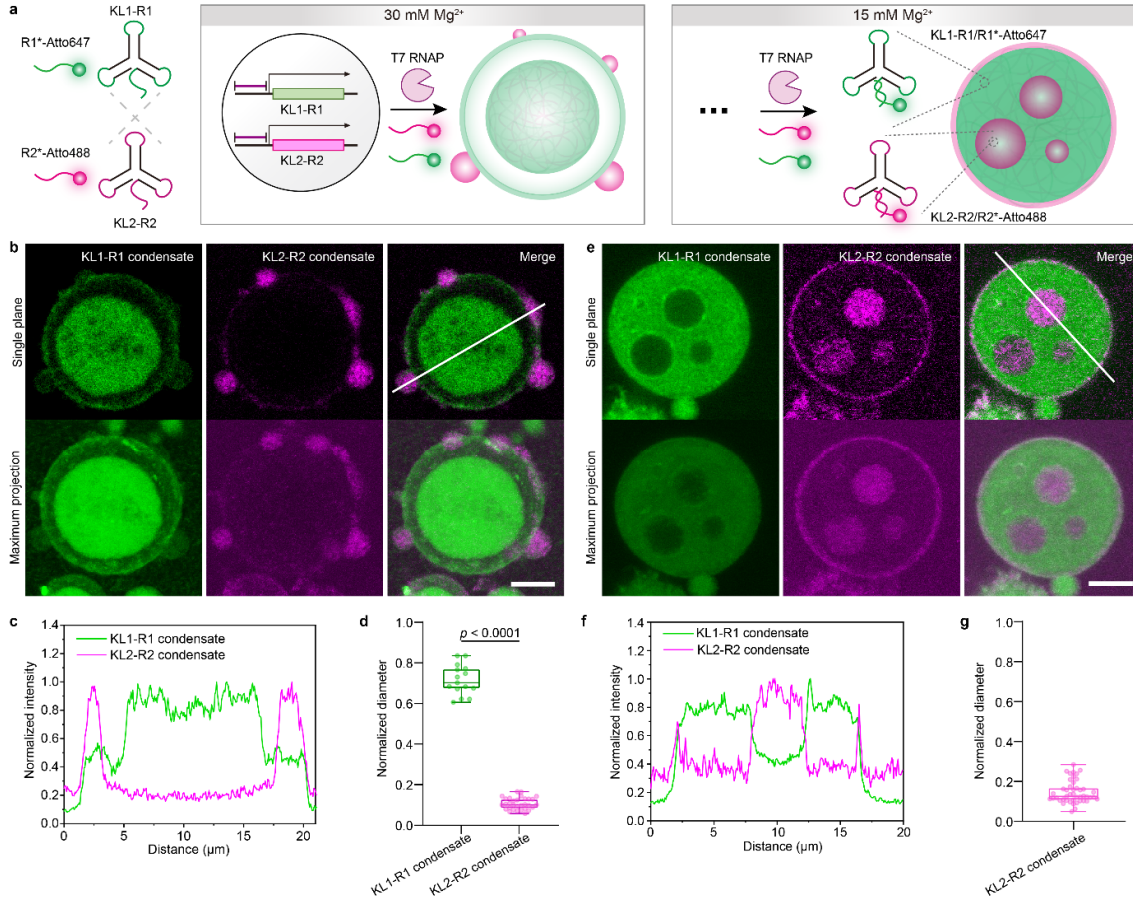


Figure 4-5. Formation of orthogonal transcriptional KL condensates in PN. **a**, Scheme showing orthogonal transcription and condensation of KL1-R1 and KL2-R2 in the same PN at different salinity. KL1-R1 is identical to KL1 in its nanostar framework, but with an additional recognition tail (R1) for R1*-Atto647 labelling. KL2-R2 shares the same stem sequence as KL1 but has orthogonal kissing loop sequences and a distinct recognition tail (R2) for R2*-Atto488 labelling. R1*-Atto647 and R2*-Atto488 are added during transcription. **b**, Representative single-plane CLSM image and maximum intensity projection of z-stacked CLSM image showing orthogonal transcriptional condensates of KL1-R1 (green channel) and KL2-R2 (magenta channel) in PN at 30 mM Mg²⁺ ([NTP] : [R1*] : [R2*] : [T_{KL1-R1}] : [T_{KL2-R2}] : [p] = 3.6 : 1.8 : 1.8 : 0.5 : 0.5 : 1). **c**, Normalized intensity profiles of line segment analyses corresponding to the white line in **b** for both channels. **d**, Diameter of formed orthogonal condensates at 30 mM Mg²⁺, normalized to the diameter of the host PN. *N* = 15 for KL1-R1 condensate, *N* = 31 for KL2-R2 condensate, both from 3 independent experiments. **e**, Representative single-plane CLSM image and maximum intensity projection of z-stacked CLSM image showing orthogonal transcriptional condensates of KL1-R1 and KL2-R2 in PN at 15 mM Mg²⁺ ([NTP] : [R1*] : [R2*] : [T_{KL1-R1}] : [T_{KL2-R2}] : [p] = 3.6 : 1.8 : 1.8 : 0.5 : 0.5 : 1). **f**, Normalized intensity profiles of line segment analyses corresponding to the white line in **e** for both channels. **g**, Diameter of formed KL2-R2 condensates at 15 mM Mg²⁺, normalized to the diameter of host PN. Note that the diameter of KL1-R1 condensates cannot be quantified due to their hollow shape. *N* = 46 for KL2-R2 condensate, from 3 independent experiments. Box plot (**d**, **g**): median (central line), interquartile range (box), min-max (whiskers). Two-sided *t*-test (**d**). Scale bars: 5 μm (**b**, **e**).

At 15 mM Mg²⁺, the transcriptional KL1-R1 occupies the major PN space, while KL2-R2 forms multiple condensates in the PN (Figure 4-5e–g). This can be attributed to weakened interactions between KL1-R1 and the PN at low salinity, allowing KL2-R2 to occupy some of the available volume in the PN to form condensates. Note that a minor distribution of KL1-R1 in KL2-R2, or KL2-R2 in KL1-R1 (Figure 4-5e, f), can be observed, possibly due to the slow diffusion of the produced KLs in the crowded condensates. Hence, the combined effect of

Mg²⁺ on changing the viscoelastic properties and modulating KL interactions as well as KL-to-PN interactions again shows a profound effect. We can conclude that phase segregation of KL1-R1 is energetically favored to be retained in the PN. A mixing of both KL phases does not occur.

To verify the competitive interaction between KL1-R1 and KL2-R2 with the polyA in the PN matrix, we performed competitive partition experiments of A₂₀-o or A₂₀-o/o* with pure transcriptional KL1-R1 and KL2-R2 condensates grown in solution. The results show preferential partitioning of A₂₀-o into the KL1-R1 condensates, whereas A₂₀-o/o* is excluded by both condensates (Supplementary Figure 4-15). This confirms a higher affinity of KL1-R1 condensates to the PN matrix and explains the different condensate architectures formed in the PN. NUPACK simulations further indicate that the origin of the preferred interaction between KL1-R1 and PN matrix is rooted in the tail structure (Supplementary Figure 4-16).

Additionally, transcriptional KL2-R2 condensates grown in solution show a less spherical structure compared with transcriptional KL1-R1 ones (Supplementary Figure 4-15), suggesting stronger condensation interactions for KL2-R2 than KL1-R1, consistent with the higher melting temperature of KL2 interactions than KL1 interactions provided in literature²¹. This also helps to explain the overall architecture of the multiphase structures in Figure 4-5b, e from the perspective of surface tension (γ) of the ternary system of PN, KL1-R1, and KL2-R2^{4,37}. Since KL2-R2 has a higher T_m regarding the KL interactions, the binding strength among KL2-R2 in condensates is stronger and therefore KL2-R2 condensates possess higher surface tension than KL1-R1 condensates. In addition, KL1-R1 has a stronger binding interaction with the PN matrix than KL2-R2. This means $\gamma_{PN/KL2-R2} > \gamma_{PN/KL1-R1}$ applies, so that KL2-R2 condensate are preferably enveloped by KL1-R1 or expelled from PN to minimize its contact with the PN matrix. In summary, these results reveal that, in addition to salinity effects, subtle variations in RNA composition and sequence modulate their interaction with the DNA matrix of PN in a competitive environment, leading to profoundly different condensation processes and resulting in distinct multi-phase co-condensate architectures in PN.

4.3 Conclusions

We have introduced a versatile nucleus-mimicking DNA condensate platform, a protonucleus, that enables localized transcription and the study of phase-separation of transcribed RNA nanostars in crowded and highly concentrated DNA environments. Since the strategy builds on our previous work on all-DNA synthetic cells^{23-25,28}, our approach shows how specific components from a completely different area of research, that is synthetic artificial cell research, can be effectively repurposed into new application domains. These protonuclei offer a highly programmable platform for introducing short genes for transcription while also enabling control over properties such as gene density and the dynamic behavior of the matrix^{23,24}. Transcription inside these crowded PN proceeds with satisfying efficiency up to

high salt concentration. To study transcriptional folding and phase segregation in the crowded, nuclear-mimetic environment, we focused on KL condensates formed by ssRNA nanostars. We have identified that ionic strength is one key parameter for cross-regulating transcription efficiency, viscoelasticity of the PN, and KL-PN affinity. These effects in turn affect the nucleation of condensates from binodal to spinodal or viscoelastic phase separation^{26,30,31}, resulting in tunable artificial nuclear architectures inside the PN. The non-specific interactions between KL and PN matrix turns out to be crucial for retaining KL transcripts inside PN via KL-PN co-condensation. We show how such interactions can be efficiently modulated using DNA nanoscience approaches in such synthetic settings, ultimately leading to a repulsion and exclusion of the KL condensates from the PN.

We have further studied co-transcription and condensation of orthogonal KLs systems within the same PN, which results in distinct structures arising from competitive interactions between different RNA nanostars and the PN matrix. This highlights the potential of using our PN platform to study subtle interactions between RNA and DNA, as well as competitive interactions among RNAs in a DNA-enriched environment. Finally, at proper conditions, multiphase condensate structures can be built, which are further regulated by salinity through the cross-regulation of the viscoelastic environment, transcription efficiency, and competitive KL-PN interaction.

Looking into the future, our work opens new perspectives for constructing artificial nuclear architectures in synthetic model systems with DNA nanoscience tools. While we focus on a rather artificial and well controllable system of KL condensates, this work lays an important cornerstone to study more sophisticated phase separation processes, such as in case of polymerase II that forms rich condensate architectures with helper proteins, e.g., P-TEFb that are involved in transcription elongation, and those which are implicated in disease and ageing^{36,38}. In addition, the modulable KL-PN interactions within protonucleus could serve as simplified models for transcriptional condensates in living cells, which are dynamically forming and dissolving, and essential for transcription regulations^{11,36}. Moreover, from the perspectives of molecular systems engineering, synthetic biology, and artificial cell research, we have identified important pathways to transcriptionally regulate structure formation processes towards multiscale condensates that can be selectively addressed in their compartments. We anticipate that this system will serve as a valuable platform and toolkit for DNA nanoscience and synthetic biology.

4.4 Experimental

4.4.1 Materials

ssDNA were purchased from Biomers and Integrated DNA Technologies (IDT). Supplementary Table 4-1 and Supplementary Table 4-2 summarize all sequences used in this study. T4 DNA Ligase (2 U/ μ L), Exonuclease I (40 U/ μ L), Exonuclease III (200 U/ μ L), and Φ_{29} polymerase (10 U/ μ L) were purchased from Lucigen. Thermostable Inorganic Pyrophosphatase (2 U/ μ L), T7 polymerase (50000 U/mL) and nuclease-free water were bought from New England BioLabs (NEB). Deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP) (100 mM), Aminoallyl-dUTP-XX-ATTO-643 (1 mM), Aminoallyl-UTP-Atto488 (1 mM), and Aminoallyl-UTP-Atto630 (1 mM) were purchased from Jena Bioscience. Hexadecane, sodium chloride, magnesium chloride, Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), Trizma base, acetic acid, and Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), were purchased (as bioreagent grade if available) from Sigma-Aldrich. RNase Inhibitor (40 U/ μ L), RNase-free TE buffer (Invitrogen, 10 mM Tris and 1 mM EDTA, pH 8.0, 500 mL) were purchased from Thermo Fisher Scientific. 384-well high-content imaging glass bottom microplates were purchased from Corning.

4.4.2 Instruments

All thermal annealing and heating ramps were performed on a TPersonal Thermocycler (Analytik Jena). Incubation with shaking was carried out on an Eppendorf ThermoMixer C with heated lid. DNA concentration was determined by a DS-11 Spectrophotometer (DeNovix). Confocal laser scanning microscopy (CLSM) was performed on a Leica Stellaris 5 (software: LAS X, v4.3.0.24308) with an oil-immersion $\times 63$ objective.

4.4.3 Methods

Synthesis of circular ssDNA templates and long ssDNA polymers

The synthesis of the circular DNA template and its corresponding ssDNA polymer can be found in our previous reports²³. In short, the linear ssDNA template and the corresponding ligation strand were firstly mixed at concentration of 1 μ M in 100 μ L TE buffer containing 100 mM NaCl. The solution was heated to 85 $^{\circ}$ C for 5 min before cooling to 25 $^{\circ}$ C with a cooling rate of 0.01 $^{\circ}$ C/s for hybridization. Afterwards, 20 μ L of 10 $\times$ Ligase buffer (500 mM Tris-HCl, 100 mM MgCl_2 , 50 mM dithiothreitol and 10 mM ATP (Lucigen)), 70 μ L of nuclease-free water, and 10 μ L of T4 DNA Ligase (2 U/ μ L (Lucigen)) were introduced into the reaction mixture at room temperature for 3 h reaction. The solution was then heated to 70 $^{\circ}$ C for 20 min to deactivate the enzyme. Then, 10 μ L of Exonuclease I (40 U/ μ L (Lucigen)) and 10 μ L of Exonuclease III (200 U/ μ L (Lucigen)) were added into the reaction mixture for further overnight reaction at 37 $^{\circ}$ C to remove the ligation strands and any non-circularized templates in solution. Afterwards, the

reaction mixture was heated to 80 °C for 40 min to deactivate the enzymes. To obtain the final circular ssDNA templates, the reaction mixture was washed with 400 µL TE buffer and filtrated using Amicon Ultra-centrifugal filters with a 10 kDa cut-off (Merck Millipore) for three times. The concentrations of the collected circular ssDNA templates were measured by the DS-11 Spectrophotometer (DeNovix), and the templates were stored in TE buffer at -20 °C.

For the synthesis of the long ssDNA polymers, we used rolling circle amplification (RCA). 5 µL of circular template (1 µM in TE buffer) and 1 µL of exonuclease resistant primer (10 µM in TE buffer) were mixed with 76 µL of nuclease-free water, 10 µL of commercial 10× polymerase buffer (500 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 40 mM dithiothreitol, 100 mM MgCl₂ (Lucigen)), 2 µL of Φ₂₉ DNA polymerase (10 U/µL (Lucigen)), 1 µL of thermal stable inorganic pyrophosphatase (2 U/µL (NEB)) and 5 µL of adjusted deoxyribose nucleoside 5'-triphosphate mix (100 mM, the mix contains pure dATP, dTTP, dCTP, and dGTP solutions mixed in corresponding proportions of the exact composition of the desired ssDNA polymer repeating units (Jena Bioscience)). Note that for the synthesis of ssDNA polymers with in-chain fluorophores of Atto643, we replaced 2 mol% of the dTTP in the mix with Aminoallyl-dUTP-XX-ATTO-643 for random insertion of the dye into the ssDNA chains during RCA. The reaction mixture was kept at 30 °C for 50 h before thermal cleavage at 95 °C for 15 min to shorten the ultrahigh molecular weight of the synthesized DNA polymer²³. The final products were purified by rinsing with 400 µL TE buffer and filtration in Amicon Ultra-centrifugal filters with 30 kDa cut-off (Merck Millipore) three times. The concentrations of the collected final ssDNA polymers were measured using the DS-11 Spectrophotometer (DeNovix), and the DNA polymers were stored in TE buffer at -20 °C.

Preparation of all-DNA PN embedded with T7 promoter sequence.

The preparation of the PN is adapted from our previous reports²³ with modifications for the formation of PN containing T7 promoter sequence. Adenine-rich DNA polymers (poly(A₂₀-p)_n + poly(A₂₀-o)_n in a ratio of 1:9) (0.5556 g/L) and poly(T₂₀-k)_n (0.0694 g/L) were mixed in TE buffer without any salt at a final volume of 9 µL. The solution mixture was heated at 95 °C for 15 min for thermal cleavage to further reduce the chain length of the ssDNA polymers. Afterwards, 1 µL of TE buffer containing 500 mM MgCl₂ was introduced into the reaction mixture. The solution containing finally 0.5 g/L mixture of polyA and 0.0625 g/L poly(T₂₀-k)_n with 50 mM MgCl₂ was heated to 95 °C for 20 min (3 °C/s) and cooled down to room temperature (3 °C/s), yielding core-shell PN. Finally, the 10 µL of solution containing the PN was diluted 5 times by adding 40 µL of TE buffer containing various amounts of MgCl₂ to reach desired salinity. The obtained 50 µL of DNA condensates solution (as 5× diluted) has 0.1 g/L polyA mixture and 0.0125 g/L poly(T₂₀-k)_n, corresponding to ca. 0.8 µM p barcode, ca. 7.2 µM o barcode and ca. 1 µM k barcode, respectively, in total solution. The solution was then stored in a fridge at 4 °C for 1 week for equilibration before usage.

Spatially controlled transcription assay in PN.

For transcription in PN monitored by plate reader, 3.125 μ L of 5 $\times$ diluted PN (90% o barcode + 10% p barcode) was further diluted into 25 μ L of solution containing 1 $\times$ RNA polymerase buffer (40 mM Tris-HCl, 6 mM MgCl₂, 1 mM DTT, 2 mM spermidine), 100 nM template (T_{Rep^*}), 1 μ M prehybridized fluorophore-quencher reporter (Rep/Rep' dsDNA), 2 U/ μ L T7 RNAP, 0.02 U/ μ L Thermostable Inorganic Pyrophosphatase, 1 U/ μ L RNase Inhibitor. MgCl₂ concentration was adjusted in different settings as noted in each figure caption. At the end, 2 μ L of NTP mix (to reach 2 mM of ATP, GTP, CTP, and UTP each) was added into the solution to trigger the transcription reaction at different temperatures ranging from 25–30 °C. The final promoter sequence concentration in the solution is at 100 nM. As for control, transcription with pure promoter ssDNA (p) and poly(A₂₀-p)_n ssDNA polymer was also performed to compare the transcription efficiency.

For kinetic experiments under CLSM, T_{x^*} was loaded into the PN at a final concentration of 100 nM. Reporter was not used, instead, we further added 0.0833 mM Aminoallyl-UTP-Atto488 so that the transcribed RNA can be fluorescently labeled and observed under CLSM. For each plate reader experiment, we have included a reference sample containing 1000 nM DNA-fluorophore conjugate. We calculate the intensity ratio between individual samples and the reference sample to yield the transcribed RNA concentration by following equation:

$$[RNA] = 1000 \text{ nM} \times \frac{\text{Intensity (sample)}}{\text{Intensity (reference)}} \quad (1)$$

[NTP] calculation

[NTP] is defined as the maximum amount of transcripts that can be produced per template given the nucleotide concentrations in the NTP mix in relation to the sequence of the transcript. For example for [NTP] : [template] = 5 : 1, the [NTP] concentration is set in a way to at least allow for 5 full transcripts from 1 template based on the most abundant nucleotide in the transcript. Other NTPs will be in a slight excess as the NTP mix has equal stoichiometry for all four needed NTPs.

Transcriptional KLs condensates formation.

2.5 μ L of 5 $\times$ diluted PN (90% o barcode + 10% p barcode) was further diluted into 20 μ L solution containing 1 $\times$ RNA polymerase buffer (40 mM Tris-HCl, 6 mM MgCl₂, 1 mM DTT, 2 mM spermidine), 100 nM dsDNA template ($T_{KL1}/T_{KL1'}$, $T_{KL1-BrA}/T_{KL1-BrA'}$, $T_{KL1-R1}/T_{KL1-R1'}$, or $T_{KL2-R2}/T_{KL2-R2'}$; or 50 nM $T_{KL1-R1}/T_{KL1-R1'}$ + 50 nM $T_{KL2-R2}/T_{KL2-R2'}$), 2.5 U/ μ L T7 RNAP, 0.02 U/ μ L Thermostable Inorganic Pyrophosphatase, 1 U/ μ L RNase Inhibitor, and 0.048 mM NTP mix (0.048 mM of ATP, GTP, CTP, and UTP each at [NTP] : [T_{KL1}] = 3.6 : 1, for maximum amount of KL1 produced, which is 3.6-fold of T_{KL1} , adjusted in different settings as noted in figure captions). MgCl₂ concentration was adjusted to 30 mM unless otherwise specified in figure captions. The mixture was incubated with shaking at 30 °C for 18 h reaction. The final promoter sequence concentration in the solution was at 100 nM. As control, transcription of

KLs with pure promoter oligo was also performed with corresponding NTP concentration. For transcription of KLs with covalent label, 1 mol% of UTP was replaced by either Aminoallyl-UTP-Atto488, or Aminoallyl-UTP-Atto630 in transcription system. For KL1-BrA transcription, 0.05 mM DFHBI was added to the solution. For KL1-R1 or KL2-R2 transcription, 360 nM R1*-Atto647 or R2*-Atto488 sequence was added to the system, respectively. For transcriptional KL1 condensate formed in solution as a control, 100 nM promoter ssDNA was added to system, instead of PN.

Fluorescence recovery after photobleaching (FRAP) experiments.

FRAP experiments were performed by applying 3 times bleaching in a small circular region of interest (ROI) with diameter of 2 μm by 100 % laser intensity. Post-bleaching images were recorded over different periods at 30 °C. The intensities within the circular ROI (I_{ROI}), and intensities in a circular region of the same size away from bleached region within the condensates (I_{ref}), in pre- and post-bleaching images were measured in ImageJ for performing double normalization in bleached regions by:

$$I_{\text{Norm}}(t) = \frac{I_{\text{ROI}}(t)}{I_{\text{ROI}}(t_0)} \times \frac{I_{\text{ref}}(t_0)}{I_{\text{ref}}(t)} \quad (2)$$

to quantify the recovery kinetics over time. Note that $I(t_0)$ represents the intensity measured in the first image before bleaching.

Statistics and reproducibility

Statistical parameters including the definitions and exact value of N (e.g., total number of experiments, PN, or condensate), deviations, p values, and the types of the statistical tests are reported in the figures and corresponding figure legends. Statistical analysis was carried out using GraphPad Prism 10.3.1 (509). Statistical analysis was conducted on data from three or more independent experimental replicates. Comparisons between groups were planned before statistical testing and target effect sizes were not predetermined. Error bars displayed on graphs represent the mean $\pm$ standard deviation (s.d.) or mean $\pm$ standard error of the mean (s.e.m.) from at least three independent experiments, as indicated in the corresponding figure legends. Statistical significance was analyzed using an unpaired Student's t -test for two groups. $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ were considered significant. For Figure 4-2c,k and Figure 4-4e, mean values were presented from two independent experiments with similar results. All representative CLSM images were obtained from three independent experiments with similar results. CLSM images were analyzed by ImageJ (v2.9.0; 64-bit). Data analysis was performed on Microsoft 365 Excel (Version 2307) or MATLAB (R2023a). Origin 2023 (v10.0.0.154) or GraphPad Prism 10.3.1 (509) was used for plot.

4.4.4 Supplementary Tables

	Name	Sequence (5' → 3')	Purification	Modification	Supplier
Template and RCA	Tp(A ₂₀ -p)	/Phosphate/ATA GTG AGT CGT ATT ATT TTT TTT TTT TTT TTT TTT ATC CCT	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -o)	/Phosphate/TAC CTC AAT GCT TTT TTT TTT TTT TTT TTT TTG GGA GCA ACA A	HPLC	5'-Phosphorylation	Biomers
	Tp(T ₂₀ -k)	/Phosphate/ATC CTC TAA AAT CAA AAA AAA AAA AAA AAA AAA GTA AAA CCA CAC G	HPLC	5'-Phosphorylation	Biomers
	ligation-p	TAA TAC GAC TCA CTA TAG GGA T	HPLC	None	Biomers
	ligation-o	GCA TTG AGG TAT TGT TGC TCC CA	HPLC	None	Biomers
	ligation-k	TTT TAG AGG ATC GTG TGG TTT T	HPLC	None	Biomers
	primer-p	TAA TAC GAC TCA CTA TAG GG*A*T	Desalting	Phosphorothioated twice (*)	IDT
	primer-o	GCA TTG AGG TAT TGT TGC TC C*C*A	Desalting	Phosphorothioated twice (*)	IDT
	primer-k	TTT TAG AGG ATC GTG TGG TT*T*T	Desalting	Phosphorothioated twice (*)	IDT
Label	Atto565-p*	/ATTO565/ATC CCT ATA GTG AGT CGT ATTA	HPLC	5'-Atto565	Biomers
	Atto647-o*	/ATTO647N/TGG GAG CAA CAA TAC CTC AAT GC	HPLC	5' Atto647N	Biomers
	Atto488-k*	/ATTO488/ AAA ACC ACA CGA TCC TCT AAA A	HPLC	5' Atto488	Biomers
	Atto647-k*	/ATTO647N/ AAA ACC ACA CGA TCC TCT AAA A	HPLC	5' Atto647N	Biomers
	Atto565-A ₂₀ -o	/Atto565/ AAA AAA AAA AAA AAA AAA AA GCA TTG AGG TAT TGT TGC TCCCA	HPLC	5' Atto565	Biomers
	Atto647-A ₂₀ -o	/Atto647N/ AAA AAA AAA AAA AAA AAA AA GCA TTG AGG TAT TGT TGC TCCCA	HPLC	5' Atto647N	Biomers

Supplementary Table 4-1. Oligomers for PNs and labels, with their names, sequences, purification methods, modifications, and suppliers.

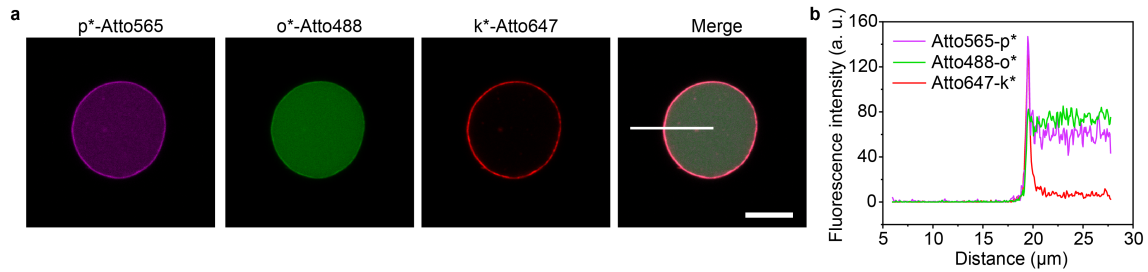
Chapter 4. Constructing Synthetic Nuclear Architectures in a DNA Protonucleus

	Name	Sequence (5' → 3')	Purification	Modification	Supplier
Transcription	P (promoter)	TAA TAC GAC TCA CTA TAG GGA T	HPLC	None	Biomers
	Rep	/6-FAM/CTA CAT CCA CAT ACT A	HPLC	5'-6-FAM	Biomers
	Rep'	GTT AAT TAG TAT GTG GAT GTAG/BMN-Q1/	HPLC	3'-BMN-Q1	Biomers
	R1 Rep	/6-FAM/ATC TGA ACG AGT AAG G	HPLC	5'-6-FAM	Biomers
	R1 Rep'	TGG GGT CCT TAC TCG TTC AGA T/BMNQ1/	HPLC	3'-BMN-Q1	Biomers
	R2 Rep	/6-FAM/GTG GCT TAT TTA CAG G	HPLC	5'-6-FAM	Biomers
	R2 Rep'	CTA ACG CCT GTA AAT AAG CCA C/BMNQ1/	HPLC	3'-BMN-Q1	Biomers
	T _{Rep} * for plate reader	GTT AAT TAG TAT GTG GAT GTA GAT CCC TAT AGT GAG TCG TAT TA	HPLC	None	Biomers
	T _x * for CLSM	TTA GGA TAG ATA TAC GGG TTC ATC CCT ATA GTG AGT CGT ATT A	HPLC	None	Biomers
Kissing Loop	T _{KL1}	CACTCATAGCACTGTGCTTTCGCGATGCACAATGCTAC GAGTGACGCGTACCTCAAAGGACTTTCGCGATGTCCTC TGAGGCACGCGAGCAGCTAGAGCCGCTCTTTCGCGA TGAGCGACTCTAATGTGCATCCCTATAGTGAGTCGTAT TA	HPLC	None	IDT
	T _{KL1} '	GCACATTAGAGTCGCTCATCGGAAAGAGCGGCTCTA GTGTGCTCGCGTGCCTCAGAGGACATCGCGAAAGTCC TTTGAGGTACGCGTCACTCGTAGCATTGTGCATCGCGA AAGCACAGTGCTATGAGTGATGAGTCGTATGAGTG	HPLC	None	IDT
	T _{KL1-R1}	TGGGGTCTTACTCGTTCAGATGCACTCATAGCACTGT GCTTTCGCGATGCACAATGCTACGAGTGACGCGTACCT CAAAGGACTTTCGCGATGTCCTCTGAGGCACGCGAGC ACACTAGAGCCGCTCTTTCGCGATGAGCGACTCTAATG TGCATCCCTATAGTGAGTCGTATTA	HPLC	None	IDT
	T _{KL1-R1} '	GCACATTAGAGTCGCTCATCGGAAAGAGCGGCTCTA GTGTGCTCGCGTGCCTCAGAGGACATCGCGAAAGTCC TTTGAGGTACGCGTCACTCGTAGCATTGTGCATCGCGA AAGCACAGTGCTATGAGTGATGAGTCGTATGAGTG CCCCA	HPLC	None	IDT
	T _{KL2-R2}	CTAACGCCTGTAATAAGCCACCCACTCATAGCACTGT GCTTGTGCACTGCACAATGCTACGAGTGACGCGTACCT CAAAGGACTTGTGCACTGTCTCTGAGGCACGCGAGC ACACTAGAGCCGCTCTTGTGCACTGAGCGACTCTAATG TGCATCCCTATAGTGAGTCGTATTA	HPLC	None	IDT
	T _{KL2-R2} '	GCACATTAGAGTCGCTCAGTCGACAAGAGCGGCTCTA GTGTGCTCGCGTGCCTCAGAGGACAGTCGACAAGTCC TTTGAGGTACGCGTCACTCGTAGCATTGTGCACTGCAC AAGCACAGTGCTATGAGTGAGTGAGTATTTACAGGC GTTAG	HPLC	None	IDT
	Atto647-R1*	/ATTO647N/TCC TTA CTCG	HPLC	5'-Atto647N	Biomers
	Atto488-R2*	/ATTO488/CGC CTG TAAA	HPLC	5'-Atto488	Biomers
	T _{KL1-BrA}	GGAGCCCACTCTACTCAACAGGCAACATTTTGTGC CTGGACCCGACCGTCTCAACACTCATAGCACTGTGCT TTCGCGATGCACAATGCTACGAGTGACGCGTACCTCAA AGGACTTTCGCGATGTCCTCTGAGGCACGCGAGCACA CTAGAGCCGCTCTTTCGCGATGAGCGACTCTAATGTGC ATCCCTATAGTGAGTCGTATTA	HPLC	None	IDT
	T _{KL1-BrA} '	GCACATTAGAGTCGCTCATCGGAAAGAGCGGCTCTA GTGTGCTCGCGTGCCTCAGAGGACATCGCGAAAGTCC TTTGAGGTACGCGTCACTCGTAGCATTGTGCATCGCGA AAGCACAGTGCTATGAGTGTTGGAGACGGTCGGGTCC AGGCACAAAAATGTTGCCTGTTGAGTAGAGTGTTGGGC TCC	HPLC	None	IDT

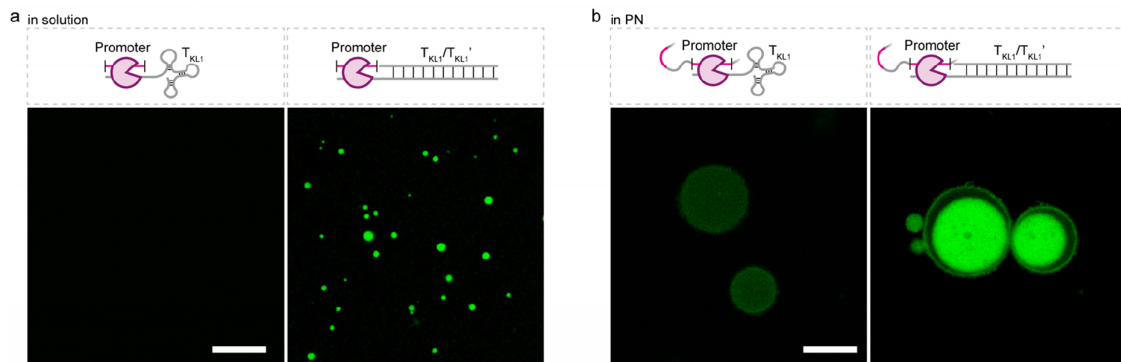
Supplementary Table 4-2. Oligomers for kissing loop condensate transcription, with their names, sequences, purification methods, modifications, and suppliers.

* Represents a fully complementary sequence; ' denotes a partially complementary sequence with a toehold.

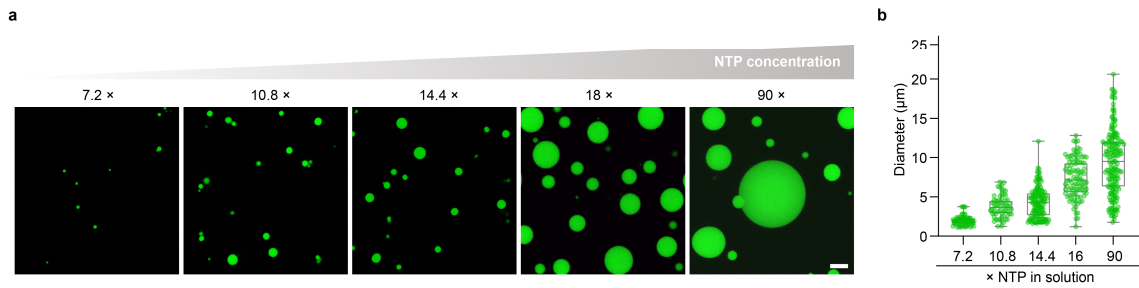
4.4.5 Supplementary Figures



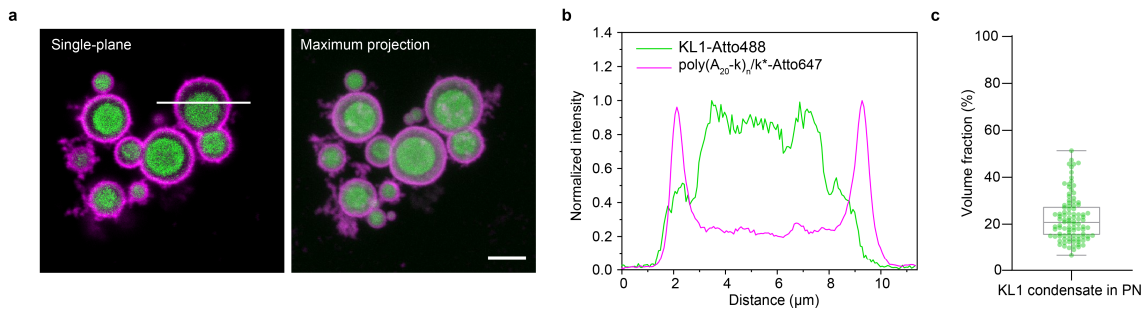
Supplementary Figure 4-1. Preparation of PNs containing promoter sequences (p) inside. **a**, Representative CLSM images of the PN containing different core (p and o) and shell (k) barcodes, labeled by their complementary ssDNA strands (k*-Atto647, p*-Atto565, and o*-Atto488). **b**, Fluorescence intensity profiles corresponding to the line segment analysis along the white line in **a** in three channels, showing a core-shell structure with poly(A₂₀-p)_n and poly(A₂₀-o)_n colocalized at the interior of the PN, and poly(T₂₀-k)_n at the PN shell. Scale bar: 10 μm.



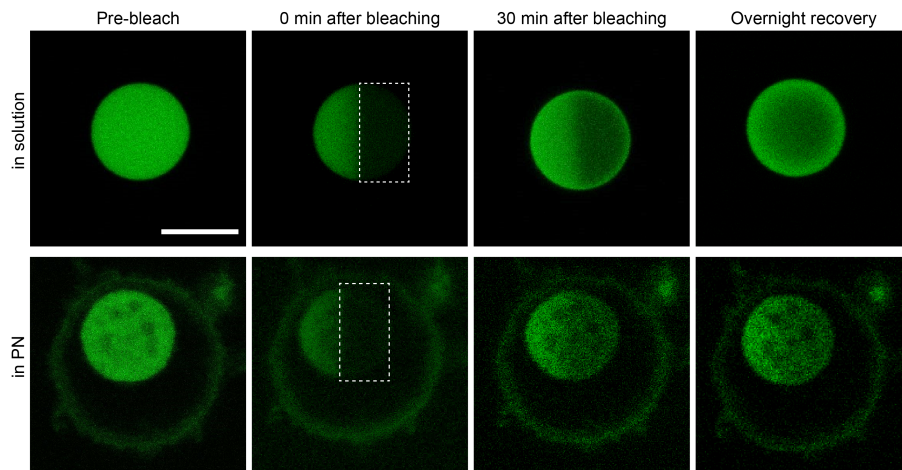
Supplementary Figure 4-2. ssDNA template hinders the formation of transcriptional KL condensates in both solution and PN. **a**, Representative CLSM images of transcriptional KL condensate in solution with [NTP] : [TKL1] = 7.2, transcribed by promoter ssDNA, which is hybridized with TKL1 as a ssDNA template or TKL1/TKL1' as a dsDNA template ([TKL1] : [p] = 1 : 1, 30 °C, 30 mM Mg²⁺, 2.5 U/μL T7 RNAP). Note that [NTP] is set to a concentration where condensation in solution appears (see Supplementary Figure 4-3). **b**, Representative CLSM images of transcriptional KL in PN with ssDNA template or dsDNA template for transcription ([NTP] : [TKL1] = 3.6, 30 °C, 30 mM Mg²⁺, 2.5 U/μL T7 RNAP). Scale bar: 10 μm.



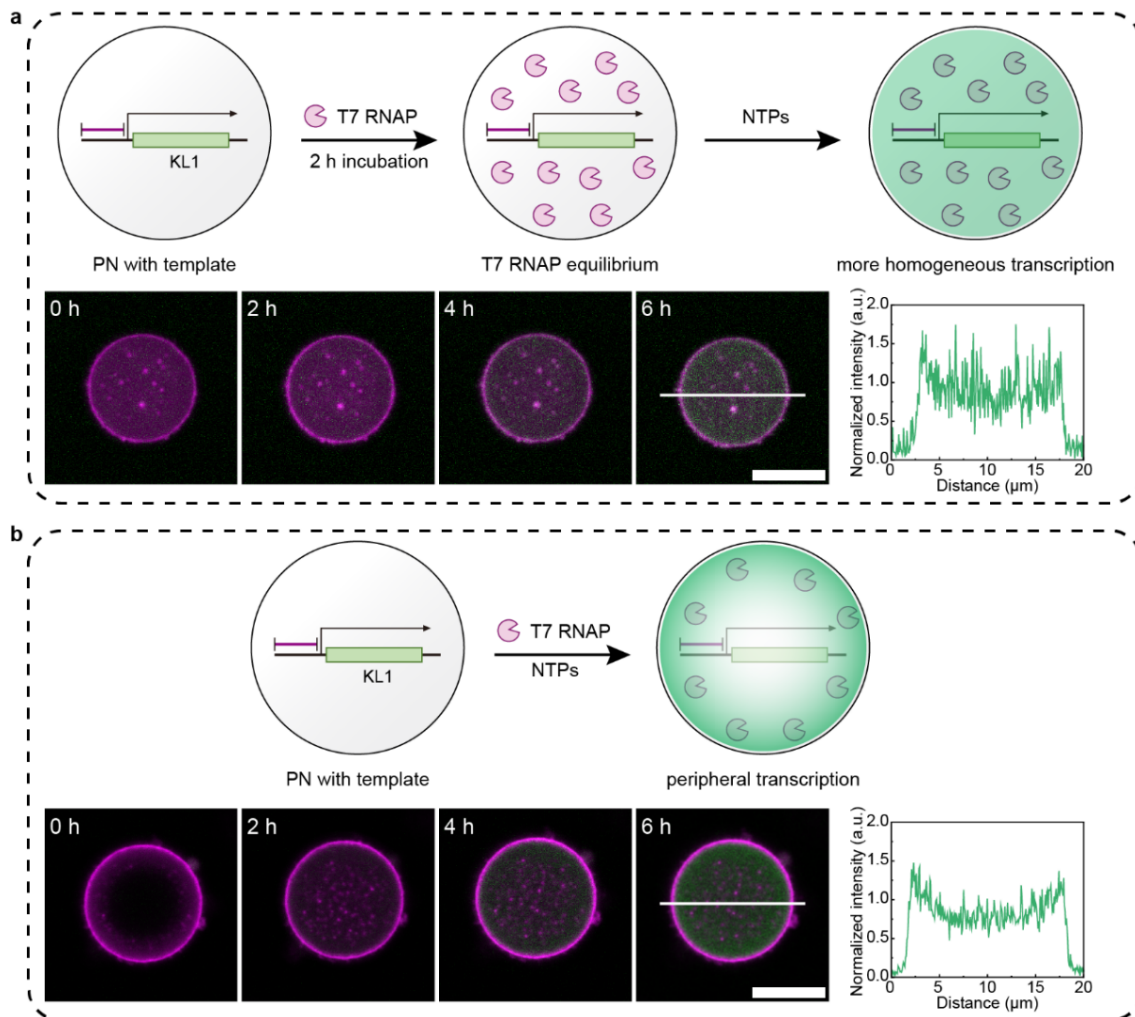
Supplementary Figure 4-3. Formation of transcriptional KL1 condensates in solution at different NTP concentrations. **a**, Representative CLSM images of in-solution transcriptional KL1 condensates at various [NTP] : [TKL1] ratios from 7.2 to 90, transcribed by promoter ssDNA ([TKL1] : [p] = 1 : 1). **b**, Diameter distribution at different NTP concentrations. Box plot (**b**): median (central line), interquartile range (box), min-max (whiskers). $N = 61 - 129$ from 3 independent experiments. Scale bar: 10 μm.



Supplementary Figure 4-4. Formation of single transcriptional KL1 condensates in PNs. **a**, Representative single-plane CLSM image and maximum intensity projection of z-stacked CLSM images showing the formation of single KL1 condensates in each PN ([NTP] : [TKL1] = 3.6 : 1, 30 °C, 30 mM Mg²⁺, 2.5 U/μL T7 RNAP, 18 h reaction). Green channel: KL1 condensate (labeled by UTP-Atto488); Magenta channel: PN shell (labeled by k'-Atto647). Scale bar: 5 μm. **b**, Normalized intensity profiles corresponding to the line segment analysis along the white line in **a** showing the distribution of KL1 condensates with a spongy structure, and the PN shell. **c**, Volume fraction of single transcriptional KL1 condensate relative to the host PNs. This figure relates to Figure 4-2e in the main text.

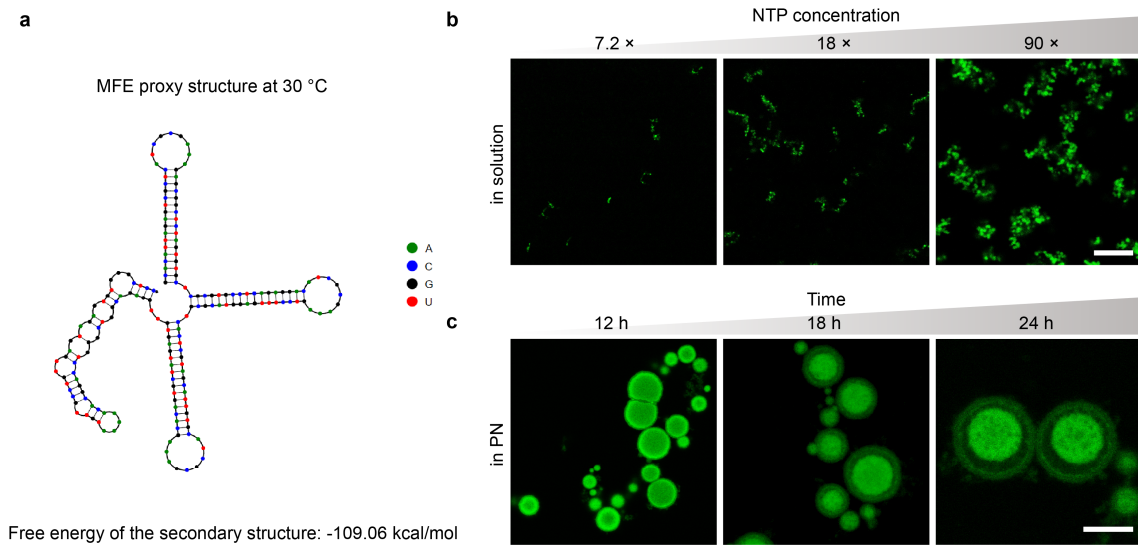


Supplementary Figure 4-5. Half-bleaching experiment on KL1 condensates in solution and in PN. Representative CLSM images showing the transcriptional KL1 condensates in solution and in PN, before and after bleaching at different times. The bleached regions are indicated by the white dashed rectangle. Scale bar: 5 μm.

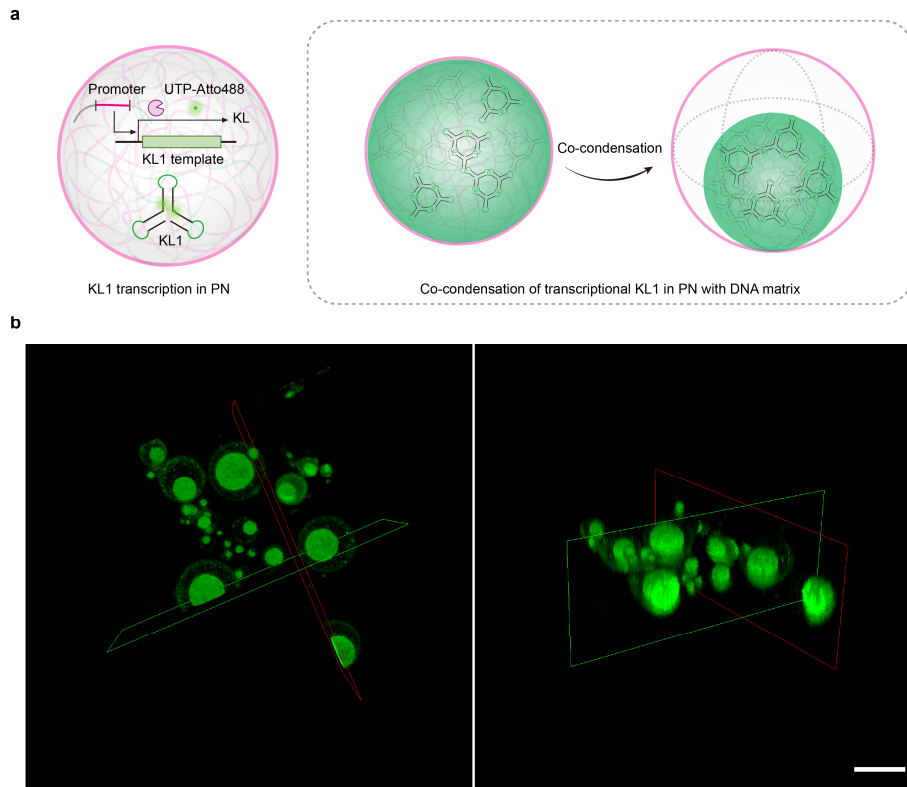


Supplementary Figure 4-6. Pre-equilibrium of T7 RNAP enables a more homogenous transcription inside PN.

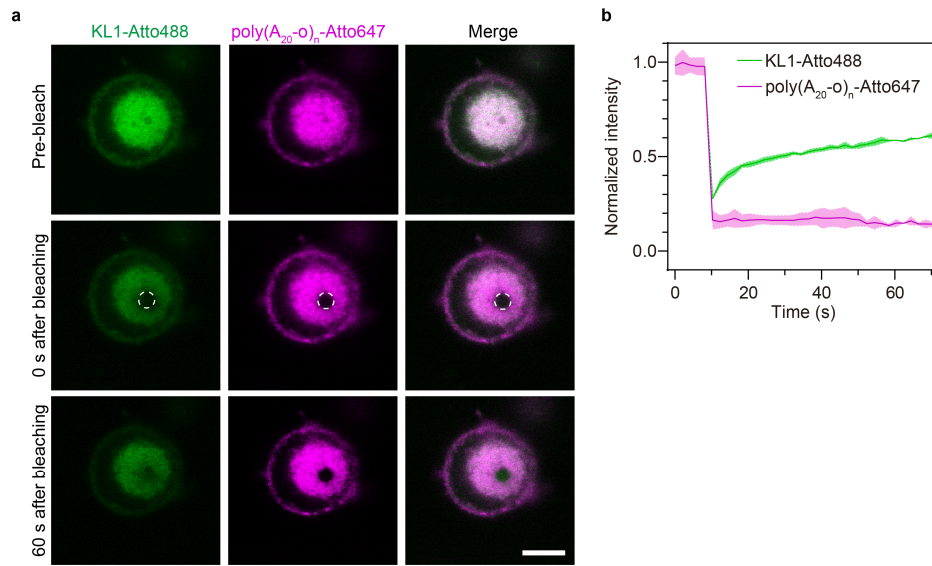
Rationale: To investigate if the diffusion of T7 RNAP into PN has an impact on the peripheral transcription, we performed transcription of KL1 inside PN equilibrated with T7 RNAP prior the addition of NTPs. **a**, scheme and representative CLSM images of KL1 transcription in PN pre-equilibrated with T7 RNAP for 2 h, before the addition of NTPs to trigger transcription. The plot shows cross-sectional line profile along the white line in the CLSM image at 6 h, demonstrating a more homogenous transcription inside the PN. **b**, scheme and representative CLSM images of KL1 transcription in PN with simultaneous addition of T7 RNAP and NTPs (standard conditions used in the main text). The plot shows cross-sectional line profile along the white line in the CLSM image at 6 h, demonstrating a non-homogenous and peripheral transcription inside the PN. Green channel: KL1 condensate labeled by UTP-Atto488; Magenta channel: PN shell (poly(T₂₀-k)_n labeled with k*-Atto647). [NTP] : [TKL1] = 3.6 : 1, 1 mol% UTP-Atto488, 30 °C, 2.5 U/ μL T7 RNAP, 30 mM Mg²⁺. Note that we only focus on the first 6h of co-transcriptional condensation, because afterwards it's rather the maturation of the structure/co-condensate. Scale bars: 10 μm .



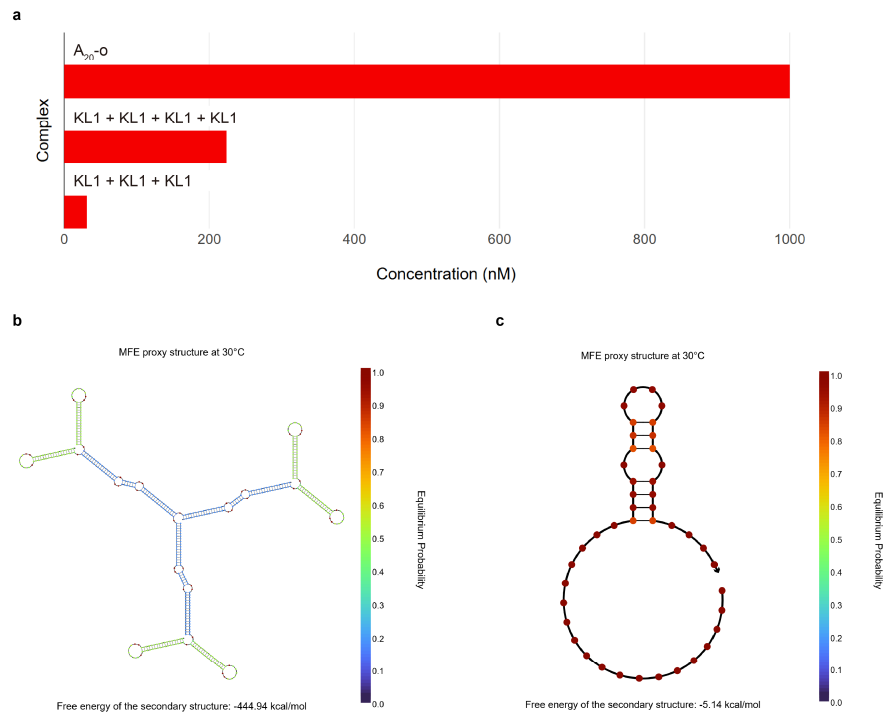
Supplementary Figure 4-7. Design and transcription of KL1-BrA in solution and in PN. **a**, NUPACK-simulated structure of KL1-BrA at 30 °C. **b**, Transcription and assembly of KL1-BrA in solution at varying [NTP] : [TKL1] ratios from 7.2 to 90 ([TKL1] : [p] = 1 : 1, 30 °C, 30 mM Mg²⁺, 24 h reaction). **c**, Formation of transcriptional KL1-BrA condensates in PN over 12–24 h ([NTP] : [TKL1] : [p] = 3.6: 1 : 1, 30 °C, 30 mM Mg²⁺, 2.5 U/μL T7 RNAP). Scale bars: 10 μm.



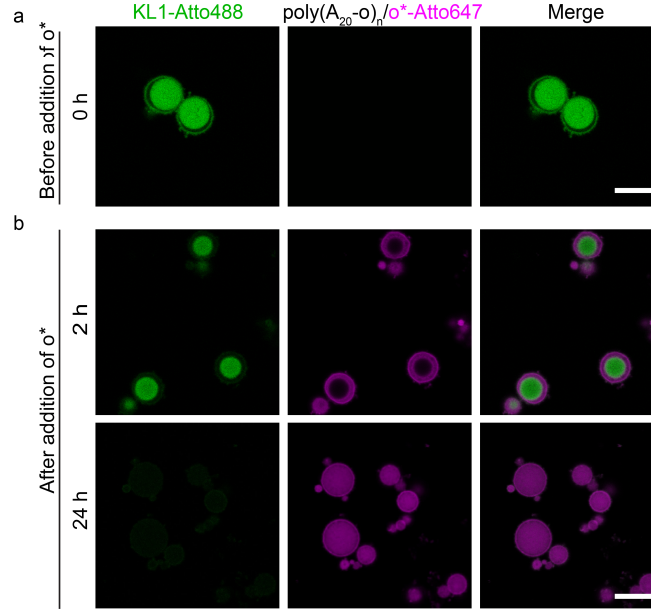
Supplementary Figure 4-8. Formation of transcriptional KL1-PN co-condensate deposited at the bottom of host PN. **a**, Schematic illustration of KL1-PN co-condensation between KL1 transcripts and DNA matrix of the host PN induced by localized transcription of KL1 in the PN. **b**, Representative 3D CLSM images showing the top (left) and front (right) views of co-condensates in PN. Scale bar: 10 μm.



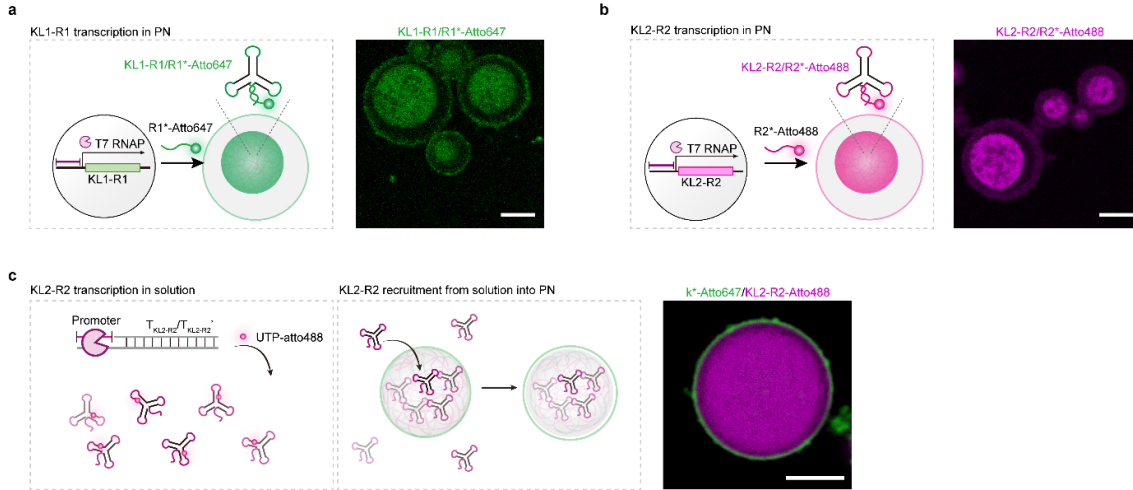
Supplementary Figure 4-9. Dynamic properties of KL1 phase and PN matrix phase in the KL1-PN co-condensate quantified by FRAP. **a**, Representative CLSM images showing KL1 condensate phase (green channel) and PN matrix phase (magenta channel) of a co-condensate in PN before and after bleaching at 30 °C. The white dashed circles indicate the bleached regions. **b**, Normalized fluorescence recovery kinetics in the bleached regions in **a** for the KL1 phase and the PN matrix phase, quantifying the molecular diffusivity of co-condensates in PN at 30 °C. Intensity values were normalized to pre-bleached levels. Data are presented as mean $\pm$ SD ($N = 4$ independent experiments). Scale bar: 5 μ m.



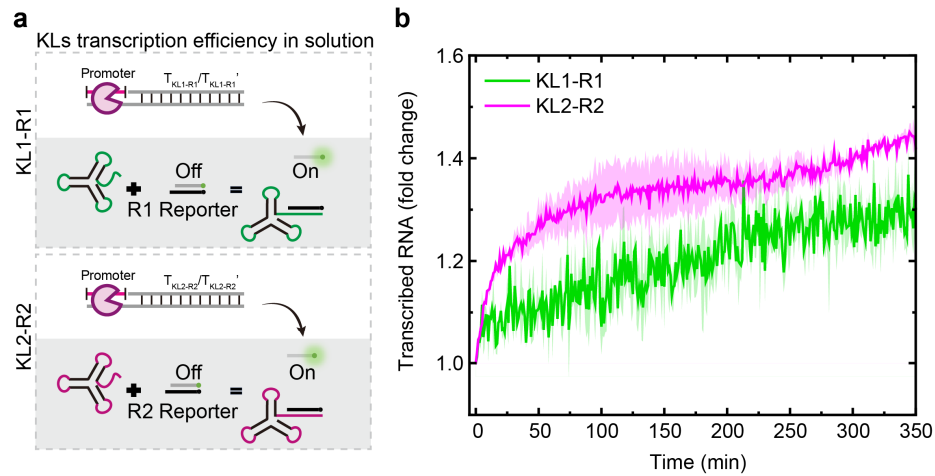
Supplementary Figure 4-10. NUPACK simulation showing the absence of specific interaction between A₂₀-o ssDNA and KL1 sequence. **a**, Simulated complex structures formed by 1 μ M A₂₀-o (ssDNA) and 1 μ M KL1 (ssDNA) at 30 °C. **b**, NUPACK-simulated structures of KL1 + KL1 + KL1 + KL1. **c**, NUPACK-simulated structures of A₂₀-o. No complex structure between A₂₀-o and KL1 is formed. Note that all simulations are based on DNA sequences.



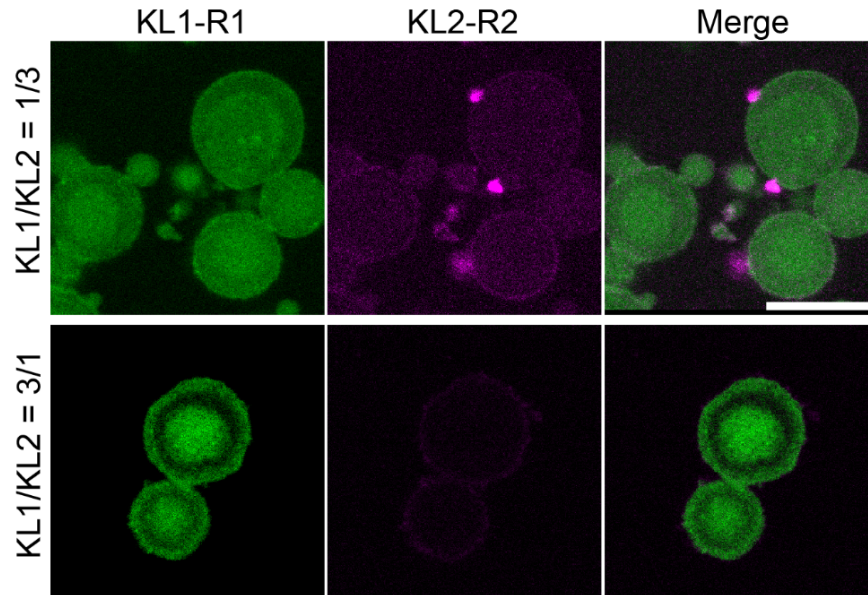
Supplementary Figure 4-11. Invasion of o*-Atto647 at 100 mM Mg²⁺ shows slower disassembly of KL-PN co-condensate compared to 30 mM Mg²⁺. **a**, Representative CLSM images of KL-PN co-condensate formed by KL1 transcription in PN with 30 mM Mg²⁺ (KL1-Atto488, green channel). The overall Mg²⁺ concentration was then increased to 100 mM, and the solution was incubated for 24 h before the addition of o*-Atto647 to hybridize with poly(A₂₀-o)n in PN (magenta channel). **b**, Representative CLSM images of KL-PN co-condensate at 2 or 24 h after o*-Atto647 addition at 100 mM Mg²⁺. Scale bar: 10 μm.



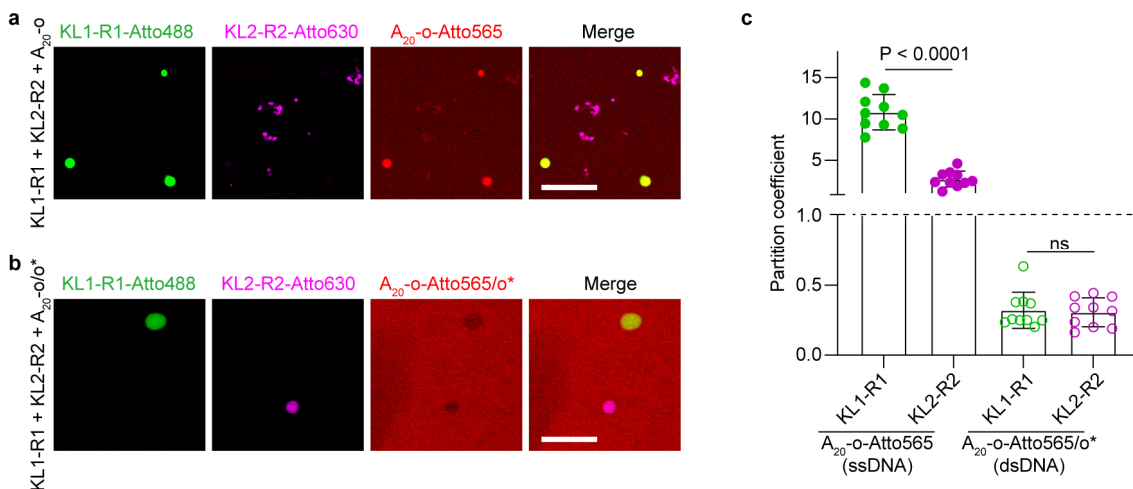
Supplementary Figure 4-12. Formation of KL1-R1 and KL2-R2 condensates in PN versus KL2-R2 transcribed in solution and recruited into PN. **a**, Scheme and representative CLSM image showing the formation of single condensates in PN by localized transcription of KL1-R1. The condensate is labeled by R1*-Atto647 (green channel). **b**, Scheme and representative CLSM image showing the formation of single condensates in PN by localized transcription of KL2-R2. The condensate is labeled by R2*-Atto488 (magenta channel). 2.5 U/μL T7 RNAP, 30 mM Mg²⁺, 30 °C, [NTP] : [R1* or R2*] : [TKL1-R1 or TKL1-R1] : [p] = 3.6 : 3.6 : 1 : 1, 18 h reaction for both **a** and **b**. **c**, Scheme and representative CLSM images for the transcription of KL2-R2 in solution, which gets recruited into the PN. KL2-R2 was first transcribed in solution with UTP-Atto488 feeding ([NTP] : [TKL2-R2] = 3.6 : 1, 1 mol% UTP-Atto488, 30 °C, 2.5 U/μL T7 RNAP, 30 mM Mg²⁺). Pristine PN was added and incubated with the existing KL2-R2 for 18 h for recruitment at 30 °C with 30 mM Mg²⁺. Green channel: PN shell labeled with k*-Atto647; Magenta channel: KL2-R2-Atto488. Scale bars: 5 μm (**a**, **b**); 10 μm (**c**).



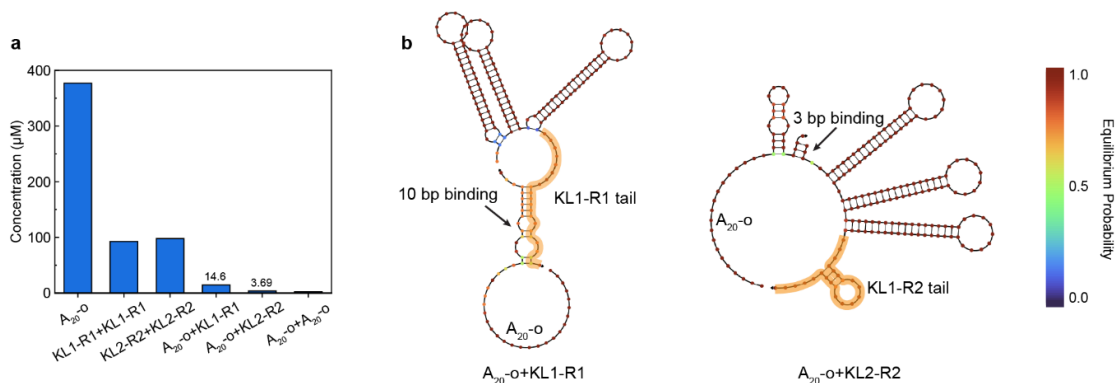
Supplementary Figure 4-13. Transcription kinetics of KL1-R1 and KL2-R2 in solution. **a**, Scheme showing transcription of KL1-R1 (top) or KL2-R2 (down) in solution characterized by plate reader. To quantify the transcribed KLs RNA, dsDNA reporters with fluorophore-quencher pairs are present in solutions to react with dangling strand of transcribed KLs (R1 Reporter for KL1-R1, R2 Reporter for KL2-R2) by SDR, generating fluorescent signals. **b**, Transcription kinetics for KL1-R1 and KL2-R2 in solution with free promoter oligonucleotide, monitored by plate reader ([NTP] : [Rx Reporter] : [TKLx-Rx] : [p] = 100 : 10 : 1 : 1, 30 °C, 6 mM Mg^{2+} , 2.5 U/ μ L T7 RNAP, x = 1 or 2). Data are presented as mean $\pm$ SD from $N = 2$ independent experiments.



Supplementary Figure 4-14. Effect of asymmetric template concentrations for orthogonal KLs on the formation of condensates in PN. Representative CLSM images of orthogonal KLs transcription in PN with 1/3 ratio (top) and 3/1 ratio (bottom) of TKL1-R1/TKL2-R2 at 30 Mg^{2+} ([NTP] : [R1*] : [R2*] : [TKL1-R1] : [TKL2-R2] : [p] = 3.6 : 1.8 : 1.8 : 0.75 : 0.25 : 1). Green channel: KL1-R1; Magenta channel: KL2-R2. Scale bar: 10 μ m.



Supplementary Figure 4-15. Preferred partitioning of A20-o ssDNA into KL1-R1 condensates and exclusion of A20-o/o* dsDNA from both KL1-R1 and KL2-R2 condensates. **a**, Representative CLSM images of mixed transcriptional KL1-R1 and KL2-R2 condensates in solution, with the addition of ssDNA A₂₀-o-Atto565 for 1 h. **b**, Representative CLSM images of mixed transcriptional KL1-R1 and KL2-R2 condensates in solution with the addition of dsDNA A₂₀-o-Atto565/o* for 1 h. In **a** and **b**, both transcriptional condensates were initially prepared in separate reactions and subsequently mixed. ([NTP] : [TKL1-R1 or TKL2-R2] : [p] = 14.4 : 1 : 1, 30 °C, 30 mM Mg²⁺, 18 h reaction). KL1-R1 and KL2-R2 transcripts are labeled with 1 mol% UTP-Atto488 or UTP-Atto630, respectively. Green channel: KL1-R1 condensate; Magenta channel: KL2-R2 condensate; Red channel: A₂₀-o-Atto565 ssDNA in **a** and A₂₀-o-Atto565/o* dsDNA in **b**. **c**, Partition coefficient of ssDNA A₂₀-o-Atto565 and dsDNA A₂₀-o-Atto565/o* in different KL condensates, quantified by normalizing their intensity in KL condensates to their intensity in solution. *N* = 10 from 3 independent experiments. Data are presented as mean ± s.e.m. The statistical data were analyzed using a two-sided t-test. *p* = 0.789 for KL1-R1 vs KL2-R2 with addition of A₂₀-o-Atto565/o*. Scale bars: 10 μm (**a**, **b**).



Supplementary Figure 4-16. Binding interaction between A₂₀-o, KL1-R1, and KL2-R2. **a**, Simulated concentration distribution of complex sequences in a pool of A₂₀-o (400 μ M), KL1-R1 (200 μ M), and KL2-R2 (200 μ M) at 30 $^{\circ}$ C based on rna06 model. Salt: 1 M Na⁺. **b**, Predicted minimum of free energy (MFE) proxy structure at 30 $^{\circ}$ C of A₂₀-o + KL1-R1 and A₂₀-o + KL2-R2. Tails of KL1-R1 and KL2-R2 are highlighted in orange. The binding between A₂₀-o and KLs are indicated by arrows.

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Preliminary note: This chapter is based on a manuscript ready for submission by **Weixiang Chen**, Rahmetullah Demirci, Miao Xie & Andreas Walther*. Minor changes have been made concerning formatting style.

I thank Dr. P. Swinkels for fruitful discussion on DNA computing.

Abstract: DNA strand displacement reactions of synthetic oligonucleotides have enabled the development of DNA reaction networks for various functions such as biosensing, molecular machines, and molecular computing. However, the need for high sequence orthogonality poses a major challenge to the modular integration and scaling of DNA networks into more complex systems capable of advanced or parallel functions. Here, we propose the use of liquid-like DNA condensates with addressable barcodes for confining DNA reaction networks for parallel and selective operation of near-identical circuits that would otherwise show undesired crosstalk in homogenous solution. By introducing a Transducer module, specific inputs can be recognized by corresponding condensates and converted to a unified Messenger for triggering downstream DNA processing locally. This allows orthogonal execution of DNA reactions of the same sequence design in different compartments in parallel without crosstalk and interference. This strategy addresses the challenges of modularity and scalability in DNA reaction network design. We believe our findings will significantly advance the development of DNA reaction networks, leading to more sophisticated and complex functionalities.

5.1 Introduction

Compartmentalization is an important concept at every level of living systems that possess complex functions.^{1–3} For example, even in the basic units of life, e.g., a eukaryotic cell, functions and processes are organized in space using sub-cellular structures such as phase-separated organelles, mitochondria, and the endoplasmic reticulum.^{2,4,5} This allows competing or interfering processes to operate more efficiently or to operate at all in parallel.

The advent of DNA nanoscience has given rise to the programmable construction of nanostructures, switchable nanodevices, as well as to information processing through DNA-based reaction networks (DNA-RNs).^{6–10} Among the latter, toehold-mediated DNA strand displacement reactions (SDR) have enabled concepts for disease diagnostics, switchable materials and self-assemblies, as well as computation.^{11–19} However, two major challenges presently constrain further development of DNA-RNs: Sequence overlap and interference limit the scale and complexity of larger systems.²⁰ The modular integration of different DNA-RNs into a single system is challenging because it often requires major changes or even a complete redesign of the sequences. For example, in a recent study reporting a general-purpose DNA computation system consisting of hundreds of DNA strands, the DNA-RN had to be executed in individual subunits of selected DNA gates, separated in different vials.²¹ Signal transduction between individual parts of the DNA-RNs required additional steps, including DNA registration on a DNA origami scaffold and manual purification and transfer. Overcoming these issues requires establishing robust compartmentalization strategy to identify approaches for running otherwise interfering DNA-RNs in compartmentalized objects that have access to a global input-output space, but prevent unwanted crosstalk. In this sense, parallelized computing in water-in-oil droplets would not be useful, as the water droplets cannot access the same input-output space. Indeed, there have been a few attempts using

DNA origami to physically separate DNA-RNs, demonstrating possible reusability of the same sequence design for different DNA-RNs in the same solution.^{22,23} However, a DNA origami can only fix limited number of DNA strands, which possesses little diffusivity to only react with neighbor DNA strands. This limits the development of more complex DNA-RNs on a DNA origami.

Thinking about an ideal compartmentalization strategy for DNA-RNs, the following criteria should be met: (1) Permeability and material exchange between inside and outside, for receiving signals to execute DNA-RNs and release outputs. (2) Enrichment of desired DNA strands, rejection of undesired ones, and prevention of diffusive loss of important DNA strands, thus achieving high effective concentrations. (3) High mobility of DNA strands inside the compartments for a dynamic reaction. Here, we propose the use of liquid-like DNA condensates as a powerful design for compartmentalizing DNA-RNs. By physically isolating individual DNA-RNs within distinct condensates, interference and crosstalk are efficiently suppressed, thereby eliminating the need for orthogonal sequence design. To address the condensates selectively in parallel, we design an input-selective signal transduction mechanism that enables condensates to parallelly use the same DNA sequence design to respond to different DNA inputs.

5.2 Results and Discussion

5.2.1 Design of Three-Component DNA Reaction Network

To exemplify the effect of compartmentalization on running interfering SDR circuits in parallel and orthogonal, we resort to simple three-component systems composed of Inputs, Transducers, and Reporters (Figure 5-1a): We use two DNA reporter duplexes (Reporter A and Reporter B) that are identical in sequence but have different fluorophore-quencher pairs. They can report a strand (Messenger) ejected by the Transducer. We use two Transducers that have distinct 4-nt toeholds, but are otherwise identical and can eject an identical Messenger if the correct Input is added. This overall allows to engineer a sequence diversity of $4^4 = 256$ distinct Input signals (See Supplementary Figure 5-1 for details of sequence design). Note that the Messenger has a non-bonded loop region in the transducer to ensure that the Inputs cannot directly react with the Reporters.

Critically, when placing both Reporters and both Transducers into a bulk solution, there is unavoidable crosstalk, as either of the two Inputs can activate both Reporters, because diffusion happens globally and the Transducer produces a unified Messenger for both Inputs (Figure 5-1b). However, when compartmentalizing pairs of Reporter/Transducer orthogonally into condensates, the downstream SDR can be confined within each condensate because parts of the modules are tethered to the compartment (Figure 5-1c).

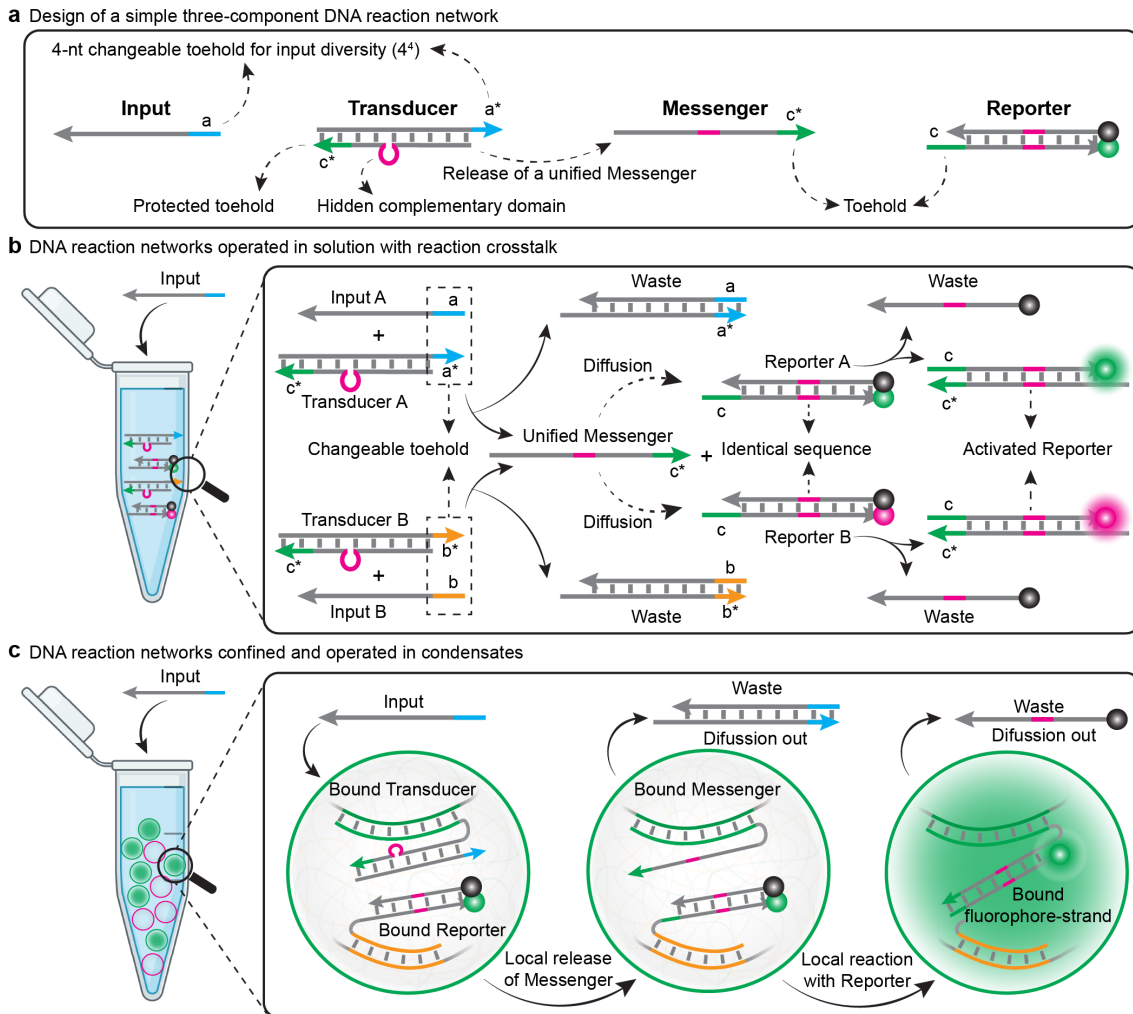


Figure 5-1. Design of three-component DNA reaction networks. **a**, Scheme showing the design of the three-component system, including an Input, a Transducer, and a Reporter. The input can react with the Transducer by toehold-mediated SDR and release a Messenger, which can react with the Reporter. **b**, Scheme of the DNA reactions in solution containing two sets of the three-component system, where any of the two Inputs (Input A and Input B) reacts with its corresponding Transducer, which releases a unified Messenger for activating both Reporters A and B. **c**, Scheme of the DNA reaction in condensates, where the Input reacts with the Transducer to locally release the Messenger, which activates the Reporter in the same condensate. Created with BioRender.com

5.2.2 DNA Reaction Networks Operated in Solutions

We first analyze selectivity and crosstalk in solution in the absence of any condensates. Figure 5-2a–d demonstrate that the correct Input/Transducer combination allows to activate either of the two Reporters. For instance, Figure 5-2a, c display activation of Reporter A and Reporter B, respectively, when Input A is combined with Transducer A. However, when Input B is added to the same system, there is no Reporter activation because Input B cannot react with Transducer A. No input as a control leads equally to no reaction. Similarly, Figure 5-2b, d show the inverted system, where Input B with Transducer B leads to activation of either reporter,

whereas Input A results in no activation. This underscores that the selectivity of the system is encoded in the 4-nt toehold overlap of the Input/Transducer recognition region.

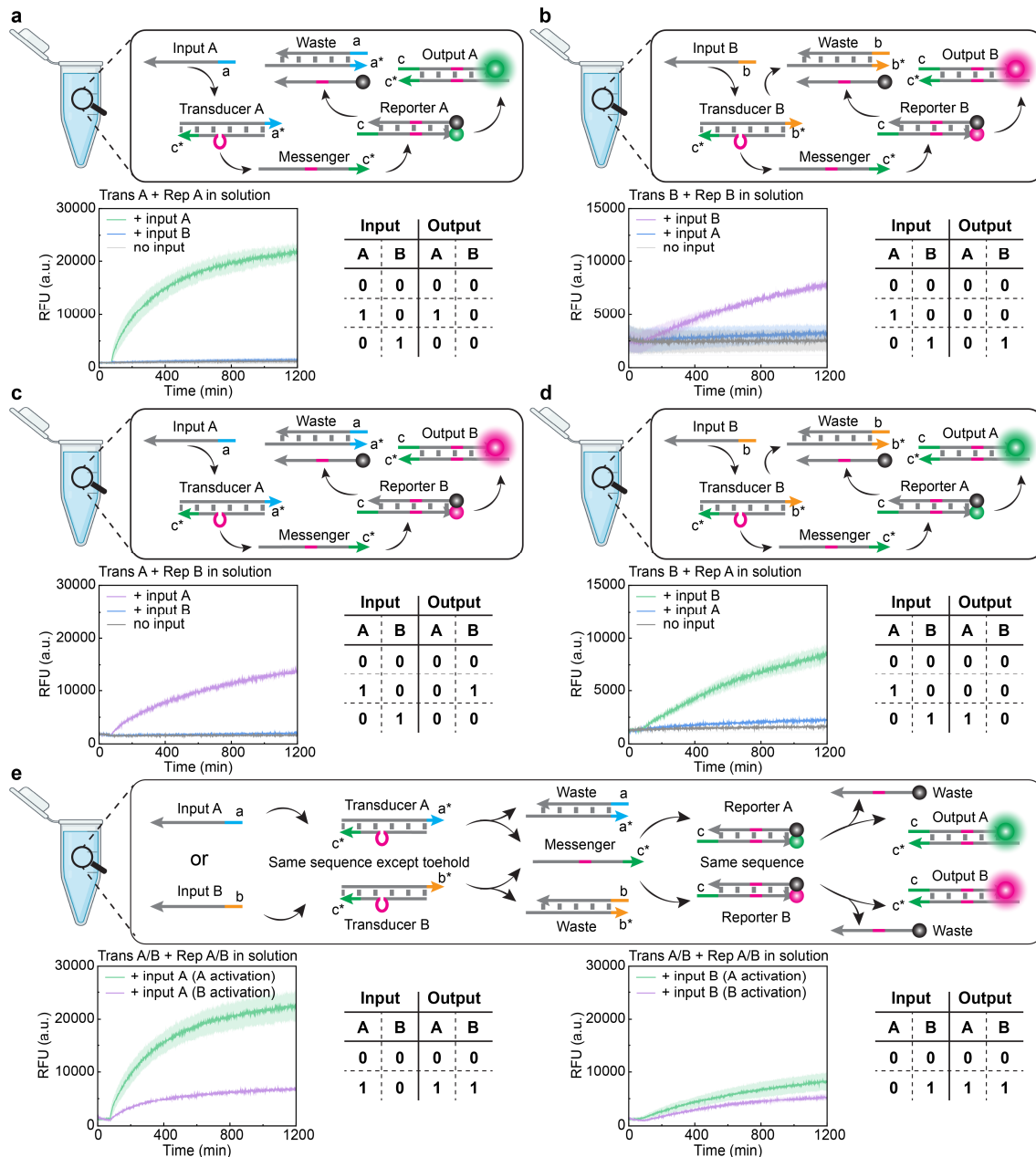


Figure 5-2. In-solution DNA-RNs with corresponding truth table. **a**, Input A reacts with Transducer A to release a Messenger, which activates Reporter A to produce fluorescence signal. **b**, Input B reacts with Transducer B to release a Messenger, which activates Reporter B to produce fluorescence signal. **c**, Input A reacts with Transducer A to release a Messenger, which activates Reporter B to produce fluorescence signal. **d**, Input B reacts with Transducer B to release a Messenger, which activates Reporter A to produce fluorescence signal. **e**, In case of Transducers A, B and Reporters A, B in the same solution, any of both Inputs A and B reacts with its corresponding Transducer to release a Messenger in the whole solution, which activates both Reporters A and B. Shaded areas represent standard deviation. Sample size (n) = 2 for plate reader experiments. Created with BioRender.com

Most importantly, when both Transducers A and B are integrated in the same solution, interference and crosstalk appear (Figure 5-2e). Upon adding any of the two Inputs (Input A or B), the corresponding Transducer is activated to release its Messenger into the whole solution that then activates both Reporters (See Supplementary Figure 5-2 for details of reaction mechanism). It therefore becomes impossible to distinguish which Input is added. This minimalistic system highlights, by specific design, the general limitations of in-solution DNA-RNs, where sequence interference and crosstalk can occur between different DNA-RNs. Parallel execution of Input-specific DNA-RNs would require redesign of the whole sequence set.

5.2.3 DNA Reaction Networks Operated in DNA Condensates

Now let us compartmentalize the systems to overcome this limitation. We use micron-sized core-shell DNA condensates formed through liquid-liquid phase separation of long ssDNA, that, after fabrication, feature a liquid-like interior with specific ssDNA barcodes to immobilize other DNA components while providing high intrinsic mobility.^{24–27} The shell is permeable and allows large macromolecules to penetrate.^{25,28,29} Using a recently developed co-phase separation approach, it is possible to incorporate different ssDNA barcodes into the condensates at desired concentrations.³⁰ We designed and prepared two different DNA condensates (Supplementary Figure 5-3). Condensate A contains a 1:1 ratio of barcodes m and p, whereas condensate B contains a 1:1 ratio of barcodes n and q. The local concentration of each barcode is around 200 μM .²⁸

We then immobilized Transducer A and Reporter A into condensate A and Transducer B and Reporter B into condensate B via specific barcode binding (Figure 5-3a, e). The immobilization is done in a way that the important Messenger component remains tethered to the DNA condensate material and cannot escape the condensate by diffusion. Similarly, the dye-labelled strand of the Reporter is attached to simplify imaging. FRAP experiments reveal high internal fluidity to execute DNA-RNs based on the tethered strands (Supplementary Figure 5-4). We then evaluate the response of each condensate to Inputs A and B. Both in-condensate DNA-RNs exhibit a positive response to their corresponding Inputs, resulting in activation of the embedded Reporter via a fluorescence signal (Figure 5-3b–d, f–h). As expected, the non-complementary Input does not activate the Reporter. These results demonstrate that DNA reactions can be efficiently carried out within the DNA condensates. The kinetics are comparable to those in solution, as efficient enrichment occurs through the permeable shells and on account of the dynamic condensate interior.

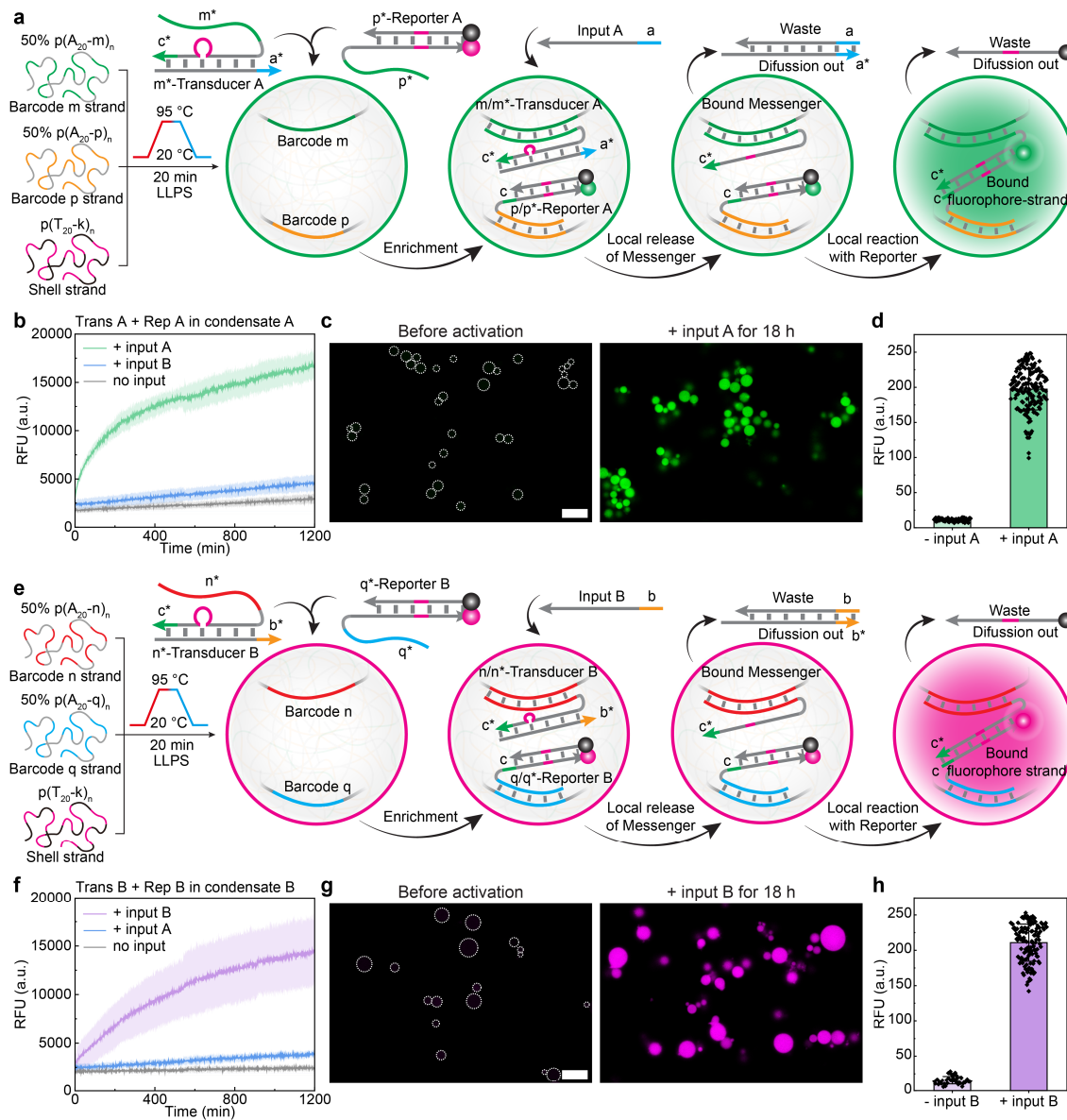


Figure 5-3. In-condensate DNA-RNs. **a**, Scheme of a DNA condensate A containing m and p barcodes for encapsulating Transducer A and Reporter A with complementary overhang strands (m^* and p^*). Addition of Input A activates Transducer A to release a Messenger locally, which subsequently reacts with Reporter A in the same condensate. **b**, Activation kinetics of Report A inside condensate A in response to different input. **c**, Representative CLSM images showing the activation of Reporter A in condensate A, triggered by input A. **d**, Fluorescence intensity inside DNA condensate A before and after adding input A, as quantified from CLSM data in **c**. **e**, Scheme of a DNA condensate B containing n and q barcodes for encapsulating Transducer B and Reporter B with complementary overhang strands (n^* and q^*). Addition of Input B activates Transducer B to release a Messenger locally, which subsequently reacts with Reporter B in the same condensate. **f**, Activation kinetics of Report B inside condensate B in response to different input. **g**, Representative CLSM images showing the activation of Reporter B in condensate B, triggered by input B. **h**, Fluorescence intensity inside DNA condensate B before and after adding input B, as quantified from CLSM data in **g**. Shaded areas and error bars represent standard deviation. $n = 2$ for CLSM and plate reader experiments. Scale bars, 10 μm (**c**, **g**).

5.2.4 Parallel Operation of the Same DNA Reaction Networks in Different Condensates

Finally, we test the system behavior when both condensates are present with their embedded DNA-RNs in the same solution. Strikingly, upon addition of Input A, only condensate A population processes the input to activate the embedded Transducer A, which then triggers a local reaction with the internal Reporter A (Figure 5-4a, b). In contrast, Reporter B embedded in the condensate B population remains dormant.

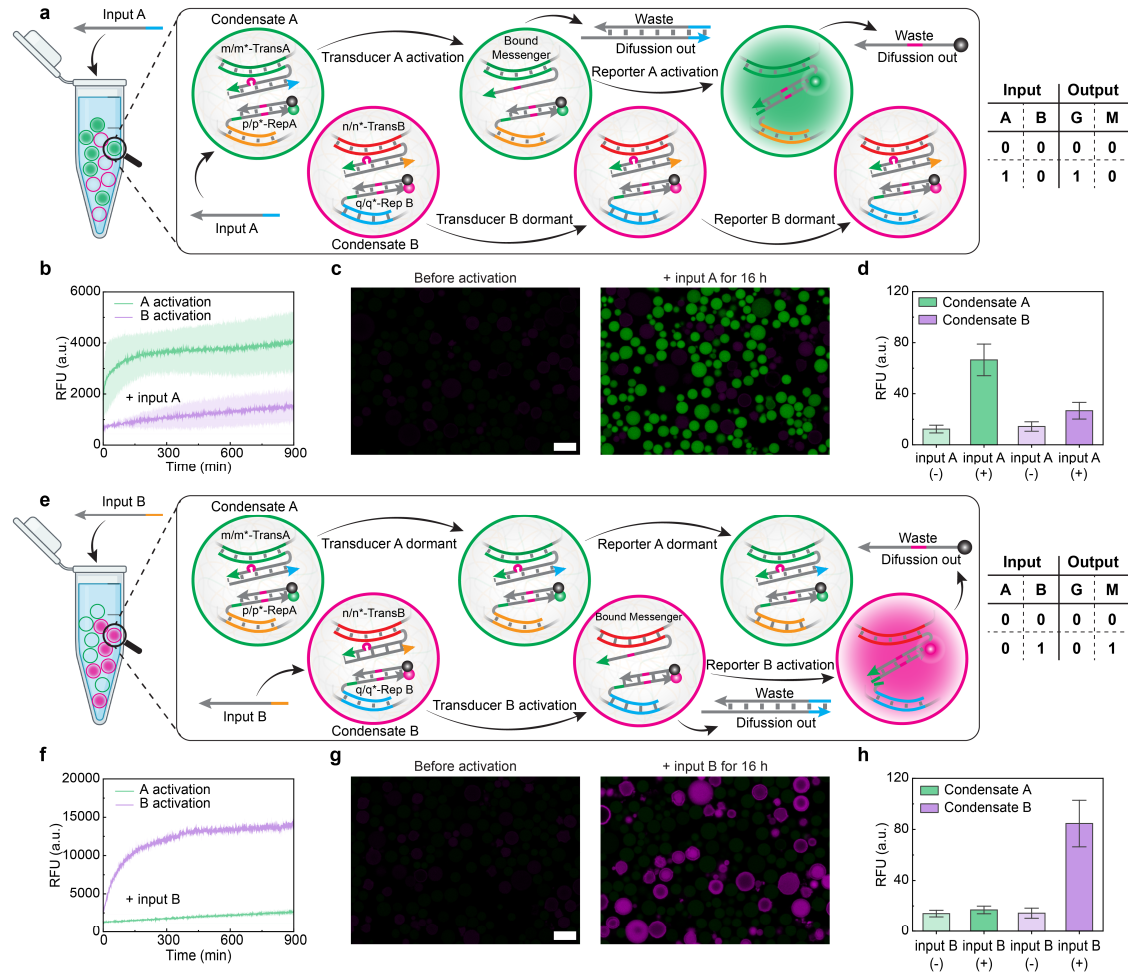


Figure 5-4. DNA condensates enable spatial confinement of DNA-RNs. **a**, Scheme of the parallel DNA reactions in two different DNA condensate populations, triggered by input A, with corresponding truth table. **b**, Addition of Input A activates Reporter A embedded in condensate A with an increase in fluorescence signal, whereas Reporter B in condensate B remains dormant. **c**, Representative CLSM images show that input A specifically activates Reporter A in condensate population A, whereas Reporter B in condensate population B remains dormant. **d**, Activation of Reporters A and B in both condensate populations in response to input A, as quantified from CLSM data in **c**. **e**, Scheme of the parallel DNA reactions in two different DNA condensate populations, triggered by input B, with corresponding truth table. **f**, Addition of Input B activates Reporter B embedded in condensate B with an increase in fluorescence signal, whereas Reporter A in condensate A remains dormant. **g**, Representative CLSM images show that input B specifically activates Reporter B in condensate population B, whereas Reporter A in condensate population A remains dormant. **h**, Activation of Reporters A and B in both condensate populations in response to input B, as quantified from CLSM data in **g**. Error bars represent standard deviation. $n = 2$ for CLSM and plate reader experiments. Scale bars, 10 μm (**c**, **g**). Created with BioRender.com

Complementary CLSM experiments further confirm these findings with selective appearance of green fluorescence in condensate A while condensates B remain dark (Figure 5-4c, d). Conversely, addition of Input B triggers magenta fluorescence exclusively in condensates B, with condensates A remaining dark (Figure 5-4e–h). This result is now profoundly different from the in-solution experiment (Figure 5-4e), where either of the two Inputs activates both Reporters due to crosstalk on the Messenger level. These results unequivocally demonstrate that the compartmentalization in dynamic DNA condensates provides effective physical separation of interfering DNA-RNs, enabling parallel and spatially confined DNA signal processing within the same solution. The localized activation of the Transducer and “release” of the tethered intermediate Messenger ensure that DNA reactions occur only within condensates able to process the correct Input, and that any further signal processing is confined to within the compartment.

5.3 Conclusions

To summarize, we developed compartmentalized DNA-RNs by using liquid-like DNA condensates as hosts. Hybridization between condensate barcodes and DNA-RN parts effectively governs localized DNA reactions within condensates and prevents the diffusive leakage of key components. This allows us to orthogonally operate DNA-RNs with the same sequence design in different compartments without interference and crosstalk. Our strategy therefore eliminates the need for high orthogonality of DNA sequences in a large system. By introducing a Transducer module, different condensate-specific Inputs can be converted to a unified Messenger for triggering downstream DNA-RNs locally. This can be further exploited to couple with developed DNA-RNs and facilitate their modular integration into a single system. In particular, we anticipate our research to stimulate development of DNA-RNs with complex functions and DNA computing systems capable of operating a large number of DNA gates in the same solution.²⁰ Intriguingly, in the context of system chemistry, our methodology also demonstrates a simple design principle for engineering kinetic asymmetry and selectivity in reaction pathways, by exploiting the proximity effect in compartmentalized systems.

5.4 Experimental

5.4.1 Materials

Supplementary Table 5-1 summarizes oligonucleotide used in this study, which were purchased from Biomers and Integrated DNA Technologies (IDT). T4 DNA Ligase (2 U/ μ L), Exonuclease I (40 U/ μ L), Exonuclease III (200 U/ μ L), and Φ_{29} polymerase (10 U/ μ L) were purchased from LGC Genomics. Thermal stable inorganic pyrophosphatase (2 U/ μ L) and nuclease-free water were bought from New England BioLabs (NEB). Deoxynucleotide triphosphate (dATP, dTTP, dGTP and dCTP) (100 mM) were purchased from Jena Bioscience. Hexadecane, sodium chloride and magnesium chloride, and Magnesium acetate solution (1M

in water) were purchased (as bioreagent grade if available) from Sigma-Aldrich. RNase-free TE buffer (Invitrogen, 10 mM Tris and 1 mM EDTA, pH 8.0, 500 mL) were purchased from ThermoFisher Scientific. Phosphate-buffered saline (PBS) were purchased from CARL ROTH. 384 well high-content imaging glass bottom microplates for microscopy and 384 well microplates for plate reader were purchased from Corning.

5.4.2 Instruments

All thermal annealing and heating ramps were performed on a TPersonal Thermocycler (Analytik Jena). Incubation was carried out on an Eppendorf ThermoMixer C. DNA concentrations were determined by a DS-11 Spectrophotometer (DeNovix). Fluorescence measurements were performed by a plate reader (Tecan). Confocal laser scanning microscopy (CLSM) was performed on a Leica Stellaris 5.

5.4.3 Methods

Synthesis of circular ssDNA templates and long ssDNA polymers

Synthesis of circular DNA template and its corresponding ssDNA polymer is adapted from our previous reports. The linear ssDNA template and the corresponding ligation strand were firstly mixed at concentration of 1 μ M in 100 μ L TE buffer containing 100 mM NaCl. The solution was heated to 85 $^{\circ}$ C (3 $^{\circ}$ C/s) for 5 min before cooling to 25 $^{\circ}$ C (0.01 $^{\circ}$ C/s) for complete hybridization. Afterwards, 20 μ L of 10 $\times$ Ligase buffer (500 mM Tris-HCl, 100 mM MgCl₂, 50 mM dithiothreitol and 10 mM ATP (Lucigen)), 70 μ L of nuclease-free water and 10 μ L of T4 DNA Ligase (2 U/ μ L (Lucigen)) were introduced into the reaction mixture and gently mixed before leaving at room temperature for 3 h. The reaction mixture was then heated to 70 $^{\circ}$ C for 20 min to deactivate the enzyme. Then, 10 μ L of Exonuclease I (40 U/ μ L (Lucigen)) and 10 μ L of Exonuclease III (200 U/ μ L (Lucigen)) were added into the reaction mixture to react overnight at 37 $^{\circ}$ C for degradation of the ligation strands and any non-circularized templates in solution. Afterwards, the reaction mixture was heated to 80 $^{\circ}$ C for 40 min to deactivate the enzymes. To obtain the final circular ssDNA templates, the reaction mixture was washed by adding 400 μ L TE buffer and filtrated using Amicon Ultra-centrifugal filters with a 10 kDa cut-off (Merck Millipore) for three times. The concentrations of the collected circular ssDNA templates were measured by the DS-11 Spectrophotometer (DeNovix), and the templates were stored in TE buffer at -20 $^{\circ}$ C.

For synthesis of long ssDNA polymers, we used rolling circle amplification (RCA). 5 μ L of circular template (1 μ M in TE buffer) and 1 μ L of exonuclease resistant primer (10 μ M in TE buffer) were mixed with 76 μ L nuclease-free water, 10 μ L of commercial 10 $\times$ polymerase buffer (500 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 40 mM dithiothreitol, 100 mM MgCl₂ (Lucigen)), 2 μ L of Φ ₂₉ DNA polymerase (10 U/ μ L (Lucigen)), 1 μ L of thermal stable inorganic pyrophosphatase (2 U/ μ L (NEB)) and 5 μ L of adjusted deoxyribose nucleoside 5'-triphosphate mix (100 mM, the mix contains pure dATP, dTTP, dCTP, and dGTP solutions mixed in

corresponding proportions of the exact composition of the desired ssDNA polymer repeating units (Jena Bioscience)). The reaction mixture was kept at 30 °C for 65 h before thermal cleavage at 95 °C for 15 min to shorten the ultrahigh molecular weight ssDNA. The final products were purified by rinsing with 400 μ L TE buffer and filtration in Amicon Ultra-centrifugal filters with 30 kDa cut-off (Merck Millipore) for three times. The concentrations of the collected final ssDNA polymers were measured using the DS-11 Spectrophotometer (DeNovix), and the DNA polymers (cleaved at 95 °C for 15 min) were stored in TE buffer at -20 °C.

Preparation of DNA condensates via *co*-phase separation

To prepare DNA condensate containing 50% m barcode and 50% p barcode, 0.222 g/L of $p(A_{20-m})_n$, 0.222 g/L of $p(A_{20-p})_n$, and 0.111 g/L $p(T_{20-k})_n$ were mixed in TE buffer without any salt at a final volume of 18 μ L. The solution mixture was then heated at 95 °C for 15 min (in addition to the first thermal cleavage during ssDNA synthesis mentioned above) for thermal cleavage to further reduce the chain length of the ssDNA polymers. Afterwards, 2 μ L of TE buffer containing 500 mM $MgCl_2$ was introduced into the reaction mixture. The solution containing finally 0.2 g/L $p(A_{20-m})_n$, 0.2 g/L $p(A_{20-p})_n$, and 0.1 g/L $p(T_{20-k})_n$ with 50 mM $MgCl_2$ was heated to 95 °C for 20 min (to induce $p(A_{20-m})_n$ and $p(A_{20-p})_n$ phase separation) and subsequently cooled down to room temperature, during which the $p(T_{20-k})_n$ localized as shell on the periphery of the $p(A_{20-m})_n$ and $p(A_{20-p})_n$ core by A_{20}/T_{20} hybridization. The heating and cooling rates were both 3 °C/s. Finally, the 20 μ L solution containing the core-shell DNA condensates was diluted 2 times by adding 20 μ L PBS buffer. The obtained 40 μ L DNA condensate solution (as 2 times diluted) has 0.1 g/L $p(A_{20-m})_n$, 0.1 g/L $p(A_{20-p})_n$, and 0.05 g/L $p(T_{20-k})_n$, corresponding to ca. 8 μ M m barcode, 8 μ M p barcode, and 4 μ M k barcode, respectively, in total solution. The solution was then stored in a fridge at 4 °C for 1 week for equilibration. Before usage, the DNA condensate solution was always slightly vortexed to redisperse the DNA SCs homogenously in the solution. For preparation of DNA condensates containing 50% n barcode and 50% q barcode, the $p(A_{20-m})_n$ and $p(A_{20-p})_n$ were replaced by $p(A_{20-n})_n$ and $p(A_{20-q})_n$. The successful integration of the desired barcodes in the DNA condensates was then confirmed by CLSM (Supplementary Figure 5-3).

Fluorescence recovery after photobleaching

FRAP experiments were performed independently on various labeled barcodes (m, p, n, q) of different DNA condensates to probe the internal dynamics of DNA condensates. Condensate barcodes are labeled by 2 $\times$ stoichiometric amount of DNA strands tagged with different fluorophores (Atto488-m*, Atto565-p*, Atto565-n*, Atto425-q*). Condensates were firstly imaged with low laser intensity 5 times (1 s/frame) as pre-bleaching images, before 5 times bleaching by 100% laser intensity in a circular ROI with a diameter of 1.5 μ m in the center of condensates. Then, 60 frames of post-bleaching images were recorded at a rate of 1 s/frame. For quantification, we measured intensities within the bleached circular ROI (I_{ROI}), and intensities in a circular ROI away from bleached region within the condensates (I_{ref}), in pre-

and post-bleaching images. We applied double normalization by $I_{\text{Norm}}(t) = \frac{I_{\text{ROI}}(t)}{I_{\text{ROI}}(t_0)} \times \frac{I_{\text{ref}}(t_0)}{I_{\text{ref}}(t)}$, where $I(t_0)$ represents the intensity measured in the first image before bleaching and $I(t)$ is the intensity measured over time.

Preparation of DNA Transduction layer and reporter

Double-stranded DNA duplexes such as transduction layer and reporter are prepared by heating a solution containing 20 μM of each of the needed strands in TE buffer containing 15 mM MgCl_2 to 85 $^{\circ}\text{C}$ for 5 min and then cooling down to 20 $^{\circ}\text{C}$ (0.1 $^{\circ}\text{C}/\text{min}$). The formed DNA duplex solutions were stored at -20 $^{\circ}\text{C}$ for further experiments.

DNA reaction networks in solution and in DNA condensates

Isolated DNA reaction networks in solutions were prepared by mixing 400 nM Transduction layer A (or B), 400 nM Reporter A (or B) in 20 μL PBS buffer containing 15 mM MgAc_2 in well plate. After 1h incubation, the reaction networks were triggered by the addition of 800 nM (2 $\times$ stoichiometric) input A (or B) and monitored by plate reader.

Combined DNA reaction networks in solution were prepared by mixing 400 nM Transduction layer A, 400 nM Transduction layer B, 400 nM Reporter A, and 400 nM Reporter B in 20 μL PBS buffer containing 15 mM MgAc_2 in well plate. After 1h incubation, the reaction networks were triggered by the addition of 800 nM (2 $\times$ stoichiometric) input A (or B) and monitored by plate reader.

Isolated DNA reaction networks in DNA condensates were prepared by mixing 1 μL of condensate A (or B) stock solution (corresponding to 400 nM of each barcode in final solution) with 400 nM Transduction layer A (or B), 400 nM Reporter A (or B) in 20 μL PBS buffer containing 15 mM MgAc_2 in well plate. The samples were incubated in well plate for 6h before the addition of 800 nM (2 $\times$ stoichiometric) input A (or B) for triggering the system and monitored by plate reader and CLSM.

Combined DNA reaction networks of both systems in DNA condensates were prepared by mixing 2 μL of condensate A stock solution and 2 μL of condensate B stock solution with 800 nM Transduction layer A, 800 nM Transduction layer B, 800 nM Reporter A, and 800 nM Reporter B in 20 μL PBS buffer containing 15 mM MgAc_2 in well plate. The samples were incubated in well plate for 6h, before 4 times washing process by adding 20 μL PBS buffer containing 15 mM MgAc_2 , centrifugation, and removal of 20 μL supernatant to remove excess DNA duplexes and potentially free DNA polymers in solution. Afterwards, the system was triggered by an addition of 800 nM (1 $\times$ stoichiometric) input A (or B) and monitored by plate reader and CLSM.

Note that the washing process is essential to remove all excess strands to avoid interference and crosstalk and ensure parallel DNA reactions in different DNA condensates.

Statistical analysis

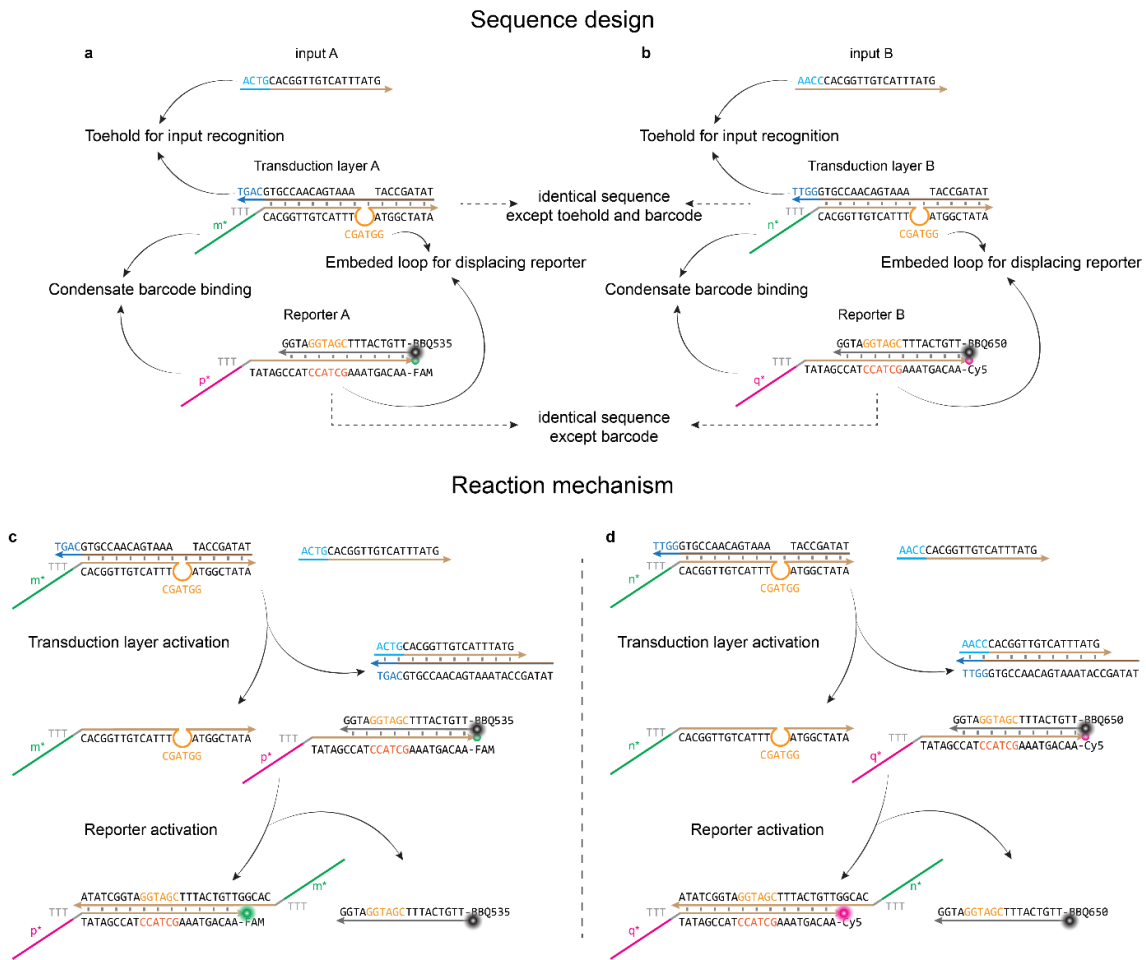
All data points featuring statistical analysis are presented as mean $\pm$ standard deviation. The sample size (n) for each statistical analysis is reported in the relevant figure legend. Statistical analysis was carried out using Origin 2023 and Microsoft Excel.

5.4.4 Supplementary Tables

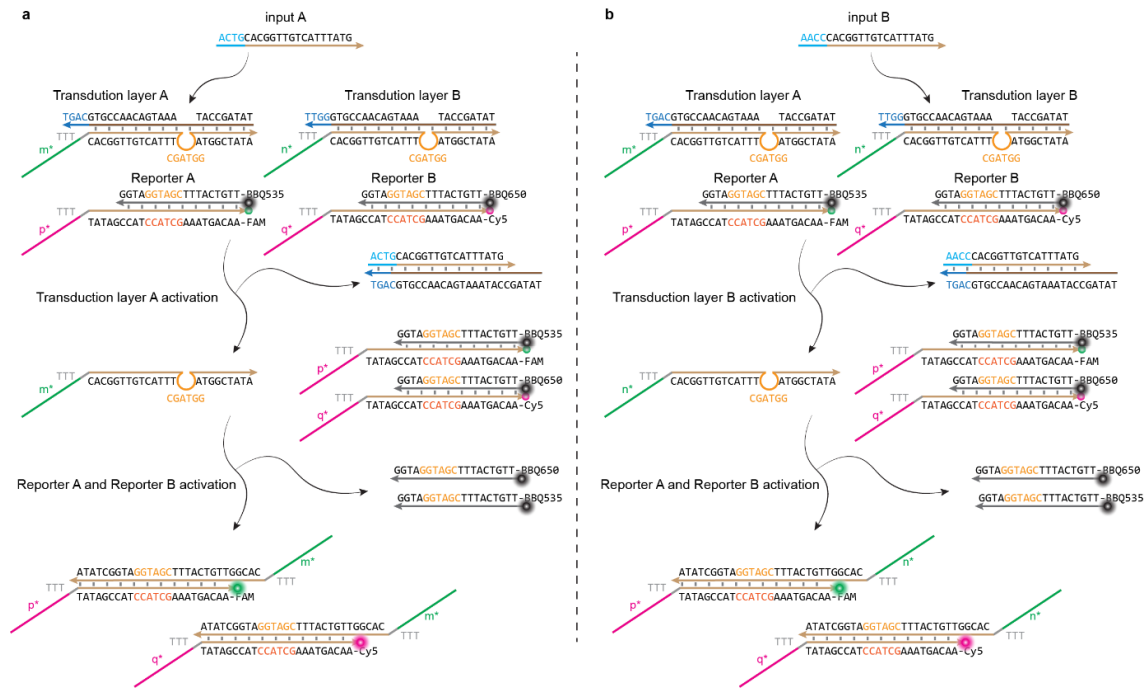
	Name	Sequence (5' $\rightarrow$ 3')	Purification	Modification	Supplier
Template and RCA	Tp(A ₂₀ -m)	/Phosphate/ATC TAT CCT AAT TTT TTT TTT TTT TTT TTT TGA ACC CGT AT	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -p)	/Phosphate/ATA GTG AGT CGT ATT ATT TTT TTT TTT TTT TTT TTT ATC CCT	HPLC	5'-Phosphorylation	Biomers
	Tp(A ₂₀ -n)	/Phosphate/TCG TTC AGA TGT TTT TTT TTT TTT TTT TTT TTT GGG TCC TTA C	HPLC	5'-Phosphorylation	Biomers
	Tp(T ₂₀ -q)	/Phosphate/TAT AAG TGC CAT TTT TTT TTT TTT TTT TTT TTT TTC TTC CGA CAG C	HPLC	5'-Phosphorylation	Biomers
	Tp(T ₂₀ -k)	/Phosphate/ATC CTC TAA AAT CAA AAA AAA AAA AAA AAA AAG TAA AAC CAC ACG	HPLC	5'-Phosphorylation	Biomers
	ligation-m	TTA GGA TAG ATA TAC GGG TTC	HPLC	None	Biomers
	ligation-p	TAA TAC GAC TCA CTA TAG GGA T	HPLC	None	Biomers
	ligation-n	CAT CTG AAC GAG TAA GGA CCC CA	HPLC	None	Biomers
	ligation-q	TGG CAC TTA TAG CTG TCG GAA GA	HPLC	None	Biomers
	ligation-k	TTT TAG AGG ATC GTG TGG TTT T	HPLC	None	Biomers
	primer-m	TTA GGA TAG ATA TAC GGG T*T*C	Desalting	Phosphorothioated twice (*)	IDT
	primer-p	TAA TAC GAC TCA CTA TAG GG*A*T	Desalting	Phosphorothioated twice (*)	IDT
	primer-n	CAT CTG AAC GAG TAA GGA CCC*C*A	Desalting	Phosphorothioated twice (*)	IDT
	primer-q	TGG CAC TTA TAG CTG TCG GAA*G*A	Desalting	Phosphorothioated twice (*)	IDT
	primer-k	TTT TAG AGG ATC GTG TGG TT*T*T	Desalting	Phosphorothioated twice (*)	IDT
Barcode label	Atto488-m*	/ATTO488/TGA ACC CGT ATA TCT ATC CTA A	HPLC	5'-Atto488	Biomers
	Atto565-p*	/ATTO565/ATC CCT ATA GTG AGT CGT ATT A	HPLC	5'-Atto565	Biomers
	Atto565-n*	/ATTO565/-TGG GGT CCT TAC T	HPLC	5'-Atto565	Biomers
	Atto425-q*	/ATTO425/ TCT TCC GAC AGC TAT AAG TGC CA	HPLC	5'-Atto425	Biomers
	Atto647-k*	/ATTO647N/AAA ACC ACA CGA TCC TCT AAA A	HPLC	5'-Atto647N	Biomers
Transduction layer and reporter	A1 (Transduction A)	TAT AGC CAT AAA TGA CAA CCG TGC AGT	HPLC	None	Biomers
	m*-TTT-A2 (Transduction A')	GAA CCC GTA TAT CTA TCC TAA TTT CAC GGT TGT CAT TTC GAT GGA TGG CTA TA	HPLC	None	Biomers
	p*-A3-FAM (Reporter A)	ATC CCT ATA GTG AGT CGT ATT ATT TTA TAG CCA TCC ATC GAA ATG ACAA/6-FAM/	HPLC	3'-6-FAM	Biomers
	A4 (Reporter A')	/BBQ535/TTG TCA TTT CGA TGG ATG G	HPLC	5'-BBQ535	Biomers
	A5 (Input A)	ACT GCA CGG TTG TCA TTT ATG	HPLC	None	Biomers
	B1 (Transduction B)	TAT AGC CAT AAA TGA CAA CCG TGG GTT	HPLC	None	Biomers
	n*-TTT-B2 (Transduction B')	TGG GGT CCT TAC TCG TTC AGA TGT TTC ACG GTT GTC ATT TCG ATG GAT GGC TAT A	HPLC	None	Biomers
	q*-B3-Cy5 (Reporter B)	CTT CCG ACA GCT ATA AGT GCC ATT TTA TAG CCA TCC ATC GAA ATG ACAA/Cy5/	HPLC	3'-Cy5	Biomers
	B4 (Reporter B')	/BBQ650/TTG TCA TTT CGA TGG ATG G	HPLC	5'-BBQ650	Biomers
	B5 (Input B)	AAC CCA CGG TTG TCA TTT ATG	HPLC	None	Biomers

Supplementary Table 5-1. Oligomers for condensates, labels, and DNA reaction networks, with their names, sequences, purification methods, modifications, and suppliers.

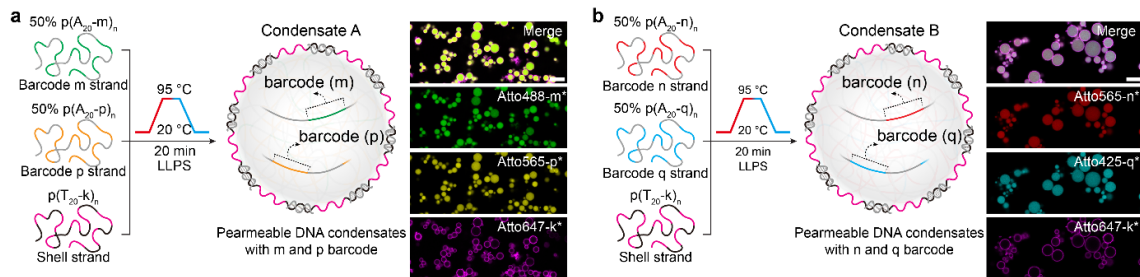
5.4.5 Supplementary Figures



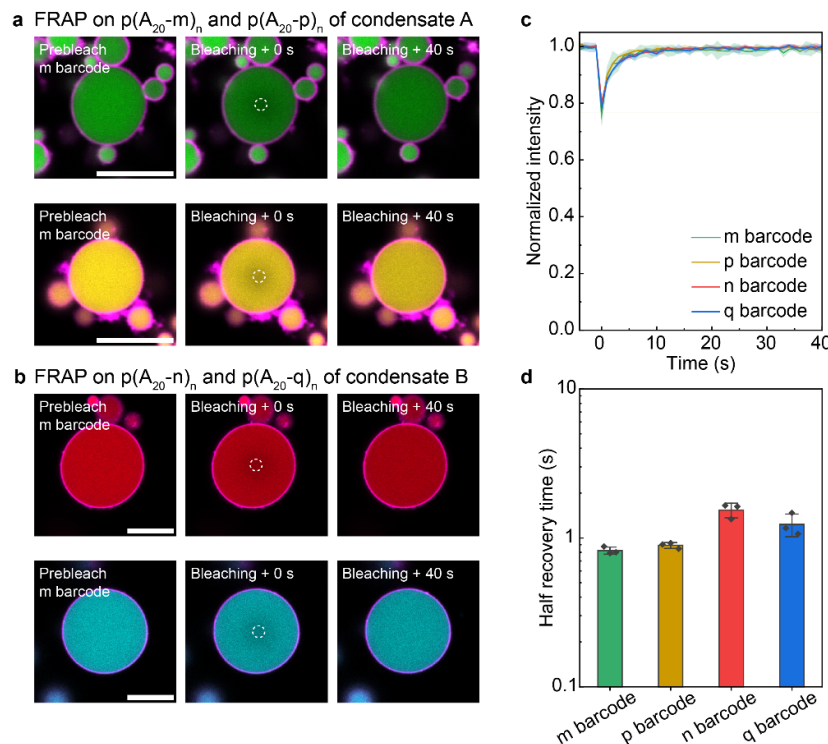
Supplementary Figure 5-1. Sequence design and reaction mechanism for transduction layer and reporter. a, Sequence design for Transduction layer A and Reporter A. **b,** Sequence design for Transduction layer B and Reporter B. **c,** Reaction mechanism for Transduction layer A and Reporter A in isolated solution. **d,** Reaction mechanism for Transduction layer B and Reporter B in isolated solution.



Supplementary Figure 5-2. Reaction mechanism when executing both system A and system B in the same solution. a, Scheme illustrating that addition of input A will result in the activation of both system A and system B. **b,** Scheme illustrating that addition of input B will result in the activation of both system A and system B.



Supplementary Figure 5-3. Preparation of DNA condensates containing different barcodes. a, Scheme and CLSM images for the DNA condensate A with m and p barcodes, labeled by Atto488-m* and Atto565-p*, respectively. **b,** Scheme and CLSM images for the DNA condensate B with n and q barcodes, labeled by Atto565-n* and Atto425-q*, respectively.



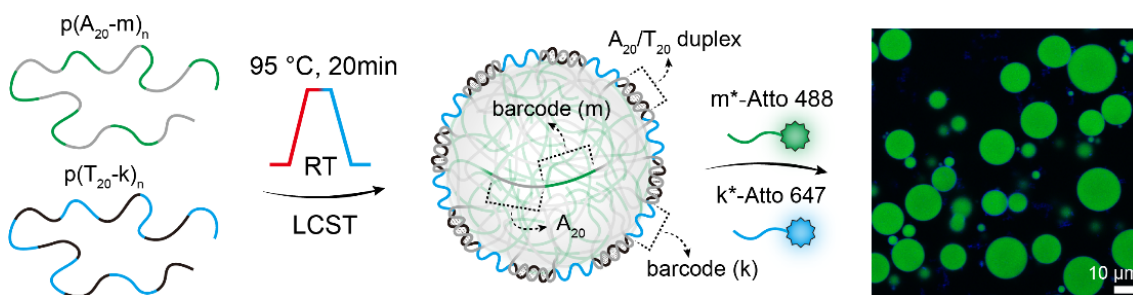
Supplementary Figure 5-4. Dynamic properties of DNA condensates. **a**, Fluorescence recovery after photobleaching (FRAP) experiments on $p(A_{20}-m)_n$ and $p(A_{20}-p)_n$, labeled by Atto488-m* and Atto565-p*, respectively, in a DNA condensate A. **b**, Fluorescence recovery after photobleaching (FRAP) experiments on $p(A_{20}-n)_n$ and $p(A_{20}-q)_n$, labeled by Atto565-n* and Atto425-q*, respectively, in a DNA condensate B. **c**, Normalized intensity in the bleached areas in **a** and **b**, as indicated by white dashed circles, for different DNA polymers inside different condensates, showing fast recovery and very dynamic interior property. **d**, Half recovery time ($t_{1/2}$) extracted from the FRAP curves in **c** for different DNA polymers inside different condensates. Shaded areas and error bars represent standard deviation. $n = 3$ for FRAP experiments. Scale bars, 10 μm .

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6. Temperature-Induced Phase Separation of Nucleic Acids: Mechanisms, Properties, and Applications



Preliminary note: This chapter is based on a review article currently in preparation, by **Weixiang Chen[#]**, Johann Fritzen[#] & Andreas Walther*. ([#]Equal contribution). Minor changes have been made concerning formatting style.

Author contributions: W.C., J.F., and A.W. conceptualized the review. W.C. and J.F. researched data for the review, wrote the first draft. In particular, W.C. wrote the sections on condensate properties and applications, while J.F. wrote the section on the mechanism of phase separation of nucleic acid polymers and part of condensate applications for studying FUS phase separation. W.C., J.F., and A.W. reviewed and edited the manuscript.

I acknowledge the dedication and creativity of all team members who contributed to the research on DNA condensate phase separation.

Abstract: Nucleic acids are essential biological macromolecules bearing genetic information and being important in post-transcriptional regulation. Given their high programmability based on Watson–Crick–Franklin base-pairing interactions, synthetic DNA and RNA oligonucleotides have become versatile building blocks for programmable assembly of nanostructures, nanomachines, and macroscopic materials. Recent discoveries have shown that long-chain nucleic acids can undergo temperature-induced phase separation, enabling rapid and facile formation of micro-sized, nucleic acid-rich condensates. Unlike conventional DNA/RNA nanotechnology, which relies primarily on base-pairing interactions, phase separation leverages the intrinsic polymeric nature of nucleic acids. While it expands the scope of DNA/RNA nanotechnology for new applications, nucleic acid phase separation also provides a fresh perspective for how compartmentalization may have emerged in the prebiotic RNA world during the origin of life. In this Minireview, we discuss the current mechanistic understanding of temperature-induced phase separation of DNA and RNA. We highlight strategies for controlling the physical and chemical properties of DNA condensates and review the progress and advances in developing them for various applications.

6.1 Introduction

70 years ago, based on the famous photo 51, Franklin, Watson, and Crick deciphered the double helix structure of B-DNA based on specific base-pairing interaction.^{1,2} This work is now widely believed to be one of the most important scientific discoveries of the 20th century, revealing how genetic information is stored in living organisms. The double helix structure of DNA has since set the foundation for establishing the structural basis for the central dogma in molecular biology.³ In the 1980s, Nadrian Seeman envisioned to take DNA out of its biological context and use synthetic DNA oligonucleotides to construct self-assembled nanostructures by predictable base-pairing interactions.⁴ Seeman's group's pioneering works for DNA junctions, cubes, DNA double-crossover tiles, initiated a new research direction — DNA nanotechnology.^{5–7} DNA has since become a versatile material and attracted the interest of researchers from different disciplines. Most notably, pioneering works such as DNA origami by Rothemund,⁸ DNA-based logic computation and DNA-based logic circuits by Seeman and Winfree,^{9,10} DNA crystals by Mao and Seeman,¹¹ and DNA walking devices by Seeman,^{12,13} have profoundly shaped DNA nanotechnology research, from DNA nanostructures and DNA crystal to DNA computation and DNA nanomachines.¹⁴

While molecular recognition via base pairing enables programmable nanostructure formation with high precision, the technology is largely limited to the nano world without further hybridization to other components. New interactions were needed to bring all-DNA materials to higher length scale. In 2018, we discovered that synthetic long-chain single-stranded DNA (ssDNA) can undergo sequence-dependent phase separation (PS) at elevated temperatures, leading to the formation of micro-sized DNA condensates on the mesoscale.¹⁵ In 2023, a

similar temperature-induced PS was discovered for RNA.¹⁶ This behavior resembles the lower critical solution temperature (LCST) phenomenon known for synthetic polymers.¹⁷ These works highlight the polymeric nature of DNA and RNA. In particular, RNA PS is of high biological relevance due to the formation of many transcriptional and RNA-containing protein condensates in cells.^{18–20} It may also provide a perspective on the emergence of compartmentalization in a prebiotic RNA world, potentially shedding light on the origin of life.^{21,22}

Leveraging the LCST-type phase separation, we and other groups have developed DNA (and to a lesser extent RNA) condensate materials into synthetic cells (SCs) with a range of functions, including internal secondary structures, prototissue formation, metabolic and signaling capacity, and even cellular regulation.^{15,23–30} Moreover, the high concentration of long-chain nucleic acids within the condensates creates a macromolecularly crowded and viscoelastic environment, which recapitulates conceptually the interior of natural cells.^{31–33} Alongside, a series of studies has begun to elucidate the mechanisms underlying PS of nucleic acid polymers.^{16,34,35} As a result, DNA and RNA condensates formed through PS have emerged as a promising new class of nucleic acid materials with broad application potential.

In this Minireview, we revisit the current mechanistic understanding of temperature-induced phase separation of nucleic acids and highlight the unique properties of DNA/RNA condensates as a new class of nucleic acid materials. Strategies developed to control and engineer their properties are summarized, along with emerging applications of DNA condensates across diverse fields, with particular emphasis on their use as model systems for SC research. Finally, we conclude with a perspective on future directions for nucleic acid condensate research, from mechanistic studies of nucleic acid PS, and their role in SC systems, to their broader impact on DNA and RNA nanotechnology.

6.2 Mechanism for the Temperature-Induced Phase Separation of Nucleic Acids

To understand the mechanism of nucleic acid PS, we first highlight the structural differences between DNA and RNA. Subsequently, we illustrate the influence of intrinsic factors, such as purine–pyrimidine composition and molecular weight, as well as extrinsic factors, such as type and concentration of salt present during the process. The interplay between these parameters can give rise to partial or complete percolation transitions, thereby modulating the dynamics of the resulting condensates.

6.2.1 Structures of DNA and RNA – Similarities and Differences

In terms of structure, DNA and RNA are very similar macromolecules, yet their minute differences make them selectively recognizable in biological systems. Both consist of a linear polymer chain containing phosphodiester-linked nucleotides as repeating units. DNA uses

deoxyribose as the central sugar, whose 1'-position is connected to one of the four nucleobases: adenine (A), cytosine (C), guanine (G) or thymine (T). Phosphodiester bonds are formed between the 3'-hydroxyl group of one sugar and the 5'-hydroxyl group of the next. Each nucleobase pairs specifically with its complementary partner through hydrogen bonding: A with T and C with G. Consequently, every DNA strand has an exactly matching complementary strand that aligns in an antiparallel orientation to form the double helix.

RNA differs in three major points: (1) It uses ribose as sugar, consequently providing a free 2'-hydroxyl group in each nucleotide. (2) Instead of thymine, uracil (U) is used as a nucleobase. (3) RNA is rarely found as a perfect double helix, but rather it possesses intramolecular folds, and long RNAs can form complex 3D structures which can even possess enzymatic activity. While this function is partially due to the 3D structure, it also arises from the catalytic contributions of the free 2'-hydroxy group (2'-OH) of RNA, serving as internal nucleophile to promote intramolecular transesterification reactions. RNA is more prone to hydrolysis, especially in alkaline conditions, and is chemically less stable than DNA.³⁶

6.2.2 Temperature-Induced Phase Separation

Despite different structures, DNA and RNA exhibit similar behavior in temperature-induced PS: Long nucleic acid strands phase separate with increasing temperature and divalent salt concentration. Ultimately, the similarities between DNA and RNA, most prominently the phosphate backbone, contribute majorly to the PS propensity. Figure 6-1 discusses all-atom molecular dynamics (MD) simulations of simple inorganic polyphosphate (poly(P)) by Wadsworth et al.¹⁶ They demonstrate that poly(P) features a distribution of more collapsed conformations at higher temperatures in the presence of 100 mM MgCl_2 (Figure 6-1a). At elevated temperatures, a higher amount of bound Mg^{2+} , which are even bound with higher coordination number, indicates the transition towards intramolecular crosslinking of poly(P) on account of a preference for multivalent Mg^{2+} interactions (Figure 6-1b, c, e, f). Desolvation of the backbone occurs for Mg^{2+} coordinated at higher coordination number (Figure 6-1d). Consequently, the polymer chain collapses to a compacted dense phase in an entropy-driven transition. Complementary experiments of temperature-induced PS of poly(P) confirm the simulation data.¹⁶

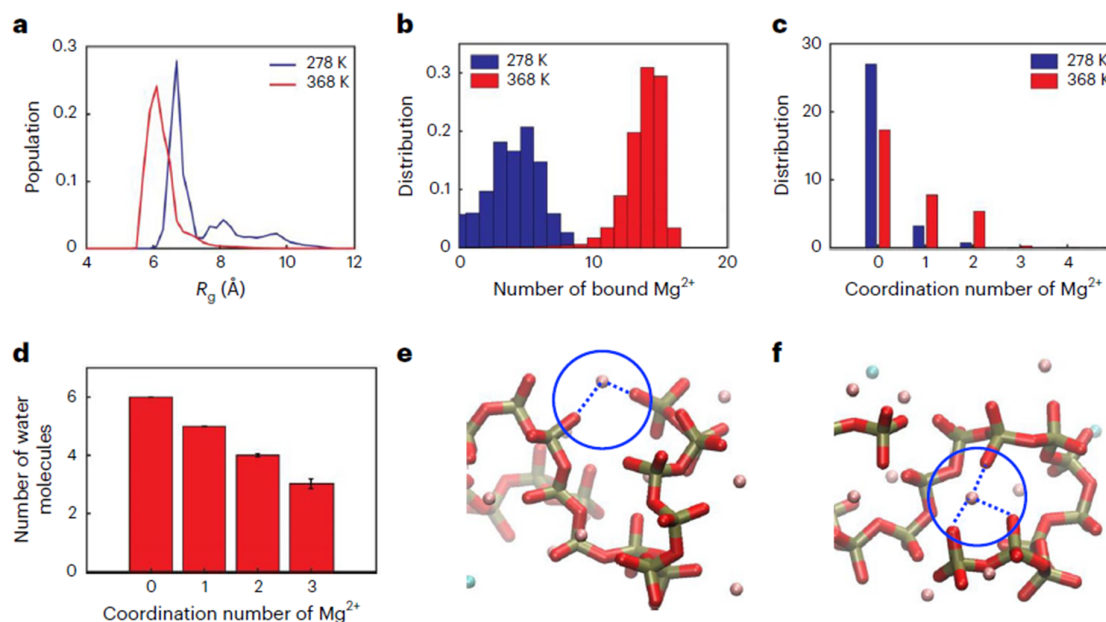


Figure 6-1. Mg^{2+} bridging induces compaction of poly(P) chains. **a**, All-atom MD simulations of poly(P) in the presence of 100 mM MgCl_2 shows more compact conformations at 368 K than at 278 K. **b**, Distribution of Mg^{2+} ions bound to poly(P) at 278 K and 368 K and **c**, the respective coordination number of the Mg^{2+} ions. **d**, Effect of Mg^{2+} coordination to oxygen on the first solvation shell. **e**, **f**, Snapshots of different Mg^{2+} binding modes to two or three oxygen atoms. Figures are adapted from ref. 16 with permission. Copyright 2023, The Authors, under exclusive license to Springer Nature Limited.

6.2.3 Purine-Rich Sequences Drive Phase Separation

While temperature-induced backbone desolvation is a common driver of LCST behavior, homo-polymeric DNA and RNA exhibit a more complicated PS process. After charge neutralization of the backbone and the entropically driven expulsion of water, the interaction between polymer chains increases. The newly formed hydrophobic environment guides base–base interactions, such as hydrogen bonding and π – π interactions, also referred to as base stacking. These additional interactions determine the cloud point temperature (T_{cp}). Because of their larger aromatic surfaces, purines show more pronounced base stacking than pyrimidines, resulting in lower T_{cps} for purine-rich sequences. Additionally, G, A and C have a high propensity for hydrogen bonding, which is an additional contribution to base–base interactions (Figure 6-2a).

These trends have been identified by us for DNA (Figure 6-2b),¹⁵ and by Wadsworth et al. for RNA (Figure 6-2c).¹⁶ In agreement with the order of base-stacking propensity, both nucleic acids show earlier PS for poly(A) and poly(G). While poly(A) shows reversible PS, poly(G) does not: once the backbone desolvation has induced base stacking at high temperature, the resulting interactions, possibly accompanied by G-quadruplex formation, become so strong that PS persists even upon cooling. This is not the case for the pyrimidine bases, such as C and

T/U, where higher levels of Mg^{2+} are needed for PS to occur, and even then the PS remains reversible.

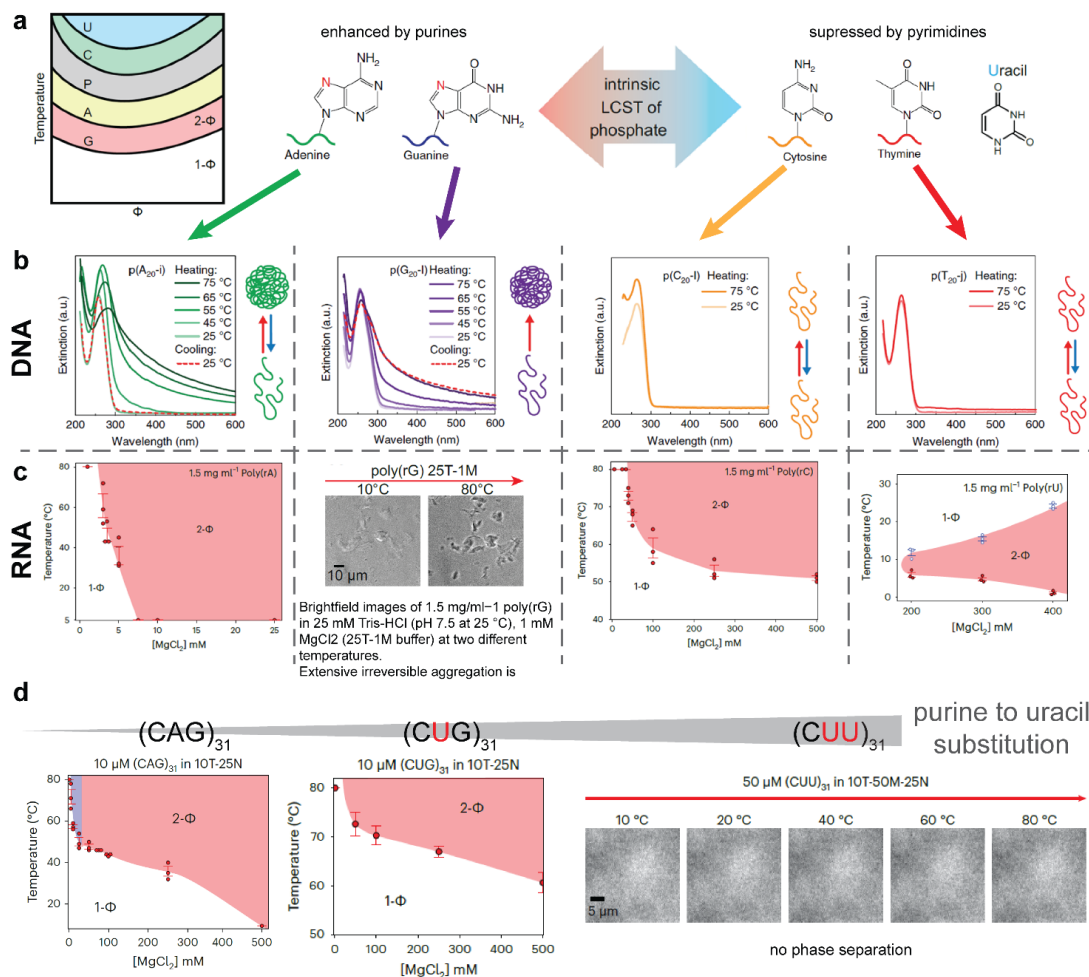


Figure 6-2. Influence of nucleobases (A, G, C, T, and U) on DNA and RNA PS behavior. **a**, Molecular structures of DNA and RNA nucleobases and schematic ranking of their PS propensity. **b**, **c**, Comparison of nucleobase-dependent temperature-induced PS behavior of DNA and RNA. **(b)** DNA sequences have a blocky structure of alternating homopolymer-blocks and a specific sequence (here, i, j, or l). The PS behavior has been characterized by UV-Vis measurements. **(c)** Phase diagrams of homopolymer RNA sequences and brightfield images of poly(G) showing aggregation. **d**, PS of (CAG)₃₁ in comparison with purine-to-uracil variants (CUG)₃₁ and (CUU)₃₁. Scale bars, 10 μm (**c**) and 5 μm (**d**). Figures **a**, **c**, **d** are adapted from ref. 16 with permission. Copyright 2023, The Authors, under exclusive license to Springer Nature Limited. Figure **b** is adapted from ref. 15 with permission. Copyright 2018, The Authors.

As a remarkable exception, poly(U) RNA shows a closed-loop reentrant behavior, with LCST < UCST. This special case can be explained by the careful balance between the two aforementioned mechanisms for PS: Approaching the LCST from low temperatures, the backbone is desolvated until PS occurs. In the two-phase region the effect of backbone desolvation and the enthalpic stabilization from base stacking and hydrogen bonding complement each other. Because the enthalpic stabilization of poly(U) is so low, this interaction can be overpowered at higher temperatures by entropic effects, such as increased

conformational freedom of the polymer chains, leading to condensate dissolution. Comparison of the PS behavior of a (CAG)₃₁ with its mutant (CUG)₃₁ and double mutant (CUU)₃₁ indeed shows that purine-to-uracil (pyrimidine) substitutions lead to an increase in T_{cp} , ultimately suppressing PS for the double mutant at high concentration and 80 °C (Figure 6-2d). In conclusion, while the phosphate backbone drives LCST behavior, purines clearly increase PS propensity, whereas pyrimidines rather suppress it.

6.2.4 Influence of Molecular Weight and Salinity

The polymeric nature of DNA and RNA is clearly important for their LCST behavior, but how does their molecular weight influence PS behavior? Rolling circle amplification (RCA) allows the production of very long ssDNA polymers to investigate this question. Figure 6-3a, b shows an agarose gel electrophoresis characterization of RCA-produced ssDNA, which can be shortened to desired length by heat-induced cleavage.³⁴ Figure 6-3c illustrates that the T_{cp} decreases with increasing DNA length, and ssDNA with very low molecular weight does not undergo phase separation.¹⁵

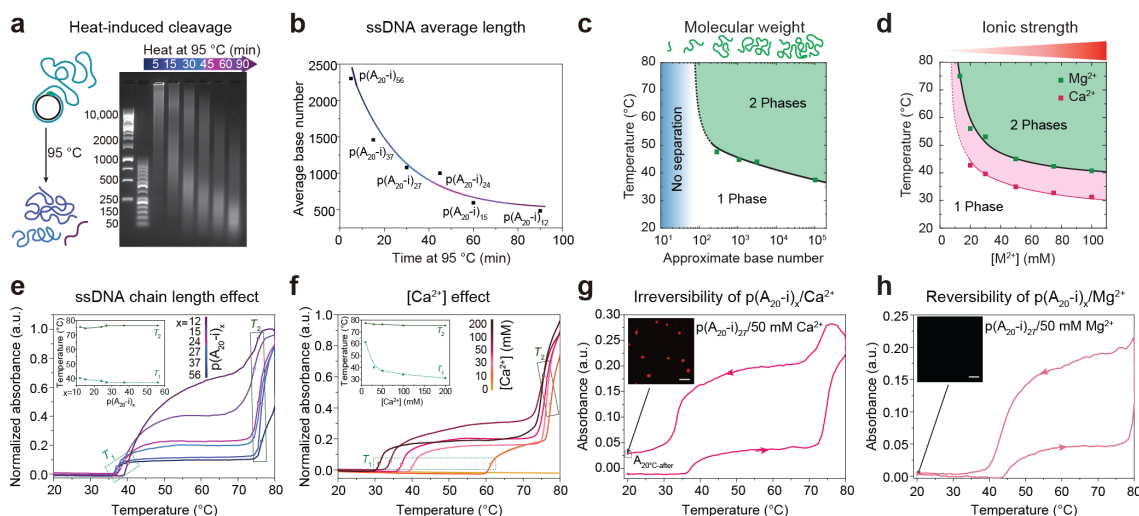


Figure 6-3. Molecular weight and salt identity determine LCST and reversibility of PS. **a**, Agarose gel electrophoresis for the thermal cleavage of poly(A_{20-i})_n (n = repeating units) by heating to 95 °C for different incubation times (5, 15, 30, 45, 60, 90 min). **b**, Average length of poly(A_{20-i})_n as a function of incubation time. **c**, T_{cp} of 0.06 g/L solutions of p(A_{20-i})_n as a function of molecular weight (in the presence of 50 mM MgAc₂). Solid and dotted lines are guides to the eye. **d**, T_{cp} of 0.06 g/L solutions of p(A_{20-i})_n as a function of counterion concentration (MgAc₂ and CaCl₂). Solid and dotted lines are guides to the eye. **e**, Temperature-dependent absorbance at 350 nm of poly(A_{20-i})_n with different molecular weights in the presence of 50 mM Ca²⁺. The inset shows T_1 and T_2 as a function of molecular weight. **f**, Temperature-dependent absorbance at 350 nm of poly(A_{20-i})₂₇ at varying Ca²⁺ concentrations. The inset shows T_1 and T_2 as a function of Ca²⁺ concentrations. **g**, Cyclic temperature ramp showing absorbance at 350 nm of poly(A_{20-i})₂₇ in the presence of 50 mM Ca²⁺. The inset shows a CLSM image after the heating/cooling cycle. **h**, Cyclic temperature ramp showing absorbance at 350 nm of poly(A_{20-i})₂₇ in the presence of 50 mM Mg²⁺. The inset shows a CLSM image after the heating/cooling cycle. Temperature ramp rate: 1 °C/min. Scale bars, 10 μ m (**g**, **h**). Figures **a**, **b**, **e**–**h** are adapted from ref. 34 with permission under the terms of the Creative Commons CC BY-NC 4.0 license. Copyright 2022, The Authors. Figures **c**, **d** are adapted from ref. 15 with permission. Copyright 2018, The Authors.

The PS is also controlled by salt identity and concentration.¹⁵ This is evident from Figure 6-3d, which shows DNA PS as a function of Mg^{2+} and Ca^{2+} concentrations. Clearly, higher salt concentrations lead to lower T_{cps} on account of stronger counterion condensation and reduced electrostatic repulsion. In general, Ca^{2+} shows a stronger effect than Mg^{2+} in promoting PS of DNA.

Interestingly, temperature-dependent turbidity measurements of the PS process for DNA of various chain lengths reveal two distinct transition temperatures, T_1 and T_2 . This holds for both Mg^{2+} and Ca^{2+} (Figure 6-3e–h).³⁴ In agreement with dynamic light scattering data, these transitions can be assigned to nucleation (T_1) and growth/coalescence (T_2) phases. At constant $[\text{Ca}^{2+}]$, the ssDNA chain length strongly influences the absorbance level between both transition temperatures, with shorter chains showing progressively higher turbidity, likely indicating the formation of larger nuclei. At the same time, both transition temperatures stay almost fully constant. Modulating $[\text{Ca}^{2+}]$ shows little effect on turbidity levels, but the onset of nucleation (T_1) occurs much earlier at higher $[\text{Ca}^{2+}]$ (Figure 6-3f). Additionally, heating ramps in the presence of 50 mM Ca^{2+} lead to irreversible PS (Figure 6-3g), where condensates remain insoluble after cooling. In contrast, in the presence of 50 mM Mg^{2+} , T_1 and T_2 are shifted towards higher temperatures and the absorbance returns to baseline levels after cooling, confirming reversible PS (Figure 6-3h). Taken together, the identity of the divalent cation plays a critical role in controlling the LCST behavior and determining the fate of the phase-separated state. While both Mg^{2+} and Ca^{2+} lead to phase separation of DNA polymers, this process is irreversible with Ca^{2+} but reversible with Mg^{2+} , indicating a tighter binding of Ca^{2+} compared to Mg^{2+} .

6.2.5 Phase Separation Coupled to Percolation

The PS process of nucleic acids can be linked to system spanning networks, that is, a percolation transition mediated by interchain interactions.³⁷ This is sketched in Figure 6-4 for different levels of percolation. While a percolation transition can occur independently of PS, the two processes are often coupled with each other, as depicted in the phase diagrams in Figure 6-4a. For nucleic acid condensates, these transitions typically occur within the dense phase after phase separation, and the percolated networks can persist upon cooling from the two-phase region. Non-percolating nucleic acids (left diagram of Figure 6-4a) lack an overlap between the two-phase regime and the percolation regime, meaning that no system composition will lead to a dense phase below the percolation temperature (T_{prc}) for percolation transition. In the case of weakly percolating nucleic acids, a small overlap between the two regimes exists (shown in orange in the middle diagram of Figure 6-4a). As a result, all system states within the overlap domains correspond to dense phase compositions below T_{prc} and therefore promote percolation transition. For strongly percolating nucleic acids, the overlap domain is much larger, meaning that more system states lead to dense phase percolation and thus to irreversible aggregate formation.

The difference between weakly and strongly percolated morphologies is illustrated in Figure 6-4b.³⁷ Weakly percolated condensates can still achieve minimum-energy shape through slow relaxation and chain rearrangement, whereas strongly percolated condensates are dynamically arrested. Consequently, weakly percolated condensates possess more liquid-like and dynamic behavior, such as reconfiguration under stress, whereas strongly percolated condensates behave like solids.

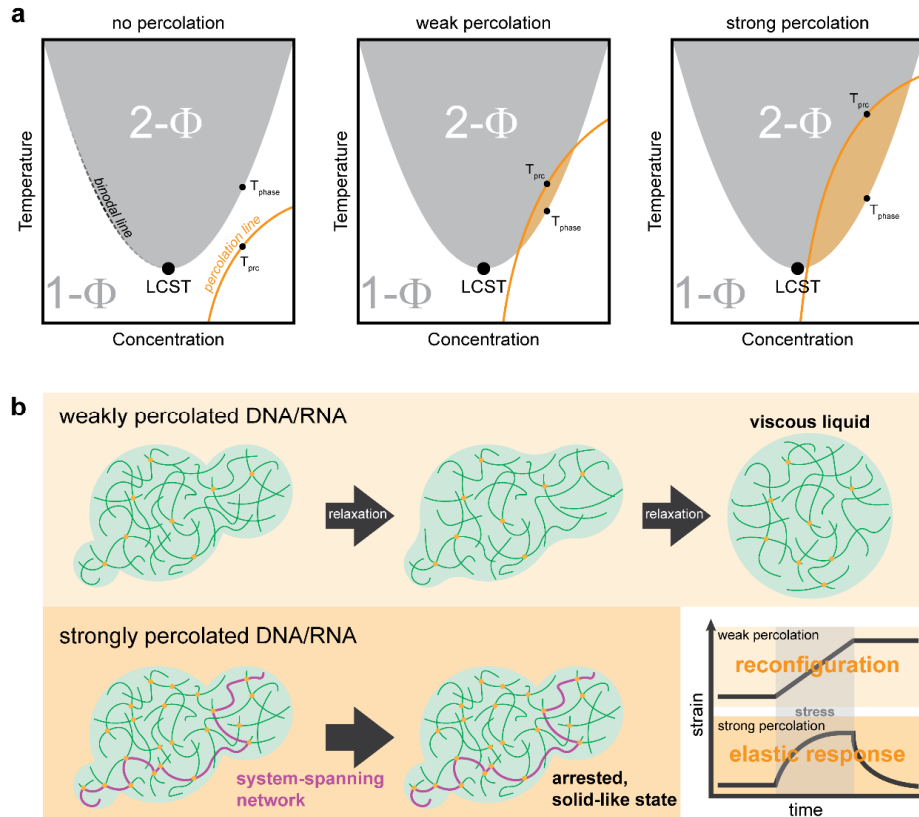


Figure 6-4. Comparison of weakly and strongly percolated condensates. **a**, Schematic phase diagrams for nucleic acids with different tendencies of percolation. **b**, Different chain relaxation and dynamic states for weakly and strongly percolating nucleic acid condensates and idealized representation of stress-strain behavior of such condensates. Figures are adapted from ref. 37 with permission. Copyright 2024, Elsevier Inc.

6.3 Engineering Structures and Dynamics of DNA Condensates

While the understanding of the nucleic acid PS behavior is concurrently pursued for both DNA and RNA, the focus on engineering them for molecular systems has largely been on DNA. DNA condensates can be engineered in their structures and dynamics by applying different phase separation strategies, using different DNA polymers, and adjusting salinity. It is possible to obtain both membraneless and core-shell condensates with tunable localization, defined ssDNA sequences at core and shell (serving as recognition barcode for binding complementary ssDNA), and with adjustable condensate dynamics.

6.3.1 Structure and Barcode Engineering of DNA Condensates

Stabilization of DNA condensates for their use below their T_{cp} can be achieved via three main approaches (Figure 6-5a): (1) Systems engineered to exhibit strong percolation (high G or A content, high salinity, Ca^{2+} preferred over Mg^{2+}) remain stable after heating above their T_{prc} and subsequent cooling below the original T_{cp} .³⁷ (2) Incorporating specifically engineered, self-crosslinking palindromic repeats (XL) into phase-separating ssDNA polymers promotes intra- and intermolecular crosslinking during cooling, which allows to stabilize membraneless condensates with tunable properties on account of the XL interactions.¹⁵ (3) Heating mixtures of poly(A) and poly(T) leads to the formation of core-shell condensates.

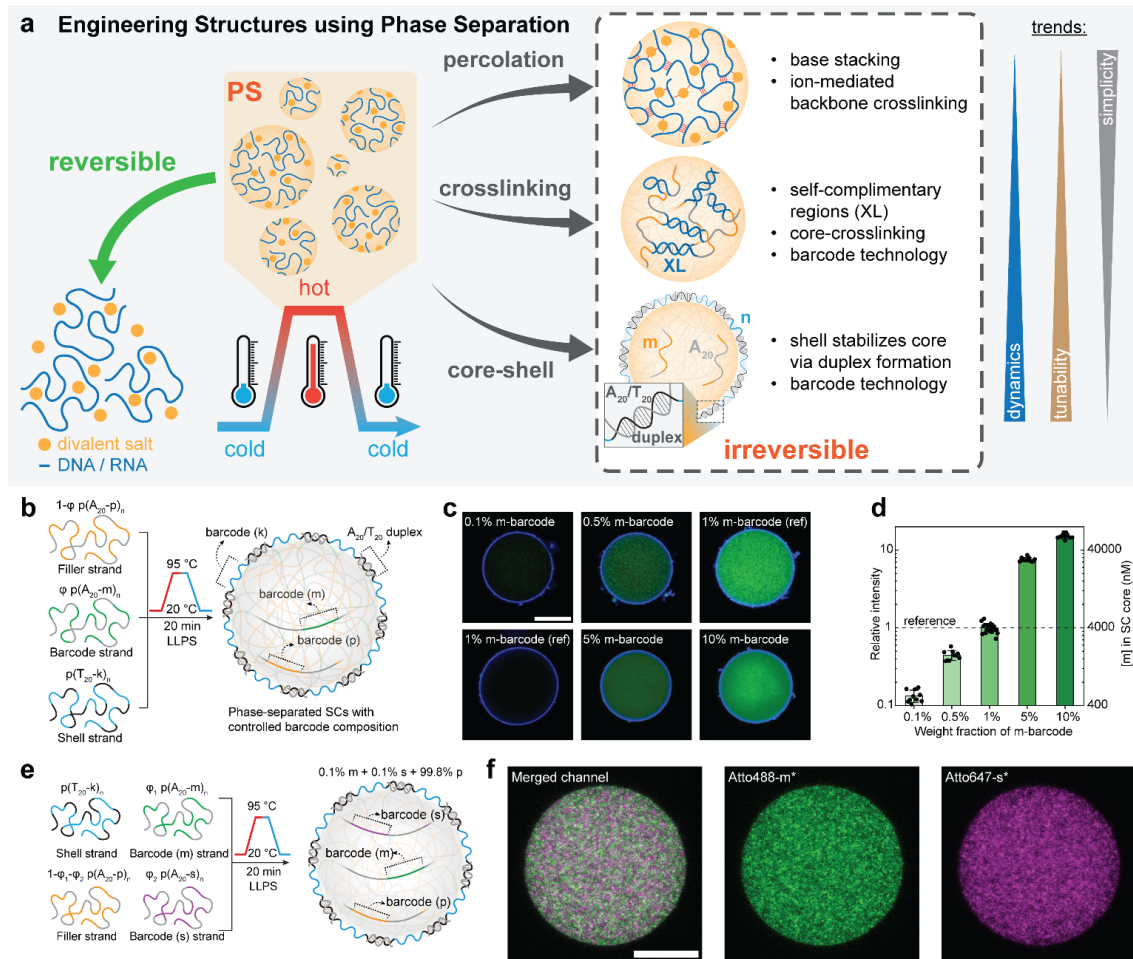


Figure 6-5. Structure and barcode engineering of DNA condensates. **a**, Schematic comparison of different approaches to engineering structures based on LCST behavior of nucleic acids. **b**, Scheme for preparation of condensates containing 2 barcodes through co-phase separation of several ssDNA polymers. **c**, Representative CLSM images showing the control of m barcode concentration inside condensates, labelled by m*-Atto488. Note that for each row of the CLSM images, laser settings are the same for direct comparison of internal fluorescence intensity to quantify relative m barcode concentrations ([m]). **d**, Quantification of [m] inside the condensate core as a function of weight fraction of p(A_{20} -m)_n used during preparation. **e**, Scheme for the dilution of two barcodes (m and s) at a stoichiometric ratio in a “matrix” of a third poly(A) ssDNA polymer (with barcode p) inside condensates. **f**, Representative CLSM images showing a condensate containing 0.1% m and 0.1% s barcode, labelled by Atto488-m* and Atto647-s*. Scale bars, 10 μ m (**c**, **f**). Figures **b–f** are adapted from ref. 27 with permission under the terms of the Creative Commons CC BY license. Copyright 2025, The Authors.

In particular for the core-shell condensate formation process, poly(A) undergoes phase separation upon heating, whereas poly(T) forms a hydrogel shell on the poly(A) condensates during cooling through surface hybridization.¹⁵ The resulting thin shell is permeable, allowing diffusion of large macromolecules, including nucleic acids, proteins, synthetic polymers, as well as enzymes such as T7 RNA polymerase, glucose oxidase, and urease^{15,23,25,27,28,38}. As we will discuss in the following sections, such core-shell condensates have been widely applied for applications in the context of SCs.

Towards controlling the composition, we recently showed that multiple poly(A) ssDNA polymers, each bearing distinct barcode sequences, can undergo co-phase separation into the same condensate phase (Figure 6-5b-d)²⁷. Figure 6-5c-f depict images and analysis routines for DNA condensates composed of binary and ternary mixtures, demonstrating strategies for controlling the local concentration of desired barcodes (Figure 6-5c, d), or for diluting two barcodes at a stoichiometric ratio within a “matrix” of a third poly(A) ssDNA polymer (Figure 6-5e, f).

6.3.2 Engineering of Condensate Dynamics and Related Transport Phenomenon

Depending on compositional factors (sequence and length), environmental conditions (salt type and concentration), and phase separation parameters (molecule concentration and temperature), stable condensates formed after the temperature ramp can exist in distinct dynamic states. We take core-shell DNA condensates, prepared via temperature-induced PS of poly(A) and poly(T) mixtures as an example, which is of highest relevance to SC research and among the most deeply investigated system.

Let us first focus on the simple influence of salinity on the condensate dynamics investigated by fluorescence recovery after photobleaching (FRAP) measurements. At high $[Mg^{2+}]$, the condensate core is in an arrested state with no FRAP recovery, whereas reducing $[Mg^{2+}]$ below 17.5 mM converts the condensate to a dynamic state with an appreciable FRAP recovery (Figure 6-6c-e).³⁹ This gives an indication of how the persistence of percolation networks after cooling can be modulated (Figure 6-4).

Now one can ask the question, what happens if we start hybridizing the ssDNA barcodes in the core with complementary ssDNA strands (invader strands)? This process revealed some real surprises. In fact, we recently discovered that the invasion process of invader strands into the condensate core proceeds via an unprecedented transport mechanism that deviates from classical Fickian diffusion.³⁹ We observed that a sharp, high-intensity invader front propagates linearly into the condensate over time, while the condensate simultaneously swells approximately fourfold in volume (Figure 6-6a, b). Given these characteristics, we named this phenomenon “ballistic wave diffusion”. This linear time dependence and accumulation of invader strand at the front is strikingly different from Fickian diffusion, characterized by a square-root time dependence and blurred front.

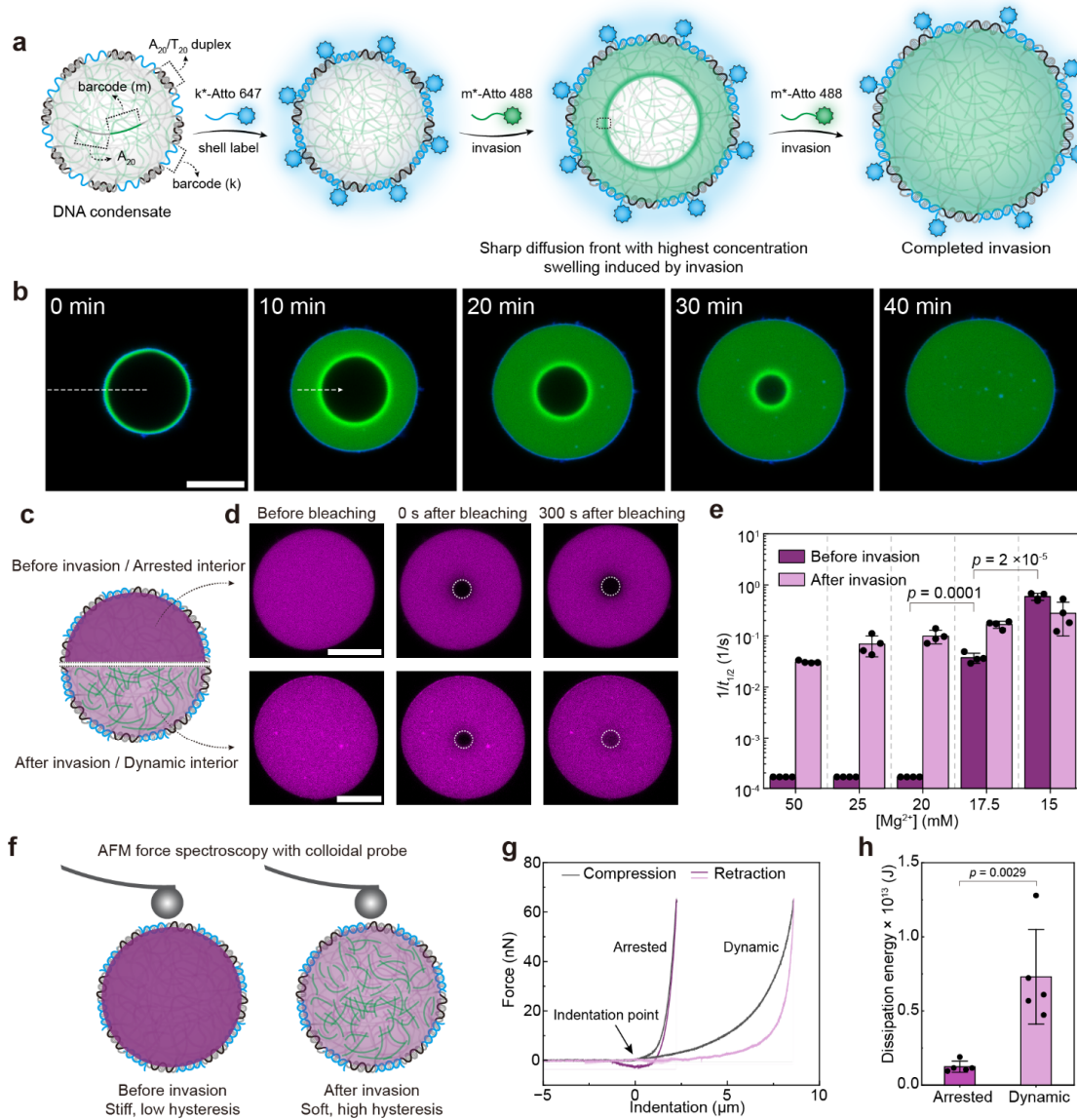


Figure 6-6. Arrested or dynamic properties of DNA condensates determined by matrix hybridization and salinity. **a**, Scheme for ballistic wave diffusion in a DNA condensate, which converts the condensate from an arrested to a dynamic state. **b**, Representative time-series CLSM images showing the ballistic wave diffusion in a DNA condensate. **c**, Scheme for different dynamic properties of DNA condensate before and after invasion. **d**, FRAP experiments showing different recovery in bleached area (indicated by white dashed circle) before and after invasion. **e**, Reciprocal of half recovery time ($1/t_{1/2}$) as a quantification for different dynamic properties of DNA condensates before and after invasion at various Mg^{2+} concentrations. **f**, Scheme for AFM force spectroscopy experiments on DNA condensates before and after invasion. **g**, Representative force spectroscopy in a full compression–retraction cycle of DNA condensates before and after invasion, showing different mechanical behaviors and hysteresis. **h**, Quantification of dissipation energy from hysteresis of force spectroscopy measurement of DNA condensates before and after invasion. Scale bars, 10 μm (**b**, **d**). Figures are adapted from ref. 39 with permission under the terms of the Creative Commons CC BY license. Copyright 2025, The Authors.

The cause for this “ballistic wave diffusion” is rooted in a critical transition of the condensate properties from an arrested to a dynamic state and the presence of defined supramolecular interaction between the invader and condensate core (barcode binding). Upon binding of the

invader to the arrested core, a transition from ssDNA to double-stranded DNA (dsDNA) occurs. This conformational transition substantially increases the persistence length of the DNA polymers, which induces local swelling due to volumetric expansion and possibly breaks Mg^{2+} -mediated percolation bridges between individual ssDNA polymers. We verified this transition using FRAP measurement: prior to invasion, the bleached region shows no recovery, while after invasion, significant recovery occurs, indicating enhanced molecular mobility inside condensates (Figure 6-6c, d). Complementary atomic force microscopy (AFM) force spectroscopy confirmed these findings. Pristine condensates exhibit purely elastic behavior with no hysteresis in compression–retraction cycles typical of solid-like materials in an arrested state, whereas invaded condensates show clear hysteresis, a characteristic feature of viscous and liquid-like behavior (Figure 6-6f–h).

Regulating condensate properties is crucial for implementing structures and interactions in the context of building functional SCs. As we will discuss below, achieving a liquid-like and dynamic core is essential for enabling the self-assembly of molecular building blocks, such as DNA tiles, and for supporting enzymatic processes within the condensates. Enhanced fluidity promotes efficient material transport and biochemical conversion, both of which are fundamental to the development of functional SCs.

6.4 DNA Condensates for Various Applications

DNA condensates have taken the lead in nucleic acid condensates when it comes to their utilization in systems chemistry, SC models, or various application-oriented research. From the perspective of traditional DNA nanotechnology, they represent a novel class of mesoscopic DNA materials that expand beyond existing methodologies.^{8,10,14,40–42} Given their DNA nature, these condensates can be readily integrated with established DNA assembly strategies to construct higher-order superstructures, modulate internal architectures and properties, and couple with nucleic-acid-modifying enzymes or dynamic DNA reaction networks based on strand displacement reactions. Furthermore, due to their densely packed, macromolecularly crowded interiors (ca. 5–10 g/L), DNA condensates can serve as models for SCs, complementing classical liposome-based systems, which typically feature a dilute aqueous lumen.²⁷ In addition, the DNA-rich interior of these condensates offer a nucleus-mimetic environment for investigating protein–nucleic acid interactions and to study processes such as transcription and phase separation occurring in the nucleus. Finally, DNA condensates hold strong potential as next generation life-like materials capable of interacting and communicating with living cells for cellular regulations. In this chapter, we review how DNA condensates intersect with various research fields and highlight their development as SCs.

6.4.1 Hierarchical DNA Colloidal Superstructure

Exploiting their all-DNA nature, these condensates are inherently compatible with traditional DNA nanotechnology approaches that use sequence-specific hybridization to drive superstructure formation or to embed functional DNA-conjugated materials. As an example, we demonstrated early on that two distinct DNA condensate species (green and red) could be co-assembled into core–satellite structures or into so-called proto-tissues (Figure 6-7a).¹⁵ Furthermore, centrifuging and reheating core–shell condensates allowed to create cellularly compartmentalized hydrogels (Figure 6-7b). Finally, functionalization of the shells of both membraneless and core–shell DNA condensates with T₂₀-HS-functionalized gold nanoparticles allowed to leverage photo-thermal effects for spatiotemporal actuation and controlled disruption of the condensate shell and ejection of the liquid core materials (Figure 6-7c).

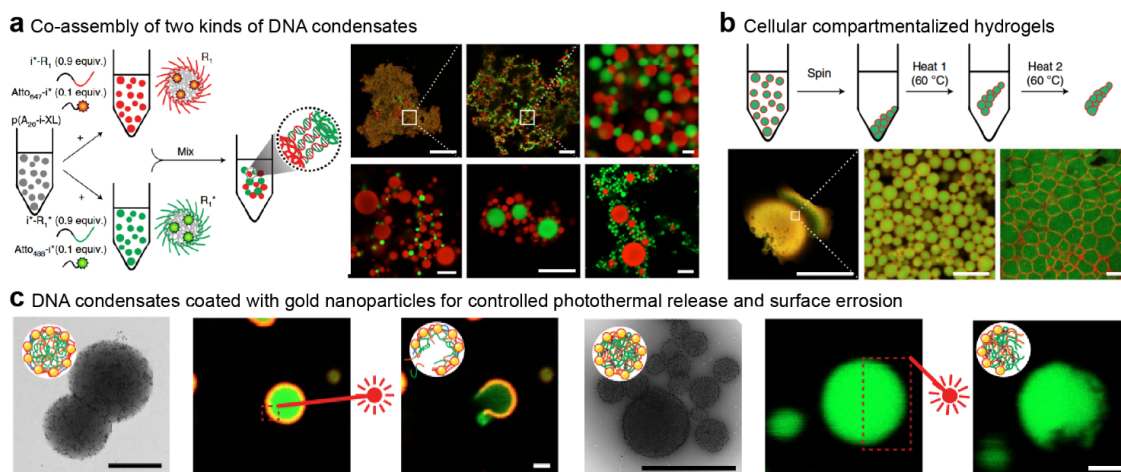


Figure 6-7. Formation of mesostructured DNA condensates and superstructures. **a**, **b**, Scheme and representative CLSM images showing the co-assembly of two different membraneless condensates into **(a)** colloidal assemblies, asymmetric core–satellite structures, and **(b)** millimeter-scale supra-particulate hydrogels. **c**, TEM and CLSM images showing membraneless condensates and core–shell condensates coated with gold nanoparticles at the shell. Upon laser irradiation, core–shell condensates release the internal material as a result of shell breakage, whereas membraneless condensates only show selective erosion due to their arrested core. Scale bars are 20, and 2 μm for **(a)** in different zoomed-in images (left to right), 500, 5, and 5 μm for **(b)** in different zoomed-in images (left to right), and 1 μm for **(c)**. Figures are adapted from ref. 15 with permission. Copyright 2018, The Authors.

6.4.2 Synthetic Cells with Artificial Secondary Structure

One of the most appealing features of core–shell DNA condensates is their potential as a novel SC model that contrasts traditional liposome or polymersome models.^{43–45} In short, DNA condensate SCs provide extensive opportunities for programmable functionalization of both the core and shell through barcode recognition. They are permeable to large molecules up to the level of enzymes, and feature a tunable, macromolecularly crowded interior that can be adjusted to mimic the dynamic and mechanical complexity of the cytoplasm.

If we first look at structural complexity of cells, one of the intriguing questions that can be raised is on the construction of artificial substructures—such as organelles or cytoskeletons—and how their formation is regulated by the crowded DNA condensate confinement. To achieve switchable artificial organelles inside SCs, we embedded a thermo-responsive ssDNA-*b*-polyethylene glycol acrylate (*i**-*b*-pEGA) block copolymer via the *i* barcodes into the core of rationally designed DNA core–shell SCs.³⁸ Upon heating, the pEGA block underwent phase separation through an initial co-continuous morphology, which subsequently coarsened and eventually developed into a concentric ring structure (Figure 6-8a, b). This subcompartment within the SC is hydrophobic and can be used as a reaction crucible for sequestering hydrophobic molecules and promoting chemical reactions, similar to the functions of membraneless organelles inside cells. As a proof of concept, a retro-Diels–Alder reaction between protected dansylfuran and DMAP was demonstrated to occur locally within the subcompartments of SCs, yielding a fluorescent product. A threefold acceleration in reaction rates compared to the same reaction in pristine SCs underscores the importance of constructing such compartments to increase reactivity on demand (Figure 6-8e–g).

Regarding artificial cytoskeletons, we recently reported the growth of DNA nanotubes (DNTs) within such SCs.²⁷ By programming hybridization interactions between DNT monomers and core barcodes, it was possible to achieve local enrichment of these monomers and facilitate their growth into DNT cytoskeletons (Figure 6-8c, d). A key factor facilitating controlled enrichment and proper cytoskeleton growth was the reduction of “active target barcode” concentration within a matrix of passive barcodes inside SC, as discussed previously in Figure 6-8b–d. Super-resolution microscopy revealed extensive branching within these cytoskeletal structures, indicating a non-classical growth mechanism driven by end-to-end joining of nucleated short fibrils, rather than the conventional nucleation and growth mechanism typically operating in dilute solutions (Figure 6-8d). Extensive studies on temperature dependence, matrix crosslinking, and DNT tile concentration revealed the critical role of modulating condensate dynamics in promoting or suppressing cytoskeleton growth. AFM force spectroscopy further showed that the cytoskeleton-containing SCs exhibit an elastic modulus two orders of magnitude higher than that of pristine SCs, reaching the modulus of natural cells (Figure 6-8h, i).

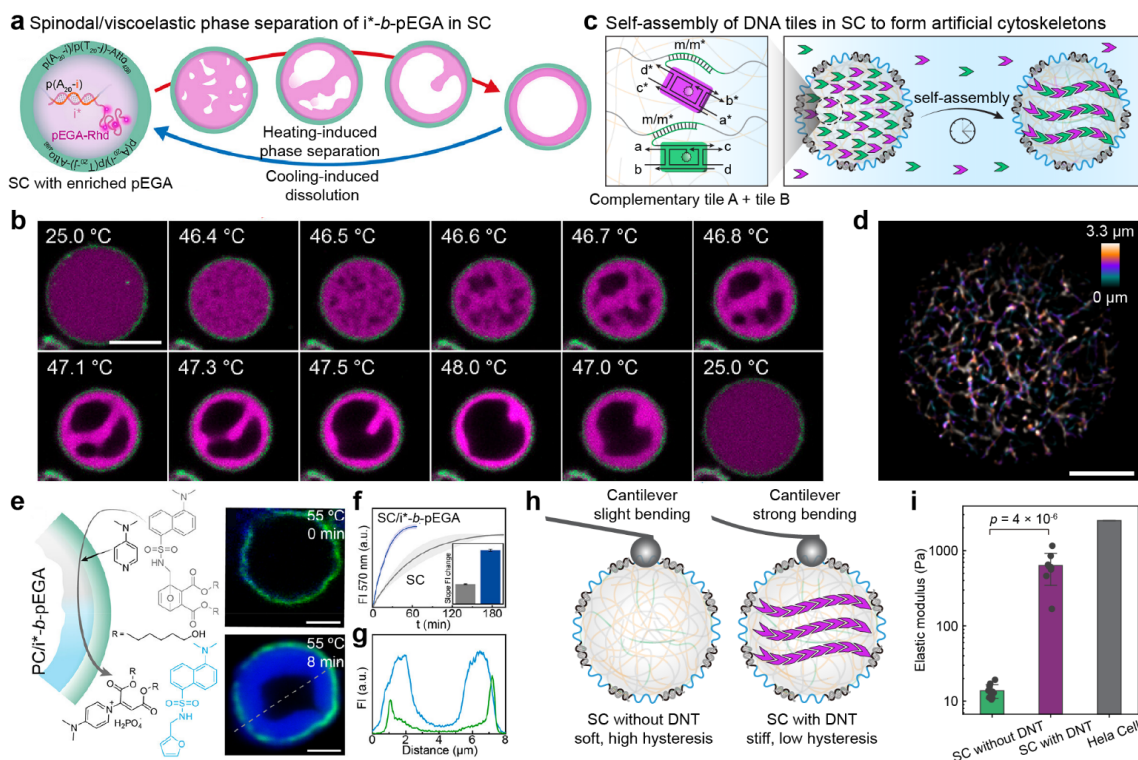


Figure 6-8. Formation of artificial substructures inside DNA for improved functionality and mechanical properties. **a**, Scheme of the thermo-reversible phase separation of i^*-b -pEGA in a SC for artificial organelle formation. **b**, CLSM images showing the thermo-reversible phase separation of i^*-b -pEGA (magenta channel) within a SC (green shell), following spinodal/viscoelastic phase separation. **c**, Scheme of the barcode-mediated enrichment of DNA tiles and cytoskeleton formation in a SC. **d**, Structured illumination microscopy (SIM) image of artificial cytoskeletons formed in a SC. The color-coded z-projection image reveals branching morphology. **e**, Scheme and CLSM images showing the use of the hydrophobic subcompartments for promoting a retro-Diels–Alder reaction. **f**, Time-dependent fluorescence of the retro-Diels–Alder reaction within SC/ i^*-b -pEGA hybrids vs. normal SCs without i^*-b -pEGA. **g**, Line segment analysis along the dashed line in (e). **h**, Scheme of the AFM force spectroscopy measurements on SCs with and without artificial cytoskeletons inside. **i**, Comparison of elastic modulus between SC with DNT, SC without DNT, and HeLa cells. Scale bars, 5 μm (b, d), and 2 μm (e). Figures a, b, e–g are adapted from ref. 38 with permission. Copyright 2023, American Chemical Society. Figures c, d, h, i are adapted from ref. 27 with permission under the terms of the Creative Commons CC BY license. Copyright 2025, The Authors.

6.4.3 Synthetic Cells with Metabolic Functions

In addition to constructing complex internal structures, we also developed SCs with integrated metabolic functions. In a pioneering study, we demonstrated the encapsulation of an artificial metalloenzyme using biotin–streptavidin interactions (Figure 6-9a)²³. This endowed the SCs with catalytic activity, enabling them to perform an uncaging reaction that produces the fluorescent reporter umbelliferone (Figure 6-9b). Owing to the nucleic acid-rich environment within the SCs, the artificial metalloenzyme exhibited enhanced catalytic efficiency compared to that in dilute solution, a result of macromolecular crowding. In addition, point mutations of the amino acid sequence—in particular relocating cationic amino acid residues—proved vital to boost the catalytic efficiency of the artificial metalloenzymes. More intriguingly, the

production of umbelliferone inside the SCs triggered a morphological response of SCs, including swelling, membrane softening, and ultimately fusion with neighboring SCs (Figure 6-9c). This represents a significant advance in DNA-based SC systems, demonstrating the ability to metabolize signals and undergo adaptive morphological changes.

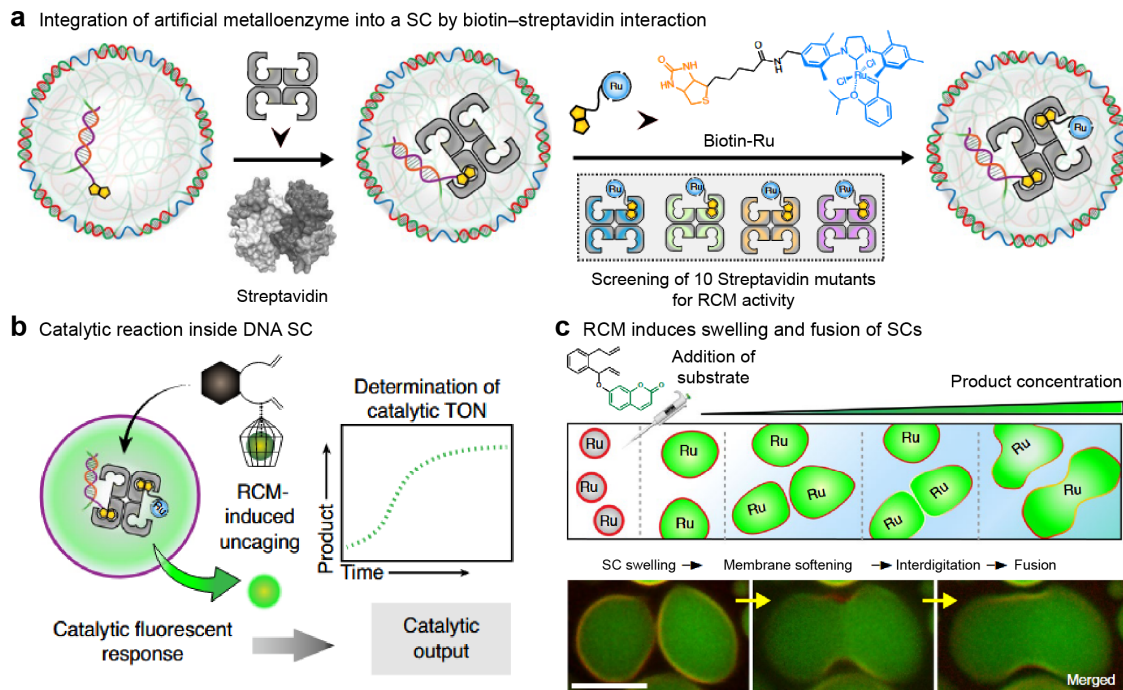


Figure 6-9. DNA SCs with encapsulated artificial metalloenzymes. **a**, Scheme for encapsulation strategy of artificial metalloenzyme into SCs by biotin–streptavidin interactions. **b**, The artificial metalloenzyme catalyzes a DNA-orthogonal uncaging reaction by ring-closing metathesis (RCM), yielding a primary fluorescent signal. **c**, Scheme and representative CLSM images of RCM-induced morphological transformation and fusion of SCs. Scale bar, 5 μm (c). Figures are adapted from ref. 23 with permission. Copyright 2020, The Authors, under exclusive license to Springer Nature Limited.

In a complementary approach, we encapsulated DNAzymes inside the SCs via barcode-mediated interactions, endowing them with catalytic activity to cleave specific DNA substrates (Figure 6-10a).²⁵ Similar to the artificial metalloenzymes, fluorescence assays on the catalytic activity revealed a more powerful activity when confining the DNAzymes into the SC (Figure 6-10b, c). By further engineering the systems to catalytically produce a strand in the core, which then translocates to the shell to induce multivalent interactions, it was possible to achieve signal-mediated prototissue formation through the encoded metabolic activity. (Figure 6-10d, e).

Taken together, these two strategies illustrate the modular incorporation of diverse metabolic functions into DNA-based SCs. Looking forward, we envision that these SCs could be developed into fully modular systems, allowing diverse structures and functions to be independently incorporated as needed. This will pave the way towards truly intelligent and functional SCs.

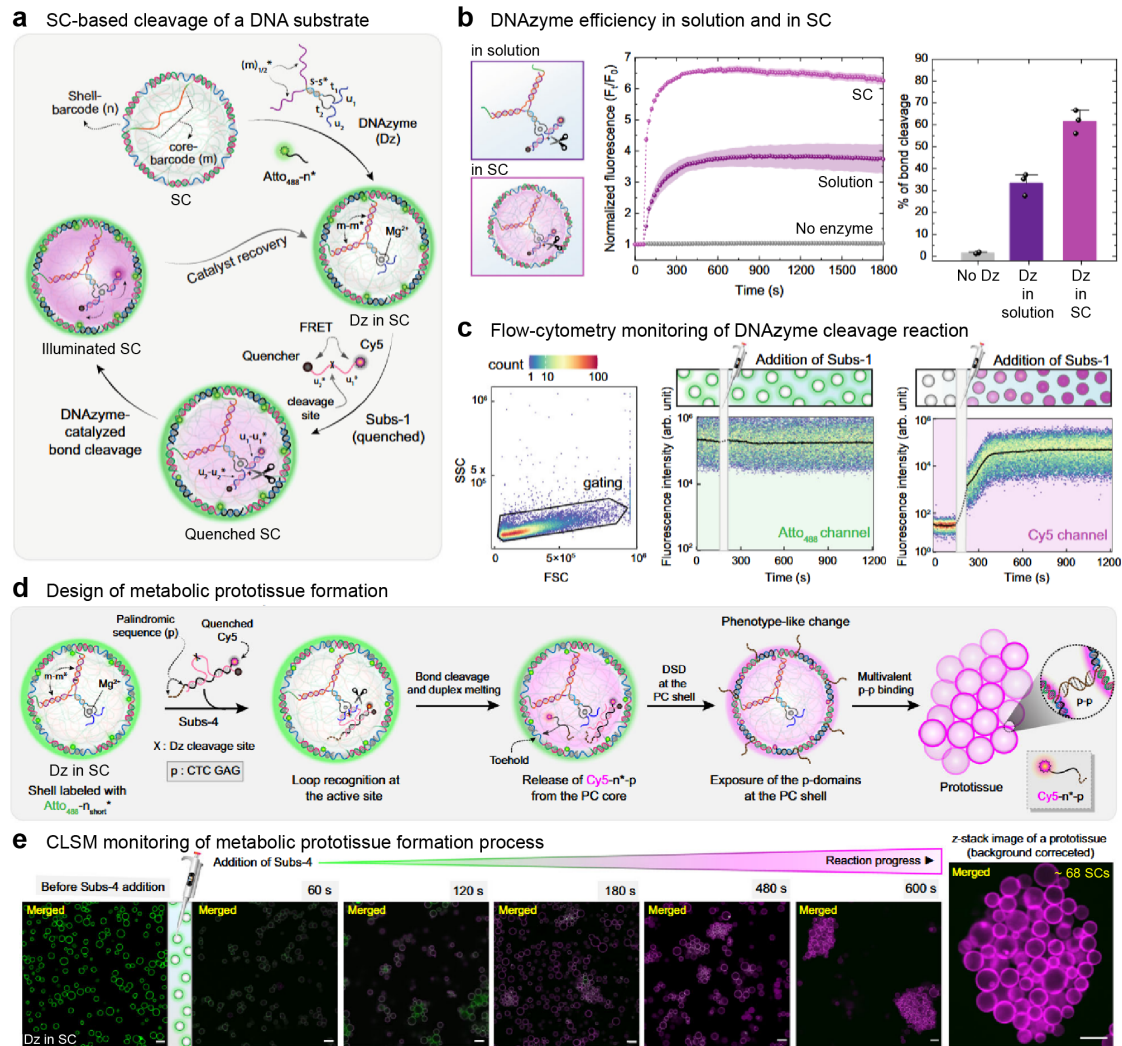


Figure 6-10. DNA SCs with encapsulated DNAzymes. **a**, Scheme illustrating the encapsulation of DNAzymes into SCs to cleave substrates and generate fluorescent signals. **b**, Scheme and experiments comparing DNAzyme cleavage reaction in solution and in SCs. **c**, Flow cytometry monitoring of the DNAzyme cleavage reaction inside SCs. **d**, Scheme for metabolic prototissue formation based on DNAzyme-catalyzed substrate cleavage in SCs. **e**, Time-series CLSM images showing the shell transformation and prototissue formation triggered by DNAzyme reaction in SCs. Scale bars, 5 μ m (**e**). Figures are adapted from ref. 25 with permission under the terms of the Creative Commons CC BY license. Copyright 2022, The Authors.

6.4.4 Synthetic Protonucleus with Transcription Function

With a DNA-rich interior, it is natural to think about repurposing core-shell DNA condensates as a nucleus-like compartments or protonucleus (PN), which enables the study of nuclear functions such as DNA replication, RNA transcription, and phase separation in environments reminiscent of the cell nucleus. To integrate transcriptional functionality into the PN system, we embedded a T7 RNA polymerase (T7 RNAP) promoter sequence as a barcode in the SC core. Providing the corresponding template with the promoter binding site then enabled transcription to occur inside the SCs (Figure 6-11a).²⁸ Using light-up aptamers and

fluorescently labeled nucleotides, it was possible to confirm the localized transcription inside the PN using CLSM (Figure 6-11b, c). The observed transcription activity inside the PN is comparable to those in plain solution (Figure 6-11d).

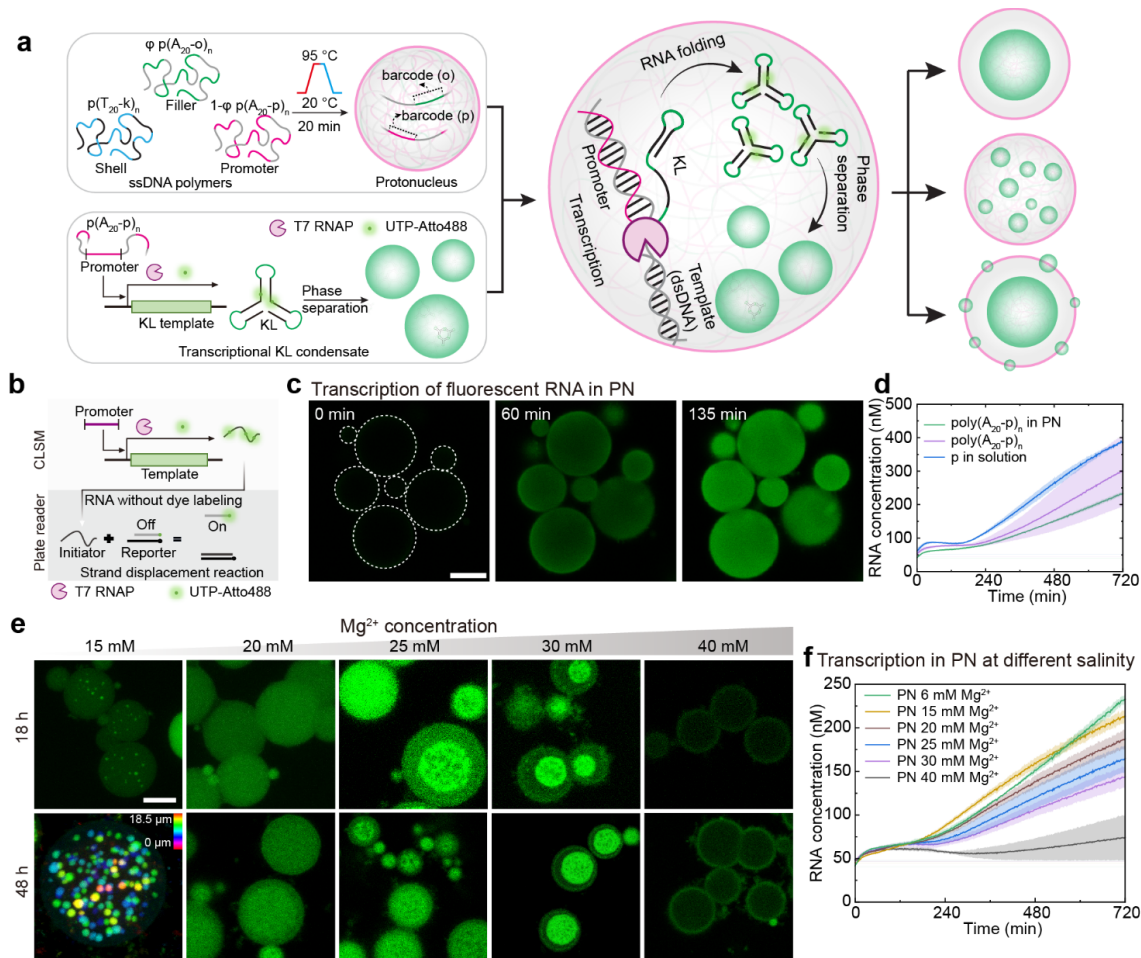


Figure 6-11. DNA condensates as protonuclei for transcriptional function and RNA condensation. **a**, Using co-phase separation approach to form protonucleus (PN) with embedded promoter sequence to gain transcription function. Transcription of RNA kissing loops (KLs) inside PN induces the formation of distinct nuclear patterns via different nucleation and condensation processes. **b**, Scheme showing transcription in PN characterized by CLSM and plate reader. For CLSM, UTP-Atto488 is added to the NTP mix for transcript labeling. For plate reader, dsDNA reporters with fluorophore-quencher pairs are present in solution to react with transcribed RNA by strand displacement reaction (SDR), generating fluorescent signals. **c**, Representative CLSM images showing the localized transcription of fluorescent RNA (green, labeled by UTP-Atto488 during transcription) inside PN at different times. **d**, Transcription kinetics inside PN, in solution with free poly(A₂₀-p)_n, and in solution with free promoter (p) oligonucleotide, monitored by SDR of the reporter in a plate reader. **e**, Representative CLSM images showing the effects of [Mg²⁺] on transcriptional KL condensate formation in PN after 18 and 48 h reaction. **f**, Effects of [Mg²⁺] on transcription kinetics in PN, monitored via RNA-triggered SDR of the reporter. Figures are adapted from ref. 28 with permission under the terms of the Creative Commons CC BY license. Copyright 2025, The Authors.

More intriguingly, when using a DNA template encoding RNA kissing-loop motifs, which are intermolecular binding segments, the transcribed RNA can spontaneously assemble into transcriptional condensates inside the PN. Because Mg²⁺ ions influence both the transcription rate and the strength of RNA kissing-loop interactions, a variation of [Mg²⁺] results in distinct

RNA condensate morphologies and densities inside the PN (Figure 6-11e, f). At low $[Mg^{2+}]$, transcription is faster, leading to the formation of multiple small RNA condensates due to higher local RNA concentrations and widespread nucleation. In contrast, at high $[Mg^{2+}]$, slower transcription and stronger kissing-loop interactions favor the formation of a single large condensate in each PN through co-continuous phase separation and compaction. This study demonstrates the versatility of DNA condensates as synthetic platforms for investigating processes and functions in nucleus-mimetic environments.

6.4.5 Emergent Platform to Study Protein Phase Separation in the Nucleus

DNA condensate-based protonuclei also provide a nucleus-mimetic environment to study the PS behavior of nucleic-acid-binding proteins. In addition to the locally high DNA concentration, they offer a complex environment with non-specific binders, crowding effect, compositional heterogeneity, and tunable viscoelasticity. We investigated the PS of the fused in sarcoma (FUS) protein within PN, which is linked to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).^{46–48} A strong dependence of the phase-separated condensate morphology on the loading ratio of FUS exists. Figure 6-12a shows that the protein forms condensates at low loading ratios, whereas higher loading ratios lead to co-continuous network morphologies.

The PN platform allows for core modifications via the barcode hybridization strategy. This is schematically shown in Figure 6-12b, where binding of specific DNA or RNA oligonucleotides to the barcode *m* or the A_{20} sequences introduces additional nucleic acid components to the PN core. Detailed studies comparing electrophoretic mobility shift assays (EMSA; Figure 6-12c) to quantify the affinities of different sequences to FUS in solution, alongside measurements of FUS uptake (Figure 6-12d) and analyses of the phase-segregated morphologies, revealed striking differences between affinities determined in solution and in PN. The partitioning value (*P*) for FUS in pristine PN (**1**, *P* = 410) and T_{20} -Atto647N-hybridized PN (**4**, *P* = 743) is much higher than that for the remaining samples, although EMSA does not show the same trend. On the contrary, the sequence with the highest affinity (*m**-RNA), tested in EMSA, shows a rather low partitioning value (*P* = 84). This discrepancy is a result of the complex PN environment, which cannot be replicated by solution assays such as EMSA. It highlights the importance of developing *ex vivo* assays with engineered crowded environment, allowing the influence of non-specific interactions and macromolecular crowding to be properly assessed. Furthermore, by tuning the viscoelastic properties of the PN—very similar to what has been established in DNA hydrogels⁴⁹—through partial substitution of $p(A_{20}-m)_n$ with $p(A_{20}-m-XL)_n$ (XL = self-crosslinking domain), it was shown that FUS phase separation is progressively suppressed with increasing amount of $p(A_{20}-m-XL)_n$ in PN (Figure 6-12e). This demonstrates how modulation of condensate dynamics can possibly prevent aberrant phase transitions in disease.

Chapter 6. Temperature-Induced Phase Separation of Nucleic Acids (Review)

The combination of modulating PN sequence composition and core viscoelasticity positions these nuclear mimics as a tunable synthetic platform to investigate PS of proteins, peptides, and their mutants. This is in particular relevant for protein sequence mutations and their impact on PS behavior, which remain a major focus of current research in neurodegenerative diseases.

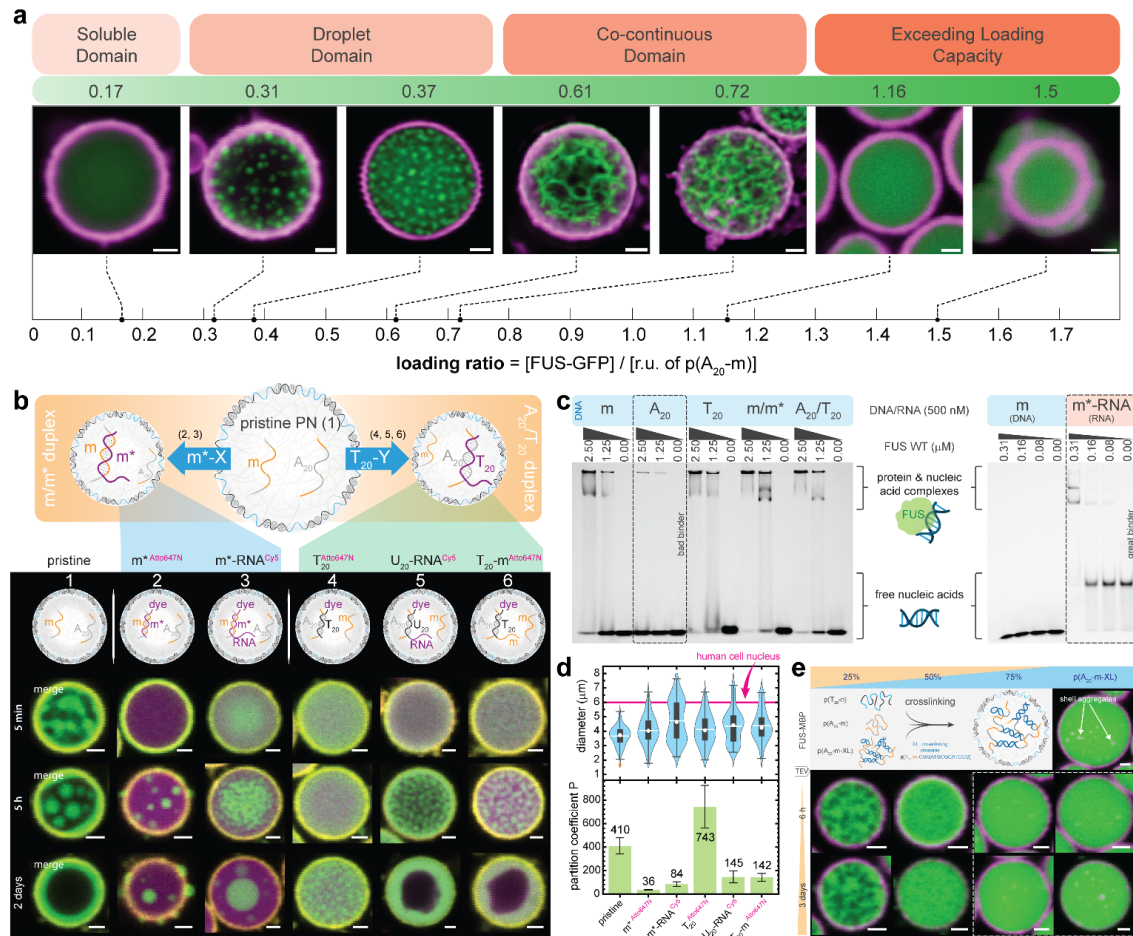


Figure 6-12. DNA condensates as protonuclei for investigating FUS protein PS in a nucleus-mimetic environment. **a**, Representative CLSM images showing that different loading ratio of FUS-GFP determines the PS morphology in PN. **b**, Representative CLSM images showing that PN core can be modified with various DNA and RNA sequences, which in turn show different PS kinetics and morphologies. **c**, EMSAs of the most important PN domain sequences. **d**, Average diameters of PNs used in (**b**), as well as the partitioning of MBP-FUS-GFP before TEV addition. **e**, Influence of crosslinking on FUS-GFP PS. MBP: Maltose-binding protein; GFP: Green fluorescent protein; TEV: Tobacco Etch Virus protease. Scale bars, 1 μm for (**a**, **b**, **e**). Figures are from illustrations courtesy of Johann Fritzen (unpublished work).

6.4.6 Synthetic Cell-Natural Cell interface

One of the most exciting applications of DNA-based SCs lies in their potential to build interfaces with natural cells for targeted interaction, communication, and cellular regulation. Studies are still in early stages, but we wish to highlight two important approaches.

First, our group has explored how the enhanced mechanical stability of cytoskeleton-containing SCs facilitates the construction of an SC–cell mechano-interface by functionalizing the SC shell with Cyclic Arginyl-Glycyl-Aspartic acid peptides (cRGD) that bind integrin receptors on cells (Figure 6-13a).²⁷ Once in contact with cells, the internal artificial cytoskeletons provided sufficient mechanical integrity to stabilize the SCs under cellular forces and prevented their rupture (Figure 6-13a).

Second, nucleic acid-based aptamers have been employed to target specific cell surface receptors and modulate cellular functions. In a recent study, Nie and coworkers utilized the similar DNA SCs and made them responsive to Zn^{2+} and photo-thermal heating.²⁹ The Zn^{2+} response was instigated by activating Zn^{2+} -responsive DNAzyme, which cleaved the SC shell and exposed MET-targeting aptamers embedded in the core. This aptamer then binds to MET receptors on the A549 cell surface, establishing targeted cell recognition (Figure 6-13b).

Furthermore, the inclusion of gold nanorods (AuNRs) allowed near-infrared (NIR) irradiation to locally heat and disrupt the SC shell, exposing the embedded aptamers and facilitating similar interaction with living A549 cells. Downstream signaling in the target A549 cell could be induced, resulting in the translocation of extracellular signal-regulated kinases (ERK) from the nucleus to the cytoplasm (Figure 6-13c). Remarkably, the Zn^{2+} -mediated and NIR-mediated aptamer release mechanisms can operate orthogonally in the same solution, enabling selective and independent regulation of ERK translocation in distinct cell populations—A549 (triggered by Zn^{2+}) and NIH3T3 (triggered by NIR)—each recognized by a specific aptamer embedded in distinct SCs (Figure 6-13d).

Both works highlight the potential for further expansion of the DNA SC platform towards more advanced communication networks. Through co-phase separation of multiple barcode ssDNA polymers and assigning distinct functions to individual barcodes, one can envision the creation of multifunctional SCs capable of executing complex tasks and engaging in programmable interactions with living systems.

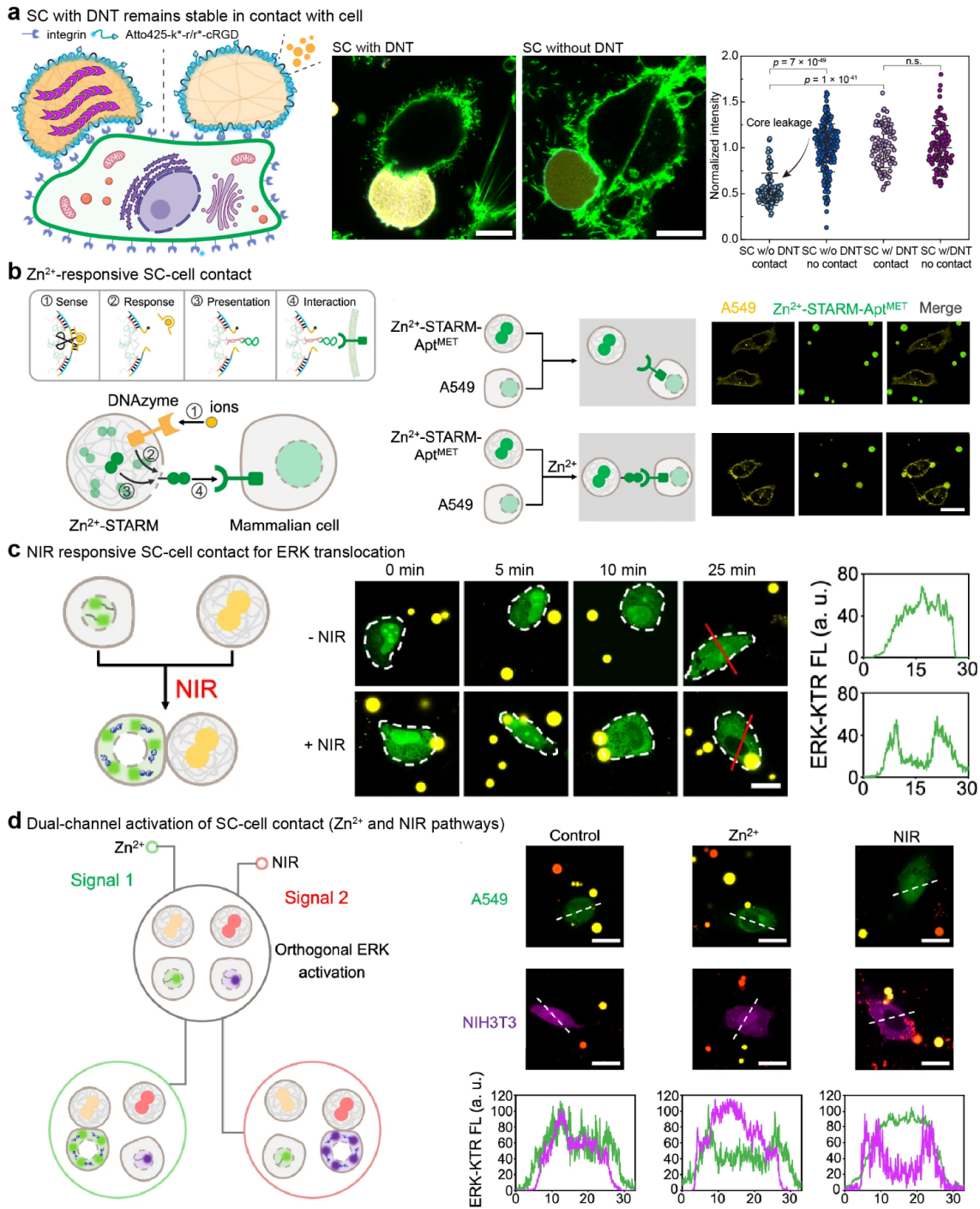


Figure 6-13. Construction of SC-cell interacting interface. **a**, Scheme and CLSM images showing the SC–cell contact mediated by cRGD–integrin binding. The DNTs provide mechanical stability to SCs, ensuring a durable SC–cell interface. In absence of the internal cytoskeleton, significant leakage of liquid SC core material occurs. **b**, Scheme and CLSM images showing the SC–A549 cell interaction through Zn²⁺-mediated activation of DNAzyme, which cleaves the SC shell and releases internal aptamer for cellular receptor binding. In absence of Zn²⁺, SC–A549 cell interaction cannot be observed. **c**, Scheme and CLSM images showing NIR-triggered SC–A549 cell interaction, leading to ERK translocation. **d**, Scheme and CLSM images showing the dual-channel activation of SC–cell interactions and orthogonal ERK activation by Zn²⁺ (targeting A549 cells) and NIR light (targeting NIH3T3 cells). Scale bars, 10 μ m (**a**) and 20 μ m (**b–d**). Figure **a** is adapted from ref. 27 with permission under the terms of the Creative Commons CC BY license. Copyright 2025, The Authors. Figures **b–d** are adapted from ref. 29 with permission under the terms of the Creative Commons CC-BY-NC-ND license. Copyright 2025, The Authors.

6.5 Conclusions

Owing to their polyelectrolyte nature and unique chemical structures, the PS behavior of nucleic acids is influenced by factors such as purine/pyrimidine composition, salinity, and molecular weight. The presence of divalent cations, such as Mg^{2+} , plays a crucial role in facilitating intra- and intermolecular bridging between phosphodiester backbones during DNA and RNA phase separation.¹⁶ Within the dense phase of the condensates, interchain interactions, such as base stacking, hydrogen bonding, and sequence hybridization, can further lead to the formation of a system-spanning network, known as a percolation transition.⁵⁰ Although general dependencies of PS on nucleic acid composition and environmental parameters have been investigated from experiments, we are still far from truly understanding the molecular grammar on PS of nucleic acids.^{15,16} Deciphering these sequence dependencies will likely require high-throughput experimentation combined with machine learning analysis.

Condensates can be stabilized at room temperature by various methods, most simply by percolation-induced network formation following PS or by combination of PS with base-pairing hybridization technologies to access defined metastable structures.^{15,16} The resulting material properties of nucleic acid condensates can be tuned between arrested and dynamic states, particularly through salinity adjustments and hybridization approaches converting coiled ssDNA into more expanded dsDNA.³⁹

In terms of applications, owing to their stability, ease of preparation, and compatibility with DNA nanotechnology, DNA condensates have outpaced RNA condensates in various areas, such as constructing complex colloidal materials, creating synthetic cells with defined structures and functions, and studying physical and chemical processes in DNA-rich environments.^{15,23,27,28} The field now stands at a pivotal point to fully realize the potential of this emerging class of DNA-based materials. For example, DNA condensates can be exploited as confined compartments for spatially organizing DNA reaction networks within distinct regions. Such spatial organization could be particularly powerful in DNA computing, where encapsulating DNA logic gates in separate condensates helps prevent reaction crosstalk and interference.⁵¹ Moreover, DNA condensates can serve as molecular enrichment platforms that facilitate desired reactions through proximity effects, offering promising opportunities for diagnostic applications.

With the development of more complex SCs, one particularly exciting application lies in further exploiting SC–cell interfaces for cellular regulation. Achieving this will require the creation of intelligent SC systems with embedded reaction networks capable of sensing and processing cellular signals and responding accordingly. We envision that multi-barcode SCs can be engineered to integrate distinct functional modules and reaction networks, thereby enabling multifunctional SCs with embedded intelligence and empowering SCs to sense, process, and respond to molecular signals from living cells, while simultaneously generating regulatory cues that modulate cellular processes and functions.

Finally, the potential of nucleic acid condensates extends far beyond the applications explored so far and discussed above. Positioned at the intersection of chemistry, biology, physics, and materials science, they offer a versatile platform for innovative research across diverse fields. We anticipate nucleic acid condensates to make more impactful applications in DNA/RNA nanotechnology, soft matter, synthetic biology, and biomaterials research.

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7. Conclusions and Outlooks

This final chapter provides comprehensive concluding remarks of the key results obtained in my research on DNA condensate materials for various applications. It concludes with a forward-looking perspective that highlights open questions and opportunities for further advancing DNA condensate materials across diverse fields, including soft matter, synthetic biology, and DNA nanotechnology.

7.1 Concluding Remarks

In this thesis, I investigated the unique physical and chemical properties of DNA condensates. I developed various methods for engineering DNA condensates and coupled them with DNA nanostructures, synthetic biology tools, and DNA-RNs, for achieving DNA-based materials and systems with various functions.

In Chapter 2, DNA condensates were used as a model system to study transport phenomena in biomolecular condensates. We identified a new diffusion mechanism, termed ballistic wave diffusion, in soft matter in general and in DNA condensates in particular. Unlike Fickian diffusion, ballistic wave diffusion is characterized by an ultrasharp diffusion front that propagates linearly with time. The invading molecules accumulate in a non-equilibrium high-intensity front, representing a transition interface that relaxes with time. This mechanism arises from molecular recognition and the transition of condensate states from arrested to dynamic during macromolecule invasion. Guided by numerical model for reaction–diffusion simulation, we identified invader concentration, salinity, and polymer length are key parameters for controlling front velocity and front shape. Decrease of the salinity can dynamize the DNA condensates and alter the diffusion mechanism from ballistic wave diffusion to Fickian diffusion. Although we revealed ballistic wave diffusion in purely synthetic system, molecular recognition and different dynamic states are ubiquitous features of biological condensates.^{1–5} Sharp diffusion fronts have been observed in biological studies of condensates based on coiled-coil interactions, but they have not been linked to a specific diffusion mechanism.⁶ Our study provides an explanation for these observations through the concept of ballistic wave diffusion. This mechanism also suggests a potential route for cells to regulate condensate dynamics, preventing aging or aggregation and maintaining normal function. It could potentially guide therapeutic solutions for reversing solid-to-liquid transition of relevant biomolecular condensates, such as hnRNPA1, TDP43, and FUS.^{2,7,8} Overall, these findings offer a new perspective to comprehend the intricate interplay of dynamics and chemical kinetics in biomolecular condensates and their relation to cellular function.

In Chapter 3, DNA condensates were employed as synthetic cells, leveraging their macromolecularly crowded and viscoelastic interiors and intrinsic compatibility with DNA nanotechnology. We used co-phase separation approach to control the barcode concentration inside SCs at desired concentrations, which regulated the growth of enriched DNA tiles into artificial cytoskeletons at different densities. In contrast to classical crowding conditions, a

bundling of formed cytoskeletons was not observed.⁹ Mechanistically, cytoskeleton growth proceeds primarily through end-to-end joining of fibrils, rather than by tile addition as commonly observed in solution.^{10–12} By tuning the viscoelastic property of the SC matrix via crosslinking, temperature, and concentration, we can modify the dynamics of the coupled SC matrix and influence the cytoskeleton assembly kinetics. With ternary barcode SC systems, different stimuli pathways, and orthogonal tiles, we realized metabolic growth of cytoskeletons inside SCs, as well as controlled formation and destruction of multiple cytoskeletons inside the same SC. At a system level, we could use these SCs to selectively enrich desired monomers from a common pool in a diverse SC community and grow distinct structures. Finally, we built a mechano-interface between SCs and Hela cells by functionalizing SC shells with cRGD, which can bind to the integrin at cell membrane. Artificial cytoskeletons enhance the SC stiffness and lead to stable SC-cell mechano-interface. From a broader perspective, unlike classical SCs constructed from liposomes or polymersomes with simple aqueous compartments,^{9,13} or coacervates exhibiting only a certain degree of crowding,^{14,15} our SC represents an advance toward mimicking real cells. It features not only macromolecular crowding, but also a viscoelastic environment, programmable uptake, and controlled local enrichment, which are ubiquitous in biological systems but remain rare in synthetic cells. Our SC can thus serve as a modular molecular system for studying self-assembly and structure formation under cell-like environments. In addition, our co-phase separation approach endows SCs with highly flexible and programmable barcodes in their cytoplasm-mimetic matrix, which can be modularly utilized to design and functionalize SCs with higher degree of complexity and functionality.

In Chapter 4, DNA condensates were repurposed as protonuclei with transcription functions. These PN offer a highly programmable platform for introducing short genes for transcription with satisfying efficiency up to high salt concentration. To study transcriptional folding and phase segregation in the crowded, nuclear-mimetic environment, we focused on KL condensates formed by ssRNA nanostars.¹⁶ We identified that ionic strength is one key parameter for cross-regulating transcription efficiency, viscoelasticity, and KL-PN affinity. These effects in turn affect the nucleation process of condensates from binodal to spinodal or viscoelastic phase separation, resulting in tunable artificial nuclear architectures inside the PN. The non-specific interactions between KL and PN matrix are crucial for retaining KL transcripts inside PN via KL-PN co-condensation. Such interactions can be efficiently modulated when SC matrix is hybridized with complementary DNA strands, leading to a repulsion and exclusion of the KL condensates from the PN. We have further studied co-transcription and condensation of orthogonal KLs within the same PN, which results in distinct structures arising from competitive interactions between different RNA nanostars and the PN matrix. Insights from this study could guide the investigations into more sophisticated phase separation process of proteins and nucleic acids in DNA-enriched environments, such as RNA repeats (CAG) and HP1a protein involved in repeat expansion disorders and heterochromatin domain formation in nucleus.^{17,18} Collectively, this platform enables the construction of controlled nuclear

patterns, advances understanding of RNA condensation in nuclear environments, and provides strategies for engineering nucleus-mimetic systems with precise architectural control.

In Chapter 5, DNA condensates were explored as microreactors for compartmentalizing DNA-RNs. We anchored two sets of Transducer-Reporter system into DNA condensates through programmed hybridization with condensate barcodes. The Transducers were designed to receive specific Inputs and release a unified Messenger for triggering both Reporters of the same sequence but different fluorophore-quencher pairs. Combining both Transducer-Reporter systems in the same solution results in unavoidable sequence interference. In contrast, the in-condensate DNA reaction effectively governs a localized reaction and confines diffusive leakage of key components. This allows the orthogonal operation of DNA-RNs containing interfering strands in different compartments without crosstalk. Compared to traditional DNA computing systems in homogeneous solution, our strategy eliminates the need for high sequence orthogonality in large-scale systems.^{19,20} Furthermore, in contrast to DNA reactions on DNA origami that rely on proximity effects, dynamic DNA condensates host reaction networks with freely diffusing components that remain accessible throughout the condensate, thus preserving the benefits of solution-based reactions.²¹ Finally, the Transducer module can be further exploited to couple with various downstream DNA-RNs and facilitate their modular integration into a unified system for achieving multifunctional DNA-based systems.

Finally, in Chapter 6, a review for current understanding and development of DNA condensates was presented, focusing on their physical and chemical properties and their applications across various fields. In the context of DNA condensate engineering, we have established robust strategy to modulate barcode content and concentration inside DNA condensates via co-phase separation of adenine-rich DNA polymers carrying different barcodes. We also identified salinity to be the critical parameter to regulate the dynamic properties of DNA condensates for various applications. In DNA nanotechnology, DNA condensates have been explored for assembling hierarchical DNA materials and structures. In synthetic biology, they have served as synthetic cell models that feature viscoelastic property and macromolecular crowding. DNA condensate-based SCs have been shown to host enzymatic and transcriptional functions, albeit only in isolated studies where each function was introduced separately. Looking ahead, we highlight opportunities to advance DNA condensates into multifunctional synthetic cells and to harness them for compartmentalizing DNA reaction networks, thereby enabling large-scale DNA-RNs with advanced functions.

7.2 Future Perspectives

DNA condensates possess unique physical and chemical properties, including ballistic wave diffusion, solid-liquid transitions, and programmable DNA barcodes. They also represent versatile and compatible materials for integration with DNA nanotechnology and synthetic

biology to achieve diverse functions. Harnessing these features will open avenues for creating new materials and functionalities across a range of applications.

Specifically, in the context of responsive materials and systems, ballistic wave diffusion and solid–liquid transitions can be harnessed to program a gating mechanism in DNA condensates, in response to signaling molecules.²² In the solid-like state, condensates exclude large macromolecules from entering their core, whereas the liquid-like state permits their diffusion. Since ballistic wave diffusion governs the solid-to-liquid transition, certain invaders can be employed as triggers to activate macromolecule recruitment into the condensate core. This recruitment can then be further engineered to initiate downstream reactions and functions inside DNA condensates, thereby establishing a responsive condensate system. Additionally, since molecular recognition and solid–liquid states are ubiquitous in biological systems, it will be intriguing to investigate whether ballistic wave diffusion and sharp diffusion fronts also occur in natural biomolecular condensates and to explore their potential cellular functions.^{23,24}

Along the lines of DNA condensate-based synthetic cells, barcode engineering via co-phase separation has enabled the integration of multiple barcodes within the same synthetic cell, a strategy thus far applied only to the growth of multiple artificial cytoskeletons. Looking forward, these barcodes could be harnessed to modularly incorporate diverse functional modules, enabling synergistic interactions between them. Such advances may allow us to develop more complex and multi-functional synthetic cells with the ability to adapt, self-replicate, and evolve.

DNA condensates can also act as microreactors for detection and diagnostics. For example, when targeting diluted species such as specific mRNAs, condensates can concentrate them through barcode recognition. The resulting local enrichment and proximity effect then trigger reactions with pre-loaded DNA duplexes carrying fluorophore–quencher pairs, producing a fluorescence signal. Moreover, by pre-loading distinct condensates with multiple distinct fluorophore–quencher duplexes, different mRNAs associated with diseases can be detected simultaneously, thereby enabling diagnostic applications. Such system can be further coupled with high-throughput methods such as flow cytometry to enable rapid diagnostics.

DNA condensate-based microreactors can also be employed for large-scale screening of DNA reactions. For instance, condensates can be prepared with varying barcode concentrations, while DNA-RNs of interest are designed with overhangs complementary to these barcodes. Upon mixing, individual condensates selectively enrich DNA-RN components, such that each condensate hosts the same DNA-RN, but individual DNA-RN components are at distinct concentrations across different condensates. When triggers are added, these in-condensate DNA-RNs are activated simultaneously in the same solution, proceeding with different kinetics defined by their local concentrations. This allows us to use only one single reaction to explore a large parameter space. Coupled with flow cytometry, the reaction outcomes can be

efficiently obtained and compared, enabling rapid identification of optimal reaction conditions.

DNA condensates coupled with DNA-RNs also offer a promising route toward more complex DNA computing systems. When embedded in condensates, DNA-RNs become modular and insulated from interference and sequence crosstalk. This largely simplifies the design principles in large-scale DNA-RNs.²⁵ For example, the same DNA Adder gate array can be distributed across 16 condensates, each equipped with appropriate signal transduction mechanisms, to realize 16-bit adders. In contrast, constructing a 16-bit Adder in solution would require 16 distinct Adder circuits with orthogonal sequences to prevent interference. This modularization strategy can be extended to assemble DNA-RNs with diverse signal-processing functions by embedding different RNs into separate condensates. Such DNA condensate system can be further programmed to interact with living cells, processing cell-released signals and in turn regulating cellular processes and functions through in-condensate computing and signal processing.

In summary, DNA condensates represent a versatile and still largely unexplored class of materials with many open opportunities for further development across diverse applications. Featuring macromolecularly crowded interiors and inherent compatibility with both DNA nanotechnology and synthetic biology, DNA condensates emerge as promising synthetic cell models. Their unique capacity for compartmentalization and modularization further expands the design space of materials and systems for DNA nanotechnology. We anticipate that DNA condensates will play an increasingly important role in shaping the future of soft matter materials, DNA nanotechnology, and synthetic cell research.

7.3 References

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