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Review Article (Invited)

Pioneering artificial cell-like structures with DNA nanotechnology-based liquid-liquid phase separation

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Recent studies have revealed that liquid-liquid phase separation (LLPS) plays crucial roles in various cellular functions. Droplets formed via LLPS within cells, often referred to as membraneless organelles, serve to concentrate specific molecules, thus enhancing biochemical reactions. Artificial LLPS systems have been utilized to construct synthetic cell models, employing a range of synthetic molecules. LLPS systems based on DNA nanotechnology are particularly notable for their designable characteristics in droplet formation, dynamics, properties, and functionalities. This review surveys recent advancements in DNA-based LLPS systems, underscoring the programmability afforded by DNA's base-pair specific interactions. We discuss the fundamentals of DNA droplet formation, including temperature-dependence and physical properties, along with the precise control achievable through sequence design. Attention is given to the phase separation of DNA nanostructures on two-dimensional closed interfaces, which results in spatial pattern formation at the interface. Furthermore, we spotlight the potential of DNA droplet computing for cancer diagnostics through specific microRNA pattern recognition. We envision that DNA-based LLPS presents a versatile platform for the exploration of cellular mimicry and opens innovative ways for the development of functional synthetic cells.

Key words: DNA nanostructure, DNA computing, DNA droplets, water-in-oil emulsion

◀ *Significance* ▶

This review introduces the frontier of DNA-based liquid-liquid phase separation (LLPS), emphasizing its potential in constructing artificial cells with programmable functionalities. By harnessing the precision of base-specific interactions, fusion/fission of DNA droplets, pattern formation at a two-dimensional interface, and information processing can be achieved. The exploration of DNA LLPS systems provides a designable model for biological LLPS but also paves the way for advanced artificial cell-like structures.

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Introduction

Liquid-liquid phase separation (LLPS) phenomenon generates droplets of water-soluble molecules in aqueous solution. The LLPS are classified into two types: segregative- or associative [1]. The segregative LLPS is mainly driven by the immiscibility of the different components under given conditions. The aqueous two-phase system of polyethylene glycol (PEG) and Dextran mixtures is a typical example of the segregative LLPS. In associative LLPS (coacervation), the interaction between components induces the formation of condensates, leading to the formation of a molecular dense phase and a diluted phase.

A cell is a complex and sophisticated molecular system in which a wide variety of components are enclosed within a single microscopic space. Within the cell, biopolymer molecules, such as nucleic acids and proteins, can form droplet-like condensates via the associative LLPS. These droplets are called membraneless organelle and have roles to concentrate specific molecules to increase the efficiency of biochemical reactions [2]. The LLPS also contribute to gene expression in the cell nucleus, where heterochromatin and euchromatin form spatial structural patterns near the nuclear membrane [3]. The functions, physical properties, and principles of formation of the structures formed by LLPS have been the subject of numerous biophysical studies.

The LLPS exhibited by natural biopolymer molecules, especially proteins, is an essential research target to elucidate the basis of cellular functions and mechanisms. On the other hand, the phase separation exhibited by synthetic molecules is also an important research target from the viewpoint of artificially creating and understanding cells in a bottom-up manner. Although the LLPS of PEG/Dextran is often adopted in artificial cell studies, it is necessary to establish LLPS systems with more designable molecules to create artificial cells whose behavior can be programmed. In these viewpoints, DNA nanotechnology provides a promising approach for the bottom-up construction of artificial cell-like structures [4]. Due to the Watson-Crick base pairing, artificially synthesized sequence-designed DNAs hybridize with specific strand pairs. This feature is best used to construct designed-shaped nanostructures and molecular computing systems. In recent years, the programmability of DNA has been extended to the LLPS [5,6], attracting worldwide attention in artificial cell studies. The LLPS of DNA nanostructures was observed when branched DNA nanostructures, such as “Y-shape” and “X-shape,” were mixed in a solution. The self-assembly of the branched-DNA nanostructures into macroscale hydrogels was reported by Um et al. in 2006 [7]. Then, the phase separation of the DNA nanostructures into DNA-rich and DNA-poor phases was first reported by Biffi et al. in 2017 [8], and the formation of liquid-like droplets via the LLPS was reported by Nguyen et al. in 2017 [9]. Then, our research group demonstrated the programmability of the DNA-based LLPS system in 2020 [10]. In this review, we overview the LLPS of DNA nanostructures and examples of research contributing to the construction of artificial cells based on the DNA-based LLPS system. This review article is an extended version of the Japanese article [11].

Liquid–Liquid Phase Separation of DNA Nanostructures and Formation of DNA Droplets

LLPS of DNA nanostructures can be observed by changing the temperature of solutions containing DNA nanostructures with multiple interaction sites (sticky ends) [10]. Y-shaped DNA nanostructures (Y-motifs) in a buffer solution containing salt self-assembled into droplet-like condensates (DNA droplets) via the LLPS by decreasing the solution temperature (Fig. 1a). The DNA droplet exhibit coalescence when two of them collide with each other. The droplet formation process occurs during the transition from the "dispersed state" to the "droplet state" of the Y-motif. Further temperature decrease lead to the transition from droplets to hydrogels (Fig. 1b). It should be noted that the transition from the dispersed state to the droplet state occurs rapidly below the transition temperature, while the transition from the droplet state to the gel state is a gradual process with temperature changes. The DNA droplets are composed of only DNA nanostructures, but they can uptake DNA-tagged proteins. When streptavidin tagged with DNA whose sequence is complementary to the sticky end was mixed with the DNA droplets, the streptavidin accumulated in the DNA droplets. This accumulation depends on the DNA sequence; streptavidin tagged with non-complementary sequences is not accumulated. It shows the applicable potential of DNA droplets as artificial organelles that accumulate target molecules for efficient chemical reactions.

The advantage of the DNA-based LLPS system is its tunability, which was demonstrated by the changes in the droplet formation temperature [10] and the physical properties of the DNA droplets [12]. As for the droplet formation temperature, a shorter sticky end sequence, i.e., a less stable sequence, resulted in a lower droplet formation temperature (Fig. 1c). This result showed that the molecular design approach can offer the means for tuning the droplet formation temperature. The physical properties of the droplet, including viscosity and surface tension, were also varied with the sticky end sequence. In comparing the fusion dynamics of the droplets at the droplet formation temperature (Fig. 1d). it was suggested that the sticky end stability affected the viscosity but had more impact on the surface tension. It is proposed that the surface tension of the DNA droplets is dominated by the unbound sticky end at the droplet’s surface, which will generate a surface excess free energy. Indeed, the experiment performed at 22°C suggested the relationship between the expected free energy of the sticky ends and the amount of unbound sticky ends [13]. On the other hand, the experiment performed at

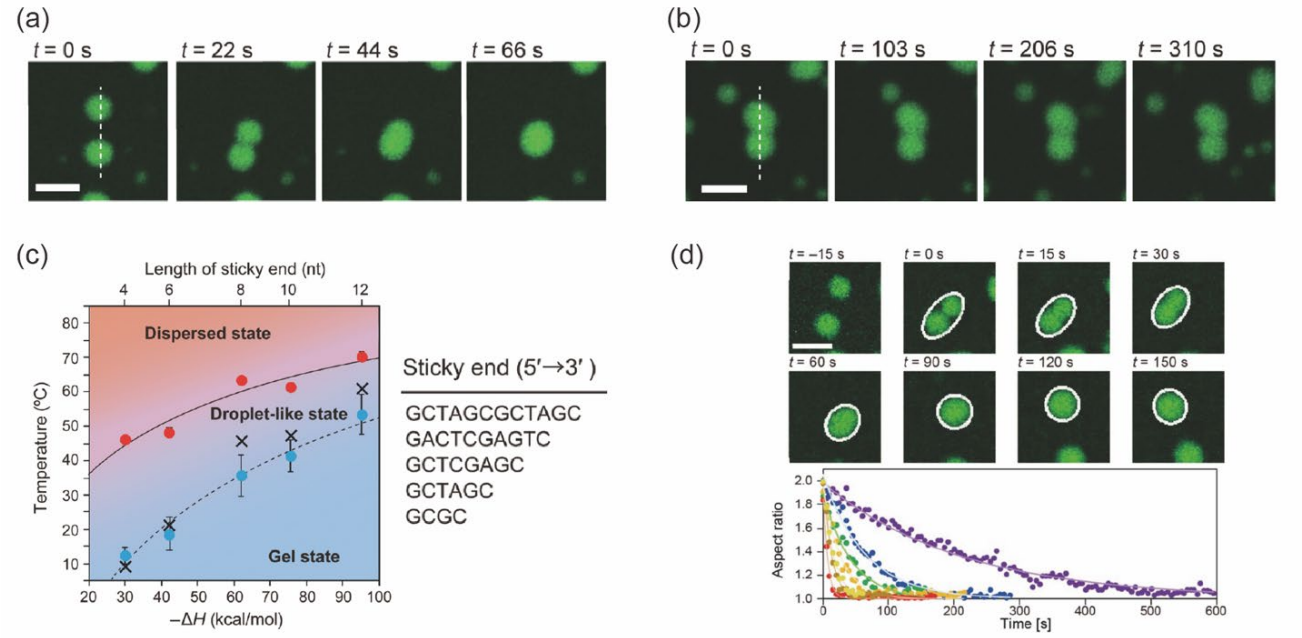

Figure 1 (a) and (b) Confocal microscope images of DNA droplets at droplet state (a) and hydrogel state (b). Scale bars: 10 μm. (c) State diagram representing state-change temperatures of Y-motifs with different sticky end sequences listed on the right-hand side. Red and blue dots represent mean values of dispersed-droplet and droplet-gel transition temperatures, respectively. (d) (Top) Sequential images of the fusion process for DNA droplets composed of the Y-motif with a sticky end "GCTCGAGC", obtained at 63 °C, droplet formation temperature. The white ellipses indicate the fitting results used to analyze the aspect ratio of the droplets undergoing fusion. Scale bar: 10 μm. (Bottom) Aspect ratios of the DNA droplets with different sticky end designs over time. All analyses were performed at each droplet formation temperature. (a)-(c) Reproduced under the terms of the Creative Commons CC BY-NC 4.0 license.[10] Copyright 2020, The Authors, published by American Association for the Advancement of Science. (d) Reproduced from Ref. [12] with permission from the Royal Society of Chemistry.

the droplet formation temperature, much higher than the melting temperature (T_m) of sticky end hybridization, did not show the correlation between the free energy and the surface tension. A deepening understanding of the hybridization dynamics under such unstable conditions, i.e., much higher than T_m, would explain how the sticky end interaction alters the physical properties.

Rational design of the sticky end sequences can achieve the selective fusion and fission of DNA droplets [10]. When two types of DNA nanostructures (Y-motif and orthogonal Y-motif: ^{orth}Y-motif), whose sequences are orthogonal (non-complementary), each nanostructure form DNA droplets that show selective and exclusive fusion (Fig. 2a). Namely, Y-motif forms the DNA droplets composed of only Y-motifs (Y-motif droplets), and ^{orth}Y-motif forms the DNA droplets composed of only ^{orth}Y-motif (^{orth}Y-motif droplets). The Y- and ^{orth}Y-motif droplets can fuse only with the same types of droplets but never show the fusion between Y- and ^{orth}Y-motif droplets. This immiscibility between the two different DNA droplets can be eliminated by adding cross-linker DNA nanostructures that can bind to both types of the motifs (Fig. 2b). The mixed DNA droplets can exhibit fission when the cross-linker DNA nanostructures are cleaved into two portions so that their cross-linking ability is lost (Fig. 2c). Thus, the LLPS of DNA nanostructures can control various dynamic behaviors, such as the immiscibility between the DNA droplets and the droplet fission, based on the base sequences and the nanostructure design.

The advantage of the DNA-based LLPS system is not limited to the sequence-dependent interaction. A nanostructure size, structural flexibility, and the number of interaction sites impact the DNA-based LLPS system. Growth rate of DNA droplets was found to be slower for smaller motifs and faster for larger motifs [14] (Fig. 3a). When single-stranded and double-stranded DNA were combined to create motifs to prepare different flexibility of interaction sites, the more flexible motifs were less likely to form the droplets by LLPS and vice versa [15]. In addition, by varying the number of interaction sites, a larger number of the interaction sites led to a higher droplet formation temperature [10] (Fig. 3b). These findings provide various design parameters of the DNA-based LLPS system in addition to the sequence design of DNA.

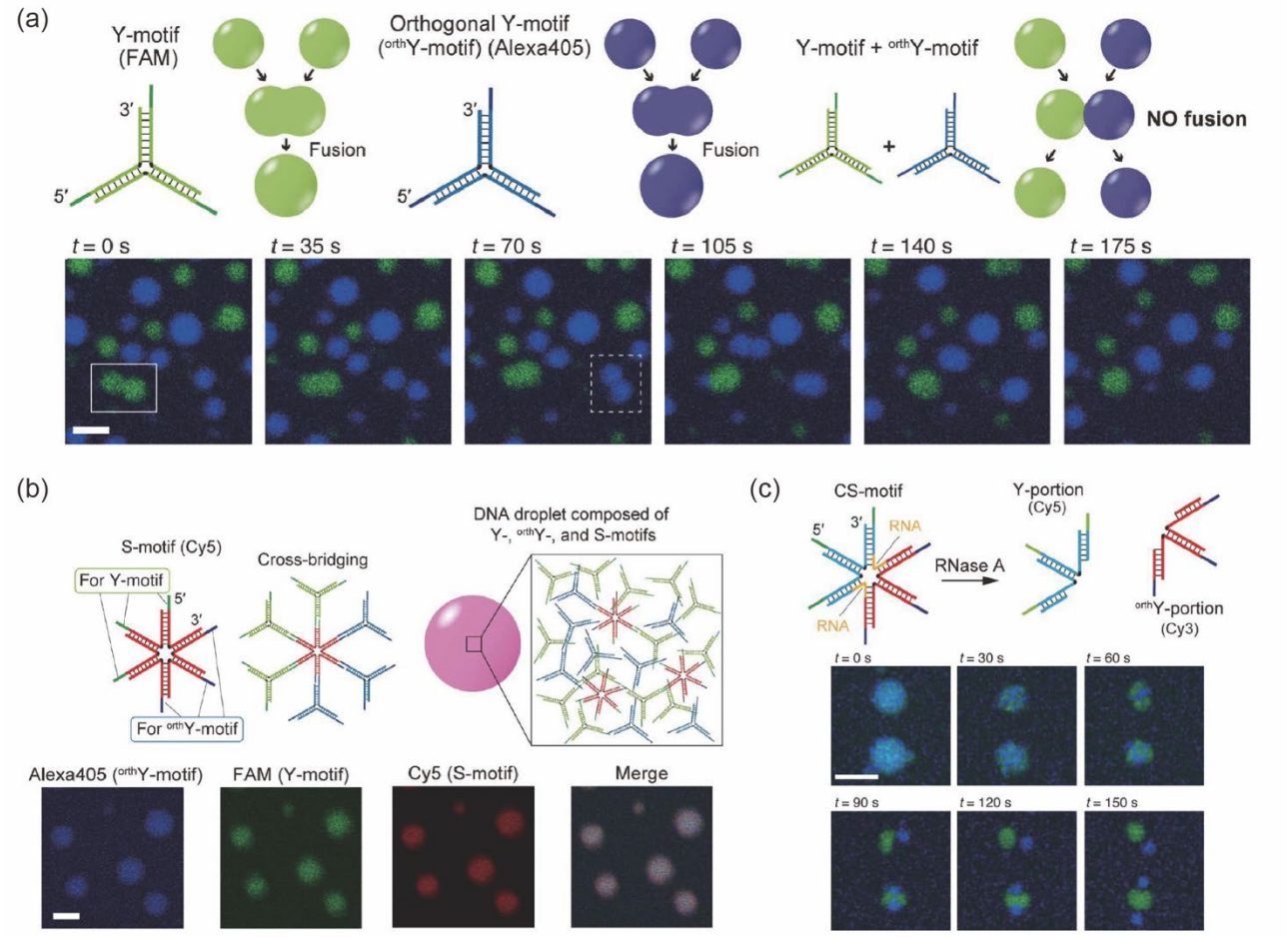

Figure 2 (a) Schematics of selective fusion in DNA droplets composed of Y-motif or orthogonal Y-motif droplets and its observation results with a confocal microscope. Scale bar: 10 μm. (b) Elimination of the immiscibility between the two different DNA droplets by adding cross-linker DNA nanostructures (Six-junction motif: S-motif). Scale bar: 10 μm. (c) DNA droplets' fission driven by the cleavage of DNA-RNA chimera S-motif (CS-motif). Scale bar: 20 μm. Reproduced under the terms of the Creative Commons CC BY-NC 4.0 license.[10] Copyright 2020, The Authors, published by American Association for the Advancement of Science.

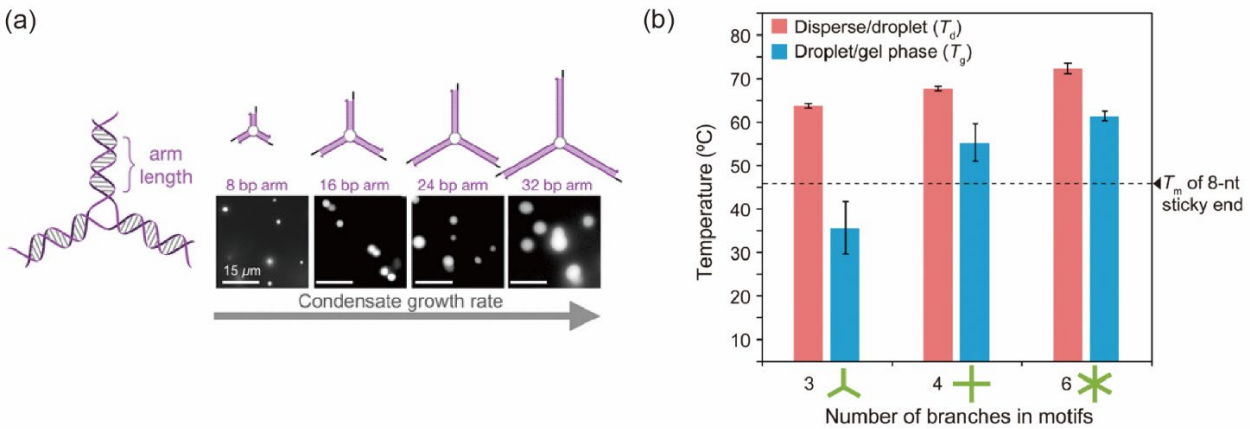

Figure 3 (a) The growth rate differences depending on the motif size. (b) Differences in the droplet formation temperature depending on the number of branches in motifs. (a) Reprinted with permission from Ref [14]. Copyright 2022 American Chemical Society. (b) Reproduced under the terms of the Creative Commons CC BY-NC 4.0 license.[10] Copyright 2020, The Authors, published by American Association for the Advancement of Science.

Phase Separation of DNA Nanostructures on a Two-dimensional Closed Interface

The phase separation of DNA nanostructures described above was observed in bulk solution. As we described in the Introduction, chromatin in the cell nucleus exhibits a spatial phase-separation pattern in the vicinity of the nuclear membrane. Several studies revealed that the LLPS of the DNA nanostructures also shows pattern formation on a two-dimensional closed interface.

Because DNA is a negatively charged polymer molecule, when cell-sized water-in-oil (W/O) droplets were prepared with positively charged amphiphilic molecules, DNA nanostructures were adsorbed on the water/oil interface. When the W/O droplet solution, whose aqueous phase contains the Y-motif, was heated and cooled gradually, the porous pattern was formed [16] (Fig. 4a). When the temperature change was faster, the porous pattern was not formed. The research revealed that there are two routes for the pattern formation: (1) simple viscoelastic phase separation (VPS) and (2) cluster-cluster aggregation after the VPS (interaction of coacervate particles). This phenomenon was explained by three factors: accumulation of the motifs at the interface, (2) concentration of the accumulated motifs, and (3) diffusion of the motifs. The detailed mechanism of the phenomena is described in the reference paper [16].

Although the porous pattern was observed in only the Y-motif solution in the cell-sized W/O droplets, more various patterns can be formed in the Y- and ^{orth}Y-motif combined system [17] (Fig. 4b). In the bulk solution, Y- and ^{orth}Y-motif individually form two types of DNA droplets. On the other hand, when the motifs are accumulated at the interface by electrostatic interaction, the two types of droplets did not form, but patterns of Y- and ^{orth}Y-motif-rich regions were formed (DNA phase-separated capsule). The pattern formation is thought to be due to that the immiscible behavior between the two types of DNA droplets was defined by the boundary of the oil-water interface. In bulk solution, the Y- and the ^{orth}Y-motif are not spatially defined and form droplets independently. However, by restricting the position of the motifs to the interface through electrostatic interaction, the phase separation changes to a lateral phase separation, as seen in lipid bilayer membranes, leading to the formation of patterns. Detailed investigation of the relationship between the space size and the pattern formed, as well as the pattern formation process, may lead to the construction and control of artificial cell nuclei.

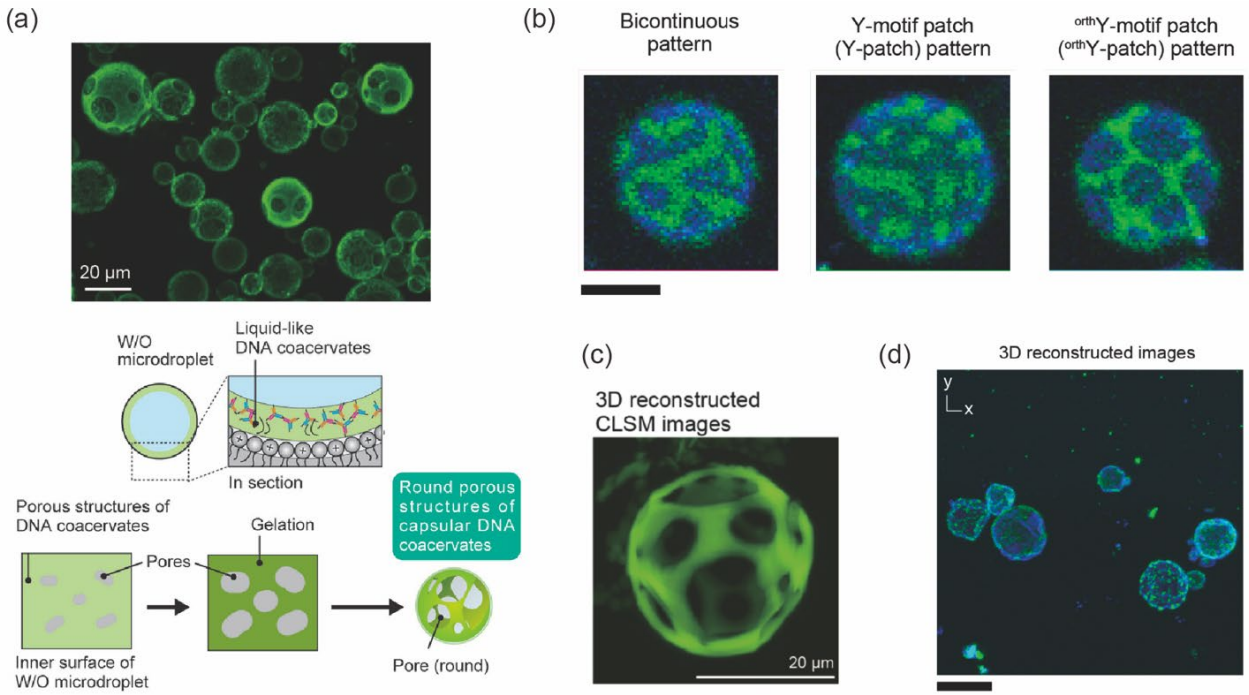

Figure 4 (a) Pattern formation of Y-motifs on the water-in-oil (W/O) interface caused by viscoelastic phase separation (top) and the proposed mechanism (bottom). (b) Pattern formation of Y- and orthogonal Y-motifs on the interface by the immiscible phase separation of the two motifs. Scale bar: 10 μm. (c) and (d) Extraction of gelated capsule-like structures from W/O droplets into aqueous solution. Scale bar in (d): 20 μm. (a) and (c) Reproduced under the terms of the Creative Commons CC BY-NC 4.0 license.[16]. (b) and (d) Reproduced under the terms of the Creative Commons CC BY-NC 4.0 license.[17] Copyright 2020, The Authors, published by American Association for the Advancement of Science.

An important achievement in the above works is that the formed structures on the W/O droplet interfaces can be extracted to aqueous solution [16,17] (Fig. 4c and 4d). Because the Y-motifs formed hydrogels at room temperature, the formed structures on the interface is not fluid but stable. Both works successfully demonstrated the extraction of the "capsule-like" DNA hydrogels with patterns formed by the VPS or immiscibility of two different Y-motifs. On the other hand, the extracted structures were not completely spherical shape. The mechanism is discussed to be that the hydrogel is distorted due to its softness and excess surface area when it is released from adsorption on the interface [18]. Since various functional DNA nanostructures for sensing or actuation were developed, the cell-sized DNA microcapsules would possibly be adopted as a compartment for artificial cells functionalized with the DNA devices.

"Intelligent" Droplets: microRNA Pattern Detection by DNA Droplets

One of the major differences between the DNA droplets and other polymer droplets is the ability to use the "information" encoded in the DNA base sequences, just as genetic information is encoded in the base sequence. In the field of DNA computing [18,19], the base-specific interaction is used to construct logic gates and information processing devices. Because the DNA droplets were assembled from the sequence-designed DNAs, DNA droplets could have the capability of information processing. The potential was demonstrated as a microRNA (miRNA) detection function [20]. Cancer cells show a particular miRNA expression pattern. By using the miRNA pattern as an input for computing, the detection of a specific combination of the four miRNAs, which are known as a set of cancer markers (Fig. 5). As an output of the detection, fission of the DNA droplets was used. In the reported DNA droplet computing system, three types of Y-motifs, each sequence is orthogonal, are connected by the two types of cross-linker DNA nanostructures. The cross-linkers can accept miRNAs and can split into two portions depending on the miRNA sequences, resulting in the fission of DNA droplets. As the demonstration, the expression patterns of four types of miRNAs (miRNA-1, miRNA-2, miRNA-3, miRNA-4) = (yes, yes, yes, no), specific to breast cancer and determines the possibility of breast cancer, was used as the input and the DNA droplets successfully exhibited the fission only when the specific patterns were given as the input signals. For details of the principle and achievements, please refer to Reference [20]. Although there are many points that need to be improved in this system, such as sensitivity to input, the successful demonstration would lead to constructing a molecular system that shows practical functions by the combination of DNA computing and the DNA-based LLPS indicates the possibility of constructing intelligent artificial cells and their applications.

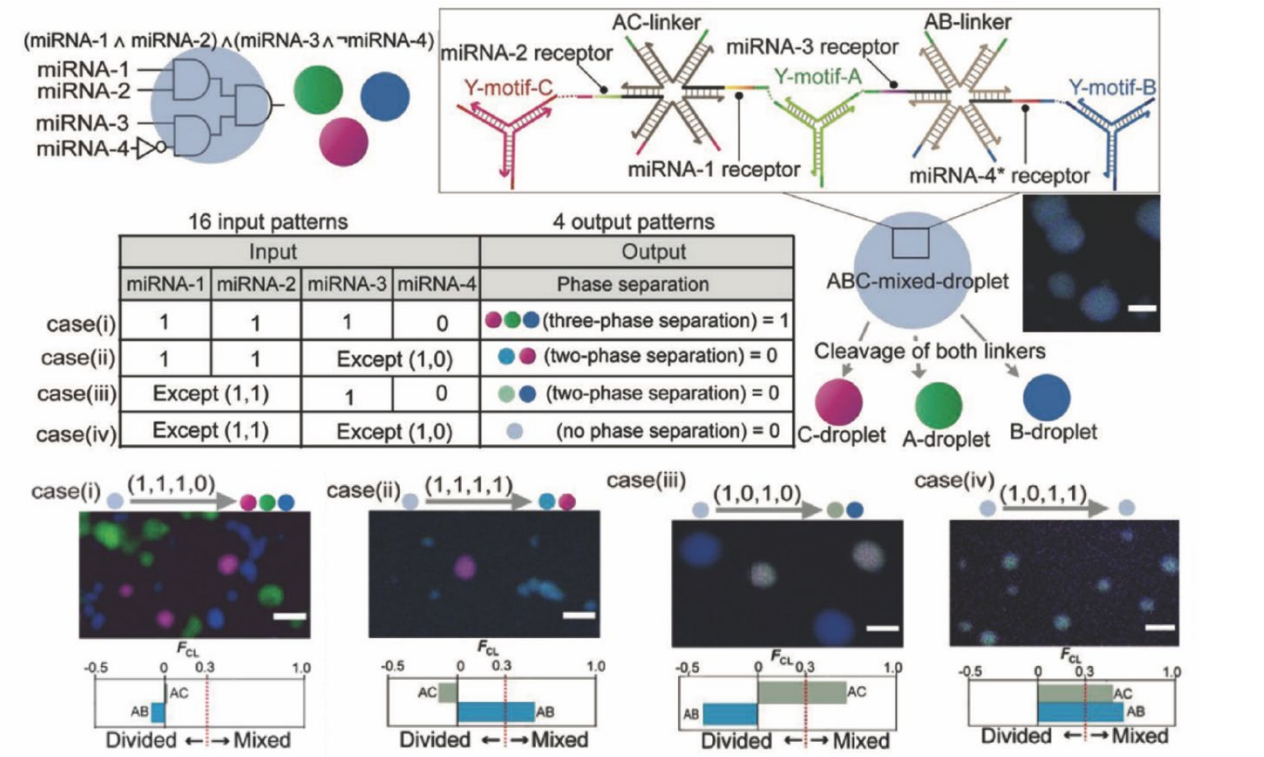

Figure 5 DNA droplet computing capable of recognizing microRNA (miRNA) sequence inputs. Expression of miRNA patterns on breast cancer, (miRNA-1 ∧ miRNA-2) ∧ (miRNA-3 ∧ ¬miRNA-4), was recognized and output was generated as fission into three different DNA droplets (three phase separation). Scale bars: 10 μm. Reproduced under the terms of the Creative Commons CC BY-NC 4.0 license [20].

Conclusion

As overviewed in this review, phase separation of DNA nanostructures can be designed based on molecular design. Fusion and fission of the DNA droplets can be well controlled by the base sequence designs. Patterns derived from phase separation are formed by regulating the spatial area of the phase separation to a two-dimensional closed space. By combining DNA computing techniques and the phase separation, the DNA droplets can have information processing function. These achievements are expected to contribute significantly to the technology for constructing artificial cells. On the other hand, the design technology of amino acid sequences has been rapidly growing in recent years [21]. Additionally, de novo protein design can be assisted with a neural network [22]. In the near future, the combination of the phase separation of designed proteins and DNA nanostructures would possibly emerge as a novel artificial LLPS system. Such a hybrid LLPS system will emerge as the new technique for the construction of functional artificial cells, such as programmable gene expression regulation based on phase separation.

Conflict of Interest

The authors declare no competing financial interests.

Author Contributions

Y.S. wrote the manuscript draft and prepared figures. Y.S. and M.T reviewed and edited the manuscript.

Data Availability

The all data used in this Review article are originated in the corresponding referred articles.

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