Active coacervate droplets as a model for membraneless organelles and a platform towards synthetic life

Carsten Donau,¹ Fabian Spaeth,¹ Marilyne Sosson,¹ Brigitte Kriebisch,¹ Fabian Schnitter,¹ Marta Tena-Solsona,¹,² Hyun-Seo Kang,¹,³ Elia Salibi,⁴ Michael Sattler,¹,³ Hannes Mutschler,⁴ Job Boekhoven¹,²,*

Affiliations

¹ Department of Chemistry, Technical University of Munich, Lichtenbergstrasse 4, 85748 Garching, Germany
² Institute for Advanced Study, Technical University of Munich, Lichtenbergstrasse 2a, 85748 Garching, Germany
³ Institute of Structural Biology, Helmholtz Zentrum München, Ingolstädter Landstrasse 1, 85764 Neuherberg
⁴ Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried

Abstract

Membraneless organelles like stress granules are active liquid-liquid phase-separated droplets that are involved in many intracellular processes. Their active and dynamic behavior is often regulated by ATP-dependent reactions. However, how exactly membraneless organelles control their dynamic composition remains poorly understood. Herein, we present a model for membraneless organelles based on RNA-containing active coacervate droplets regulated by a fuel-driven reaction cycle. These droplets emerge when fuel is present, but decay without. Moreover, we find these droplets can transiently up-concentrate functional RNA, and that this up-take is accelerated by the chemical reaction cycle. Finally, we show that in their pathway towards decay, these droplets self-divide asymmetrically. Self-division combined with emergence, decay, rapid exchange of building blocks, and functionality are all hallmarks of life, and we believe that our work could be a stepping stone towards its synthesis.
Introduction

Membraneless organelles, which include the nucleoli and P granules, rely on liquid-liquid phase separation in order to compartmentalize biochemical reactions. They typically comprise proteins and RNA held together by entropic and ion-pairing effects in a process called complex coacervation, resulting in an organelle with liquid-like properties. Recent work has demonstrated that some of these organelles are actively regulated by ATP-dependent chemical reaction cycles. These findings suggest that some of the membraneless organelles are active droplets, i.e., these droplets exist out of equilibrium and are regulated by chemical reactions. However, how the cell regulates phase separation of these droplets remains poorly understood.

Numerous synthetic complex coacervate-based droplets exist, which are used to investigate the characteristics of membraneless organelles. Examples of reversible complex coacervate droplets have been reported that form or dissolve upon a change in pH, temperature, salt or polymer concentration, or in response to an enzymatic reaction. These models provide valuable information on the formation or dissolution of droplets. However, these droplets evolve from one equilibrium state to another (e.g., from droplets to solution) in response to a change in their environment. An ideal model to study how dynamic assembly and disassembly affect droplet behavior would include the fuel-driven behavior as observed in biology. Such a model could reveal insights on how chemical reactions regulate droplet growth or how kinetics can regulate droplet composition. Moreover, chemically fueled active coacervate droplets have been proposed as a protocell model capable of self-division.

The field of fuel-driven self-assembly has recently gained traction. Here, the self-assembly of molecules is regulated by a chemical reaction cycle that comprises two reactions: a building block activation and deactivation reaction. In the activation reaction, a precursor molecule is activated for self-assembly by the irreversible consumption of a chemical fuel. In the deactivation reaction, the product is reverted to the precursor. Consequently, the self-assembling product is present for a finite amount of time, and the assembly’s properties are regulated by the kinetics of the reaction cycle. Such kinetic regulation is inspired by biology’s mode of controlling assemblies like the membraneless organelles. Examples of synthetic dissipative assemblies include fibers driven by photochemical or fuel-driven reaction cycles, vesicles driven by ATP or carbodiimides, and others.

In this work, we thus introduce a model for membraneless organelles based on complex coacervate droplets regulated by a fuel-driven chemical reaction cycle. We find that droplet behavior is regulated by the conversion of a chemical fuel. For example, these droplets form spontaneously and decay in the absence of fuel. Moreover, they take up components from their surroundings much faster compared to droplets in-equilibrium. Finally, we show that functional RNA, like ribozymes, can be transiently up-concentrated in these droplets.
Results and Discussion

The active droplets are based on the complexation of RNA and a peptide. The RNA component of the droplets is homo-polymeric RNA (poly-U, ±2,500 bases), which is frequently used in coacervate-based studies.\textsuperscript{14,19,37,38} The droplet dynamics are regulated by a peptide of which the RNA affinity is controlled by a chemical reaction cycle. The activation reaction converts a negatively charged aspartate into its corresponding anhydride driven by the hydrolysis of EDC (1-ethyl-3-(3-
dimethylaminopropyl)carbodiimide, fuel) to its urea (EDU, Figure 1A). In the deactivation reaction, the anhydride product spontaneously hydrolyzes to the precursor peptide. The result is a transient anhydride at the expense of EDC.

The design criteria for the peptide were that in the anhydride state (product), it can induce complex coacervation with poly-U (RNA), but not the aspartate state (precursor). We tested several peptide designs (Table S1), but the most successful sequence was: Ac-FRGRGRGD-OH (phenyl alanine (F), arginine (R), glycine (G) and aspartate (D), Figure 1A). In our design, three cationic arginines were required to form salt-bridges with the anionic RNA. We purposefully terminated the sequence with an aspartate. Thus, in the precursor state, the overall charge of the peptide was +1, but in the product state, it was +3. In that way, droplet formation via complex coacervation is expected for the product but not the precursor (Figure 1B).

**Transient formation of droplets.** We dissolved 23 mM of the peptide precursor in an aqueous 200 mM MES buffer solution at pH 5.3 with 4.1 mM RNA (expressed in [Uracil nucleotides]). Isothermal titration calorimetry (ITC) showed a weak interaction between the precursor and the RNA with a dissociation constant ($K_d$) of 2.9 mM (Figure S1). The precursor solution was optically clear (Figure 2A), pointing towards the absence of droplets.

In contrast, upon the addition of 25 mM EDC (fuel), the solution immediately turned turbid (Figure 2A) as a result of droplet formation (Figure 2B). The fuel-induced turbidity faded rapidly, and the solution was clear after 18 minutes. Control experiments confirmed that the transient droplet formation was a result of fuel, RNA, and peptide combined (Figure S2). In addition, droplets re-emerged after the addition of a second batch of fuel (Figure S3). A plate reader-study confirmed the rapid increase of turbidity after the addition of fuel to a maximum reached after 3 minutes (Figure 2C, black trace). After 18 minutes, the turbidity decayed to its original level.

The concentrations of the product and fuel were measured by HPLC, and the data was used to develop a kinetic model that predicts the concentration of fuel, precursor, and product throughout the cycle (Figure 2C, S4, and Table S2). The concentration of fuel rapidly decayed (Figure S4), leading to the transient presence of product (Figure 2C). The time at which the fuel was depleted coincided with the time at which we detected no more product. Therefore, fuel was present throughout the cycle and, thus, activation and deactivation were always operating simultaneously. We noted a strong correlation between HPLC data and the turbidity: at the highest product concentration, the turbidity peaked, and, when the product and fuel were exhausted, the samples returned to transparent. We refer to these droplets as “dynamic droplets” since their presence and absence seemed to be dynamically regulated by the kinetics of the reaction cycle.

The droplets behaved differently when more fuel (60 mM) was added (Figure 2D). While HPLC showed that the fuel and product were absent after 30 minutes (Figure S4), the turbidity of the solution persisted for 76 minutes. The latter observation was a strong indication that these droplets were in a kinetically arrested state; despite being
transient, the dynamics of disassembly were much slower than the kinetics of the reaction cycle allowing the droplets to persist for longer. We refer to these droplets as metastable droplets.

We performed the same experiments with various fuel concentrations to make a phase diagram (Figure 2E, S5). We found that more than 7.5 mM fuel was required to induce an increase in turbidity, which corresponds to 0.9 mM product. This result suggests a critical coacervation concentration of 0.9 mM anhydride product. Between 7.5 mM and 40 mM fuel, we found dynamic droplets. Above 40 mM of fuel, the droplets were metastable. We tested the effect of the RNA concentration on the droplet’s behavior (Figure 2F, S6-7). For any RNA concentration, no increased turbidity was observed below 7.5 mM fuel, indicating that the RNA concentration was not the limiting factor for

**Figure 2. Macroscopic analysis of the droplets.** A) Photographs of solutions of 23 mM precursor, 25 mM EDC (fuel), and 4.1 mM poly-U (RNA), before, 2 minutes after and 18 minutes after the addition of fuel. B) Bright field-micrograph of a solution described in A after 2 minutes. C-D) Absorbance of 600 nm of light as a measure of turbidity (left y-axis, black traces) and anhydride product concentration in the solution (right y-axis, red traces) as a function of time for the same condition as described in A with 25 mM fuel (C) or 60 mM fuel (D). Dynamic droplets show a strong correlation between the concentration of product and the turbidity. E) The maximum turbidity as a function of fuel for samples with 4.1 mM poly-U. The three regimes are shaded: no droplets (white), dynamic droplets (green) and metastable droplets (red). F) Behavior of the droplets as a function of RNA monomer concentration and amount of fuel. All error bars show the standard deviation from the average (N=3).
droplet formation. At lower RNA concentrations, less fuel was required to obtain the metastable droplets, e.g., 25 mM fuel or more for 1.4 mM RNA. We hypothesized that these metastable droplets can complex most RNA in the solution and that addition of more fuel results in more peptide product per RNA. The increased ratio of peptide to RNA results in an increased packing density compared to dynamic droplets, which may increase the energy barrier for disassembly. We thus measured the RNA concentration in the phase outside of the droplets. We used fluorescently labeled RNA to form dynamic droplets (25 mM fuel, 4.1 mM RNA), and, after 2 minutes, the droplet-containing solution was centrifuged to separate the two phases. Fluorescence spectroscopy showed that 10% of the initially added RNA remained in the supernatant (Figure S8). In contrast, no RNA was found in the supernatant when we did the same experiment with metastable droplets (60 mM fuel). The data from these experiments indicate that dynamic droplets leave behind some RNA in the outer phase, whereas metastable droplets capture all RNA.

To understand where the peptide activation and deactivation were taking place, we filtered the turbid solution to remove the droplets and measured the concentration of fuel and product of the filtrate by HPLC. This data, combined with the dataset without filtration, was used to calculate the respective concentrations inside and outside of the droplet phase. For both dynamic and metastable droplets, almost all fuel was present in the phase outside of the droplets at all times (Figure S9). This observation suggests that the activation of our reaction cycle predominantly occurs outside of the droplets in accordance with our design in Figure 1. After 2 minutes, roughly 1.1 mM product (47 % of the total product in solution) was inside the dynamic droplets, while about 1.9 mM (31 %) was present in metastable droplets (Figure S9). This result suggests that metastable droplets comprised more product than dynamic droplets. Considering that the amount of RNA in the droplet phase was almost similar in these experiments (vide supra), the ratio of product to RNA was significantly higher in metastable droplets. Specifically, the ratio of peptide cations to nucleic acid anions after 2 minutes was 1.4 for the metastable droplets compared to 0.9 for the dynamic droplets. The increased ratio for metastable droplets could explain their increased stability.

We further studied the emergence, evolution, and decay of the dynamic and metastable droplets by confocal microscopy using Cy3-tagged A15 hybridized with RNA (Figure 3A-B). In the first part of the cycle of dynamic droplets, the droplets were spherical and polydisperse (Figure 3A). Halfway through the cycle, vacuoles (holes) started to appear in the remaining droplets. Towards the end of the cycle, the droplets suddenly fragmented in smaller, not perfectly spherical droplets. These final fragmented droplets finally dissolved. We quantified the above described trend by calculating the droplet number, their size, and the RNA partitioning coefficient (Figure 3C-E, black traces). Within a minute after addition of 25 mM fuel, we observed a high number of droplets (Figure 3A and C). In line with the turbidity measurements, the droplet number decayed after 3 minutes. Towards the end of the cycle, the number of droplets suddenly increased (Figure 3C). The droplet volume immediately increased...
early in the cycle and started to decrease after 12 minutes (Figure 3D). We calculated a high RNA partitioning coefficient that rapidly decayed as time progressed (Figure 3E). The decay in the partitioning coefficient points to the fast release of RNA as droplets collapse.

When we performed the same experiments with the metastable droplets, we also found a high number of spherical, polydisperse droplets in the first minutes (Figure 3B). Again, the droplet number rapidly decreased (Figure 3C, red traces). However, droplets were still present even after complete fuel consumption (e.g., 60 minutes). After 20 minutes, the average volume of the metastable droplets remained constant (Figure 3D). The most obvious difference between dynamic and metastable droplets was in their RNA partitioning (Figure 3E). In dynamic droplets, the partitioning coefficient decreased as the cycle progressed while in metastable droplets, it did not change significantly throughout the cycle. We conclude that the RNA remained in

Figure 3. Microscopy analysis of dynamic and metastable droplets. A-B) Confocal micrographs of solutions of 23 mM precursor and 4.1 mM poly-U (Cy3-A15 hybridized) after the addition of 25 mM EDC (A) or 60 mM EDC (B). C-E) Statistical analysis of confocal micrographs of the solutions described in A and B for the number (C), volume (D), and the RNA partitioning (E) of the respective droplets after addition of fuel. All error bars show the standard deviation from the average (N=3). The lines are added to guide the eye.
metastable droplets, long after all product had hydrolyzed (e.g., 40 minutes). In other words, the peptide is able to hold together the droplet to some degree after it has been deactivated.

Confocal microscopy time-lapses with higher acquisition rates revealed further dynamic behavior in the dynamic droplets (Figure 4A, Movie S1). We could not observe the droplet nucleation, likely because it occurred rapidly after fuel addition (20 mM fuel). In the first minutes, we did observe a frequent fusion of the small droplets (Figure 4B), which is common for coacervate-based droplets.\(^{43}\) As time progressed, fusion became less apparent. After 12 minutes, we observed the first formation of vacuoles. In some cases, we found multiple vacuoles per droplet that grew or fused until they became the major component of the droplet (Figure 4C). Interestingly, as the vacuoles grew, the droplet itself also increased in size, likely because of water uptake (Movie S2). We hypothesize that vacuoles are a result of deactivation of the peptide and the consequent efflux with RNA. The efflux is hindered by a diffusion barrier formed by the remaining part of the droplet, which yields in the accumulation of RNA at the droplet periphery. Moreover, studies of membraneless organelles in vitro\(^ {17}\) and in vivo\(^ {44}\) showed the dynamic formation of similar vacuoles. Around 14 minutes after fuel addition, we observed the first dissolution events of droplets (Figure 4D-E). The droplet lost its roundness, and protrusions formed. Excitingly, these protrusions pinched off and formed their own daughter-droplets (Figure 4E, Movie S3, and Movie S4). In some cases, these daughter droplets drifted off by Brownian motion and could survive for tens of seconds before finally dissolving. The daughter droplet formation would be a form of asymmetric self-division, which occurs as the mother droplet is losing integrity from the loss of anhydride product, which we will further study in future work.

From these combined experiments, we conclude that dynamic droplets almost immediately nucleate and take up RNA rapidly in response to fuel. Then, their average volume grows, while their number decreases due to fusion events. When product deactivation is faster than peptide activation, the product concentration starts decreasing, and the droplets release RNA, as can be concluded from the decline in the partitioning coefficient. In some cases, this release of RNA is hindered by the droplet itself and results in vacuole formation, i.e., areas with low RNA surrounded by a diffusion barrier with high RNA concentration. Towards the last minutes of the cycle, the droplets rapidly collapsed resulting in fragmentation and further release of the RNA. The dynamic uptake and release of RNA show similarities with some membraneless organelles. For example, stress-granules are organelles of proteins and mRNA that form when a cell is under stress.\(^ {45}\) These granules also rapidly exchange RNA with the cytosol, and this process has been demonstrated to be accelerated by the hydrolysis of ATP. Similarly, nucleoli, another type of membraneless organelles, have been shown to be up to ten times more dynamic under ATP-dissipating conditions.\(^ {8}\)
Inspired by the rapid dynamics of membraneless organelles, we tested the uptake of RNA by our dynamic droplets with Cy3-tagged poly-U (red RNA). We added fuel,
waited 2 minutes for red droplets to form, and spiked the reaction with a batch of Cy5-tagged poly-U (green RNA). After another 2 minutes, we observed that all droplets that contained red RNA also had taken up green RNA, even though some droplets had clearly taken up more than others (Figure 5A, S10). There were also droplets that contained exclusively green RNA. We explain the green droplets by the presence of excess fuel and precursor peptides that was still present outside of the droplets, which can complex the newly introduced green RNA. After 10 minutes, we found that most droplets had taken up large amounts of green RNA and were homogeneously mixed, resulting in yellow-orange droplets.

As a control, we carried out a similar experiment on droplets that were not fuel-driven. We acidified the precursor and (red) RNA solution to pH 3, thus protonating the aspartic acid carboxylates and neutralizing the negative charges, which resulted in immediate droplet formation. We added green RNA to these droplets and found minor uptake after 10 minutes (Figure 4B and S10). Finally, we conducted an identical experiment with metastable droplets (60 mM fuel). Here, we also found the formation of new green droplets after 2 minutes (Figure S10). We also found that all red droplets had taken up some green RNA. However, after 10 minutes, the droplet population remained a heterogeneous mixture of red, green and some yellow droplets.

Two pathways result in uptake of the RNA among the droplet population: fusion of red droplets with green droplets, or exchange of the RNA between the droplets and the phase outside of the droplets. Although droplet fusion was observed in all three experiments, it seems unlikely that all droplets have fused resulting in the yellow-orange droplets (Figure 5A). Moreover, the controls (Figure S10B-C) demonstrate that fusion alone cannot explain the rapid homogenization of dynamic droplets. Therefore, we conclude that the fluxes of the peptide in and out of the droplets combined with the droplets' fuel-driven nature result in the rapid uptake of RNA by the dynamic droplets.

Complex coacervate droplets containing RNA have frequently been proposed as a potential protocell that emerged at the origin of life.\textsuperscript{12,46} To demonstrate that our dynamic droplets can indeed up-concentrate functional RNA during their limited lifespan, we investigated the evolution of partitioning of functional RNA sequences into the dynamic droplets (Figure 5C-E). We used a ligating ribozyme, a cleaving ribozyme, and a fluorophore-binding aptamer (SunY,\textsuperscript{47} Hammerhead\textsuperscript{12} and Broccoli,\textsuperscript{48} respectively). We added fuel to the solutions containing the precursor, poly-U, and one of the functional RNA sequences, and imaged the droplets by confocal microscopy. Five minutes after the addition of fuel (25 mM), we observed that all three functional RNA sequences had partitioning coefficients larger than ten. (Figure 5D, Table S3). In line with other reports, the partitioning seemed to be size-dependent; i.e., the longest sequence had the highest partitioning coefficient (SunY, 187 nucleotides, $K = 37$), while the shortest strand had the lowest (Hammerhead, 44 nucleotides, $K = 13$). As the cycle was progressing, an increasing amount of functional RNA was observed.
outside of the droplets until the droplets dissolved (Figure 5E). Taken together, upon emergence, our droplets partition the functional RNA, and release it upon decay, resulting in the transient up-concentration of functional RNA driven by a chemical reaction cycle. In future work, the combination with self-replicating RNA strands could result in the up-concentration and activation of catalytic RNA sequences whose activity helps to sustain the droplets.

**Conclusions**

We made complex coacervation a fuel-driven process. RNA-containing droplets emerge in response to fuel and decay when the fuel is depleted. We envision that our
droplets could serve as a powerful model for membraneless organelles, which are also regulated by chemical reactions. Indeed, we show the active nature of these droplets accelerates the rate of RNA uptake from their surrounding liquid. We also believe these droplets can serve as a great model for protocells, and we show that they transiently partition functional RNA. More excitingly, we show the first signs of spontaneous asymmetric division towards the end of a coacervate droplet cycle. Emergence, decay, rapid exchange of building blocks, functionality, and self-division are all hallmarks of life. In future work, we work to combine these properties in our droplets with the aim to set steps towards the synthesis of life.

Acknowledgments

This project was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Project-16 364653263 – TRR 235. J.B. is grateful for funding by the Technical University of Munich – Institute for Advanced Study, funded by the German Excellence Initiative and the European Union Seventh Framework Programme under grant agreement n° 291763.

Keywords: dissipative self-assembly, membraneless organelles, chemically fueled self-assembly, active droplets

Author Contributions

J.B. and C.D. conceived the research and wrote the manuscript. J.B., C.D., M.T.S., Mic.S., and H.M. designed the experiments. C.D., F.S., Mar.S., B.K., F.S., M.T.S., H-S.K., E.S., and J.B. performed experiments and analyzed the data.

Conflict of interest

The authors declare no competing interests.

Methods section

Materials. The peptides were synthesized in house. For the RNA, we used polyuridylic acid potassium salt from Sigma Aldrich (P9528). Other chemicals were mostly ordered from Sigma Aldrich and used without purification. See SI for more details.

Peptide synthesis and purification. Synthesis was performed on a CEM Liberty microwave-assisted peptide synthesizer and purified using preparative HPLC. See SI for more details.

General sample preparation. For most experiments, we used these standard conditions: 23 mM Ac-FRGRGGRG-OH (tri-trifluoroacetic acid salt), 4.1 mM poly-U (uridine units), 200 mM MES, pH 5.3, 25 mM EDC (in case of dynamic droplets) or 60 mM EDC (metastable).

Stock solutions of the precursor (100 mM, tri-trifluoroacetic acid salt), MES buffer (500-1000 mM), poly-U (800 kDA, ~2-15 µg/µL) and EDC (1-2 M) were prepared in nuclease-free water. The pH of the peptide and MES stock were adjusted to 5.3. Typically, stock solutions of EDC and poly-U as well as the resulting peptide-poly-U
solution in MES were prepared freshly for each experiment. Reaction networks were started by addition of EDC to the peptide-poly-U solution in MES.

Further description of the used methods is given in the SI.

References


