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Research Article
doi.org/10.1002/syst.202400013

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Unveiling the Liquid-Liquid Phase Separation of Benzene-1,3,5-Tricarboxamide in Water

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Dedicated to Prof. E. W. (Bert) Meijer to honor his seminal contributions to the field of supramolecular polymers. Shikha Dhiman has been nominated for the special collection Systems Chemistry Talents by Board Member Subhabrata Maiti

The intricate interplay between self-assembly and phase separation orchestrates biomolecular organization inside cells, thereby dictating the formation of vital structures such as protein assemblies and membraneless organelles (MLOs). However, in the context of supramolecular polymerization, these fundamental processes have traditionally been studied separately. This study reevaluates the supramolecular polymerization process to unveil the presence of phase-separated droplet state. Utilizing the well-studied benzene-1,3,5-tricarboxamide (BTA) supramolecular motif, we explore its thermally driven liquid-liquid phase separation (LLPS). Thermodynamic and kinetic analysis, employing temperature-dependent spectroscopic and microscopic techniques, elucidates the distinct BTA states and their evolution towards the thermodynamic fiber state. This research sheds light on the existence of hidden phases of supramolecular monomers, emphasizing the delicate balance of non-covalent interactions among monomers and with solvents in governing self-assembly vs. phase separation. This is particularly important in comprehending phase separation in the biological realm such as in MLOs, and for applications such as condensate-modifying therapeutics.

Introduction

Supramolecular chemistry finds its conceptual roots in the intricacies of biological systems, where the orchestration of structural and functional attributes relies on non-covalent interactions.[1] These interactions, which govern many dynamic cellular processes such as receptor-ligand binding, protein assembly, enzyme reactions, cell motility, and division, also serve as the pillars of supramolecular polymerization.[2] Supramolecular polymers are dynamic assemblies of monomers connected via non-covalent intermolecular interactions, possessing versatile mechanical, biological, and optoelectronic properties.[3] Akin to protein assemblies, supramolecular polymers may exhibit a complex energy landscape, owing to the diverse possibilities of non-covalent bonds and arrangements between monomers, endowing them to access different structural and functional states.[4,5,6] Recent studies revealed that intrinsically disordered proteins (IDPs) such as FUS, α-Synuclein, and tau, undergo liquid-liquid phase separation (LLPS), en route to their self-assembly.[7,8] This metastable phase-separated state serves as a precursor, facilitating the nucleation process.[9] These liquid droplets, also known as biocondensates,[10] have been associated with pathogenic processes such as plaque formation in detrimental neurodegenerative diseases like Alzheimer's,[11] but they also contribute to beneficial applications such as the formation of spider silk, known for its exceptional tensile strength.[12]

Phase separation is well-studied in large polymers alone or with small multivalent molecules,[13] however, in contrast, the phenomenon of phase separation preceding self-assembly in small molecules has remained largely elusive until recent times.[9] A limited number of small molecules have now been observed to undergo phase separation prior to their supramolecular polymerization.[9,14] This observation prompts inquiry into whether only specific monomers undergo phase separation or if collective intermolecular non-covalent interactions among monomers bypass the metastable phase-separated state. Alternatively, rapid nucleation processes and the absence of suitable probes to investigate the supramolecular polymerization with high spatiotemporal resolution may have hindered the detection of such phase-separated states. Consequently, there is a critical need to revisit the supramolecular polymerization processes, aiming to identify and understand the existence and role of phase separation. This is pertinent, given the growing interest in comprehending phase separation in the biological realm such as in membraneless organelles (MLOs), and for its applications in developing compartments for artificial cells,[15,16,17] drug/RNA delivery systems,[18,19,20] and exploring condensate-modifying therapeutics.[21,22] Moreover, achieving a delicate balance between non-covalent interactions and solubility is imperative for controlling the formation of

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/syst.202400013

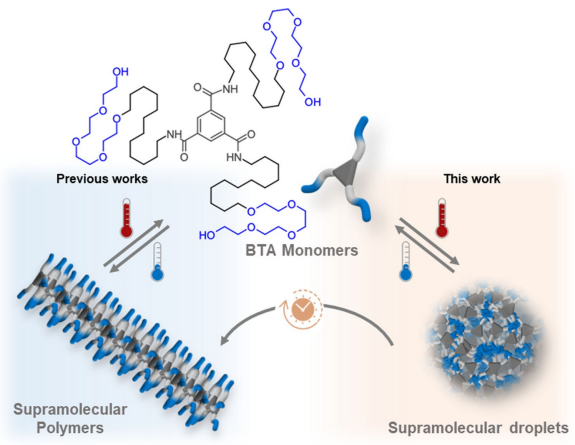
liquid droplets, and avoiding their irreversible transition to supramolecular polymers.

Based on this conceptual framework, benzene-1,3,5-tricarboxamide (BTA) is the selected supramolecular motif of interest. BTAs, a unique monomer class, have undergone extensive study in the past decade, notably by Meijer, Palmans, and co-workers.[2,24] A variety of derivatives have been studied to understand the fundamental aspects of supramolecular polymers, including chirality,[25] defects,[26,27] dynamics,[28] and their applications in supramolecular (bio)materials.[29] A recent study revealed the emergence of a droplet phase in BTA derivative when surfactant cetrimonium bromide (CTAB) was introduced and the solution was subsequently diluted.[30] At high concentrations, CTAB interfered with BTA self-assembly, and on dilution, CTAB was ejected leading to droplet formation en route to self-assembly. This raises the question of whether the existence of the droplet phase remained unnoticed due to the aforementioned reasons or if it is a surfactant-induced property.

Hence, in the present study, we demonstrate the existence of a droplet state within the well-studied BTA system. Our investigation comprises thermodynamic and kinetic analysis, revealing a distinctive phase segregation process at lower critical solution temperature (LCST) giving rise to metastable droplets at elevated temperatures (Scheme 1). Remarkably, this droplet phase undergoes a subsequent transition from a liquid to a fiber state through a cooperative supramolecular polymerization process. This study emphasizes the significance of reevaluating the mechanisms underlying supramolecular polymerization to comprehend the role of phase separation in the overall process.

Results and Discussion

BTA with three amphiphilic tails consisting of dodecyl and tetra(ethylene glycol) (TEG) was synthesized following the previously reported protocol (Scheme 1).[25] This BTA monomer undergoes supramolecular polymerization into micrometer-long fibers in aqueous environments, facilitated by the benzene core engaging in π-π stacking, three amide groups forming triple hydrogen bonds, and three amphiphilic tails containing water-shielding dodecyl hydrophobic spacers followed by water solubilizing TEG. Generally, TEG in BTA and other such monomers are acknowledged for conferring water solubility and are part of the amphiphilic design.[31,32] While poly(ethylene glycol) (PEG) has been extensively studied for its LCST behavior, it is noteworthy that shorter oligoethylene glycols also manifest LCST characteristics.[33,34,35] Consequently, the incorporation of TEG into BTA may confer a thermoresponsive LLPS phenomenon, possibly culminating in the formation of phase-separated liquid droplets. Preliminary evidence of this LCST phenomenon was detected in an early BTA report almost a decade ago, however, comprehensive investigation to unravel any supramolecular droplet formation was not pursued.[25]



Scheme 1. Chemical structure of BTA and schematic illustration of BTA states: monomer, supramolecular polymers, and phase separated supramolecular droplets.

To investigate LLPS behaviour, a solution containing 40 μM BTA in Milli-Q (MQ) water was heated to 85 °C for 15 minutes under continuous stirring, followed by cooling to ambient temperature and equilibration overnight. The UV-Vis absorption spectrum displayed the characteristic absorption peaks at ~210 and ~225 nm, indicative of the formation of supramolecular polymers of BTA through π-π-stacking, triple hydrogen bonding, and hydrophobic interactions, consistent with previous reports (Figure S1).[25] Next, the sample was subjected to controlled heating with a slow temperature ramp of 1 °C/min, while monitoring absorbance at 225 nm to observe change in BTA organization (Figure 1a, S2).[25] Up to 55 ± 1 °C, negligible changes in absorption were observed, demonstrating the stability of the supramolecular polymers. However, between 55–69 ± 1 °C, a gradual decrease in absorbance occurred, indicating a disassembly process. A plateau was then evident up to 80 ± 1 °C, followed by a gradual increase in absorbance. Simultaneously, the solution was observed to transition from transparent to translucent, suggesting the attainment of cloud point temperature (T_{cp}) and the onset of LLPS.

To corroborate this, absorption spectra were taken at ascending temperatures at 1 °C/min. In between 20 °C to 60 °C, the spectra depicted negligible changes in shape and intensity, after which at 60 °C, a pivotal transition is observed (Figure 1b, c). Here, a new state emerges displaying substantially reduced absorption at the peak of 225 nm and 210 nm. Concomitantly, absorption at 200 nm exhibited enhancement (Figure 1b). From this, it can be inferred that there is a shift from the polymeric state to a molecularly dissolved state similar to that observed in good solvent methanol (Figure S3). Next, from 70 °C to 80 °C the new state seems equilibrated since no differences in absorption occur, however, at 85 °C a second transition emerged characterized by increasing absorption at all wavelengths, indicating the evolution of the system into a new state. Since the absorption at 400 nm, far away from any molecular absorption band, is rising, it can be deduced that scattering effects manifest. Hence, the LCST has been reached and phase separation of BTA occurs. To corroborate this, temperature-dependent emission spectra using the polarity-sensitive Nile red (NR) dye were performed (Figure 1c, S5) When NR is in a hydrophobic environment of BTA supramolecular

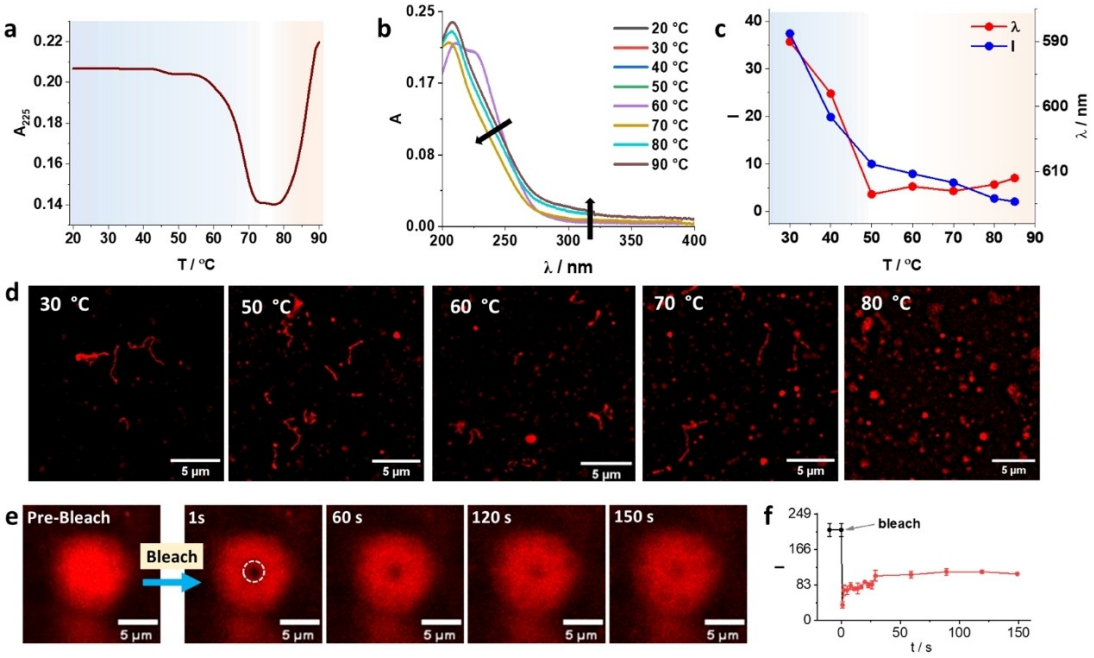


Figure 1. a. Temperature-dependent changes in absorbance at 225 nm during the heating cycle with a temperature ramp of 1 °C/min. **b.** Temperature-dependent absorption spectra were observed during the heating cycle. **c.** Change in fluorescence intensity and emission maximum with temperature of BTA with NR. **d.** SIM images at increasing temperatures. **e.** CLSM images obtained during FRAP of a supramolecular droplet. **f.** Corresponding FRAP kinetics of supramolecular droplet. [BTA] = 40 μM.

polymer, its emission is blue-shifted. On increasing temperature, due to depolymerization NR is removed from the hydrophobic environment of BTA supramolecular polymer to water, as a result, a red shift in its emission is observed. At 85 °C, there is a slight blue shift in its emission, which is absent in control (i.e. only water), which may be due to NR experiencing a new, comparatively hydrophobic environment in the condensed liquid droplet phase of BTA. These spectroscopic studies indicate the existence of three states of BTA (Figure S5). To ascertain whether the LLPS induces macro phase separation or microphase separated droplets, *in situ* temperature-variable Structured illumination microscopy (SIM) was conducted. SIM was employed as an imaging modality since this technique yields an enhanced resolution of *ca.* 100 nm as compared to conventional fluorescence microscopy whilst no manipulations to the sample preparation are required.[32]

To visualize BTA under SIM, samples were prepared with 5 mol% of fluorescent dye labeled BTA using a modified protocol (See SI).[28] The sample was added to the VAHEAT microscopy chamber and the VAHEAT setup was used for an *in situ* precise temperature control. At 30 °C (lower limit restricted by the instrument) the anticipated supramolecular fibers were observed which remained present until 60 °C (Figure 1d, S7–12). Although spectroscopic analysis revealed that depolymerization is initiated at 60 °C, due to the high polydispersity of BTA fibers any size reduction was challenging to discern from SIM images (Figure 1d, S10). At 80 °C, a significant morphological change occurred, with numerous circular structures populating the surface and the complete disappearance of fibers. This is indicative of disassembly of BTA fibers and subsequent formation of BTA droplet beyond T_{cp}. To probe the liquid nature of these droplets, Fluorescence Recovery After Photobleaching (FRAP) was performed (Figure 1e, f). A fast initially recovery within 25 seconds after photobleaching in 150 seconds was observed (See SI for protocol). Complete recovery was hindered by the rapid photobleaching of the Cy5 dye (Figure S13) and its instability at higher temperature (sample preparation requires 15 minutes heating at 85 °C). Moreover, when the sample is sample is observed, the droplets have very high mobility (Supporting Video 1) due to which it takes at least 5 minutes before the sample can be analysed. Furthermore, FRAP was conducted on a confocal microscope where in situ temperature control was not possible. Consequently, during FRAP, temperature decrease over time resulted in the gradual gelation of these droplets (vide infra, Figure 2, S14). We also observed the droplets wetting the surface of the well-plate (Supporting Video 2) depicting the liquid-like nature of these droplets. Nevertheless, this observation confirms the formation of BTA droplets at elevated temperatures facilitated by the LCST of TEG chains along with intra and intermolecular non-covalent interactions.

To elucidate the energy landscape of BTA self-assembly,[37,38] a heat-cool cycle for 40 uM BTA in water was conducted at a temperature ramp of 1 °C/min while monitoring three distinct wavelengths. At 200 nm, the monomer was tracked, 225 nm depicted the polymeric state of BTA, and 400 nm measured sample turbidity and LLPS (Figure 2a). A consistent observation across all three wavelengths revealed a broad hysteresis for both LLPS and supramolecular polymerization processes. The LLPS onset during the heating cycle was observed at 80 ± 1 °C,

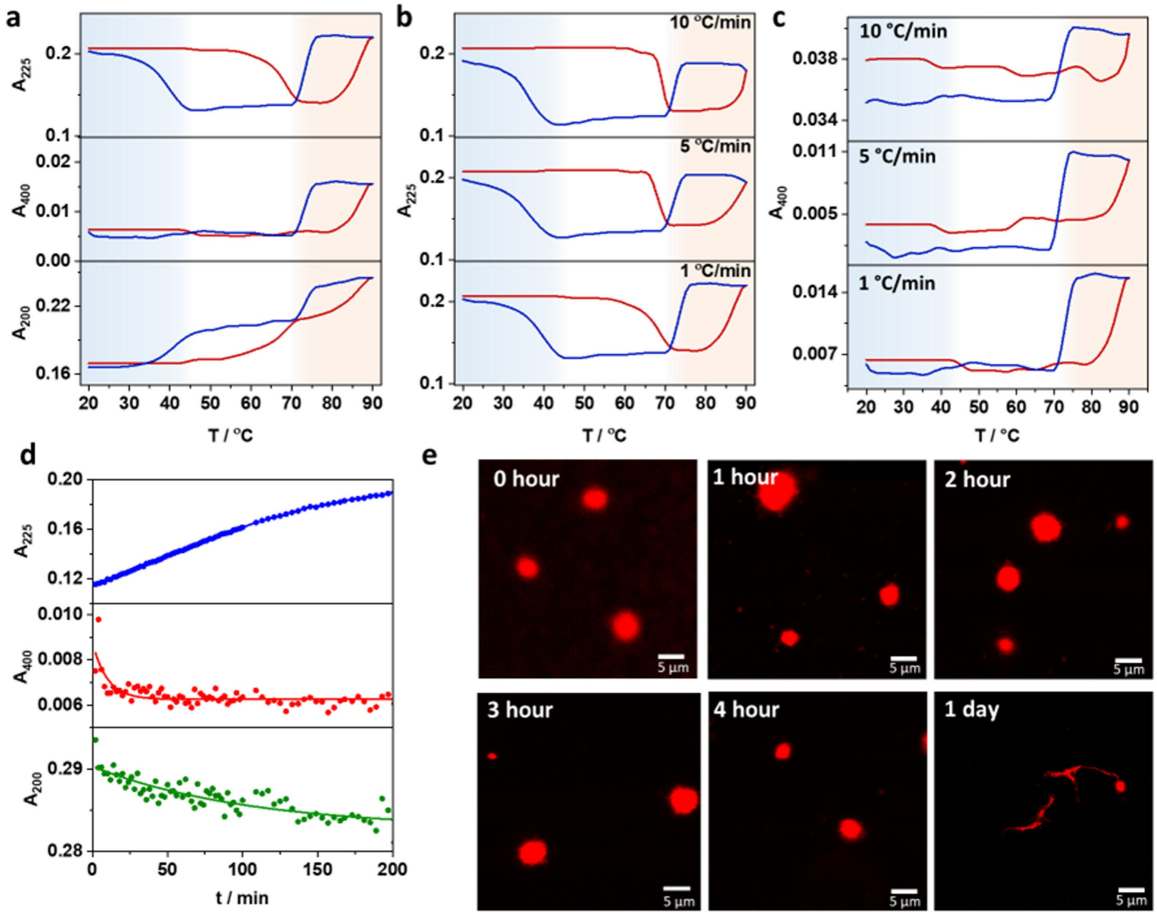


Figure 2. a. Temperature-dependent changes in absorbance at 225, 200, and 400 nm during the heating (red) and cooling (blue) cycle with a temperature ramp of 1 °C/min. **b.** Temperature-dependent change in absorbance at 225 nm during heating (red) and cooling (blue) cycles with different temperature ramps. **c.** Time-dependent absorbance changes after quenching of sample from 90 to 20 °C. **d.** Temperature-dependent absorbance changes at 400 nm during heating (red) and cooling (blue) cycles with different temperature ramps. **e.** CLSM at different time intervals after quenching of the sample. [BTA] = 40 μM.

while the LLPS disappearance during the cooling cycle finished at 70 ± 1 °C, particularly evident at 400 nm. The observed hysteresis in the heat-cool cycle can be attributed to BTA engaging in intra and intermolecular hydrogen bonding in the droplet phase.[39] Consequently, the transition from droplet to monomer during the cooling cycle proceeds through a slower rehydration process, leading to hysteresis. This hysteresis is indicative of a kinetically controlled process.[40,41] The supramolecular polymerization on cooling follows a cooperative growth as confirmed by fitting to the nucleation-elongation model.[42] Remarkably, the onset of supramolecular depolymerization during the heating cycle was observed at 59 ± 1 °C and finished *ca.* 73 ± 1 °C, while supramolecular polymerization during the cooling cycle was initiated at 45 ± 1 °C and wasn't completed by 20 °C. This broad hysteresis in supramolecular polymerization, indicates pathway complexity, emphasizing that BTA is not in equilibrium even during the slow cooling rate of 1 °C/min (Figure 2a). Additionally, a discernible difference in absorption spectra between pre- and post-heating further supports the contention that the samples have not reached thermodynamic equilibrium (Figure S16). Consequently, it is recommended to slow down the temperature ramping to better probe thermodynamically controlled processes. However, due to instrumental limitations, this was not feasible in the current study. To delve deeper into pathway complexity, we augmented the rate of heating and cooling from 1 °C/min to 5 and 10 °C/min. An increase in the heating and cooling rates led to a reduction in the extent of the LLPS, possibly attributable to the diminished time available to achieve equilibria (Figure 2b,c). Consequently, broader hysteresis and sharper transitions were observed in both the phase separation and supramolecular polymerization processes. The comparison of melting temperature (T_m, temperature at 50% depolymerization), revealed a gradual increase with an accelerated temperature ramp, while the elongation temperature (T_e, temperature where elongation started, Figure S17–19) demonstrated a corresponding decline. This trend signifies that a higher temperature ramp causes the system to be under kinetic control. Importantly, at the conclusion of the heating-cooling cycle, samples subjected to a faster rate did not reach the equilibrium state as observed by the difference in absorption at 20 °C between the heating and cooling cycles, as expected (Figure 2b, c).

In an attempt to capture the kinetic state at a lower temperature, an even faster cooling rate was employed, where-

by a sample heated to 85 °C for 15 minutes was rapidly quenched to 20 °C (average temperature ramp of 70 °C/min), and time-dependent spectra were recorded (Figure S20). The absorption spectrum at the initial time point (t0) revealed the presence of multiple small absorption bands absent at elevated temperatures perhaps interfered by scattering due to phase separation (Figure S21). These bands indicate a coexistence of diverse states within the sample. Over time, a gradual transition towards a supramolecular polymer was observed as depicted by the evolution of distinct bands at 210 and 220 nm (Figure S20, 2d). However, in comparison to the sample cooled at a slower rate of 10 °C/min, equilibrium had not yet been reached even after 3 hours. At a higher concentration of 100 μM BTA, a temperature ramp of 10 °C/min resulted in a complete recovery, however through this quenching kinetically trapped state was observed (Figure S22–23). Confocal microscopy revealed that droplets at 40 μM BTA were kinetically trapped on rapid quenching, and these spherical structures were still observed even after 4 hours at 20 °C (Figure 2e, S26–30). However, BTA in these droplets at 20 °C are unstable and undergo stacking to form supramolecular polymers. Due to the condensed nature of BTA in these droplets, BTA doesn't form single fibers but a metastable gel state.[30] However, over time monomer exchange dynamics between dense and dispersed phase allow BTA monomers to come out of these spherical gels to form thermodynamically stable supramolecular polymers in water as observed from time-dependent imaging (Figure 2e) and FRAP (Figure S14). This observation underscores the influence of cooling rates on the morphological and equilibrium characteristics of the supramolecular polymer, shedding light on the interplay between temperature modulation and the resulting polymerization outcomes.

The recent discovery of the metastable droplet phase of BTA, primarily occurring at elevated temperatures, prompts a reevaluation of our previous report on dilution-induced self-assembly of BTA.[26] In this study, a metastable droplet phase was observed at room temperature, whose factors for formation were unclear. It is plausible that the surfactant, serving as a competitive guest for BTA, played a role in diminishing intermolecular interactions among BTAs. This, in turn, could create opportunities for other intra- and intermolecular interactions, particularly among TEG, to exert influence on the polymerization pathway.

Conclusions

We systematically reevaluated the self-assembly landscape of BTA through a comprehensive thermodynamic and kinetic analysis. This investigation unveiled a hidden phase-separated state of BTA at elevated temperature, droplets, which could also be kinetically trapped at room temperature by employing a thermal quenching method. This study emphasizes the need to reassess self-assembly mechanisms,[43] shedding light on the pivotal role of phase separation in supramolecular polymerization – a concept well-established in conventional covalent polymers and gaining prominence in cellular contexts. Moreover, substrate-liquid and liquid-liquid interfaces can also alter the LLPS behaviour and kinetics of supramolecular polymers as recently reported by Meijer and coworkers thus yielding the study of phase separation and self-assembly inherently entangled.[44] The formation of these liquid droplets within a minimalistic system holds promise for applications in artificial cells, serving as mimics for MLOs and offering model systems to comprehend the role of phase separation in regulating protein content in both monomeric and aggregated states within biological environments, as well as in materials and optoelectronic applications.[45]

Acknowledgements

M. K. and J. N. S. H. contributed equally to this work. We thank Prof. E. W. Meijer for providing BTA and for the valuable discussions. Special thanks to Prof. A. Walther, Prof. P. Besenius, and Prof. S. Seiffert for the spectroscopy and microscopy facilities. Funding from the DFG (Deutsche Forschungsgemeinschaft) is acknowledged: S. D. is member of the GRK 2516 (project No. 405552959) and M. K. is the recipient of a doctoral position within the GRK 2516 program. Elyra7 (SIM microscope) was funded by the Gutenberg Research College and Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 497845157. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Self-assembly · Supramolecular polymers · Phase separation · Bioinspired · Supramolecular droplets

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Manuscript received: February 6, 2024
Accepted manuscript online: April 23, 2024
Version of record online: May 22, 2024