Multicomponent supramolecular hydrogels are promising scaffolds for applications in biosensors and controlled drug release due to their designer stimulus responsiveness. To achieve rational construction of multicomponent supramolecular hydrogel systems, their in-depth structural analysis is essential but still challenging. Confocal laser scanning microscopy (CLSM) has emerged as a powerful tool for structural analysis of multicomponent supramolecular hydrogels. CLSM imaging enables real-time observation of the hydrogels without the need of drying and/or freezing to elucidate their static and dynamic properties. Through multiple, selective fluorescent staining of materials of interest, multiple domains formed in supramolecular hydrogels (e.g., inorganic materials and self-sorting nanofibers) can also be visualized. CLSM and the related microscopic techniques will be indispensable to investigate complex life-inspired supramolecular chemical systems.

1. Introduction

1.1. Stimulus-Responsive Supramolecular Hydrogels

Low-molecular-weight gelators (LMWG) self-assemble into fibrous structures that entangle with each other to form supramolecular hydrogels.[1–5] Precise molecular design of hydrogelators can provide stimulus responsiveness towards heat, pH, and reactive molecules. A wide variety of functional molecules (proteins, catalysts, and nanomaterials) can also be incorporated into hydrogels without loss of function (termed multicomponent hydrogel systems), enabling applications in controlled drug release and delivery systems and regenerative medicine.[6–10] To achieve rational construction of such multicomponent supramolecular hydrogels, their in-depth characterization is crucial. To date, spectroscopic methods including circular dichroism (CD), absorbance, and fluorescent spectroscopy have been widely used, particularly to evaluate molecular packing in the nanofibers.[11] However, these spectroscopic analyses give only ensemble (average) structural information. Microscopy methods such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) are able to visualize individual nanofiber shapes at nanometer resolution. Although microscopic techniques are powerful, there is a requirement for drying and/or freezing during the sample preparation, which may cause artifacts that change the self-assembled structure.[12] Furthermore, these techniques cannot discriminate chemical species in multicomponent systems. Recently, confocal laser scanning microscopy (CLSM) has emerged as a powerful tool for structural analysis of multicomponent supramolecular hydrogel systems (Figure 1).[13–19] CLSM not only allows observation of supramolecular nanofibers and hydrogels without the need for a drying process but can also distinguish between chemical species through the use of appropriately designed fluorescent probes. In this mini-review, we briefly explain design principles of fluorescent probes for supramolecular nanofibers and introduce representative examples of CLSM imaging of supramolecular nanofibers/hydrogels. For more detail on the mechanism of CLSM and examples of CLSM imaging of other self-assembled functional materials, please refer to other excellent reviews.[20,21]

1.2. Confocal Laser Scanning Microscopy and Fluorescent Probe Design

CLSM shows higher spatial resolution and contrast than conventional wide-field fluorescent microscopy based on reducing the contribution of fluorescence from non-focal plane with a pinhole. The use of lasers as a light source enables selective, high-resolution, and bright excitation of the materials of interest. As demonstrated in the field of cellular biology, CLSM has the following advantages for hydrogel observation over SEM, TEM, and AFM: (1) a drying process is not required for sample preparation; (2) three-dimensional structures can be reconstructed from multiple two-dimensional images obtained...
Supramolecular hydrogel

- No need for drying and freezing
- 3D z-stack imaging
- Time-lapse imaging for dynamic processes
- Multiple fluorescent staining
- A wide range of field of view (µm-mm)

Confocal scanning laser microscopy

1. Nanofiber imaging  2. Fluidity

Photo bleaching

3. Monomer exchange  4. Formation/degradation

5. Cellular environment  6. Structural transformation

7. Multiple domain  8. Self-sorted nanofibers

Time

Figure 1. (Top) Illustration of CLSM imaging of a supramolecular hydrogel and advantages of CLSM imaging. (Bottom) Applications of CLSM imaging in supramolecular nanofibers and hydrogels.

at different focal planes; (3) time-lapse imaging enables visualization of dynamic processes in a real-time manner (e.g., formation and collapse of nanofibers); (4) distinct chemical species can be distinguished by staining with fluorescent

Ryō Kubota was born in 1985 in Tokyo, Japan. He received his Ph.D. from the University of Tokyo under the supervision of Prof. Mitsuhiko Shionoya in 2013. During his Ph.D. course, he joined Prof. Luisa De Cola’s group as a visiting student. He then carried out his postdoctoral research in the group of Prof. Itaru Hamachi at Kyoto University. He is currently an assistant professor at Kyoto University. His research interests include life-inspired supramolecular chemistry, coordination chemistry, and chemical biology.

Keisuke Nakamura obtained his M.S. degree from Kyoto University in 2019. He is currently a PhD student under the supervision of Prof. Itaru Hamachi at Kyoto University. His research interests include multi-component supramolecular materials.

Shogo Torigoe was born in 1994 in Okayama, Japan. He obtained his B.Sc. in Kyoto University in 2018. He is now a master course student in the group of Prof. Itaru Hamachi at Kyoto University. His research fields are chemical biology and functional polymer.

Itaru Hamachi obtained Ph.D. at Department of Synthetic Chemistry of Kyoto University in 1988 under the supervision of Prof. Iwao Tabushi. He started his academic carrier as an assistant professor in Prof. Toyoki Kunitake’s group at Kyushu University in 1988, and then moved to Seiji Shinkai’s lab in the same department as an associate professor In 2001, he became a full professor at Kyushu University and then moved to Department of Synthetic Chemistry and Biological Chemistry of Kyoto University in 2005. Currently, he is also a research director of JST ERATO project termed Innovative Molecular Technology for Neuroscience. His interest has now been extended to chemical biology and organic chemistry in living systems, and supramolecular biomaterials.
probes selective for each component; (5) the use of objectives with different magnification allows for observation with a wide range of field of views from the sub-micro to millimeter scale. One drawback of CLSM is a higher diffraction-limited spatial resolution than those of TEM, SEM, and AFM owing to the Abbe limit of resolution (lateral resolution: ~ 200 nm); however, recently developed super-resolution microscopic techniques [stimulated emission depletion (STED), photo activated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and structured illumination microscopy (SIM)] have enabled acquisition of images at higher spatial resolutions (lateral resolution: less than 100 nm).

To visualize supramolecular nanofibers by CLSM, the targets of interest should be stained with fluorescent probes. As shown in Figure 2, fluorescent-staining methods are divided broadly into three categories: (1) use of fluorescently-labeled monomers, (2) use of fluorescent probes that interact with supramolecular nanofibers, and (3) use of fluorescent probes that contain self-assembled moieties of the hydrogelators. As a representative example of (1), Xu et al. used peptide hydrogelators that were covalently linked with an environmentally-sensitive fluorescent dye, NBD. These fluorescently-modified hydrogelators are well suited for use in complex chemical environments such as live cells. In the case of methods (2) and (3), non-fluorescent hydrogelators are stained by a small amount of a fluorescent probe (typically ~ 0.1 mol%), which tends to have negligible effects on the properties of the supramolecular nanofibers/hydrogels. As a typical example of (2), we designed a fluorescent coumarin probe tethering a guanidium group to interact with peptide hydrogelators/nanofibers that have a carboxylate group at the C-terminus. As an example of (3), van Esch et al. reported a probe that had a fluorescent dye at the terminus of one of ethylene glycol linkers. Such fluorescent probes are particularly useful for selectively staining multiple orthogonal supramolecular nanofibers.

2. CLSM Imaging of Single Component Supramolecular Hydrogels

This section provides examples of CLSM imaging of supramolecular hydrogels that contain one type of hydrogelator (defined as single component supramolecular hydrogels). CLSM imaging can visualize fiber network structures and reveal the dynamic properties of supramolecular nanofibers in real time.

2.1. CLSM Imaging of Supramolecular Hydrogels/Fibers

In 2002, we reported the first example of supramolecular nanofibers made of LMWG, which could be visualized by CLSM. Glycolipid-based nanofibers were labeled with an environment-sensitive fluorescence probe (HANDB), which enabled 3D networks of bundled nanofibers (diameter ca. 0.2–0.6 μm) to be directly imaged by CLSM (Figure 3). Following this report, Schneider et al. used CLSM to image a β-hairpin type amphiphilic peptide-based supramolecular hydrogel that was labeled with a hydrophobic fluorescence probe (DiO). They observed the heterogeneous microdomains of a fibrillar network inside the hydrogel.

Figure 2. Three distinct design guidelines of fluorescent probes: (a) use of fluorescently-labeled monomers; (b) use of fluorescent probes interacted with nanofibers; (c) use of fluorescent probes that contain self-assembled moieties of the hydrogelators.
2.2. Dynamic Properties of Supramolecular Nanofibers

CLSM imaging allows for elucidation of dynamic behaviors such as nanofiber formation/degradation in supramolecular hydrogels because no drying processes are required during the sample preparation. In this section, representative examples of real-time observations of dynamic properties of supramolecular nanofibers based on time-lapse CLSM imaging are introduced.

2.2.1. Monomer Diffusion in the Nanofibers Determined by Fluorescent Recovery After Photobleaching (FRAP)

Fluorescence recovery after photobleaching (FRAP) is a powerful method for investigating the mobility of supramolecular nanofibers. In FRAP experiments, a fluorescence probe embedded in the nanofiber is photobleached over a limited small region by an intense laser pulse and the fluorescent intensity recovery owing to inflow of unbleached fluorescence probe, is monitored (Figure 4a). The recovery curve gives the diffusion coefficient and mobile fraction. In 2010, we analyzed the fluidic properties of glycolipid-based supramolecular nanofibers co-assembled with a fluorescent glycolipid analogue by FRAP (Figure 4b).

This nanofiber showed immediate recovery of the fluorescent intensity and the diffusion coefficient was estimated to be $0.12 \pm 0.03 \ \mu m^2 s^{-1}$, which is comparable to that of a biomembrane. This fluidic nanofiber can control the directional motion of attached proteins and nanobeads along the fiber. Furthermore, FRAP analysis was used to identify the properties of individual nanofibers in the multi-component hydrogel.

--

Figure 3. The first example of CLSM imaging of a supramolecular hydrogel. (a) Chemical structures of a hydrogelator and a fluorescent probe. (b) CLSM image. Adapted from Ref. 32 with permission. Copyright 2002 American Chemical Society.

Figure 4. (a) Schematic illustration of FRAP experiment. (b) Time-lapse CLSM imaging of FRAP experiment of supramolecular nanofibers. Adapted from Ref. 36 with permission. Copyright 2010 Springer Nature.
2.2.2. Determination of the Monomer Exchange Kinetics

Time-lapse CLSM imaging also revealed the kinetics and pathway of monomer exchange.\textsuperscript{40–43} In 2014, Meijer et al. successfully quantified the exchange kinetics of fluorescent probes between supramolecular nanofibers by STORM.\textsuperscript{40} They measured time-lapse imaging after mixing two distinct supramolecular nanofibers, each of which was stained with Cy3- or Cy5-tethered probes. As incubated, exchange of the fluorescent probes gradually proceeded, thereby both fluorescent probes began to colocalize on the same nanofibers and reached the equilibrium state within 24 h. The quantitative analysis suggested that the monomer exchange took place on the mechanism of homogenous exchange along the polymer backbone. This mechanism is unlike that of biological supramolecular fibers or cytoskeletons, where monomer exchange proceeds on “polymerization-depolymerization at the end” and/or “fragmentation-recombination” mechanisms. Stupp and Meijer et al. conducted similar experiment with a peptide amphiphile.\textsuperscript{41} In this case, the exchange kinetics depends on the position of supramolecular nanofibers, which suggests a heterogeneous packing structure of the supramolecular nanofibers.

2.2.3. In Situ Imaging of Seeded Supramolecular Polymerization

Time-lapse in situ CLSM imaging of supramolecular systems is invaluable for elucidating their formation processes. On the basis of temperature-dependent CD spectra data, the supramolecular nanofibers made of peptide-based gelators are assumed to form via a cooperative nucleation-elongation mechanism.\textsuperscript{30,44–50} We directly visualized the cooperative elongation process of peptide-based nanofiber by CLSM (Figure 5). The nanofibers were gradually observed with a delay of 5 min in the heat-and-cooling preparation protocol without seeds (Figure 5b, bottom).\textsuperscript{30} In contrast, the fibers immediately formed from the seeds and grew linearly in the presence of seeds (Figure 5b, top). Moreover, CLSM observations clarified that the morphologies of the nanofibers were markedly different in the presence and the absence of the seed. These imaging studies clearly suggest that the growth of the peptide-based nanofiber mainly occurred via a cooperative process. Such a clear conclusion cannot be drawn from the CD spectral data that provide only ensemble data for all of the nanofibers.

2.2.4. Transient Formation of Supramolecular Nanofibers by Fuel-Driven Dissipative Self-Assembly

Fuel-driven dissipative (out-of-equilibrium) self-assembly has recently drawn considerable attentions for developing life-inspired active, autonomous, adaptive soft materials.\textsuperscript{51–61} Time-lapse CLSM imaging can clarify the real-time dynamics of fuel-driven self-assembly. In 2015, van Esch et al. succeeded in transient formation of supramolecular nanofibers upon treatment of a hydrogelator precursor, having carboxylate groups, with dimethyl sulfate, a strong methylating reagent.\textsuperscript{56} CLSM imaging demonstrated that the nanofibers stochastically collapsed from their tips with velocities of up to 15 μm/min. Furthermore, both growth and collapse of nanofibers simultaneously proceeded in the same field of view, indicating spatially-heterogeneous, non-synchronized formation, and degradation of nanofibers. This unexpected behavior is similar to the nonlinear dynamics and dynamic instability of microtubule filaments.

![Figure 5](image-url)
2.2.5. Imaging of Supramolecular Block Copolymer and Covalent Block Copolymer Self-Assembly

One of the unique strengths of CLSM is the ability to spatially discriminate different chemical species from fluorescence spectra. Meijer et al. directly observed a supramolecular block copolymer with the use of CLSM, based on the use of spontaneous physisorption of the dyes to the polymer (iPAINT).

The mixture of two preassembled homopolymers made of triarylamine triamide monomer S-1 or S-2 led to thermodynamically stable supramolecular block copolymers (Figure 6a). These two different homopolymers were modified by physisorption of distinct fluorescent probes before mixing, and the bicolored contiguous fibers composed of block copolymers were clearly observed by STORM, a super-resolution imaging mode (Figure 6b). Manners et al. successfully visualized living crystallization-driven self-assembly of covalent block copolymer consisting of ferrocenyldimethylsilane and methylsiloxane monomers.

In this research, they prepared the two different polymers covalently grafted with distinct fluorophores. The partial difference in fluorescence of the CLSM observations revealed that the supramolecular structures, such as microfibril or micelles formed stepwise and grew from the different polymer components.

2.2.6. Spectral Imaging of Time Course Change of Supramolecular Packing Structures

Spectral imaging combined with a linear unmixing in CLSM is a highly useful technique to distinguish fluorescent probes and assemblies with quite similar emission properties by measuring a fluorescent spectrum at each pixel. Through the use of spectral imaging, De Cola et al. succeeded in real-time imaging of the dynamic assembly process of a neutral Pt\(^{4+}\) complex.

The Pt\(^{4+}\) complex formed molecular assemblies comprising two kinetic aggregates (spherical aggregates (A) and ribbon-like intermediates (B)) and one thermodynamic nanofiber (C), which showed distinct emission properties. These emission features could be used as unique fingerprints (maximum emission wavelength: 615, 575, and 464 nm, respectively). Spectral imaging in CLSM successfully uncovered complex dynamics of the assembly evolution, that is, after formation of spherical aggregates A, both assemblies B and C appeared to grow anisotropically by consuming the neighboring spherical aggregates A, and then the ribbon-like intermediate B slowly converted into the thermodynamic nanofibers C. In situ imaging showed that all the assemblies formed in dynamic equilibrium with monomeric species, which is similar to biological infection processes, such as those of prion diseases.

2.3. Self-Assembly in the Complex Cellular Environment

CLSM imaging allowed for direct observation of the formation of supramolecular structures in live cells. Xu et al. developed supramolecular hydrogelators that formed into hydrogels by enzymatic dephosphorylation and ester hydrolysis (Figure 7a). They prepared a gelator-derivative modified fluorescent probe and observed the fibrous aggregates in vitro and in live cells by CLSM (Figure 7b). Maruyama et al. also observed the formation of the supramolecular fibers on the tumor cell surface, upon cleavage of a gelator precursor by metalloprotease.

![Figure 6.](image-url)
confirmed the fiber formation by slow recovery of the fluorescence intensity in particular areas of live cells. Furthermore, supramolecular structures in animals have been observed by CLSM. Rao et al. demonstrated self-assembly of a fluorescent small molecule responsive to GSH and caspase 3/9 in apoptotic cells of mice transplanted human cancer cells. The self-assembled structure was produced by an intramolecular cyclization reaction triggered by enzymatic reactions in cells. The use of 3D-SIM, super-resolution microscopy, enabled clear mapping of the distribution of the supramolecular structures in cancer tumors.

3. CLSM Imaging of Multi-Domain Supramolecular Hydrogels

Living eukaryotic cells have sophisticated functions based on an elaborate interplay among multiple organelles and assemblies (cytoskeletons). Multicomponent supramolecular hydrogels with chemically-distinct nano- or micro-sized domains and environments (defined as multi-domain supramolecular hydrogels) show promise for imitating biological fascinating functions, such as multiple stimulus-response and biosensing. In situ CLSM imaging is also a powerful tool for in-depth structural evaluation of the multi-domain supramolecular hydrogels.

3.1. Imaging of Supramolecular Hydrogels Containing Inorganic Materials

Encapsulation of (inorganic) materials that create nanospaces in supramolecular hydrogels has allowed construction of multi-component hydrogels consisting of distinct micro/nano-environments such as the intracellular organelles of live cells, and these hydrogels have been demonstrated to be applicable for semi-wet biosensors. The spatially distinct domains and the translocation of the fluorescence probe between these domains in a multi-component hydrogel can be imaged by choosing appropriate probes capable of selectively staining each domain.

In 2009, we developed a polyanion-selective fluorescence sensing material by hybridization of a glycolipid-based supramolecular hydrogel, mesoporous silica particles (NH$_2$-MCM41) and phosphatase together with a fluorescence probe (Figure 8a). There are three distinct domains in the hydrogel matrix, that is the cationic cavity of NH$_2$-MCM41, the hydrophobic domain of the interior of the supramolecular nanofiber, and the water phase containing phosphatase. An anionic phosphorylated coumarin probe (P-Coum) that was loaded in the inside of NH$_2$-MCM41 was released to the water phase by the addition of polyanions through an ion-exchange process, where P-Coum was hydrolyzed by phosphatase (Figure 8b). The hydrolyzed P-Coum became hydrophobic and was thus translocated into the hydrophobic domain of the nanofibers. Fluorescence resonance energy transfer (FRET) was induced from the translocated probe to a BODIPY-tethered probe embedded in the nanofibers, which made it possible to detect the polyanion from the fluorescence color change. CLSM
imaging clearly demonstrated the spherical fluorescence of P-Coum in NH₂-MCM41 and the fiber-like fluorescence of the BODIPY-type probe in the nanofibers arranged orthogonally in the system (Figure 8c). Upon addition of a polyanion such as chondroitin sulfate, the fluorescence of P-Coum merged well with the fluorescence of the BODIPY-probe in the nanofibers, as clearly imaged by in situ CLSM. We also observed translocation of a cationic fluorescence probe from the interlayer space of montmorillonite (MMT) to supramolecular fibers upon addition of polyamines in the MMT-hydrogel hybrid. [78]

3.2. Imaging of Supramolecular Hydrogels Containing Liposomes

In 2012, van Esch et al. reported in situ imaging of a supramolecular hydrogel containing an enzyme-embedded liposome (Figure 9a). [29] Supramolecular nanofibers and liposomes were stained with an FITC-modified hydrogelator and NBD-modified phosphatidylethanolamine, respectively (Figure 9b). A multicomponent hydrogel was successfully constructed by addition of a pre-organized liposome containing rhodamine-modified chymotrypsin to a hydrogelator solution. CLSM imaging of the resultant hydrogels clearly demonstrated orthogonal formation of supramolecular nanofibers and liposomes entrapped in the nanofiber network (Figure 9c). Furthermore, the rhodamine-modified chymotrypsin localized in the inner water layer of the liposome, suggesting that the inner water layer was a physically-different domain from the bulk water layer.

3.3. Supramolecular Double Network Hydrogels Comprising Self-Sorting Nanofibers

Multiple selective fluorescent staining is also ideal for in situ visualization of self-sorted supramolecular nanofibers [30,31,37,82–93] whose structural evaluation is extremely difficult by transmission/scanning electron microscopy owing to the similar structural morphology. We recently reported that peptide- and lipid-type hydrogelators are an excellent self-sorting pair (Figures 10a, b). [30] To evaluate the self-sorting behavior, we designed two distinct fluorescent probes that have self-assembled motifs of the target hydrogelators (Figure 10b). CLSM imaging of a self-sorted supramolecular hydrogel comprising two hydrogelators and two fluorescent probes showed an orthogonal double network structure, where peptide- and lipid-type nanofibers were well entangled but not completely overlapping each other (Figures 10c, d). Super-resolution microscopy such as STED and Airyscan [94] techniques enabled visualization of the 3D structure of the self-sorted network at much higher resolution. Furthermore, we succeeded in in situ imaging-based evaluation of the self-sorting behavior of peptide- and lipid-type nanofibers. [91] CLSM imaging enabled us to determine whether the hydrogelator pair form self-sorted or co-assembled nanofibers in a high throughput manner. Imaging-based screening approaches allowed us to estimate the important chemical properties for self-sorting phenomena such as chemical structures, charge, and hydrophobicity.
3.4. Phase Separation Within Supramolecular Hydrogels

The use of objectives with different magnification allows for visualization of a wide range of specimens with sizes of up to mm order. Notably, van Esch et al. successfully visualized the phase-separated compartmentalized structure in supramolecular hydrogels of tris-hydrazone gelators that formed from a soluble hydrazide, neutral and anionic aldehydes (Figure 11a).\cite{92,93} By mixing three building blocks in a phosphate buffer, a turbid gel was produced. 3D CLSM imaging of the hydrogel stained with an anionic fluorescein probe demonstrated that the resultant supramolecular hydrogels consisted of a sheet-like structure with a width of 100 μm (Figure 11b). The authors concluded that a phase-separated sheet-like structure was composed of mainly anionic nanofibers rather than neutral nanofibers because the sheet-like structure could be stained with Hoechst33342, which does not stain neutral nanofibers.

3.5. Real-Time Imaging of Formation Process of Multiple Domain Supramolecular Hydrogels

Through the use of time-lapse CLSM imaging, the formation process of multicomponent supramolecular hydrogels can be clearly evaluated. We successfully monitored the formation process of the self-sorted double network structure.\cite{30} CLSM clarified that the formation kinetics of peptide- and lipid-type nanofibers is substantially different (Figure 12a). Lipid-type nanofibers had already formed at the start of time-lapse imaging, whereas peptide-type nanofibers began to form after an induction time of 20 min. These distinct formation kinetics are considered to be an important aspect contributing to self-sorting phenomena, as also described by Adams et al.\cite{83} In the case of observations of the formation process in the presence of seeds of peptide-type nanofibers, we found that the seed surface was stained by a lipid-type fluorescent probe, whereas the peptide nanofibers that grew from the seeds showed good self-sorting ability (Figure 12b). This result suggested that the self-sorting ability of the seed surface was attenuated. van Esch et al. also observed the formation process of a phase-separated sheet-like structure by time-lapse CLSM imaging.\cite{92} Their CLSM movie demonstrated that a neutral homogeneous domain initially formed and after several hours anionic sheet-like structures gradually formed. These results suggest that phase separation proceeded via a kinetic self-sorting mechanism probably induced by the higher critical aggregation concentration value of anionic than neutral nanofibers.

4. Conclusions

We have highlighted applications of in situ CLSM imaging of supramolecular nanofibers and hydrogels. As described above, CLSM imaging is one of the most powerful methods available for evaluating dynamic chemical processes that take place in stimulus-responsive supramolecular hydrogels. We believe that in situ imaging-based materials design is a promising way to rationally design and evaluate multicomponent supramolecular systems. However, despite its usefulness, CLSM imaging generates artifacts that could lead to misinterpretation of experimental results: (1) photobleaching can occur in long-time imaging, (2) local temperature increases under laser irradiation, and (3) fluorescent crosstalk can occur in a multicolor imaging. Therefore, appropriate control experiments should be always conducted. In the near future, other emerging imaging techniques such as FLIM (fluorescent lifetime imaging microscopy),\cite{95} TIRF (total internal reflection fluorescence),\cite{96} and light-sheet fluorescent microscopy\cite{97} will offer new ways for in-depth analysis of multicomponent supramolecular systems. Confocal fluorescent imaging and the related microscopic techniques will provide opportunities to investigate complex life-inspired chemical systems that are realized by combination of supramolecular chemistry, systems chemistry, and out-of-equilibrium chemistry.

---

Figure 9. (a) A multicomponent supramolecular hydrogel with enzyme embedding liposome. (b) Chemical structures of a hydrogelator and a fluorescent probe. (c) CLSM imaging of the multicomponent supramolecular hydrogel containing chymotrypsin embedding liposome. Liposome was labeled with NBD-modified phosphatidylethanolamine and rhodamine-labeled chymotrypsin was used. Red: liposomes, green: supramolecular nanofibers, blue: chymotrypsin. Adapted from Ref. 29 with permission. Copyright 2012 American Chemical Society.
Figure 10. (a) Schematic illustration of supramolecular hydrogel consisting of self-sorting nanofibers. (b) Chemical structures of peptide- and lipid-type hydrogelators and fluorescent probes. (c) STED images of self-sorted supramolecular hydrogel. (left) Oregon-green channel, (middle) Alexa fluor546 channel, and (right) merged images. (d) Line plot analysis along a white line shown in (c). Adapted from Ref. 30 with permission. Copyright 2016 Springer Nature.

Figure 11. Phase-separated supramolecular hydrogel. (a) Chemical structures of hydrogelators and a fluorescent probe. (b) CLSM imaging of the phase-separated supramolecular hydrogel. (left) fluorescein channel, (middle) Hoechst33342 channel, and (right) merged images. Adapted from Ref. 92 with permission. Copyright 2019 American Chemical Society.
Competing financial interests

The authors declare no competing financial interests.

Acknowledgement

This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas “Chemistry for Multimolecular Crowding Biosystems” (JSPS KAKENHI Grant JP17H06348), JST ERATO Grant Number JPMJER1802 to I.H., and by a Grant-in-Aid for Young Scientists (JSPS KAKENHI Grant JP18K14333) to R.K. We thank Andrew Jackson, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Keywords: confocal microscopy · gels · self-assembly · stimulus response · supramolecular chemistry

Figure 12. Real-time imaging of formation process of the self-sorted supramolecular hydrogel in the (a) absence and (b) presence of peptide-type seeds. Adapted from Ref. 30 with permission. Copyright 2016 Springer Nature.

[1] T. Aida, E. W. Meijer, S. I. Stupp, Science 2012, 335, 813–817.
[2] X. Du, J. Zhou, J. Shi, B. Xu, Chem. Rev. 2015, 115, 13165–13307.
[3] D. J. Cornwell, D. K. Smith, Mater. Horiz. 2015, 2, 279–293.
[4] E. R. Draper, D. J. Adams, Chem 2017, 3, 390–410.
[5] D. B. Amabilino, D. K. Smith, J. W. Steed, Chem. Soc. Rev. 2017, 46, 2404–2420.
[6] H. Shigemitsu, I. Hamachi, Acc. Chem. Res. 2017, 50, 740–750.
[7] K. Sato, M. P. Hendricks, L. C. Palmer, S. I. Stupp, Chem. Soc. Rev. 2018, 47, 7539–7551.
[8] M. J. Webber, E. A. Appel, E. W. Meijer, R. Langer, Nat. Mater. 2016, 15, 13–26.
[9] G. A. Silva, C. Czeisler, K. L. Niece, E. Beniash, D. A. Harrington, J. A. Kessler, S. I. Stupp, Science 2004, 303, 1352–1355.
[10] E. R. Draper, D. J. Adams, Chem. Soc. Rev. 2018, 47, 3395–3405.
[11] L. L. E. Mears, E. R. Draper, A. M. Castilla, H. Su, Zhuo, B. Dietrich, M. C. Nolan, G. N. Smith, J. Doutch, S. Rogers, R. Akhtar, H. Cui, D. J. Adams, Biomacromolecules 2017, 18, 3531–3540.
[12] K. Sugiyasu, N. Fujita, S. Shinkai, Angew. Chem. Int. Ed. 2004, 43, 1229–1233.
[13] A. D. Guerzo, A. G. L. Olive, J. Reichwagen, H. Hopf, J.-P. Desvergne, J. Am. Chem. Soc. 2005, 127, 17984–17985.
[14] X. Lin, M. Hiroto, T. Seki, H. Kurata, T. Karatsu, A. Kitamura, D. Kuzuhara, H. Yamada, T. Ohaba, A. Saeki, S. Seki, S. Yagai, Chem. Eur. J. 2013, 19, 65661–6565.
[15] A. Sarker, S. Dhiman, A. Chalishzar, S. J. George, Angew. Chem. Int. Ed. 2017, 56, 13767–13771.
CLSM observation has been used for visualizing domain structures in polymer hydrogels. Hashimoto firstly applied CLSM imaging to the polymer hydrogels. Science 1976, 16, 1055–1069.

J. A. Wang, X. Feng, B. Xu, Angew. Chem. Int. Ed. 2019, 58, 10332–10337.

J. Zhou, X. Du, J. Li, N. Yamagata, B. Xu, Angew. Chem. Int. Ed. 2017, 56, 1021–1027.

M. Tena-Solsona, B. Ried, R. K. Grötsch, F. C. Lohrer, C. Wancke, B. Kasdorff, A. R. Bausch, P. Müller-Buschbaum, O. Lieleg, J. Boekhoven, Nat. Commun. 2017, 8, 15895.

M. Tena-Solsona, C. Wancke, B. Riess, A. R. Bausch, J. Boekhoven, Nat. Commun. 2018, 9, 2044.

A. Mishra, D. B. Korlepara, M. Kumar, A. Jain, N. Jonnalagadda, K. K. Bejagam, S. Balasubramanian, S. J. George, Nat. Commun. 2018, 9, 1295.

J. Leira-Iglesias, A. Tassoni, T. Adamchi, M. T. Hermans, Nat. Nanotechnol. 2018, 13, 1021–1022.

B. Adelizzi, A. Alou, A. J. Markvoort, H. M. M. Ten Eikelder, I. K. Voets, A. R. A. Palmans, E. M. Meijer, J. Am. Chem. Soc. 2018, 140, 7168–7175.

B. Adelizzi, A. Alou, N. J. Van Zee, A. R. A. Palmans, E. M. Meijer, I. K. Voets, ACS Nano 2018, 12, 4431–4439.

Z. M. Hudson, D. J. Lunn, A. Manners, Nat. Commun. 2014, 5, 5321–5330.

Z. M. Hudson, C. E. Boot, M. E. Robinson, P. A. Rupar, M. A. Winnik, I. Manners, Nat. Commun. 2014, 5, 893–898.

H. Qiu, Z. M. Hudson, A. Manners, Science 2015, 347, 1329–1332.

H. Qin, Y. Gao, C. E. Boot, O. E. C. Gould, R. L. Harniman, M. J. Miles, S. E. D. Webb, M. A. Winnik, I. Manners, Science 2016, 352, 697–701.

X-H. Jin, M. B. Price, J. R. Finnegan, C. E. Boot, J. M. Richter, A. Rao, S. M. Menke, R. H. Friend, G. R. Whittell, I. Manners, Science 2018, 360, 897–900.

H. Koh, A. H. Williams, R. S. Bang, S. D. Stoyanov, O. D. Velez, Nat. Mater. 2019, 18, 1315–1320.

T. Zimmermann, J. Riedtorf, R. Pepperkok, FEBS Lett. 2003, 546, 87–92.

A. Aliprandi, M. Mauro, L. De Cola, Nat. Chem. 2015, 7, 8–10.

H. Wang, Z. Feng, B. Xu, Angew. Chem. Int. Ed. 2019, 58, 10423–10432.

J. Zhou, X. Du, J. Li, N. Yamagata, B. Xu, J. Am. Chem. Soc. 2015, 137, 10040–10043.

D. Ye, A. J. Shuhendler, L. Cui, L. Tong, S. S. T. Gee, G. Tikhomirov, D. W. Felscher, J. Rao, Nat. Chem. 2014, 6, 519–526.

F. Versluis, D. M. van Eilands, S. Mytryn, D. L. Perrier, F. Trausel, J. M. Poolman, C. Mait, V. A. A. Le Sage, S. I. van Kasteren, J. H. van Esch, R. Eelkema, J. Am. Chem. Soc. 2011, 133, 1670–1673.

A. Heeres, C. Van der Pol, M. Stuart, A. Friggeri, B. L. Ferina, J. van Esch, J. Am. Chem. Soc. 2003, 125, 14522–14525.

A. Brizard, M. Stuart, K. Van Bommel, A. Friggeri, M. de Jong, J. van Esch, Angew. Chem. Int. Ed. 2008, 47, 2063–2066.

S. L. Higashi, A. Shibata, Y. Kitamura, K. M. Hirosawa, K. G. N. Suzuki, K. Matsuura, M. Ikeda, J. Am. Chem. Soc. 2019, 141, 13719–13725.

M. Safton-Sempere, G. Fernández, F. Würthner, Chem. Int. Ed. 2011, 50, 5784–5814.

K. L. Morris, L. Chen, J. Raeburn, O. R. Sellick, P. Cotanda, A. Paul, P. C. Griffiths, S. M. King, R. K. O'Reilly, L. C. Serpell, D. J. Adams, Nat. Commun. 2013, 4, 1480.

C. Colquhoun, E. R. Draper, E. G. B. Eden, B. N. Catto, K. L. Morris, L. Chen, T. O. McDonald, A. E. Terry, P. C. Griffiths, L. C. Serpell, D. J. Adams, Nat. Nanotechnol. 2014, 6, 13719–13725.

E. R. Draper, E. G. B. Eden, T. O. McDonald, D. J. Adams, Nat. Nanotechnol. 2015, 7, 848–852.

E. R. Cross, S. Sproulés, R. Schweins, E. R. Draper, D. J. Adams, Angew. Chem. Int. Ed. 2018, 57, 8667–8670.

J. Wang, Z. Wang, J. Gao, L. Wang, Z. Yang, D. Kong, Z. Yang, J. Mater. Chem. 2009, 19, 7892–7896.

D. Kirya, M. Ikeda, H. Onoe, M. Takinoue, H. Komatsu, Y. Shimoyama, I. Hamachi, M. Takeuchi, Angew. Chem. Int. Ed. 2012, 51, 1553–1557.

M. Lovrak, W. E. J. Hendriksen, C. Mait, S. Mytryn, V. van Steijn, R. Eelkema, J. Phys. Chem. B 2008, 112, 6985–6995.

B. A. Grzybowska, W. T. S. Huck, Nat. Nanotechnol. 2016, 11, 585–592.

I. R. Epstein, B. Xu, Nat. Nanotechnol. 2016, 11, 312–319.

R. Merindol, A. Waltherr, Chem. Rev. 2017, 116, 5588–5561.

J. Kang, D. Miyajima, T. Mori, Y. Inoue, Y. Itoh, T. Aida, Science 2015, 347, 646–651.

T. Fujii, S. Kawai, S. Fujimoto, Y. Morimoto, T. Honjo, D. Koda, M. Goto, T. Maruyama, J. Am. Chem. Soc. 2015, 137, 770–775.

D. Axelrod, D. E. Koppel, J. Schlessinger, E. Elson, W. W. Webb, Biophys. J. 1976, 16, 1035–1069.

F. R. A. Chivers, D. K. Smith, R. E. Draper, E. G. B. Eden, T. O. McDonald, A. E. Terry, P. C. Griffiths, L. C. Serpell, D. J. Adams, Nat. Nanotechnol. 2014, 6, 13719–13725.

J. Wang, Z. Wang, J. Gao, L. Wang, Z. Yang, D. Kong, Z. Yang, J. Mater. Chem. 2009, 19, 7892–7896.
[91] R. Kubota, S. Liu, H. Shigemitsu, K. Nakamura, W. Tanaka, M. Ikeda, I. Hamachi, Bioconjugate Chem. 2018, 29, 2059–2067.
[92] Y. Wang, M. Lovrak, Q. Liu, C. Maity, V. A. A. Le Sage, X. Guo, R. Eelkema, J. H. van Esch, J. Am. Chem. Soc. 2019, 141, 2847–2851.
[93] Y. Wang, R. M. de Kruijf, M. Lovrak, X. Guo, R. Eelkema, J. H. van Esch, Angew. Chem. Int. Ed. 2019, 58, 3800–3803.
[94] J. Huff, Nat. Methods 2015, 12, 1205.
[95] M. Y. Berezin, S. Achilefu, Chem. Rev. 2010, 110, 2641–2684.
[96] D. Axelrod, J. Cell Biol. 1981, 89, 141–145.
[97] J. Huisken, J. Swoger, F. D. Bene, J. Wittbrodt, E. H. K. Stelzer, Science 2004, 305, 1007–1009.

Manuscript received: November 6, 2019
Revised manuscript received: December 15, 2019