Supplementary Information for

Supramolecular Localization in Liquid-Liquid Phase Separation and Protein Recruitment in Confined Droplets

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Materials and Methods

Reagents and materials

2,2'-Azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, ovalbumin (OVA), horseradish peroxidase (HRP), tetramethylrhodamine isothiocyanate–Dextran (TRITC-Dex) were purchased from Sigma Aldrich (St. Louis, MO, United States). Dextran 200,000 (Dex), dextran 60,000, glucose oxidase (GOx), lysozyme, and glucose were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). Polyethylene glycol #6,000 (PEG) and polyethylene glycol #20,000 were purchased from Nakarai tesque (Kyoto, Japan). 5-TAMRA N-hydroxysuccinimidyl (NHS) ester was obtained from Funakoshi (Tokyo, Japan). Dylight™ 405-NHS ester, dextran 500,000, and Amplex™ Red reagent were purchased from Thermo Fisher Scientific (Waltham, MA, United States). Cy5-NHS ester was purchased from Cytiva (Marlborough, MA, United States).

Synthesis of Cya-PA and Mel-NBD and preparation of co-assembly

Cya-PA and Mel-NBD were synthesized through the same procedure reported in our previous papers[1]. The concentrations of Cya-PA and Mel-NBD were determined using UV-vis (V-750, JASCO, Tokyo, Japan) and NMR measurements (JNM-ECZ400, JEOL, Tokyo, Japan). After mixing Cya-PA and Mel-NBD in PBS solution in 1:1 molar ratio, we sonicated the solution for 30 min, followed by heating at 60 °C for 30 min for complete dissolusion. After heating, the temperature was decreased to 37 °C by 1 °C /min and incubated at the temperature until use.

Preparation of fluorescence-labeled proteins

HRP was labeled with 5'-TAMRA NHS ester or Dylight™ 405-NHS ester by the standard procedure. GOx and lysozyme were reacted with 5'-TAMRA NHS ester, and OVA was labeled with Cy5 NHS ester. Briefly, protein (10 mg/mL) and fluorescence probes (molar ratio of protein : fluorescence probe = 1 :0.5) were mixed in 100 mM carbonate buffer (pH = 9.3) for 1.5 h at room temperature, followed by purification and desalination by a PD-10 desalting column packed with Sephadex G-25 resin (Cytiva) using pure water as an eluent to get fluorescence-labeled proteins. Concentrations of the labeled proteins were determined by the absorbance at 280 nm using a NANO Drop-2000c (Thermo Fisher Scientific, Waltham, MA, USA) and the extinction coefficient predicted by ExPAsy ProtParam.

Observations of co-assemblies with microscopies

Morphologies of co-assemblies were observed with transmission electron microscopy (TEM) using a JEM-2010 (JEOL, Tokyo, Japan), scanning probe microscopy (SPM) using a Dimension Icon or PeakForce (Bruker, Billerica, MA, United States) in a tapping mode, and confocal laser scanning microscopy (CLSM) using a Zeiss LSM700 (Carl Zeiss, Oberkochen, Germany).

For TEM observation, 1.0 mM of Cya-PA/Mel-NBD co-assemblies were drop-cast onto a STEM grid with an elastic carbon film (Okenshoji, Tokyo, Japan). The samples were stained with 2% uranyl acetate, dried in a vacuum, and imaged at an accelerating voltage of 120 kV. For SPM, the mixtures prepared at 1.5 mM were diluted to 0.5 mM using PBS and drop-cast onto freshly cleaved mica substrate, washed with water, and dried under ambient conditions. The images were obtained using SCANASYST-AIR probes and microcantilevers (SI-DF3P2, Hitachi High-Technologies, Tokyo, Japan). For CLSM observation, co-assembly samples were cast in a multi-well glass-bottom dish (Matsunami Glass Ind, Osaka, Japan). For imaging, diode laser (488 nm for NBD) was used.

Fourier-transform infrared (FT-IR) spectroscopy

Fourier-transform infrared (FT-IR) spectra were recorded on a Spectrum Two (PerkinElmer, Waltham, MA, USA) at 2 cm^{-1} resolution using an attenuated total reflection (ATR) mode. Cya-PA/Mel-NBD co-assemblies (1.0 mM) in PBS were freeze-dried over 2 d to obtain lyophilized powders for the analysis.

Zeta-potential measurement

Zeta-potentials of Cya-PA3/Mel-NBD co-assembly and proteins were measured with a Nano-ZSP (Malvern Panalytical, Worcestershire, United Kingdom). Samples (Cya-PA3/Mel-NBD at 0.4 mM or proteins at 1.0 mg/mL) were prepared in phosphate buffer (10 mM, pH = 7.4). Samples were loaded in the disposable folded capillary cells (Malvern Panalytical) for the measurement.

Bio-layer interferometry analysis with BLItz

Bio-layer interferometry was performed with a BLItz (Fortebio, CA, United States) to analyze the adsorption of co-assemblies on GOx or HRP. Aminopropyl silane (APS) biosensors (Sartorius AG, Göttingen, Germany) were hydrated in Milli-Q for at least 30 min before use. After the baseline step with Milli-Q and PBS, 4 μL of protein solutions (0.1 mg/mL in PBS) were added to the drop holder to allow the association of proteins with the APS biosensor surfaces for 300 s and then the sensors were exposed to PBS for 120 s to remove the excess proteins. After the baseline step with PBS for 30 s, the association of samples (1.0 mM of co-assemblies or each component) with the proteins on the tips for 200s and the dissociation step for 200s by soaking in PBS solution were applied.

Construction of the aqueous two-phase system

Liquid-liquid phase separation (LLPS) environment used in the present study was composed of polyethylene glycol (PEG) and dextran (Dex)[s2]. Stock solutions of PEG (20 wt%) and Dex (20

wt%) were prepared in PBS. Simple mixing of the stock solutions of PEG and Dex at final concentration of 5 wt% each resulted in the PEG-rich continuous phase and Dex-rich droplets with the average diameter of over 10 μm.

Observation of the localization of co-assemblies in liquid-liquid phase separation (LLPS)

LLPS based on PEG-Dex was used to form cell-sized droplets for observation by CLSM.[s2] Concentrated polymer solution ([PEG] = [Dex] = 20 wt%) was added to pre-mixed Cya-PA and Mel-NBD solutions to prepare an LLPS solution ([PEG] = [Dex] = 5 wt%) and incubated at 37°C under shaking (1000 rpm, cute mixier CM-1000, EYELA, Tokyo, Japan). After gentle pipetting, samples were transferred to a microscope slide and covered with a coverslip (Matsunami Glass Ind.) with a 0.12 mm-thick spacer (Grace Bio-Labs SecureSeal™ imaging spacer, Sigma Aldrich) between them. For imaging, diode lasers (405 nm for Dylight405, 488 nm for NBD, 555 nm for TAMRA, 639 nm for Cy5) were used.

Evaluation of the distribution coefficient

The distribution coefficient of each component of the co-assemblies was estimated by evaluating the concentrations of molecules in each phase. Macroscopic phase separation (PEG-rich upper phase and the Dex-rich lower phase) was obtained after centrifugation (2000 g, 5 min). After sampling the solutions of each phase, the solutions were diluted by more than 1000-fold using PBS to avoid the influence of the co-existence of polymers (PEG or Dex) for the analysis. The distribution coefficients of HRP was determined by a standard enzymatic reaction using Amplex Red as a substrate, while for GOx and glucose, GOx-HRP cascade reaction was used. The distribution coefficients of ABTS, Cya-PA, Mel-NBD, and Cya-PA/Mel-NBD were detemined by a high performance liquid chromatography (HPLC) (JASCO, Tokyo, Japan) on a Inertsil ODS-3 column (GL Science, Tokyo, Japan).

Evaluation of enzymatic cascade reaction

The activity of the cascade reaction of cascade enzymes GOx and HRP was evaluated by the initial production rate of ABTS-diradical using its UV-absorbance at 418 nm (ε_{418} = 36800 $M^{-1}cm^{-1}$) recorded with a plate reader SpectraMax i3x (Molecular devices, CA, United States). GOx, HRP, co-assemblies, and ABTS were mixed in a 96-well half-area polystyrene plate (Greiner Bio-One, Frickenhausen, Germany) under the different conditions (with/without LLPS and with/without co-assemblies). After adding glucose, time-dependent production of ABTS-diradical was monitored at 418 nm throughout 1000 s. The final concentrations of GOx, HRP, ABTS, glucose, and co-assemblies were 0.08 μg/mL, 0.22 μg/mL, 2.0 mM, 40 mM, and 0.38 mM, respectively, and the total volume was 50 μL.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Significance was evaluated by one-way analysis of variance, followed by Tukey's post hoc test for multiple comparisons. $P < 0.05$ was considered statistically significant. Data are expressed as the mean ± standard error (S.E.) or mean ± standard deviation (S.D.).

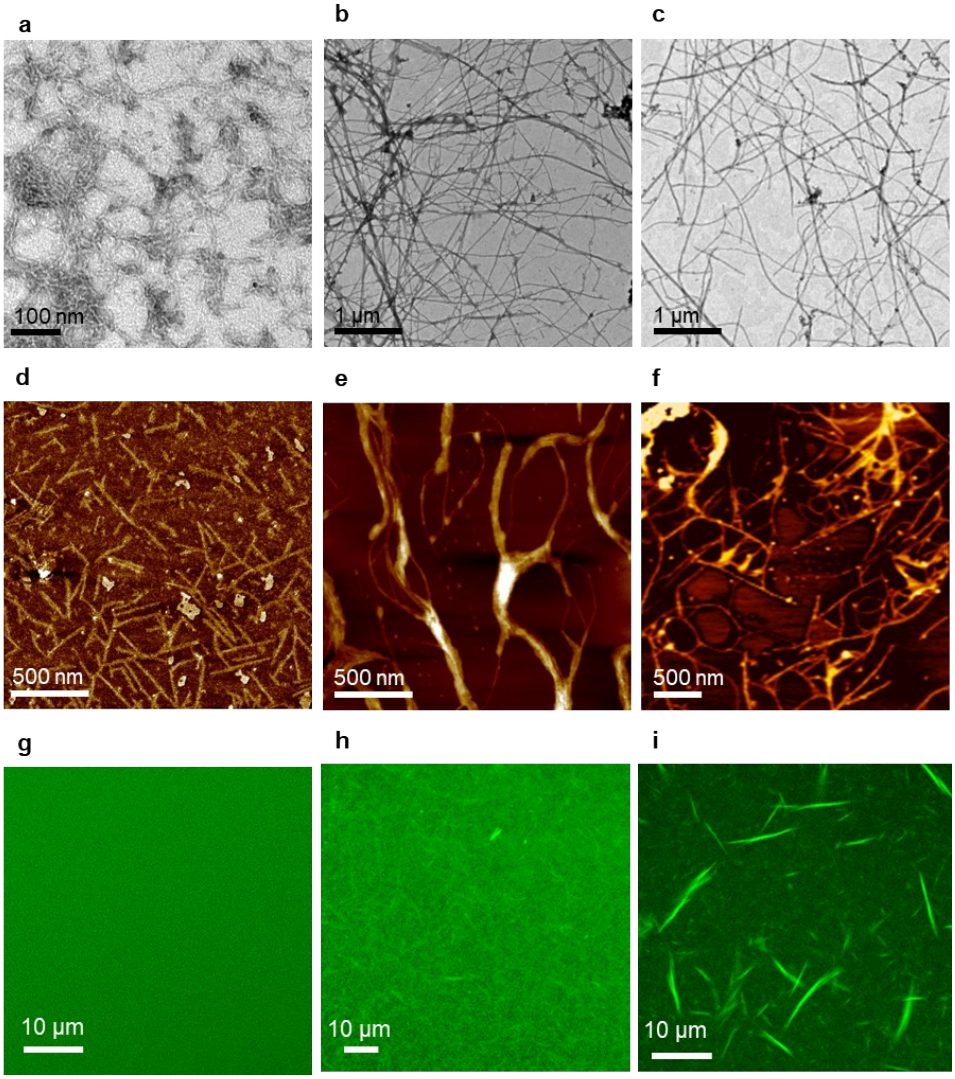

Fig. S1 Morphological observations of co-assemblies of Cya-PA1/Mel-NBD (a, d, g), Cya-PA2/Mel-NBD (b,e,f), and Cya-PA3/Mel-NBD (c,f, i). (a-c) Co-assemblies (1.0 mM) observed with Transmission electron microscope (TEM). (d-f) Co-assemblies (0.5 mM) observed with scanning probe microscope (SPM). (g-h) Co-assemblies (1.5 mM) observed with confocal laser scanning microscope (CLSM). Cya-PA1/Mel-NBD forms rods of several hundreds of nanometers long, while Cya-PA2/Mel-NBD and Cya-PA3/Mel-NBD form micron-scale fibrous assemblies.

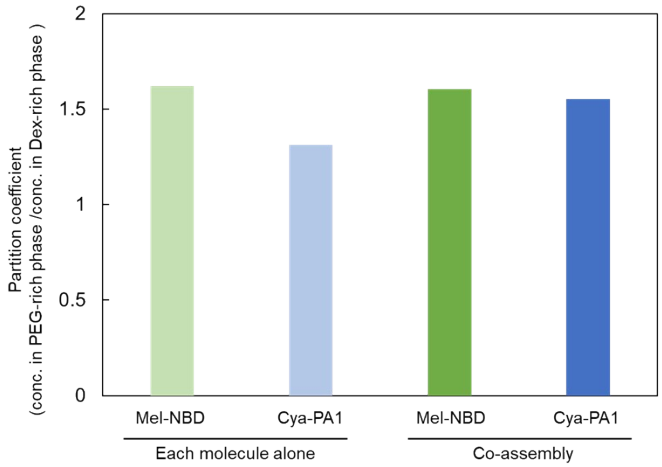

Fig. S2 Partition coefficient (conc. In PEG-rich phase /conc. In Dex-rich phase) of Mel-NBD and Cya-PA1 before and after the formation of co-assembly determined by HPLC.

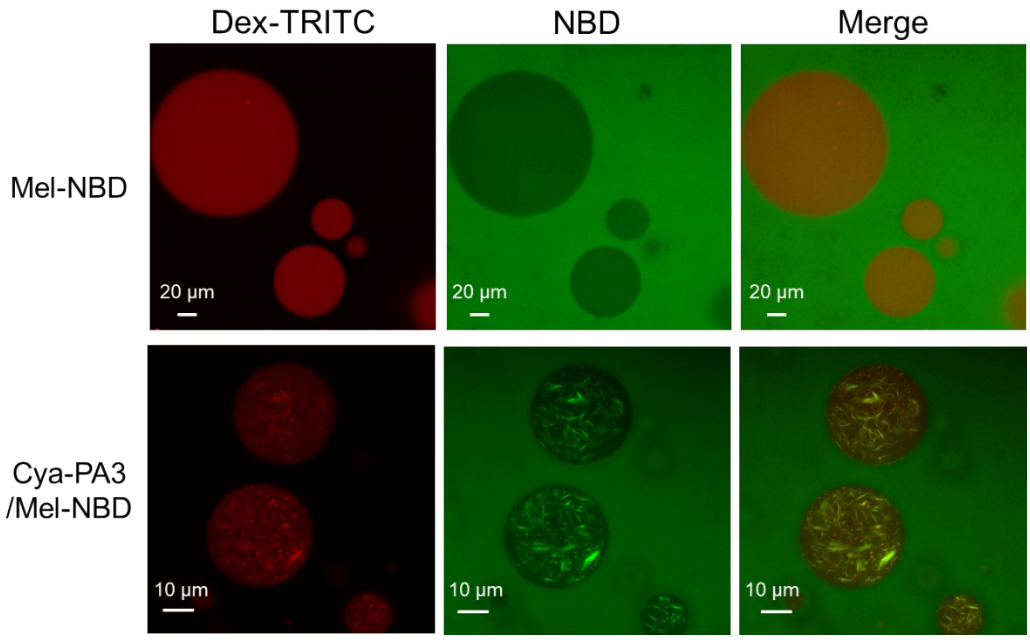

Fig. S3 CLSM observation of localizations of Mel-NBD alone and Cya-PA3/Mel-NBD under LLPS condition stained with TRITC-Dex. [Cya-PA] = 1.5 mM, [Mel-NBD] = 1.5 mM, [PEG] = [Dex] = 5 wt%, [TRITC-Dex] = 0.1 mg/ml.

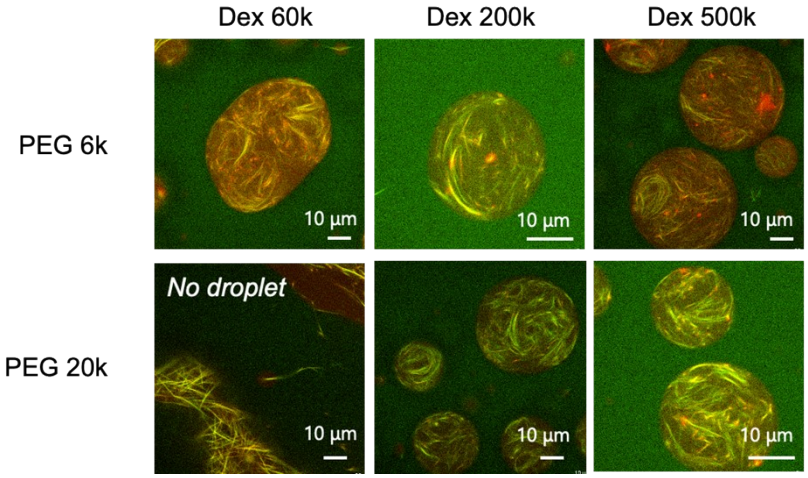

Fig. S4 Localization of fibrous structures of Cya-PA3/Mel-NBD in different LLPS conditions constructed by a series of polymers (PEG 6k, PEG 20k, Dex 60k, Dex 200k, and Dex 500k). [PEG] = [Dex] = 5 wt%, [TRITC-Dex] = 0.1 mg/mL, [Cya-PA3/Mel-NBD] = 0.75 mM.

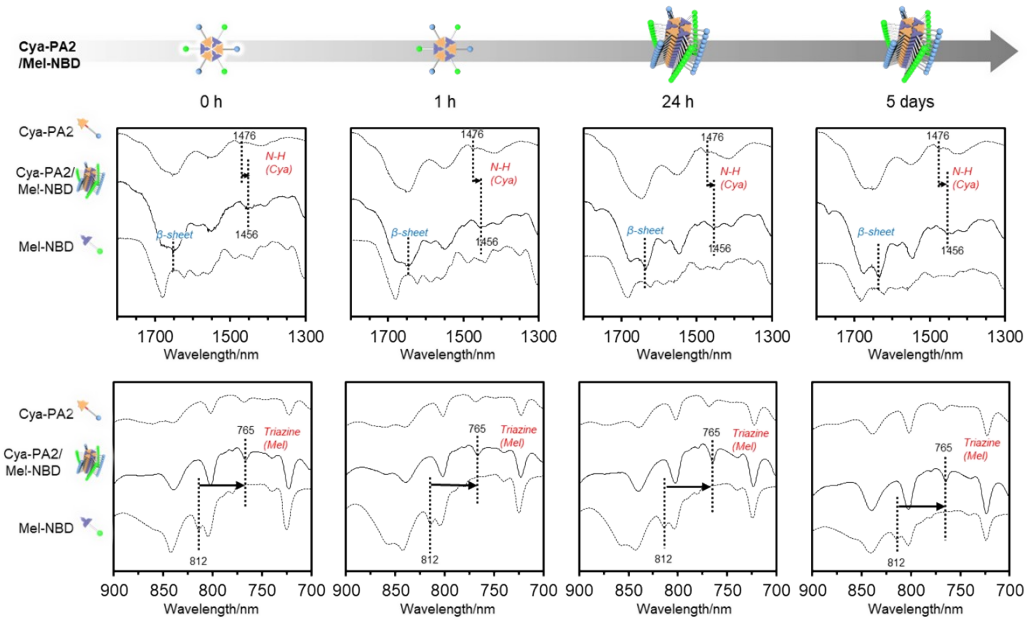

Fig. S5 FT-IR spectral changes of Cya-PA2/Mel-NBD, Cya-PA2 alone, and Mel-NBD alone as aging. Just after mixing (0h incubation), only complementary interation between Cya and Mel was suggested. After 24h incubation, a peak belonging to β-sheet formation between peptides appeared, suggesting the hierarchical assembly.

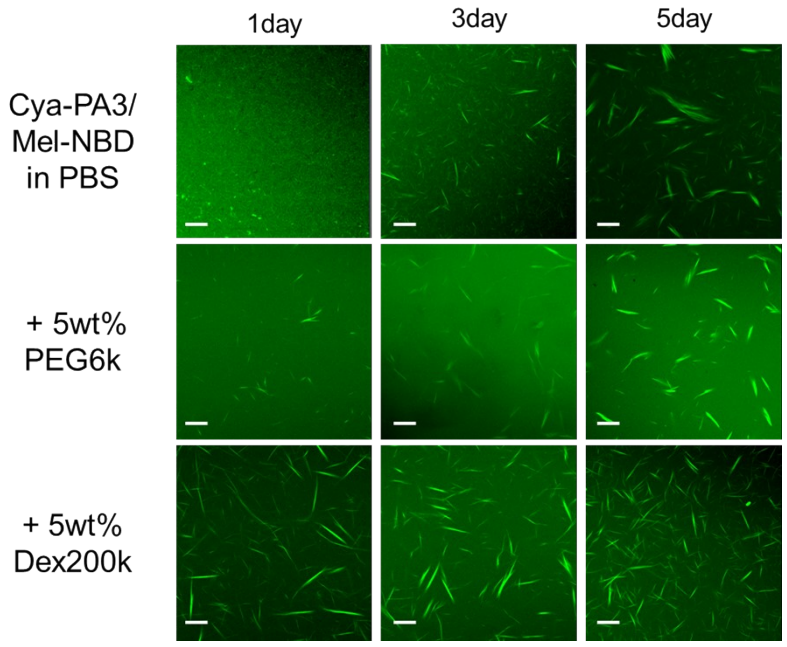

Fig. S6 Time-dependent hierarchical assembly behavior of Cya-PA3/Mel-NBD in PBS (top), in 5 wt% PEG/PBS (middle), and in 5 wt% Dex/PBS (bottom). [Cya-PA3/Mel-NBD] = 1.5 mM, bars: 10 μm.

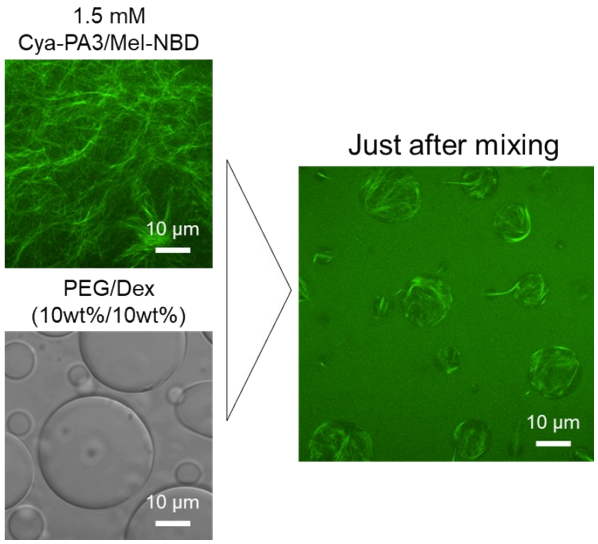

Fig. S7 Phase-selectivity of pre-formed Cya-PA3/Mel-NBD assemblies observed just after mixed with equal amount of a LLPS solution. Final concentrations of Cya-PA3/Mel-NBD, PEG, and Dex are 0.75 mM, 5 wt%, and 5 wt%, respectively.

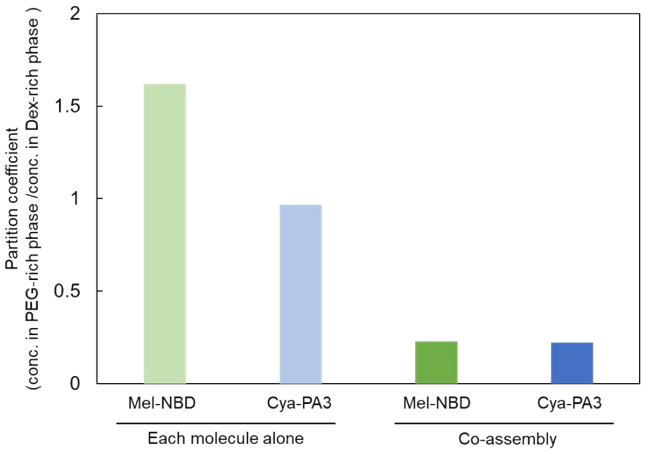

Fig. S8 Partition coefficient (conc. in PEG-rich phase /conc. in Dex-rich phase) of Mel-NBD and Cya-PA3 before and after the formation of co-assembly determined by HPLC.

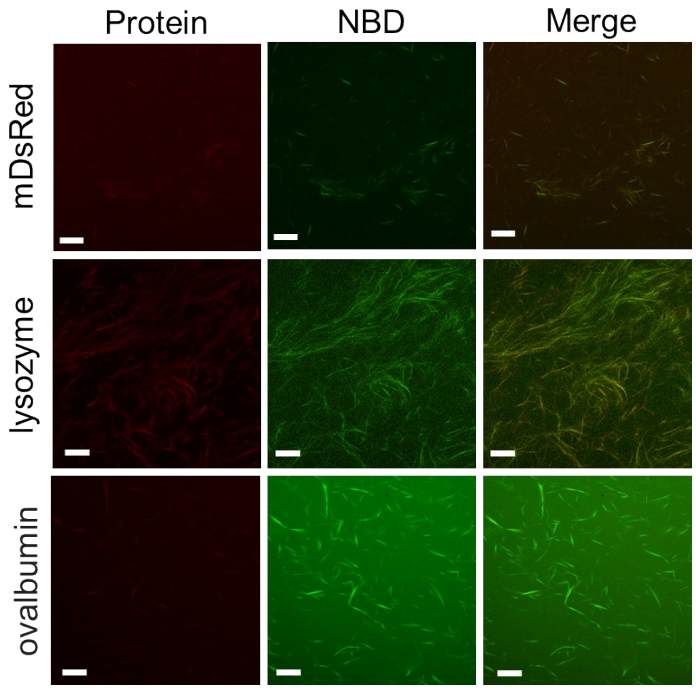

Fig. S9 Physical adsorption of several types of proteins on Cya-PA3/Mel-NBD. Final concentration of Cya-PA3/Mel-NBD, mDsRed, TAMRA-lysozyme, and Cy5-OVA are 0.75 mM, 7.5 μM, 5 μM, and 0.25 mg/ml, respectively. Bars: 10μm

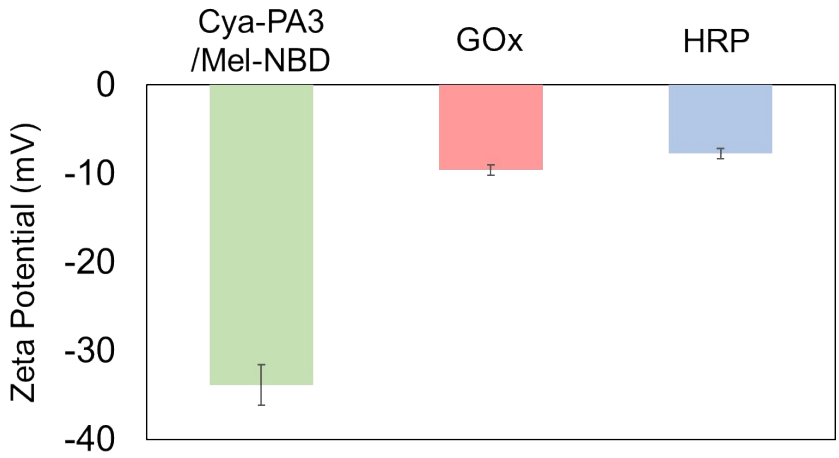

Fig. S10 Zeta-potential measurements of Cya-PA3/Mel-NBD, GOx and HRP in phosphate buffer (pH 7.4). [Cya-PA/Mel-NBD] = 0.4 mM, [GOx] = [HRP] = 1 mg/mL. N = 3, mean ± SD.

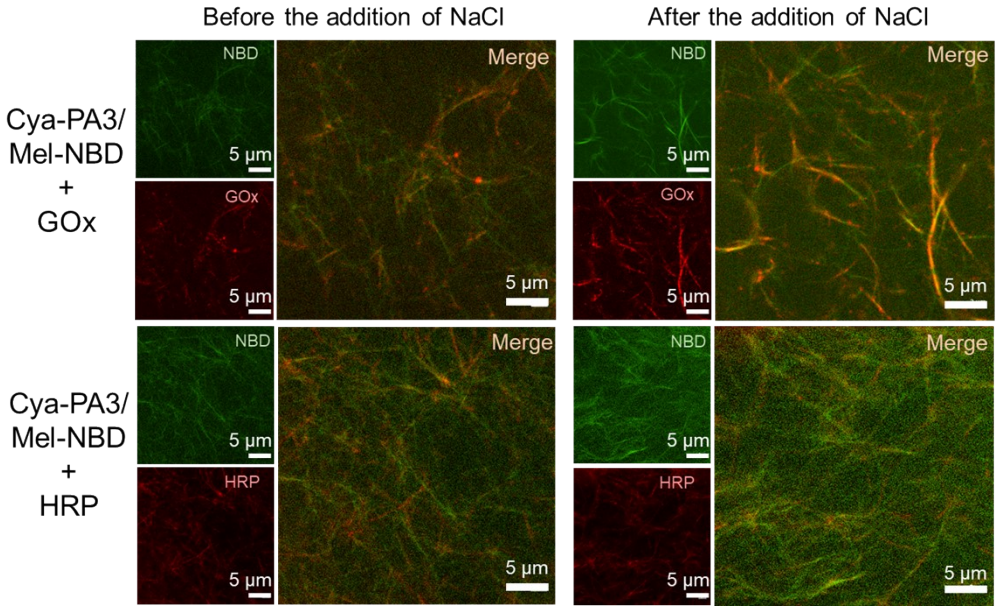

Fig. S11 Effect of salt addition on the physical adsorption of proteins on the Cya-PA3/Mel-NBD co-assembly. Final concentrations of NaCl, Cya-PA3/Mel-NBD, TAMRA-HRP, and TAMRA-GOx are 0.5 M, 0.45 mM, 88 μg/mL, and 64 μg/mL, respectively.

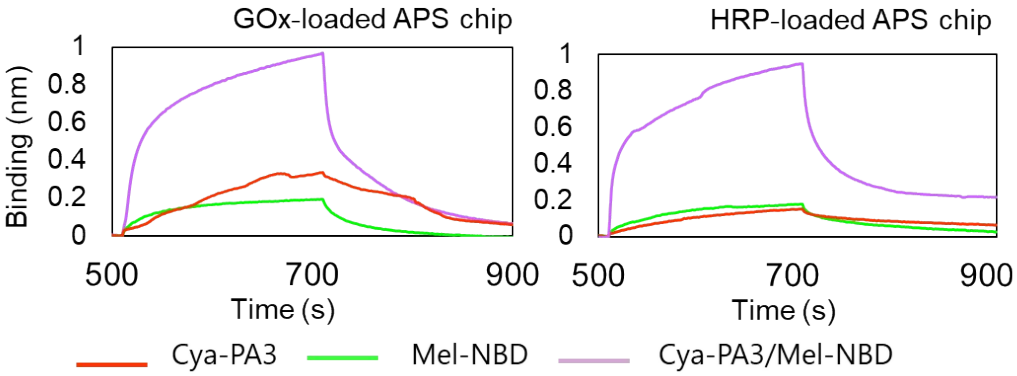

Fig. S12 Association and dissociation curves of Cya-PA3 alone, Mel-NBD alone, or Cya-PA3/Mel-NBD with the GOx or HRP-loaded APS chips measured by biolayer interferometry. [HRP] = [GOx] = 0.1 mg/ml, [Cya-PA] = [Mel-NBD] = 1.0 mM.

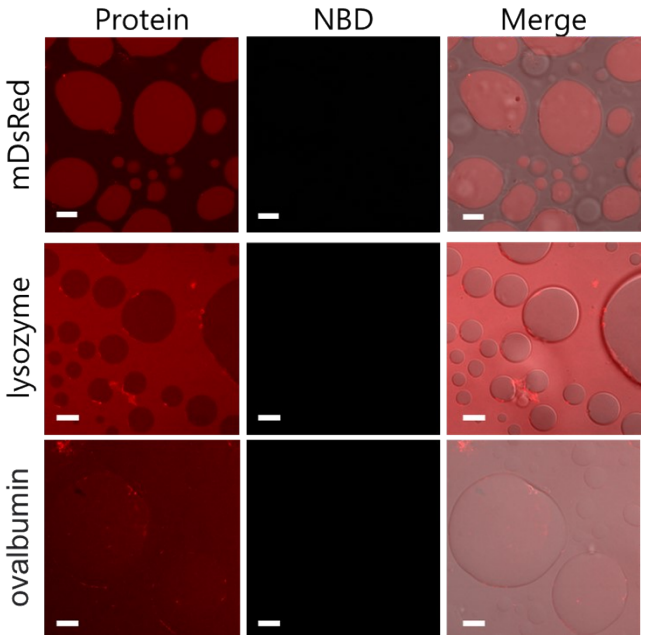

Fig. S13 Phase-selectivity of mDsRed (top), lysozyme (middle), and ovalbumin (bottom) in LLPS condition. [mDsRed] = 7.5 μM, [TAMRA-lysozyme] = 5 μM, [Cy5-OVA] = 0.25 mg/ml, [PEG] = 5 wt%, [Dex] = 5 wt%, Bars: 10 μm.

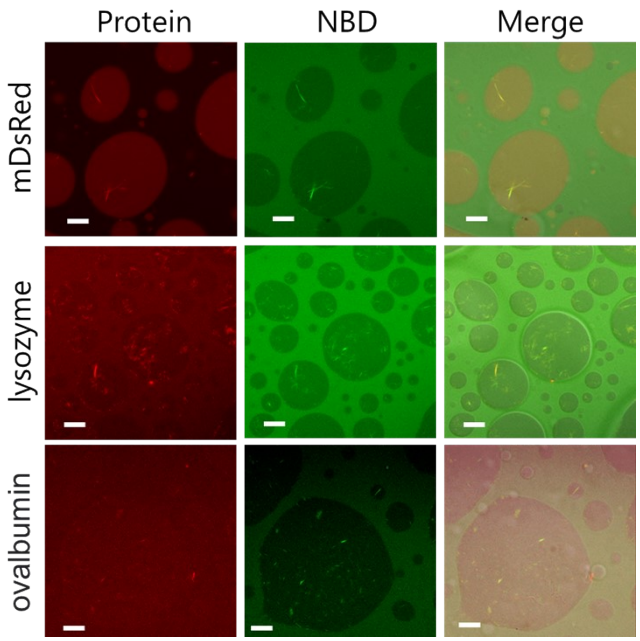

Fig. S14 Localization of mDsRed (top), lysozyme (middle), and ovalbumin (bottom) in LLPS conditions under the presence of Cya-PA3/Mel-NBD. [Cya-PA3/Mel-NBD] = 0.75 mM, [mDsRed] = 7.5 μM, [TAMRA-lysozyme] = 5 μM, [Cy5-OVA] = 0.25 mg/ml, [PEG] = 5 wt%, [Dex] = 5 wt%. Bars: 10 μm.

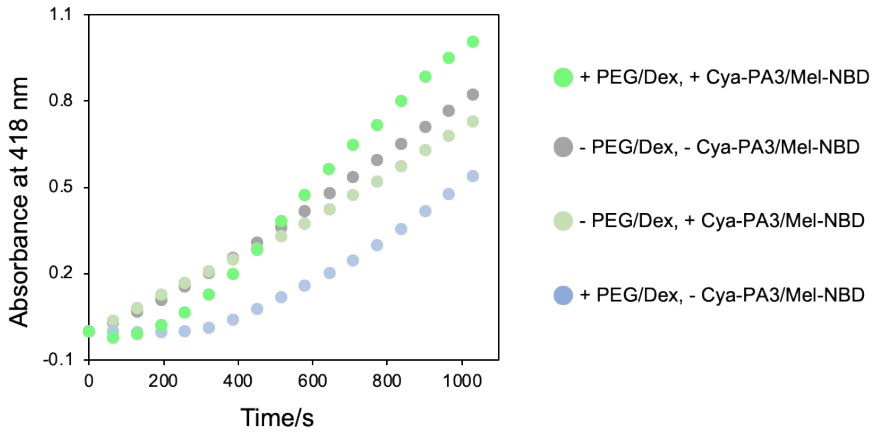

Fig. S15 Time profiles of the absorption at 418 nm (derived from oxidized ABTS) in different environments. Representative profile for each condition is shown. [Mel-NBD] = [Cya-PA] = 0.38 mM, [PEG] = [Dex] = 5 wt%, [GOx] = 0.08 μg/mL, [HRP] = 0.22 μg/mL, [ABTS] = 2.0 mM, [glucose] = 40 mM.

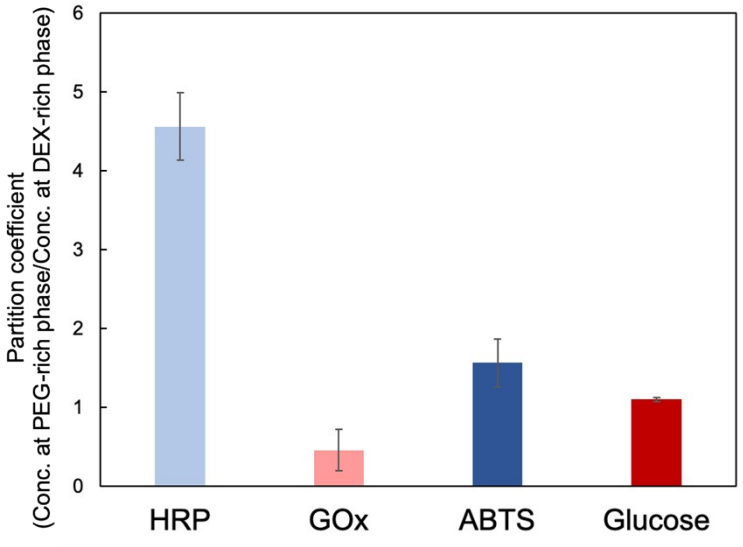

Fig. S16 Partition coefficient (conc. at PEG-rich phase/conc. at DEX-rich phase) of enzymes and substrates for cascade reaction. N = 3, mean ± SD.

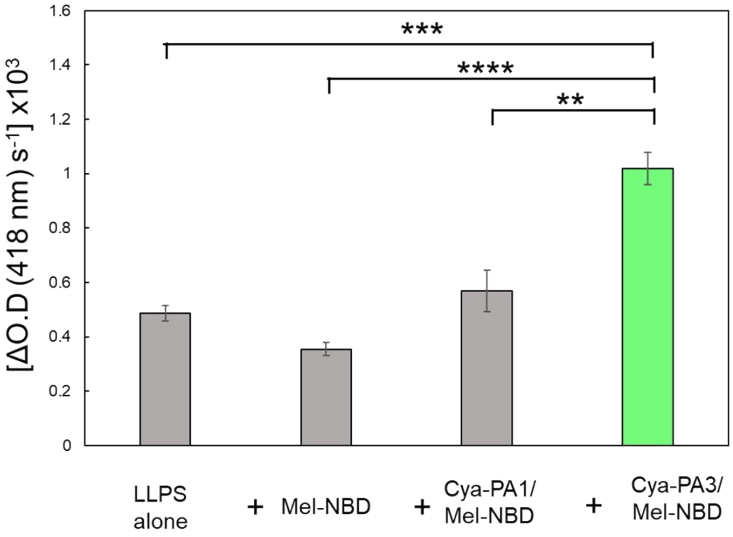

Fig. S17 Influence of the presence of molecules/supramolecules on the initial reaction rate of enzymatic cascade reaction. [Mel-NBD] = [Cya-PA] = 0.38 mM, [PEG] = [Dex] = 5 wt%, [GOx] = 0.08 μg/mL, [HRP] = 0.22 μg/mL, [ABTS] = 2.0 mM, [glucose] = 40 mM. N = 3, mean ± SE, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

References

s1. R. Wakabayashi, H. Obayashi, R. Hashimoto, N. Kamiya and M. Goto, *Chem. Commun.*, 2019, **55**, 6997–7000.

s2. N. Nakatani, H. Sakuta, M. Hayashi, S. Tanaka, K. Takiguchi, K. Tsumoto and K. Yoshikawa, *ChemBioChem*, 2018, **19**, 1370–1374.