Supplementary Information (SI) for Nanoscale.

Supplementary Information

Coacervate Microenvironments Modulate Fluorescent Dye Behaviour and Förster Energy Transfer Dynamics

Mohit Kumar,† Minea Kapidžić † and Shikha Dhiman*

Department of Chemistry, Johannes Gutenberg University Mainz, Duesbergweg 10–14, Mainz, 55122 Germany

E-mail: shikha.dhiman@uni-mainz.de, Homepage: https://www.dhimanlab.com

† These authors contributed equally.

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1. General Methods:

Materials

Poly-L-lysine (PLL, average Mw=22500) and Sulforhodamine B (SRh) were obtained from Sigma- Aldrich. Pyranin (PN) and adenosine triphosphate (ATP) were purchased from Carl Roth. Cyanin5 NHS (CY5) was from Lumiprobe. Poly (ethylene glycol) brushes were purchased from abcr GmbH (2-[Methoxy(polyethyleneoxy)propyl] trimethoxysilane, 90%, 6-9 PEG-units). All the chemicals were used without further purification.

Confocal laser scanning microscopy (CLSM)

Imaging was performed at room temperature using Leica TCS SP8 confocal microscope. Leica oil-immersion objectives were used, specifically the HC PL APO CS2 63x with a numerical aperture (NA) 1.40. The acquired images were processed using LAS X (Leica) and ImageJ software and custom python code. Images were acquired using coverslips modified with PEG brushes mount on glass slides.

Ultraviolet-visible (UV-Vis) Spectroscopy

UV-Vis spectra were recorded at JASCO V-750 in Quartz cuvettes (Hellma) with a path length of 2 mm. The measurements were performed with a scan speed of 200 nm/min, and a data interval of 1 nm.

Fluorescence spectroscopy

Spectra were recorded on a JASCO FP-800 fluorescence spectrometer. Samples were prepared as stated and measured in Quartz cuvettes (500 µL Hellma Analytics) with a path length of 10×2 mm. The sample was excited at λ_{ex}= 458 nm, and the emission spectrum was recorded at λ_{em}= 478- 750 nm.

Zeta potential

Zeta potential measurements were performed on a Malvern Zetasizer Advance equipped with a maximum 10 mW He-Ne laser, emitting at 633 nm. Malvern disposable polycarbonate folded capillary cells with gold plated beryllium-copper electrodes (DTS1070), which were rinsed with ethanol and MQ water before filling, were used. All measurements were at 25 °C, and the sample's temperature was allowed to equilibrate for 120 seconds.

Microplate reader assays

To investigate coacervation behaviour of the system, we conducted turbidity-based titrations by incremental addition of PLL (0-80 µM) to PN solution (0.5, 1, 3, and 6 mM). The absorbance was measured by the Byonoy Absorbance 96 plate reader at 600 nm, where neither of the components absorbs. The absorbance was processed into turbidity as:

$$\%\text{Turbidity} = 100 - 100 \cdot 10^{-A}$$

2. Experimental Protocols

Coacervate preparation

PN-PLL coacervates: To a PN solution (0.5, 1, 3, and 6 mM) in MQ water (pH=4.91), required amounts of PLL stock solution were added to achieve a range of PLL concentrations (0-80 µM).

ATP-PLL coacervates: To prepare coacervates, PLL was added from a stock solution (5 mM) to the ATP solution (5 mM, pH=7.01) to achieve final PLL concentration of 15 µM.

Coverslip modification

Prior to PEGylation, glass-coverslips were rinsed with ethanol and MQ water to remove any dust particles. Coverslips were then incubated for 48 hours in a 5 vol% solution of 2-[Methoxy(polyethyleneoxy)propyl] trimethoxysilane in toluene and finally rinsed with ethanol and dried under nitrogen.

Fluorescence recovery after photobleaching (FRAP)

For successful FRAP experiments, 0.1 µl (100 mM) of Cyanine 5 **(**Cy5) was added to the sample. In CLSM, FRAP was measured within a region of interest with a diameter of 2 µm. The region of interest was subjected to 100% bleaching power of the λ_{ex}= 633 nm laser for 3 seconds. Every 1.6 seconds an image was recorded for 5 minutes. Image analysis performed in ImageJ and LAS X (Leica). The fluorescence intensity was normalized by intensity of a reference area, following the equation, where i(t) is the normalized fluorescent intensity at time t, I(t) and I (0) are the fluorescence intensities of the region of interest at time t and t=0.

$$\mathrm{i(t)}=\frac{I(t)\,/\,R(t)}{I(0)\,/\,R(0)}$$

Energy transfer sample preparation

To assess energy transfer, PN was added (3 mM) to a SRh solution (0.03, 0.06, 0.09, 0.12, and 0.15 mM), with subsequent addition of PLL (40 µM) to induce coacervation.

Intensity calculation of two-channel images through Python

The fluorescence intensity of the two-channel CLSM images PN (donor) and SRh (acceptor) was quantified using a custom Python script.[1]

- The CLSM images corresponding to the two fluorescence channels, PN (donor) and SRh (acceptor), were saved in .tif format. The brighter reference channel should be named as 1_ref.tif, 2_ref.tif, etc (reference image) while the other channel images can be named sequentially as 1.tif, 2.tif, etc. (main image).
- The script identifies coacervates in the reference image by first applying a Gaussian blur to reduce noise, followed by Otsu thresholding to separate coacervates from the background. Small artifacts are then removed through morphological filtering, and coacervate boundaries are detected using contour finding. The positions these coacervates are used to create masks that define regions of interest. These same masks are then applied to the corresponding paired (main) image to extract the mean fluorescence intensity from identical coacervate locations, ensuring accurate comparison of signal intensity between the two channels.

- For each coacervate detected in the reference image, the script calculates the mean fluorescence intensity within that coacervate region. The same coacervate masks are then applied to the corresponding paired image to determine the mean fluorescence intensity at the identical locations.
- The output includes two text files: ref_particle_data_i.txt, which contains the mean intensities of coacervates from the reference image (i_ref.tif), and main_intensity_data_i.txt, which lists the mean intensities of the corresponding coacervates from the paired image (i.tif). Additionally, two quick-check plots are generated one showing the overlay of detected coacervate outlines on the main image and another visualization displaying per-coacervate intensity mapping.

Calculation of Energy Transfer Efficiency (Φ_{ET})

Energy-transfer efficiency (Φ_{ET}) was calculated through CLSM images using the equation:

$$\Phi_{ET} = 1 - I_{DA} / I_D$$

where I_{DA} is the average fluorescence intensity of the donor within the coacervates in the presence of the acceptor, and I_D is the corresponding average donor intensity in the absence of the acceptor.[2]

Calculation of the Partition coefficient (K_p)

The Partition coefficient of fluorescent molecules was calculated using the formula:

$$K_p = (I_{coacervate} - I_{background})/(I_{dilute\ phase} - I_{background}).$$

A blank was obtained with a sample containing only MQ water, and the averaged intensity was taken as the background intensity.[3]

3. Supplementary table and figures

Table S1: Average sizes of coacervates measured by CLSM and DLS at SRh 0-0.15 mM.

SRh (mM)	d_{avg} CLSM (µm)	d_{avg} DLS (µm)
0	2.9	2.8
0.03	2.7	2.5
0.15	2.3	2.9

Table S2: Average zeta potential measured for PN-PLL coacervates with increasing SRh concentration.

SRh (mM)	ζ (mV)
0	7.229 ± 0.025
0.03	6.787 ± 0.595
0.15	8.41 ± 0.369

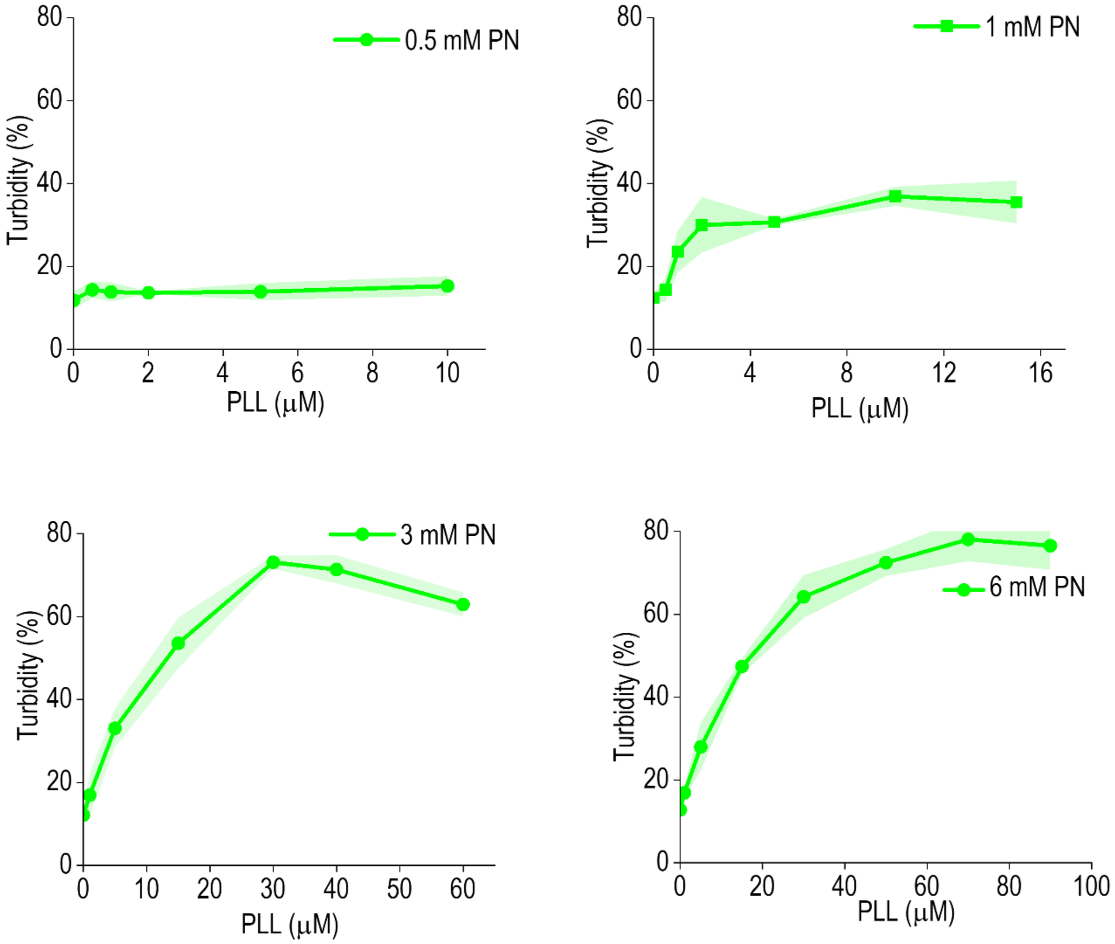

Figure S1: Turbidity measurements for titration of PN with PLL (a) 0.5 mM PN, (b) 1 mM PN, (c) 3 mM PN, (d) 6 mM PN.

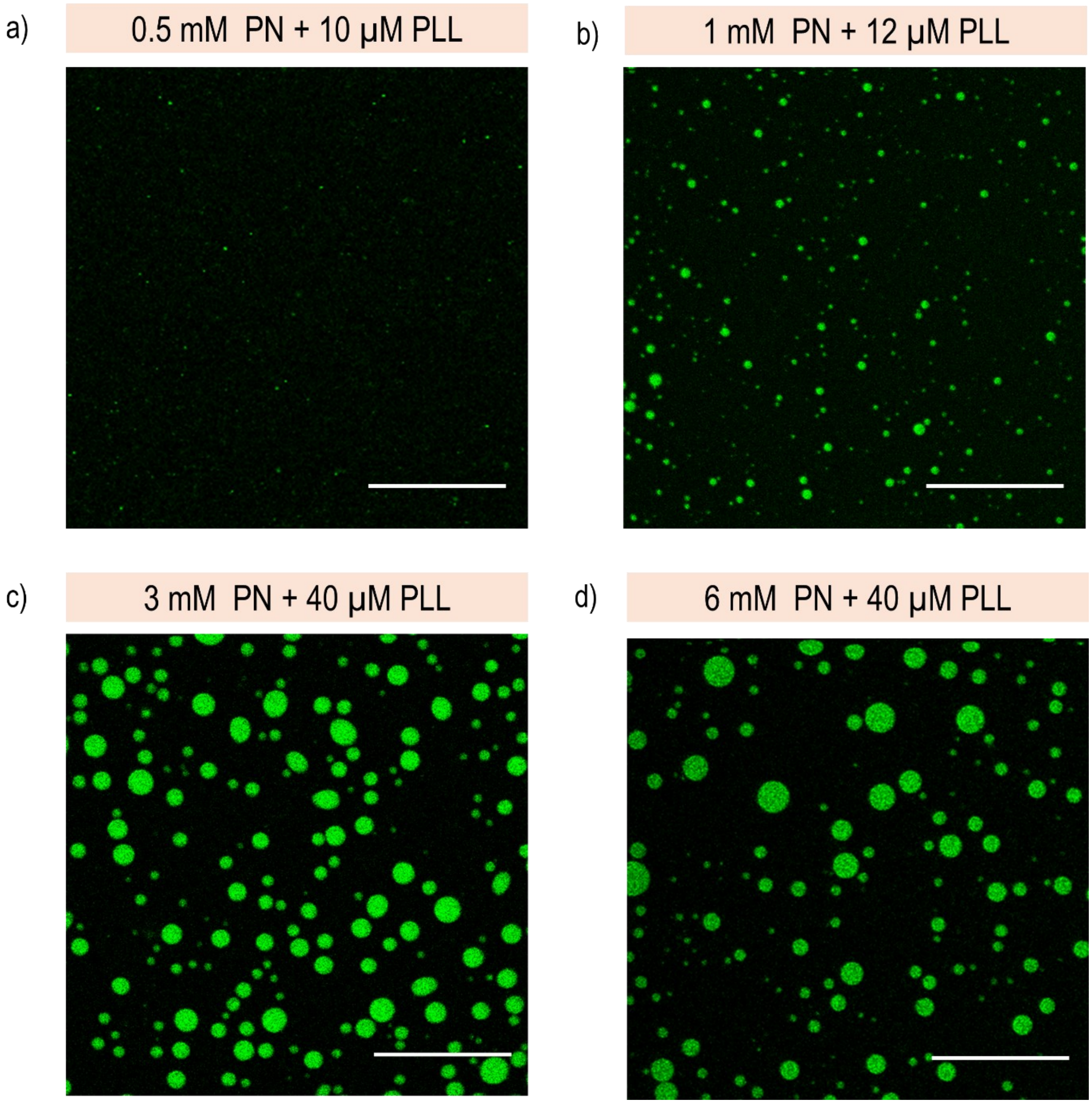

Figure S2: CLSM images of PN-PLL coacervates at (a) 0.5 mM PN + 10 µM PLL, (b) 1 mM PN + 12 µM PLL, (c) 3 mM PN + 40 µM PLL, and (d) 3 mM PN + 40 µM PLL. Scale bar = 20 µm.

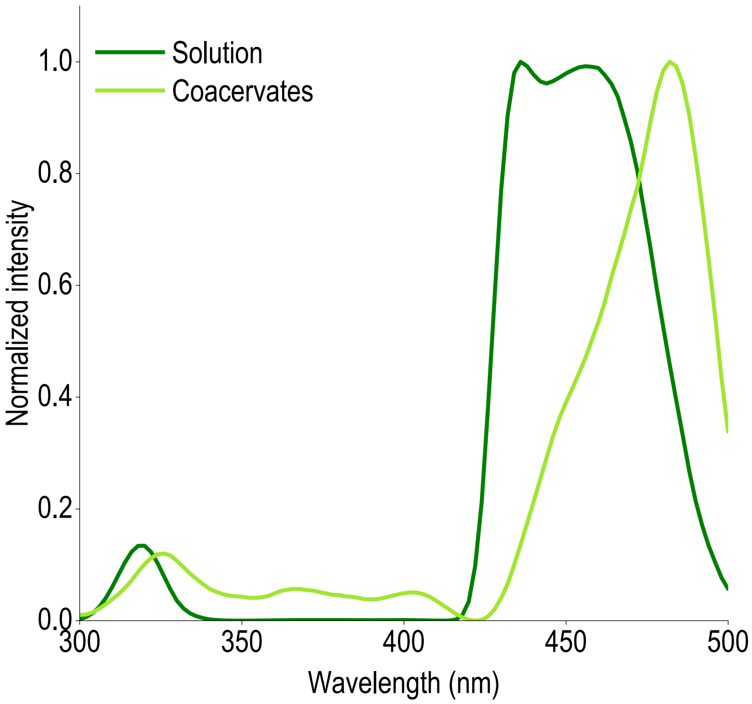

Figure S3: Excitation spectra of 3 mM PN in solution and coacervate state at λ_{em} = 512 nm, showing a pronounced red shift upon coacervate formation.

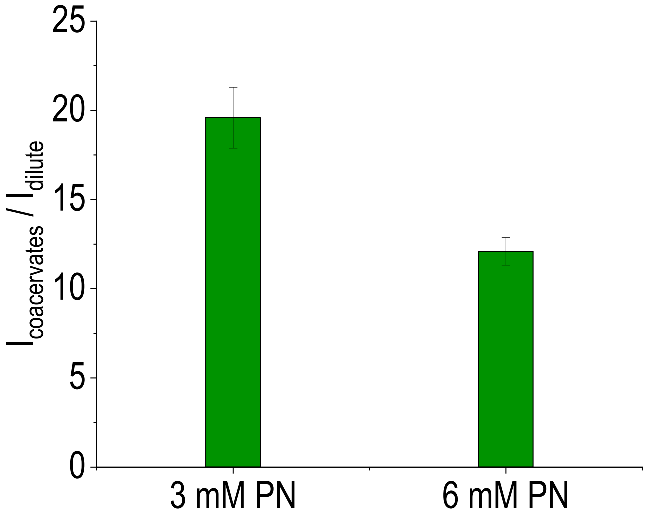

Figure S4: Comparison of $I_{coacervates}$ /I_{dilute} at 3 mM PN and 6 mM PN with 40 µM PLL.

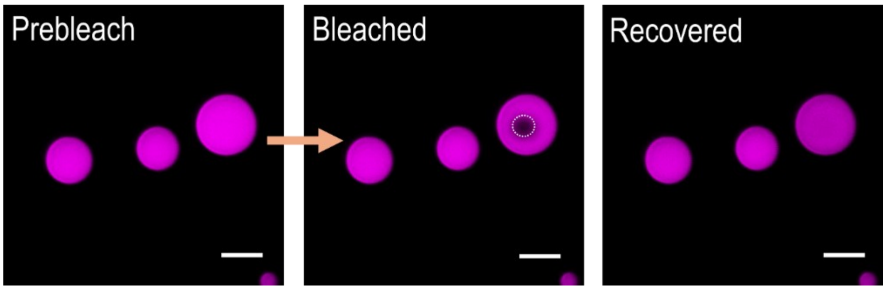

Figure S5: CLSM images of PLL-PN coacervates obtained during FRAP. [PN] = 3 mM, [PLL] = 40 µM, [Cy5] = 0.02 mM. Scale bar = 5µm.

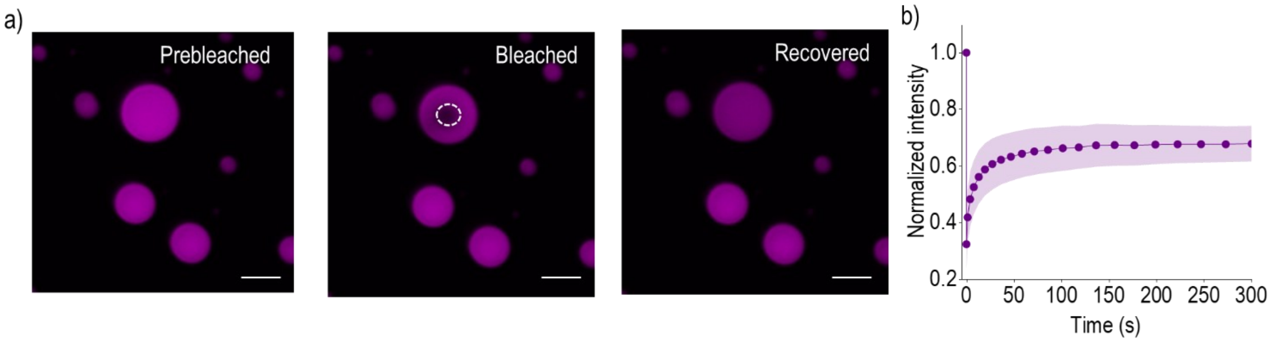

Figure S6: (a) CLSM images of PLL-PN coacervates obtained during FRAP, (b) Corresponding FRAP kinetics. [PN] = 6 mM, [PLL] = 40 µM, [Cy5] = 0.02 mM. Scale bar = 5µm.

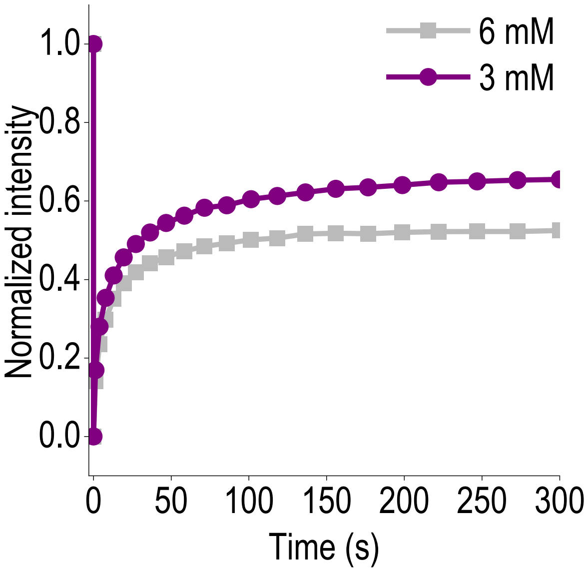

Figure S7: FRAP kinetics of PN-PLL coacervates at 3 mM PN and 6 mM PN with Cy5 dye (0.02 mM).

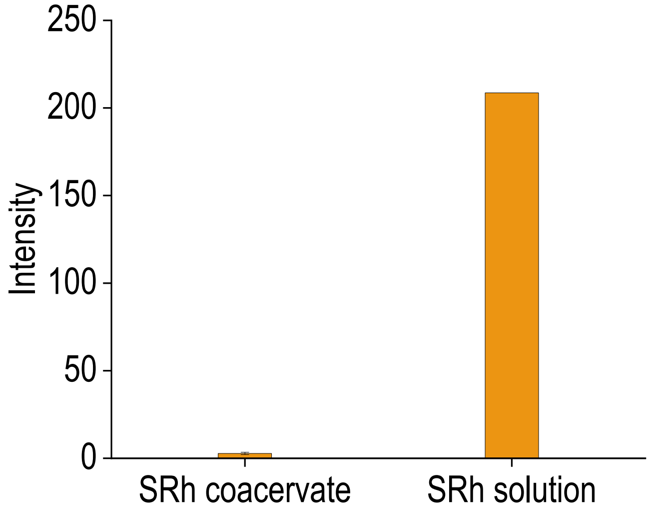

Figure S8: Comparison of SRh fluorescence intensity in PN-PLL coacervates and solution. [SRh] = 0.06 mM.

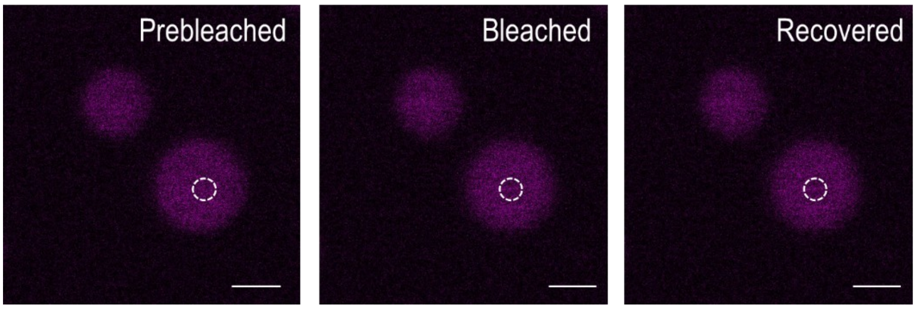

Figure S9: CLSM images of PLL-ATP coacervates obtained during FRAP. [ATP] = 5 mM, [PLL] = 15 μM, [Cy5] = 0.02 mM. Scale bar = 5μm.

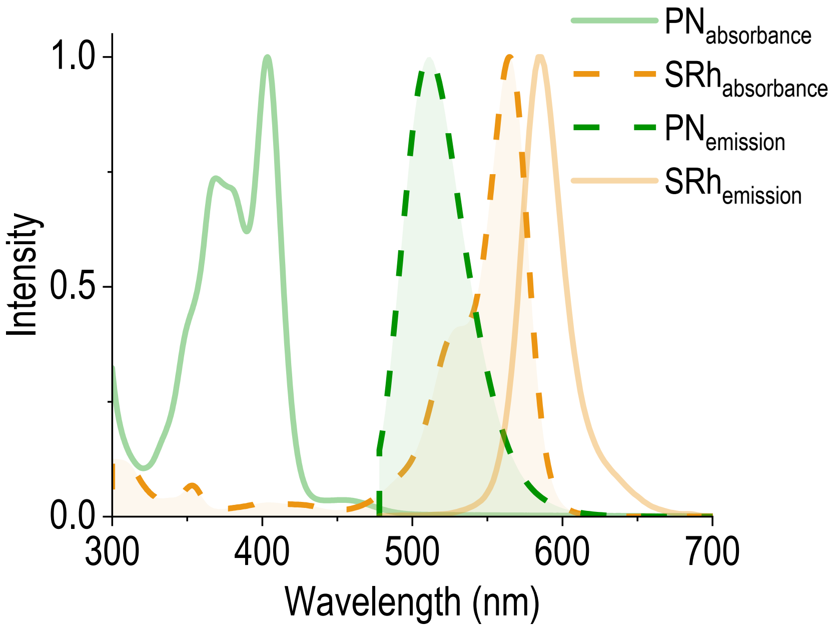

Figure S10: Absorbance and emission spectra of PN and SRh showing the spectral overlap between PN emission and SRh absorbance. [PN] = [SRh] = 0.3 mM.

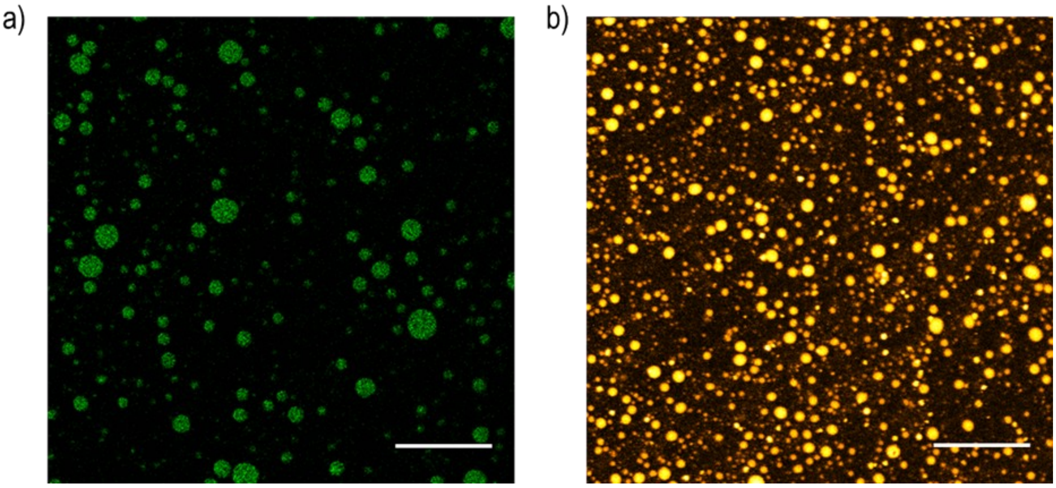

Figure S11: CLSM images of PN-PLL coacervates encapsulating (a) PN, λ_{ex} = 458 nm and (b) SRh, λ_{ex} = 561 nm. [PN] = 3 mM, [SRh] = 0.06 mM. Scale bar = 20µm.

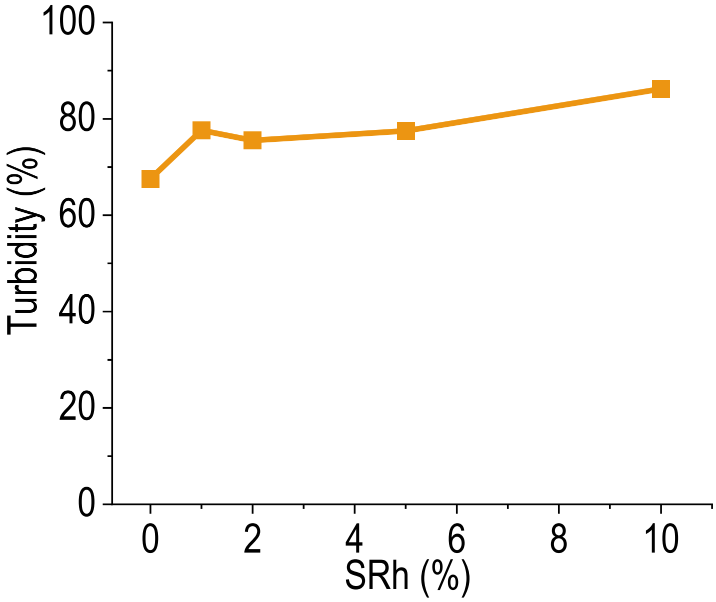

Figure S12: Turbidity measurements of PN–PLL coacervates with increasing concentrations of SRh (0.03 to 0.15 mM).

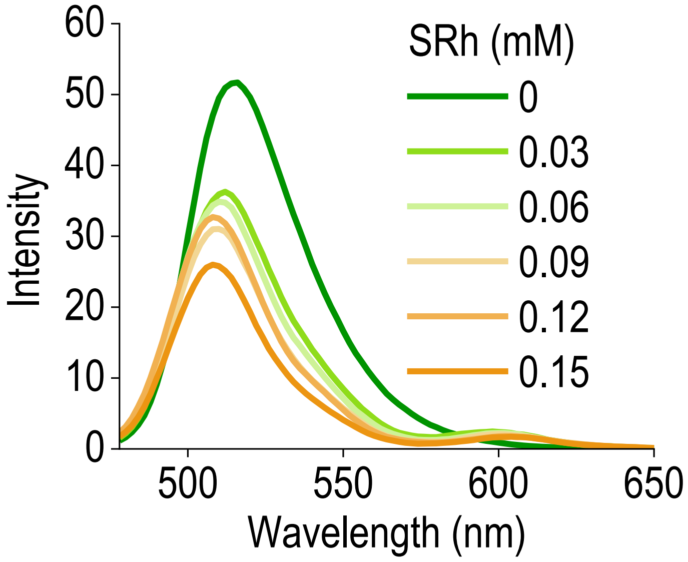

Figure S13: Fluorescence spectra of PLL–PN coacervates with increasing concentrations of SRh (0–0.15 mM), showing quenching of PN emission, λ_{ex} = 458 nm.

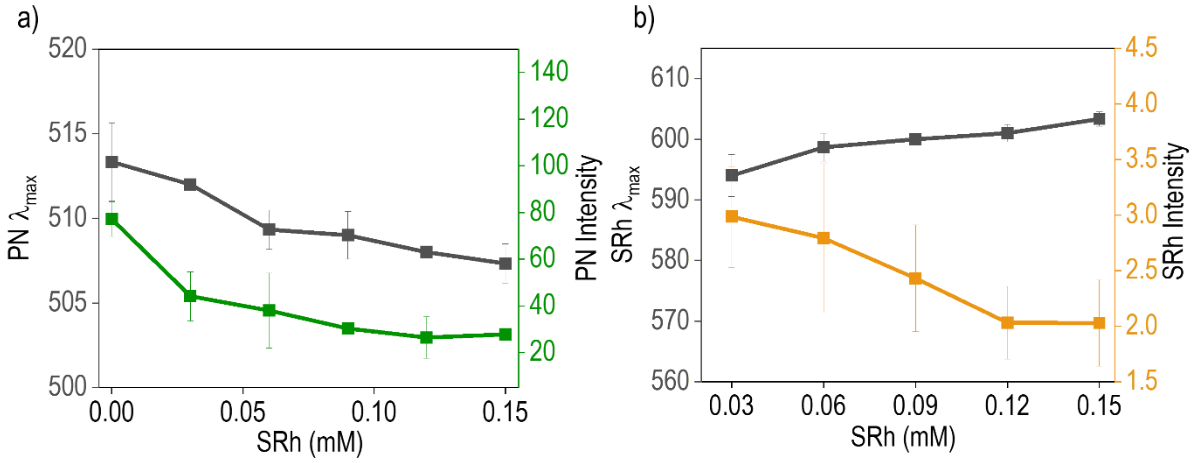

Figure S14: (a) Change in PN emission and λ_{max} with increasing concentration of SRh in the coacervate state. (b) Change in SRh emission and λ_{max} with increasing concentration of SRh in the coacervate state. [PN] = 3 mM.

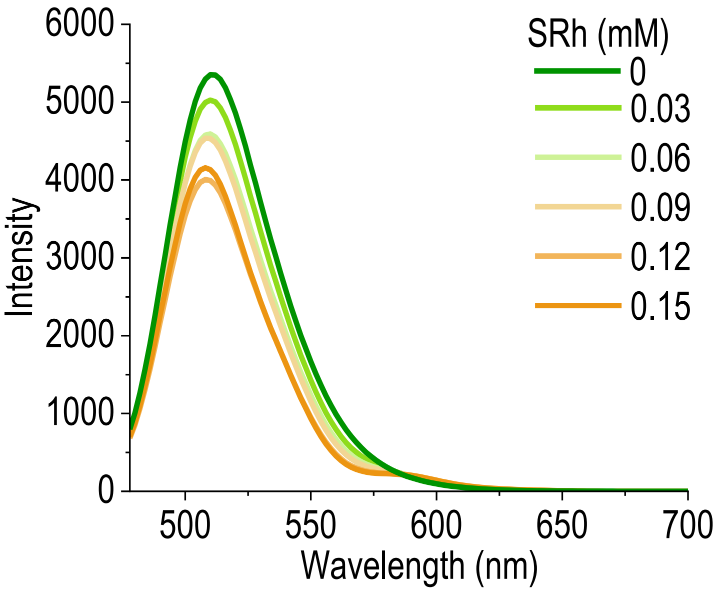

Figure S15: Fluorescence spectra of PN with increasing concentrations of SRh (0–0.15 mM), λ_{ex} = 458 nm in solution. [PN] = 3 mM.

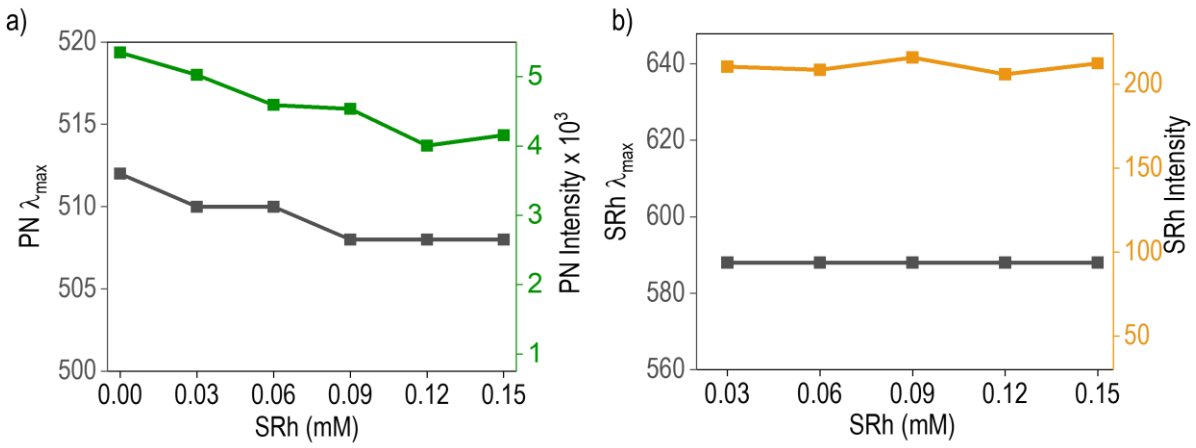

Figure S16: (a) Change in PN emission intensity and λ_{max} with increasing concentration of SRh in the solution. (b) Change in SRh emission intensity and λ_{max} with increasing concentration of SRh in the solution. [PN] = 3 mM.

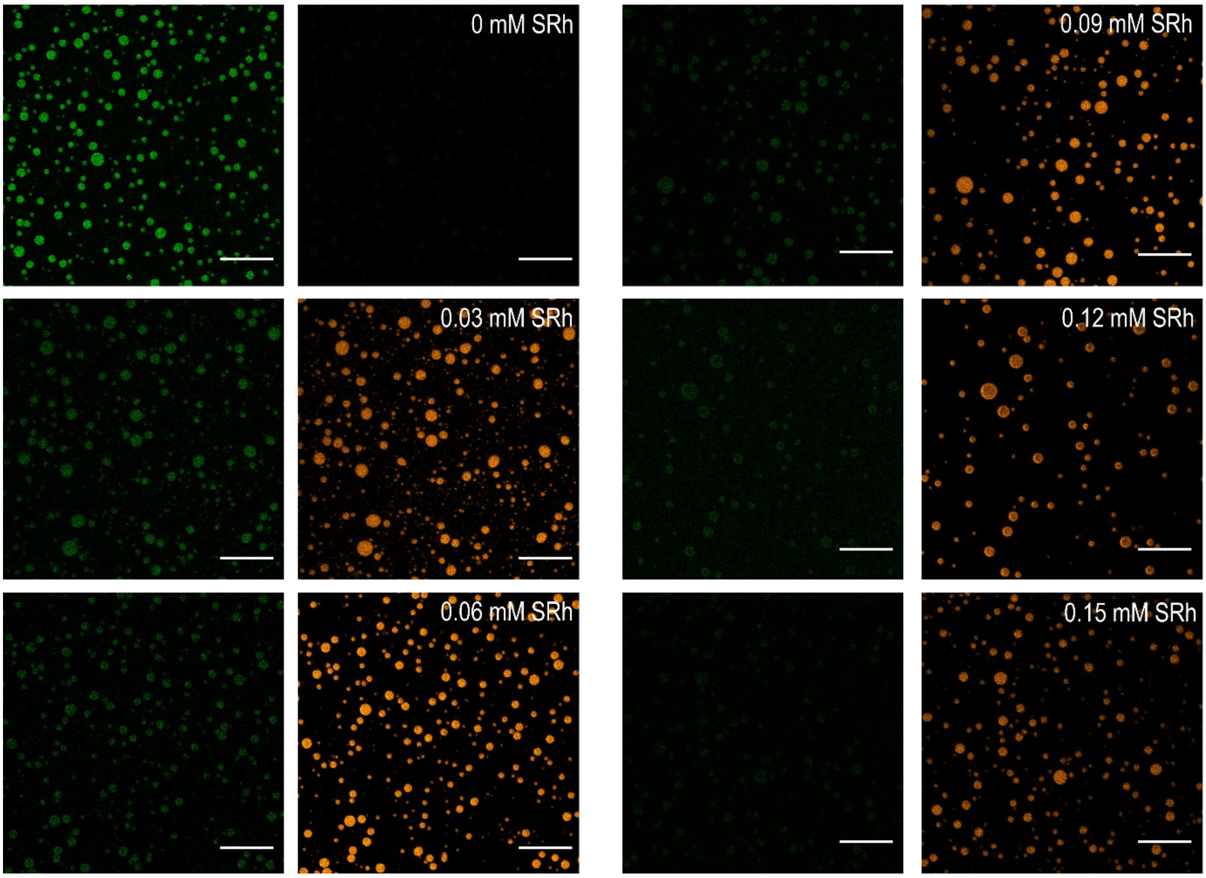

Figure S17: Two channel CLSM images of PN–PLL coacervates with increasing concentrations of SRh, showing energy transfer evidenced by the decrease in PN emission with rising SRh concentration. Green = PN; Orange = SRh. [PN] = 3 mM; [SRh] = 0–0.15 mM, λ_{ex} = 458, Scale bar = 20 µm.

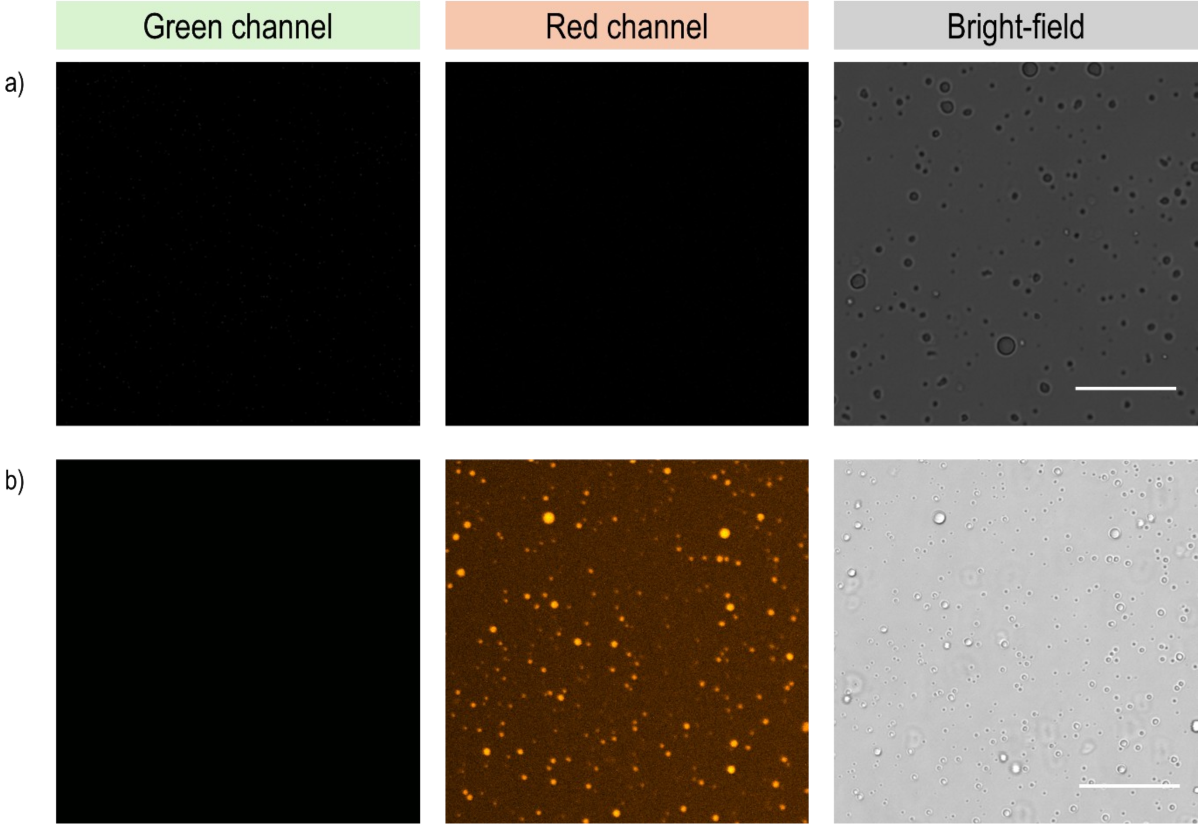

Figure S18: (a) CLSM images of PLL–ATP coacervates encapsulating SRh under indirect excitation at λ~~ex~~ = 458 nm showing no SRh emission, indicating the absence of energy transfer in the absence of PN. (b) CLSM images under direct excitation of SRh λ_{ex} = 561 nm showing characteristic SRh emission. Green channel = PN, Red channel = SRh. [SRh] = 0.06 mM, Scale bar = 20 µm.

4. References:

1. Mokumar16/Dual-channel-intensity-calculation https://github.com/Mokumar16/Dual-channel-intensity-calculation
2. T. Xiao, L. Zhang, H. Wu, H. Qian, D. Ren, Z. Y. Li, X. Q. Sun, *Chem. Commun.,* 2021,**57**, 5782-5785.
3. K. K. Nakashima, M. H. I. van Haren, A. A. M. André, I. Robu, E. Spruijt, *Nat Commun*, 2021, **12**, 38.