Supplementary Information

Protein migration via dissolution of ATP-stabilized condensate in gradient of AMP

Shikha Shikha, Sakshi Juneja, Subhabrata Maiti*

Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Knowledge City, Manauli 140306, India.

*E-mail: smaiti@iisermohali.ac.in

1. Experimental Setup

MATERIALS AND METHODS

All commercially available reagents were used without additional purification. Adenosine-5'-Diphosphate Disodium salt from Tokyo Chemical Industry (TCI). Adenosine 5'-Monophosphate disodium salt (AMP-Na_2), Adenosine 5'-triphosphate disodium salt (ATP-Na_2), Bovine Serum Albumin (BSA), Fluorescein-5-Maelimide (F5M), were purchased from Sigma-Aldrich. Agarose from Himedia. 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), Polyethylene Glycol (PEG9000), Magnesium Chloride ($MgCl_2$), Rhodamine B were purchased from Sisco Research Laboratory (SRL).

UV-Visible Spectroscopy

A Varian Cary 60 spectrophotometer (Agilent Technologies) was used to conduct UV-Vis experiments. The total volume was 500 µL, and a cuvette with a path length of 1 cm was employed throughout the studies. All measurements have been performed at 25 ^{o}C. Milli-Q (Merck Millipore) type-I water was used for all the experiments.

General sample preparation

The stock solutions were prepared either by weighing or by UV-visible spectroscopy using their molar extinction coefficients. In water: ε_{280} = 43,824 $M^{-1}cm^{-1}$ for BSA; ε_{260} = 15,400 $M^{-1}cm^{-1}$ for AMP, ADP & ATP; ε_{380} = 16,000 $M^{-1}cm^{-1}$ for CNP; ε_{400} = 75 $M^{-1}cm^{-1}$ for CNP in demountable cuvette; ε_{405} = 1300 $M^{-1}cm^{-1}$ for CNP in hybridization chamber; ε_{550} = 106,000 $M^{-1}cm^{-1}$ for Rhodamine B; ε_{495} = 83000 $M^{-1}cm^{-1}$ for Fluorscein-5-Maelimide.

Preparation of F5M-tagged BSA

BSA was labelled with Fluorescein Maleimide (F5M) following a previously reported procedure [Ref 2]. A 50 µM BSA solution was prepared at room temperature in 10 mM HEPES buffer (pH = 7.0). 40 mM of F5M stock solution was prepared in DMSO (fresh F5M solution has always been used). 250 µM of F5M solution was added to the BSA solution and was kept at room temperature for 3 hrs. After that, the conjugation was purified using Milli-Q water and gel filtering (Sephadex G-25). The fractions mentioned above were subjected to UV scan spectra, and it was found that BSA-F5M showed peaks at 280 nm for BSA and 495 nm for F5M. The following formula was used to determine the bioconjugate's dye/protein ratio (D/P) = $(A_{max}/\varepsilon_{dye}) \times (\varepsilon_{protein}/A_m)$, where $A_m = A_{280} - c.A_{max}$ (for the BSA-F5M conjugate). Here, A_{280} is the absorbance of the BSA-F5M conjugate at 280 nm; A_{max} is the absorbance of the conjugate at the absorption maximum of the corresponding F5M at 495 nm; c is the correction factor for the BSA-F5M conjugate; and ε_{prot} and ε_{dye} are the molar extinction coefficients for the protein and the F5M, respectively.

Synthesis of 5-nitrobenzisoxazole

According to the literature, it has been synthesized [Ref. 1]. At room temperature, 5 ml of concentrated sulfuric acid was used to dissolve 0.5 g of 1,2- benzisoxazole. The concentrated sulfuric acid (0.2 mL) and concentrated nitric acid (0.6 mL) were then mixed, and 0.5 mL was added to the mixture while stirring for 40 minutes at room temperature. This mixture was added to a 20 mL mixture of ice and water, which was continuously swirled and thawed for ten minutes. The white precipitate was filtered, cleaned with cold ethanol, dried under high vacuum, and then recrystallized in absolute ethanol to obtain a white, crystalline solid.

Synthesis of 2-cyano-4-nitrophenol

According to the literature [Ref. 1], it has been synthesized. After preparing a solution of 5-nitrobenzisoxazole (0.1 g) in ethanol (2 mL) and water (1 mL), 3 mL of 2 M NaOH was added, and the mixture was let to stand for ten minutes. In order to bring the pH of the solution to 1, HCl was added gradually while being continuously stirred. Dichloromethane was then used to extract the solution, and a high vacuum was used to dry it. The product was a solid that was pale yellow in colour.

Fluorescence Microscopy

A Zeiss Axis Observer 7 microscope equipped with AxioCam 503 Mono 3 Mega pixels and ZEN 2 software was used to record the fluorescence microscopic images.

Confocal microscopy

A ZEISS LSM 980 Elyra 7 super-resolution microscope with a 63× oil-immersion objective (NA 1.4) and an Airyscan 2 detector with 32 GaAsP channels was used for the confocal imaging. The AxioCamMRm Rev.3 FireWire(D) high-resolution monochrome-cooled camera was utilized. A 488 nm excitation laser and a 519 nm emission filter were used to image samples containing F5M-tagged BSA. ZEN Blue 3.2 software was used to analyze the images. ImageJ software was used to analyze the fluorescence intensity of confocal images containing F5M. A ZEISS Axio Observer 7 microscope with an AxioCam 503 Mono 3 Megapixel camera and ZEN 3 software was used for fluorescent microscopy. An excitation wavelength of 488 nm was used to image F5M tagged BSA (λ_{em} = 530 nm). For FRAP, a circular region with diameter 0.6 to 1 µm was selected for bleaching inside coacervate droplets, for which a 488 nm laser diode was used on FITC-tagged proteins. Fluorescence recovery curves were normalized, background-corrected, plotted and fitted using the Origin software.

Synthesis of BSA PEG Condensates

BSA-PEG condensates were prepared by mixing 25 µM of BSA and 20 % (w/v) PEG9000 followed by the addition of 1 mM $MgCl_2$ in 10 mM HEPES Buffer (pH = 7). After mixing all the constituents, the solution became turbid, condensates formed within 30 s, and condensate formation was confirmed by microscopy. We used F5M tagged BSA for a confocal microscopy study.

Turbidity measurement

Turbidity was measured using a UV-visible spectrophotometer. 500 µl of the sample was used for performing all the turbidity measurements at 492 nm. The study employed 25 µM BSA and 20% (w/v) PEG in 10 mM HEPES buffer with varying amounts of AMP, ADP, ATP, and Mg^{2+}.

Kinetics Study

We incubated three samples of volume 1000 µl that contains 25 µM BSA, 20% (w/v) PEG, 1mM $MgCl_2$ & 250 µM NBI with (a) Buffer (b) 1mM AMP (c) 1mM ATP in 10 mM HEPES Buffer (pH = 7) then we measured the CNP formation at 380 nm after 1 min, 15 min, 30 min & 60 min. Before taking the reading the samples were diluted ten times to avoid any scattering due to turbidity.

Activity Measurement in Hybridization chamber

A Merilyzer EIAQuant plate reader was used for performing kinetics study in hybridisation chamber of dimension 20 mm diameter and 0.8 mm height the CNP formation was monitored at 405 nm. For this study 0.5 % (w/v) of three agarose gel were prepared that contains only Buffer, 50 mM AMP & 50 mM ATP respectively. Two gels were placed at a distance of 10 mm and CNP formation was measured near the gel till 8 hours.

Fig. S1: Representative setup showing the hybridisation chamber used in the turbidity & kinetics study having diameter 20 mm and height 0.8 mm.

Activity Measurement in Demountable cuvette

A Merilyzer EIAQuant plate reader was used for performing kinetics study in demountable cuvette of dimension 35 mm length and 0.05 mm height the CNP formation was monitored at 405 nm. We filled the bottom of the cuvette with 40µL of 250 µM NBI in Buffer or AMP in 10 mM HEPES Buffer (pH = 7) with 1mM Mg^{2+} and from the arm hole 15 µL of 25 µM BSA with 2 mM ATP, 1mM Mg^{2+} in 20 % PEG & 10mM HEPES buffer (pH = 7) was added and the data was recorded at two points one point near the armhole and another point which is away from the armhole and the distance between these two points is 1 cm.

Microfluidics Study

Microfluidics studies were performed using a syringe pump (World Precision Instruments) and microfluidics channels made up of PMMA material with dimension of 1.7 cm x 600 µm in length and width respectively.

For 2-inlet 1-outlet experiment, we injected 25 µM of BSA (10 % labelled + 90% unlabelled), 1 mM Mg^{2+} & 20% PEG in 10 mM HEPES Buffer (pH=7) through the top inlet and different concentrations of ATP or AMP with 1 mM Mg^{2+} & 20% PEG in 10 mM HEPES Buffer (pH=7) was injected through bottom inlet using syringe pump. For each inlet flow speed was 150 µL/h. We calculated Fluorescence intensity profile data using ImageJ software.

For 3-inlet 1-outlet experiment, we injected 25 µM of BSA (10 % labelled + 90% unlabelled), 1 mM Mg^{2+} & 20 % PEG in 10 mM HEPES Buffer (pH = 7) through the middle inlet and different concentrations of AMP with 1 mM Mg^{2+} & 20% PEG in 10 mM HEPES Buffer (pH=7) was injected through the top inlet & ATP with 1 mM Mg^{2+} & 20% PEG in 10 mM HEPES Buffer (pH=7) through the bottom inlet using the syringe pump. For each inlet flow speed was 150 µL/h. We calculated Fluorescence intensity profile data using ImageJ software.

Glass slide experiment

First, we centrifuged the BSA-condensate at 2000 rpm for 30 minutes to separate the condensed and dilute phases then we pipetted out 35ul of the dilute phase and placed it on the glass slide. The glass slide was covered with a cover slip so that the solution is evenly distributed. From one side of the cover slip we added 1ul of the condensed phase. The microscopic images were captured and analysed via ImageJ software.
Microscopic images were taken after 10 seconds and 120 seconds after the addition of the condensed phase from the side of the cover slip, as shown in the figure. We performed three set of experiments in which 1 µl condensed phase (BSA-ATP-Mg^{2+} condensate which is fluorescent due to presence of Rhodamine B (RhB)) was added from the side of the coverslip. On the glass slide added dilute phase consisted of (a) BSA-Mg^{2+} condensate (b) BSA-AMP-Mg^{2+} condensate (c) BSA-ATP-Mg^{2+} condensate. In these experiments, we observed that the drift of BSA-ATP-Mg^{2+} condensate is towards more dissolution-favored zone.
Drift velocity was calculated using Tracker software. The average and standard deviation particle velocity in AMP gradient was determined with 10 individual particles.

Tryptophan fluorescence emission

The spectra were recorded at an excitation wavelength of 280 nm and a slit width of 5 nm for both excitation and emission using an Agilent Cary Eclipse Fluorescence Spectrophotometer in a quartz cuvette with a fixed pathlength of 1 cm. 25 µM BSA, 1mM Mg^{2+} in 10 mM HEPES (pH = 7) with varying concentration of AMP, ADP, ATP, Adenosine, Na_2HPO_4 was used for this study.

2. **Turbidity analysis:**

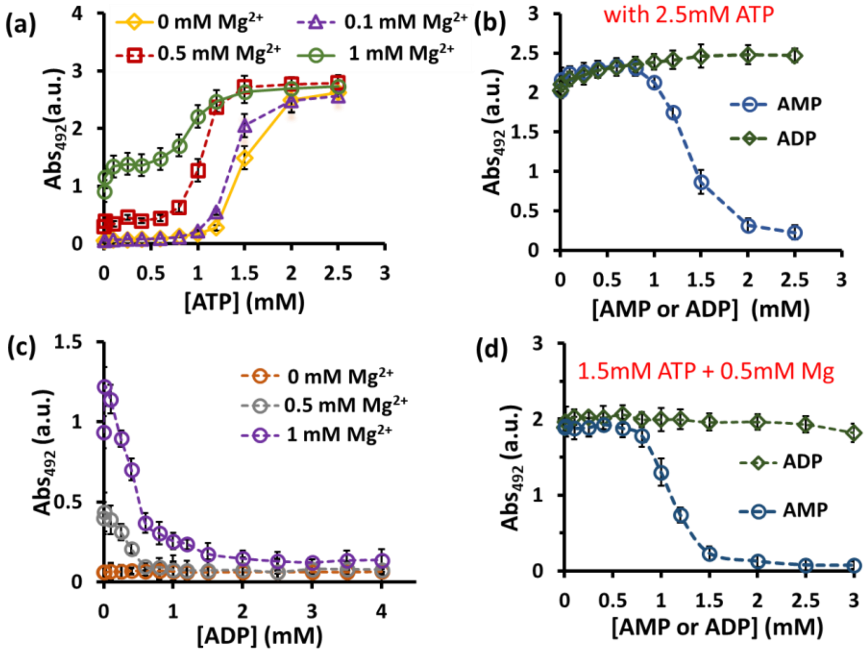


Fig. S2: Plots showing the turbidity of (a) BSA condensates as a function of ATP concentration (0 to 2.5 mM) in presence of fixed Mg^{2+} (0, 0.1, 0.5 and 1 mM); (b) BSA-ATP condensates with varying concentration of AMP and ADP; (c) BSA condensates with varying concentration of ADP and (d) BSA-ATP condensates (without PEG) with varying concentration of AMP & ADP. Experimental Conditions: [BSA] = 25 µM, [PEG] = 20 % (w/v) in 10 mM HEPES buffer, pH = 7 at 25 °C.

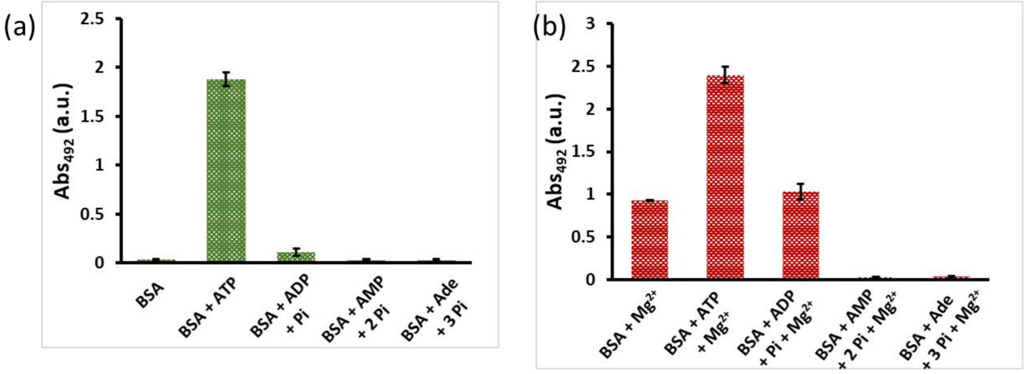


Fig. S3: Turbidity values in (a) absence and (b) presence of Mg^{2+} (1 mM). Experimental Conditions: [BSA] = 25 µM, [ATP] = [ADP] = [AMP] = [Ade] = [Pi] = 2 mM; [PEG] = 20 % (w/v) in 10 mM HEPES buffer, pH = 7 at 25 °C. 2 Pi and 3 Pi mean 4 and 6 mM Pi, respectively.

For keeping similar ionic strength, we use hydrolyzed product of ATP (2 mM) to ADP (2 mM) + Pi (4 mM) or AMP (2 mM) + Pi (4 mM) or Ade (2 mM) + Pi (6 mM). We found presence of specific ATP significantly increase the turbidity. In presence of Mg^{2+}, it is even higher. Whereas other combinations do not increase the turbidity values. Turbidity values is a qualitative measure of condensate formation.

Turbidity experiments with Lysozyme in PEG media

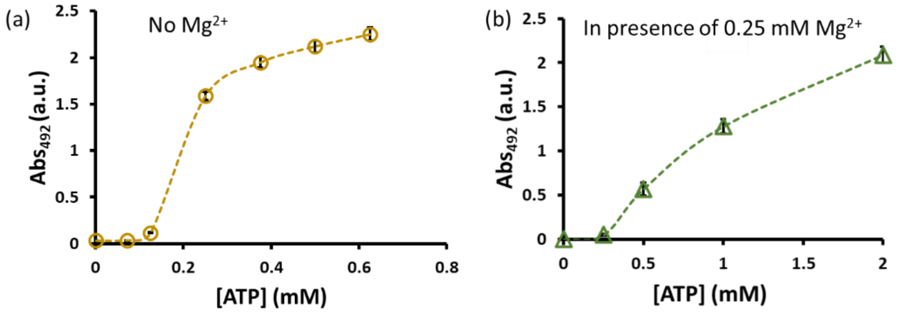


Fig. S4. Plot showing the Lysozyme condensates formation by varying the concentration of ATP in the (a) absence and (b) presence of Mg^{2+}. Experimental Conditions: [Lysozyme] = 50 µM, [PEG] = 20 % (w/v), [Mg^{2+}] = 250 µM in 10 mM HEPES buffer, pH = 7 at 25 °C.

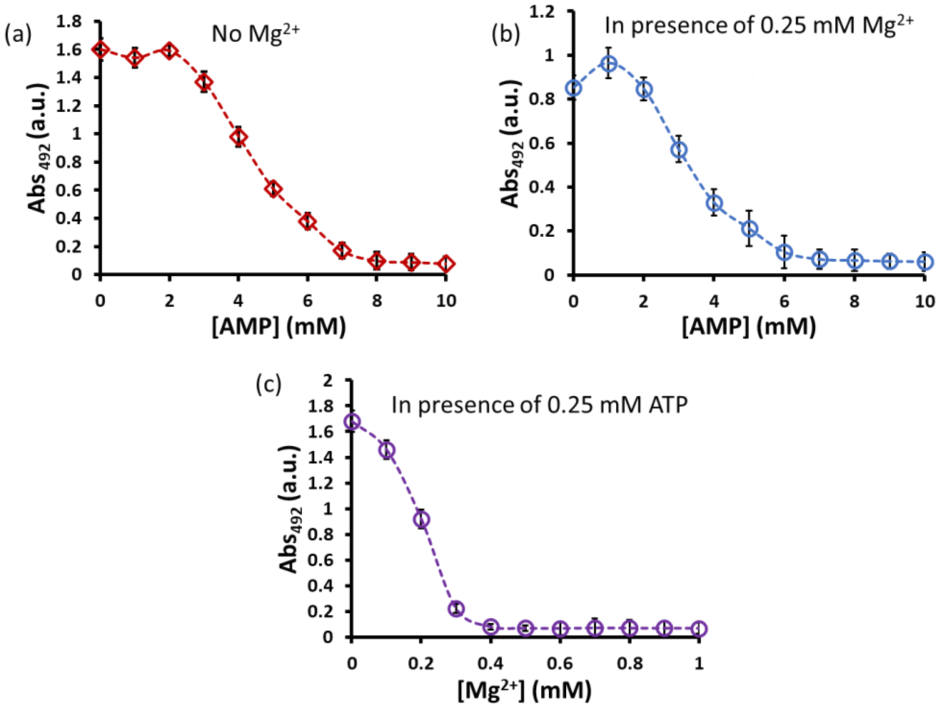


Fig. S5. Plot showing the Lysozyme condensates dissolution by varying the concentration of AMP in the (a) absence, (b) presence Mg^{2+} and (c) by varying the concentration of Mg^{2+} when ATP concentration is fixed at 0.5 mM. Experimental Conditions: [Lysozyme] = 50 µM, [PEG] = 20 % (w/v), [ATP] = 500 µM, [Mg^{2+}] = 250 µM in 10 mM HEPES buffer, pH = 7 at 25 °C.

Here also we observed ATP promotes LYS-condensate formation in 20% PEG 9000 (w/v) and the ATP-stabilized LYS-condensate dissolves in presence AMP. However, unlike BSA-condensate, LYS-ATP stabilized condensate dissolves with increasing Mg^{2+} concentration, probably due to the cationic nature of the protein as observed in Fig. S5c. Here turbidity of the solution decreased with increasing Mg^{2+}. At present, we have performed only turbidity study with LYS-condensate as the main work is related BSA-condensate. Microscopic characterization and physical nature of the LYS-condensate will be explored in future work.

3. Thioflavin T (ThT) Fluorescence Study

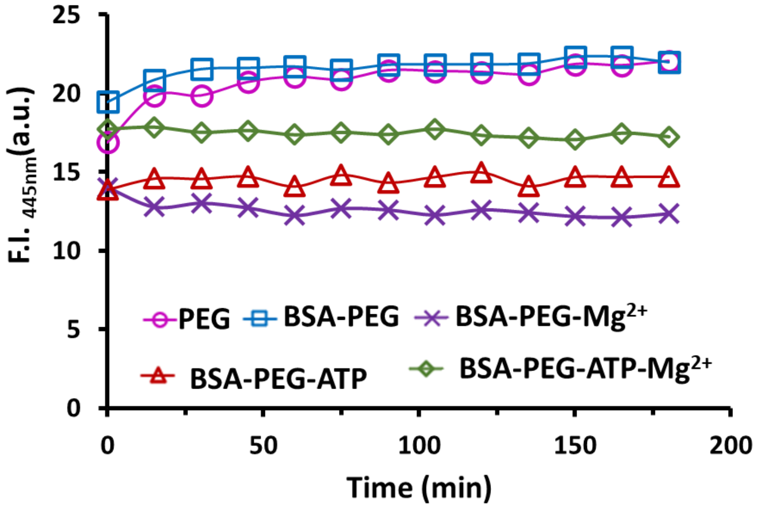


Fig. S6. Representative plot of ThT Fluorescence spectra. Experimental condition: [BSA] = 25 µM, ex = 385 nm, em = 445nm, ex/em slit width = 5/5 nm, [PEG] = 20 % (w/v), $[Mg^{2+}]$ = 1mM, in 10 mM HEPES buffer, pH = 7 at 25 °C.

4. Confocal Fluorescence Microscopic Images

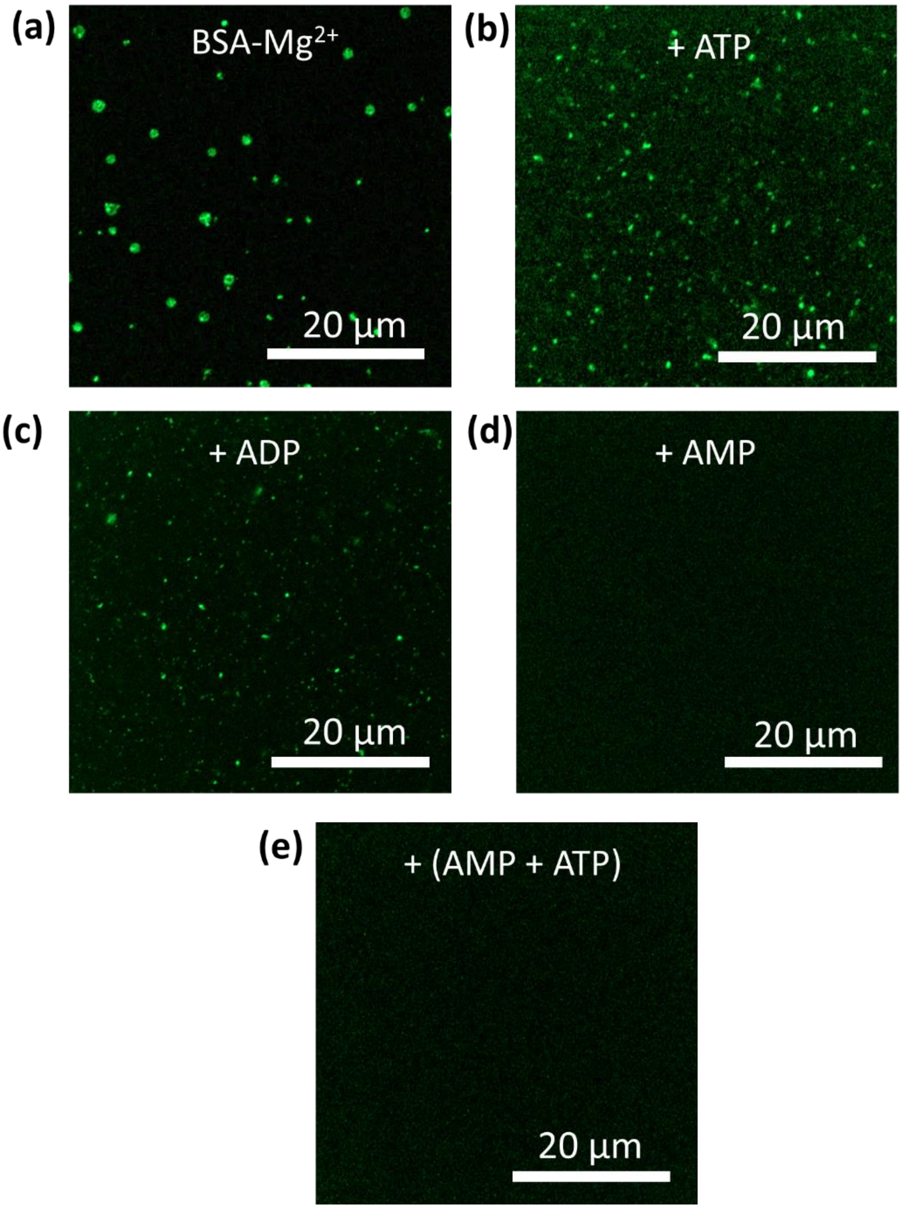
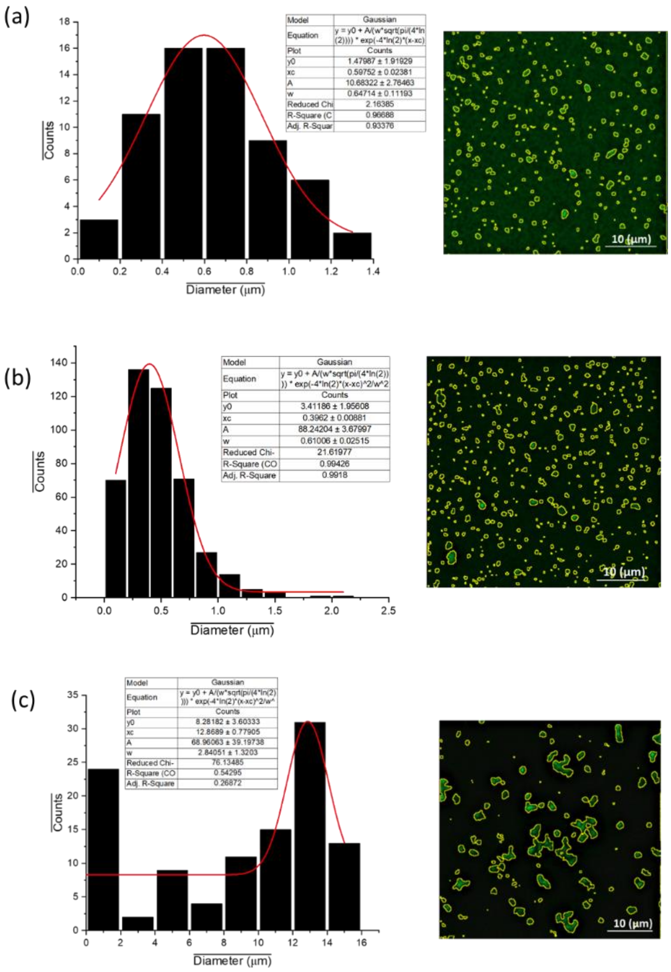


Fig. S7: Confocal fluorescence microscopic images of BSA-PEG-Mg^{2+} condensates in the presence of (a) Buffer only (b) ATP (c) (d) AMP (e) ATP & AMP. Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1mM, [ATP] = 0.5 mM, [ADP] = 1 mM, [AMP] = 1mM in 10 mM HEPES buffer, pH = 7 at 25 °C.



Model	Gaussian
Equation	y = y0 + A/(w*sqrt(pi/(4*ln(2)))) * exp(-4*ln(2)*(x-xc)
Plot	Counts
y0	1.47987 ± 1.91929
xc	0.59752 ± 0.02381
A	10.68322 ± 2.76463
w	0.64714 ± 0.11193
Reduced Chi	2.16385
R-Square (C	0.96688
Adj. R-Squar	0.93376

Model	Gaussian
Equation	y = y0 + A/(w*sqrt(pi/(4*ln(2)))) * exp(-4*ln(2)*(x-xc)^2/w^2
Plot	Counts
y0	3.41186 ± 1.95608
xc	0.3962 ± 0.00881
A	88.24204 ± 3.67997
w	0.61006 ± 0.02515
Reduced Chi-	21.61977
R-Square (CO	0.99426
Adj. R-Square	0.9918

Model	Gaussian
Equation	y = y0 + A/(w*sqrt(pi/(4*ln(2)))) * exp(-4*ln(2)*(x-xc)^2/w^
Plot	Counts
y0	8.28182 ± 3.60333
xc	12.8689 ± 0.77905
A	68.96063 ± 39.19738
w	2.84051 ± 1.3203
Reduced Chi-	76.13485
R-Square (CO	0.54295
Adj. R-Square	0.26872

Fig. S8. Histogram representing the size distribution pattern for the corresponding confocal images of BSA-PEG-Mg^{2+} condensates in the presence of (a) Buffer only (b) 1mM ATP (c) 5mM ATP. Size distribution of the BSA-PEG-Mg^{2+} condensates was first analysed by the ImageJ software and the corresponding size distribution histogram were plotted using Origin software. Experimental Conditions: [BSA] = 25 μM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1mM, [ATP] = 1 and 5 mM, in 10 mM HEPES buffer, pH = 7 at 25 °C.

The size distribution histogram was plotted using a Gaussian Function, given by:

$$y = y_0 + \frac{A}{w\sqrt{\frac{\pi}{4\ln 2}}} \exp\left[-\frac{4\ln 2\,(x - x_c)^2}{w^2}\right]$$

Here, x_c = mean particle diameter; A = area under the curve; Y_0= baseline offset; w = Full width half Maxima

Here, we find in presence of 1 mM ATP, number density of BSA-Mg^{2+} condensates increased by ~8-fold, although dimension remains unaltered and in presence of 5 mM ATP, the condensates merged and increased in size.

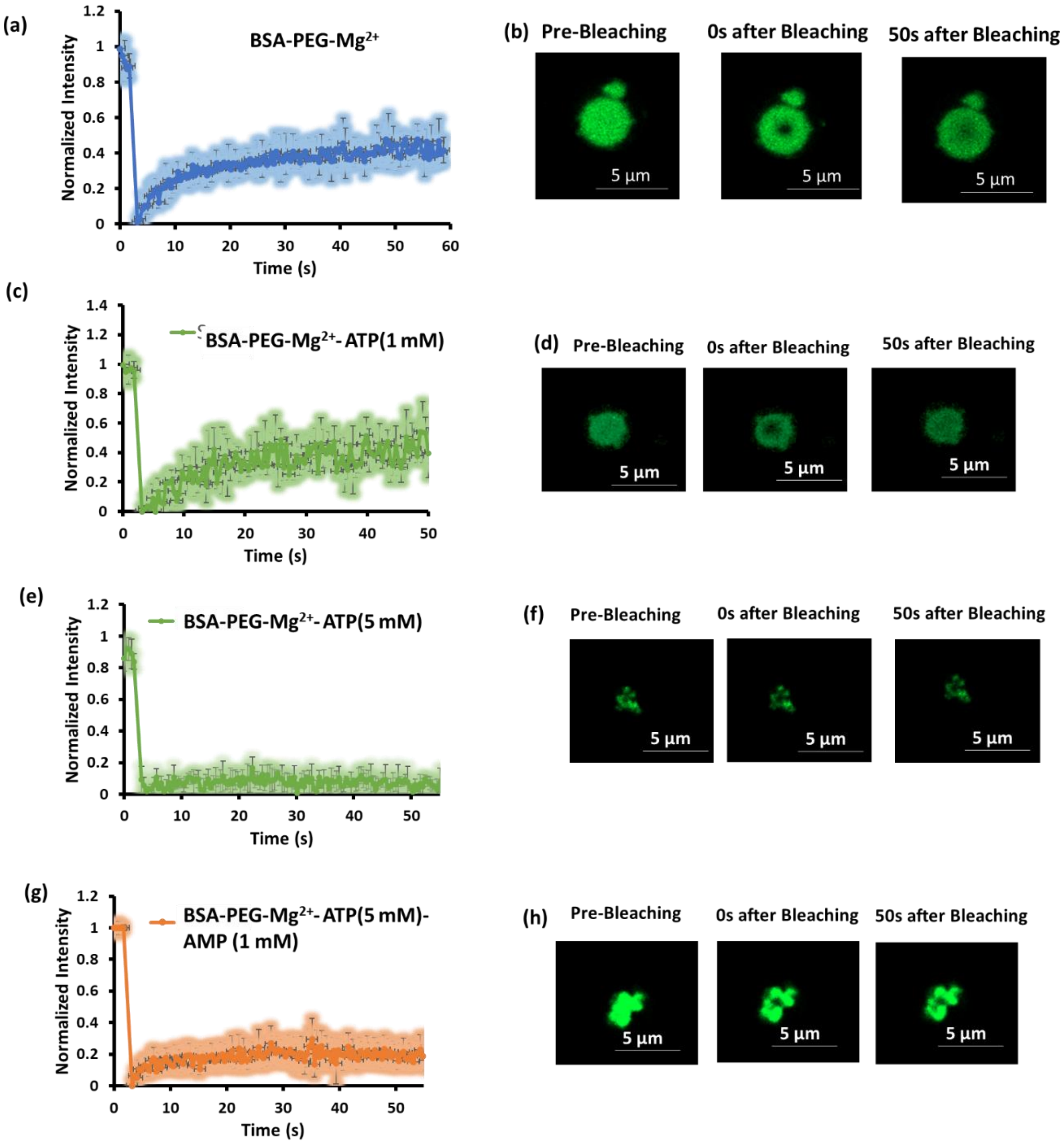
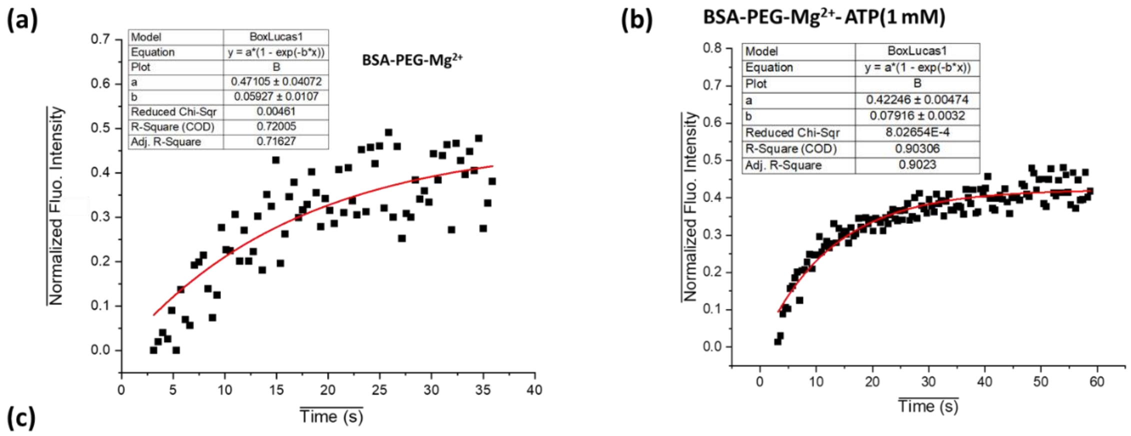


Fig. S9. Fluorescence recovery plot and representative images during the FRAP experiments done on BSA-PEG-Mg^{2+} condensates in the presence of (a,b) only buffer; (c,d) 1 mM ATP; (e,f) 5 mM ATP and (g,h) 5 mM ATP + 1 mM AMP Scale bar: 5 µm Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), 10 mM HEPES buffer, pH = 7 at 25 °C.



(c)

	b (s^{-1})	r (µm)	τ (s)	D_{app} (µm^2/s)
BSA-PEG-Mg^{2+}	0.05927 ± 0.01	0.30303	16.87	0.005442 ± 0.000637
BSA-PEG-Mg^{2+}- ATP(1 mM)	0.07916 ± 0.003	0.29275	12.63	0.006784 ± 0.000908

Fig. S10. (a-c) Calculated D_{app} values corresponding to the recovery plots in (a) and (c). D_{app} values were obtained by fitting the normalized FRAP data against the exponential equation with recovery function, $f(t) = A\,(1-e^{-t/\tau})$, which was used previously in the literature (*Commun. Chem.*, 2024, **7**, 242; *Proc. Natl Acad. Sci. USA*, 2015, **112**, 7189) Here, τ is the recovery timescale, from which D_{app} was calculated using the formula: $D_{app} \approx r^2/\,\tau$.

5. Microfuidics Experiments

1. In two-inlet Channels

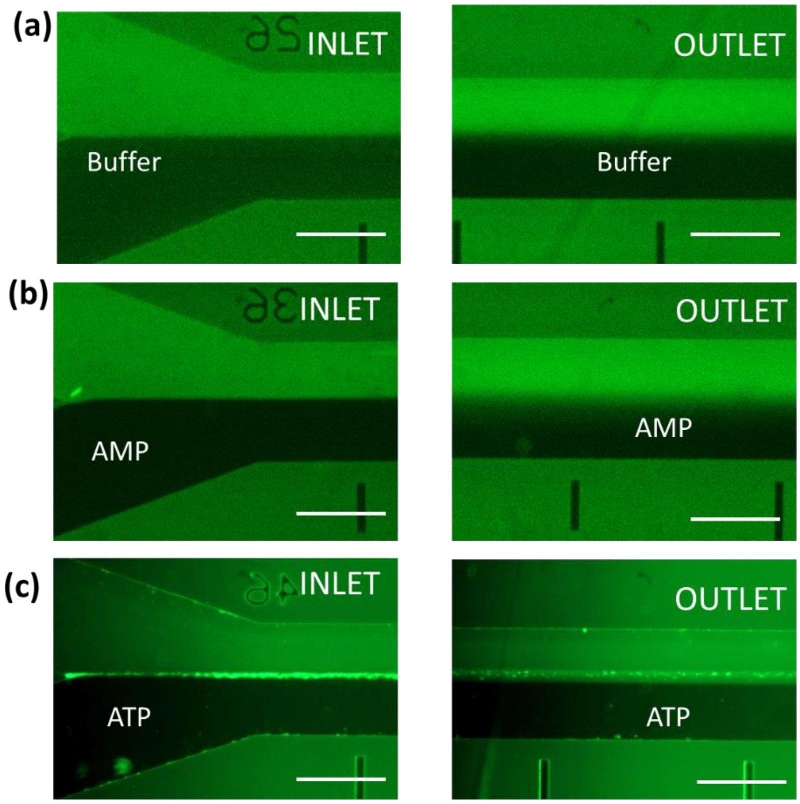


Fig. S11: Fluorescence microscopic images of 2-inlet 1-outlet microfluidics channel in which BSA-PEG-Mg^{2+} condensates were injected through the top inlet and from the bottom inlet (a) PEG-Mg^{2+} (b) AMP-PEG-Mg^{2+} (c) ATP-PEG-Mg^{2+} was injected. Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1mM, [ATP] = 5 mM, [AMP] = 5 mM, Flow rate = 150 µL/hr in 10 mM HEPES buffer, pH = 7 at 25 °C. (Scale bar-350 µm)

2. In three-inlet channels

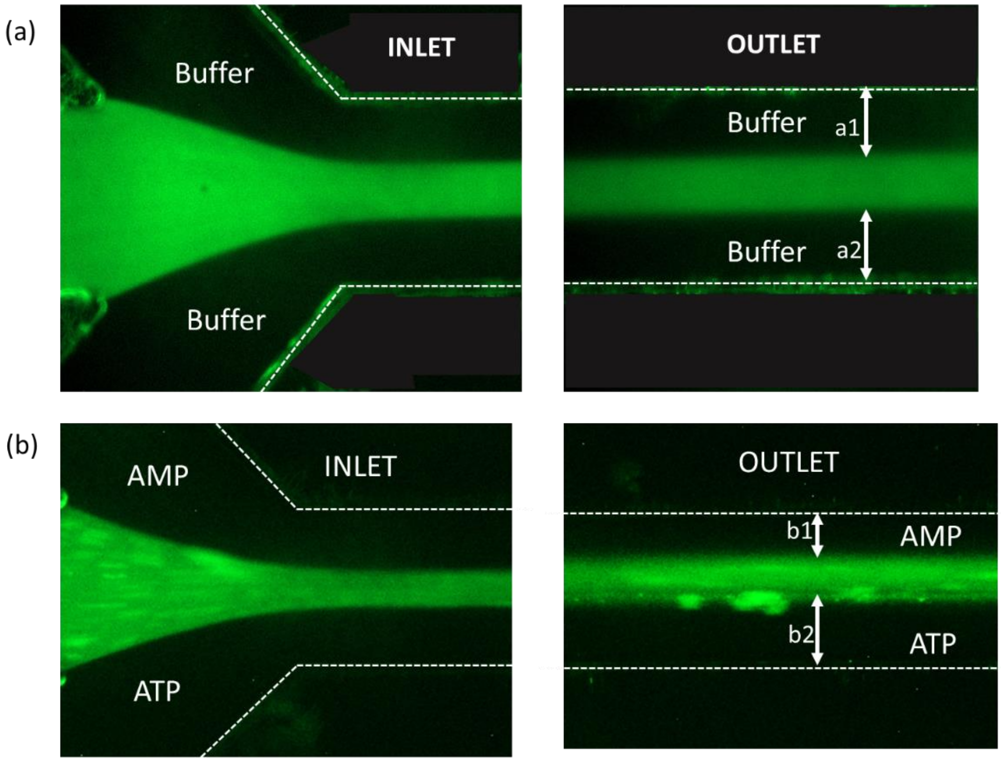


Fig. S12: Fluorescence microscopic images showing the 3-inlet 1-outlet microfluidics channel in which BSA-PEG-Mg2+ condensates were injected through the middle inlet and from the top & bottom inlet (a) only buffer and (b) AMP-PEG-Mg^{2+} & ATP-PEG-Mg^{2+} were injected respectively. Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1mM, [ATP] = 5 mM, [AMP] = 5 mM, Flow rate = 150 µL/hr in 10 mM HEPES buffer, pH = 7 at 25 °C. Channel width = 0.6 mm.

Herein, we observed the drift of BSA towards AMP side, suggesting dissolution-based migration of BSA and BSA-ATP condensate. In figure a, in case of buffer, in the outlet arrow a1 and a2 have almost equal width; whereas for panel b, the outlet arrow b1 is significantly smaller than b2; suggesting fluorescent protein migration towards AMP side. After considering (a1 – b1), the drift is around ~80 µm towards AMP side.

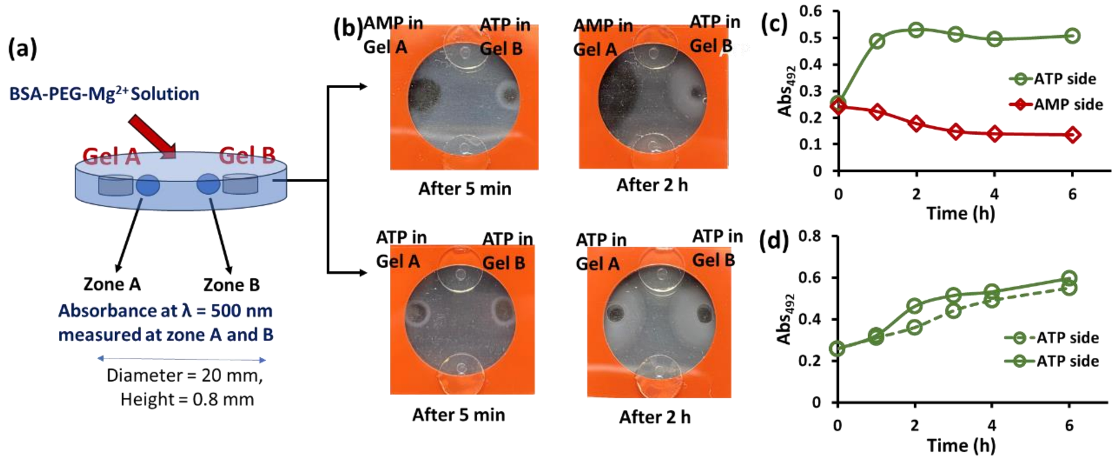


Fig. S13. a) Schematic representation of turbidity study inside the hybridisation chamber near the AMP or ATP gel. b) Representing the hybridisation chamber with (AMP, ATP) & (ATP, ATP) gel in the presence of BSA-PEG-Mg^{2+}solution. (c and d) are the representative plots of turbidity near the AMP & ATP gel. Experimental Conditions: [BSA] = 25 µM, [PEG] = 20 % (w/v), [Mg^{2+}] = 1 mM, [ATP] = 50 mM, [AMP] = 50 mM, [Agarose gel] = 0.5 % (w/v) in 10 mM HEPES buffer, pH = 7 at 25 °C.

Herein, we used a secure seal hybridization chamber of 20 mm diameter and 0.8 mm height, and we added two gels (made up of agarose, volume = 10 µl) at two ends of the hybridization chamber, as shown in Figure S8a. In one case, gel A was filled with AMP, and gel B with ATP while in the other case, both gel A and B were filled with ATP (Figure S8b). We also performed one experiment with both gel AMP. Then the BSA-Mg^{2+} condensate in PEG solution (~200 µl) was added to the chamber to fill the space. Nucleotides from the gel will leach into the PEG solution, creating a gradient near the gel.[S1] Only the BSA-Mg^{2+} solution is slightly turbid with a turbidity value ~0.22 at 492 nm in our experimental set up. We measured the turbidity of the solution with time near gels A and B by keeping the hybridization chamber inside a plate reader. Interestingly, we observed that near the AMP gel, the turbidity was decreasing, and near ATP-containing gel, the turbidity was increasing with time. This experiment provides a visual proof of our system that in the AMP-rich zone, BSA-condensate dissolves, and in the ATP-rich zone, it becomes more condensed.

6. Steady-state Fluorescence Experiment

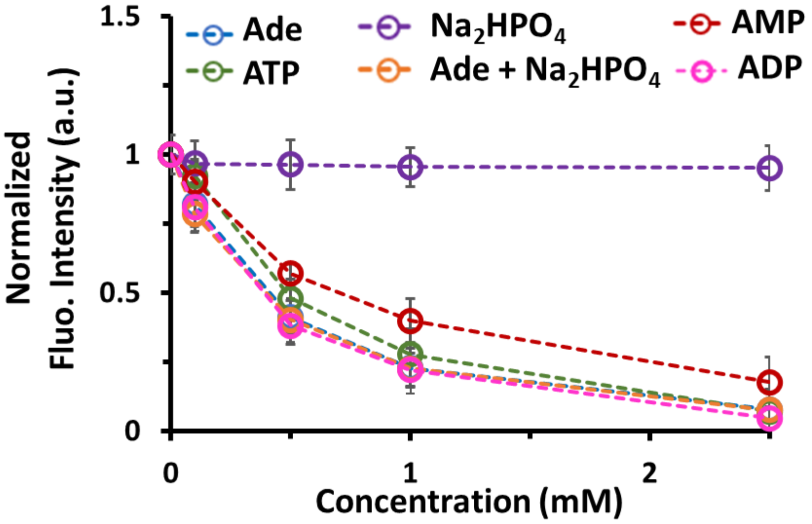


Fig. S14: Representative plot showing the fluorescence spectra of tryptophan emission of BSA excited at 280 nm wavelength measured with different concentrations of AMP, ADP, ATP, Adenosine and phosphates. Experimental condition: [BSA] = 25 µM, ex = 280 nm, ex/em slit width = 5/5 nm, [AMP] = 0.1, 0.5, 1, 2.5 mM, [ADP] = 0.1, 0.5, 1, 2.5 mM, [ATP] = 0.1, 0.5, 1, 2.5 mM, [Adenosine] = 0.1, 0.5, 1, 2.5 mM, [Na_2HPO_4] = 0.1, 0.5, 1, 2.5 mM, [$MgCl_2$] = 1 mM in 10 mM HEPES buffer, pH = 7 at 25 °C.

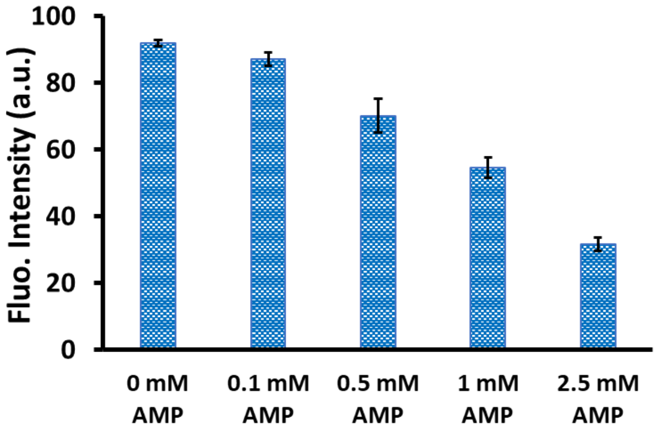


Fig. S15: Representative bar diagram showing the tryptophan emission of BSA at excitation wavelength of 280 nm. We observed that in BSA-ATP complex, the emission intensity decreases on increasing the concentration of AMP. Experimental condition: [BSA] = 25 µM, ex = 280 nm, ex/em slit width = 5/5 nm, [AMP] = 0.1, 0.5, 1, 2.5 mM, [ATP] = 1 mM, [$MgCl_2$] = 1 mM in 10 mM HEPES buffer, pH = 7 at 25 °C.

7. BSA-ATP-Mg^{2+} condensate drift analysis under static conditions

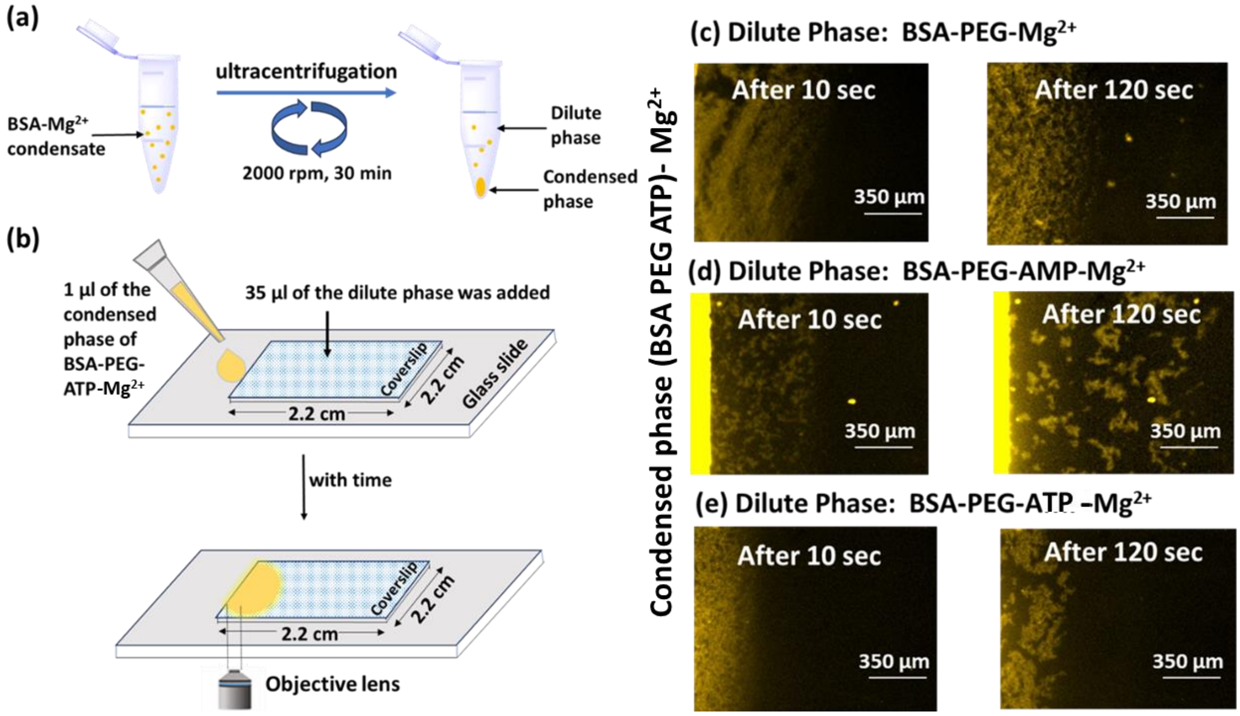


Fig. S16: Schematic representation of (a) ultracentrifugation experiment to get the dilute and condensed phase (b) experimental setup for studying the drift of condensed phase in the presence of dilute phase (dilute phase is under the coverslip & condensed phase added from the side of the coverslip. Fluorescence microscopic images showing the movement of condensed phase under the coverslip in the presence of different dilute phase (c) BSA-PEG-Mg^{2+} (d) BSA-PEG-AMP-Mg^{2+} (e) BSA-PEG-ATP-Mg^{2+}. Experimental Conditions: [BSA] = 25 µM, [PEG] = 20 % (w/v), [ATP] = 5 mM, [Mg^{2+}] = 1 mM, [AMP] = 5 mM, [RhB] = 1 µM in 10 mM HEPES buffer, pH = 7 at 25 °C.

Here, firstly, we centrifuged the BSA-condensate, either made of ATP, AMP, or buffer. We added the dilute phase (35 µl) on top of a glass slide and covered it with (2 cm x 2 cm) coverslip. Therefore, the height of the fluid was ~0.07 mm under the slide. Then, we used another set of BSA-ATP-Mg^{2+} condensate samples which were added to and doped with Rhodamine B (RhB), and centrifuged. We collected the condensed phase, which is fluorescent due to the presence of RhB. Next, 1 µl of the condensed phase of BSA-ATP-Mg^{2+} condensate was added to the side of the coverslip. After that, we observed the migration of the protein condensate inside the coverslip. We experimented three times and, in each case, we found fragmented condensate spread more when the dilute phase of the BSA-AMP-Mg^{2+} solution was added at the bottom of the coverslip. The spreading is least for the dilute phase of BSA-ATP-Mg^{2+}-condensate. Overall, both experiments clearly suggested that dissolution of BSA-ATP condensate results in motion of condensate fragments towards a more dissolution-favored zone.

8. Fluorescence microscopic images of demountable cuvette experiment

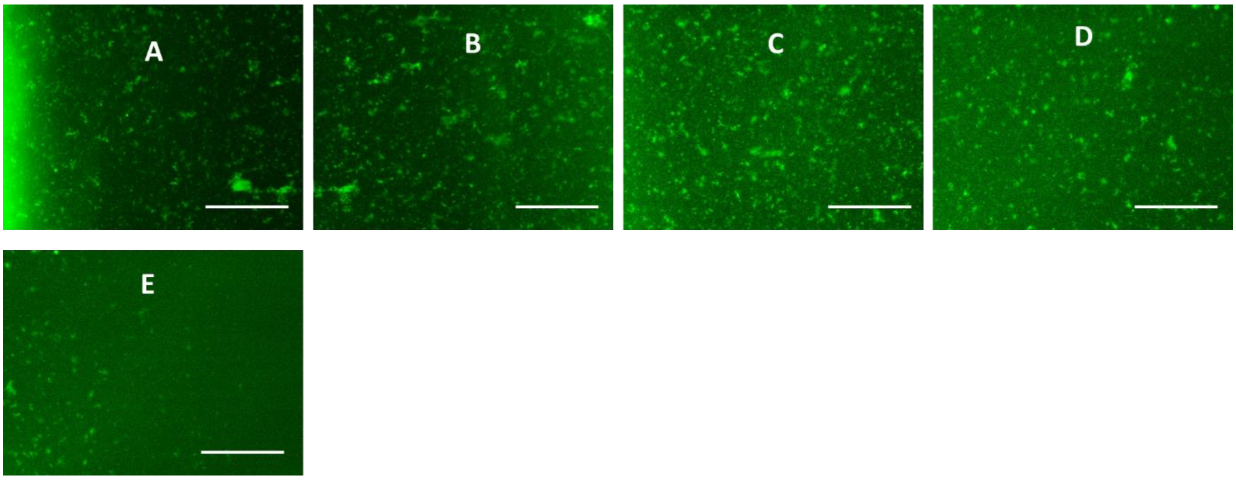


Fig. S17: Fluorescence microscopic images of demountable cuvette experiment in which drift of BSA-PEG-ATP-Mg^{2+} condensates was calculated after 10 minutes in the presence of (b) only PEG-Mg^{2+}. Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1mM, [ATP] = 2.5 mM in 10 mM HEPES buffer, pH = 7 at 25 °C. (Scale bar: 200 µm)

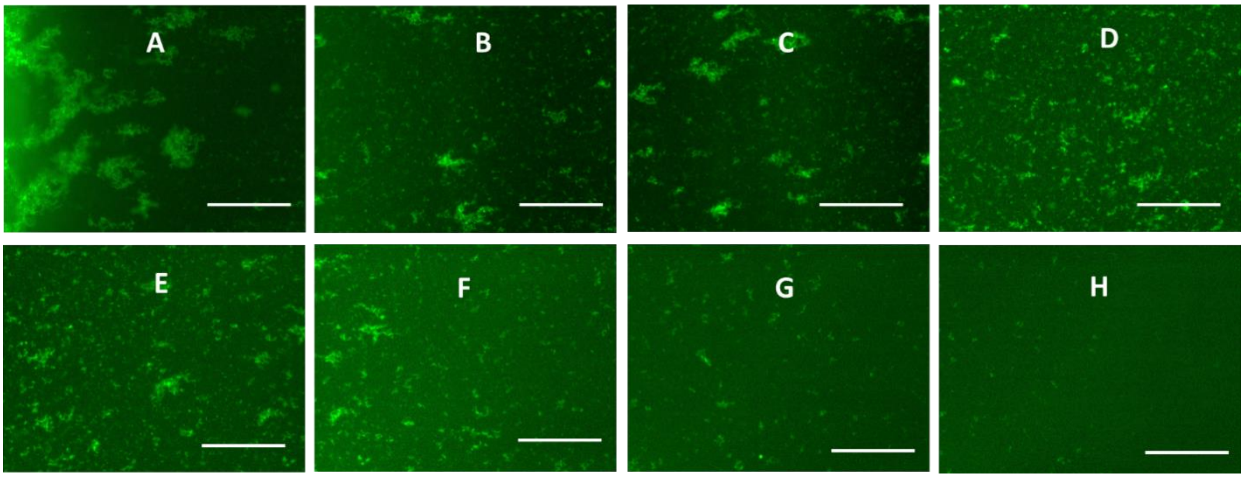


Fig. S18: Fluorescence microscopic images of demountable cuvette experiment in which drift of BSA-PEG-ATP-Mg^{2+} condensates was calculated after 10 minutes in the presence of (b) AMP-PEG-Mg^{2+}. Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1mM, [ATP] = 2.5 mM, [AMP] = 0.1 mM in 10 mM HEPES buffer, pH = 7 at 25 °C. (Scale bar: 200 µm)

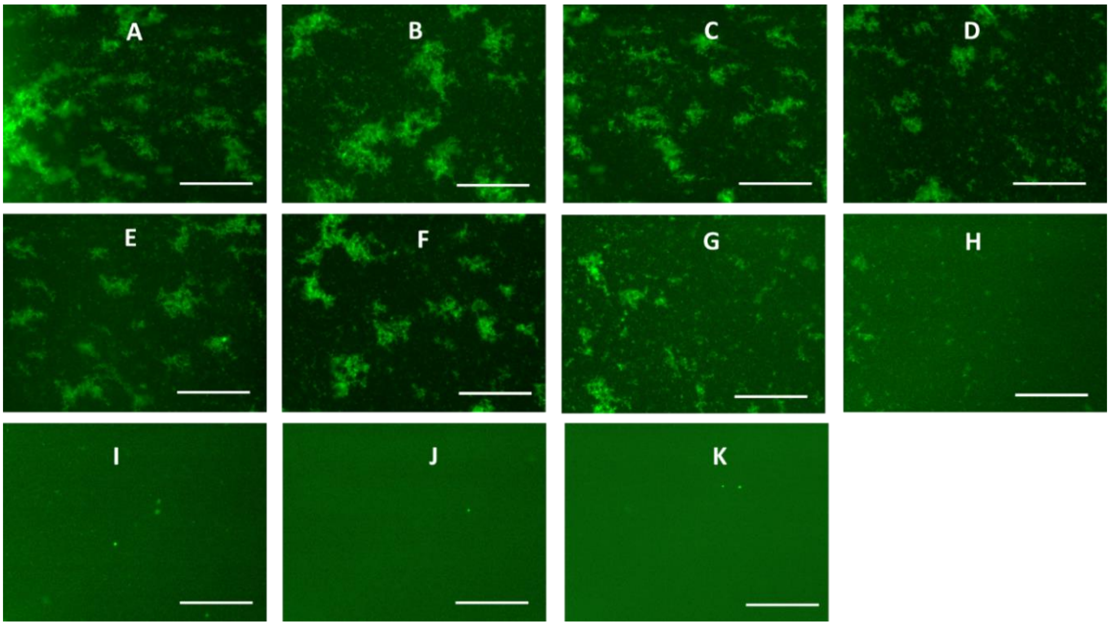


Fig. S19: Fluorescence microscopic images of demountable cuvette experiment in which drift of BSA-PEG-ATP-Mg^{2+} condensates was calculated after 10 minutes in the presence of (b) AMP-PEG-Mg^{2+}. Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1 mM, [ATP] = 2.5 mM, [AMP] = 0.5 mM in 10 mM HEPES buffer, pH = 7 at 25 °C. (Scale bar: 200 µm)

Supporting Video Captions:

SV1: Real-time recording of the dissolution of BSA-ATP-Mg^{2+} condensates in the presence of AMP. The scale bar is 200 µm. Experimental Conditions: [BSA] = 25 µM (10% labelled + 90% unlabelled), [PEG] = 20 % (w/v), [Mg^{2+}] = 1 mM, [ATP] = 5 mM, [AMP] = 5 mM in 10 mM HEPES buffer, pH = 7 at 25 °C. The experiment was performed in the demountable cuvette as described in Fig. 3d (main manuscript). Here in the bottom BSA-ATP-Mg^{2+} condensate was kept and AMP solution was added from one of the arm and recording started near the arm (at zone A) immediately.

SV2: Real-time recording of the BSA-ATP-Mg^{2+} condensates movement in the demountable cuvette as described in Fig. 3d (main manuscript). The video shows the movement of condensates in the absence of AMP. The scale bar is 200 µm.

SV3: Real-time recording of the BSA-ATP-Mg^{2+} condensates movement in the demountable cuvette as described in Fig. 3d (main manuscript). The video shows the movement of condensates in the presence of 0.1 mM AMP. The scale bar is 200 µm.

Here we observed with increasing amount of ATP condensate size increased. Then the dissolution rate and corresponding flow velocity mediated by AMP started to decrease. For example, in case of BSA-Mg^{2+}-ATP condensate with ATP concentration 1.5, 2.5 and 5 mM, the velocity decreased from 18 ± 4, 10 ± 3 and 3 ± 1.5 µm/s, in presence of fixed 0.5 mM AMP. In fact, size of the condensate can play key role in modulating spatiotemporal reactivity.

9. UV-Kinetics Study of CNP formation

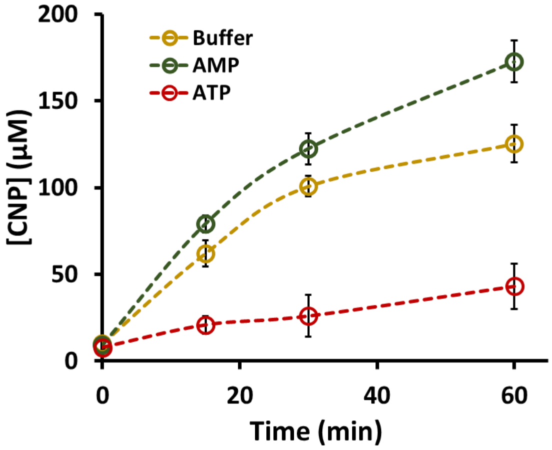


Fig. S20: Representative plot showing the amount of CNP formation in the presence of BSA-PEG-Mg^{2+}condensates in the presence & absence of AMP or ATP on UV-visible spectrophotometer at 380 nm. Experimental Conditions: [BSA] = 25 µM, [PEG] = 20 % (w/v), [NBI] = 250 µM, [Mg^{2+}] = 1 mM, [ATP] = 1 mM, [AMP] = 1 mM in 10 mM HEPES buffer, pH = 7 at 25 °C.

10. Activity Measurement in Demountable cuvette

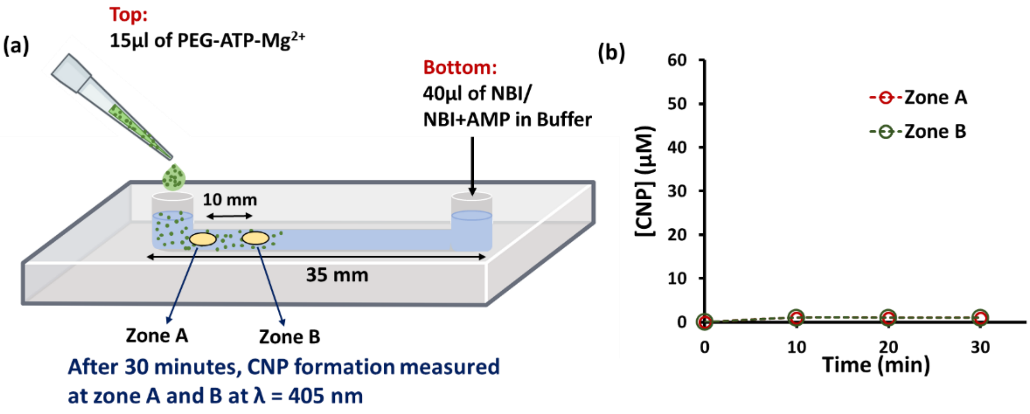


Fig. S21: (a) Schematic representation of the experimental setup for the demountable cuvette study (b) Representative plot showing the amount of CNP formation in the presence of PEG-ATP-Mg^{2+} int the demountable cuvette at zone A & zone B on the plate reader at 405 nm. Experimental Conditions: [PEG] = 20 % (w/v), [Mg^{2+}] = 1 mM, [NBI] = 250 µM, [ATP] = 2.5 mM, [AMP] = 2 mM in 10 mM HEPES buffer, pH = 7 at 25 °C.

References:

S1. S. Rani, B. Dasgupta, G. K. Bhati, K. Tomar, S. Rakshit, S. Maiti, *ChemBioChem* 2021, **22**, 1285.

S2. J. S. Nanda, J. R. Lorsch, *Methods Enzymol.* 2014, **536**, 79.

S3. S. Maiti, O. E. Shklyaev, A. C. Balazs, A. Sen, *Langmuir* 2019, **35**, 3724.