Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Condensates of short peptide and ATP for temporal regulation of cytochrome c activity

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Table of Contents

Materials:

All Fmoc protected amino acids, diisopropylcarbodiimide (DIC), oxyma, piperidine, triethylsilane (TES), potato apyrase (PA) were purchased from Sigma Aldrich. Cytochrome C (oxidized) was purchased from SRL India. ATP and pyrogallol was obtained from TCI, Japan. Fmoc-Rink amide MBHA Resin, hydrogen peroxide (30%, w/v solution) and all solvents were purchased from Merck. Milli-Q Water was used throughout the study.

EXPERIMENTAL METHODS

Synthetic procedure

Peptides were synthesized on Rink amide MBHA resin using standard Fmoc (9-fluorenylmethoxycarbonyl) solid phase peptide synthesis (SPPS) strategy in automated peptide synthesizer (Aapptec Eclipse Peptide Synthesizer, USA).[1] In a standard procedure, Fmoc-Rink Amide MBHA Resin was swollen using dimethylformamide (DMF) for 30 minutes. 20% piperidine in DMF containing 10% oxyma was used for Fmoc deprotection. Fmoc-amino acid coupling was carried out by diisopropylcarbodiimide (DIC) as activator and oxyma in DMF, and the coupling step was performed by heating. The peptide loaded resin was filtered and washed with dichloromethane (DCM) and dried under reduced pressure. Thereafter, peptides were treated with ca. 5 mL of trifluoroacetic acid (TFA) and 100 µL of triethylsilane (TES) for 3 hours at room temperature. The cleaved peptide-TFA solution was then filtered, and precipitated from to ice cold (-20°C) diethyl ether. The peptide precipitate was centrifuged at 7000 rpm for 10 min, and the pellet was washed three times with cold diethyl ether. Finally, the peptide was

lyophilized to get the crude dry peptide. Dried peptide was dissolved in minimal volume of 40% acetonitrile/H_2O containing 0.1% trifluoroacetic acid. The peptide was purified by RP-HPLC using a Waters Semi Preparative binary HPLC system fitted with a C18-reverse phase column with an acetonitrile-water solvent system containing 0.1% trifluoroacetic acid. Molecular weights were confirmed by Bruker Mass Spec Q-tof systems (m/z) calculated for $[M+2H^{2+}]$: 553.3535; found: 553.3577 and for $[M+3H^{3+}]$: 369.2385; found: 369.2429. For Ac-KVRVRVK-NH_2 peptide, calculated $[M+H^{+}]$: 925.6428; found: 925.6474.

Preparation of coacervates

Coacervate was prepared by addition of equal volume of peptides with the equal volume of ATP in different molar ratios. The final concentration of peptide was 1 mM, and concentration of ATP was varied from 1 mM to 30 mM.

For preparation of Methylene Blue (MB) sequestered coacervates, 0.25 mM of MB was incubated with coacervates for 20 min and subsequently centrifugation at 7500 rpm was done.

Optical microscopy

Optical images of coacervates were obtained using Olympus IX81 epifluorescence inverted microscope. Phase contrast images of different molar ratios of peptide/ATP coacervates were carried out within 1-2 hours after preparation. Briefly 20 μL solutions of coacervates or controls were casted on glass slides with cover slip and images were recorded with 10x resolution.

Transmission Electron Microscopy (TEM)

TEM experiments were performed by casting the samples on carbon coated copper grid (200 mesh Cu grid from Agar Scientific, UK). In a typical experiment, 10 μL of sample was taken out and diluted from the sample solution (10 % ACN-H_2O, 15 mM). The samples were then casted on the grid and allowed to adsorb for 45 s followed by wicking off the excess samples with filter paper. Grid were dried for few hours before imaging. Images were recorded in JEOL JEM-2100F electron microscopes.

For **1**; nanofibers were imaged when the aqueous solution (1 mM) was aged for ca. 3 days.

Turbidity measurements

UV spectrophotometer (BIOBASE) was employed for the turbidity measurements at 500 nm where none of the components absorb light. Turbidity was defined as (100-%T), where %T is transmittance.[2] Turbidity measurements at 500 nm were carried out by varying the ATP concentration with respect to a fixed concentration of **1** and **2** (1 mM). This particular wavelength was chosen as none of the peptide and ATP absorbed at this region. For the measurement of turbidity of **Ac-KVRVRVK-NH_2**/ATP system, ATP concentrations were varied with respect to peptide concentration. Turbidity of the **2**/ATP systems were also measured at different pH solution (3 to 14) as well as with different NaCl salt concentrations (0.005 M to 3.5 M). Turbidity of coacervates in presence of H_2O_2 were also determined in similar way. The data were presented as an average of triplicate measurements.

Zeta potential measurement

Zeta potential measurements were performed on HORIBA SZ-100 nanopartica at different molar ratio of ATP and **2** coacervates. Briefly, peptides with the equal volume of ATP in different

molar ratios were used to make the coacervates. The final concentration of peptide was 1 mM, and concentration of ATP was varied from 1 mM to 30 mM. Centrifugation at 7500 rpm was done followed by the redispersion in buffer. Then the samples were used for checking the zeta potential of the systems.

Fluorescence spectroscopy study

Fluorescence spectra of ANS sequestrated coacervates at different molar ratios were recorded using JASCO FP-8600 fluorimeter. In 50 μL of samples, 1 μL of ANS (final conc. of ANS in solution 0.1 mM) was added, and corresponding fluorescence spectra were monitored. The system was excited at 380 nm and emission range was set from 400 nm to 600 nm. Fluorescence intensity at λ_{max} for ANS mediated coacervate systems were noted and plotted against different **2**/ATP molar ratios.

Preparation of CytC bound coacervates and protein estimation studies

In order to prepare the CytC bound coacervates, varying amounts of CytC was incubated for 30 mins to the **2**/ATP coacervates (200 μM each). The solution was then centrifuged at 7500 rpm for 10 min at 4^{o}C and redispersed in 10 mM buffer solution at pH 5.5. The red pellet of CytC containing coacervates was washed twice by repeating the same procedure of centrifugation and redispersion. The supernatants after each centrifugation were collected for CytC estimation by Bradford assay following the standard protocol.[1] Briefly, known concentrations of BSA solutions and unknown concentrations of CytC in supernatants and washes were mixed with Bradford reagent in separate vials, stirred and incubated for 30 min. The absorbance was subsequently measured by UV spectrophotometer at 595 nm wavelength. The BSA concentrations and

corresponding absorbance were recorded for the plot of standard curve. The unknown concentration of CytC was calculated using the standard curve. From the curve it was found that the amount reached up to 924.24 μg when 2400 μg of CytC was exposed to the coacervates. This suggested a loading of 924.24 μg per 165.58 μg of the coacervate (2 and ATP combined, Fig. S9).

In control experiments, supernatant solutions without CytC was subjected to the same centrifugation followed by redispersion process and the supernatants were subjected to Bradford assay which showed negligible absorbance at wavelength = 595 nm.

Leaching of CytC were checked on 70.2 and 243.1 μg CytC loaded coacervates. Briefly, cytochrome c loaded coacervates were taken in different micro centrifuge tubes and incubated in buffer solution. Leaching of CytC were analysed taking each supernatant of centrifuged samples at different time intervals and subsequently checking the absorbance spectra at 410 nm wavelength.

Circular Dichroism

CD spectra were recorded using a JASCO J-810 circular dichroism spectrometer fitted with a Peltier temperature controller to maintain the temperature of 25 °C. 300 μL solution of the CytC samples (33.33μM) were placed into a 1 mm quartz cuvette with 10 mm path length. Each spectrum was obtained by scanning wavelength from 550 nm to 190 nm at a scanning rate of 100 nm/min. Five successive wavelength scans were taken to average for each sample. CD spectra were also recorded of CytC bound 1:1 coacervate samples. Secondary structure of CytC in coacervates was plotted by subtracting background of coacervates medium.

Fourier-Transform Infrared (FTIR) spectroscopy

For the measurements of IR spectra of CytC in coacervates, 100 μL of CytC solution (2.4 mg) was added to 500 μL (1:1, 0.4 mM each) of **2**/ATP coacervates solution. The solution was then lyophilized for 24 hours. For native CytC, 500 μL of 2.4 mg of CytC solution was lyophilized. Spectra of the lyophilized samples were recorded by using Bruker (model no: Alpha, Platinum ATR) in ATR mode with 512 times accumulation at a resolution of 2 cm^{-1} with intervals of 1 cm^{-1}.[3]

Activity measurements:

To measure activity, 107 μL of CytC bound coacervates (different loadings) was taken and 2.75 μL pyrogallol (final conc. 25 mM) was added to micro volume cuvette (110 μL) of 10 mm path length. To initiate reaction, 1.1 μL H_2O_2 (final conc. 30 mM) was added and rate of the formation of purpurogallin was monitored. The absorbance change was monitored at λ_{max} = 420 nm for the initial 1-2 min (ε420 nm is 2640 M^{-1} cm^{-1} in water). In similar way, peroxidase activity of controls such as ATP, peptide, buffer etc. were measured. One unit of specific activity of the enzyme-condensate system is defined as 1.0 μmole of pyrogallol is oxidised in one second by 1 μg of enzyme.

Dissipative coacervates formation and activity measurement

To monitor transient nature of coacervates, initially ATP was added to a solution containing **2** along with 0.33 U mL^{-1} PA. The final concentration of both **2** and ATP were maintained 200

μM. At different time intervals, turbidity was measured at 500 nm wavelength. After 45 min when the turbidity values decreased, 110 μM ATP was added again. The cycle was repeated for three times, with the same amount of ATP added at different time interval (Figure 4d of main text).

To monitor the peroxidase activity in the transient coacervates the following protocol was used. Briefly, to a CytC (70.2 μg mL^{-1}) loaded coacervates of **2**/ATP, PA (0.33 U mL^{-1}) was added. Oxidation reaction was monitored by adding 25 mM pyrogallol and 30 mM H_2O_2 at different time intervals. Whenever the decline of oxidation rate was observed, a fresh batch of 110 mM ATP was added to the system and subsequent activity was monitored. The cycle was repeated up to three times.

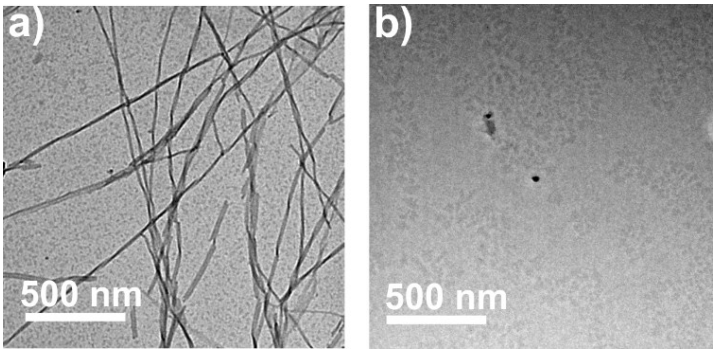

Figure S1: TEM micrograph of VVVRRKK (**1**) and KVRVRVK (**2**) peptide solution.

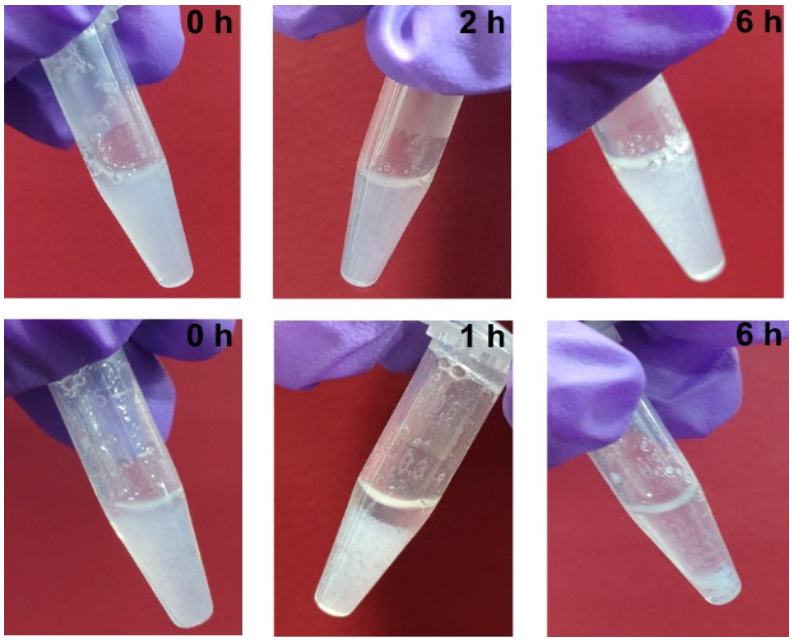

Figure S2: Time course images of **2** (1 mM)/ATP (10 mM) solution (top) and **2** (1 mM)/ATP (20 mM) solution (bottom).

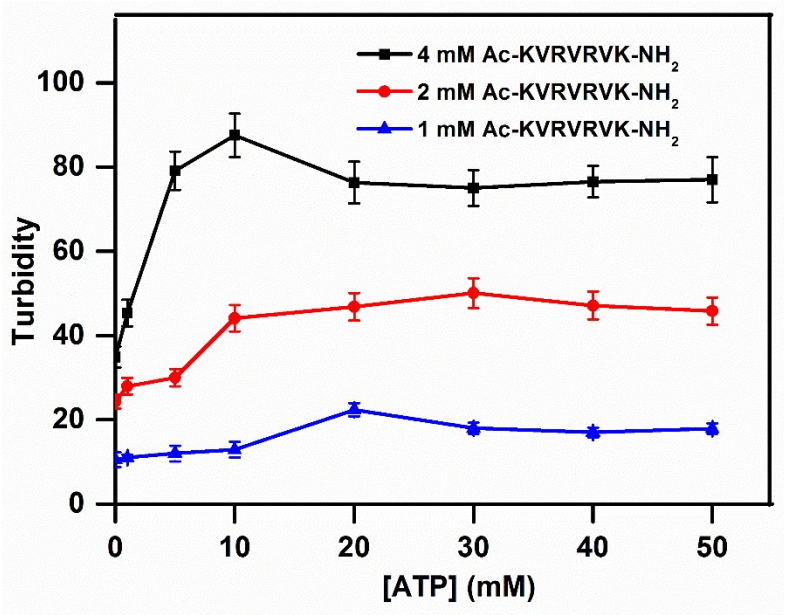

Figure S3. Turbidity plots of acetylated peptide **Ac-KVRVRVK-NH$_2$**/ATP systems in varying concentrations of the components.

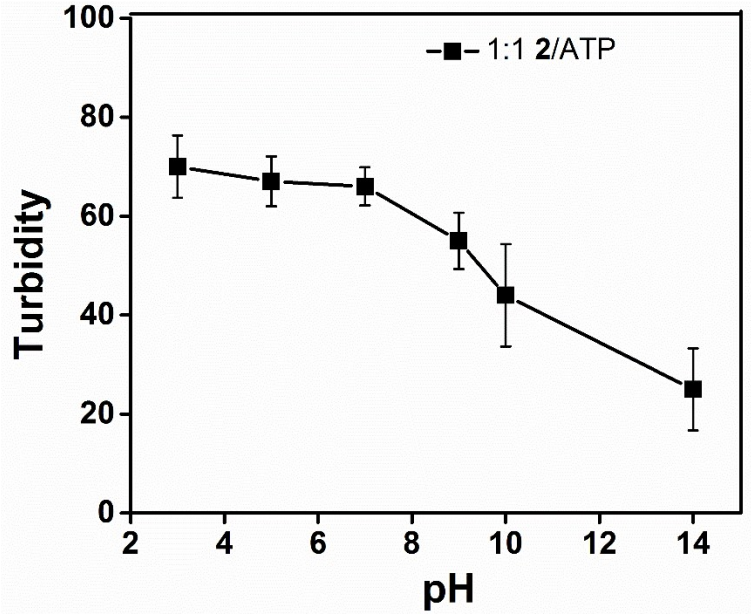

Figure S4. Effect of the pH of the medium on turbidity of the **2**/ATP system.

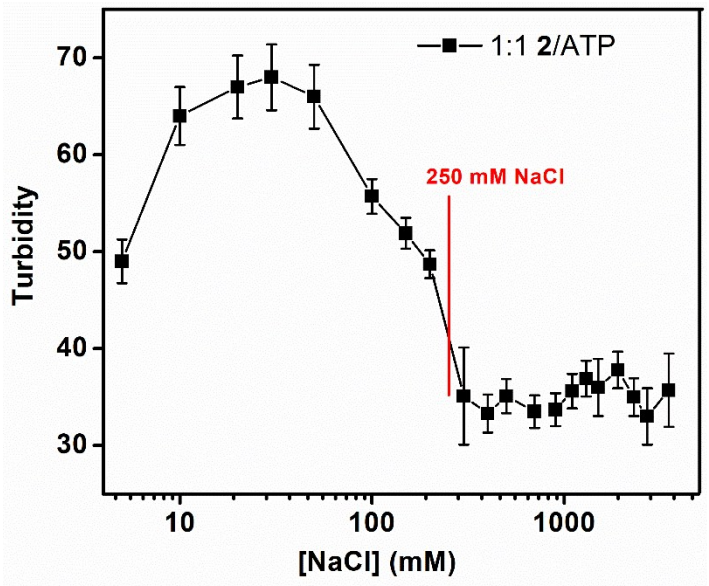

Figure S5. Effect of salt concentrations on turbidity of the **2**/ATP system. Red line shows the critical salt concentration of the system.

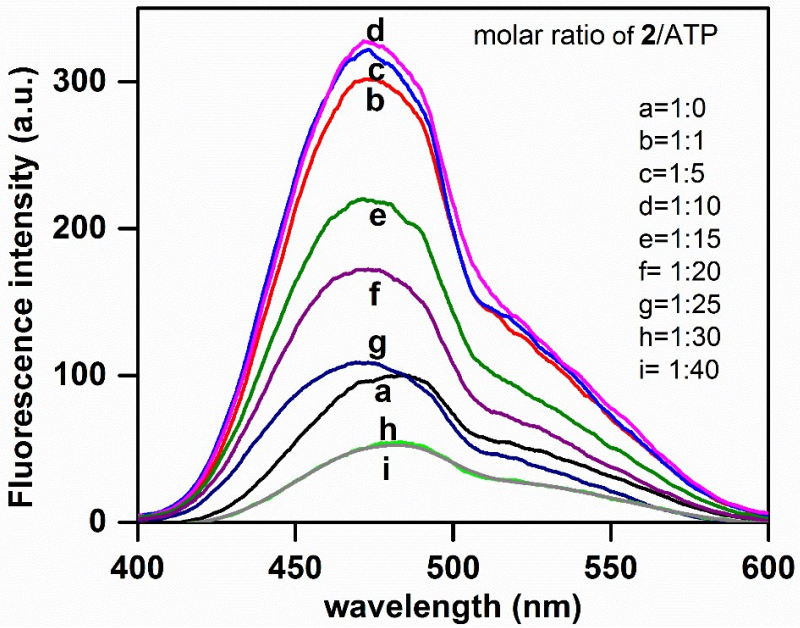

Figure S6: Fluorescence spectral changes of ANS bound coacervates formed by varying molar concentration of ATP with fixed concentration of **2** (1 mM).

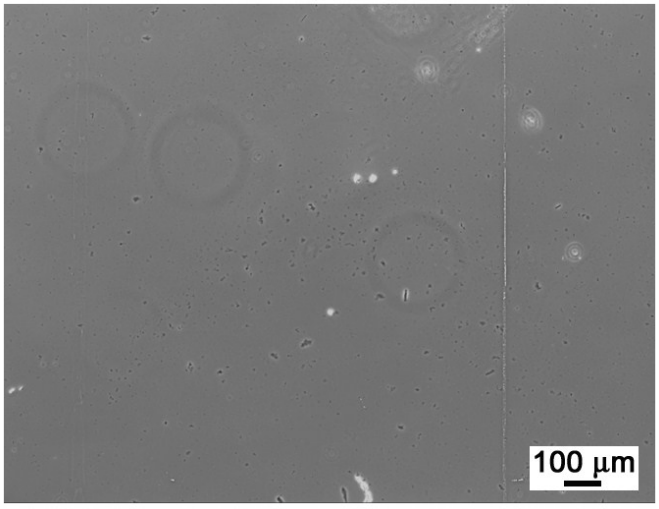

Figure S7: Optical image of **2** (1 mM)/AMP (5 mM) solution.

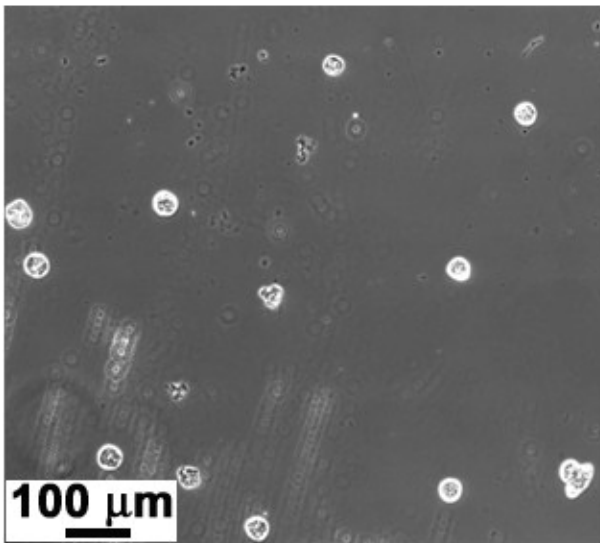

Figure S8: Optical image of coacervates formation after refueling with 110 mM ATP.

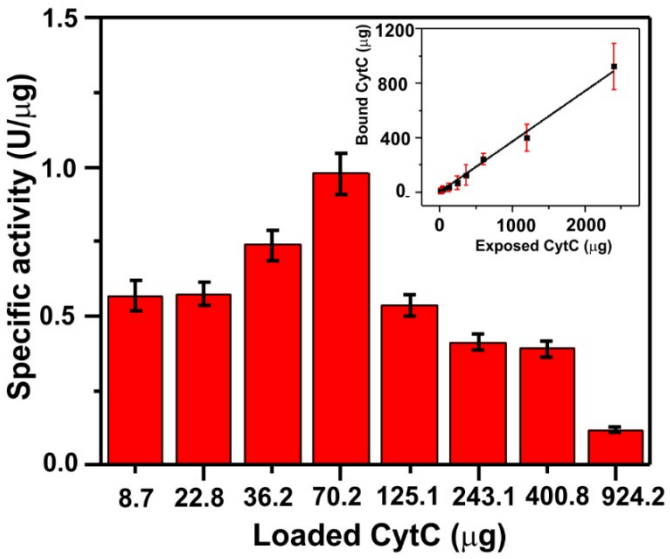

Figure S9: Bar diagram of peroxidase activity at different loading concentrations of CytC in coacervates (combined amount of **2** and ATP was 165.58 µg). Inset depicts the loading profile of CytC on coacervates, obtained from Bradford Assay.

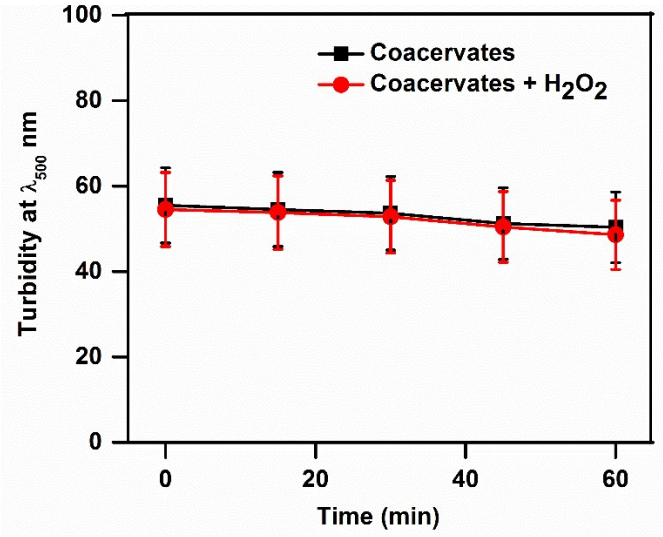

Figure S10: Effect of the turbidity of the system (1 mM **2**/5 mM ATP) in presence of H_2O_2.

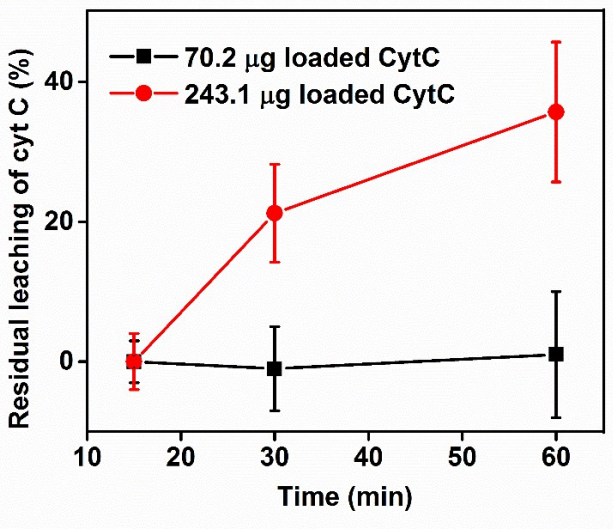

Figure S11: Time course leaching of CytC bound coacervates.

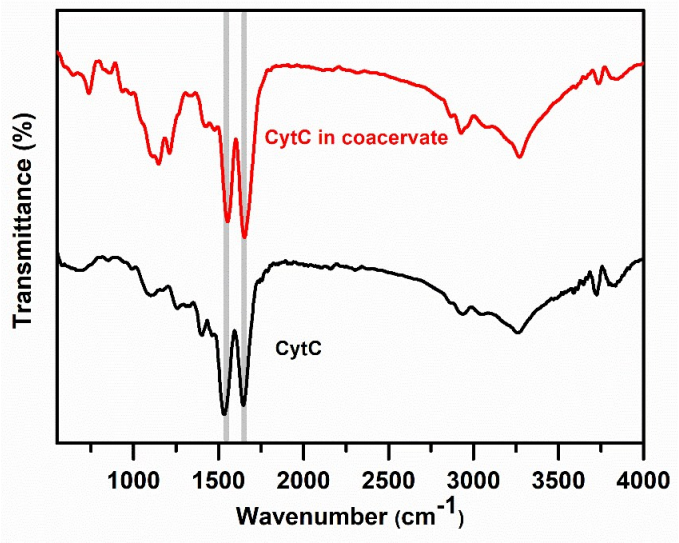

Figure S12. FTIR spectrum of CytC and CytC entrapped coacervates.

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1. N. Kapil, A. Singh and D. Das, *Angew. Chem. Int. Ed.*, 2015, **54**, 6492– 6495.

2. A. M. Marianelli, B. M. Miller and C. D. Keating, *Soft Matter,* 2018, **14**, 368-378.

3. Z. Li, H. Xia, S. Li, J. Pang, W. Zhu and Y. Jiang, *Nanoscale*, 2017, **9**, 15298–15302.