Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

For

Construction of coacervate in proteinosome hybrid microcompartments with enhanced cascade enzymatic reactions

Junbo Li, Mei Zhu, Shengliang Wang, Zhengyu Tao, Xiaoman Liu* and Xin Huang*

MIIT Key Laboratory of Critical Materials Technology for New Energy Conversion and Storage, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, Harbin, 150001, China

*E-mail: liuxiaoman@hit.edu.cn; xinhuang@hit.edu.cn

Contents of Supporting Information

1. Supplementary Experimental Section

S1. Materials and reagents

Dextran (MW 500kDa, Sigma), Polydiallyldimethyl ammonium chloride solution (PDDA, MW 100-200 kDa, 20 wt.%), Albumin from bovine serum (BSA, Sigma, 98%), Peroxidase from horseradish (HRP, Sigma), Uricase (Macklin), DNA (MW 90 kDa, Sigma), RNA (MW 20~30 kDa, Sangon Biotech), mPEG-SPA (MW 5 kDa, Biomatrik), NHS-PEG_{16}-NHS (MW 1061.14, Biomatrik), Succinic anhydride (Aladdin, 99%), Dimethyl suifoxide (DMSO, Energy sulfoxide), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride crystalline (EDAC, Sigma-Aldrich, 98%), 4-(dimethylamino) pyridine (DMAP, Sigma), FITC (Fluorescein isothiocyanate isomer I, Sigma 90%), RBITC (Rhodamine B isothiocyanate, Sigma), Atto 425 NHS ester (Sigma), uric acid (Sigma, 99%), SYBR Green I, and Amplex Red were used as received without further purification. Milli-Q water was used to prepare all the solutions in this study.

S2. Instrument and characterization

UV-vis spectra were measured on a PerkinElmer spectrophotomer (Lambda 750S, USA). Fourier transform infrared (FTIR) spectroscopic measurements were performed on a Nicolet iS5 spectrometer (Thermo Fisher Scientific, Waltham, MA). Flow cytometry characterizations were performed on a CytoFLEX Flow cytometer (Beckman Coulter). Zeta-potential measurements were conducted using Malvern Zetasizer Nano-ZSP. Optical microscopy image was performed on a Leica DMI8 manual inverted fluorescence microscope. Confocal images were obtained on a Leica SP5-II confocal laser scanning microscope attached to a Leica DMI 6000 inverted epifluorescence microscope.

S3. Synthesis of succinylated dextran (Su-Dex)

Succinylated dextran (Su-Dex) was prepared by dissolving 100 mg of dextran (500 kDa) and 185 mg of succinic anhydride in 15 mL of DMSO at 60 °C. After complete dissolution of the dextran, 5 mg of DMAP was added and the reaction mixture was left to stir for 16 hours. After the reaction, the mixture was diluted with 30 mL water and dialysed extensively against Milli-Q water using dialysis tube with 3.5 kDa. At last, freeze drying to obtain the final product.

S4. Synthesis of PEGylated BSA/PNIPAAm conjugates (mPEG-BP)

PEGylated BSA/PNIPAAm conjugates was synthesized by BSA/PNIPAAm (BP) and mPEG-SPA, of which BP was synthesized according to the previously Huang's reported method. [1] Specifically, a solution of BP (10 mg) was prepared with 10 mL PBS buffer with pH 8.0. And 10 mL mPEG-SPA (10 mg) solution was added dropwise to the stirred above protein-polymer solution and was stirred for a further 12 h. Then the reaction solution was dialyzed extensively against Milli-Q water using dialysis tube

with 12–14 kDa to remove unreacted mPEG-SPA. At last, freeze drying to obtain the final product.

S5. Synthesis of RBITC-labelled mPEG-BSA/PNIPAAm, FITC-labelled Su-Dex, FITC-labelled GOx and Atto-labelled HRP

A solution of PEGylated BSA/PNIPAAm was prepared with pH 8.5 buffer. A solution of RBITC (1 mg/mL, DMSO) was added dropwise to the above solution and was stirred for a further 12 h. After that, the solution was dialyzed (dialysis tube 3.5 kDa MWCO) extensively against Milli-Q water to remove unreacted dyes. At last, freeze drying to obtain the final product. FTTC-labelled Su-Dex, FITC-labelled GOx and Atto-labelled HRP were synthesized with the same method as RBITC-labelled PEGylated BSA/PNIPAAm.

S6. Preparation of coacervate droplets

PDDA and Su-Dex were dissolved separately in pH 7.0 PBS at a concentration 5 mg/mL and coacervation was induced by mixing the solutions of PDDA and Su-Dex in a volume ratio of 4:1. The specific procedure was as following: 100 μL of a solution of PDDA added dropwise into 25 μL of Su-Dex solution. As a result, coacervation was immediately and readily observed with the solution turned to be turbid. FITC-labelled coacervates were prepared with PDDA and Su-Dex with small amount of FITC-labelled Su-Dex.

S7. Preparation of proteinosomes

PEGylated BSA-NH_2/PNIPAAm proteinosomes were prepared by according to the previously Huang's protocol.[1] In brief, 0.5 mg NHS-PEG_{16}-NHS as cross-linker for reacting with free primary amine groups of BSA-NH_2 was added into 20 μL of aqueous PEGylated BSA-NH_2/PNIPAAm (10 mg/mL, pH 8.0 PBS buffer), and immediately followed by 400 μL of 2-ethyl-1-hexanol (aqueous/oil volume fraction, Φw, of 0.05). Then shake the mixture by hand for 10s and leave for 12 h sedimentation. To transfer the cross-linked proteinosomes into water, the upper clear oil layer was discarded and 1mL 75% ethanol was added to dissolve the sediment and the emulsion gently shaken. Repeat this procedure three times to ensure the remove of oil completely. And last time wash instead with Milli-Q water to complete the phase transfer process. Proteinosomes comprising encapsulated FITC-dextran (500 kDa) were prepared following the above procedures except that the encapsulants were added to the aqueous PEGylated BSA-NH_2/PNIPAAm solution before mixing with the oil phase.

S8. Permeability measurement of proteinosomes

The cutoff molecular weight of the membrane of the proteinosomes was evaluated indirectly by using different molecular weight of FTIC-dextran as probes which was added into the solution, and after 10 min incubation, the concentration difference of the FITC-dextran inside and outside of the proteinosomes was calculated based on the analysis of light intensity by the software of Image J. In detail, the experiment was first carried out by adding 0.5 μL of different molecular weight of FITC-Dextran (1 mg mL^{-1}) to 5 μL of proteinosomes aqueous solution. Then the corresponding fluorescence confocal microscopy images were captured under the same conditions in the presence of FITC-Dextran with molecular weights of 4, 10, 20, 40, 70, 150, 500 or 2000 kDa, respectively. Then use the light intensity analysis software Image J to analyse the fluorescence intensity difference between the internal and external environment of the microcapsule, and defined it as follows: when FITC-Dextran with a molecular weight of 2000 kDa cannot permeate the proteinosomes, the difference between the internal and external light intensity of the proteinosomes is the largest, and the transmittance is zero which also means 100% impenetrable. In contrast, when the fluorescence intensity inside the proteinosomes was the same with the external fluorescence intensity, the transmittance is 100%. Then the transmittance of FITC-Dextran with different molecular weight can be calculated by using the difference value of FITC-Dextran permeating proteinosomes with a molecular weight of 2000 kDa as the standard. A plotting was generated by using FITC-Dextran with different molecular weights as abscissa and transmittance as ordinate, and then the cutoff molecular weight could be read when the transmittance was 50% through the diffusion percentage curve. As a result, we can know the cutoff molecular weight of the proteinosomes used here was *ca.* 150 kDa.

S9. Preparation of nested coacervate-in-proteinosomes

The solution of 8 μL PDDA and 2μL Su-Dex (5, 25, or 50 mg/mL, 1.6 M NaCl above PBS buffer with pH 8.0) were mixed firstly and then implement the above procedures for proteinosomes. The method for phase transfer was similar with proteinosomes except that instead Milli-Q water at the last step with 400 mM NaCl above 50 mM PBS buffer with pH 8.0 and proteinosomes containing an individual coacervate droplet were obtained finally.

S10. Determination of the number of PNIPAAm and mPEG on the surface of mPEG-BP

Sample solutions of BSA were prepared with a series of concentration from 0.25 mg/mL to 4 mg/mL. A standard absorbance curve was performed according to the UV-vis spectra absorption at 278 nm. Record BSA-NH_2/PNIPAAm (2 mg/mL) absorbance value at 278 nm and put it into the standard curve to estimate the protein and polymer

content in protein-polymer nanoconjugates, respectively. The number of PNIPAAm per BSA-NH_2/PNIPAAm was determined to be *ca.* 2.75.

As for mPEG-BP, first record the absorbance value and estimate the protein content in mPEG-BP and then calculate the content of PNIPAAm according to the above result per BSA-NH_2/PNIPAAm. The content of mPEG can be obtained by the minus of the protein and polymer content in total protein-polymer and the number of mPEG per BSA-NH_2/PNIPAAm was determined to be *ca.* 4.22.

S11. Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) experiments were undertaken for FITC-labelled coacervate droplets encapsulated in proteinosome and in bulk, respectively. Bleaching of the coacervate droplets was achieved using a 488 nm diode laser at 100% power. Imaging was carried out using a 488 nm laser for excitation of the FITC dye at λ_{FITC} = 488 nm with an emission wavelength of λ_{FITC} = 500-590 nm. For each experiment, pre-bleaching images were acquired before bleaching. The fluorescence recovery was then recorded by imaging for 20 s every 3 seconds in the FITC channel.

Raw fluorescence data was obtained from Image J and normalized in Origin. Recovery profiles were fit to a double exponential curve to obtain the time constants $\tau_{1/2}$ - half time of recovery. Diffusion coefficients (D) was estimated according to simplified equation: $D = 0.25 \frac{r_{BR}}{\tau 1/2}$,[2] where r_{BR} is the radius of bleaching region and $\tau_{1/2}$ is the half time of recovery. Viscosity was calculated using the Stokes-Einstein equation: $\eta = \frac{kBT}{6\pi DrDP}$,[3] where k_B is the Boltzmann's coefficient, T is the temperature, D is the diffusion coefficient and r_{DP} is the radius of the diffusing particle.

2. Supplementary Table and Figures

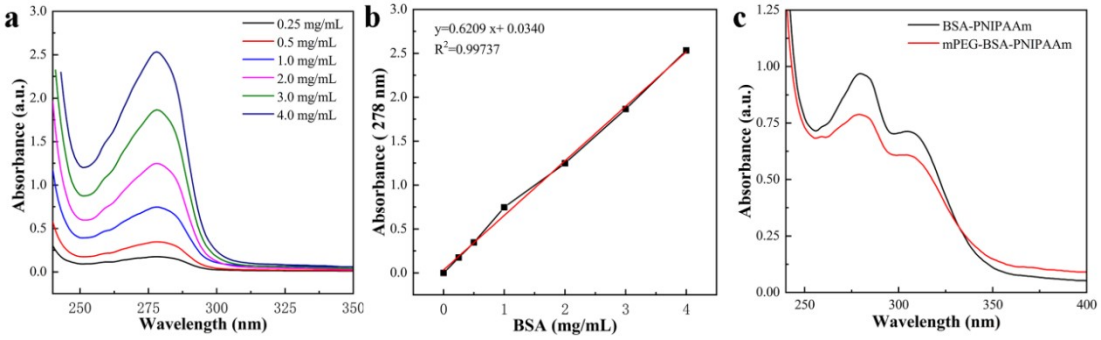

Figure S1. UV-Vis absorption spectra obtained for BSA with a series of concentration (**a**) and corresponding calibration curve based on plotting the absorbance at 278 nm (**b**). (**c**) UV-Vis absorption spectra obtained for BSA-NH_2/PNIPAAm (black) and mPEG-BP with the same concentration (red).

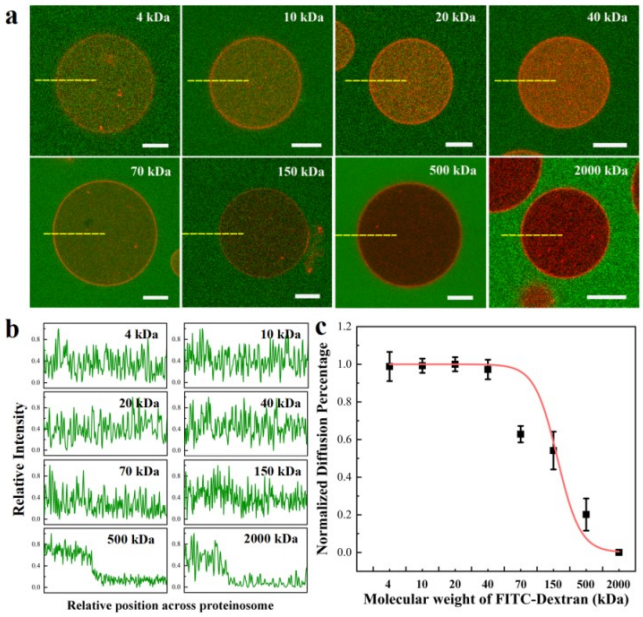

Figure S2. Study of the cutoff molecular weight of the constructed proteinosomes based on the diffusion of FITC-dextran with molecular weights from 4 to 2000 kDa. (**a**) The corresponding fluorescence confocal microscopy images were captured under the same condition in the presence of FITC-dextran with a molecular weight of 4, 10, 20, 40, 70, 150, 500 or 2000 kDa, respectively. (**b**) Corresponding fluorescence intensity line profiles of selected proteinosomes shown in the fluorescence images. (**c**) The plot showing the diffusion percentage of different molecular weight FITC-dextran after incubating with proteinosomes.

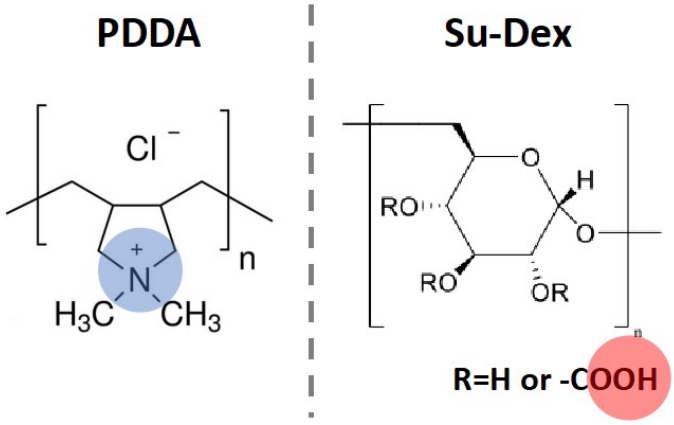

Figure S3. Chemical structures of the coacervate molecules, polycation Polydiallyldimethyl ammonium chloride solution (PDDA) and polyanion succinylated dextran (Su-Dex) that undergo coacervation at pH 8.

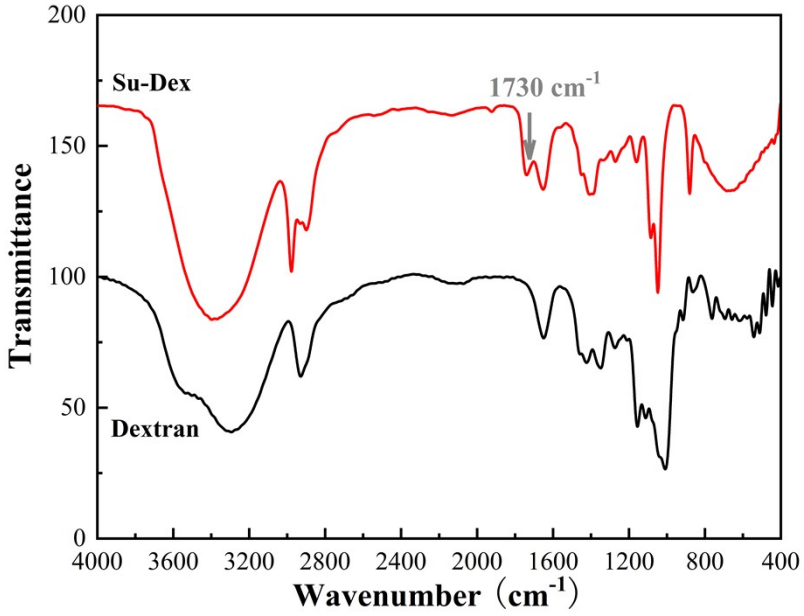

Figure S4. FTIR spectra of dextran and succinylated dextran.

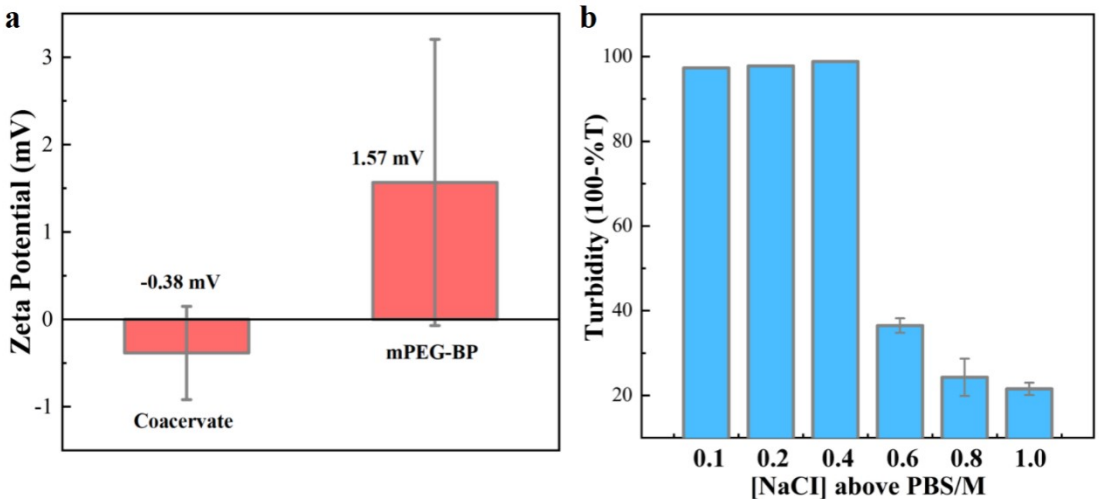

Figure S5. Turbidity measurements of PDDA/Su-Dex coacervates under different [NaCl] above PBS buffer with pH 8.0 (**a**). Zeta-potential measurement of PDDA/Su-Dex coacervate droplets and mPEG-BP conjugates (**b**).

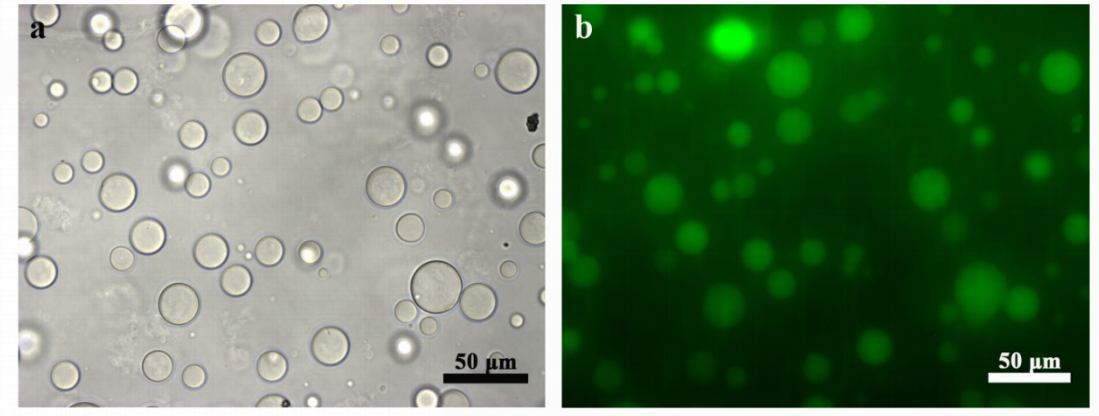

Figure S6. Optical (**a**) and fluorescence (**b**) microscope images of coacervate sequestrated Calcein.

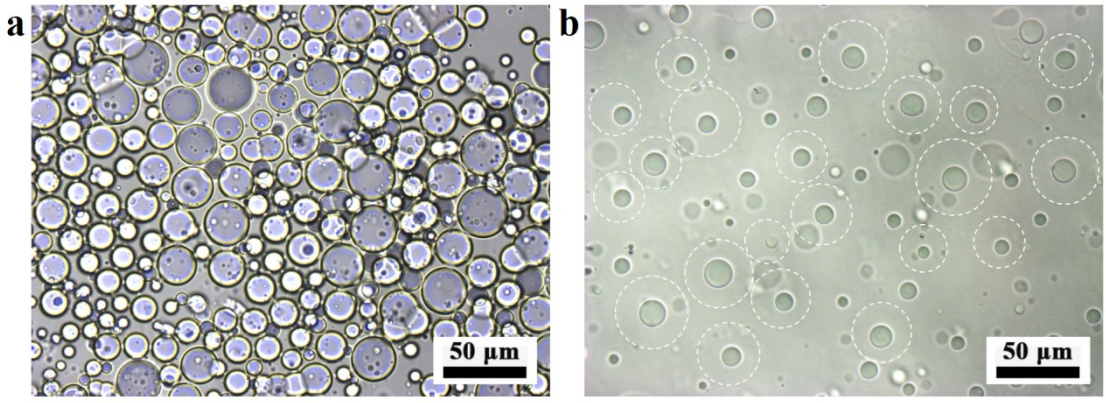

Figure S7. Optical microscopy images of coacervate-in-proteinosome protocells in oil phase (**a**), and in aqueous phase (**b**).

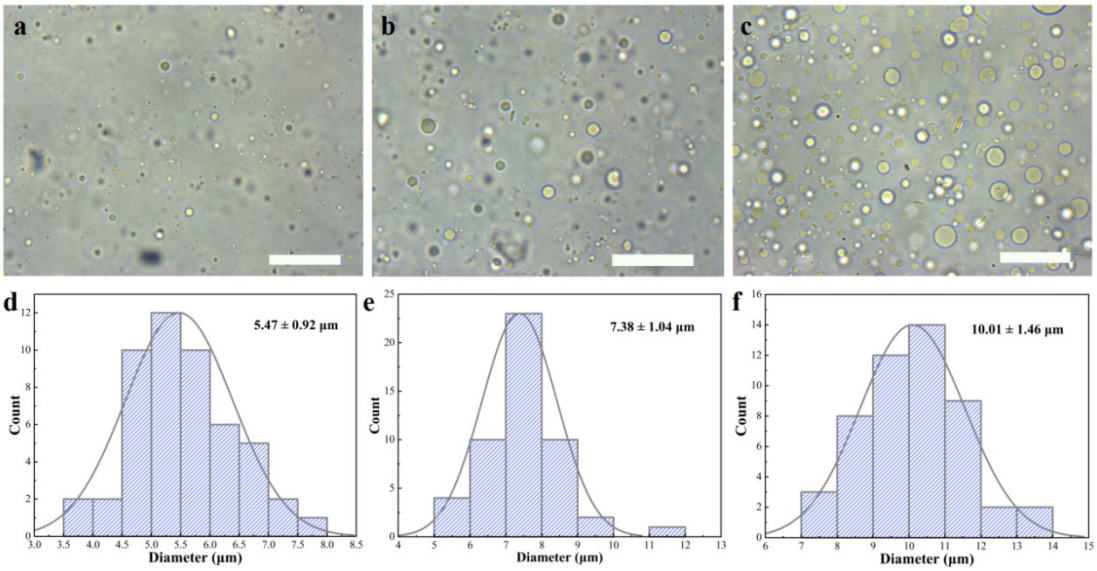

Figure S8. Optical microscopy images (**a-c**) show the size of coacervate inside proteinosome increase with the rise of concentration for PDDA and the corresponding size distribution under different concentration (**d**–**f**). Scale bars: 50 µm.

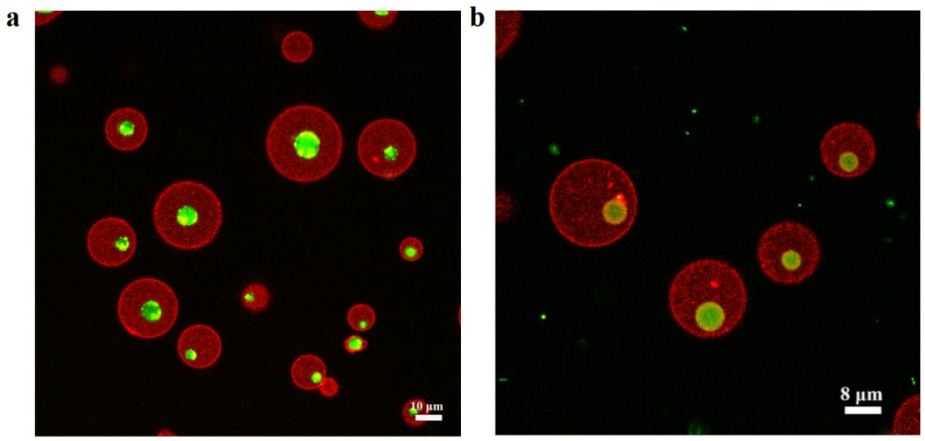

Figure S9. Confocal fluorescence images showing the sequestration for DNA (**a**) and RNA (**b**) within coacervate-in-proteinosome protocells.

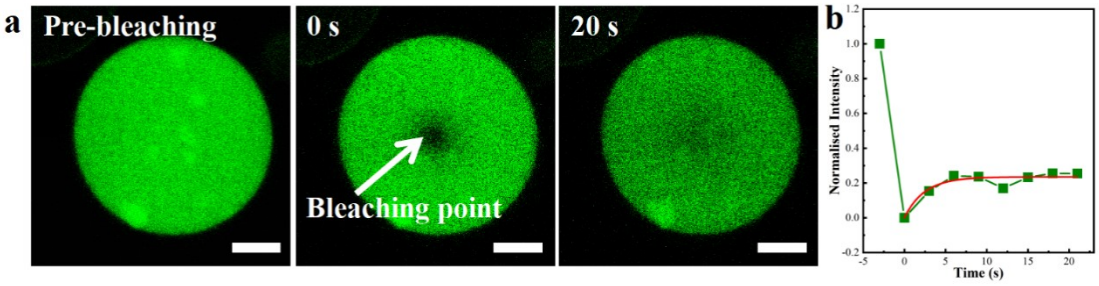

Figure S10. FRAP of coacervate in bulk. (**a**) Confocal fluorescence images of coacervate in bulk before bleaching, during bleaching (0 s), and after recovery (20 s). (**b**) Corresponding FRAP recovery curve for the coacervate. Scale bars: 3 μm.

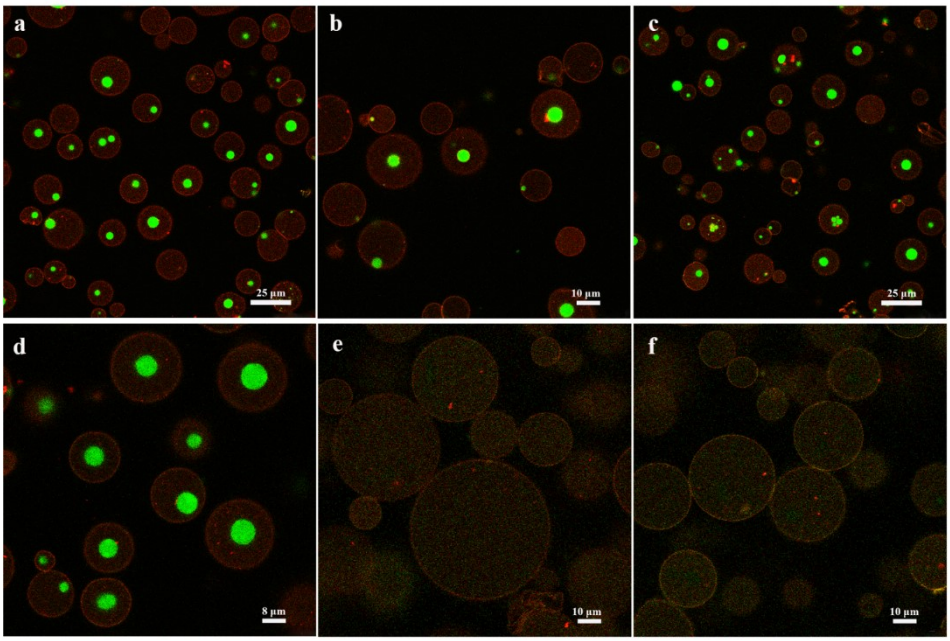

Figure S11. Confocal fluorescence microscopy images of coacervate-in-proteinosome obtained by phase transfer with 0 M (**a**), 0.1 M (**b**), 0.2 M (**c**), 0.4 M (**d**), 0.6 M (**e**) and 0.8 M (**f**) NaCl above PBS buffer with pH 8.0.

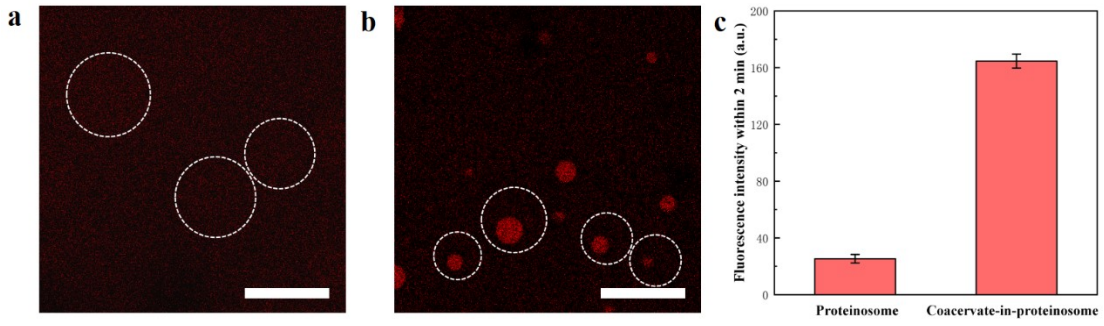

Figure S12. Confocal fluorescence microscopy images of proteinosome (**a**) and coacervate-in-proteinosome (**b**) after two minutes reaction. (**c**) The corresponding fluorescence intensity in the proteinosme and that in the coacervate. Scale bars: 25 μm.

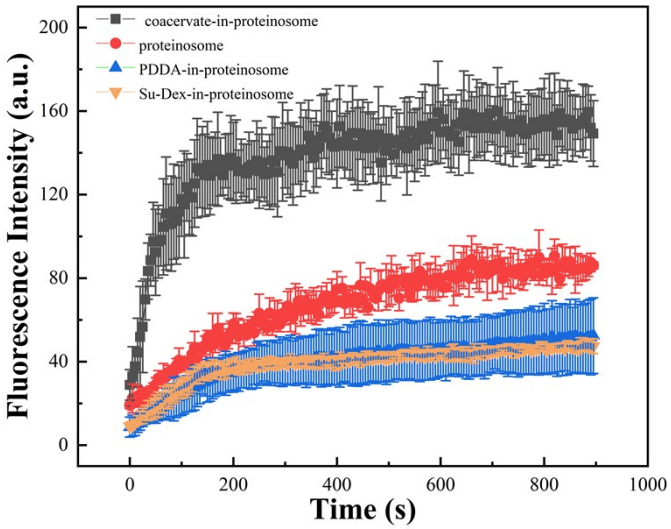

Figure S13. Time profile showing the increase in fluorescence intensity for uricase/HRP cascade reactions in coacervate-in-proteinosome (black), empty proteinosome (red), proteinosome encapsulated only PDDA (blue) and proteinosome encapsulated only Su-Dex (yellow), respectively.

Table S1. Relative content of BSA, PNIPAAm and mPEG in protein-polymer and PEGylated nanoconjugates.

	BSA-NH_2/PNIPAAm (2mg/mL)	mPEG-BSA-NH_2/PNIPAAm (2 mg/mL)
278 nm	0.968	0.788
BSA (mg/mL)	1.5	1.21
PNIPAAm (mg/mL)	0.5	0.403
mPEG (mg/mL)	——	0.387

Table S2. Diffusion coefficient (D), and viscosity (η) calculated for coacervate in proteinosome, and coacervate in bulk, respectively.

	Coacervate in Proteinosome	Coacervate in Bulk
Fitted equation	y=(0.24979±0.01)[1-exp(-0.23348±0.03556)x]	y=(0.23457±0.01488)[1-exp(-0.408012±0.15078)x]
D ($\mu m^2/s$)	0.33±0.0005	0.31±0.008
η (mPa.s)	30.3±0.21	32.3±6.95

3. Reference

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3 M. A. Vibhute, M. H. Schaap, R. J. M. Maas, F. H. T. Nelissen, E. Spruijt, H. A. Heus, M. M. K. Hansen and W. T. S. Huck, *ACS Synth. Biol.*, 2020, **9**, 2797–2807.