Electronic Supplementary Information for Quantum Yield Enhancement of Firefly Bioluminescence with Biomolecular Condensates

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1. Experimental details

Materials

Adenosine 5′-triphosphate disodium salt hydrate (ATP) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). D-Luciferin, AkaLumine-HCl, 2-amino-2-methyl-1,3-propanediol, 3,3-dimethylglutaric acid, 2-amino-2-hydroxymethyl-1,3-propanediol, and magnesium chloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Luciferase from *Photinus pyralis* (firefly), seMpai, and polyethylene glycol (PEG, average mol wt 8000) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Poly-L-lysine hydrochloride (PLL; MW = 8200), poly-L-lysine hydrochloride (PLL; MW = 1600), and poly-L-arginine hydrochloride (PLL; MW = 9600) were obtained from Alamanda Polymers, Inc. (Huntsville, AL, USA). Dehydroluciferin was obtained from Biosynth Carbosynth, Ltd., (Newbury, United Kingdom). Alexa Fluor™ 680 C2 Maleimide was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Preparation of stock solutions

Stock solutions were prepared as follows: luciferase solution (30 μM) in 50 mM GTA buffer (pH 7.4) with 10% glycerol; D-luciferin solution (25 mM) in DMSO; AkaLumine solution (25 mM) in DMSO; seMpai solution (50 mM) in MilliQ; dehydroluciferin solution (25 mM) in DMSO; ATP solution (100 mM) in 50 mM GTA buffer (pH 8); $MgCl_2$ solution (100 mM) in 50 mM GTA buffer (pH 8); peptide (PLR-50 mer or PLL-50 mer or PLL-10 mer) solution (100 mM) in 50 mM GTA buffer (pH 8); and FITC-PLL solution (25 mM) in 50 mM GTA buffer (pH 7.4). FITC-PLL was prepared according to the procedures described by Branchini et al.[1] The solutions were diluted to appropriate concentrations using 50 mM GTA buffer (pH 8). Protein concentration was determined by measuring the absorbance at 280 nm on a spectrophotometer (NanoDrop OneC; Thermo Fisher Scientific, Inc.).

Bioluminescence intensities and turbidity

ATP, luciferase, D-luciferin, and peptide (PLR-50 mer, PLL-50 mer, or PLL-10 mer) were added into 384-well microplates (Greiner Bio-One, Kremsmünster, Germany) to prepare 40 μL of the suspension (1.875 mM ATP, 5 nM luciferase, 0.0625–250 μM D-luciferin or 0.0625–12.5 μM D-luciferin analogues, 5.63 mM [monomer units] peptide, 50 mM GTA [pH 8 or pH 6.7]). After incubation at room temperature for 5–50 min, 10 μL of 5 mM $MgCl_2$ solution was added into the suspension, and luminescence signals were immediately read for 104 s using a plate reader (Spark

Cyto; TECAN, Männedorf, Switzerland). The kinetic profiles (K_m and V_{max}) were determined from the integrated values of the luminescence signals for 104 s and calculated by using GraphPad Prism9. Turbidity was determined using UV-vis absorption at 600 nm measured by the plate reader.

Bioluminescence intensities in the presence of dehydroluciferin

To 40 μL of the suspension (1.875 mM ATP, 5 nM luciferase, 0.0125–125 μM dehydroluciferin, 125 μM D-luciferin, 5.63 mM [monomer units] peptide, 50 mM GTA [pH 8]) in 384-well microplates (Greiner Bio-One), 10 μL of 5 mM $MgCl_2$ solution was added, and luminescence signals were immediately read for 104 s using a plate reader (Spark Cyto).

Bioluminescence spectra

To 40 μL of the suspension (1.875 mM ATP, 5 nM luciferase, 125 μM D-luciferin, 5.63 mM [monomer units] peptide, 50 mM GTA [pH 8 or pH 6.7]) in PCR tubes, 10 μL of 5 mM $MgCl_2$ solution was added, and luminescence spectra were immediately read using LumiFL SpectroCapture (ATTO, Tokyo, Japan), with the following settings: measurement mode, single; measurement time, 180 s; slit width, 0.5 mm; camera gain, high; diffraction grating, 150 lines/mm; and shutter for measurement, automatic.

Fluorescence microscopic images

Bright-field and fluorescence images of the 50 μL suspension (1.5 mM ATP, 40 nM Alexa680 conjugated luciferase, 100 μM D-luciferin, 4.5 mM [monomer units] peptide, 1 mM $MgCl_2$, 50 mM GTA [pH 8]) were captured using a plate reader (Spark Cyto), with green channel (excitation/emission: 461–487/500–530 nm) and far-red channel (excitation/emission: 626–644/661–800 nm). Alexa680 conjugated luciferase was prepared according to the reported procedures.[1] As for fluorescence images with dehydroluciferin, AkaLumine, and seMpai, the green channel was used.

Bioluminescence microscopic images

To 40 μL of the suspension (1.875 mM ATP, 50 nM luciferase, 125 μM D-luciferin, 5.63 mM [monomer units] PLR-50, 50 mM GTA [pH 8]), 10 μL of 5 mM $MgCl_2$ solution was added, and bright-field and bioluminescence images were captured using a IXplore Live for Luminescence (Olympus, Japan). The exposure time for bioluminescence images is 5 min.

Bioluminescence quantum yields

A custom-built luminometer with a photomultiplier tube (H11890-01; Hamamatsu Photonics, Shizuoka, Japan) was calibrated according to the protocol established by us and used to detect bioluminescence signals in photon.[2] The L-L reaction was initiated by the addition of 5 mM $MgCl_2$ solution (10 μL) into the mixture solution (1.875 mM ATP, 500 nM luciferase, 0.025 μM D-luciferin, 0 μM or 5.63 μM [monomer units] peptide, 50 mM GTA [pH 8]) in 5 mL polypropylene tube (Corning, One Riverfront Plaza, NY, USA). The bioluminescence signals were monitored until the L-L reaction was completed.

2. Supporting figures and table

Coacervates that consisted of a peptide and ATP

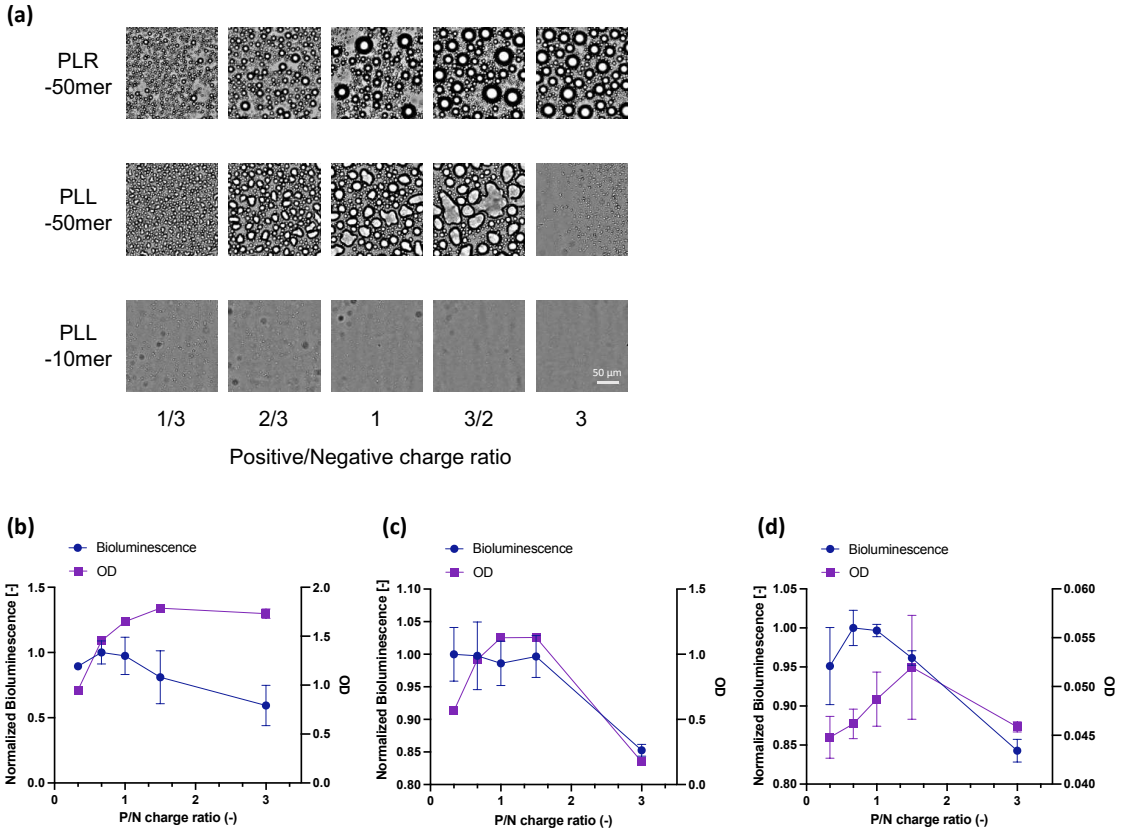

Fig. S1 (a) Microscopic images of coacervates that consisted of oppositely charged peptide (1.5–13.5 mM) and ATP (1.5 mM): 50 μm. Positive/negative charge ratio indicates the charge ratio of the peptide to ATP. Relative bioluminescence intensities and turbidites in the presence of coacervates that consisted of (b) 50-mer PLR (1.5–13.5 mM) and ATP (1.5 mM), (c) 50-mer PLL (1.5–13.5 mM) and ATP (1.5 mM), and (d) 10-mer PLL (1.5–13.5 mM) and ATP (1.5 mM). The bioluminescence intensities were measured using 100 μM D-luciferin.

Time course of PLR/ATP coacervate generation

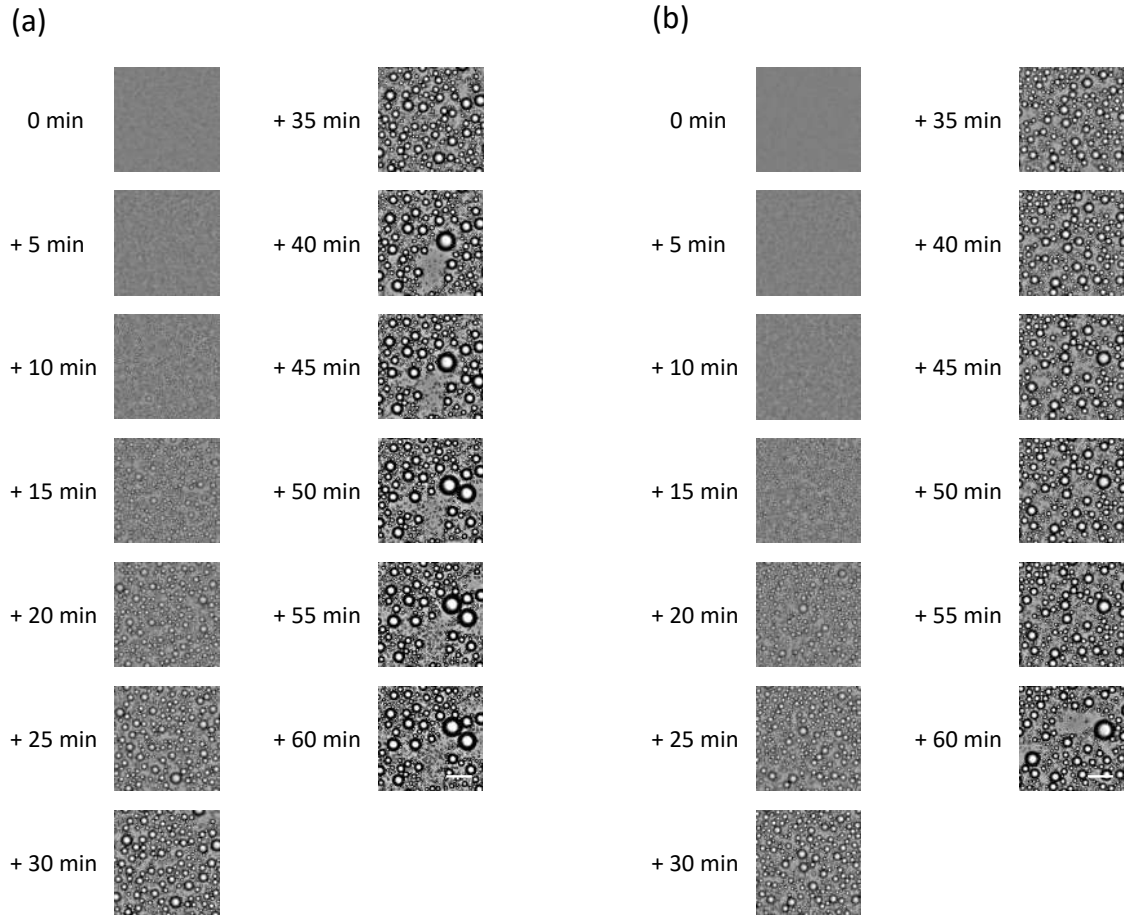

Fig. S2 Time course of PLR/ATP coacervate formation (a) with 1.0 mM $MgCl_2$ and (b) without 1.0 mM $MgCl_2$. The solution contained 4.5 mM PLR (50 mer), 1.5 mM ATP, 4 nM FLuc, and 100 μM D-luciferin in 50 mM GTA buffer (pH 8); scale bar: 50 μm.

Bioluminescence microscopic images

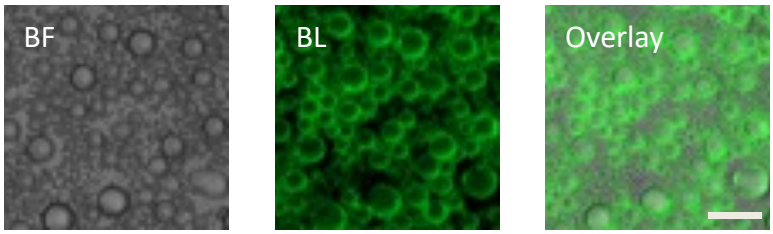

Fig. S3 Bioluminescence images of 50-mer PLR/ATP coacervate systems; scale bar: 50 μm.

Firefly bioluminescence in the presence of PEG

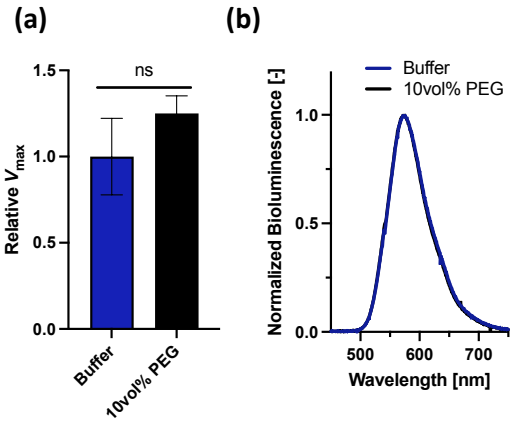

Fig. S4 (a) Relative V_{max} was calculated by normalizing the V_{max} value of each luciferin in GTA buffer (50 mM, pH 8) to 1.0. (b) Bioluminescence spectra in the presence or absence of PEG.

Time course of PLL/ATP coacervate generation

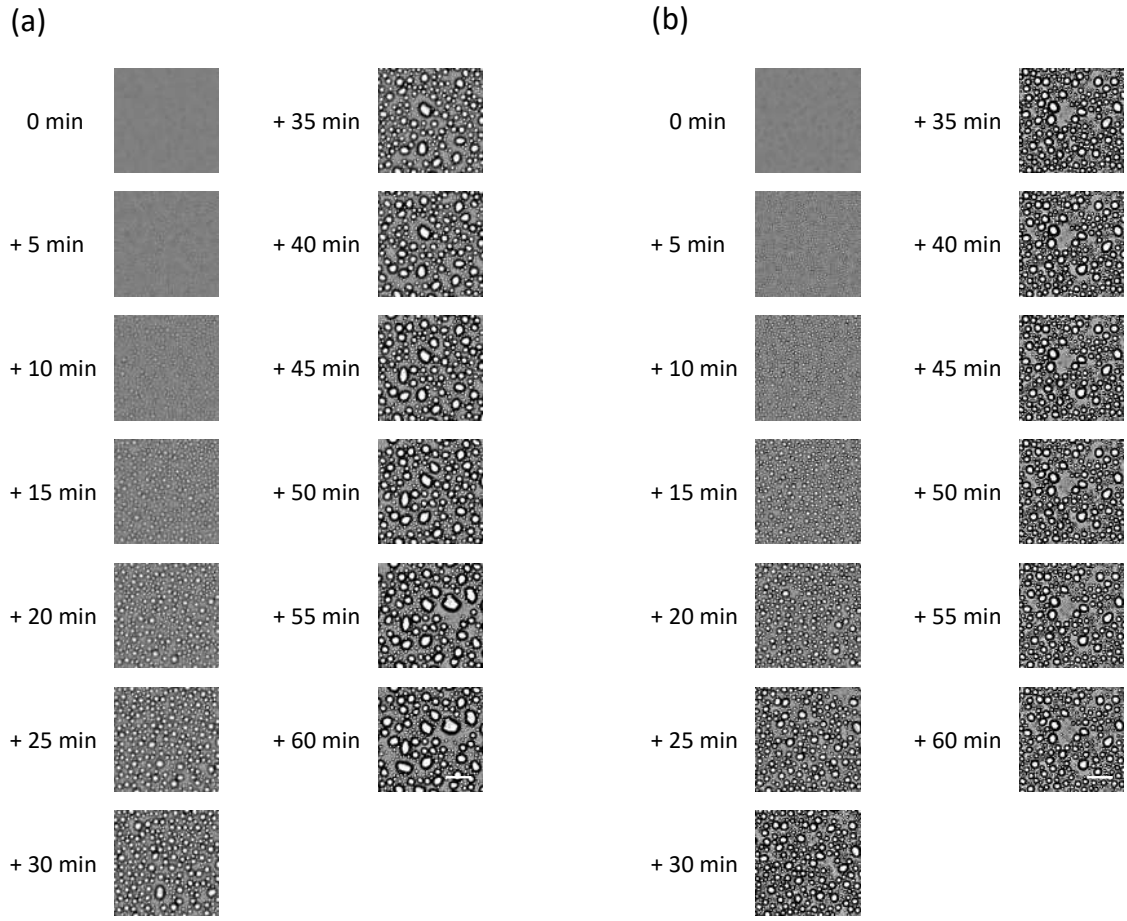

Fig. S5 Time course of PLL-50-mer/ATP coacervate formation (a) with 1.0 mM $MgCl_2$ and (b) without 1.0 mM $MgCl_2$. The solution contained 4.5 mM PLL (50-mer), 1.5 mM ATP, 4 nM FLuc, and 100 μM D-luciferin in 50 mM GTA buffer (pH 8); scale bar: 50 μm.

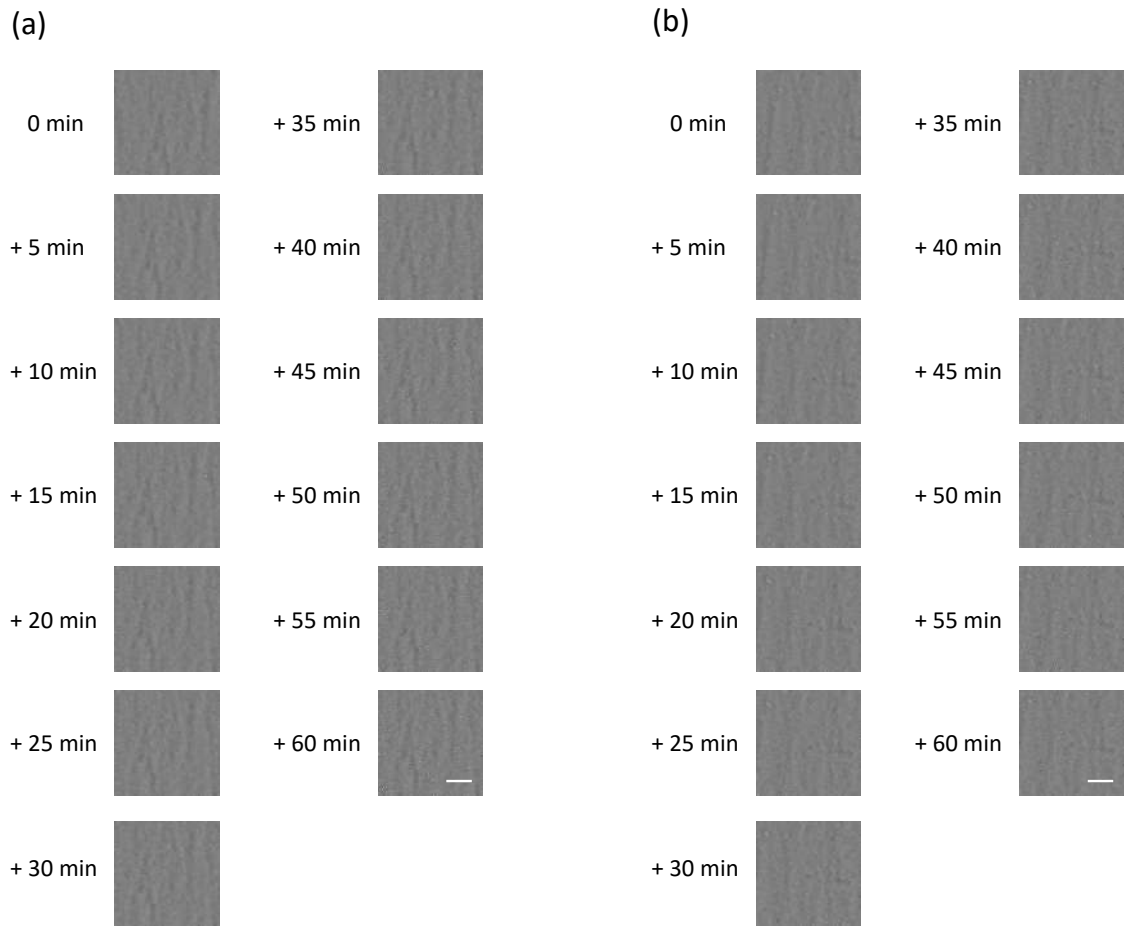

Fig. S6 Time course of PLL-10-mer/ATP coacervate formation (a) with 1.0 mM $MgCl_2$ and (b) without 1.0 mM $MgCl_2$. The solution contained 4.5 mM PLL (10-mer), 1.5 mM ATP, 4 nM FLuc, and 100 μM D-luciferin in 50 mM GTA buffer (pH 8); scale bar: 50 μm.

Firefly bioluminescence at acidic pH

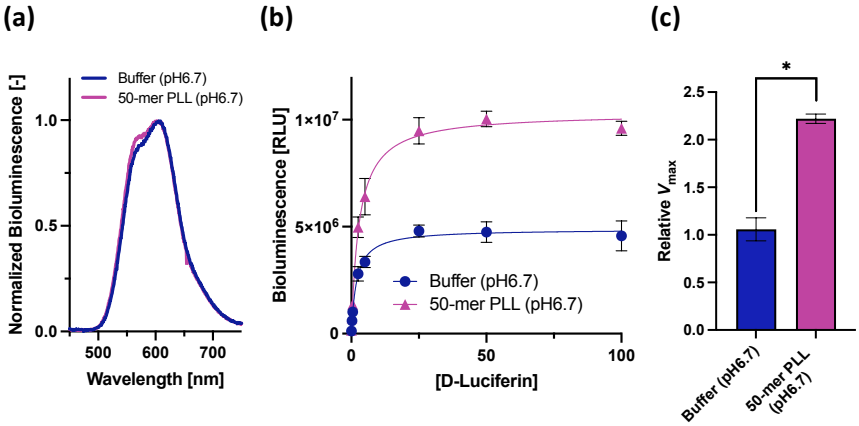

Fig. S7 (a) BL spectra in the presence or absence of 50-mer PLL at pH 6.7. (b) Dose-dependent BL intensities in the presence or absence of 50-mer PLL at pH 6.7. Data were fitted into the Michaelis–Menten equation in GraphPad Prism9. Error bars represent the standard deviation of three measurements. (c) The relative V_{max} was calculated by normalizing the V_{max} value of each substrate in GTA buffer (50 mM, pH 6.7) to 1.0. *P < 0.04 (*t*-test).

Firefly bioluminescence in the presence of CoA

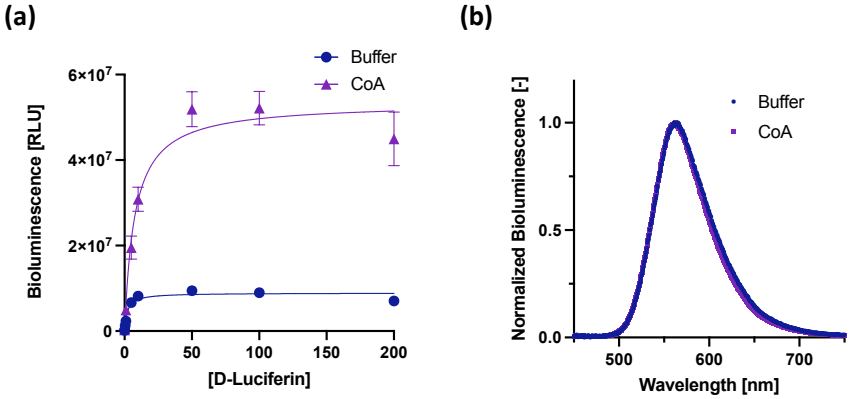

Fig. S8 (a) Dose-dependent bioluminescence intensities: 0.05–200 μM luciferin in the presence of 1.5 mM ATP, 4 nM luciferase, 1 mM $MgCl_2$, and 100 μM CoA, or in the absence of CoA. Data were fit into the Michaelis–Menten equation in GraphPad Prism9. Error bars represent the standard deviation of three measurements. (b) Bioluminescence spectra in the presence or absence of CoA.

Fluorescence microscopic images with dehydroluciferin

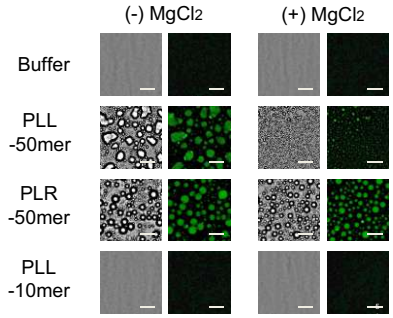

Fig. S9 Fluorescence images of 10 μM dehydroluciferin in the presence of droplets; excitation/emission: 461–487/500–530 nm, scale bar: 50 μm.

Bioluminescence of D-luciferin analogues

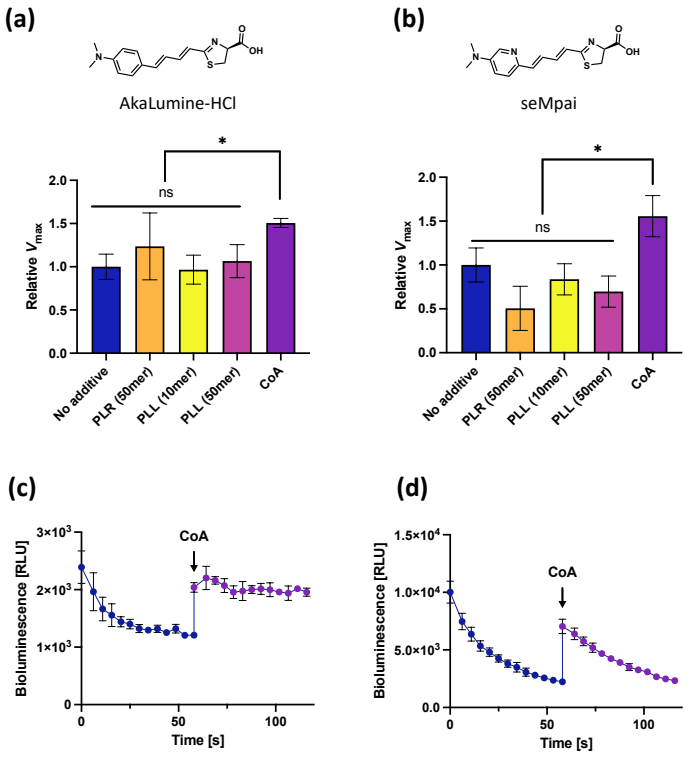

Fig. S10 (a) Chemical structure of AkaLumine-HCl and its relative V_{max}. (b) Chemical structure of seMpai and its relative V_{max}. *P < 0.04 (*t*-test). ns, not significant. Effect of the addition of CoA (100 μM) during the bioluminescence reaction. Injection of CoA enhanced the bioluminescence after the initial burst of light emission with (c) AkaLumine and (d) seMpai. A secondary emission peak was observed possibly due to the conversion of L-AMP into L-CoA for these analogues. Error bars represent the standard deviation of three measurements.

Fluorescence microscopic images with D-luciferin analogues

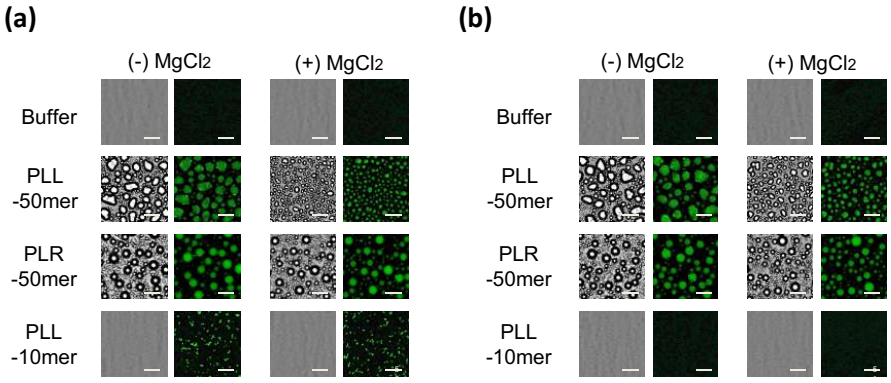

Fig. S11 Fluorescence images of 10 μM D-luciferin analogue (a: AkaLumine-HCl, b: seMpai) in the presence of droplets; excitation/emission: 461–487/500–530 nm, scale bar: 50 μm.

Kinetic profiles of firefly bioluminescence

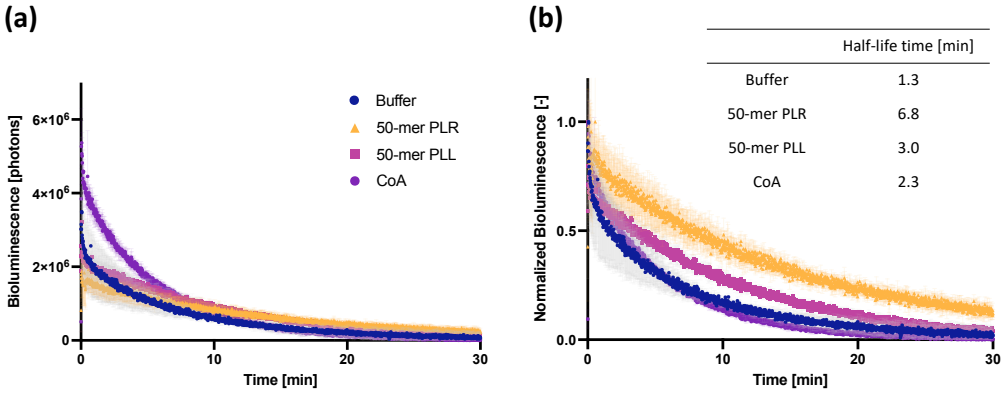

Fig. S12 The light emission time profiles of firefly bioluminescence. The time-course data shown in (b) are normalized by the bioluminescence values at 0 min in (a). The coacervate system and CoA system exhibited a more prolonged and less flash-type emission than that of the buffer system due to a reduced affinity of inhibitors to the enzyme.

Bioluminescence quantum yield under molecular crowding conditions

Table S1. Firefly bioluminescence quantum yield in the presence of PEG

Non-luminescence cofactor added	φ_{BL}	$\pm\sigma*$
+ 10% PEG	0.34	0.046

*Standard deviation of the φ_{BL} measurement.

3. References

1. B. Branchini, D. Ablamsky and J. Rosenberg, *Bioconjugate Chem.,* 2010, **21**, 2023-2030.

2. Y. Ikeda, M. Tanaka, R. Nishihara, Y. Hiruta, D. Citterio, K. Suzuki and K. Niwa, *J. Photochem. Photobiol. A Chem.,* 2020, **394**, 112459.