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# Quantum Yield Enhancement of Firefly Bioluminescence with Biomolecular Condensates

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**The enzymatic luminescence reactions of fireflies are accelerated in the presence of biomolecular condensates comprising a positively charged peptide and ATP. We revealed that this acceleration is caused by the enrichment of reaction elements, local pH changes, and promotion of inhibitory intermediate dissociation, improving the bioluminescence quantum yield by approximately 10%.**

Bioluminescence (BL) is the natural light emitted via a luminescent enzymatic reaction, typically a luciferin-luciferase reaction (L-L reaction) between the substrate luciferin and the enzyme luciferase. BL imaging (BLI) is an optical imaging technique used to visualize luciferase-fused targets *in vitro* and *in vivo* without relying on the excitation light source used in fluorescence methods.[1] In 2018, an absolute BLI technique coupled with a reference LED light source was developed,[2] with results indicating the possibility of counting the number of luciferase molecules (and its conjugated biomolecules) expressed in a single cell based on the BL quantum yield ($\varphi_{BL}$) value. The $\varphi_{BL}$ value is an index of brightness defined as the probability of photon emission per luciferin molecule reacted[3] and is one of the key indicators in the selection of luciferase as an imaging probe.

Firefly luciferase (Fluc) has been commonly used as a reporter protein for BLI applications such as cancer cell imaging[4] because it has a higher $\varphi_{BL}$ (41%)[3] compared to luciferase in other luminescent organisms (30% for *Cypridina* and 16% for *Aequorin*[5]). However, all reported $\varphi_{BL}$ values have been determined under ideal conditions consisting of isolated key components: luciferin, luciferase, and cofactors. Furthermore, the effect of non-luminescent reactants and other biomolecules on the L-L reaction has not been sufficiently considered. These issues are closely related to the reliability of the absolute quantitation values of BLI and hence must be addressed promptly.

In this study, the enzymatic rate and $\varphi_{BL}$ value of Fluc from the common eastern firefly *Photinus pyralis* were evaluated in high-density environments containing (bio)molecules. Here, we compared macromolecular crowding conditions and protein droplets (also called coacervates or biomolecular condensates) mimicking 'membrane-less organelles (MLOs)'. MLOs are liquid-like condensates formed through liquid–liquid phase separation (LLPS) of biomolecules, which has been considered to regulate intracellular enzymatic reactions by spatiotemporal compartmentation (concentration) of enzymes and their substrates.[6] However, the effect of these dense conditions on the L-L reaction remains elusive even though MLOs are ubiquitous in cells.

Fluc has been extensively investigated since its L-L reaction was first demonstrated in 1917.[5] The luminescence reaction involves a two-step chemical reaction (Fig. 1a)—the activation of D-luciferin ($LH_2$) to form an enzyme-bound luciferyl adenylate ($LH_2$-AMP) and pyrophosphate (PPi) (Fig. 1a: equation [1]) and the oxidative decarboxylation of the bound $LH_2$-AMP and production of AMP, $CO_2$, and oxyluciferin ($OxyLH_2$) in the excited state to generate light (Fig. 1a: equation [2]).

First, the effects of MLOs were investigated. Coacervates, as models of MLOs, can be formed using well-designed peptides.[7] Encapsulation of key components into the peptide-based coacervates enhances the enzymatic reaction rates, including ribozymes,[8] multi-enzyme complexes, and sequential reactions *in vitro*.[9] Here, we used the coacervates mainly formed through the electrostatic interactions between cationic homopeptides and anionic nucleotides, which can concentrate enzymes as guest molecules without the loss of their function[7].

A cationic homopeptide, poly-L-arginine (PLR), and ATP pair are known to form coacervates via cation-π interactions for arginine-adenosine in addition to electrostatic interactions.[7]

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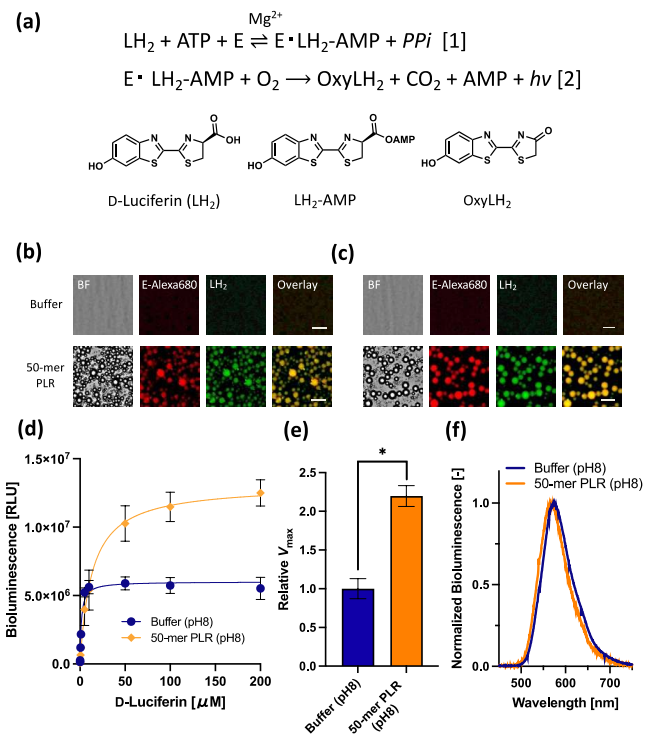


**Fig. 1** (a) $LH_2$-Fluc reaction scheme of D-luciferin to oxyluciferin. 'E' indicates Fluc. Microscopic images of coacervates before (b) and after (c) the $LH_2$-Fluc reaction. Scale bar: 50 μm. (d) Dose-dependent BL intensities in the presence or absence of 50-mer PLR at pH 8. Data were fitted into the Michaelis–Menten equation in GraphPad Prism9. Error bars represent the standard deviation of three measurements. (e) The relative $V_{max}$ was calculated by normalising the $V_{max}$ value of each substrate in GTA buffer (50 mM, pH 8) to 1.0. *P < 0.04 (*t*-test). (f) BL spectra in the presence or absence of 50-mer PLR at pH 8.

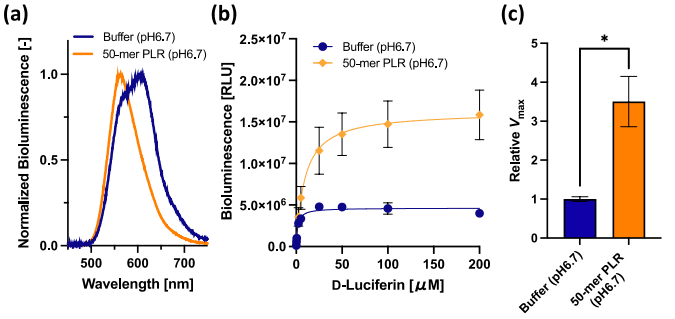


**Fig. 2** (a) BL spectra in the presence or absence of 50-mer PLR at pH 6.7. (b) Dose-dependent BL intensities in the presence or absence of 50-mer PLR at pH 6.7. Error bars represent the standard deviation of three measurements. (c) The relative $V_{max}$ was calculated by normalising the $V_{max}$ value of each substrate in GTA buffer (50 mM, pH 6.7) to 1.0. *P < 0.04 (*t*-test).

To determine appropriate assay conditions, we examined the effect of weight ratios of PLR (50-mer, i.e., 50 arginine repeats) and ATP on the increase in turbidities due to coacervate formation and on firefly BL intensities. Aqueous suspensions of coacervate droplets were prepared at pH 8 by the addition of PLR at various concentrations into the mixture of D-luciferin, Fluc, and ATP. Consequently, highly turbid suspensions were obtained under close-to-neutral charge conditions, even in the presence of the Fluc reaction system (Fig. S1). Since the spherical assemblies observed under these conditions were fused over time (Fig. S2), these were coacervates produced via LLPS.

The $LH_2$-Fluc reaction was then initiated by adding magnesium solutions to the suspensions. The firefly BL with coacervates exhibited the highest BL intensities under near-neutral charge (Fig. S1). In this assay condition, the morphology of coacervates was not affected by the reaction (Fig. S2). With these results, the following experiments were carried out under neutral charge conditions (concentration of 3 equiv. of arginine monomers of PLR relative to trivalent ATP) with an excess of ATP to ensure that D-luciferin is fully consumed in the oxidative luminescence reaction.

The localizations of Fluc and D-luciferin were characterized using the fluorescence of Fluc labelled with Alexa-680 and D-luciferin itself, which revealed that these luminescent components were encapsulated in coacervates during the $LH_2$-Fluc reaction (Fig. 1b-c). BL imaging showed that the luminescent enzymatic reaction proceeded primarily in the coacervate phase rather than in the continuous phase (Fig. S3).

Next, we determined the $LH_2$-Fluc reaction rate with reference to initial BL intensities for 104 s to investigate the effect of encapsulation of the BL reaction system in PLR-ATP coacervates. At higher luciferin concentrations (>50 μM), the reaction velocity was higher in the coacervate system than in the buffer solution alone (Fig. 1d), with the apparent maximum reaction rate ($V_{max}$) of the enzyme in the coacervate system reaching approximately twice that of the buffer (Fig. 1e). The apparent $K_m$ in the coacervate system was 13.0 μM, which is higher than that in the buffer system ($K_m$: 1.46 μM), probably because of the binding of ATP to PLR via cation-π interaction and electrostatic interactions, resulting in a low concentration of the cofactor ATP, which is involved in the enzymatic reaction. Furthermore, there was only a negligible difference in the BL spectra between the two systems at pH 8 (Fig. 1f).

Firefly BL is known to be pH-sensitive, emitting green-yellow luminescence at basic pH and red-shifted luminescence at acidic pH.[10] At pH 6.7, the buffer system showed red emission with an emission maximum ($\lambda_{max}$) at 607 nm, while the coacervate system showed green-yellow emission ($\lambda_{max}$: 562 nm) (Fig. 2a). The difference in emission spectra between the two systems indicated that the apparent pH inside the coacervates is more basic than that in the buffer system. In addition, the difference in pH also had a significant impact on the reaction rates, with the $V_{max}$ of the coacervate system being 3.5-times higher than that of the buffer (Fig. 2b-c). The coacervate phase contains highly concentrated charged portions of the scaffold molecules (PLR and ATP), such as amines, carboxylates, and phosphate groups. These dense charged functional groups may have altered the proton availability in the coacervate,[7] resulting in local pH changes around the enzymes.

In contrast, the changes in enzymatic rates and BL spectra of Fluc could not be observed even with 10 vol% polyethylene glycol (PEG), a neutral, inert macromolecule commonly used to mimic an intracellular crowding environment (Fig. S4). As described above, we uncovered that more intense BL emission occurred when the $LH_2$-Fluc reaction system was encapsulated in coacervates, unlike the macromolecular crowded environment.

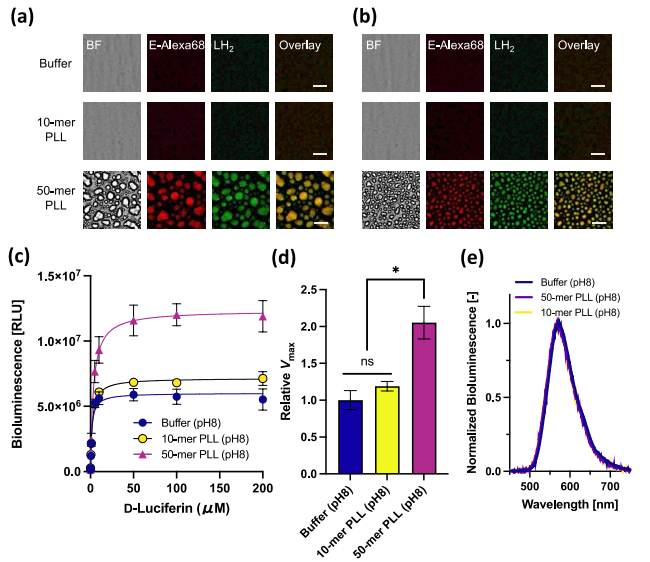


**Fig. 3** Microscopic images of coacervates before (a) and after (b) the $LH_2$-Fluc reaction. Scale bar: 50 μm. (c) Dose-dependent BL intensities in the presence or absence of PLL at pH 8. Error bars represent the standard deviation of three measurements. (d) The relative $V_{max}$ was calculated by normalizing the $V_{max}$ value of each substrate in GTA buffer (50 mM, pH 8) to 1.0. *P < 0.04 (*t*-test). (e) BL spectra in the presence or absence of PLL at pH 8.

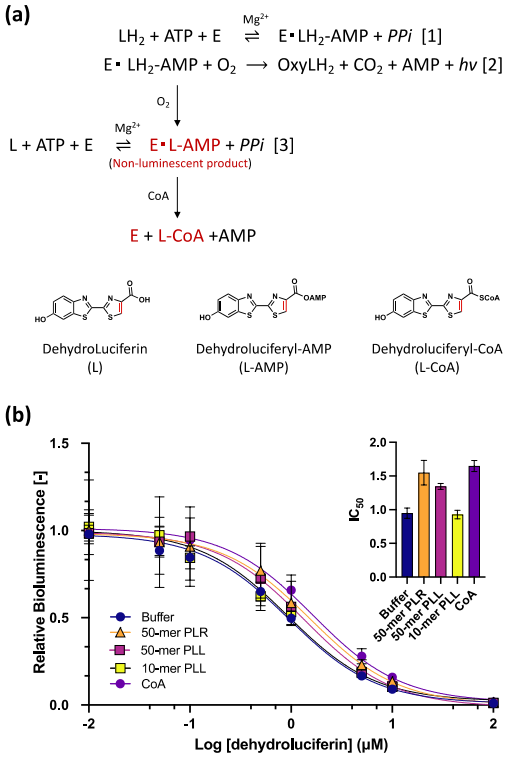


**Fig. 4** (a) $LH_2$-Fluc reaction scheme in the presence of CoA; CoA chemically converts E ˙ L-AMP complex to L-CoA and free E. (b) Dose-response analysis of L for firefly BL at pH 8. Relative BL intensities were determined relative to the control reaction with no L. Data were fitted into the dose-response curve in GraphPad Prism9. The inset indicates the $IC_{50}$ values calculated from the fitted curves. Error bars represent the standard deviation of three measurements.

To gain insight into its mechanism, we further investigated the effects of other polymeric scaffolds, inhibitory intermediates, substrates, and quantum yields.

In addition to 50-mer PLR, poly-L-lysine with 50 or 10 lysine repeats (50-mer and 10-mer PLL, respectively) were used as scaffolds for the coacervates. The 50-mer PLL formed coacervates, and luminescent molecules were concentrated internally before and after the $LH_2$-Fluc reaction (Fig. 3a-b and Fig. S5-6). Coacervates consisting of 50-mer PLL, as opposed to 50-mer PLR, exhibited higher BL intensity in the coacervate system than in the buffer at all substrate concentrations (up to approximately two-fold; Fig. 3c-d) without changes in its spectrum at pH 8 (Fig. 3e). Unlike PLR, PLL forms coacervates with ATP mainly by electrostatic interaction alone. Therefore, the higher concentration of ATP, which was involved in the reaction because of the weak interactions, may have caused the apparent $K_m$ value = 5.40 μM of PLL to be smaller than that of PLR (Fig. 3c). In contrast, the 10-mer PLL did not form coacervates under the current conditions, and no significant difference in $V_{max}$ or apparent $K_m$ value (2.09 μM) was observed (Fig. 3c-d). At pH 6.7, the shoulder at 560 nm of the emission spectrum with 50-mer PLL was slightly higher than that in the buffer system (Fig. S7), suggesting that the local pH was higher even within the coacervate composed of different polymers. Although the apparent $K_m$ varies with the polymeric scaffold, these results indicated that at least the increase in BL emission is likely due to the encapsulation into the coacervates.

In firefly BL, dehydroluciferin (L) and dehydroluciferyl adenylate (L-AMP), an oxidative product of $LH_2$-AMP, are known to act as antagonistic inhibitors of D-luciferin (Fig. 4a). Based on the fact that (i) these inhibitory effects are reduced at basic pH[11, 12] and (ii) emission spectra similar to those produced at basic pH were observed even at pH 6.7 in the presence of PLR-based coacervates (Fig. 2), we assumed that the affinity of inhibitors toward Fluc decreased in the coacervate systems due to its higher local pH than that of the buffer.

To clarify this point, we focused on coenzyme A (CoA), one of the several non-substrate cofactors, which accelerates the firefly BL reaction.[13] CoA eliminates L-AMP through the thiolytic chemical reaction between CoA and L-AMP, which gives rise to a weaker inhibitor, dehydroluciferyl-CoA (L-CoA), resulting in a high reaction rate with no influence on the BL spectrum (Fig. 4a and Fig. S8).[14] The action of CoA (100 μM) on the $LH_2$-Fluc reaction seemed to be similar to that of coacervates at pH 8. Then, the $IC_{50}$ values (an index of inhibition) of dehydroluciferin (L) were compared between coacervates and CoA (Fig. 4b and S9). The $IC_{50}$ of L for Fluc was almost the same in the buffer alone (0.94 μM) and 10-mer PLL (i.e., no coacervate; 0.92 μM), whereas it was higher in 50-mer PLR, 50-mer PLL, and CoA (1.55 μM, 1.34 μM, and 1.64 μM, respectively; Fig. 4b). These results suggested that coacervates improve reaction efficiency by decreasing the affinity of L toward Fluc due to increased pH, or possibly competing with the inhibitor L for Fluc, like CoA.

Next, we investigated whether the acceleration of enzymatic reaction rates by coacervates was common to other luciferins with different enzyme affinities. Fluc can also catalyse D-luciferin analogues, AkaLumine-HCl and seMpai, with red-shifted BL emission suitable for *in vivo* BLI studies.[15] These analogues have a higher affinity for Fluc than D-luciferin;[16, 17] the $K_m$ values for D-luciferin, AkaLumine-HCl, and seMpai

**Table 1** Quantum yield ($\varphi_{BL}$) in the presence or absence of cationic peptide/CoA

| Non-luminescence cofactor added | $\varphi_{BL}$ | $\pm\ \sigma*$ |
|---|---|---|
| Buffer | 0.37 | 0.001 |
| 50-mer PLR | 0.51 | 0.035 |
| 50-mer PLL | 0.49 | 0.019 |
| CoA | 0.50 | 0.022 |

Standard deviation of the three $\varphi_{BL}$ measurements.

calculated in this study were 1.46 μM, 0.12 μM, and 0.59 μM, respectively. Interestingly, no significant changes in apparent $V_{max}$ were observed between the buffer and the coacervate systems (Fig. S10), although these analogues were also highly partitioned into coacervates (Fig. S11). In general, the enrichment of enzymes and/or substrates in the interior plays a significant role in the acceleration of enzymatic reactions by coacervates.[6] However, these results of our $LH_2$-Fluc reaction on high-affinity substrates suggest that enrichment in coacervates is not always sufficient to enhance the enzymatic reaction rates.

Finally, to quantitatively evaluate the brightness of firefly BL with coacervates or CoA, the $\varphi_{BL}$ values were determined with a calibrated luminometer, which reads BL intensities in photons.[18] The $\varphi_{BL}$ values were calculated from the total number of photons emitted and the total number of luciferin molecules reacted in the presence of an excess amount of luciferase (Table 1 and Fig. S12). The $\varphi_{BL}$ value of firefly BL in the buffer solution was 37%, which agreed with the reported value within the uncertainly.[3] Interestingly, the $\varphi_{BL}$ values were improved by more than 10% in the presence of coacervates, that is, 51% for PLR and 49% for PLL. In addition, the molecular crowding environment with 10% PEG did not affect the $\varphi_{BL}$ value (Table S1), indicating that only coacervates improve the brightness of firefly BL. Until now, there have been no reports of increasing the $\varphi_{BL}$ values of firefly BL without modifying the structures of luciferin and luciferase.[19] We are currently pursuing to elucidate the detailed mechanism of this increase, and to achieve more rational control of BL using coacervates.

In conclusion, the detailed characterization of firefly BL with coacervates suggested that the (i) concentration of luciferin and luciferase into the coacervate phase, (ii) local pH changes in the coacervate, and (iii) decreasing affinity of the enzyme reaction inhibitor and Fluc were possible mechanisms of the acceleration of the $LH_2$-Fluc reaction. Importantly, we demonstrated for the first time that coacervates can increase the $\varphi_{BL}$ value of firefly BL by more than 10%. Our enzymatic study with coacervates uncovered essential features of the BL system that were not captured in dilute systems with only isolated components, which should be crucial in the interpretation of BL imaging. Fluc is concentrated in peroxisomes and vesicles of lantern cells, and its $LH_2$-Fluc reaction is controlled by nitric oxide,[20] but the control mechanisms of the reaction in such luminous organisms are not well understood. Detailed characterization of firefly BL with coacervates may greatly contribute to the elucidation of its reaction mechanism in lantern cells and the extension of the absolute BLI platform in molecular and cell biology.

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## Conflicts of interest

There are no conflicts to declare.

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