ELECTROCHEMICAL CONTROL OF BIOLUMINESCENCE FOR PROTEIN BINDING ASSAY-BIOLUMINESCENCE OF BACTERIAL LUCIFERASE

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Light emission by living organisms has long been well known and interested by many peoples. Among the various kinds of light-emitting organisms, firefly is probably the most popular one (Table-1). Jerry-fish has become popular through the 2008 Nobel Prize awarded to Dr. Shimomura for his study of the bioluminescence of jerry-fish.[1] The bioluminescence reaction is one of the enzyme reactions which is catalyzed by the enzyme named as **luciferase** (L). Luciferase oxidizes the substrate **luciferin** (Luc) with the aid of molecular oxygen and other substances as shown in the eq. 1.

$$\mathrm{Luc} + \mathrm{O_2} \xrightarrow{\mathrm{L}} \mathrm{O-Luc} + \mathrm{Light}$$

Where, O-Luc is the oxidation product of luciferin, oxyluciferin. Although most of bioluminescence reactions can be expressed in the eq. 1, luciferase and luciferin are the generic names of light-emitting enzyme and substrate, respectively, but not the names of specific substances. The structures and reaction mechanisms of the luciferase and luciferin differ widely among the light-emitting organisms as shown in Figure 1. The firefly luciferase (FFL) is extracted from the lantern of firefly tail and the reaction mechanism of its bioluminescence has been most extensively studied. The reaction mechanism of the luminescence of bacterial luciferase was studied intensively by Hastings and his research group.[2]

Table 1. Taxonomic list of luminous organisms

Kingdom	Class	Luminous organisms
Monera	Bacteria	Photobacterium
Fungi	Fungi	Luminous fungi
Protists	Protozoa	Noctiluca scintillans
Animals	Porifera	Sponge
	Cnidaria	Jerry-fifh
	Annelida	Earthworm
	Mollusca	Firefly-fish, Latia
	Arthropoda	Firefly, Sea-firefly
	Fish	Luminous fish

Luminescence reactions of FFL and BL
FFL: FFL requires Mg^{2+} and adenosine triphosphate (ATP), in addition to firefly luciferin and molecular oxygen to promote the luminescence reaction. In the course of the reaction cycle, ATP is hydrolyzed to adenosine monophosphate (AMP) and luciferin is oxidized to oxyluciferin (Fig. 2).

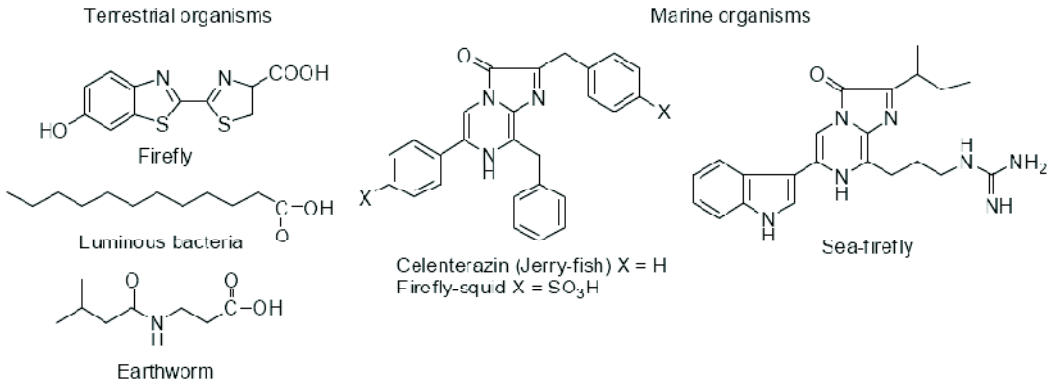

Figure 1. Structures of some well known luciferins

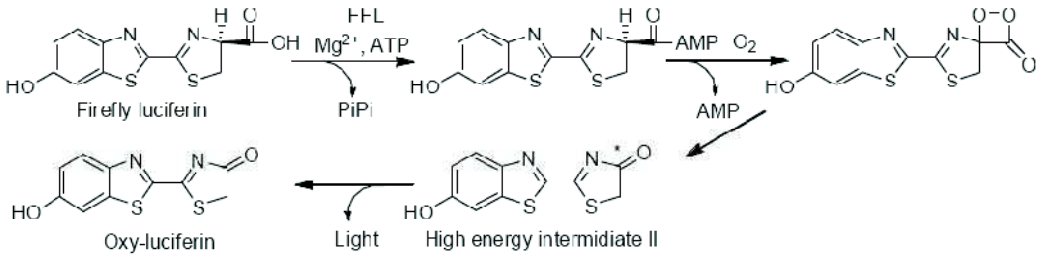

Figure 2. Reaction scheme of firefly bioluminescence

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This reaction is highly sensitive to the concentration of ATP and then utilized for the detection of bioactivities, because all of the living organisms use ATP as their energy source. Although FFL luminescence has been used for the various fields including biotechnology, food inspections and medical purposes, the application to the analysis of the anesthetic action on protein seems to be one of the most interesting examples. General anesthetics inhibit the FFL luminescence in proportional to their anesthetic potencies to animals as shown in Figure 3.[3] The excellent correlation between the inhibitory potency to FFL luminescence and anesthetic potency to animals gives us the idea that general anesthetics should act on the active site in FFL with a similar mechanism to the anesthetic action on the nerve system proteins of animals. Then, it has also been considered that the mechanism of general anesthesia can be elucidated from the study of the action of these agents on FFL.

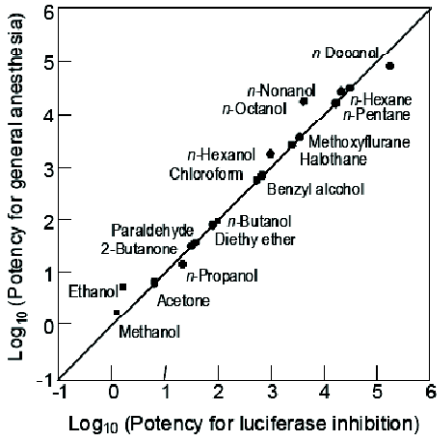

Figure 3. Correlation between anesthetic potency and FFL inhibition

In spite of the extensive studies using FFL, it has several weak points for using in vitro luminescence study. FFL is rather hydrophobic protein and has low solubility and stability in aqueous solution. To break down these problems, some agents such as electrolyte, EDTA and albumin are added to the FFL solution. However, the addition of these agents may affect on the luminescence reaction of FFL and may cause the misunderstanding of the action mechanisms of target agent.[4]

BL: In contrast to FFL, BL has higher solubility and stability in aqueous solution. Moreover, the BL reaction utilizes a hydrophobic alkylaldehyde as the substrate of the reaction as shown in Figure 4. Many of medical agents including general anesthetics also have the hydrophobic nature. Then it can be expected that hydrophobic agents will interfere the BL reaction in competing with the substrate alkylaldehyde. This feature seems to be a great advantage of BL system to analyze the action mechanisms of hydrophobic agents.

Electrochemical control of BL luminescence

BL reaction uses a FMNH2, which is a reduced form of a flavin mononucleotide (FMN), as a cofactor of BL in addition to the substrate alkylaldehyde and molecular oxygen (Fig. 4). However, $FMNH_2$ is readily oxidized by dissolved oxygen with eq. 2, so it is required to reduce the FMN to $FMNH_2$ to promote the BL luminescence reaction (eq. 3).

$FMNH_2 + O_2 \rightarrow FMN + H_2O_2$ (2)

$FMN + 2H^+ + 2e^- \rightarrow FMNH_2$ (3)

For this purpose, photobacteria enzymatically reduce the FMN by using the enzyme FMN reductase. In vitro study, chemical reducing agents such as sodium hydrosulfite have been used so far. However, the reducing agents may affect the action of target agents to BL.

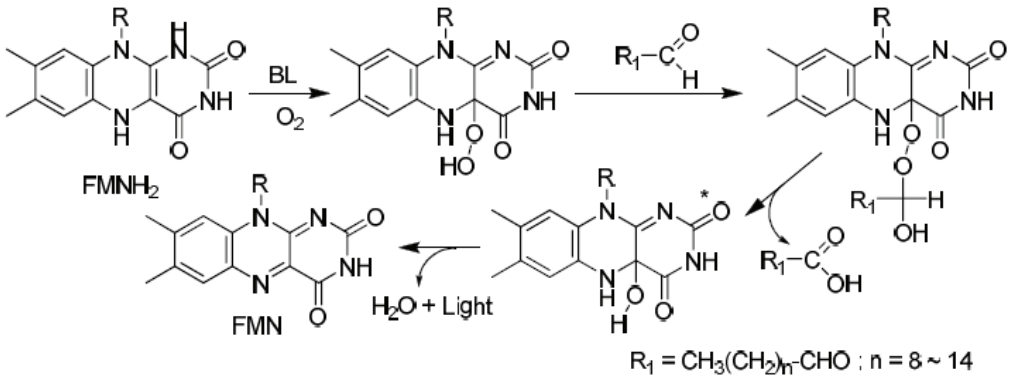

Figure 4. Reaction scheme of bacterial luciferase bioluminescense

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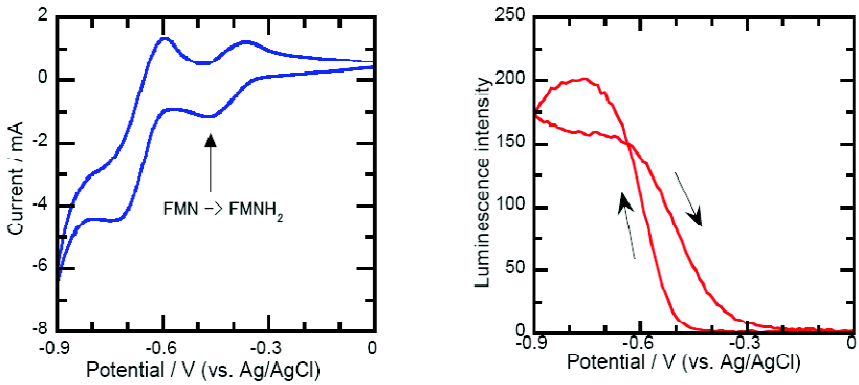

Figure 5. Cyclic voltammogram of FMN solution (left) and light intensity-potential curve of FMN and BL solution (right)

Moreover, it can not be used to realize the steady-state BL luminescence, because the reducing agents are rapidly consumed by the substances other than FMN. To keep off these problems, we planed to build-up the electrochemical FMN reducing system.

Figure 5 shows the cyclic voltammogram of 1.0 mmol dm^{-3} FMN solution (left) and light intensity-potential curve of 1.0 μmol dm^{-3} BL and 30 μmol dm^{-3} FMN solution (right). FMN is reduced to $FMNH_2$ at the potential about -0.5 V (vs. Ag/AgCl). The BL light intensity increased in accordance with the regeneration of $FMNH_2$ confirms that the BL system functions well.5 Since the BL system was confirmed to be function well, we tried to build-up the flow-electrolysis luminescence (FEL) system shown in Figure 6 to attain the steady-state BL luminescence. In this system, the BL solution containing FMN is continuously introduced by peristaltic pump into the FEL cell and then the FMN is electrochemically reduced at Pt or Au mesh electrode to generate $FMNH_2$. The resulting light emission is monitored by photo-multiplier (PMT) and recorded on a digital recorder. All of the system is controlled with personal computer.

Figure 7 shows the BL luminescence observed in this system with repeating the application of electrolysis potential is on and off. The light intensity increased rapidly after

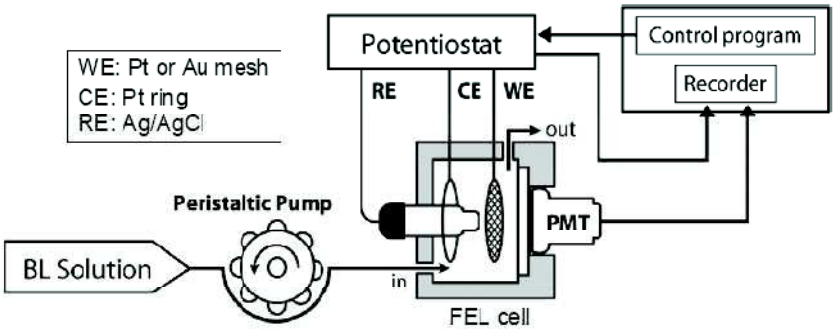

Figure 6. Schematic illustration of the flow-electrolysis luminescence (FEL) system

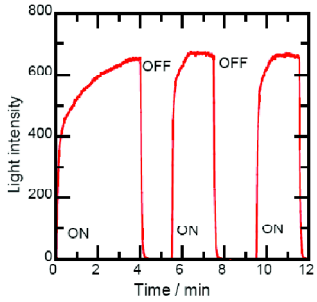

Figure 7. Effect of potential application on the light intensity for FEL system

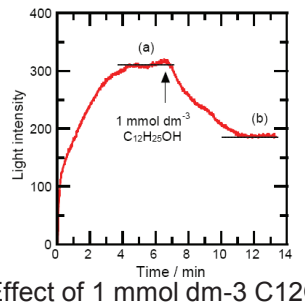

Figure 8. Effect of 1 mmol dm-3 C12OH on the BL light intensity

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the application of -0.7 V (vs. Ag/AgCl) and reached to constant value. On the other hand, the luminescence dropped off when turned off the potential application

As we could confirm that steady-state and repeated BL luminescence has been attained with the FEL system, we then applied this system to analyze the effects of hydrophobic molecules on the BL luminescence reaction.[6]

Effects of hydrophobic molecules on BL reaction

Figure 8 shows the effect of dodecylalcohol ($CH_3(CH_2)_{11}OH$) on the BL light intensity observed with the FEL system. After reaching the light intensity to a constant value at (a), 1 mmol dm-3 of dodecylalcohol was introduced into BL solution and resulting change of the light intensity was recorded at (b). Then the relative light intensity was obtained as the ratio of the intensities at (a) to (b). From this result, we can see that dodecylalcohol inhibit the BL liminescence reaction.

For the hydrophobic effectors on BL reaction, we chose terminally-substituted alkanes Cn-X, in which Cn is the hydrocarbon chain of carbon number n and X is the terminal unit substituted with hydroxyl (OH), amide ($CONH_2$), carboxyl (COOH) and amino (NH_2) units. OH and $CONH_2$ have no net charge, COOH has negative charge and NH2 has positive charge at neutral pH. So, we can examine the effects of hydrophobicity and charge on the BL reaction by changing the carbon number n and terminal unit X, respectively, with these alkanes.

Figure 9 shows the results of the effects of $CnCONH_2$, CnOH, CnCOOH and C_nNH_2, respectively, on the BL luminescence intensity. $CnCONH_2$ inhibited the BL reaction for the all of chain length examined. Longer chain CnOH also inhibited the BL reaction, however, shorter chain C_nOH enhanced the reaction. Longer chain C_nCOOH inhibited the reaction, though shorter chain C_nCOOH scarcely affected the reaction. On the other hand, CnNH2 enhanced the reaction for the all of chain length examined. These results are in quite contrast to the previous report in which chemical reduction method was used to generate $FMNH_2$.[2] In the present study, electrochemical method is was used for this purpose. What is the difference between these two methods? In the case of chemical method, enzymatic turn-over of BL is limited to single because of the slow dissociation of FMN from BL after the light emission step as compared with the consumption of chemical

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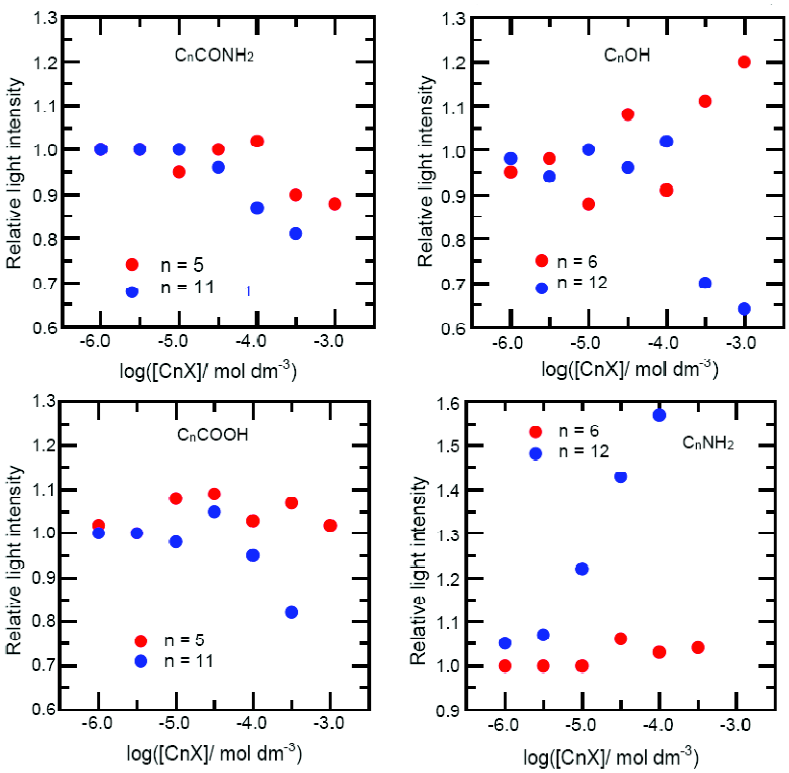

Figure 9. Effects of Cn-X on the light intensity of BL luminescence measured with FEL system

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reductant. In the case of electrochemical method, in contrast, the electrode always ready for regenerate the FMNH2 from FMN irrespective of the speed of the BL reaction cycle. FEL method is the multi turn-over system for the enzyme reaction of BL luminescence. If C_n-X acts on the successive steps after the light emission step, the effect of this action will be reflected on the light intensity of FEL system but not on the intensity of chemical reduction system. The difference in the effects of C_n-X on BL reaction between two methods can then be attributed to the difference of the turn-over number for the two $FMNH_2$ regeneration methods. The increase of the light intensity observed in shorter chain C_nOH and C_nNH_2 will be a consequence of the enhanced dissociation rate of the FMN from BL after the light emission step due to the action of these alkanes.

In the seminar, I will also show you some examples of our current efforts to enhance the luminescence intensity of BL reaction

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