Chemiluminescence assay for quinones based on generation of reactive oxygen species through the redox cycle of quinone

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Abstract

A sensitive and selective chemiluminescence assay for the determination of quinones was developed. The method was based on generation of reactive oxygen species through the redox reaction between quinone and dithiothreitol as reductant, and then the generated reactive oxygen was detected by luminol chemiluminescence. The chemiluminescence was intense, long-lived and proportional to quinone concentration. It is concluded that superoxide anion was involved in the proposed chemiluminescence reaction because the chemiluminescence intensity was decreased only in the presence of superoxide dismutase. Among the tested quinones, the chemiluminescence was observed from 9,10-phenanthrenequinone, 1,2-naphthoquinone and 1,4-naphthoquinone, whereas it was not observed from 9,10-anthraquinone and 1,4-benzoquinone. The chemiluminescence property was greatly different according to the structure of quinones. The chemiluminescence was also observed for biologically important quinones such as ubiquinone. Therefore, simple and rapid assay for ubiquinone in pharmaceutical preparation was developed based on the proposed chemiluminescence reaction. The detection limit (blank + 3SD) of ubiquinone was 0.05 μM (9 ng/assay) with an analysis time of 30 seconds per sample. The developed assay allowed the direct determination of ubiquinone in pharmaceutical preparation without any purification procedure.

Keywords:

Luminol chemiluminescence, quinone, semiquinone radicals, redox cycle, ubiquinone

Introduction

Quinones have an important role to maintain biological functions of animal and plant. Quinones including ubiquinone (coenzyme Q10) acts as electron carrier in mitochondrial electron transport chain [1], besides take part in the blood clot action as phylloquinone (vitamin K_1) which is naphthoquinone derivative [2]. Recently, pyrroloquinoline quinone (PQQ), which is a redox cofactor of bacterial dehydrogenases, was reported as a vitamin for mice [3]. Moreover, in the process of photosynthesis cycle in plant and bacteria, it is well known that plastoquinone has an essential role. In addition to these biological roles, quinones can be applied to wide variety of industrial usage. Quinones are exploited as herbicide, bleaching reagent and cosmetic [4]. Some drugs have quinone structures, such as daunorubicin and doxorubicin, which are used as anti-tumor drugs [5]. Moreover, quinones existed in environment, including 9,10-phenanthrenequinone, have become the focus of attention owing to their potential harmful effects on human health [6-8]. It is reported that quinones serves as a potent inhibitor of some biological functions such as nitric oxide synthase activity [9] and progesterone secretion [10]. Quinones are capable of generating reactive oxygen species (ROS) in biological system [11-13], which cause oxidative stress to living body. From these aspects, a sensitive and selective determination method is required to clarify the activity and disposition of quinones in various fields.

Several techniques have been developed for the determination of quinones including spectrofluorometry [14], electron spin resonance (ESR) assay [15], flow-injection analysis (FIA) with ultraviolet (UV) detection [16], gas-chromatography with

mass spectrometry (GC-MS) [17], high-performance liquid chromatography (HPLC) with UV [18-20], fluorescence (FL) [21], chemiluminescence (CL) [22] and electrochemical (ECD) detection [23-25]. However these techniques have disadvantages in the aspect of sensitivity, rapidity and simplicity. Generally, UV absorbance method is less sensitive and less selective. Because the quinones have no (or extremely weak) fluorescence, fluorometric methods require conversion reaction of quinone to fluorescent compound. Although the HPLC-ECD method is sensitive and is often used for the determination of quinones, the quinones should be reduced beforehand, thus the methods lack the simplicity and rapidity. The GC-MS method needs complicated derivatization procedures to convert quinones to volatile compounds. For the ESR analysis, special and expensive instrument is required. Recently, we reported the novel determination methods for quinones by HPLC-FL [26] and CL methods [27]. Although these methods have great sensitivity and selectivity, the HPLC-FL method requires the pre-column derivatization reaction and the HPLC-CL method requires photoreactor for the photochemical reaction.

In the present study, we focused on the phenomenon that the quinones produce reactive oxygen species (ROS) through their redox reaction cycle. The quinones are reduced by dithiothreitol (DTT) as reductant to form semiquinone radical. This semiquinone radical is oxidized to quinone under aerobic conditions. In this way, dissolved oxygen is converted to ROS. On the other hand, it is known that luminol emits strong luminescence by the reaction with ROS. These reactions were combined to develop a CL assay for quinones. We studied the mechanism and properties of the CL by

using typical quinones. As an application studies of proposed CL assay, we developed the rapid assay for the determination of ubiquinone in pharmaceutical preparations.

Experimental

Material and reagents

9,10-Phenanthrenequinone, 1,2-naphthoquinone, 9,10-anthraquinone, 1,4-benzoquinone and catalase from bovine liver (1600 U/mg) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 1,4-Naphthoquinone, phylloquinone, DTT and ethanol were obtained from Nacalai Tesque (Kyoto, Japan). Luminol and superoxide dismutase (SOD) from bovine erythrocytes (4470 U/mg) were purchased from Sigma (St. Louis, MO, USA) and sodium hydroxide (NaOH) was from Merck (Tokyo, Japan). Acetonitrile (HPLC grade), PQQ and ubiquinone were from Kanto Chemical (Tokyo, Japan). Purified water was prepared by a Simpli Lab UV (Millipore, Bedford, MA, USA) water device. The ubiquinone containing drugs (tablet and granules) were obtained from Eisai Pharmaceuticals (Tokyo, Japan). Other chemicals were of extra pure grade. Stock solutions of quinones except for ubiquinone were prepared in acetonitrile and diluted appropriately with acetonitrile to prepare the working solutions. Stock solution of ubiquinone was prepared in ethanol and diluted with ethanol. Solutions of DTT and luminol were prepared in acetonitrile and aqueous NaOH solution, respectively, just before analysis.

Assay procedure for typical quinones

In a small test tube, 100 µL of quinone solution and 100 µL of 150 µM luminol dissolved in 6 mM NaOH*aq.* was mixed. The test tube was placed in Lumat LB-9507 luminometer (Berthold) and then 100 µL of 50 µM DTT acetonitrile solution was injected automatically, and the produced CL was measured for 600 s. The total intensity was defined as the area under the CL delay curve. Data was expressed as the mean of triplicate measurements.

Determination of ubiquinone in pharmaceutical preparations

The tablets were ground and equivalent amount to one tablet was dissolved in 5.8 mL of ethanol and then a 20 µL aliquot of the solution after filtration with 0.45 µm pore size membrane filter was diluted 100 times with ethanol. A 10 µL of the diluted solution was transferred to small tube and diluted 20 times with ethanol. A 200 µL of 400 µM luminol dissolved in 160 mM NaOH*aq.* was added in the small tube containing 200 µL of standard ubiquinone solution or diluted sample and then 200 µL of 20 mM of the DTT solution was automatically injected into the tube in luminometer LB-9507. The produced CL was measured for 30 s and the total CL intensity was integrated. For granules, thirty-five mg of granules was dissolved in 20 mL of ethanol and then treated by the similar procedures to tablets.

Results and discussion

Mechanism of CL reaction

A strong CL was emitted after the addition of DTT to the mixture of luminol and 9,10-phenanthrenequinone, and continued for more than 10 min. Figure 1 shows the time profiles of CL emission obtained at different 9,10-phenanthrenequinone concentrations. As increasing the 9,10-phenanthrenequinone concentration, the CL intensity increased and the time needed to reach the maximum CL intensity reduced accordingly. The higher concentration of 9,10-phenanthrenequinone enhanced the CL emission and accelerated the CL reaction. It was thought that some ROS had been generated in the reaction mixture because luminol emits CL when it reacts with ROS. In addition, since the significant CL was not observed in the absence of 9,10-phenanthrenequinone or DTT (Fig. 2), both 9,10-phenanthrenequinone (quinone) and DTT (reductant) should play a key role in the proposed CL reaction. Therefore, we considered that ROS was generated from the reaction between 9,10-phenanthrenequinone and DTT.

It has been reported that quinones are reduced to semiquinones by NAD(P)H cytochrome P450 reductase and these semiquinones can be recycled to the original quinones along with the formation of ROS [7]. Kumagai et al. reported that superoxide anion was generated during the reaction of 9,10-phenanthrenquinone with DTT [13]. Yamashoji and Takeda developed the CL assay for bacteria viability, which is based on the generation of ROS through the reduction of menadione (2-methyl-1,4-naphthoquinone) by NAD(P)H-quinone reductase in bacteria [28]. The generated ROS

were detected by luminol CL and the CL intensity was proportional to the viability of bacteria.

In order to elucidate the ROS in relation to proposed CL reaction, we investigated the quenching of CL of 9,10-phenanthrenequinone in the presence of selective ROS scavenger (Table 1). ROS scavenger dissolved in water (20 μL) was added in the mixture of 9,10-phenanthrenequinone, DTT and luminol, and then the CL was measured. The CL intensity was significantly diminished in the presence of SOD that decomposes the superoxide anion selectively. On the other hand, the CL was not quenched drastically in the presence of mannitol, sodium azide (NaN_3) and catalase. Therefore, we concluded that superoxide anion was the main ROS generated through the redox reaction of 9,10-phenanthrenequinone with DTT.

In consideration of the above-mentioned studies, we presume that the possible CL mechanism as follows (Fig. 3): (1) the quinones are reduced by DTT to generate semiquinone radicals. (2) semiquinone radicals convert dissolved oxygen to superoxide anion, and are oxidized to the original quinones (3) CL was emitted by the reaction between the superoxide anion and luminol. The long-lived CL may be attributed to the continuous generation of superoxide anion by the repetition of redox cycle reaction of quinones.

CL properties

As a preliminary study, we investigated the effect of the different kinds of thiol reductants such as DTT, glutathione and penicillamine on CL intensity. Among the

examined reductants, DTT gave the strongest CL and was selected as reductant. The effect of DTT concentration on the CL reaction of 9,10-phenanthrenequinone was investigated. The CL intensity increased as increasing the DTT concentration. However, the time for maximum CL intensity was delayed in the presence of higher concentration of DTT (Fig. 4). The higher concentration of DTT compared with 9,10-phenanthrenequinone might reduce the semiquinone radical to quinol (hydroquinone), and subsequently quinol interacted with quinone to yield the semiquinone radicals [29]. This reaction route might be a reason for the delay of the CL. The concentration ratio of 9,10-phenanthrenequinone and DTT may determine the rate of the CL reaction. On the other hand, the concentrations of luminol and NaOH did not significantly affect the kinetic profile of CL of 9,10-phenanthrenequinone.

Under the experimental conditions described in assay procedure for typical quinones, the CL intensities of other typical quinones such as 1,2-naphthoquinone, 1,4-naphthoquinone, 9,10-anthraquinone and 1,4-benzoquinone were measured. The CL intensities obtained from 1,2-naphthoquinone and 1,4-naphthoquinone were approximately 0.7 and 0.4 times of that from the same concentration of 9,10-phenanthrenequinone, respectively. In contrast, no significant CL was observed from 9,10-anthraquinone and 1,4-benzoquinone. The order of CL intensities is 9,10-phenanthrenequinone>1,2-naphthoquinone>1,4-naphthoquinone, which is in agreement with the order of ROS production capacity reported by Lemaire and Livingstone [12]. They also reported that the rate of ROS production of 9,10-anthraquinone and 1,4-benzoquinone was much lower than those of 9,10-phenanthrenequinone, 1,2-naphthoquinone and 1,4-naphthoquinone. The CL of 9,10-anthraquinone and 1,4-

benzoquinone might not be detected within 600 s because of their slow ROS production rates. In addition, Rodriquez et al. reported that 9,10-anthraquinone and 1,4-benzoquinone could not exhibit toxicity associated with the ROS generation [30]. Figure 5 shows the time profiles of CL emission obtained from 1,2-naphthoquinone and 1,4-naphthoquinone. The CL time profiles were apparently different between these structural isomers. It has been reported that *p*-quinone is reduced to semiquinone radical by single-electron reduction [31] whereas *o*-quinone is reduced to quinol by two-electron reduction [29, 32]. The difference of reduction behavior between *p*-quinone and *o*-quinone might influence the formation rate of semiquinone radical. Slow CL reaction of *o*-quinone such as 1,2-naphthoquinone might be explained from that certain amount of *o*-quinone was reduced to *o*-quinol and then covert to *o*-semiquinone radical. On the other hand, *p*-quinone such as 1,4-naphthoquinone gives a fast CL reaction because *p*-semiquinone radical might return rapidly to *p*-quinone without reducing to *p*-quinol. This might attribute to the difference between the CL profiles obtained from 1,4-naphthoquinone and 1,2-naphthoquinone. However, the further experiments will be required to elucidate the relationship between CL property and structure of quinone.

The CL of the biologically important quinones such as ubiquinone, phylloquinone and PQQ was also measured by the proposed CL assay and the intense CLs were observed for these quinones. Figure 6, 7 and 8 show the time profiles of CL emission obtained from ubiquinone, phylloquinone and PQQ, respectively, under the optimized conditions for each quinone. The proposed method can also be applied to the determination of these biological important quinones.

Application of CL assay for ubiquinone to pharmaceutical preparations

The proposed CL assay was applied to the determination of ubiquinone in the pharmaceutical preparations. It is well known that ubiquinone is prescribed for heart disease such as congestive heart failure. For the routine quality control of ubiquinone preparations, rapid and reliable analytical methods are desirable. As shown in Figure 6, though the maximum CL intensity was reached at about 300 s, the integration time of the CL intensity was set at 30 s in consideration the rapidity of the assay.

To obtain strong CL intensity and maximum signal to blank (S/B) ratio, the reagent concentrations were examined. The effects of DTT concentrations ranging from 5 to 30 mM on CL intensity and S/B ratio were examined, and 20 mM of DTT was selected because the concentration gave the highest CL intensity and S/B ratio. The effects of luminol concentration ranging from 100 to 700 μM on CL intensity and S/B ratio were investigated. It was found that CL intensity increased by increasing the luminol concentration whereas S/B ratio increased till the concentration with 400 μM and then decreased. Therefore, 400 μM luminol was employed. Moreover, the optimum concentration of NaOH was investigated ranging from 75 to 275 mM. The CL intensity increased with increasing NaOH concentration but the S/B ratio was reached maximum at 160 mM and thus 160 mM of NaOH was selected.

A calibration curve was prepared for standard ubiquinone solution; a good linear relationship (r=0.997) between concentration and CL intensity was obtained in the concentration ranges of 0.3-6.0 μM. The regression equation (mean ± SD, n=3) of the calibration curve was $I = (362.6 \pm 8.6) \times 10^3 C - (65.7 \pm 3.6) \times 10^3$, where I and C

represent the integrated CL intensity and the ubiquinone concentration, respectively. The detection limit (blank + 3 SD) of ubiquinone was 0.05 μM (9 ng/assay). The sensitivity of the method for ubiquinone was approximately 60-, 25 and 2 times higher than those of ESR assay [15], spectrophotometry [33] and voltammetry [34], respectively. Moreover, the proposed CL method allowed rapid determination of ubiquinone. The repeatability of the method was determined using three levels of ubiquinone. As shown in Table 2, the relative standard deviations (RSD) for within-day analyses were less than 2.6% and between-day analyses were less than 6.8%, the sufficient repeatability of the assay was proved.

The effect of some possible additives used in pharmaceutical preparations on CL intensity of ubiquinone was studied. The CL of 1.0 μM of ubiquinone was measured in the absence and presence of 20 μL of the possible additives (Table 3). There are no significant interferences even in the presence of 500 or 1000 times molar excess of additives against ubiquinone. Finally, the proposed method was applied to the determination of ubiquinone in two forms of pharmaceutical preparations. As shown in Table 4, it was clear that the measured values determined by the proposed CL method were in good agreement with both the indicated values and the values determined by HPLC-UV method described in the Japanese Pharmacopoeia [35].

Conclusion

A unique and novel CL assay for quinones was established. By mixing quinones with DTT and luminol, strong and long-lived CL was observed and the intensity was

proportional to the concentration of quinones. We concluded that the superoxide anion was generated through the redox reaction of quinones initiated by DTT and could be detected by luminol CL. The proposed CL assay for quinones should be selective because the most chemicals do not undergo a reversible redox cycle that produces the ROS by the reaction with reductant such as DTT. Actually, possible excipient additives and several ROS scavengers except for SOD did not affect the CL of quinones. Furthermore, the background signal is extremely low because the proposed assay does not require the addition of any oxidant, which reacts with luminol and causes high background signal. As an application study, the developed CL assay allowed a simple and rapid analysis of ubiquinone in pharmaceutical preparations. The proposed CL reaction should be useful in a variety fields such as biological, pharmaceutical and environmental analyses.

Acknowledgement

This work was supported in part by Grant-in-Aids for Encouragement of Young Scientist (B) (No. 17790100) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Figure captions

Fig. 1. Time profiles of CL emission obtained from 0.4 to 0.9 μM of 9,10-phenanthrenequinone. The concentrations of DTT, luminol and NaOH are 50 μM, 150 μM and 6 mM, respectively.

Fig. 2. CL emission obtained from a mixture of (A) 0.9 μM 9,10-phenanthrenequinone, 50 μM DTT and 150 μM luminol; (B) 0.9 μM 9,10-phenanthrenequinone and 150 μM luminol; (C) 50 μM DTT and 150 μM luminol. CL was not observed at all in the absence of luminol.

Fig. 3. Deduced CL mechanism for quinones.

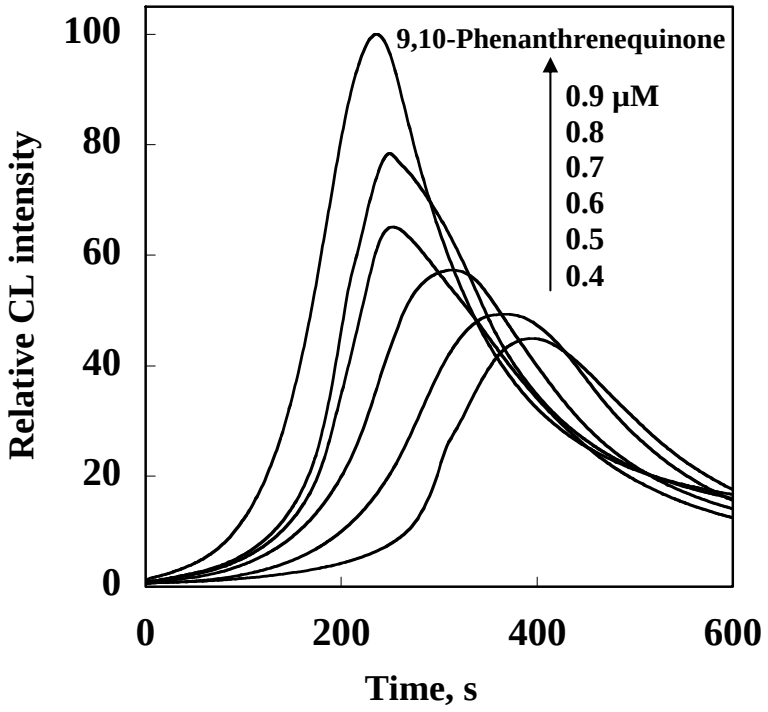
Fig. 4. Effect of DTT concentration on time profile of CL emission for 1.0 μM of 9,10-phenanthrenequinone. DTT concentrations are (A) 1.0, (B) 5.0 and (C) 50 μM. The concentrations of luminol and NaOH are 150 μM and 6 mM, respectively.

Fig. 5. Time profiles of CL emission obtained from 0.8 μM of (A) 1,2-naphthoquinone and (B) 1,4-naphthoquinone. The concentrations of DTT, luminol and NaOH are 50 μM, 150 μM and 6 mM, respectively.

Fig. 6. Time profiles of CL emission obtained from 0.8 μM of ubiquinone. The concentrations of DTT, luminol and NaOH are 20 mM, 400 μM and 160 mM, respectively.

Fig. 7. Time profiles of CL emission obtained from 10 μM of phylloquinone. The concentrations of DTT, luminol and NaOH are 5 mM, 350 μM and 70 mM, respectively.

Fig. 8. Time profiles of CL emission obtained from 0.1 μM of PQQ. The concentrations of DTT, luminol and NaOH are 20 μM, 150 μM and 6 mM, respectively.



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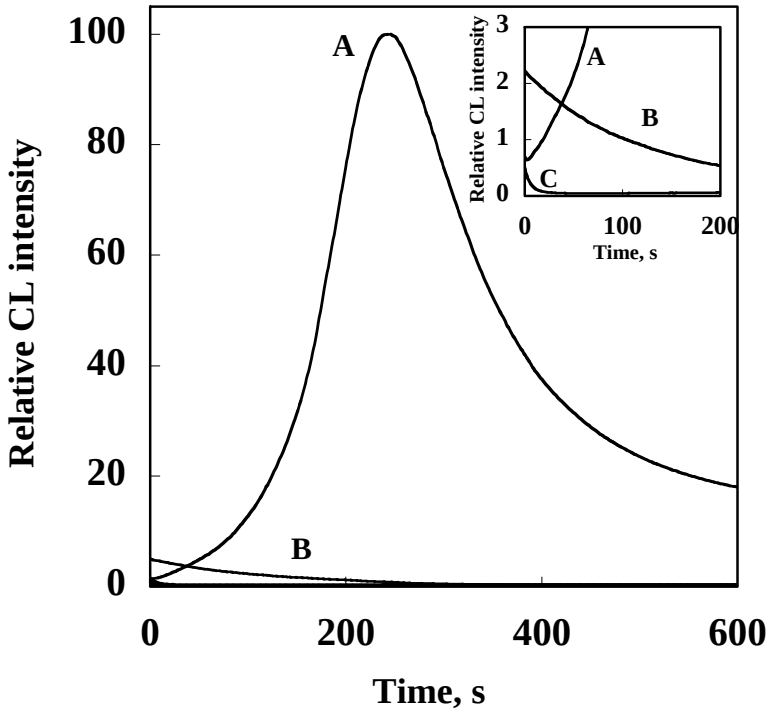

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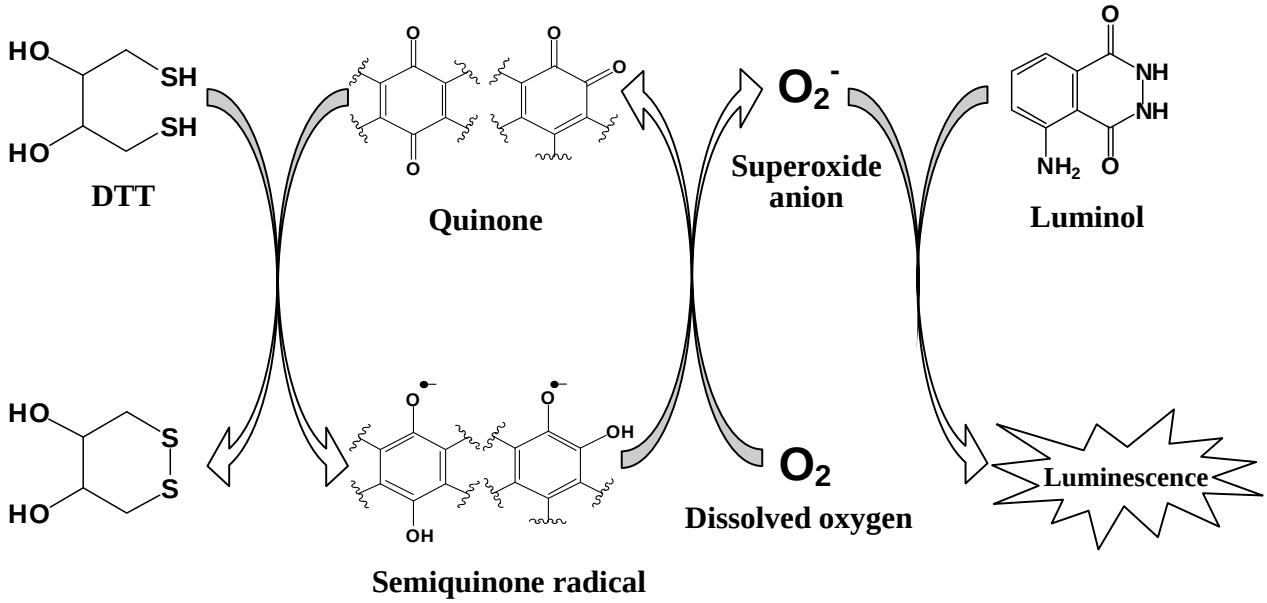

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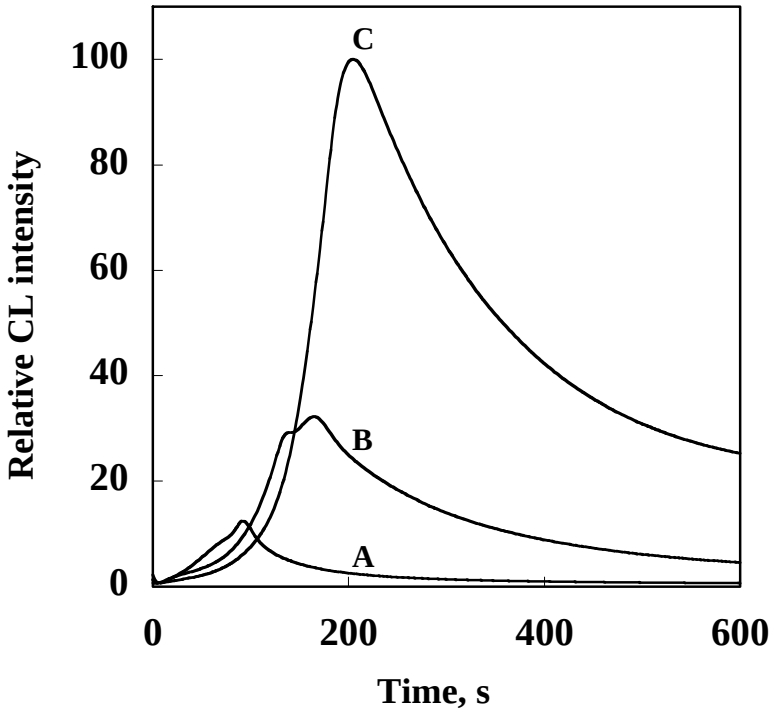
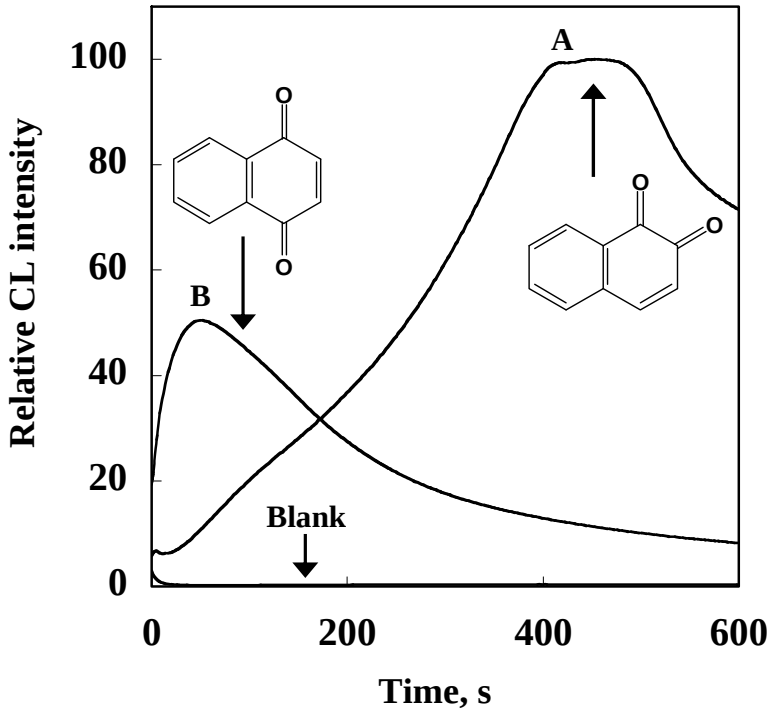


Fig. 4. Effect of DTT concentration on time profile of CL emission for 1.0 µM of 9,10-phenanthrenequinone. DTT concentrations are (A) 1, (B) 5 and (C) 50 µM. The concentrations of luminol and NaOH are 150 µM and 6 mM, respectively.



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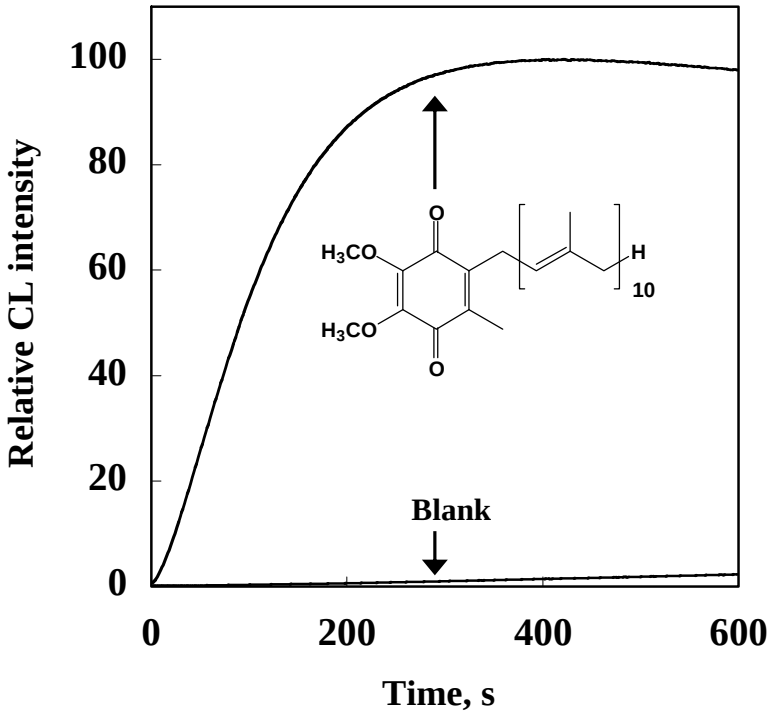

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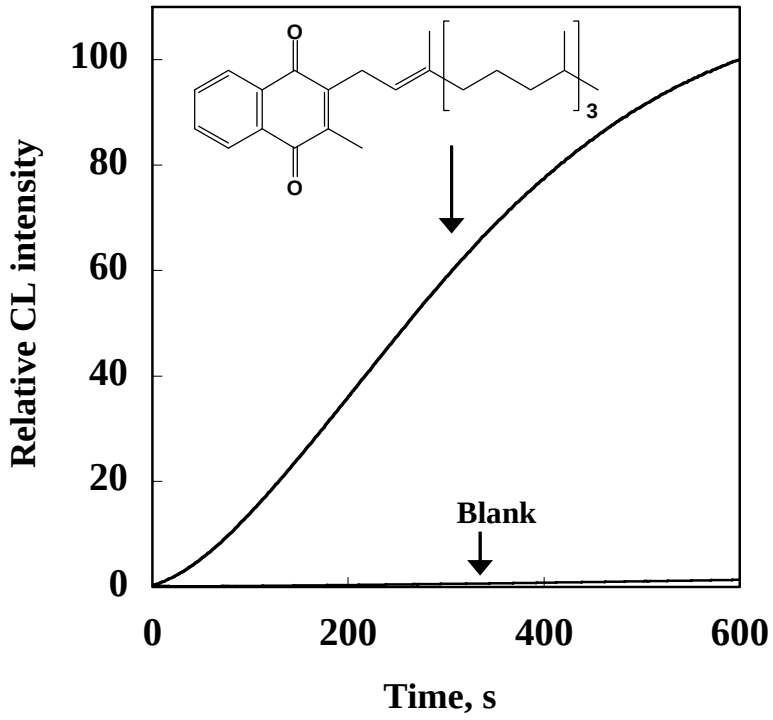

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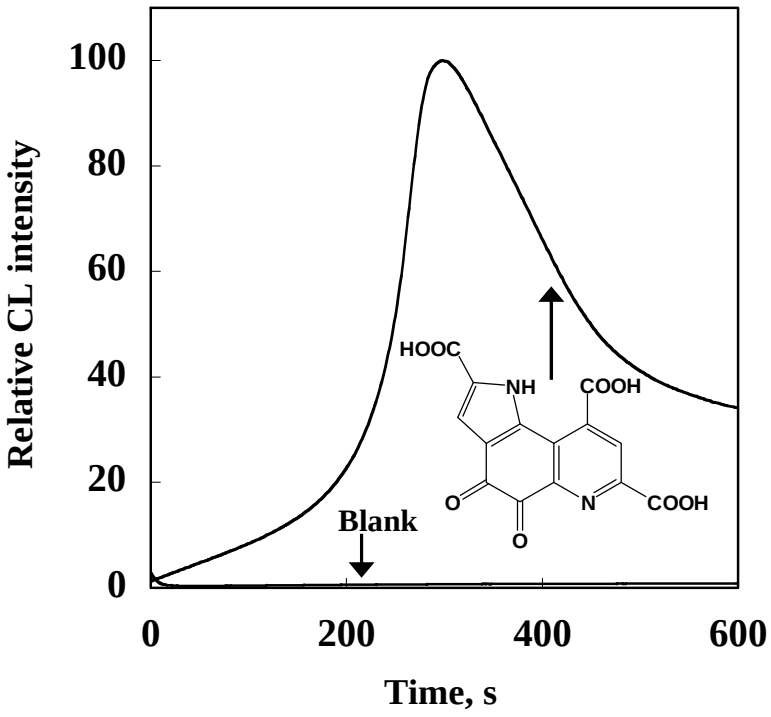

Fig. 8. Time profiles of CL emission obtained from 0.1 µM of PQQ. The concentrations of DTT, luminol and NaOH are 20 µM, 150 µM and 6 mM, respectively.

Table 1 Effect of ROS scavengers on CL of 9.10-phenanthrenequinone

Scavenger	Concentration, μg/mL	RCI[a]
without scavenger		100
SOD	0.21 (1 U/mL)	44
	2.1 (10 U/mL)	13
Catalase	0.63 (1 U/mL)	96
	6.3 (10 U/mL)	94
Mannitol	0.18 (1 μM)	102
	18 (100 μM)	88
NaN_3	0.07 (1 μM)	97
	6.5 (100 μM)	93

[a]CL intensity of 1.0 μM of 9,10-phenanthrenequinone without scavenger was taken as 100.

Table 2 Method repeatability for ubiquinone

Ubiquinone, μM	Precision (RSD, %)	
	Within-day (n=5)	Between-day (n=3)
0.3	1.2	6.8
1.0	2.6	5.4
6.0	2.2	3.3

Table 3 Effect of possible additive on CL of ubiquinone

Additive[a]	Concentration, mM	RCI[b]
Without addtitive		100
KCl	5	100
$MgCl_2$	5	100
$CaCl_2$	5	100
Alanine	10	104
Stearic acid	10	102
Lactose	10	99

[a]Stearic acid was dissolved in ethanol, and others were dissolved in water.

[b]CL intensity of 1.0 μM of ubiquinone without additive was taken as 100.

Table 4 Ubiquinone in pharmaceutical preparations

Formuation	Measured value, mg (mean ± SD, n=5)		Indicated value, mg
	Proposed CL method	HPLC-UV method[c]	
Tablet[a]	9.9 ± 0.6	10.0 ± 0.3	10
Granules[b]	10.0 ± 0.5	10.0 ± 0.2	10

[a] 10 mg/tablet.

[b] 10 mg/g.

[c] Japanese Pharmacopoeia XV