

# **Evaluation of chemiluminescence reagents for selective detection of reactive oxygen species**

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## Abstract

In order to evaluate the chemiluminescence (CL) reagents for selective detection of reactive oxygen species (ROS), we comprehensively measured the CL responses of twenty CL reagents (three luminol derivatives, two imidazopyrazinone derivatives, eight lophine derivatives, six acridinium ester derivatives and lucigenin) against six types of ROS (superoxide anion:  $O_2^{\bullet-}$ , hydroxyl radical:  $\bullet OH$ , hydrogen peroxide:  $H_2O_2$ , hypochlorite anion:  $ClO^-$ , singlet oxygen:  $^1O_2$ , and nitric oxide: NO). As a result of the screening, it was found that nine CL reagents selectively detected  $O_2^{\bullet-}$  while one CL reagent selectively detected  $\bullet OH$ . However, no CL reagent had selectivity on the detection of  $H_2O_2$ ,  $ClO^-$ ,  $^1O_2$  and NO. Our screening results could help to select the most suitable CL reagent for selective determination of different ROS.

As an application study, 4-methoxyphenyl-10-methylacridinium-9-carboxylate (MMAC), one of the acridinium ester derivatives, showed high selectivity on the detection of  $O_2^{\bullet-}$ , and thus was applied to the assay of superoxide dismutase (SOD) activity. The dynamic range and detection limit of the developed CL assay were 0.1-10 and  $0.06 U ml^{-1}$ , respectively. Significant correlation ( $r = 0.997$ ) was observed between the results by the CL assay using MMAC and the spectrophotometric assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt.

Keywords: reactive oxygen species; chemiluminescent probe; screening; acridinium ester; superoxide anion dismutase

## 1. Introduction

In a living body, reactive oxygen species (ROS) generate in various biological systems such as mitochondria [1, 2], NADPH oxidase of neutrophils [3] and xanthine oxidase (XOD) of vascular endothelial cells [4]. ROS are related to signal transduction, production of energy, phagocytosis and defense mechanism against infection [5-8]. On the other hand, ROS are proved to be potentially cytotoxic and its massive increase can lead to oxidative stress, promoting aging [9] and various diseases such as arteriosclerosis [10] and cancer [11].

ROS, in broad sense, include not only free radicals, (*i.e.* superoxide anion ( $O_2^{\bullet-}$ ), hydroxyl radical ( $\bullet OH$ ) and nitric oxide (NO)), but also non radicals, (*i.e.* hydrogen peroxide ( $H_2O_2$ ), hypochlorite anion ( $ClO^-$ ) and singlet oxygen ( $^1O_2$ )). It is thought that each ROS has its own generation mechanism and lifetime, and thus the function of each ROS on the living body should be different (*e.g.*  $H_2O_2$  is an endothelium-derived hyperpolarizing factor in human and mice [12] and  $\bullet OH$  plays an important role as a second messenger in T-cell activation [13]). In addition, each ROS has its own characteristic chemical reactivity (*e.g.*  $^1O_2$  reacts with anthracene to yield endoperoxide by Diels-Alder reaction [14],  $\bullet OH$  can directly react with an aromatic ring to yield a hydroxylated product [15] and NO reacts with guanine to yield a deaminated compound [16]). Although a number of findings about ROS have been reported, it is not fully understand the function of each ROS *in vivo*. Therefore, in order to study the individual activity of ROS, it is essential to selectively detect each ROS.

Several methods have been reported for specific detection of ROS: electron spin resonance (ESR) [17, 18], spectrophotometric assay [18, 19], fluorescence assay [18, 20] and chemiluminescence (CL) assay [18, 21]. Although ESR can detect free radicals specifically and directly, its sensitivity is relatively low and it requires a specialized and expensive spectrometer. In contrast, spectrophotometric assay is widely used because of its simplicity, however, the sensitivity is much lower when compared to other methods. Some fluorescent probes that have a xanthene structure were developed for bio-imaging of ROS [22]. On the other hand, most of the CL reactions are based on oxidation reaction; therefore, a CL assay should be the useful tool for the detection of ROS. Actually, imidazopyrazinone derivatives including 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (CLA) and 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) have widely been used as a CL probe for the selective detection of  $O_2^{\bullet-}$  [23] and have also been reported to react with  $^1O_2$  [24]. Although there are several types of CL reagents, CL responses of these CL reagents against each ROS has not yet been comprehensively investigated.

In this study, in order to evaluate the CL reagents for selective detection of ROS, we measured the CL responses of twenty CL reagents shown in Fig. 1 (three luminol derivatives (1B-1D), two imidazopyrazinone derivatives (2A and 2B), eight lophine derivatives (3A-3H), six acridinium ester derivatives (4A-4F) and lucigenin (4G)) against six types of ROS ( $O_2^{\bullet-}$ ,  $\bullet OH$ ,  $H_2O_2$ ,  $ClO^-$ ,  $^1O_2$  and NO) using luminol (1A) as standard CL reagent.

In addition, because of the highly selective detectability for  $O_2^{\bullet-}$  of 4-methoxyphenyl-10-methylacridinium-9-carboxylate (MMAC, 4B, Fig. 1), one of the acridinium ester derivatives, we applied it to the assay of superoxide dismutase (SOD) activity. SOD is ubiquitously found in oxygen-metabolising organism and catalyzes the dismutation of  $O_2^{\bullet-}$  to yield molecular oxygen and  $H_2O_2$  ( $2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$ ). The CL method using MMAC successfully measured SOD activity without any interference with  $H_2O_2$  generated by the dismutation reaction.

## 2. Experimental

### 2.1. Materials

Hypoxanthine (HX), sodium bromide (NaBr) and lactoperoxidase (LPO) from bovine milk were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 8-Amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)-dione (L-012) and sodium hypochlorite (NaClO) were from Wako Pure Chemical (Osaka, Japan). Luminol, isoluminol, *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI), bis(*N*-methylacridinium)nitrate (Lucigenin), CLA and MCLA were from Tokyo Chemical (Tokyo, Japan). XOD from butter milk was from Nacalai Tesque (Kyoto, Japan). Phenyl-10-methylacridinium-9-carboxylate, ( $\pm$ )-(*E*)-4-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-1) and ethylenediaminetetracetic acid (EDTA) were from Dojindo (Kumamoto, Japan). Acridinium ester derivatives and lophine derivatives were synthesized according to the previously reported methods [25-30]. Water was deionized by Autosill WG 220 (Yamato Kagaku, Tokyo) and passed through Puric-Z (Organo, Tokyo, Japan). All other reagents and solvents were of analytical grade.

### 2.2. Measurement of CL response of CL reagent against each ROS

In this study, CL responses of different CL reagents against ROS were compared by using relative CL intensity (RCI). RCI was a percentage of CL intensity of each CL reagent against that of luminol (1A) and was calculated by the following equation:

$$RCI = 100 \times (CL_{RE} - CL_{BL}) / (CL_{LU} - CL_{BL})$$

where  $CL_{RE}$ ,  $CL_{BL}$  and  $CL_{LU}$  represent the CL intensities of CL reagent, blank and luminol, respectively. Luminol was used as a standard CL reagent because luminol have wide reactivity against various ROS [31, 32]. For the screening, the concentrations of CL reagents were set within the range of the straight line of dilution curves of each ROS. Each CL reagent was dissolved in dimethyl sulfoxide (DMSO) or *N,N*-dimethylformamide (DMF). CL responses were monitored for 1 min at room temperature by a Lumatag Analyzer Auto-250 (Berthold, Wildbad, Germany). As a blank solution, DMSO or DMF was used.

### 2.3. Evaluation of CL response

The generation of ROS was performed according to the previous method [33-37].  $O_2^{\cdot-}$  was produced during the enzymatic oxidation of HX by XOD, and  $\bullet OH$  was produced by Fenton reaction ( $H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$ ) using  $H_2O_2$  and  $FeSO_4$ .  $H_2O_2$  and  $ClO^-$  were used after dilution of commercially available  $H_2O_2$  and  $NaClO$  solution, respectively.  $^1O_2$  was produced through the reaction of  $H_2O_2$  with hypobromide ion that generated from  $NaBr$  by the enzymatic reaction of LPO. For the generation of NO, NOR-1 was used as NO donor reagent that releases NO under alkaline condition. All CL reagents were dissolved in DMSO and diluted to appropriate concentration with DMSO except for the evaluation against  $\bullet OH$ . For the  $\bullet OH$  assay, CL reagents were dissolved in DMF because DMSO has scavenging activity against  $\bullet OH$ .

#### 2.3.1. Evaluation of CL response against $O_2^{\cdot-}$

To 600  $\mu l$  of  $0.5 U ml^{-1}$  XOD in 100 mM HEPES buffer (pH 7.4, buffer A) in a test tube, 100  $\mu l$  of 1.2 mM CL reagent in DMSO and 200  $\mu l$  of buffer A were added. The reaction was started by adding 300  $\mu l$  of 1.6 mM HX in buffer A to the mixture.

#### 2.3.2. Evaluation of CL response against $\bullet OH$

To 100  $\mu l$  of 6  $\mu M$  CL reagent in DMF in a test tube, 650  $\mu l$  of buffer A and 150  $\mu l$  of 1 mM  $FeSO_4$  in water were added. The reaction was started by adding 300  $\mu l$  of 0.5 mM  $H_2O_2$  in buffer A to the mixture.

#### 2.3.3. Evaluation of CL response against $H_2O_2$

To 100  $\mu l$  of 12  $\mu M$  CL reagent in DMSO, 800  $\mu l$  of 50 mM borate buffer (pH 9.5, buffer B) was added. The reaction was started by adding 300  $\mu l$  of 7.5 mM  $H_2O_2$  in buffer B to the

mixture.

#### *2.3.4. Evaluation of CL response against $ClO^-$*

To 100  $\mu$ l of 12  $\mu$ M CL reagent in DMSO, 800  $\mu$ l of buffer B was added. The reaction was started by adding 300  $\mu$ l of 23  $\mu$ M NaClO in buffer B to the mixture.

#### *2.3.5. Evaluation of CL response against $^1O_2$*

To 300  $\mu$ l of 80 mM NaBr in 100 mM acetate buffer (pH 4.5, buffer C), 300  $\mu$ l of 10  $\mu$ g  $ml^{-1}$  LPO in buffer C, 100  $\mu$ l of 120  $\mu$ M CL reagent in DMSO and 200  $\mu$ l of buffer C were added. The reaction was started by adding 300  $\mu$ l of 120 mM  $H_2O_2$  in buffer C to the mixture.

#### *2.3.6. Evaluation of CL response against NO*

To 100  $\mu$ l of 12  $\mu$ M CL reagent in DMSO in a test tube, 700  $\mu$ l of water and 100  $\mu$ l of 2 mM NOR-1 in DMSO/100  $\mu$ M HCl $aq$  (1:1, v/v) were added. The reaction was started by adding 300  $\mu$ l of 100 mM HEPES buffer (pH 8.2) to the mixture.

#### *2.4. SOD assay by CL method by using MMAC*

To 600  $\mu$ l of 0.05 U  $ml^{-1}$  XOD in 10 mM HEPES buffer (pH 8.2) in a test tube, 100  $\mu$ l of 4  $\mu$ M MMAC in acetonitrile and 100  $\mu$ l of SOD in water were added. The reaction was started by adding 300  $\mu$ l of 200  $\mu$ M HX in 10 mM HEPES buffer (pH 8.2) to the mixture. CL responses were monitored for 1 min at room temperature by a Sirius-Luminometer (Berthold).

#### *2.5. SOD assay by WST-1 method [38]*

To 2.5 ml of 50 mM sodium carbonate buffer (pH 10.2), 100  $\mu$ l of 3 mM HX in water, 100  $\mu$ l of 3 mM EDTA in water, 100  $\mu$ l of 3 mM WST-1 in water and 100  $\mu$ l of SOD in water were added. The reaction was started by adding 100  $\mu$ l of 0.06 U  $ml^{-1}$  XOD in water. The absorbance at 438 nm was monitored for 20 min at room temperature by a UV-265FS (Shimadzu, Kyoto, Japan).

### **3. Results and discussion**

#### *3.1. CL responses of CL reagents against each ROS*

Table 1 summarizes CL reagents that showed notable CL response against each ROS

among twenty CL reagents. The values were expressed as the mean value of four measurements. Since all of the lophine derivatives tested in the study (3A-3H) did not show significant CL responses against all ROS (S/B ratio < 3.0), the data were not shown in Table 1.

In the luminol derivatives (1B-1D), CL responses of isoluminol (1B) and ABEI (1C) were smaller than those of luminol on all ROS. On the other hand, L-012 (1D) showed larger CL responses against all ROS than those of luminol and hence L-012 was the most sensitive luminol derivative for the detection of ROS. In fact, L-012 was used for measuring the generation of ROS from activated neutrophils in human blood and oral cavity, and from peritoneal cavity of the rat. Under physiological conditions, opsonized zymosan-dependent CL intensity of L-012 in human blood neutrophils was about 100 and 20 times higher than that of luminol and MCLA, respectively [39].

CLA (2A) and MCLA (2B) showed high responses against  $O_2^{\bullet-}$  and  $^1O_2$  in our study, which was good agreement with previous study [23, 24]. Moreover, the CL responses of CLA and MCLA against NO were newly observed.

Among the acridinium ester derivatives (4A-4F), it was found that 4F has unique response characteristics. The derivatives possess methoxy groups in their structure (4B-4E) showed higher responses than luminol against  $O_2^{\bullet-}$  (870-28000 times),  $H_2O_2$  (5.8-23 times) and  $ClO^-$  (5.1-51 times). On the other hand, 4F that possess the nitro group showed relatively large RCI against  $\bullet OH$  (220 times),  $^1O_2$  (57 times) and NO (21 times) compared with luminol. In this context, electron donating substituent (methoxy group) and electron withdrawing substituent (nitro group) may be responsible for the difference in CL responses.

As a result of the screening, it was found that nine CL reagents (1D, 2A, 2B, 4A-4E and 4G) showed higher selectivity on the detection of  $O_2^{\bullet-}$  and one CL reagent (4F) showed higher selectivity on the detection of  $\bullet OH$ . However, no CL reagent selectively detected  $H_2O_2$ ,  $ClO^-$ ,  $^1O_2$  and NO. The CL reagents which can selectively detect  $O_2^{\bullet-}$  and  $\bullet OH$  are expected to become an excellent CL probe for the detection of these ROS. On the other hand, luminol chemiluminescence was used for not only *in vitro* experiments but also *in vivo* experiments such as measurement of ROS generation in neutrophils [40,41]. The application of the selective CL reagents for  $O_2^{\bullet-}$  and  $\bullet OH$  into *in vivo* experiments can be adapted to identify the kinds of ROS generated from neutrophils. Also, these CL reagents should be useful to elucidate the roles of  $O_2^{\bullet-}$  and  $\bullet OH$  in biological systems.

### 3.2. SOD assay by using MMAC

MMAC (4B), an acridinium ester derivative, was employed for the assay of SOD activity because we found that MMAC could selectively detect  $O_2^{\bullet-}$  in the screening. SOD and MMAC competitively react with  $O_2^{\bullet-}$ . Therefore, SOD activity can be measured as the inhibition ratio of CL intensity. The inhibition ratio of CL intensity was calculated by the following equation:

$$\% \text{Inhibition} = 100 \times (CL_B - CL_S) / CL_B$$

where  $CL_B$  and  $CL_S$  represent the CL intensities obtained from blank (water) and SOD solution, respectively. As the concentration of SOD increased, the inhibition ratio also increased (Fig. 2). When more than  $10 \text{ U ml}^{-1}$  of SOD was used, the inhibition ratio reached almost 100%. This showed that the CL intensity of MMAC is all due to  $O_2^{\bullet-}$ . When the logarithm of SOD concentration was plotted against the logarithm of ( $\% \text{Inhibition} / (100 - \% \text{Inhibition})$ ), a linear relationship was observed over the range of  $0.1$  to  $10 \text{ U ml}^{-1}$  with a correlation coefficient of 0.998. The detection limit defined as 5% inhibition of the CL intensity of MMAC was  $0.06 \text{ U ml}^{-1}$ . The values of the relative standard deviation (RSD) of inhibition ratio were 1.8, 1.4 and 0.1% (within-day,  $n = 5$ ) and 9.3, 3.6 and 0.3% (between-day,  $n = 3$ ) at concentration levels of 0.5, 1.5 and  $10 \text{ U ml}^{-1}$ , respectively.

Figure 3 shows the correlation between the results obtained by the proposed CL method using MMAC (MMAC method) and WST-1 method in the determination of SOD activity. A good linear correlation ( $r = 0.997$ ) was observed, which supported the reliability of the MMAC method. For the analytical time, MMAC method took only 1 min while WST-1 method took 20 min.

## 4. Conclusions

In this study, we systematically screened the selective detection ability of twenty CL reagents against six types of ROS. As a result, we discovered that nine CL reagents selectively detected  $O_2^{\bullet-}$  and one CL reagent selectively detected  $\bullet\text{OH}$ . The results obtained by this comprehensive screening should be useful to select the most suitable CL reagent for selective determination of ROS.

One of acridinium ester derivatives, MMAC, was successfully applied for the determination of SOD activity. The assay results had a good correlation with those obtained by WST-1 method. Each assay in the proposed CL method was completed in 1 min; therefore, it should be useful to assay the SOD activity.



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## Figure captions

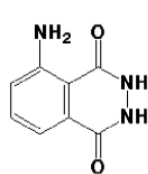
Fig. 1. Structures of CL reagents tested.

Fig. 2. Inhibition curve of SOD using MMAC method

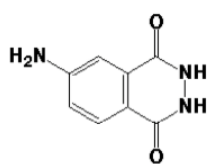
The reaction mixture contained 600  $\mu\text{l}$  of 0.05  $\text{U ml}^{-1}$  XOD in 10 mM HEPES buffer (pH 8.2), 100  $\mu\text{l}$  of 4  $\mu\text{M}$  MMAC in acetonitrile, 100  $\mu\text{l}$  of SOD in water at the concentration shown on the abscissa and 300  $\mu\text{l}$  of 200  $\mu\text{M}$  HX in 10 mM HEPES buffer (pH 8.2). The error bars mean standard deviations ( $n = 5$ ).

Fig. 3. Correlation between the SOD activity in standard solutions by CL method using MMAC and spectrophotometric method using WST-1. The error bars mean standard deviations ( $n = 5$ ).

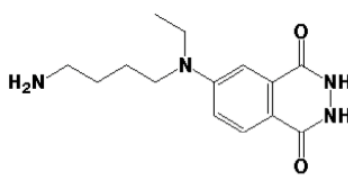
## 1, luminol derivatives



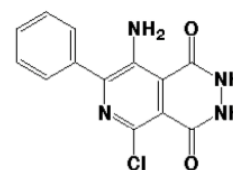
1A (luminol)



1B (isoluminol)

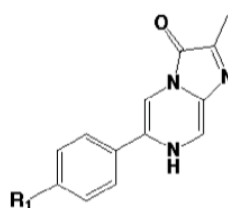


1C (ABEI)



1D (L-012)

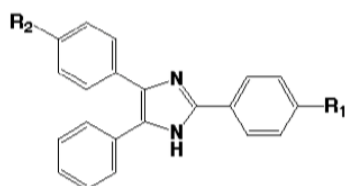
## 2, imidazopyrazinone derivatives



2A:  $R_1 = H$  (CLA)

2B:  $R_1 = OCH_3$  (MCLA)

## 3, lophine derivatives



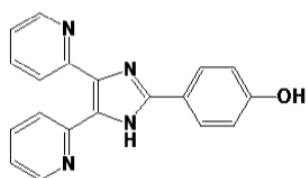
3A:  $R_1 = R_2 = H$  (lophine)

3B:  $R_1 = B(OH)_2$ ,  $R_2 = H$

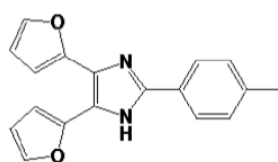
3C:  $R_1 = OH$ ,  $R_2 = H$

3D:  $R_1 = COCl$ ,  $R_2 = H$

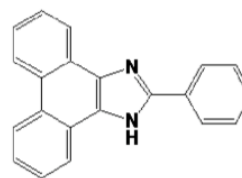
3E:  $R_1 = COOCH_3$ ,  $R_2 = N(CH_3)_2$



3F

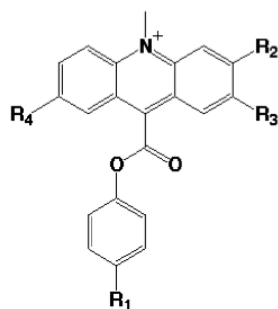


3G



3H

## 4, acridinium derivatives



4A:  $R_1 = R_2 = R_3 = R_4 = H$

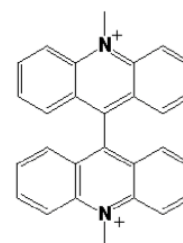
4B:  $R_1 = OCH_3$ ,  $R_2 = R_3 = R_4 = H$  (MMAC)

4C:  $R_2 = OCH_3$ ,  $R_1 = R_3 = R_4 = H$

4D:  $R_3 = OCH_3$ ,  $R_1 = R_2 = R_4 = H$

4E:  $R_3 = R_4 = OCH_3$ ,  $R_1 = R_2 = H$

4F:  $R_1 = NO_2$ ,  $R_2 = R_3 = R_4 = H$



4G (lucigenin)

Fig. 1. Yamaguchi et al.

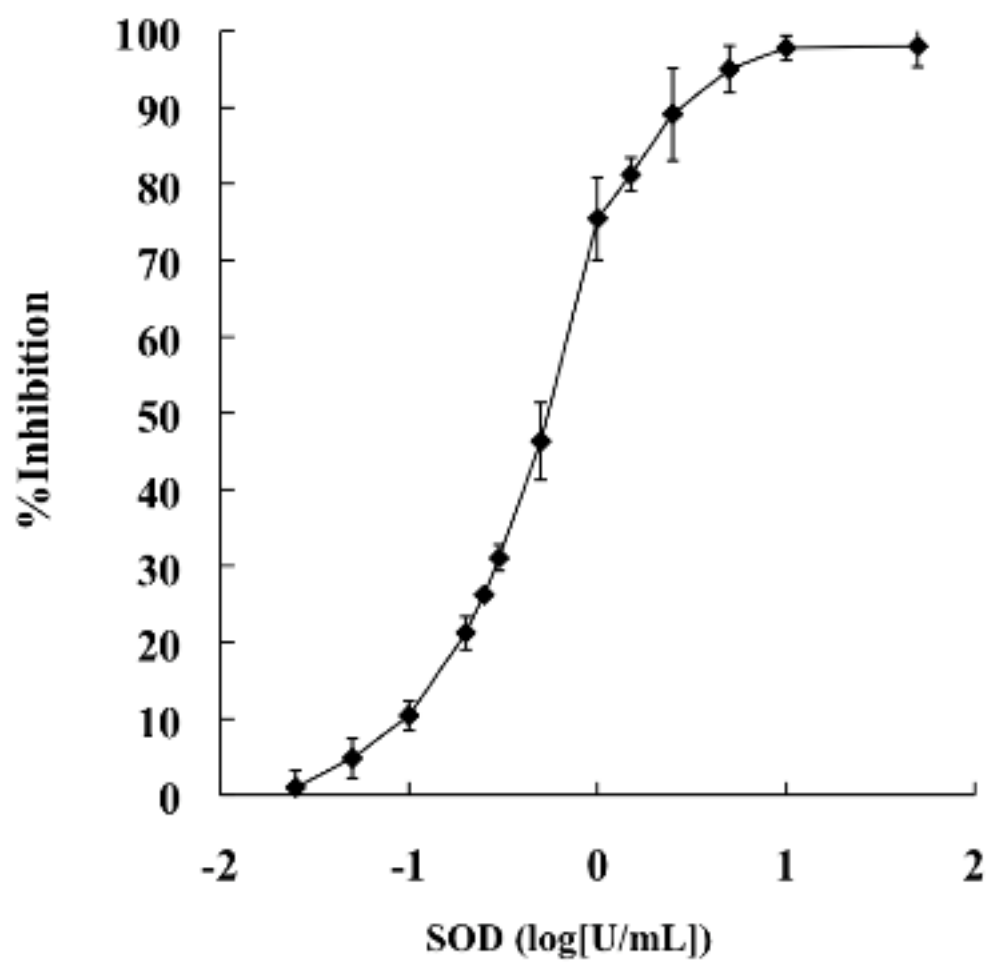


Fig. 2. Yamaguchi et al.

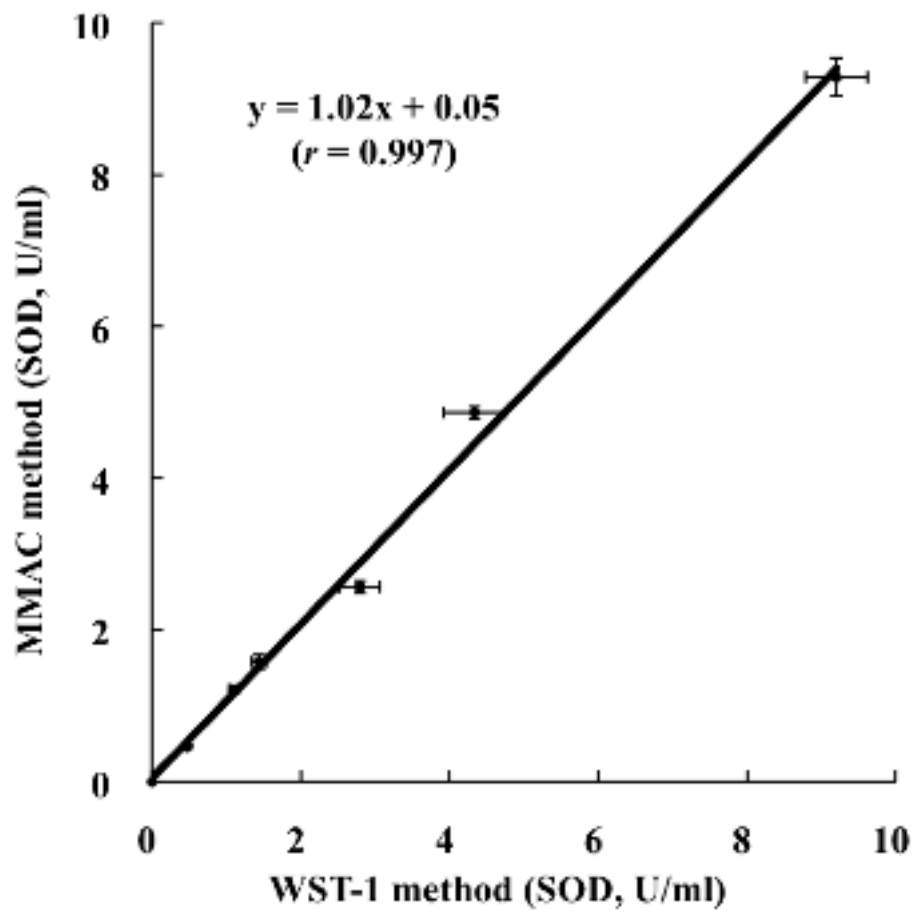


Fig. 3. Yamaguchi et al.



**Table 1 RCI of the investigated CL reagents against individual ROS (n = 4)**

Compounds	RCI <sup>a</sup>					
	O <sub>2</sub> <sup>-</sup>	•OH	H <sub>2</sub> O <sub>2</sub>	ClO <sup>-</sup>	<sup>1</sup> O <sub>2</sub>	NO
<b>1A (luminol)</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>
1B	41	–	10	15	4.5	6.1
1C	– <sup>b</sup>	–	52	38	1.7	15
1D	41000	7600	350	370	6500	8100
2A	190000	–	0.4	–	510	3700
2B	230000	–	0.4	730	1400	17000
4A	160000	82	2200	1200	100	61
4B	2500000	38	1500	870	71	11
4C	87000	–	2100	1500	3.3	2.3
4D	2800000	41	2300	5100	21	15
4E	120000	–	580	510	2.1	2.8
4F	500	22000	410	560	5700	2100
4G	6000	420	16	–	–	–

Each compound was dissolved in DMSO or DMF according to the type of ROS.

<sup>a</sup>: Relative CL intensity. The CL intensities of luminol to each ROS were assumed to be all 100.

<sup>b</sup>: Significant difference was not detected against the value of blank at the tested conditions (S/B ratio < 3.0).