

Detection of Hydrogen Peroxide *in vitro* and *in vivo* Using Peroxalate Chemiluminescent Micelles

Iljae Lee,^{†,a} On Hwang,^{†,a} Donghyuck Yoo,[†] Gilson Khang,[‡] and Dongwon Lee^{†,*,‡}

[†]Department of BIN Fusion Technology and

[‡]Department of Polymer-Nano Science and Technology, Jeonju, Chonbuk 561-756, Korea. *E-mail: dlee@jbnu.ac.kr

Received March 2, 2011, Accepted May 10, 2011

Hydrogen peroxide plays a key role as a second messenger in the normal cellular signaling but its overproduction has been implicated in various life-threatening diseases. Peroxalate chemiluminescence is the light emission from a three component reaction between peroxalate, hydrogen peroxide and fluorophores. It has proven great potential as a methodology to detect hydrogen peroxide in physiological environments because of its excellent sensitivity and specificity to hydrogen peroxide. We developed chemiluminescent micelles composed of amphiphilic polymers, peroxalate and fluorescent dyes to detect hydrogen peroxide at physiological concentrations. In this work, we studied the relationship between the chemiluminescence reactivity and stability of peroxalate by varying the substitutes on the aryl rings of peroxalate. Alkyl substitutes on the aryl ring of peroxalate increased the stability against water hydrolysis, but diminished the reactivity to hydrogen peroxide. Chemiluminescent micelles encapsulating diphenyl peroxalate showed significantly higher chemiluminescence intensity than the counterpart encapsulating dimethylphenyl or dipropylphenyl peroxalate. Diphenyl peroxalate-encapsulated micelles could detect hydrogen peroxide generated from macrophage cells stimulated by lipopolysaccharide (LPS) and image hydrogen peroxide generated during LPS-induced inflammatory responses in a mouse.

Key Words : Hydrogen peroxide, Chemiluminescence, Micelles, Peroxalate ester

Introduction

Reactive oxygen species (ROS) are very small reactive molecules that contain oxygen atoms and peroxides and form as a natural byproduct of the normal cellular metabolism of oxygen.¹ They include hydrogen peroxide, superoxide, hydroxyl radical, nitric oxide and peroxynitrite. ROS are an essential physiological regulator and serve as an important biological messenger in cell signal transduction cascades.^{2,3} However, the overproduction of ROS leads to oxidative stress, resulting in significant damages to cell structures. Accumulation of oxidative stress damages over time is associated with many life-threatening diseases including cardiovascular diseases, cancer, Alzheimer disease and related neurodegenerative diseases.⁴ In particular, hydrogen peroxide is a major ROS playing a key role as a second messenger in normal cellular signaling.⁵ Recently, numerous publications have suggested that controlled burst of hydrogen peroxide have beneficial roles for cell survival, growth, differentiation and maintenance.^{6,7} However, the overexpression of hydrogen peroxide causes oxidative damages to tissues and organs and has been implicated in inflammatory responses and afore-mentioned life-threatening diseases.⁸ Therefore, hydrogen peroxide has great potential as a diagnostic biomarker of inflammatory responses and there is increasing interest for the development of strategies to detect hydrogen peroxide at physiological concentrations.^{9,10}

A fluorescent probe, called Amplex red reagents, has been widely used to detect hydrogen peroxide released from human cells.¹¹ This probe reacts in a 1:1 stoichiometry with hydrogen peroxide to yield highly fluorescent resorufin in the presence of peroxidase. Amplex red reagents can detect as low as 10 picomoles of hydrogen peroxide. However, its application has drawbacks because of poor selectivity to hydrogen peroxide. Recently, a new methodology has been introduced to detect hydrogen peroxide which exploits peroxalate chemiluminescence reaction.^{9,12} Peroxalate chemiluminescence reaction is a three component reaction between hydrogen peroxide, peroxalate esters and fluorescent dyes.¹³ In principle, peroxalate esters react with hydrogen peroxide to generate dioxetanedione with high energy, which chemically excite fluorescent dyes, leading to the chemiluminescence (Figure 1). Peroxalate chemiluminescence is analogous to ester hydrolysis in which hydrogen peroxide instead of water serves as the substituting nucleophile.¹³

It has great advantages for the detection of hydrogen peroxide due to excellent sensitivity and selectivity to hydrogen peroxide. Bis(2,4,6-trichlorophenyl) oxalate is one of the most widely used peroxalates because of its high reactivity in oxidation by hydrogen peroxide.^{14,15} The electron-withdrawing nature of chlorine on bis(2,4,6-trichlorophenyl) oxalate was known to accelerate addition of hydrogen peroxide. However, the poor stability limits its applications under aqueous conditions because peroxalate linkages react with water to decompose. Previously, Dasari *et al.* developed chemiluminescent micelles composed of polycaprolactone-

^aBoth authors contributed equally to this work.

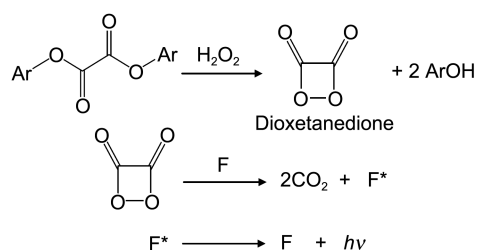


Figure 1. General mechanism of peroxalate chemiluminescence, where F is the fluorophore and F* is the singlet excited state of the fluorophore.

co-polyethylene glycol (PCL-PEG) copolymer and diphenyl peroxalate which is more stable than bis(2,4,6-trichlorophenyl) oxalate in water.¹⁶ Diphenyl peroxalate was encapsulated in the hydrophobic cores and thus protected from water hydrolysis. Diphenyl peroxalate has poor stability against water hydrolysis, but the encapsulation in the hydrophobic core of micelles increased its stability. The micelles could detect hydrogen peroxide at nanomolar concentrations. However, diphenyl peroxalate based chemiluminescent micelles exhibited a half-life of 30 min in water, which impairs its practical applications for the detection of hydrogen peroxide under aqueous environments.

In this work, we investigated the relationship between the chemiluminescence reactivity and stability by varying the substitutes on the aryl rings of peroxalate. We synthesized the dipropylphenyl peroxalate and dimethylphenyl peroxalate based on the rationale that alkyl groups in the aryl group disfavor the addition of nucleophilic attack of water, increasing the stability of oxalate ester linkages at the expense of reactivity. Chemiluminescent micelles encapsulating various peroxalates could detect hydrogen peroxide with a linear correlation between the hydrogen peroxide concentration and chemiluminescence intensity. In this report, we present the effects of alkyl substitutes on the chemiluminescence reaction, such as emission intensity and duration. We also evaluated the potential of peroxalate chemiluminescent micelles for the detection of hydrogen peroxide using activated macrophages and mice with LPS-induced inflammation.

Materials and Methods

Preparation of PCL-PEG Copolymers. PCL-PEG copolymers were synthesized as previously reported.¹⁷ Methoxy poly(ethyleneglycol) (mPEG, Mn = 2,000) (0.4 mmol) and toluene were added into a flask which was dried by heating in a vacuum. The solution was distilled by azeotropic distillation to remove water and then toluene was completely distilled off. Dry dichloromethane (DCM) was added to dried mPEG, followed by the addition of dry caprolactone (11.2 mmol) using a syringe. The polymerization was initiated by the addition of 1.0 M solution of HCl in diethyl ether (0.8 mmol) at room temperature. After 24 h, the reaction mixture was poured into cold hexane to precipitate polymers. The obtained polymer was redissolved

in DCM and filtered off. The polymer solution was concentrated by a rotatory evaporator and dried under vacuum. The chemical structure of PCL-PEG copolymer was determined by ¹H NMR: ¹H NMR (CDCl₃, 400 MHz): δ 4.05 (t, 2H), 3.63 (s, 4H), 3.37 (s, 3H), 2.30 (t, Hz, 2H), 1.64 (m, 4H), 1.37 (m, 2H). The molecular weight was determined by GPC using polystyrene standards.

Synthesis of Dipropylphenyl Peroxalate and Dimethyl Peroxalate. 4-Propyl phenol or 4-methyl phenol (24 mmol) was dissolved in 10 mL of dry DCM at 0 °C under nitrogen. Oxalic chloride (12 mmol) was added to the solution dropwise using a syringe and the mixture was stirred for 4 h at room temperature. The reaction mixture was extracted with DCM, dried on sodium sulfate, filtered and concentrated to get crude dipropylphenyl peroxalates. The crude compound was purified using silica gel chromatography (hexane/ethyl acetate = 80:20). The chemical structure was determined by ¹H NMR: ¹H NMR (CDCl₃, 400 MHz). Dipropylphenyl peroxalate: δ 7.0 (m, 3H), δ 2.55 (m, 2H), δ 1.7 (d, 2H), δ 0.9 (s, 3H) and methylphenyl peroxalate: δ 7.0 (m, 3H), δ 2.35 (m, 3H).

Preparation of Chemiluminescent Micelles. Chemiluminescent micelles were prepared *via* a solvent displacement method. Rubrene (1 mg), PCL-PEG (50 mg) and peroxalate (10 mg) were dissolved in 1 mL of acetone, to which 10 mL of phosphate buffer solution (pH 7.4, 0.1 M) was added. The mixture was vortexed for 10 sec and acetone was evaporated using a rotary evaporator to generate chemiluminescent micelles with a concentration of 2 mg/mL. The particle size of micelles in PBS was measured by dynamic light scattering using a particle analyzer (ELS-8000, Photal Otsuka Electronics, Japan).

Cytotoxicity Assay. The cytotoxicity of micelles was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. RAW 264.7 macrophage cells were seeded at a density of 1 × 10⁶ cells/well in a 24 well plate and incubated for 24 h to reach ~90% confluency. Cells were treated with various amounts of micelles (10 µg/mL to 100 µg/mL) and incubated for 1 day. Each well was given 100 µL of MTT solution and were incubated for 4 h. Two hundred microliters of dimethyl sulfoxide (DMSO) was added to cells to dissolve the resulting formazan crystals. After 10 min of incubation, the absorbance at 570 nm was measured using a microplate reader (Thermomax, Molecular Device Co.). The cell viability was obtained by comparing the absorbance of nanoparticles-treated cells to that of control cells.

Detection of Hydrogen Peroxide. Various concentrations of hydrogen peroxide solutions were prepared in 0.1 M phosphate buffer, pH 7.4. Hydrogen peroxide solutions were added to each micelle solutions (2 mg/mL) in a test tube and immediately their chemiluminescence was measured using a luminometer (FB12, Zylux, US) with an acquisition time of 10 s.

Detection of Hydrogen Peroxide Generated in Macrophages. Mouse macrophage RAW 264.7 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) in a 6-well

cell culture plate. When cells reached ~90% confluency, the medium was replaced with Krebs Ringer-buffer. Cells were treated with 1 μ L or 2 μ L of LPS (1 mg/mL) for 1 h to induce the generation of hydrogen peroxide. Culture medium of 800 μ L was taken and mixed with 200 μ L of freshly made chemiluminescent micelles and immediately the chemiluminescence intensity was measured using a luminometer.

Imaging of Hydrogen Peroxide. One milliliter of chemiluminescent micelles was placed in a 6-well cell culture plates. To each well, hydrogen peroxide solutions with various concentrations were added and the plate was gently shaken rapidly. The chemiluminescent emission from the micelles was imaged using an IVIS-200 imaging system (Xenogen, US) for 1 min acquisition time.

***In vivo* Chemiluminescence Imaging of Inflamed Mouse Ankle.** The ankle of 4 week old mice was injected with 2 μ L of LPS (1 μ g/ μ L) to induce inflammation. Twenty microliters of micelles (1 μ g/ μ L) was injected into the inflamed ankle 4 h after LPS treatment. Chemiluminescent images of inflamed ankle were made using an IVIS-200 imaging system with 1 min acquisition.

Results and Discussion

Synthesis of PCL-PEG Copolymers and Alkyl Substituted Phenyl Peroxalate. PCL-PEG copolymers were synthesized by the ring-opening polymerization of caprolactone by terminal alcohol of mPEG as an initiator in the presence of HCl-ethylene ether.¹⁷ In this reaction, HCl serves as an activator, but not as an initiator. This polymerization method has been considered as a potential alternative for simple polymerization of PCL-PEG copolymers with a well defined-structure. The PCL-PEG copolymers were obtained in almost quantitative yield. Its chemical structure was confirmed by NMR (Figure 2). The molecular weight was determined by ~4,500 Da with molecular weight distribution of ~1.2.

We developed dimethylphenyl peroxalate and dipropylphenyl peroxalate in which electron-donating alkyl substituents attached to the aryl group increase the stability against water hydrolysis (Figure 3). The reaction proceeded in dry DCM under nitrogen to generate the corresponding

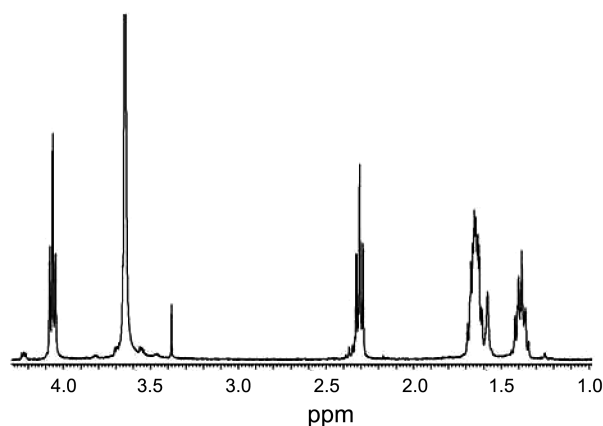


Figure 2. ^1H NMR spectrum of PCL-PEG in CDCl_3 .

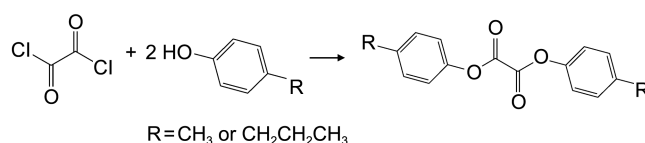


Figure 3. Synthesis of alkyl substituted phenyl peroxalate.

alkyl substituted phenyl peroxalate in ~70% yield. The products were obtained as pale yellow solids after a silica gel chromatography and drying under high vacuum and their chemical structure was confirmed by ^1H NMR.

Development of Chemiluminescent Micelles. A three component peroxalate chemiluminescence has a great potential to detect hydrogen peroxide *in vivo* and *in vitro* because of its excellent sensitivity and specificity.⁹ However, the detection of hydrogen peroxide exploiting peroxalate chemiluminescence needs nano-sized structures which can sequester oxalate esters and fluorescent dyes in close proximity and

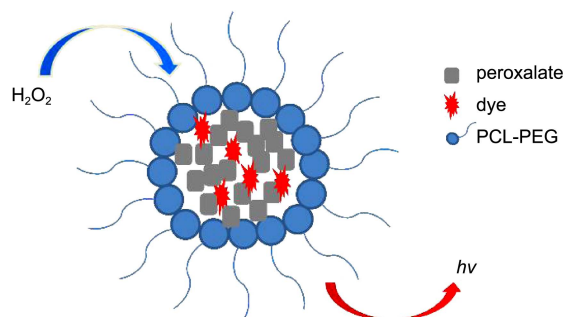


Figure 4. A schematic diagram of chemiluminescent micelles composed of PCL-PEG copolymer, peroxalate and fluorescent dye. The micelles generate light emission instantaneously in response to hydrogen peroxide.

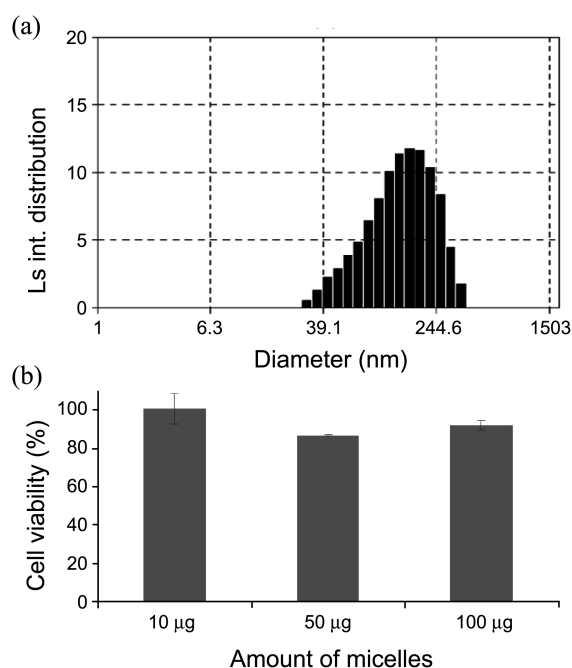


Figure 5. Characterization of chemiluminescent micelles composed of PCL-PEG copolymer, peroxalate and fluorescent dye. (a) size analysis by dynamic light scattering, (b) cytotoxicity assay of micelles.

allow them to perform peroxalate chemiluminescence instantaneously when exposed to hydrogen peroxide. Polymer micelles are a useful nanostructured material because of their hydrophobic core which can provide environment for sequestering of peroxalate esters and fluorescent dyes in close proximity, as shown in Figure 4.^{16,18}

Chemiluminescent micelles were composed of PCL-PEG, peroxalate and fluorescent rubrene. The micelles had a mean size around ~110 nm and their size was not influenced by the classification of peroxalate (Figure 5). A MTT assay was performed to evaluate the cytotoxicity of chemiluminescent micelles. No or minimal cytotoxicity was observed with micelles upto 100 μ g of micelles.

Reactivity of Chemiluminescent Micelles to Hydrogen Peroxide. Chemiluminescence of PCL-PEG micelles containing various peroxalates were examined in water by adding hydrogen peroxide or by photoexcitation. Figure 6 shows the emission spectra of chemiluminescent micelles encapsulating rubrene as a fluorophore. Photoexcitation of the micelles at 530 nm generated light emission at 560 nm which is a typical wavelength of rubrene. Upon the addition of 33.3 μ M of hydrogen peroxide, the micelles performed a chemiluminescence reaction to emit light at the same wavelength. The results demonstrate that PCL-PEG micelles sequester peroxalate and rubrene in close proximity in their hydrophobic core to allow them to perform chemiluminescence reaction in response to hydrogen peroxide. We also investigated the effects of alkyl substitutes of peroxalate on the chemiluminescence reaction. The micelles encapsulating diphenyl peroxalate showed a higher chemiluminescent emission intensity than the counterpart of dimethylphenyl peroxalate when exposed to the same concentration of hydrogen peroxide. The lower chemiluminescent intensity of dimethylphenyl peroxalate-encapsulated micelles is attributed to the presence of electron-donating nature of methyl groups which disfavor the oxidation of peroxalate with hydrogen peroxide.^{9,10,13}

The sensitivity of chemiluminescent micelles to hydrogen peroxide was measured using a luminometer. Chemiluminescent micelles luminesced instantaneously after exposure to hydrogen peroxide. As shown in Figure 7, the micelles

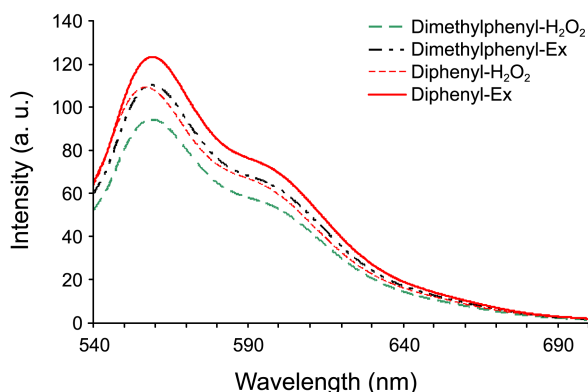


Figure 6. Photoluminescence and chemiluminescence emission spectra of rubrene-encapsulated chemiluminescent micelles.

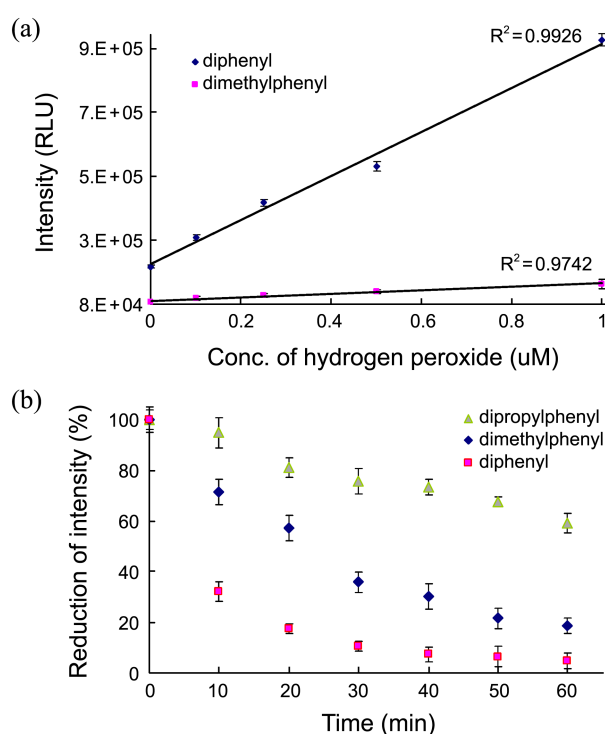


Figure 7. Reactivity of chemiluminescent micelles with hydrogen peroxide. (a) Sensitivity to hydrogen peroxide, (b) Duration of chemiluminescence reaction.

exhibited a linear correlation between chemiluminescent intensity and hydrogen peroxide concentration. However, the alkyl substitute on the phenyl groups influenced the chemiluminescence reactivity. The highest chemiluminescence intensity was observed with diphenyl peroxalate. In the chemiluminescence reaction in the micelles, dimethylphenyl peroxalate generated chemiluminescence with significantly lower intensity than diphenyl peroxalate, but much higher than dipropylphenyl peroxalate (data not shown). The lowest peroxalate chemiluminescence reactivity of dipropylphenyl peroxalate is due to the presence of electron-donating propyl groups which diminish the reactivity to hydrogen peroxide-induced oxidation.¹³

We also investigated the duration of chemiluminescence reaction in micelles by measuring the chemiluminescence intensity during the incubation with hydrogen peroxide (1 μ M). As shown in Figure 7(b), chemiluminescent micelles encapsulating diphenyl peroxalate showed the fastest reduction in chemiluminescence intensity with time, while dipropylphenyl peroxalate encapsulated micelles exhibited the least reduction. The duration of chemiluminescence reaction is highly related to the stability of peroxalate. Although diphenyl peroxalate generated strongest chemiluminescence emission, it showed very short lasting chemiluminescence reactions because of the fast reaction with hydrogen peroxide and water hydrolysis. The results indicate that the alkyl substitutes increase the stability against hydrolysis at the expense of reactivity to hydrogen peroxide.

Detection of Hydrogen Peroxide in Cells. Macrophage cells were stimulated with LPS for 1 h to produce hydrogen

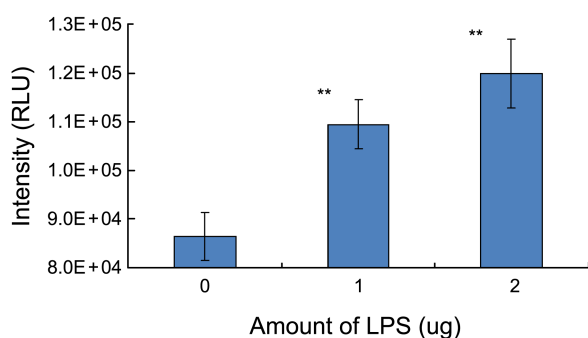


Figure 8. Detection of hydrogen peroxide generated from LPS-stimulated macrophages. ** $p < 0.01$ ($n = 3$).

peroxide in the Krebs-Ringer buffer as a culture medium.^{9,19,20} The level of hydrogen peroxide in the Krebs-Ringer buffer was measured by adding chemiluminescent micelles to the buffer and measuring the emission intensity. Figure 8 shows the chemiluminescent intensity of Krebs-Ringer buffer using micelles encapsulating diphenyl peroxalate. Cells treated with LPS produced more hydrogen peroxide, as evidenced by the higher chemiluminescent intensity. The hydrogen peroxide generation was dependent on the amount of LPS given to cells. We also used the micelles encapsulating dimethylphenyl peroxalate. The micelles generated insufficient chemiluminescence intensity to detect hydrogen peroxide generated by activated cells. It is because of the low concentration of hydrogen peroxide generated by stimulated cells and low reactivity of dimethylphenyl peroxalate to hydrogen peroxide.

To demonstrate the potential of chemiluminescent micelles for *in vivo* imaging, a mouse inflammatory disease model was used. Inflammation was induced in a mouse ankle by intra-articular injection of LPS that causes acute inflammation.^{9,18} Chemiluminescent micelles encapsulating diphenyl peroxalate were injected into the inflamed site at an early stage of inflammation. Figure 9 illustrates a representative *in vivo* chemiluminescent imaging of hydrogen peroxide in LPS-induced inflammation. Immediately after the

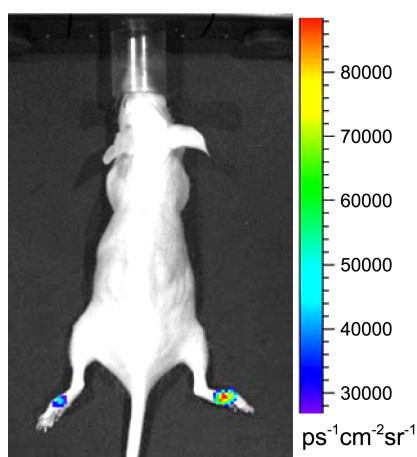


Figure 9. A representative *in vivo* imaging of hydrogen peroxide in LPS-induced inflammatory responses in a mouse.

injection of chemiluminescent micelles, chemiluminescence emission was detected from the LPS-treated inflamed site (right ankle), while LPS-negative control site showed negligible signal (left ankle). The observation demonstrate the potential of chemiluminescent micelles for the detection of hydrogen peroxide-associated inflammatory diseases.¹⁸

Conclusions

Chemiluminescent micelles were prepared by self-assembly of amphiphilic PCL-PEG which sequester peroxalate and fluorescent dyes in their hydrophobic core. The addition of hydrogen peroxide generated light emission from the micelles through the three component chemiluminescence reaction, evidenced by the spectra of chemiluminescence emission. The linear correlation was observed between the chemiluminescent intensity and the concentration of hydrogen peroxide. Chemiluminescent micelles composed of diphenyl peroxalate showed a higher emission intensity than those encapsulating alkyl substituted diphenyl peroxalate. However, alkyl substitutes increased the stability against water hydrolysis. Chemiluminescent micelles composed of diphenyl peroxalate could detect hydrogen peroxide generated from the macrophages activated by LPS. In addition, they were able to image hydrogen peroxide generated in LPS-treated inflamed joint of mice. Taken together, the chemiluminescent micelles have great potential to detect hydrogen peroxide in biological environments.

Acknowledgments. This work is supported by a grant of the National Research Foundation of Korea (2009-0063931, 2010-0021903) and World Class University Project (R31-20029) by the Ministry of Education, Science and Technology.

References

1. Azad, N.; Rojanasakul, Y.; Vallyathan, V. *Journal of Toxicology and Environmental Health-Part B-Critical Reviews* **2008**, 11(1), 1.
2. Miller, E. W.; Albers, A. E.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *Journal of the American Chemical Society* **2005**, 127(47), 16652.
3. Lee, J. Y.; Jang, Y. W.; Kang, H. S.; Moon, H.; Sim, S. S.; Kim, C. J. *Archives of Pharmacal Research* **2006**, 29(10), 849.
4. Chang, M. C. Y.; Pralle, A.; Isacoff, E. Y.; Chang, C. J. *Journal of the American Chemical Society* **2004**, 126(47), 15392.
5. Carter, W. O.; Narayanan, P. K.; Robinson, J. P. *Journal of Leukocyte Biology* **1994**, 55(2), 253.
6. Lang, J. D.; McArdle, P. J.; O'Reilly, P. J.; Matalon, S. *Chest* **2002**, 122(6), 314S.
7. Feder, L. S.; Stelts, D.; Chapman, R. W.; Manfra, D.; Crawley, Y.; Jones, H.; Minnicozzi, M.; Fernandez, X.; Paster, T.; Egan, R. W.; Kreutner, W.; Kung, T. T. *American Journal of Respiratory Cell and Molecular Biology* **1997**, 17(4), 436.
8. Park, H.; Kim, S.; Song, Y.; Seung, K.; Hong, D.; Khang, G.; Lee, D. *Biomacromolecules* **2010**, 11(8), 2103.
9. Lee, D.; Khaja, S.; Velasquez-Castano, J. C.; Dasari, M.; Sun, C.; Petros, J.; Taylor, W. R.; Murthy, N. *Nature Materials* **2007**, 6, 765.
10. Lee, D. W.; Erigala, V. R.; Dasari, M.; Yu, J. H.; Dickson, R. M.; Murthy, N. *International Journal of Nanomedicine* **2008**, 3(4),

- 471.
11. Soh, N. *Analytical and Bioanalytical Chemistry* **2006**, 386(3), 532.
12. Kamyshny, A.; Magdassi, S. *Colloids and Surfaces B-Biointerfaces* **1998**, 11(5), 249.
13. Hadd, A. G.; Lehmpuhl, D. W.; Kuck, L. R.; Birks, J. W. *Journal of Chemical Education* **1999**, 76(9), 1237.
14. Motoyoshiya, J.; Sakai, N.; Imai, M.; Yamaguchi, Y.; Koike, R.; Takaguchi, Y.; Aoyama, H. *Journal of Organic Chemistry* **2002**, 67(21), 7314.
15. Maulding, D. R.; Clarke, R. A.; Roberts, B. G.; Rauhut, M. M. *The Journal of Organic Chemistry* **1968**, 33(1), 250.
16. Dasari, M.; Lee, D.; Erigala, V. R.; Murthy, N. *Journal of Bio-medical Materials Research Part A* **2009**, 89A(3), 561.
17. Kim, M. S.; Seo, K. S.; Khang, G.; Cho, S. H.; Lee, H. B. *Journal of Biomedical Materials Research Part A* **2004**, 70A(1), 154.
18. Lim, C. K.; Lee, Y. D.; Na, J.; Oh, J. M.; Her, S.; Kim, K.; Choi, K.; Kim, S.; Kwon, I. C. *Advanced Functional Materials* **2010**, 20(16), 2644.
19. Sredni-Kenigsbuch, D.; Kambayashi, T.; Strassmann, G. *Immunology Letters* **2000**, 71(2), 97.
20. Hikosaka, K.; Koyama, Y.; Motobu, M.; Yamada, M.; Nakamura, K.; Koge, K.; Shimura, K.; Isobe, T.; Tsuji, N.; Kang, C. B.; Hayashidani, H.; Wang, P. C.; Matsumura, M.; Hirota, Y. *Bioscience Biotechnology and Biochemistry* **2006**, 70(12), 2853.
-