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Chemiluminescent and Antioxidant Micelles as Theranostic Agents for Hydrogen Peroxide Associated-Inflammatory Diseases

*Sujin Cho, On Hwang, Iljea Lee, Gayoung Lee, Donghyuck Yoo, Gilson Khang, Peter M. Kang, and Dongwon Lee**

Hydrogen peroxide (H_2O_2) is one of essential oxygen metabolites in living organisms, but is generated in large amounts during inflammatory responses. Therefore, H_2O_2 has great potential as diagnostic and therapeutic markers of several inflammatory and life-threatening diseases. Here, chemiluminescent and antioxidant micelles are reported as novel theranostic agents for H_2O_2-associated inflammatory diseases. The chemiluminescent micelles composed of amphiphilic block copolymer Pluronic F-127, hydroxybenzyl alcohol-incorporated copolyoxalate (HPOX) and fluorescent dyes perform peroxalate chemiluminescence reactions to detect H_2O_2 as low as 100 nM and image H_2O_2 generated in inflamed mouse ankles. The micelles encapsulating HPOX reduce the generation of reactive oxygen species in lipopolysaccharide (LPS)-activated macrophages by scavenging overproduced H_2O_2 and releasing antioxidant hydroxybenzyl alcohol (HBA). They also exert inhibitory effects on H_2O_2-induced apoptosis. HPOX-based chemiluminescent and antioxidant micelles have great potential as a theranostic agent for H_2O_2-associated inflammatory diseases.

1. Introduction

Reactive oxygen species (ROS) are a collective term of small reactive molecules including hydrogen peroxide (H_2O_2), superoxide and hydroxyl radicals and play important roles as a mediator in a variety of biological and pathological events.[1] Excessive and unregulated production of ROS is known to cause oxidative stress, leading to functional decline of organs and tissues.[2–4] The accumulation of oxidative stress over time is associated with debilitating conditions such as cancer, cardiovascular diseases, neurodegenerative diseases and acute and/or chronic inflammatory process.[4–8] Although H_2O_2 is an essential oxygen metabolite in living organisms and plays fundamental roles in the cellular signaling pathway[2], it is also a major source of oxidative stress and a common marker of ROS-associated diseases.[3] H_2O_2 is a precursor of highly reactive ROS such as hydroxyl radical, peroxynitrite and hypochlorite[5], and the overexpression of H_2O_2 leads to oxidative damages and has been implicated in inflammation and aging-associated diseases.[9] Therefore, selective detection and scavenging of overproduced H_2O_2 provide enormous benefits to the successful treatment of ROS-related diseases.

In chemical terms, H_2O_2 is poorly reactive and acts as a mild oxidizing agent.[7] However, in peroxalate chemiluminescence (POCL), H_2O_2 has been widely used as a key reactant due to its high reactivity to peroxalate compounds. Peroxalate compounds are oxidized by H_2O_2 spontaneously and instantaneously to form high energy 1,2-dioxetanedione intermediates that excite nearby fluorophore to generate chemically ignited fluorescence.[10,11] POCL reaction is performed by only H_2O_2, not by other ROS such as superoxide, hydroxyl radical and nitric oxide.[12] Therefore, POCL has great potential for the imaging of H_2O_2 *in vivo* as well as *in vitro* due to its excellent specificity and sensitivity to H_2O_2.[4,10,12,13] However, it is challenging to exploit POCL for *in vivo* imaging of H_2O_2 because it requires nano-sized scaffolds that sequester peroxalate fuels and fluorophore in a close proximity and allow them to perform POCL reaction spontaneously and instantaneously in response to H_2O_2. The first study to image H_2O_2 *in vivo* was achieved by chemiluminescent nanoparticles that were composed of peroxalate polymers and fluorescent dyes. The polymer possessing peroxalate ester bonds in its backbone formed scaffolds of chemiluminescent nanoparticles and also served as a peroxalate fuel in POCL. The chemiluminescent nanoparticles detected H_2O_2 at concentrations as low as 250 nM and were capable of imaging H_2O_2 generated in a peritoneal cavity of mice during the lipopolysaccharide

S. Cho, O. Hwang, I. Lee, G. Lee, D. Yoo, Prof. P. Kang, Prof. D. Lee
Department of BIN Fusion Technology
Chonbuk National University
Jeonju, 561-756, Korea
E-mail: dlee@chonbuk.ac.kr
Prof. G. Khang, Prof. D. Lee
Polymer Fusion Research Center
Department of Polymer·Nano Science and Technology
Chonbuk National University
Jeonju, 561-756, Korea
Prof. P. Kang
Cardiovascular Division
Beth Israel Deaconess Medical Center
Harvard Medical School
Boston, MA 02215, USA

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(LPS)-induced inflammatory responses. However, their physicochemical properties such as the large size and hydrophobic surface limit their intravenous applications because the large size prevents their extravasation into tissues and the hydrophobic surface promotes clearance by the reticuloendothelial system.[4,13]

Previously, we developed polymeric nanoparticulate antioxidants based on fully biodegradable copolyoxalate in which hydroxybenzyl alcohol (HBA) is chemically incorporated in its backbone.[14,15] HBA is a main active pharmaceutical ingredient in *Gastrodia elata*, a widely used herbal agent in Oriental countries for several centuries because of its protective roles against oxidative damage-related diseases including ischemic brain injury and coronary heart disease.[16,17] HBA-incorporated copolyoxalate (HPOX) was designed to contain labile peroxalate ester bonds in its backbone and degrade completely to release therapeutic HBA.[14] The fully biodegradable and biocompatible HPOX nanoparticles exerted strong antioxidant and anti-inflammatory activity by reducing the generation of ROS and tumor necrosis factor-alpha (TNF-α).[15]

In this work, by exploiting the appealing features of HPOX such as the ability to perform a POCL reaction in response to H_2O_2 as well as potent antioxidant activities, we developed chemiluminescent and antioxidant HPOX micelles as a novel theranostics of H_2O_2-associated inflammatory diseases. We report here that the multifunctional micelles are able to image H_2O_2 endogenously generated during the inflammatory responses by performing a POCL reaction in response to H_2O_2 and exert potent antioxidant and anti-inflammatory activity.

2. Results and Discussion

Chemiluminescent HPOX micelles were simply prepared by using amphiphilic Pluronic F-127 copolymers that self-assemble to form micelles to sequester HPOX and rubrene as a fluorophore in a close proximity in the hydrophobic interior, as shown in **Figure 1**a. F-127 was chosen for its biocompatibility and its approval by US-FDA.[18,19] In design of the chemiluminescent micelles, HPOX plays double roles as a peroxalate fuel to initiate POCL for imaging H_2O_2 and as a polymeric binder in the micelle formulations, forming a hydrophobic core and enhancing the nanostructure stability.[12] H_2O_2 is expected to diffuse into the core of chemiluminescent micelles and initiate POCL reaction with HPOX encapsulated.

We first prepared chemiluminescent HPOX micelles by varying the weight ratios of the components and selected an optimal ratio to formulate the stable HPOX micelles with the best H_2O_2 detection ability as previously reported.[12] The stable chemiluminescent HPOX micelles were prepared by homogenous mixing of F-127, HPOX and rubrene at a weight ratio of 100:10:0.5. Dynamic light scattering revealed that the micelles have an average hydrodynamic diameter of ~150 nm, suitable for clinical and diagnostic applications (Figure 1b).

We next examined the ability of HPOX micelles as a chemiluminescent nanoreactor to perform POCL reactions by measuring the chemiluminescent emission spectrum in the presence of H_2O_2. As shown in **Figure 2**a, upon the addition of H_2O_2, the chemiluminescent HPOX micelles instantaneously performed POCL reactions to generate light emission at 565 nm, typical emission wavelength of rubrene.[10] The emission intensity was proportional to the concentration of H_2O_2. However, no light emission was detected in the absence of H_2O_2. The results demonstrate that Pluronic F-127 copolymers sequester HPOX and rubrene in close proximity and HPOX serves as a peroxalate fuel in POCL initiated by H_2O_2 in the micelles. Emission intensity of chemiluminescent HPOX micelles in response to H_2O_2 was also measured using a luminometer. The micelles showed a linear correlation between the chemiluminescent emission intensity and the concentration of H_2O_2 in the range of physiologically relevant concentrations (Figure 2b).[2] The chemiluminescent HPOX micelles could detect H_2O_2 at concentrations as low as 100 nM, which is similar to the previously reported chemiluminescent micelles.[4]

Imaging of H_2O_2 *in vivo* has great potential to act as a diagnostic for oxidative stress-related inflammatory diseases because the overproduction of H_2O_2 has been implicated in the development of numerous diseases such as atherosclerosis, cancer, chronic obstructive pulmonary disease, arthritis, ischemic/reperfusion injury and liver hepatitis.[2,5,9] We therefore investigated the ability of the chemiluminescent HPOX micelles to image endogenously generated H_2O_2 using a murine model of LPS-induced acute inflammation. Acute inflammation in ankle joints of mice was induced by intra-articular injection of LPS.

Chemiluminescent HPOX micelles were injected into the inflammatory site at an early stage of inflammation and chemiluminescence images were captured with 1 min of acquisition time (**Figure 3**a). Negligible chemiluminescence emission was detected at the left ankle joint which was not given LPS.

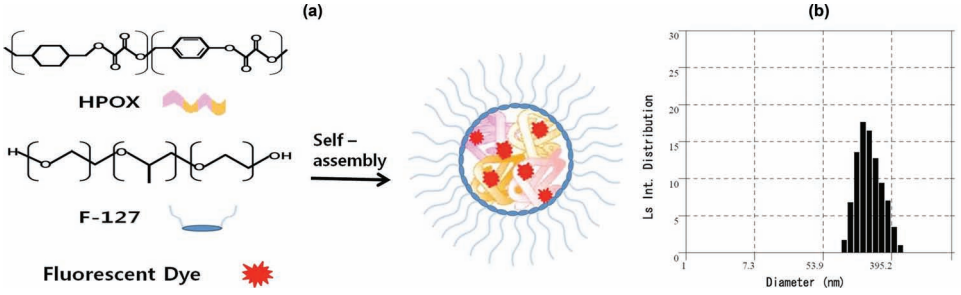

Figure 1. Chemiluminescent and antioxidant HPOX micelles for H_2O_2-associated inflammatory diseases. (a) A schematic diagram of preparation of chemiluminescent and antioxidant HPOX micelles. Amphiphilic Pluronic F-127 copolymers self-assemble to form micelles that sequester HPOX and fluorescent dyes in their hydrophobic interior. HPOX encapsulated in the micelles not only serves as peroxalate fuels in POCL to image H_2O_2 but also release therapeutic HBA with antioxidant activity. (b) A representative dynamic light scattering of the chemiluminescent HPOX micelles.

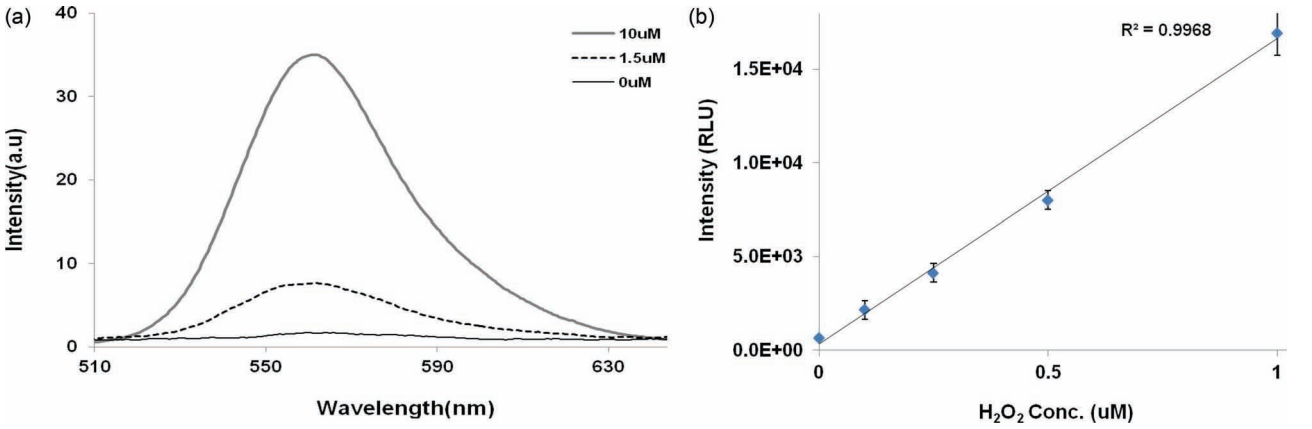

Figure 2. Detection of H_2O_2 using chemiluminescent HPOX micelles. (a) POCL emission spectra of the micelles in the presence of various concentration of H_2O_2. (b) Sensitivity of chemiluminescent HPOX micelles to H_2O_2 under physiological concentrations. The values are mean ± S.D (n = 4).

However, the LPS-treated site (right ankle joint) showed a six-fold higher chemiluminescence emission, demonstrating that chemiluminescent HPOX micelles are capable of imaging H_2O_2 endogenously generated during inflammatory responses. To further confirm the H_2O_2-specificity of chemiluminescent HPOX micelles, H_2O_2 degrading catalase was injected prior to the injection of the micelles. Pre-administration of catalase resulted in significant reduction of chemiluminescence emission from the chemiluminescent HPOX micelles at LPS-treated site (Figure 3b), suggesting that the micelles are capable of imaging H_2O_2 *in vivo* with excellent specificity over other ROS.

In a POCL reaction, H_2O_2 is consumed continuously to oxidize peroxalate compounds to generate high energy intermediate dioxetanedione. We therefore determined whether HPOX scavenges H_2O_2 by measuring the concentration of H_2O_2 solutions after incubation with HPOX micelles. Various amounts of HPOX micelles (1 mg/ml) were added to 1 mL of H_2O_2 (10 μM) solutions and the change in H_2O_2 concentration was measured after 12 h. For comparison purposes, H_2O_2 solution was mixed with 1000 μg of solid HPOX nanoparticles with a diameter of ~450 nm. As shown in **Figure 4**, HPOX micelles showed remarkable reduction of H_2O_2 concentrations in a dose-dependent manner. After 12 h of incubation, 100 μL of HPOX micelles (1 mg/mL) scavenged a majority (~80%) of H_2O_2. No further reduction in H_2O_2 concentration was observed thereafter. HPOX nanoparticles of 1000 μg showed approximately 50% reduction of H_2O_2 concentration. Surprisingly and interestingly, 100 μL of HPOX micelles which encapsulate 100 μg of HPOX exhibited a significantly stronger H_2O_2 scavenging activity than 1000 μg of HPOX in solid nanoparticles formulation. This is due probably to their higher surface area, faster diffusion of H_2O_2 in the aqueous formulations and consequent more favorable reactions with H_2O_2. The ability of HPOX micelles to scavenge H_2O_2 may play beneficial and essential roles to reduce the oxidative stress in H_2O_2-associated inflammatory diseases.

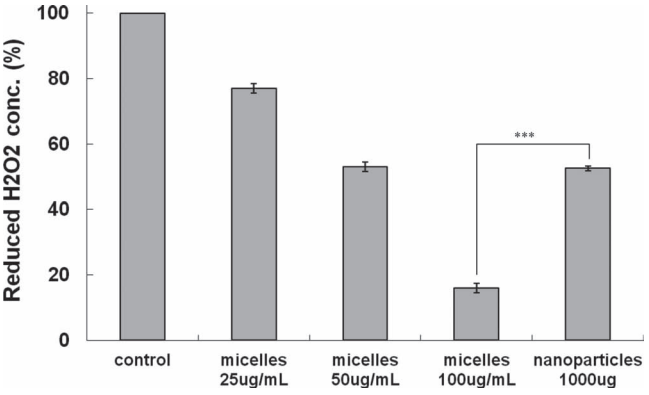

Figure 4. Scavenging of H_2O_2 by HPOX micelles. Various amounts of HPOX micelles (1 mg/mL) were added to 1 mL of 10 μM of H_2O_2 solution. HPOX nanoparticles of 1000 μg were also added to 1 mL of H_2O_2 solution for comparison purposes. $P < 0.001$ (n = 4, ±S.D).

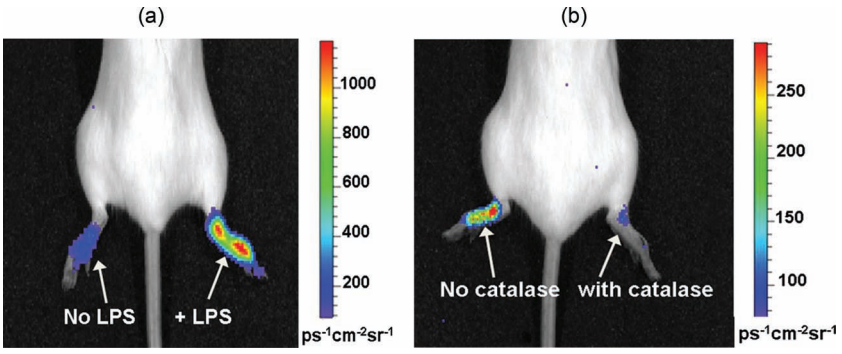

Figure 3. Imaging of H_2O_2 *in vivo* using chemiluminescent HPOX micelles. (a) Chemiluminescent image of H_2O_2 generated during LPS-induced inflammation. Inflammation was induced by the injection of 20 μL of LPS (1 μg/μL) and 40 μL of chemiluminescent HPOX micelles were injected into the inflamed site. (b) Inhibited POCL reaction of chemiluminescent HPOX micelles (1 mg/mL) by H_2O_2 degrading catalase. Inflammation was induced by the injection of 10 μL of LPS (1 μg/μL).

Cytotoxicity of HPOX micelles was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay because cytotoxicity is one of critical concerns in

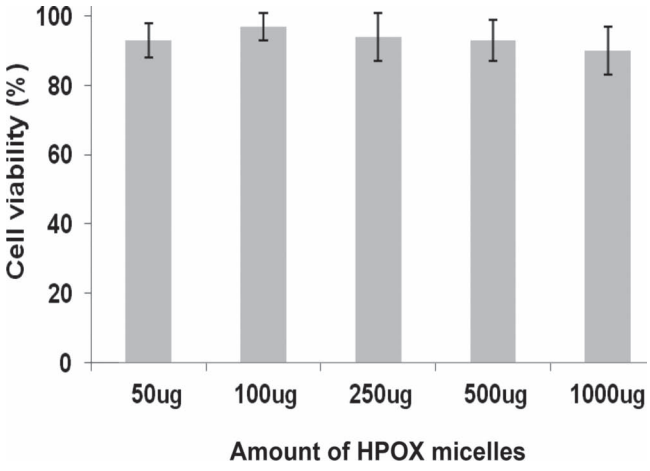

Figure 5. Cytotoxicity of HPOX micelles by MTT assay. Various amount of HPOX micelle (1 mg/mL) were added to cells.

development of biomaterials. As shown in **Figure 5**, HPOX micelles (1 mg/mL) showed negligible cytotoxicity at doses up to 1000 μL. The results demonstrate that HPOX micelles have excellent biocompatibility *in vitro* and great potential for biomedical applications.

Activated macrophages and neutrophils are known to be the major source of excessive production of ROS including H_2O_2.[10,20] We assessed the antioxidant activity of HPOX micelles by measuring the level of ROS generation in LPS-stimulated macrophages. DCFH-DA (dichlorofluorescin-diacetate) was used as a probe for intracellular ROS because it diffuses into cells and become fluorescent DCF (dichlorofluorescin) via oxidation by various intracellular ROS including H_2O_2.[15,21] During the incubation with HPOX micelles, HPOX nanoparticles or free HBA (0.5 mM), cells were stimulated with LPS and treated with DCFH-DA for 15 min. The intracellular ROS generation was determined by the shift in DCF fluorescence detected by flow cytometry (**Figure 6**a). LPS-stimulated cells showed a strong DCF fluorescence because DCFH-DA was oxidized to become fluorescent DCF by intracellular ROS-mediated oxidation. Treatment of 0.5 mM of HBA showed a moderate reduction in LPS-induced ROS generation due to its intrinsic antioxidant activity, evidenced by the leftward shift in flow cytometry.[17,22] Previously, Lim et al reported that HBA at a concentration of 0.5 mM suppressed ROS generation without cytotoxicity.[16] Theoretically, 100 μg of HPOX are expected to produce ~15 μg of HBA, which corresponds to ~0.12 mM in this experiment. Interestingly, 100 μg HPOX in both nanoparticle and micelle formulations showed a more remarkable leftward shift in DCF fluorescence than 0.5 mM of HBA, suggesting that HPOX exhibited significantly higher suppressing effects on intracellular ROS generation than free HBA. It can be explained by the dual antioxidant activities of HPOX. First, HPOX scavenges overproduced intracellular H_2O_2 through the H_2O_2-mediated oxidation of peroxalate ester bonds. Second, HPOX releases HBA during its degradation, which inhibits the generation of other intracellular ROS. In comparison with HPOX nanoparticles, HPOX micelles exhibited more remarkable reduction of intracellular ROS production in LPS-stimulated macrophages. It may be attributed to the more favorable reaction of HPOX with H_2O_2 in micelle formulations and faster release of HBA due to their higher surface area and faster H_2O_2 diffusion.

A large generation of ROS is known to induce apoptosis, which is associated with several pathological conditions, including myocardial/reperfusion injury and stroke.[2] We examined the potential of HPOX micelles to inhibit H_2O_2-mediated apoptosis of cells. Cells were stimulated with 100 μM of H_2O_2 to induce apoptosis and the apoptotic events were analyzed using Annexin V-FITC by flow cytometry. HBA exhibited moderate anti-apoptotic activity, as previously reported.[23] HPOX nanoparticles also exerted anti-apoptotic activity, but HPOX micelle formulations showed more remarkable anti-apoptotic activity (Figure 6b). The strong anti-apoptotic activity of HPOX micelles is likely attributed to the combined effects of their H_2O_2 scavenging activity and intrinsic anti-apoptotic activity of HBA released from HPOX.

As aforementioned, HPOX micelles exhibited the stronger antioxidant activity than free HBA by scavenging H_2O_2 and suppressing the generation of other intracellular ROS. It suggests that HPOX micelles are readily phagocytosed by macrophages and serve as potent antioxidants in cells. We therefore performed confocal laser scanning microscopy to confirm

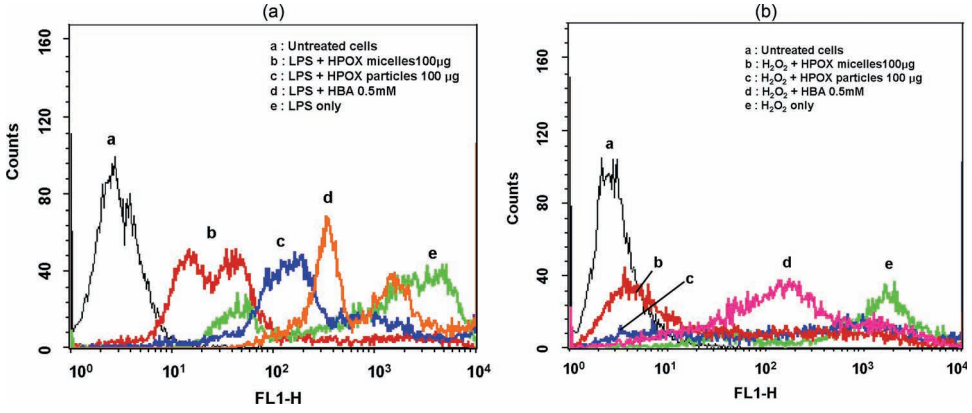

Figure 6. Antioxidant and anti-apoptotic activity of HPOX micelles. (a) Reduced generation of ROS by HPOX micelles in macrophages stimulated by LPS. (b) Inhibitory effects of HPOX micelles on H_2O_2-induced apoptosis. LPS- or H_2O_2-stimulated cells were treated with HPOX micelles which were prepared by mixing F-127 (100 mg), HPOX (10 mg) and rubrene (0.5 mg) in deionized water (10 mL).

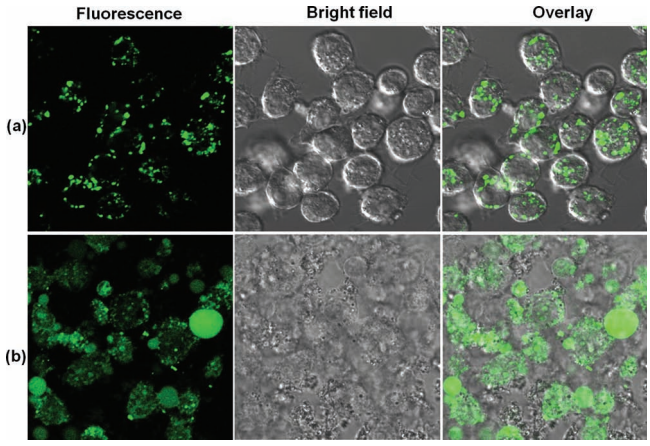

Figure 7. Confocal fluorescence micrographs showing the cellular uptake of HPOX micelles. RAW 264.7 cells were incubated with free calcein (a) or calcein-encapsulated HPOX micelles (b) for 1 h.

whether macrophages take up HPOX micelles *via* phagocytosis (**Figure 7**). Macrophages were incubated with HPOX micelles that encapsulate cell membrane impermeable calcein. Cells were also treated with free calcein for comparison purposes. Free calcein-treated macrophages showed green fluorescence with a punctuate distribution, suggesting that calcein was entrapped in the endolysosomal compartments in the periphery of cells.[24,25] In contrast, cells treated with the calcein-encapsulated HPOX micelles showed diffusive green fluorescence throughout the cytosol, demonstrating that HPOX micelles are readily phagocytosed by macrophages *via* phagocytosis and release calcein payload into the cytosol.[15]

3. Conclusions

We developed novel multifunctional HPOX micelles that are able to image H_2O_2 and serve as therapeutic agents with potent antioxidant and anti-apoptotic activity. The chemiluminescent and antioxidant HPOX micelles were formulated using amphiphilic Pluronic F-127 copolymers which self-assemble to form micelles and sequester HPOX and rubrene in a close proximity in their hydrophobic interior. They could perform POCL instantaneously in response to H_2O_2 and showed a linear correlation between POCL emission intensity and H_2O_2 concentrations, as low as 100 nM. HPOX micelles were capable of imaging specifically H_2O_2 endogenously generated in a mouse ankle joint during LPS-induced inflammatory responses. In addition, they exerted highly potent antioxidant effects by scavenging H_2O_2 and suppressing the generation of intracellular ROS. Taken together, we anticipate that HPOX micelles have considerable potential as theranostic agents for H_2O_2-associated inflammatory diseases.

4. Experimental Section

Synthesis of HPOX: 1,4-Cyclohexanedimethanol (21.96 mmol) and 4-hydroxybenzyl alcohol (5.49 mmol) were dissolved in 20 mL of dry tetrahydrofuran (THF), under nitrogen, to which triethylamine (60 mmol) was added dropwise at 4 °C. Oxalyl chloride (27.45 mmol) in dry THF (25 mL) was added to the mixture dropwise at 4 °C. The reaction was continued for 6 h under nitrogen atmosphere and the resulting polymers were obtained through the extraction using dichloromethane (DCM) and isolation by precipitating in cold hexane. The chemical structure of polymers was identified with a 400MHz ^{1}H NMR spectrometer (JNM-EX400 JEOL).

Preparation of chemiluminescent HPOX micelles and their characterization: HPOX (10 mg), F-127 (Sigma-Aldrich, 100 mg) and rubrene (Sigma-Aldrich, 0.5mg) were dissolved in DCM (1 mL) and then the solvent was removed using a rotary evaporator. Deionized water (10 mL) was added to the dried mixture to afford the micelle formation by self-assembly. Their size and distribution of micelles were measured using a particle analyzer (ELS-8000, Photal Otsuka Electronics, Japan).

Chemiluminescence of HPOX micelles: HPOX micelles were prepared in 0.1 M PBS, pH 7.4 to give a concentration of 1 mg/mL. Various amounts of a H_2O_2 solution (1 mM in PBS, 0.1M) were added to the micelles, and the chemiluminescence intensity was measured with a luminometer (Femtomaster FB12, Zylux Corporation, Huntsville, AL) with an acquisition time of 10 sec. The chemiluminescence emission spectra were obtained in the presence of various concentrations of H_2O_2 using a spectrofluorometer (RF-6500-PC, Shimadzu, Japan).

Scavenging of H_2O_2 by HPOX micelles: The ability of HPOX-containing micelles to scavenge H_2O_2 was evaluated by measuring the H_2O_2 concentration after incubation with micelles. HPOX micelles at various concentrations were prepared in H_2O_2 solutions (10 μM) and incubated at 37 °C under mechanical stirring for 12 h. After short centrifugation at 1000×*g*, the H_2O_2 concentration of the supernatant was measured using the Amplex Red assay (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Cytotoxicity assay: The cytotoxicity of HPOX micelles was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 macrophage cells in a 12 well plate were with ~90% confluency were treated with various amounts of HPOX micelles (50 μL to 1000 μL/well) and incubated for 24 h. Each well was given 100 μL of MTT solution and was incubated for 4 h. Dimethyl sulfoxide (1 mL) 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 (Synergy MX, BioTek Instruments, Inc, Winooski, VT).

Measurement of intracellular ROS: RAW 264.7 cells were treated with 0.5 mM of hydroxybenzyl alcohol, 100 μg of HPOX nanoparticles and 100 μL of HPOX micelles for 4 h and then incubated with LPS for 4 h. To measure the level of intracellular ROS, cells were treated with DCFH-DA for 15 min and analyzed by Flow Cytometry Caliber (Becton Dickinson, US).

Confocal laser scanning microscopy: Calcein (a membrane-impermeable fluorophore) was used as a tracer molecule to monitor the uptake of HPOX micelles. Calcein-encapsulated HPOX micelles were prepared by the same method as chemiluminescent HPOX micelles. RAW 264.7 cells and HEK 293 cells were cultured in a glass bottom dish (MatTek Corp. Ashland, MA) and treated with 10 μM of free calcein or 100 μL of calcein-encapsulated HPOX micelles (1 mg/mL) for 1 h. The culture media were replaced with fresh media twice. Fluorescence images of cells were made using a confocal scanning microscope (Carl Zeiss, Inc. Germany).

Imaging of hydrogen peroxide in inflamed ankles of mice: The ankle of 4 week old mice was injected with 20 μL of LPS (1 μg/μL) to induce inflammation. A volume of 40 μL of chemiluminescent HPOX micelles (1 mg/mL) was injected into the inflamed ankle 4 h after LPS treatment. Chemiluminescent images of inflamed ankle were made using an IVIS-200 imaging system (Xenogen, US) with 1 min acquisition. For another set of experiments, acute inflammation was induced by the injection of 10 μL of LPS (1 μg/μL) into an ankle. Four hours later, 20 μL of catalase (2 μg/μL) was injected into the prior to the injection of chemiluminescent HPOX based micelles. Immediately after the injection of catalase solution, chemiluminescent HPOX micelles were injected into the inflamed ankles.

Acknowledgements

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