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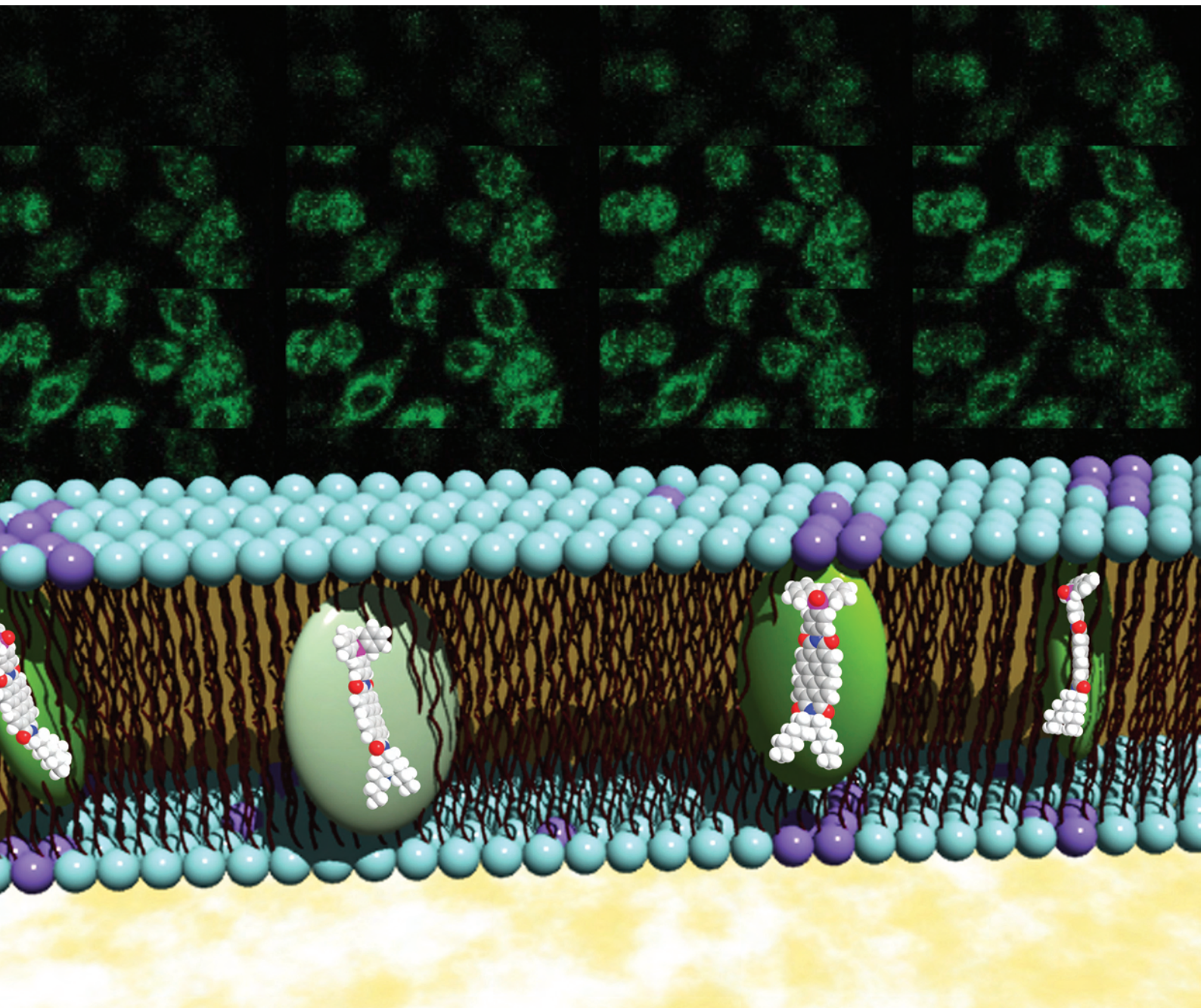

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Swallow-tailed perylene derivative: a new tool for fluorescent imaging of lipid hydroperoxides†

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A swallow-tailed perylene derivative including a triphenylphosphine moiety was synthesized and applied to the detection and the live-cell imaging of lipid hydroperoxides. The novel probe, named Spy-LHP, reacted rapidly and quantitatively with lipid hydroperoxides to form the corresponding oxide, Spy-LHPOx, which emits extremely strong fluorescence ($\Phi \sim 1$) in the visible range (λ_{em} = 535 nm, 574 nm). Spy-LHP was highly selective for lipid hydroperoxides, and the addition of other reactive oxygen species (ROS) including hydrogen peroxides, hydroxyl radical, superoxide anion, nitric oxide, peroxynitrite, and alkylperoxyl radical, caused no significant increase in the fluorescence intensity. The probe exhibited good localization to cellular membranes and was successfully applied to the confocal laser scanning microscopy (CLSM) imaging of lipid hydroperoxides in live J774A.1 cells, in which lipid peroxidation was proceeded by the stimulation of 2,2-azobis(2-amidinopropane)-dihydrochloride (AAPH). These findings establish Spy-LHP as a promising new tool for investigating the physiology of lipid hydroperoxides.

Introduction

Lipid peroxidation has attracted considerable attention as one of the causative factors for aging and various diseases including cancer and atherosclerosis.[1] Lipid hydroperoxides are the primary products in lipid peroxidation and are recognized as important markers of oxidative stress. To date, some methods including thiobarbituric acid methods,[2] iodometry methods,[3] enzymatic methods,[4] and fluorescence-based methods[5] have been developed for the determination of lipid hydroperoxides.

Although many efforts have been made to detect lipid hydroperoxides, the detection of lipid hydroperoxides in living cells still remains as a challenging task. To detect biomolecules in living cells, a fluorescent imaging technique using fluorescent probes is promising because of its facility, high sensitivity and high space resolution. The temporal and spatial information on lipid hydroperoxides in living samples using the fluorescent probe would give us important clues to enhance the understanding of the physiological roles of lipid hydroperoxides. In recent years, many fluorescent probes for detecting various reactive oxygen species (ROS) with selectivity have energetically developed.[6] As for a probe to detect lipid hydroperoxides, diphenyl-1-pyrenylphosphine (DPPP)[7] is one of the most successful. The probe was applied to the monitoring of lipid peroxidation in cell membranes,[7e,f] low-density lipoprotein (LDL),[7g] and isolated rat lungs[7h] although the excitation wavelength (~352 nm) is in a range that is short for biosamples.

Here, we reported the detection and the live-cell imaging of lipid hydroperoxides using a novel fluorescent probe, Spy-LHP (named after 'swallow-tailed perylene derivative for detecting lipid hydroperoxides'). Perylene derivative, perylene 3,4,9,10-tetracarboxyl bisimide, was used for the fluorophore of this novel phosphine compound because of its excellent fluorescent properties such as long excitation and emission wavelengths, high photochemical stability, high fluorescence quantum yield (~1), low quantum yield of intersystem crossing, and versatile reactivity,[8] although perylene derivatives have seldom been used for constructing fluorescent probes for biosamples. We have recently reported that Spy-LHP reacted with unnatural hydroperoxides such as *m*-chloroperbenzoic acid (MCPBA) to form fluorescent Spy-LHPOx.[9] We thought that the probe would have great potential to be an innovative tool for the detection of cellular lipid hydroperoxides because the fluorescent oxidative form can be excited at long wavelengths, corresponding to laser wavelengths which are frequently used in fluorescence microscopy, and emits extremely strong fluorescence in the visible range. Furthermore, the planar structure and highly hydrophobicity of the perylene derivative seems to be advantageous to localize Spy-LHP in cellular membranes. In this study, we investigated the performance of Spy-LHP as a novel fluorescent probe for detecting and imaging lipid hydroperoxides.

Results and discussion

Design, spectroscopic properties, and detection ability of Spy-LHP

The strategy for the detection of lipid hydroperoxides using Spy-LHP is based on an oxidation reaction of Spy-LHP induced by lipid hydroperoxides, which leads to the formation of highly fluorescent Spy-LHPOx (Scheme 1). Fig. 1 shows the emission

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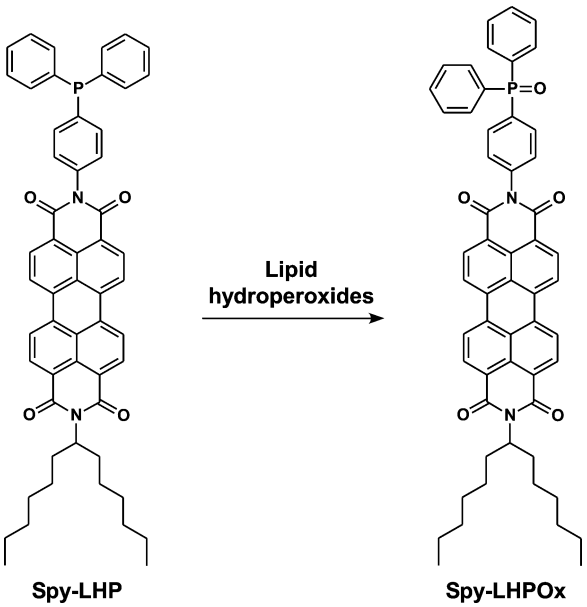
† Electronic supplementary information (ESI) available: plot showing the relationship between the ratio of the fluorescence intensity of Spy-LHP and the concentration of MeLOOH; colour version of Fig. 4; phase contrast and fluorescence images of DPPP=O in live J774A.1 cells. See DOI: 10.1039/b713223a



Scheme 1 Reaction of Spy-LHP with lipid hydroperoxides.

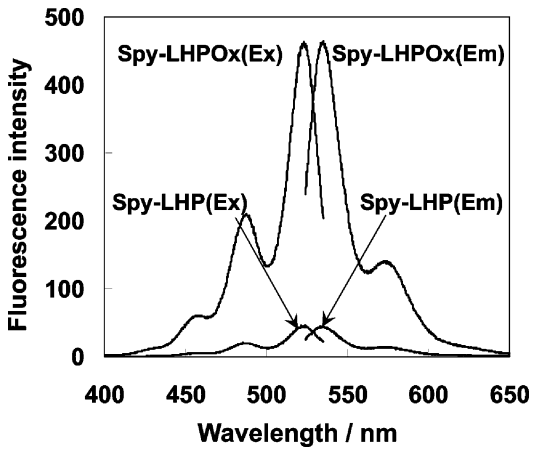


Fig. 1 Excitation and emission spectra of Spy-LHP (1 μM) and Spy-LHPOx (1 μM) at 37 °C in ethanol.

and excitation spectra for Spy-LHP and Spy-LHPOx. Peak wavelengths for both compounds in the absorption and emission spectrum are summarized with fluorescence quantum yields in Table 1. Spy-LHP is weakly fluorescent ($\Phi \sim 0.05$), whereas Spy-LHPOx emits extremely strong fluorescence ($\Phi \sim 1$). The excitation and emission wavelengths of both Spy-LHP and Spy-LHPOx are in sufficiently long range, as expected from the fluorescent properties of perylene 3,4,9,10-tetracarboxyl bisimide, which is the main skeleton of the probe dyes. Therefore, the detection using Spy-LHP can avoid undesirable effects such as damage of living cells by light irradiation and autofluorescence from biomatrices, both of which are serious problems in detection using ordinary probes such as DPPP. Although it is known that perylene-3,4,9,10-tetracarboxylic dianhydride is practically insoluble in many organic solvents, Spy-LHP and Spy-LHPOx show good solubility in various organic solvents such as chloroform and benzene, probably because the skeletons of the dyes are derivatized from the dianhydride form to the bisimide form along with a hexylheptyl group (swallow-tail).[10] According to the fluorescence properties, it can be expected that Spy-LHP is effectively excited by lasers with long wavelengths (ex. Ar laser at 488 nm). Although a window including the first peak at 535 nm is available for a collected emission range in the imaging experiments, another window including the second peak at 572 nm is also available for imaging using longer wavelengths.

Table 1 Absorbance and fluorescence properties of Spy-LHP and Spy-LHPOx[a]

	A/nm	E_m/nm	Φ_f
Spy-LHP	524, 489, 459	535, 574	0.05
Spy-LHPOx	524, 489, 459	535, 572	~ 1

[a] All data were measured in ethanol.

In order to evaluate the detection ability of Spy-LHP for lipid hydroperoxides, Spy-LHP (1 μM) was treated with various concentrations (50, 100, 150, 200 nM) of methyl linoleate hydroperoxide (MeLOOH), which is a lipid hydroperoxide of methyl linoleate (ML) and was used as a representative lipid hydroperoxide in this study. Fig. 2(a) shows the fluorescence spectra of Spy-LHP, after 10 min incubation with MeLOOH in ethanol at 37 °C. The fluorescence intensity of Spy-LHP increased with an increase in the concentration of MeLOOH, as expected. A good linear relationship ($R^2 = 0.99$) was observed between the concentration of MeLOOH and the fluorescence intensity at 535 nm, as shown in Fig. 2(b). The equivalent good linear relationship was also observed between the concentration of MeLOOH and the fluorescence intensity at 535 nm when higher concentrations of MeLOOH (up to 1 eq.) were used (incubation time: 5 min) (Fig. S1, ESI†). The results indicate that Spy-LHP is available for the quantitative determination of lipid hydroperoxides. Additionally, the fluorescence intensity of Spy-LHP (1 μM) hardly increased upon the addition of hydroperoxides (100 μM) such as *tert*-butyl hydroperoxide, *tert*-butyl perbenzoate, and di-*tert*-butyl peroxide (reactivity; 9.4%, 2.3%, 0.93%, respectively). A similar result was reported for detection using DPPP.[7b] This fact, namely, the reactivity of Spy-LHP depends on steric hindrance around a phosphorus atom, strongly suggests that the reaction mechanism of Spy-LHP with lipid hydroperoxides is based on the peroxide oxygen being attacked by the phosphine[11] as in the case of DPPP.[7a]

Fig. 3 shows the time courses for the fluorescence intensity of Spy-LHP after the addition of MeLOOH. The fluorescence intensity drastically increased upon the addition of MeLOOH and then saturated. The calculated value of the second-order rate constant for the reaction of Spy-LHP with the lipid hydroperoxides (6.4×10^3 s^{-1} M^{-1}) suggests that the reaction is diffusion-controlled. As can be seen from Fig. 3, Spy-LHP exhibits no significant increase in fluorescence intensity under light irradiation in aerobic condition (see data for 0 μM), although some phosphine compounds are known to be easily oxidized under such conditions. The results indicate that Spy-LHP has high stability and is available for the long-term monitoring of the production of lipid hydroperoxides.

Spy-LHP exhibits excellent selectivity to lipid hydroperoxides. Fig. 4(a) and (b) show the time courses for the fluorescence intensity of Spy-LHP upon the addition of various ROS (H_2O_2 (10 μM), hydroxyl radical (•OH), superoxide anion ($O_2^{-\bullet}$), nitric oxide (NO),

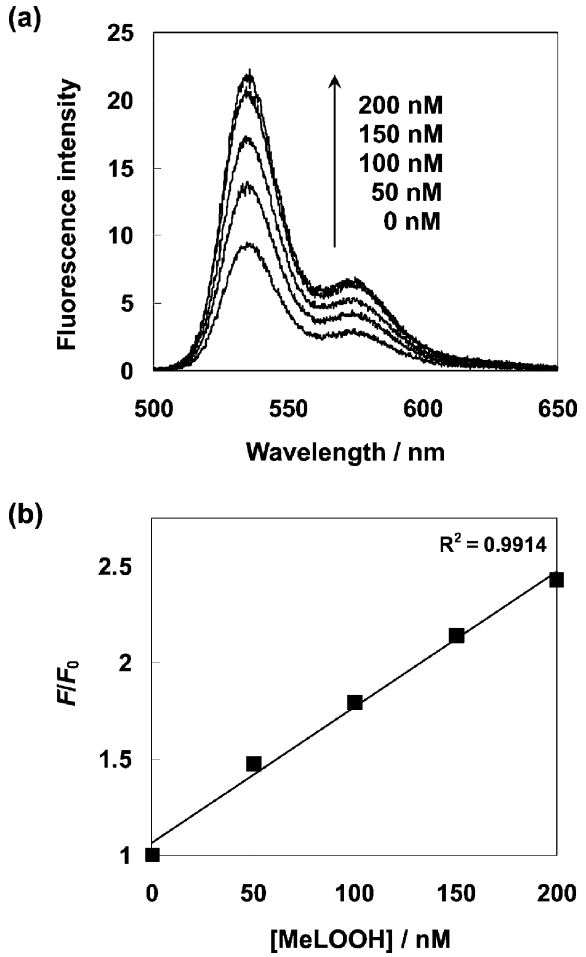


Fig. 2 (a) Emission spectra for Spy-LHP at 37 °C in ethanol in the presence of MeLOOH at various concentrations. The emission spectra were obtained 10 min after the addition of MeLOOH to the Spy-LHP (1 μM) under aerobic conditions with excitation at 465 nm. (b) Relationship between the ratio of the fluorescence intensity and the concentration of MeLOOH. F_0 and F denote the fluorescence intensity at 535 nm before and after the addition of MeLOOH, respectively.

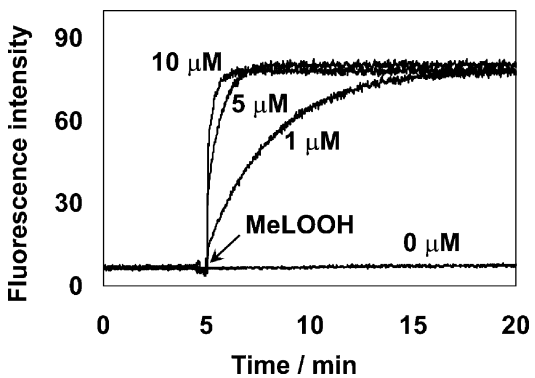


Fig. 3 Time courses of fluorescence intensity at 535 nm for Spy-LHP depending on the concentration of MeLOOH at 37 °C. A solution of MeLOOH (final 1, 5, 10 μM) was added to Spy-LHP (1 μM) in ethanol at the point indicated by the arrow.

peroxynitrite ($ONOO^-$), alkylperoxyl radical ($ROO^•$)), and the fluorescence intensities after 30 min incubation with the ROS, respectively (colour version of Fig. 4 is available as Fig. S2 in ESI†). Fenton's reagent (a mixed solution of H_2O_2 (10 μM) and $FeSO_4$ (10 μM)) was used as the donor of $^•OH$. The generation of $O_2^{-•}$ or NO was performed using potassium superoxide (KO_2) (10 μM) or 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC7) (5 μM), respectively. The reaction with $ONOO^-$ or $ROO^•$ was carried out by the addition of $ONOO^-$ solution (10 μM) (Dojindo Laboratories) or 2,2-azobis(2-amidinopropane)dihydrochloride (AAPH) (5 μM), respectively. As can be seen from Fig. 4(a) and (b), the fluorescence intensity of Spy-LHP was remarkably increased upon the addition of lipid hydroperoxides; on the other hand, no significant change in fluorescence was observed when the other ROS were added. Interestingly, the addition of H_2O_2, a kind of peroxide, did not cause significant increase in fluorescence intensity. It was reported that the addition of H_2O_2 increased the fluorescence intensity of DPPP as well as the addition of MeLOOH in chloroform–methanol (1 : 1) (3.8-fold for MeLOOH, 3.3-fold for H_2O_2) although such an increase was suppressed in liposomal membranes.[7e] Diphenylphosphine having a coumarin fluorophore (DPPEA-HC) also reacts effectively with H_2O_2 to form a corresponding fluorescent phosphine oxide and that enables the reagent to be used as a fluorescent probe for detecting H_2O_2.[12] It appears that the reactivity of these fluorescent phosphine compounds (Spy-LHP, DPPP, and DPPEA-HC) with H_2O_2 is determined by various factors such as hydrophobicity/hydrophilicity and redox

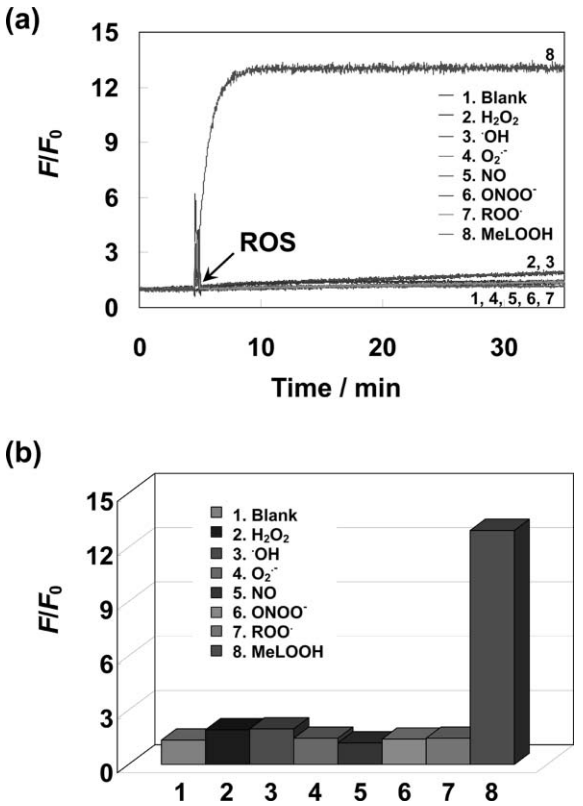


Fig. 4 (a) Time courses of relative fluorescence intensity (F/F_0) of Spy-LHP (1 μM) depending on various ROS at 37 °C in ethanol. F_0 and F denote the fluorescence intensity at 535 nm before and after the addition of various ROS, respectively. (b) F/F_0 after reaction with various ROS for 30 min.

potentials of the compounds. The results demonstrate that Spy-LHP distinguishes various ROS including H_2O_2, and reacts with lipid hydroperoxides with high selectivity.

The responsibility for lipid hydroperoxides of Spy-LHP incorporated in phospholipid bilayer was investigated as a preliminary experiment, preceding the application of the probe to living cells. The liposomal suspension of Spy-LHP was prepared using 1,2-dipalmitoyl-*sn*-glycerophosphorylcholine (DPPC) in a phosphate buffer (5 μM Spy-LHP in 100 μM DPPC) according to reference 13. The fluorescence intensity of Spy-LHP increased upon the addition of MeLOOH (25 μM) and it was confirmed that the probe reacted efficiently with lipid hydroperoxides in lipid membranes (data not shown). To investigate the ability of membrane localization, Spy-LHPOx (10 μM) was then incubated with suspensions of mouse macrophage J774A.1 cells at 37 °C for 15 min, washed with Dulbecco's phosphate-buffered saline (D-PBS) (GIBCO), and the fluorescence intensity was detected using a plate reader (Perkin Elmer, ARVO sx, $\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm, $n = 5$). As a result, the fluorescence intensity increased with an increase in the cell number of the suspension (25×10^3, 50×10^3, 100×10^3, 150×10^3, 200×10^3) (data not shown). The result supports that the probe accumulates efficiently in cell membranes.

The detection ability of Spy-LHP for lipid hydroperoxides in cell membranes was investigated. Spy-LHP (10 μM) was added to a suspension of J774A.1 cells (1×10^6 cell mL^{-1}) in Opti-MEM medium and incubated at 37 °C for 15 min. After two wash processes with D-PBS, the sample of cell suspension was reincubated with H_2O_2, ML, or MeLOOH (100 μM, each) in Opti-MEM medium at 37 °C for 15 min. Fig. 5 shows the fluorescence increments for the samples determined by the plate reader ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm, $n = 5$). As can be seen from Fig. 5, a small increase in fluorescence intensity was observed due to the incorporation of weakly fluorescent Spy-LHP (Fig. 5, sample I). The addition of H_2O_2 or ML induces no significant fluorescence intensity (Fig. 5, samples II, III), while a conspicuous increase in fluorescence intensity was observed when MeLOOH was added to the cell suspension (Fig. 5, sample IV). The results demonstrate that Spy-LHP is available for the detection of lipid hydroperoxides in living cells.

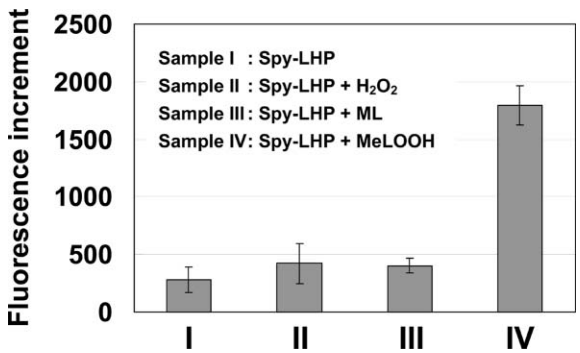


Fig. 5 Fluorescence increments in live J774A.1 cells after the incubation with Spy-LHP and H_2O_2, ML, or MeLOOH. Spy-LHP (10 μM) was incubated with a suspension of J774A.1 cells (1×10^6 cell mL^{-1}) at 37 °C for 15 min, then incubated with H_2O_2, ML, or MeLOOH (100 μM, each) at 37 °C for 15 min. The fluorescence measurements were performed using a plate reader ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm, $n = 5$). Background fluorescent intensity derived from cells or the detection system is subtracted in each sample.

Fluorescent imaging using confocal laser scanning microscopy

Our novel probe was next applied to the confocal laser scanning microscopy (CLSM) imaging using living cells. After Spy-LHPOx was incubated with J774A.1 cells cultured in a glass-bottomed dish (5×10^5 cells) at 37 °C for 15 min, the cells were washed with D-PBS, and the CLSM imaging was then performed in Opti-MEM medium. As can be seen from Fig. 6(a), ring-shaped fluorescence images along with shape of cells were observed. Similar ring-shaped fluorescence images along with the shape of cells were also observed when DPPP=O, the oxidized form of DPPP, which is used for fluorescent imaging of lipid peroxidation in cell membranes, was used (Fig. S3, ESI†). The results clearly indicate that the fluorescent dye accumulates efficiently in cell membranes. Fig. 6(b) shows a series of cross-sectional fluorescence images when the focus of the microscopy was shifted along the *z*-axis from the top of cells to the bottom at 1 μm intervals. As can be seen from Fig. 6(b), the diameters of the ring-shaped fluorescence images were gradually expanded, then gradually reduced. This result demonstrates that the fluorescent dye is distributed uniformly in the cell membranes. Introduction of Spy-LHP into the cells resulted in an observation of ring-shaped fluorescence images with

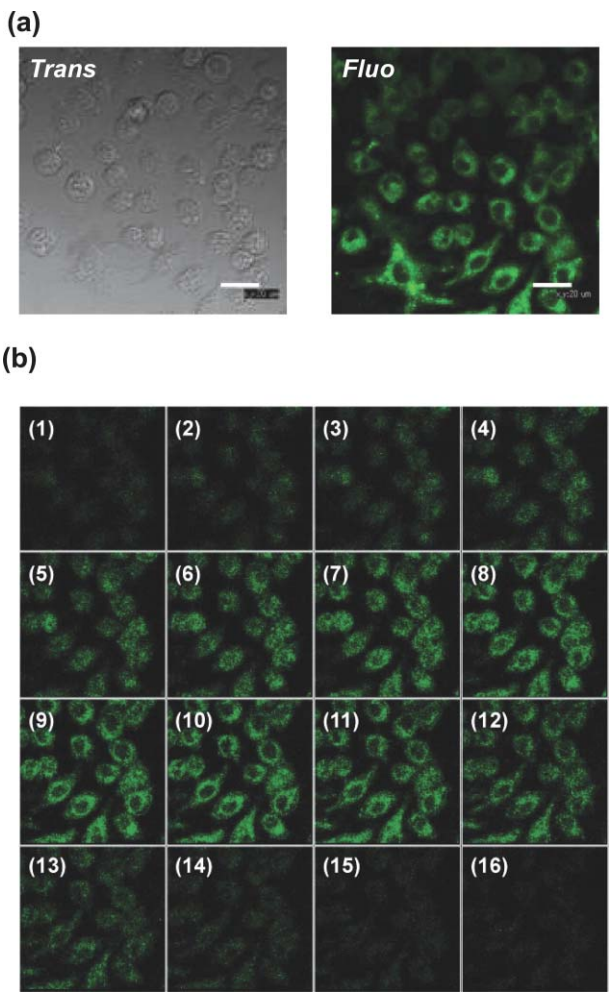


Fig. 6 (a) Phase contrasts and fluorescence images of Spy-LHPOx in live J774A.1 cells. Scale bar indicates 20 μm. (b) Fluorescence images with shifting the focus along the *z*-axis from the top of cells to the bottom at 1 μm intervals. Dichroic mirror: 505 nm, band pass filter: 515–530 nm.

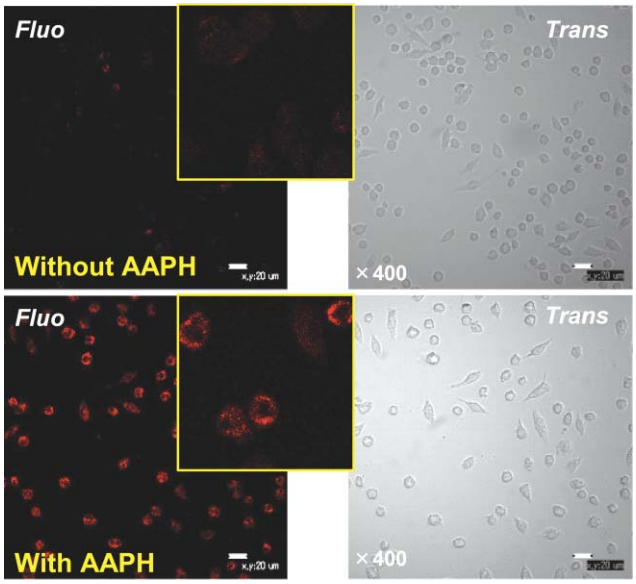


Fig. 7 CLSM imaging of lipid hydroperoxides generated in live J774A.1 cells. Spy-LHP was incubated with J774A.1 cells, which were preincubated with or without AAPH. Scale bar: 20 μm, dichroic mirror: 575 nm, band pass filter: 570LP.

very weak fluorescence intensity compared to the case of Spy-LHPOx (data not shown). The good localization of both Spy-LHP and Spy-LHPOx in cell membranes originates from the planar structure and the high hydrophobicity of the perylene bisimide.

Finally, Spy-LHP was applied to the CLSM imaging for the detection of lipid hydroperoxides generated in living cells. J774A.1 cells (5×10^5 cells per dish) were preincubated with or without AAPH (5 mM, 4 h), which induces lipid peroxidation as a radical initiator,[14] then Spy-LHP was introduced into the cells. In the experiment, an excitation light with longer wavelength (543 nm HeNe laser) and a band pass filter for longer emission (570LP band pass filter) were selected in order to reduce the background fluorescence and distinguish Spy-LHP and Spy-LHPOx more clearly. The results of CLSM imaging are shown in Fig. 7. As can be seen, no significant fluorescent change was observed for the sample without AAPH preincubation, which resulted in a very weak fluorescence image (Fig. 7, top). On the other hand, a remarkable fluorescence increase was observed for the sample with AAPH preincubation, which leads to the ring-shaped fluorescence images with strong emission (Fig. 7, bottom). In the latter case, the changes in the diameter of the ring-shaped fluorescence images, which are similar to the changes shown in Fig. 6(b), were observed according to the focus shift along the *z*-axis. Furthermore, no significant change in fluorescence intensity was observed when Spy-LHP was applied to J774A.1 cells, which were preincubated with AAPH (5 mM) for 4 h and then further incubated with glutathione peroxidase (GSH-Px) (100 unit mL^{-1}) and glutathion (1 mM) for 30 min (data not shown). The result clearly demonstrates that the fluorescence increment observed in the CLSM imaging is based on the reaction of Spy-LHP with lipid hydroperoxides.

Conclusions

In conclusion, we designed and developed a novel fluorescent probe, Spy-LHP, for detecting lipid hydroperoxides in the visible range. Spy-LHP reacted with lipid hydroperoxides selectively and quantitatively to form extremely fluorescent Spy-LHPOx. The fluorescent probe exhibits good localization to cell membranes, and can be successfully applied to the CLSM imaging of lipid hydroperoxides in living cells. Because of these unique properties, Spy-LHP shows great promise as a new high-powered tool for investigating the physiology of lipid hydroperoxides.

Experimental

Reagents

General chemicals were of the best grade available, purchased from Wako Pure Chemical Industries Co., Kishida Chemical Co., Tokyo Chemical Industry Co., or Aldrich Chemical Co. and used as received. All solvents for synthetic procedures were used after appropriate distillation or purification. Methyl linoleate hydroperoxide (MeLOOH) was prepared by mixing α-tocopherol and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeOAMVM) in toluene, according to a previous report,[15] and determined using the thiobarbituric acid method.

Tridecan-7-one oxime (2)

A suspension of 7-tridecanone (**1**) (25.0 g, 126 mmol), hydroxylamine hydrochloride (15.0 g, 215 mmol), and 85% KOH (29.3 g, 430 mmol) in 600 mL methanol was stirred at 60 °C for 24 h. After filtration, the filtrate was concentrated under reduced pressure and acidified with 2 N HCl. The resultant solution added to ether was washed with 2 N HCl and brine, dried over $MgSO_4$, and concentrated *in vacuo* to yield a pale yellow oil (27.8 g, ≥ 99%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 0.85–0.95 (t, 6H, CH_3), 1.25–1.40 (m, 12H, $6CH_2$), 1.45–1.55 (m, 4H, $2CH_2$), 2.15–2.18 (t, 4H, $2CH_2CH_2CNOH$), 2.30–2.34 (t, 4H, $2CH_2CHOH$); EI-MS: 213 $(M)^+$.

Tridecan-7-amine (3)

Compound **2** (27.6 g, 129 mmol) in absolute ethanol was heated to 60 °C. Sodium (48.5 g, 2.11 mol) was carefully added to the solution, and the reaction mixture was refluxed for 30 min. After appropriate amounts of methanol were added to consume excess sodium, the mixture was filtrated and concentrated under reduced pressure. The residue was added to ether, washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude compound was purified by vacuum distillation (15 mmHg, 120 °C) to yield a transparent, colorless oil (16.6 g, 63%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 0.8–1.0 (t, 6H, CH_3), 1.2–1.4 (m, 16H, $8CH_2$), 1.4–1.6 (m, 4H, $2CH_2CHNH_2$), 3.2 (m, 1H, $CHNH_2$); EI-MS: 199 $(M)^+$.

N,*N*′-Bis(1-hexylheptyl)perylene-3,4,9,10-tetracarboxylbisimide (5)[8b,16]

Compound **3** (1.79 g, 6.01 mmol) and perylene-3,4,9,10-tetracarboxylic dianhydride (**4**) (1.00 g, 2.55 mmol), zinc acetate (210 mg, 1.14 mmol), and imidazole (3.00 g, 44.1 mmol) were mixed and refluxed at 155 °C for 4 h. The reaction mixture was then added to conc. HCl and allowed to stand overnight. The resultant red precipitate was collected by filtration and washed

with methanol to yield a red powder (2.12 g, ≥ 99%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 0.8–0.9 (t, 12H, $4CH_3$), 1.15–1.4 (m, 32H, $16CH_2$), 1.8–1.9 (m, 4H, $2CH_2CHN$), 2.2–2.3 (m, 4H, $2CH_2CHN$), 5.15–5.25 (m, 2H, 2CHN), 8.6–8.7 (m, 8H, perylene); EI-MS: 755 $(M)^+$.

N-(1-Hexylheptyl)perylene-3,4,9,10-tetracarboxyl-3,4-anhydride-9,10-imide (6)[8b,16]

Compound **5** (1.58 g, 2.09 mmol) was added to 40 mL *tert*-butanol containing potassium hydroxide (402 mg, 7.09 mmol) and the mixture was refluxed at 80 °C. The reaction was stopped at the appropriate time, and conc. HCl was then added to the reaction mixture. After standing overnight, a red precipitate was collected by filtration, washed with water, and extracted with $CHCl_3$. After the $CHCl_3$ phase was dried over $MgSO_4$ and concentrated under reduced pressure, the crude compound was purified by chromatography on silica gel with $CHCl_3$ as the eluent to yield a red powder (506 mg, 42% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 0.8–1.0 (t, 6H, CH_3), 1.2–1.5 (m, 16H, $8CH_2$), 1.8–1.9 (m, 2H, CH_2CHN), 2.2–2.3 (m, 2H, CH_2CHN), 5.15–5.2 (m, 1H, CHN), 7.5–7.6 (d, 2H, perylene), 7.7–7.8 (d, 2H, perylene), 8.65–8.75 (d, 4H, perylene); EI-MS: 573 $(M)^+$.

4-(Diphenylphosphino)benzenamine (9)[17]

p-Iodoaniline (**7**) (4.38 g, 20.0 mmol), potassium acetate (2.39 g, 24.4 mmol), and palladium acetate (4.48 mg, 20 μmol) were dissolved in 20 mL dimethylacetamide and the solution was stirred under a N_2 atmosphere. After the addition of diphenylphosphine (**8**) (3.72 g, 20.0 mmol), the mixed solution was refluxed at 130 °C for 3 h. The resulting solution was extracted with CH_2Cl_2, dried over $MgSO_4$, and concentrated under reduced pressure. The crude compound was purified by chromatography on silica gel with $CHCl_3$ as the eluent to yield a pale yellow oil (3.23 g, 58%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 3.6–4.0 (s, 2H, NH_2), 6.6–6.7 (m, 2H, benzene), 7.1–7.2 (m, 2H, benzene), 7.25–7.35 (m, 10H, benzene); EI-MS: 277 $(M)^+$.

Spy-LHP

Compound **6** (105 mg, 182 μmol), compound **9** (60 mg, 215 μmol), zinc acetate (8.62 mg, 47 μmol), and quinoline (406 mg, 3.15 mmol) were mixed and refluxed at 155 °C for 4 h. The reaction mixture was added to 2 N HCl and allowed to stand overnight. After $CHCl_3$ was added to the mixture, the organic phase was washed with 2 N HCl and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude compound was purified by chromatography on silica gel with $CHCl_3$ as the eluent to yield a dark red powder (63.6 mg, 42% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 0.7–0.8 (t, 6H, $2CH_3$), 1.1–1.2 (m, 16H, $8CH_2$), 1.7–1.9 (m, 2H, CH_2CHN), 2.1–2.3 (m, 2H, CH_2CHN), 5.15–5.25 (m, 1H, CHN), 7.3–7.5 (m, 14H, phenyl), 8.5–8.7 (m, 8H, perylene); FAB-HRMS $(M + H)^+$: calcd. for $C_{55}H_{50}N_2O_4P$: 833.3508, found: 833.3510.

Spy-LHPOx

Spy-LHP (20 mg, 24 μmol) and MCPBA (8.3 mg, 48 μmol) were dissolved in 15 mL $CHCl_3$, and the solution was stirred at room temperature for 30 min. The resulting mixture was concentrated under reduced pressure and purified by chromatography on silica gel with $CHCl_3$–MeOH (97 : 3) as the eluent to yield a dark red powder (19.6 mg, 96% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 0.74–0.85 (t, 6H, $2CH_3$), 1.03–1.25 (m, 16H, $8CH_2$), 1.8–2.0 (m, 2H, CH_2CHN), 2.1–2.3 (m, 2H, CH_2CHN), 5.05–5.2 (m, 1H, CHN), 7.41–7.46 (m, 6H, phenyl), 7.5–7.55 (m, 2H, phenyl), 7.65–7.75 (m, 4H, phenyl), 7.8–7.9 (m, 2H, phenyl), 8.57–8.66 (m, 8H, perylene); FAB-HRMS $(M + H)^+$: calcd. for $C_{55}H_{50}N_2O_5P$: 849.3457, found: 849.3442.

Fluorometric analysis

A fluorescence spectrophotometer (Shimadzu, RF-5300PC) was used. The slit width was 1.5 nm for both excitation and emission. Relative quantum efficiencies of fluorescence for both Spy-LHP and Spy-LHPOx were obtained by comparing the area of the emission spectrum for the test sample with that for a solution of *N*,*N*′-bis(1-hexylheptyl)perylene-3,4,9,10-tetracarboxylbisimide (**5**) ($\Phi \sim 1$) excited at the same wavelength. The quantum efficiencies of fluorescence were obtained from multiple measurements ($n = 3$) with the following equation:

$$\Phi_{sample} = \Phi_{standard} \times (Abs_{standard}/Abs_{sample}) \times (\Sigma[F_{sample}]/\Sigma[F_{standard}])$$

where Abs and *F* denote the absorbance and fluorescence intensity, respectively, and $\Sigma[F]$ denotes the peak area of the fluorescence spectra, calculated by summation of the fluorescence intensity.

Imaging procedures

J774A.1 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were passed and plated on glass-bottomed culture dishes (Matsunami, D110300) one day before imaging. Opti-MEM (GIBCO) was used as a medium in the imaging experiments.

Confocal fluorescence imaging was performed with a fluorescence microscope system (C1plus, Nikon). Ar and HeNe lasers were used for excitation at 488 nm and 543 nm, respectively, and emissions were collected using appropriate band pass filters (515/530 nm, 570LP).

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