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Photoinduced electron transfer reaction in mitochondria for spatiotemporal selective photo-oxidation of lipids by donor/acceptor linked molecules†

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Donor-acceptor-linked molecules have been synthesized and utilized to induce the rapid, and site-selective lipid-oxidation in mitochondria by utilizing photoinduced intermolecular electron transfer reaction. Two water-soluble donor-acceptor molecules (**1** and **2**) were designed and synthesized for the purpose. **2** was prepared to modulate its affinity to cell membrane in mitochondria. Confocal laser microscopic experiments revealed that **1** and **2** possess high localization abilities in mitochondria. By the photoinduced electron transfer, **2** exhibited remarkable oxidation ability of lipids, mainly cardiolipin. In HeLa cells, **2** triggered mitochondrial lipid oxidation, which was followed by apoptotic cell death, under illumination within a few seconds. These results show the present molecular system is highly promising to utilize photoinduced inter molecular electron transfer reaction in a precise spatiotemporal manner in a cell by light.

Introduction

Optical control of cellular function is a fascinating methodology for cell engineering because it can achieve excellent spatiotemporal resolution in nanometer and sub-millisecond time scale.¹ In this connection, it is highly desirable to develop optical control methods that are free from potential phototoxicity of singlet oxygen (¹O₂) generated via intermolecular energy transfer (EN) from the excited states of sensitizers,² for precise and side-effect-free control of cellular functions towards future applications, such as single-cell and single-molecule measurements.³ Photoinduced electron transfer (ET) could be used as one of the methods because it frequently competes with the photoinduced EN.^{4,5} Thus, it is crucial to design donor-acceptor systems that exhibit the exclusive occurrence of photoinduced ET in biological systems.

Manipulating redox reactions in cells is an attractive approach to elucidate the biological mechanisms as well as exploit effective biological tools for cell engineering. For instance, general approaches to control redox balance in cells and redox reactions of lipids or proteins are administration of redox reagents to cells or genetic modification for regulating activity of redox related enzymes.⁶⁻⁹ However, these

approaches often face the difficulty of conducting site-specific treatments and quick response. In this regard, oxidized and reduced transient species produced by photoinduced ET in a precise spatiotemporal manner would be utilized to initiate versatile intermolecular ET reactions in cells, achieving excellent site-specificity and rapid response.¹

Meanwhile, mitochondrion is one of the most important subcellular organelles, which governs the energy generation by ATP synthesis, aminolevulinic acid synthesis, and induction of apoptotic/necrotic cell death.¹⁰ Among the lipids in mitochondria, cardiolipin (CL) usually accounts more than 20% of the mitochondrial lipid components and maintains cristae structure,^{11,12} which is responsible for various activities from mitochondria, such as acceleration/deceleration of oxidative phosphorylation and the induction of apoptotic cell death via release of cytochrome c.^{13,14} CL having multiple C-C double bonds is relatively sensitive towards oxidation.¹⁵ Therefore, we envisioned that the CL-related cell activity would be manipulated by using donor-acceptor systems that can elicit the intermolecular oxidation reaction of CL with the oxidized donor generated by photoinduced intramolecular ET.

Here, we have developed a site-selective and temporally precise method to induce intermolecular ET reaction in mitochondria to lead to lipid oxidation by donor-acceptor linked molecules.

Results and discussion

At first, 9-mesityl-10-methylacridinium perchlorate **3** was chosen as the donor-acceptor structure in which the mesityl and methylacridinium moieties are denoted as D and A⁺, respectively. It is known that photoinduced charge-shift (CS)

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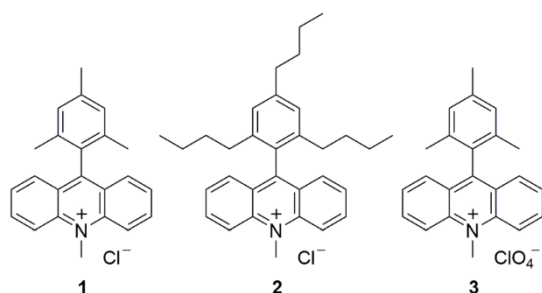


Fig. 1 Molecular structures of 1–3.

takes place from the D to the A⁺ to generate the long-lived, charge-shifted state (D^{•+}-A[•]) efficiently and selectively (quantum yield (Φ) = 98%, lifetime (τ) > 2h in frozen MeCN).¹⁶ We expected that mitochondria-specific localization of **3** and its strong interaction with anionic lipids would be achieved owing to its cationic and π -conjugated nature as reported for the mitochondria-specific fluorescent reagents.^{17,18} Because **3** was found to be insoluble in aqueous medium, its counter anion was changed from ClO₄⁻ to Cl⁻ by ion-exchange resin to afford **1** (Fig. 1). Compound **2**, where the methyl groups of the mesityl moiety are replaced with *n*-butyl ones, was also prepared to modulate its affinity to the cell membrane in mitochondria.

The UV-vis absorption spectra of **1** and **2** in Phosphate buffered saline (PBS) showed characteristic absorption bands of the acridinium moiety around 420 nm (Fig. S3 ESI[†]). The absorptions of **2** are slightly broadened and red-shifted in comparison with **1**, implying more intense intermolecular interaction in **2**. Redox potentials were recorded in PBS by the second alternating current voltammetry (Table S1). **1** and **2** were found to have the comparable oxidation potentials (**1**: 1.50 V, **2**: 1.56 V), which would be sufficiently high to arouse photo-oxidation of unsaturated lipids (mono-unsaturated CL: E_{ox} = 0.13 V, di-unsaturated CL: E_{ox} = -0.21 V)²⁰ by the oxidized donor moiety in the photoinduced charge-shifted state (Scheme S1). On the other hand, the reduction potential of **2** (-0.56 V vs. Fc/Fc⁺) was shifted considerably to a positive direction in comparison with **1** (-0.73 V), while the oxidation potentials of **1** and **2** were largely similar. Taking into account the observed absorption spectra, it is probably caused by the difference of the alkyl substituents which may increase a hydrophobic interaction of **2**.¹⁹ The more positive reduction potential of **2** would be beneficial to suppress undesirable side reductive reaction when **2** generates the photoinduced charge-shifted state.

Next, accumulation of **1** and **2** in an artificial liposome consisting of COATSOME EL-11-A (see ESI for the lipid compositions and structural information), which is similar to anionic biological membranes, were monitored respectively by size-exclusive column chromatography (Fig. S4). Remarkably, there was no significant difference in the elution profile of **1** in the absence and presence of the COATSOME in the first four fractions. Because the first fraction is expected to contain the COATSOME, which has sufficiently large size (mean diameter: 150 nm) to be eluted readily, this result suggests the weak interaction of **1** with the lipids. In the other words, although **1**

may be incorporated into the liposome, it can be released easily because of the weak interaction. In sharp contrast, more than 60% of **2** were eluted in the first fraction with the COATSOME. This difference indicates that **2** is more preferably retained in the lipid bilayer than **1**, which can be explained by the larger hydrophobicity of **2** than that of **1**.

CS properties of **1** and **2** were assessed by nanosecond time-resolved transient absorption (TA) spectra. After laser excitation at 430 nm where the acridinium moiety mainly absorbs photons, characteristic absorption appeared around 500 nm in PBS (Fig. S5). This absorption can be assigned to the acridinium radical and the aryl radical cation,^{18,23} supporting the formation of the charge-shifted state. The absorption decay profiles of **1** and **2** at 500 nm were fitted with monoexponential function (Table S2 and Fig. S6) and the kinetics of back ET (BET) to regenerate the ground state (k_{BET1} = 3.0×10^5 s⁻¹ for **1**, 2.2×10^5 s⁻¹ for **2**) were comparable to those reported for **3** in MeCN (k_{BET} = 2.8×10^5 s⁻¹ at 30 μ M).²¹ The CS yields of **1** and **2** were found to be moderate (~8 %) in PBS. This is probably due to self-quenching arising from the microscopic molecular aggregation as well as intermolecular cancellation of the charge-shifted state.^{5,16,21,22}

To get insight into the CS properties of **1** and **2** in biological membranes, the TA spectra of **1** and **2** were recorded in the COATSOME. After laser excitation, the characteristic absorption appeared around 500 nm (Figs. S7 and S8, Table S2), supporting the formation of the charge-shifted state. Much slower rate constant (k_{BET2} = 9.0×10^3 s⁻¹ for **1**, 1.7×10^4 s⁻¹ for **2**) was obtained than that of the fast-decaying component (k_{BET1} = 3.2×10^5 s⁻¹ for **1**, 3.0×10^5 s⁻¹ for **2**) which was also seen in PBS. The two decaying components may reflect different environments in the lipid bilayer. The CS yields of **1** and **2** were found to be higher (16 and 14 %) than those in PBS (8 and 7 %) (Table S2). No characteristic absorption of the triplet state around 960 nm appeared, verifying the selective formation of the charge-shifted state via photoinduced intramolecular CS.²³ As the k_{BET} values are sufficiently slow to permit the intermolecular ET reaction, **1** and **2** are suitable to facilitate the ET reactions in the lipid bilayer.

Photo-oxidation abilities of **1** and **2** towards the lipid molecules were evaluated, firstly, by test-tube experiments. Peroxidized-lipid selective fluorogenic reagent, LiperFluo®,

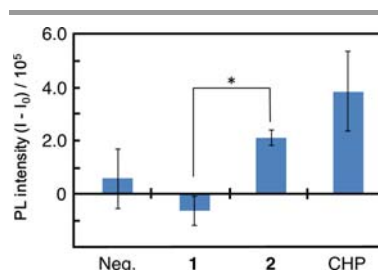


Fig. 2 Photo-oxidation experiments of CL by **1** and **2** detected by LiperFluo®. Condition; 1.0 μ M compound in PBS. *h* ν : Xe lamp 2.5 mW cm⁻², 255 sec, Ex. 425–435 nm. Detection; LiperFluo, Ex. 485 nm, Em.: 520–560 nm. Cumene hydroperoxide (CHP, 0.5 mM) was used without illumination for the control experiment. Error bars indicate S.D. Statistically significant differences are illustrated with asterisks (* p < 0.05).

demonstrated the excellent photo-oxidation ability of **2** towards CL (Fig. 2). The negligible activity of **1** may result from less interaction of **1** with CL, which is rationalized by the smaller interaction of **1** with the negatively charged COATSOME. The photo-oxidation of CL was also verified by detection of the peaks (Fig. S9d; CL-OOH), stemming from the oxidized CL together with **2** in the MALDI-TOF mass spectrum of the sample after the photoirradiation of CL in the presence of **2**. Reactive oxygen species (ROS) assays were carried out to rule out the possibility of the oxidation caused by ROS, which might be generated from photoinduced EN and ET from the excited states of **1** and **2** to O_2 during their illumination. Direct detection experiments of singlet oxygen and superoxide by fluorogenic reagents, SOSG[®] and Mito-SOX, respectively, confirmed that **1** and **2** do not generate a considerable amount of ROS during the illumination (Fig. S10). This result is rationalized by the facts that the reduction potentials of **1** and **2** are more positive than that of O_2 ($E_{red} = -0.79$ V)(Table S2), which indicates energetically unfavorable ET from the photoinduced charge-shifted states of **1** or **2** to O_2 (Scheme S1). These results support a feasible mechanism on the CL oxidation with starting from the photoinduced intramolecular CS in **2**, followed by intermolecular ET from the CL to the D^{+*} . Considering the present results and the precedent reports on the lipid-oxidation,^{20,24} we propose the photoinduced lipid-oxidation process as shown in Scheme 1. It is anticipated that **1** and **2** can work photocatalytically in the presence of proper oxidizing biomolecules and will be investigated in the future work.

The above results encouraged us to employ **1** and **2** for the artificial photocontrol of oxidation reaction in mitochondria. Confocal laser microscopic observation, after incorporation of **1** and **2** into HeLa cells, visualized their location in mitochondria (Fig. 3). Site-selective localization was attainable with the colocalization coefficients with mitochondria up to 0.95 ± 0.03 for **1** and 0.96 ± 0.01 for **2**, as anticipated from the cationic and π -conjugated nature of **1** and **2**.

Next, photoinduced lipid-oxidation was probed by using Mito-PeDPP,²⁵ i.e., a fluorogenic reagent for detecting mitochondrial lipid-peroxidation (Fig. 4). After the illumination, rapid lipid-oxidation occurred within 5 seconds by **1** and **2**, which is in marked contrast with the slow response in the positive control experiment using cumene hydroperoxide

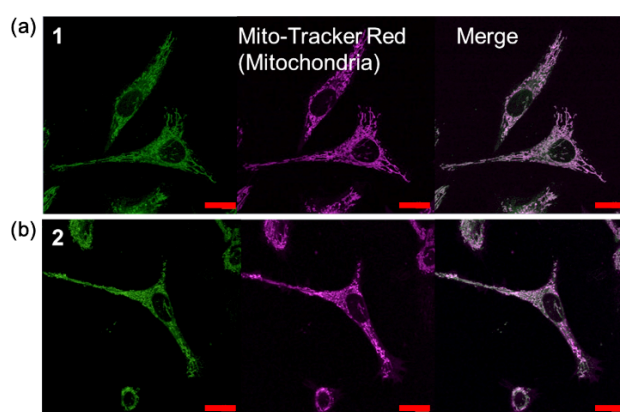


Fig. 3 Confocal laser microscopy images of HeLa cells that were treated with (a) **1** and (b) **2** (green, left) or MitoTracker[®] Red (magenta, center). The merged images are shown in the right column. Red bar indicates 20 μ m.

(CHP) (Figs. 4d and S11), i.e., a general reagent to induce mitochondrial lipid-oxidation.^{25,26} Moreover, the photo-oxidation ability of **2** was found to be higher than that of **1** (Fig. 5). Considering the similar CS properties of **1** and **2**, this difference can be explained by the higher lipophilicity of **2**, which may increase the intermolecular interaction between the lipids and **2**, than **1**, as revealed by the large accumulation of **2** into the liposomes relative to **1** (vide supra).

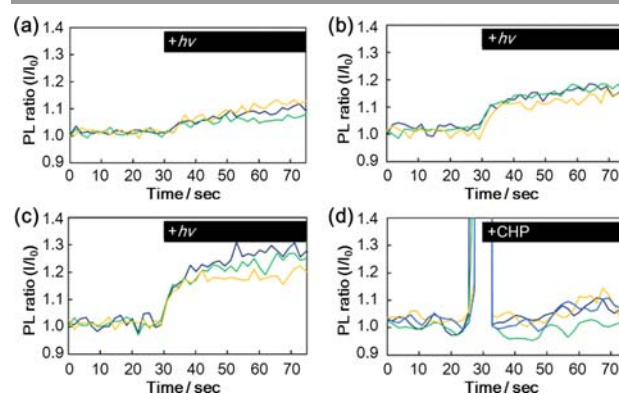
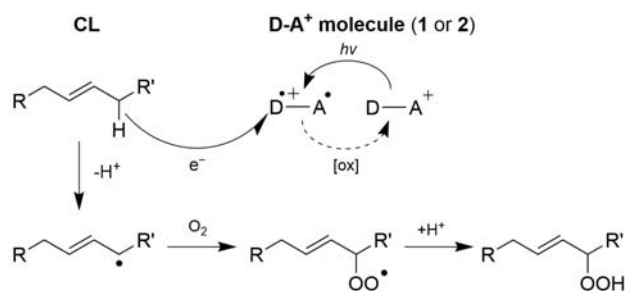


Fig. 4 CL oxidation in mitochondria of HeLa cells, which was probed by confocal laser microscope. (a) Negative control condition, (b) in the presence of **1**, (c) in the presence of **2**, and (d) 1.0 mM CHP without illumination. Large spike signal around 30 sec was caused by the room light which was used during the addition of CHP. Condition; 1.0 μ M compound. $h\nu$: Diode laser 8.0 mW cm^{-2} , Ex. 430 nm. Detection; Reagent: Mito-PeDPP, Ex.: 488 nm, Em.: Spectral imaging.



Scheme 1. Proposed photo-oxidation mechanism of CL. A partial structure of CL is herein shown. Please see Fig. S2 in ESI for the full structure of CL used in the present study.

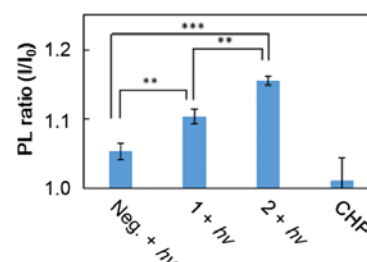


Fig. 5 CL oxidation in mitochondria of HeLa cells, which was detected by Mito-PeDPP at 5 sec after the start of illumination or the addition of 1.0 mM CHP. Error bars indicate S.D. ($n = 3-4$). Statistically significant differences are illustrated with asterisks (** $p < 0.01$, *** $p < 0.001$).

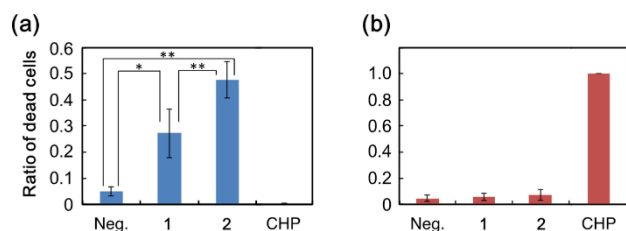


Fig. 6 Induction ability of apoptosis/necrosis of HeLa cells by the photoinduced lipid-oxidation by **1** and **2** or the addition of 5.0 mM CHP. (a) Early-apoptotic cell death detected by Annexin-V FITC conjugate and (b) late-apoptotic and necrotic cell death detected by propidium iodide. Condition; 2.5 μ M compound. *h* ν : Xe lamp 1124 mJ cm⁻², Ex. 425-435 nm. Error bars indicate S.D. Statistically significant differences between the negative control and each condition are illustrated with asterisks (**P* < 0.05, ***P* < 0.01).

Finally, cellular responses to the photoinduced oxidation by **1** and **2** were observed as induction of apoptosis. After the photo-oxidation, apoptosis was efficiently induced with the same trend as the observed lipid photo-oxidation abilities of **1** and **2** (Fig. 6).^{11,14} The apoptosis would be triggered by the release of cytochrome *c* followed by signaling from mitochondria as a consequence of the oxidation of CL.²⁴ It should be pointed out that chemical oxidation by CHP caused necrosis or late apoptosis in the same experimental condition due to its poor site-selectivity and resultant serious cell damage. These results corroborate the advantage of the present photo-control method to induce photooxidation of CL in mitochondria by **1** and **2** over conventional methods in terms of time and space.

Conclusions

The donor-acceptor linked molecules of **1** and **2** have been designed and prepared to achieve the rapid, site-specific photo-oxidation reaction of the lipids in mitochondria by selective photoinduced intramolecular CS followed by intermolecular ET between the lipids and the oxidized donor moiety. We attained the successful localization of **1** and **2** into mitochondria of HeLa cells and the resultant rapid, site-specific lipid-oxidation by light. It should be emphasized that the undesirable side effects from the ROS generation are suppressed remarkably because of the unique mechanism for the photo-oxidation as well as the rational molecular design for their site-specific delivery. By using this methodology, we will be able to develop donor-acceptor linked molecules that can be delivered to the specific sites in cells and show rapid photo-triggered redox reactions, which may be useful to modulate cellular activities as well as to create new disease model conditions with excellent spatiotemporal response.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

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