Oxidation Decomposition of Unsaturated Fatty Acids by Singlet Oxygen in Phospholipid Bilayer Membranes

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Abstract: Oxidation decomposition of unsaturated fatty acids with singlet oxygen generated from a photosensitizing agent was investigated in liposome bilayer membranes under a light irradiation condition. The liposome of which the bilayer membrane was composed of L- α -dipalmitoylphosphatidylcholine (DPPC), protoporphyrin IX (PpIX), and an unsaturated fatty acid (oleic acid, linoleic acid, α -linolenic acid, or arachidonic acid) were prepared with Bangham's method. In irradiating the liposome dispersion with light ranged from 550 to 750 nm, the unsaturated fatty acid was decomposed through an oxidation reaction with singlet oxygen. The decomposition rate constant was obeyed as the following order: arachidonic acid > oleic acid > α -linolenic acid > linoleic acid. This result indicates that oleic acid is readily degraded despite its lower unsaturated degree. In addition, micropolarity and microfluidity of the hydrocarbon region in the liposome bilayer membrane including the unsaturated fatty acid and PpIX decreased with an increase in light irradiation time. These findings suggest that interaction among the hydrocarbon chains of DPPC in the liposome bilayer membrane is promoted by migration of the oxidized unsaturated fatty acid from the hydrocarbon region, leading to form close-packed and well-ordered orientation of the hydrocarbon chains.

Key words: oxidation decomposition, unsaturated fatty acid, singlet oxygen, phospholipid bilayer membrane, protoporphyrin IX

1 INTRODUCTION

Photodynamic therapy (PDT), which is one of the most useful cancer therapies, includes administration and localization of photosensitizing agents, porphyrins, chlorines, *etc.*, into cancer tissues, followed by activation of the agent by light with a specific wavelength. The photoexcited photosensitizing agent transfers the energy to molecular triplet oxygen to form singlet oxygen¹⁾, thus leading to photochemical and photobiological processes that cause irreversible photodamage, apoptosis and necrosis, to the cancer tissues^{2,3)}.

Phospholipids are one of main compositions of biological

membranes. The phospholipid has two asymmetric hydrocarbon chains; one is saturated and the other is mono-*cis*- or poly-*cis*-unsaturated. Since the unsaturated hydrocarbon chain has a pronounced bend at a *cis*-double bond, it prevents close-packed and well-ordered orientation of the hydrocarbon chains of the phospholipids, which raises fluidity of the biological membrane⁴⁾.

Singlet oxygen readily oxidizes a *cis*-double bond of unsaturated hydrocarbon chains and introduces hydroperoxy group into them. In addition, some porphyrins employed as a photosensitizing agent in PDT temporarily accumulate in mitochondrial and cell membranes⁵⁾. Singlet

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oxygen generated from the photoexcited porphyrin then reacts to a *cis*-double bond of phospholipids in the membrane. Therefore, it is quite important in PDT to investigate oxidization of unsaturated hydrocarbon chains by singlet oxygen in phospholipid bilayer membranes and to realize influence of the oxidized unsaturated hydrocarbon chain on membrane properties.

It is well known that oxidation reactions of unsaturated fatty acids with active oxygen species are accelerated with an increase in their unsaturated degrees in homogeneous solutions. On the contrary, Yazu *et al.* have observed that oxidizability of unsaturated fatty acids solubilized in aqueous surfactant micellar phases by active oxygen species decreases with an increase in the number of their double bonds^{6,7}. Moreover, Kobayashi *et al.* have reported that unsaturated fatty acids with higher unsaturated degrees are little susceptible to their oxidation by active oxygen species in micellar phases⁸. In these studies, they used water-soluble azo compounds as an oxidation initiator and focused on the oxidizability of the unsaturated fatty acid in the surfactant micellar phase by the active oxygen species coming from the water bulk phase.

In the present study, we prepared liposomes of which bilayer membranes contained phospholipids, a photosensitizing agent, and an unsaturated fatty acid with various unsaturated degrees, and determined an oxidation decomposition rate of the unsaturated fatty acid by singlet oxygen under a light irradiation condition. We then discuss the relationship between the oxidation decomposition rate and the unsaturated degree of the unsaturated fatty acid in the phospholipid bilayer membrane. Further, physicochemical properties of the phospholipid bilayer membrane after the oxidation reaction were investigated.

2 EXPERIMENTAL PROCEDURE

2.1 Materials

L- α -dipalmitoylphosphatidylcholine (DPPC, 99.7%) was purchased from NOF. Co. Protoporphyrin IX (PpIX), which was purchased from Aldrich Co., was employed as a photosensitizing agent. They were used without further purification. Pyrene (98%) as a fluorescence probe was purchased from WAKO Pure Chemical Industries, Ltd., and was used after triple extraction with ethanol. Unsaturated fatty acids, oleic acid, linoleic acid, α -linolenic acid, and arachidonic acid, were purchased from Aldrich Co., and were used without further purification.

2.2 Preparation of liposome with phospholipid bilayer membrane including unsaturated fatty acid and PpIX

Liposomes of which bilayer membranes contained DPPC, PpIX, and an unsaturated fatty acid were prepared using Bangham's method⁹. These compounds were dissolved in 5

mL of chloroform in a test tube to obtain a given concentration: 2.5×10^{-5} M of DPPC, 1.0×10^{-5} M of the unsaturated fatty acid, and 1.0×10^{-6} M of PpIX. Chloroform was evaporated under N₂ gas, and a thin lipid film was formed on the wall of the test tube. In order to remove a residual solvent completely, the test tube was allowed to stand in a desiccator under reduced pressure. After 12 mL of distilled water was added into the test tube, the test tube was kept for 5 min at 60°C, agitated on a vortex mixer for a few minutes to give liposome dispersion. The liposome dispersion was extruded through a 10 μ m of Millipore filter (Whatman Co.) with an extruder (Lipex Biomembranes Inc.) to obtain homogeneously sized liposome dispersion. The liposome formation was confirmed with light and fluorescence microscopy.

2.3 Determination of singlet oxygen generated in liposome bilayer membrane

A 1.0 mL of 0.3 M 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TPMD, Kanto Chemical Co., Ltd.) solution was added into 1.0 mL of homogeneously sized liposome dispersion that had been prepared by using DPPC and PpIX, but without an unsaturated fatty acid. The mixture was exposed to light emitted from a metal-halide lamp for a given time at 25°C. The wavelength of the light ranged from 550 to 750 nm. Under this irradiation condition, the liposomal structure was maintained. ESR spectra of the mixture were measured with an ESR spectrometer (JES-AF200, JEOL) before and after the light irradiation. ESR spectra of the liposome dispersion with histidine, which can scavenge singlet oxygen, were also measured. In general, TPMD is oxidized by singlet oxygen and is converted to a stable nitroxide radical ($>\text{N-O}\cdot$) that gives a characteristic ESR signal¹⁰, but the ESR signal of the nitroxide radical disappears by adding histidine¹¹.

2.4 Decomposition of unsaturated fatty acid by singlet oxygen in liposome bilayer membrane

After exposing homogeneously sized liposome dispersion including PpIX and an unsaturated fatty acid to light emitted from the metal-halide lamp for a given time at 25°C, we confirmed with microscopy that the liposomal structure was maintained in the dispersion. Afterward, we broke the liposome by adding ethanol. A concentration of a remaining unsaturated fatty acid was determined with a high performance liquid chromatograph (HPLC, LaChrom, HITACHI, Ltd.) with an octadecylsilane column (250 mm in length, 4.6 mm in diameter) and an ethanol-water mixed solution (volume ratio of ethanol to water: 4:1) as eluent.

2.5 Measurement of micropolarity and microfluidity of liposome bilayer membrane using fluorescence probe method with pyrene

Homogeneously sized liposome dispersion with 6.0×10^{-3}

M of pyrene was prepared by the same method as described before. Before and after light irradiation, fluorescence spectra of pyrene in the liposome bilayer membrane were measured with a fluorescence spectrophotometer (V-530, JASCO Co.) at 25°C. The excitation wavelength was 335 nm, and the emission wavelength was in the range from 350 to 650 nm. The fluorescence peak intensity ratio of pyrene at 375 and 395 nm (I_1/I_3) reflects micropolarity around pyrene^{12,13}; the micropolarity increases with an increase in I_1/I_3 of pyrene. In addition, the face-to-face collision of pyrene monomers often forms pyrene excimers above a certain concentration of pyrene¹². The pyrene fluorescence peaks at 395 and 473 nm are assigned to pyrene monomers and excimers, respectively. The fluorescence peak intensity ratio between excimers and monomers (I_E/I_M) indicates microfluidity around pyrene¹⁴; the larger the I_E/I_M of pyrene is, the higher the microfluidity around pyrene is.

3 RESULTS AND DISCUSSION

3.1 Generation of singlet oxygen in liposome bilayer membrane

We prepared liposomes using DPPC and PpIX, but without an unsaturated fatty acid, and determined generation of singlet oxygen in the liposome dispersion under a light irradiation condition. Fig. 1 (a) and (b) show ESR spectra of the liposome dispersion with TMPD before and after the

light irradiation for 5 min, respectively. Besides, Fig. 1 (c) depicts an ESR spectrum of the liposome-TMPD mixed dispersion with histidine after the 5-min light irradiation. We detected no ESR signals before the light irradiation, but a characteristic ESR signal with three equal intensity lines (closed circles in Fig. 1 (b)) assigned to a TMPD nitroxide radical after the irradiation. When histidine was added, the ESR signal of the TMPD nitroxide radical disappeared despite the light irradiation. When exposing the water bulk phase with TMPD to the light, we detected no ESR signals (the data is not shown). In addition, fluorescence microscopy for the liposome dispersion observed the PpIX fluorescence from the liposome bilayer membrane, but not from the water bulk phase. Taking account of these findings, we consider that PpIX exists in the liposome bilayer membrane and that singlet oxygen is generated from PpIX in the membrane, but not in the water bulk phase.

Fig. 2 expresses the relative intensity of the ESR signal of the TMPD nitroxide radical against that of the manganese markers on the both sides of the ESR spectra shown in Fig. 1. The relative intensity corresponds to a quantity of singlet oxygen generated¹⁵. The relative intensity shown in Fig. 2 rises with irradiation time and becomes almost constant over 10-min light irradiation. As a result, singlet oxygen generation is restricted in light irradiation above 10 min.

Photosensitizers including PpIX are generally bleached in light irradiation. They are decomposed into their oxidants¹⁶. In order to understand the bleaching of PpIX in the liposome bilayer membrane under this experimental condition, we measured fluorescence spectra of PpIX after

Fig. 1 ESR Spectra of Liposome Dispersion with TMPD before and after Light Irradiation at 25°C: (a) and (b) Non-Light-Irradiated and Light-Irradiated Liposome Dispersion, Respectively, (c) Histidine-Containing Liposome Dispersion Irradiated with Light. Closed Circles Shows ESR Signal of TMPD Nitroxide Radical.

Fig. 2 Change in Relative Intensity of ESR Signal of TMPD Nitroxide Radical to that of Manganese with Light Irradiation Time at 25°C.

light irradiation. Fig. 3 depicts irradiation time-dependence of fluorescence peak intensity at 633 nm of PpIX in liposome dispersion excited by light at 410 nm. Here, the vertical line corresponds to the fluorescence intensity ratio of PpIX with and without the light irradiation, namely, a remaining PpIX concentration ratio in the liposome dispersion. As can be seen from Fig. 3, the concentration of PpIX in the membrane is gradually reduced with increasing light irradiation time. It is probably that this reduction induces the restriction of the singlet oxygen generation shown in Fig. 2.

3.2 Decomposition of unsaturated fatty acid by singlet oxygen in liposome bilayer membrane

Fig. 4 refers to changes in concentration of the unsaturated fatty acid in the liposome bilayer membrane with light irradiation time. Here, the vertical line corresponds to the ratio of the unsaturated fatty acid concentration at a given irradiation time against that at the initial state (irradiation time: 0). The concentration ratio decreases with increasing irradiation time in any system. We also confirmed no decomposition of the unsaturated fatty acid without light irradiation or PpIX (the data is not shown). These findings then consider that the decomposition of the unsaturated fatty acid is caused by an oxidation reaction with singlet oxygen generated through PpIX in the phospholipid bilayer membrane.

We estimate the correlation between the decomposition rate and the unsaturated degree of the unsaturated fatty acid by using oxidation kinetics. Oxidation decomposition of unsaturated fatty acids by singlet oxygen is essentially conformed to a second-order reaction for a concentration of the unsaturated fatty acid and singlet oxygen. Not

Fig. 3 Change in PpIX Fluorescence Intensity Ratio with and without Light Irradiation as a Function of Light Irradiation Time at 25°C.

detecting concentrations of singlet oxygen during the oxidation reaction in our experiment, however, we regard the oxidation reaction as an apparent first-order reaction for the unsaturated fatty acid concentration ratio shown in Fig. 4. We summarize the results obtained from the apparent first-order reaction in Fig. 5. There is a nonlinear rela-

Fig. 4 Changes in Concentration Ratio of Unsaturated Fatty Acid with and without Light Irradiation as a Function of Light Irradiation Time at 25°C.

Fig. 5 Relationship between Unsaturated Fatty Acid Concentration Ratio in Liposome Bilayer Membrane and Light Irradiation Time Evaluated by Apparent First-Order Reaction for Unsaturated Fatty Acid Concentration Ratio at 25°C. Dashed lines demonstrate slopes of the relationship at the initial state (irradiation time: 0).

tionship in any system, and the slope, or the decomposition rate constant, gradually becomes smaller with increasing irradiation time. This is probably caused by the fact that it gets hard to generate singlet oxygen with increasing irradiation time demonstrated in Fig. 2

Fig. 6 refers to change in decomposition rate constant of the unsaturated fatty acid at the initial state (irradiation time: 0) as a function of its unsaturated degree. The decomposition rate constant was estimated from the slope at the initial state, drawn by a dashed line in Fig. 5, on the assumption that the same quantity of singlet oxygen is generated at the initial state in any system. The rate constant increases with the unsaturated degree, except for oleic acid. As for oleic acid, its decomposition rate constant is comparably larger despite the lowest unsaturated degree.

Oxidizability of unsaturated fatty acids by active oxygen species in homogeneous bulk phases is accelerated with an increase in the number of their double bonds. In fact, the decomposition rate constants of oleic, linoleic, linolenic, and arachidonic acid methyl esters by singlet oxygen in pyridine are 0.7 , 1.3 , 1.9 , and $2.4 \times 10^5 \text{ M}^{-1}\text{Sec}^{-1}$, respectively¹⁷. Besides, the decomposition rate constants of linoleic, linolenic, and arachidonic acids against perhydroxyl radical in aqueous ethanoic solutions are 1.18 , 1.70 , and $3.05 \times 10^3 \text{ M}^{-1}\text{Sec}^{-1}$, respectively¹⁸. Therefore, the fast decomposition of oleic acid by singlet oxygen observed in this study is a unique phenomenon in the heterogeneous phospholipid bilayer membranes.

When solubilized by aqueous surfactant solutions, PpIX resides in the hydrocarbon region of surfactant micellar phases¹⁹. We suggest from this viewpoint that PpIX is also incorporated in the hydrocarbon region of liposome bilayer

Fig. 6 Relationship between Decomposition Rate Constant at Initial State and Unsaturated Degree of Unsaturated Fatty Acid at 25°C.

membranes in the same manner. Moreover, unsaturated fatty acids are also penetrated into the hydrocarbon region of liposome bilayer membranes in parallel with the hydrocarbon chain of the phospholipid. Therefore, the oxidation decomposition of the unsaturated fatty acid by singlet oxygen generated from the photoexcited PpIX is predicted to be closely related to properties of the hydrocarbon region of the liposome bilayer membrane.

In general, a lifetime of singlet oxygen is shortened with an increase in polarity (or permittivity) of its surroundings. For instance, lifetimes of singlet oxygen in water, acetone, and chloroform are 3.3 , 46.5 , and $60 \mu\text{s}$, respectively¹¹. Shorter lifetimes of singlet oxygen in polar solvents suppress its reactivity. When we measured I_1/I_3 of pyrene localized in the hydrocarbon region of the liposome bilayer membrane, shown in Fig. 7, micropolarity of the hydrocarbon region is almost constant in the case with any unsaturated fatty acid. Therefore, the micropolarity of the phospholipid bilayer membrane contributes little to the difference in the decomposition rate of the unsaturated fatty acid.

Increase in microfluidity of the hydrocarbon region in the phospholipid bilayer membrane accelerates mobility and diffusion of singlet oxygen and the unsaturated fatty acid in the membrane, promoting their oxidation reaction. According to the fast decomposition of oleic acid, it might be expected that microfluidity of the hydrocarbon region of the liposome bilayer membrane including oleic acid would be much greater. In measuring I_E/I_M of pyrene in the hydrocarbon region in the liposome bilayer membrane, contrary to our expectation, the microfluidity is lower with oleic acid than with any other unsaturated fatty acid, as shown in Fig. 7. The fast decomposition of oleic acid cannot be then explained by the microfluidity of the hydrocar-

Fig. 7 Changes in I_E/I_M and I_1/I_3 of Pyrene in Liposome Bilayer Membrane as a Function of Unsaturated Degree of Unsaturated Fatty Acid at 25°C.

bon region in the phospholipid bilayer membrane.

The intermolecular distance between the unsaturated fatty acid and PpIX in the liposome bilayer membrane is closely related to the decomposition rate of the unsaturated fatty acid. Unsaturated fatty acids with a *cis*-double bond have a pronounced bend, and this tendency is more remarkable for unsaturated fatty acids having higher unsaturated degrees. Hence, increasing unsaturated degree of unsaturated fatty acids spreads their molecule occupied areas, for example, 2.1 nm² for stearic acid without a double bond, and 4.1 and 4.8 nm² for oleic and linoleic acids with one and two *cis*-double bonds²⁰). In addition, since pK_a value of unsaturated fatty acids decreases with an increase in its unsaturated degree: 9.85 for oleic acid, 9.24 for linoleic acid, and 8.28 for α -linolenic acid²⁰), the unsaturated fatty acid with lower unsaturated degrees tends to be protonated around neutral pH. On the other hand, the pK_{a1} and pK_{a2} values of two carboxylic acids in PpIX evaluated from the pK_a values of dicarboxylic metalloporphyrins are approximately 5 and 7, respectively²¹), indicating that the carboxylic acids of PpIX seem to be deprotonated around neutral pH. As a result, strong ion-dipole interaction acts between the carboxylate groups of PpIX and the carboxylic acid group of the unsaturated fatty acid with lower unsaturated degrees around neutral pH. Taking into consideration the smallest molecular occupied area and the strong ion-dipole interaction of the unsaturated fatty acid with lower unsaturated degrees, therefore, we suggest that the intermolecular distance between oleic acid and PpIX is shorter in the liposome bilayer membrane and that collision frequency between the *cis*-double bond of oleic acid and singlet oxygen generated from PpIX increases; thereby, resulting in the fast decomposition of oleic acid in the phospholipid bilayer membrane. However, this suggestion will need to be verified in future study.

3.3 Characterization of liposome bilayer membrane after oxidation reaction

In order to characterize the phospholipid bilayer membranes after the oxidation of the unsaturated fatty acid by singlet oxygen, we investigated irradiation time-dependence of the membrane micropolarity and microfluidity by measuring I_1/I_3 and I_E/I_M of pyrene that was located in the hydrocarbon region of the membrane.

Changes in I_1/I_3 of pyrene in the liposome bilayer membrane with and without the unsaturated fatty acid are demonstrated as a function of light irradiation time in Fig. 8. Before this measurement, we determined no decay of the pyrene fluorescence under the light irradiation condition. As a result, the micropolarity in the hydrocarbon region of the membrane slightly decreases with increasing irradiation time in any system.

In addition, Fig. 9 shows changes in I_E/I_M of pyrene with

Fig. 8 Changes in I_1/I_3 of Pyrene in Liposome Bilayer Membrane as a Function of Light Irradiation Time at 25°C.

Fig. 9 Changes in I_E/I_M of Pyrene in Liposome Bilayer Membrane as a Function of Light Irradiation Time at 25°C.

light irradiation time. The microfluidity of the liposome bilayer membrane without the unsaturated fatty acid is almost constant, whereas the microfluidity with the unsaturated fatty acid is obviously reduced with an increase in irradiation time.

Singlet oxygen can easily react with a *cis*-double bond in unsaturated fatty acids through “ene” reaction, followed by formation of *trans* hydroperoxy group as follows²²):

Hydroperoxy group introduces hydrophilicity to the unsaturated fatty acid. If the unsaturated fatty acid with hydroperoxy group remained in the hydrocarbon region of the liposome bilayer membrane, the micropolarity of the hydrocarbon region would rise as its oxidation reaction progresses. As seen from Fig. 8, nevertheless, the micropolarity is unexpectedly reduced with an increase in irradiation time. In addition, NMR measurement of the water bulk phase in the liposome dispersion after the light irradiation provided us no peak, as well as that before the light irradiation (the data is not shown). The unsaturated fatty acid with hydroperoxy group then seems to migrate from the hydrocarbon region into the hydrophilic region of the liposome bilayer membrane. Additionally, the migration of the oxidized unsaturated fatty acid makes the interaction among the hydrocarbon chains of DPPC promoted and induces close-packed and well-ordered orientation of the hydrocarbon chains, resulting in the reduction in the microfluidity of the hydrocarbon region, as shown in Fig. 9.

4 CONCLUSION

The unsaturated fatty acid was decomposed through the oxidation reaction with singlet oxygen generated by the photoexcited PpIX in the liposome bilayer membrane. The decomposition rate constant was obeyed as the following order: arachidonic acid > oleic acid > α -linolenic acid > linoleic acid. Although having the smallest unsaturated degree, oleic acid was readily degraded. The short intermolecular distance between oleic acid and PpIX in the liposome bilayer membrane probably causes its fast decomposition. In addition, the micropolarity and microfluidity of the hydrocarbon region in the liposome bilayer membrane with the unsaturated fatty acid and PpIX decreased with an increase in light irradiation time. These results suggest that interaction among the hydrocarbon chains of DPPC in the liposome bilayer membrane is promoted by migration of the oxidized unsaturated fatty acid from the hydrocarbon region, leading to form close-packed and well-ordered orientation of the hydrocarbon chains.

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