Nanopore Formation Process in Artificial Cell Membrane Induced by Plasma-Generated Reactive Oxygen Species

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Abstract

We investigated morphological change of an artificial lipid bilayer membrane induced by oxygen radicals which were generated by non-equilibrium atmospheric pressure plasma. Neutral oxygen species, $O(^3P_j)$ and $O_2(^1\Delta_g)$, were irradiated of a supported lipid bilayer existing under a buffer solution at various conditions of dose time and distances, at which the dose amounts of the oxygen species were calculated quantitatively. Observation using an atomic force microscope and a fluorescence microscope revealed that dose of the neutral oxygen species generated nanopores with the diameter of 10 - 50 nm in a phospholipid bilayer, and finally destructed the bilayer structure. We found that protrusions appeared on the lipid bilayer surface prior to the formation of nanopores, and we attributed the protrusions to the precursor of the nanopores. We propose a mechanism of the pore formation induced by lipid oxidation on the basis of previous experimental and theoretical studies.

Keywords: Atmospheric pressure plasma; Lipid bilayer; Lipid oxidation; Reactive oxygen species; Atomic force microscope

Introduction

Reactive oxygen species (ROSs) are reactive factors and/or mediators in biological and physiological reactions shown to be either beneficial or harmful [1–3]. Atmospheric pressure plasma is a novel and valuable tool to generate ROSs intentionally, as well as reactive nitrogen species (RNSs) and electric field, and is applied in the fields of medicine and biology in these decades [4–6]. Recent studies demonstrated the multifarious applications of atmospheric pressure plasma in these fields; sterilization [7–13], selective killing of tumor cells [14,15], cellular regulation [16,17], and gene transfection [18,19]. Various kinds of ROS and RNS are generated and included in these reactions induced by atmospheric pressure plasma [12], and play crucial roles in these phenomena. Some studies showed that radical species are requisite for the transportation of materials through a cell membrane [19,20]. Recently we developed a neutral oxygen radical source on the basis of non-equilibrium atmospheric pressure plasma [21], which effectively inactivates *Penicillium digitatum* spores [22–26] and proliferates/inactivates *Saccharomyces cerevisiae* [27]. This radical source excludes the effects of electric field and lights, and supply only ROSs. The ROSs provided by this radical source are characterized and their dose amounts were quantitatively evaluated [21,25–27]. The active species critical for these reactions are attributed to the atomic oxygen at the ground state $O(^3P_j)$, and its amount is on the order of 10^{19} cm^{-3} .

In spite of the various applications of the atmospheric pressure plasma, however, what ROSs causes on cell membranes and how ROSs pass through cell membranes are still unclear. Biological cell membrane is the outermost cell organ and acts as a barrier and gate controlling the transportation of materials and information. Everything needs to pass through a cell membrane when it enters into a cell and cause an effect inside the cell. The plasma-generated ROSs also access to the cell membranes first. The critical effect of ROSs on cells may be a direct effect on cell membranes, or indirect one through physiological cascades inside cells and/or genetic damages. Some previous studies showed that cell membranes take damage and act as a barrier against plasma-induced effects on genomic damage or oxidative protein modification [7,28–31], while other studies showed that plasma effects causes the oxidation of the intracellular organelles because of the permeation of ROS through the cell wall and cell membrane, without a major deformation of the membranes [10,15,23–25]. Simple permeation of water-soluble ROSs through a cell membrane is highly unlikely, because the fundamental structure of a cell membrane is a lipid bilayer which has hydrophobic core. Physical damage to a cell membrane, such as poration, is effective for the transportation of water-soluble ROSs into cells, but such a non-selective leakage leads to the loss of membrane potential and is highly toxic for cells [32]. The oxidation of lipid induced by physiological ROSs is also related to cell damages and various pathological states [1–3]. A fundamental understanding of the effects and their mechanism of plasma-induced ROSs on a cell membrane is demanded for the further progress and establishment of safety in the medical and biological application of atmospheric pressure plasma.

Artificial lipid bilayers are biomembrane model systems useful for investigating the fundamental interaction between cell membranes and medical and biological agents [33–38]. Very recently effects of atmospheric pressure plasma irradiation to lipid bilayer membranes have been studied using lipid vesicles (liposomes) [39–44], red blood cell ghost [30], and supported lipid bilayers (SLBs) [45–47]. The studies

using lipid vesicles showed that plasma-generated ROS and RNS are prevented by the lipid bilayer effectively but partly permeate into the inside of lipid vesicles, while the structural deformation or decomposition of vesicles is limited. Hammers et al. proposed that oxidized lipid in a bilayer membrane apply a mechanical stress which leads to local rupturing of the bilayer [41]. SLB is an artificial planar lipid bilayer system formed at a solid-liquid interface [34,48,49]. The SLBs have advantages that fluid and fragile lipid membranes exist stably owing to the support of the solid substrate and that high-resolution surface scientific techniques such as atomic force microscope (AFM) are available. Recently we revealed that irradiation of atmospheric pressure plasma on the basis of dielectric barrier discharge (DBD) reduces the fluidity of a lipid bilayer at the initial stage [46], then generates pores with diameter in the order of 10 nm - 1 μ m in the lipid bilayer [45] using the SLB system. We also showed that equilibrium molecules such as hydrogen peroxide (H_2O_2) and NO_3^- does not cause these phenomena, and suggested that transient reactive species are responsible for these phenomena, [45]

In this study, we investigated effects of the oxygen radicals generated by non-equilibrium atmospheric pressure plasma on the morphology of SLB. We irradiated neutral oxygen species, $\text{O}(^3\text{P}_j)$ and $\text{O}_2(^1\Delta_g)$ of quantitatively evaluated amounts to SLB existing in a buffer solution [21,25–27]. After the irradiation of $\text{O}(^3\text{P}_j)$ and $\text{O}_2(^1\Delta_g)$, nanopores were observed in SLB with AFM, and SLB was finally decomposed almost completely. AFM observation showed that protrusions appeared on SLB before the nanopore formation. The area fraction and size of the nanopores increased with dose amount of the neutral oxygen species. We propose a mechanism of the morphological change of a lipid bilayer through two-step lipid oxidation on the acyl chain induced by hydroxyl radical.

Experimental

Irradiation of neutral oxygen radicals to supported lipid bilayer

Figure 1 shows the schematic of the experimental setup used for the irradiation of the neutral oxygen radicals to a sample cell containing the SLB on a SiO_2/Si substrate and a buffer solution, using an atmospheric pressure oxygen radical source. The oxygen radical source (Tough Plasma, Fuji Machine) was based on atmospheric pressure high-density O_2/Ar plasma, which generates electrons at a density of about 10^{16} cm^{-3} . The details about the radical source is described elsewhere [21,22]. Charged species and optical radiation from the O_2/Ar plasma were blocked by electrodes and the nozzle aperture of the radical source, so that only neutral species were supplied to the samples. The nozzle of the radical source used in this study had a slit aperture of $0.5 \times 16 \text{ mm}^2$, the same as previous studies in Refs. [23,27]. The sample cell was made of a cover glass slip and an O-ring of silicone resin with a thickness of 2 mm, and the volume of the buffer solution in the sample cell was 400 μL . The irradiation distance (d) from the nozzle of the radical source to the surface of the buffer solution was set at 8, 12, 16 or 20 mm. Prior to exposure, the treatment area was purged with the Ar gas from the radical source using a plastic cover, thereby eliminating the influence of atmospheric gases. The oxygen radical treatment was performed at a total flow rate of 5 standard liters per minute (slm) and an $\text{O}_2/(\text{Ar} + \text{O}_2)$ flow rate ratio of 0.6%, with treatment time varying from 180 s to 420 s. After the oxygen radical treatment, we immediately transferred the sample cell to a fluorescence microscope or an atomic force microscope in the same room, and started the

observation.

Preparation of lipid vesicles and supported lipid bilayer

Lipid agents in the form of chloroform solution were purchased from Avanti Polar Lipids Inc., and used without further purification. We used 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) because the diacylphosphatidylcholine, a glycerol phospholipid having phosphocoline on its hydrophilic head group, is the representative phospholipid most abundantly existing in cell membranes of Eukaryote. A vacuum-dried film of DOPC mixed with a fluorescence-labeled lipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rb-DOPE)) at the molar ratio of 200:1 was prepared from their chloroform solutions, and suspended in a buffer solution (100 mM KCl, 25 mM HEPES/NaOH (pH 7.4), chemicals were purchased from Wako Pure Chemical Industries, Ltd.) at the lipid concentration of 0.40 mM to prepare multilamellar vesicles (MLVs). The MLVs were transformed to unilamellar vesicles with an average diameter of 120 nm through freeze-and-thaw cycles and the extrusion process through a 100 nm-pore polycarbonate filter as described in previous studies [50–52]. The DOPC-SLB containing Rb-DOPE was prepared on thermally oxidized SiO₂/Si substrates by the vesicle fusion method [34]. A thermally oxidized SiO₂/Si substrate (8 × 8 mm², 0.525 mm thick) was incubated in the vesicle suspension at 45°C for 1 h. We exchanged the vesicle suspension with the buffer solution 10 times after the incubation to remove excess vesicles in the aqueous phase. Our preparation method provides uniform DOPC-SLB on whole the substrate surface reproducibly as we show in previous studies [34,45,46,50–55].

Atomic force microscope and fluorescence microscope

The SLBs were observed with an AFM (PicoPlus 5500, Agilent Technologies, Inc., formerly Molecular Imaging, Corp.), and an epi-fluorescence microscope (BX51WI, Olympus). We adopted acoustic AC mode (tapping mode) for the AFM observation using a Si₃N₄ cantilever (2 N/m, tip curvature radius of 10 nm). We used a high-pressure mercury lamp and neutral density (ND) filters for the fluorescence observation, and irradiated brighter excitation light by removing the ND filters for the observation of fluorescence recovery after photobleaching (FRAP) process [51,52,55]. We kept the SLB samples under the buffer solution during the all processes of the preparation, plasma irradiation, and observations. We used Image J software (NIH, <http://imagej.nih.gov/ij/>) for the analysis of AFM topographies. The area fraction and density of objects were evaluated as the average of at least three images for each experimental condition.

Results

Figure 2a shows the AFM topographies of DOPC-SLB on the thermally oxidized SiO₂/Si surface. The sample was settled under the O₂+Ar gas flow for 300 s without generating plasma. The SLB had uniform and flat surface with the roughness of 0.23 nm (rms) as with our previous studies [45,52,53]. Therefore the O₂+Ar gas flow did not affect the morphology of SLB. After the irradiation of neutral oxygen radicals induced by atmospheric plasma at $d = 8$ mm for 300 s, pores appeared in the SLB (Fig.

2b). The diameter of the pores was 20.8 ± 3.9 nm, and area fraction was 1.39%. The depth of the pores was approximately 2 nm. We also found some protrusions with the height of 5 - 6 nm, but its density was smaller than the nanopores. In the phase-shift image, which is the mapping of viscoelasticity, the edge of the nanopores had clear contrast compared with the flat SLB (Fig. 2c). Thickness of lipid bilayers is approximately 5 nm [52,56,57], and the nanopores in this study were observed shallower similar to our previous study [45]. One possibility is that the tip of the cantilever did not reach to the bottom of the pore, because the curvature radius of the cantilever is 10 nm as a nominal value before use. It may be also partly because of different hydrophilicity of the inside of nanopore due to some residual lipids from outside of the nanopores, artificial height appears in AFM topographies on a hydrophobic sample in an aqueous solution [57]. We note that the morphological change in DOPC-SLB such as protrusion and pores were observed from the first image after the oxygen plasma treatment. Thus the morphological change occurred during the oxygen plasma irradiation.

We observed the macroscopic morphological change using a fluorescence microscope (Fig. 3). The DOPC-SLB containing 0.2% of Rb-DOPE without the irradiation of neutral oxygen radicals was observed as a homogeneous membrane (Fig. 3a). Fluorescence recovery after photobleaching (FRAP) method [51,52] showed the fluidity and continuity of SLB (data not shown). After the irradiation of the oxygen radicals at $d = 8$ mm for 300 s, which was the same condition as Fig. 2b, we did not find a clear difference in the fluorescence image (Fig. 3b) from that before the radical irradiation (Fig. 3a). It should be noted that the size of the pores observed in the AFM topography in Fig. 2b was smaller than the resolution limit of an optical microscope using visible light. Thus the nanopores also existed in the SLB shown in Fig. 3b. Pores on the order of micrometers, which appear after the irradiation of DBD-plasma [45], were not observed in this study. After the irradiation of oxygen radicals at $d = 8$ mm for 420 s, the fluorescence signal from the DOPC-SLB almost disappeared (Fig. 3c). The result indicates that bilayer structure was deconstructed and majority of the DOPC-SLB was removed from the substrate. Nanopores of ~ 20 nm shown in Fig. 2 are large enough for the transportation of not only ROS/RNS species but also for larger molecules like DNA even if they are short-lived, and are fatal for living cell if they exist stable [32]. What important in the field of plasma medicine are these transportation phenomena and their mechanism, not the complete removal of membranes. Therefore we focus on the formation process of the nanopores in this study, and do not intend further step for the membrane destruction.

We investigated the formation process of the nanopores observed in Fig. 2b induced by the irradiation of the neutral oxygen radicals. Figure 4 shows the dependence of the DOPC-SLB morphology on the irradiation time at a fixed irradiation distance of $d = 8$ mm. We found that protrusions appeared on the DOPC-SLB surface after the irradiation for 180 s (Fig 4a). The height of the protrusion was approximately 5 - 6 nm, similar to that observed in Fig. 2b. The density of the protrusion was $22.3 \mu\text{m}^{-2}$. Small pores with the average diameter of 16.6 ± 3.7 nm also existed, and their area fraction was 0.93%. The protrusions were rarely observed after longer irradiation times (Fig. 4b: 240 s, and Fig. 4c: 300 s). The average diameter of the pores in Fig. 4b was 18.7 ± 5.5 nm, and the area fraction of the pores was 1.93%. Clear contrast at the pore edge was observed in the phase-shift image as in Fig. 2c. These results indicate that the protrusions observed in Fig. 4a were the precursor of the nanopores which were produced by the

plasma-generated oxygen species.

Figure 5 shows the dependence of the pore formation on the irradiation distance d . Figure 5a–c show the DOPC-SLB irradiated at $d = 12$ mm, 16 mm, and 20 mm, respectively, at the fixed irradiation time of 420 s. At this irradiation time, the bilayer structure was destructed in case the irradiation distance was $d = 8$ mm (Fig. 3c). Figure 5a shows the AFM topography of the DOPC after the irradiation at $d = 12$ mm. DOPC-SLB existed and nanopores of 15.6 ± 2.3 nm in diameter were observed. The area fraction of the pores was 1.92%. We also confirmed the existence of the DOPC-SLB by epi-FM (data not shown). At $d = 16$ mm, pores 46.0 ± 6.5 nm were observed (Fig. 5b), and the area fraction was 1.76%. At $d = 20$ mm, we did not found a pore in the SLB (Fig. 5c), and recognized a few protrusions. The results in Fig. 5 and Fig. 3c revealed that the rate of pore formation was attenuated depending on d , while the diameter of nanopores did not simply attenuate with d .

Discussion

Recently we reported that DBD plasma irradiation generated pores in the DOPC-SLB, and attributed the active species responsible for the nanopore formation to transient ROS species, rather than equilibrium molecules such as H_2O_2 and NO_3^- [45]. The results in the present study (Figs. 2 - 5) certify oxygen radicals were crucial for the morphological change and pore formation in SLB, because the oxygen radical source in this study provides ground-state atomic oxygen ($\text{O}(^3\text{P}_j)$), singlet oxygen molecule ($\text{O}_2(^1\Delta_g)$) and trace amount of ozone (O_3) isolating the effects of UV light and electric fields [21,22,25–27]. Excited argon atoms are also generated, but their oxidation activity is negligible [21,22]. Therefore the morphological changes observed in this study are because of the oxidation of lipid. The $\text{O}(^3\text{P}_j)$ from the gas phase generates hydroxyl radical ($\cdot\text{OH}$) and H_2O_2 as follows [27]:

$\text{O}_2(^1\Delta_g)$ turns to triplet oxygen molecule when it meets H_2O . Therefore the active oxygen species generated from $\text{O}(^3\text{P}_j)$ are mainly $\cdot\text{OH}$ and H_2O_2 , and $\text{O}_2(^1\Delta_g)$ provided dissolved O_2 in the buffer solution. A part of $\text{O}(^3\text{P}_j)$ turns to O_3 in gas phase through the three-body reaction with O_2 depending on the distance from the source nozzle. The density of O_3 might be comparable to that of $\text{O}(^3\text{P}_j)$, but is still below the detection limit in excessive amount of Ar as shown in our previous study [26]. It is possible that this trace amount of O_3 also partly contributed to the generation of $\cdot\text{OH}$, because it is well known that O_3 forms $\cdot\text{OH}$ in aqueous solution [58,59]. The dose amounts to the buffer solution in this study (400 μL) at the conditions in Figs. 2-5 were calculated on the basis of the previous study [27] and summarized in Table 1 and Table 2 accompanied with the average diameter and area fraction of the nanopores and the density of the protrusion observed in the AFM images in Figs. 2, 4 and 5. When the irradiation time was varied keeping d constant (Table 1), the dose amount of $\text{O}(^3\text{P}_j)$ and $\text{O}_2(^1\Delta_g)$ increases with time linearly at the same ratio between $\text{O}(^3\text{P}_j)$ and $\text{O}_2(^1\Delta_g)$. On the other hand, the ratio of $\text{O}(^3\text{P}_j)$ to $\text{O}_2(^1\Delta_g)$ decreases with d (Table 2).

The oxidation of lipids by ROSs under physiological condition proceeds at $\text{C}=\text{C}$ bonds of acyl chains

through two steps [2,3]: peroxidation producing hydroperoxidized lipid, and truncation resulting in a truncated-chain lipid which is terminated with aldehyde (or carboxyl) groups and fragmented fatty aldehyde (Fig. 6). Hydroxyl radical ($\cdot\text{OH}$) gives rise to primary stage of lipid hydroperoxidation generating a lipid radical, and the lipid radical triggers the peroxidation of the acyl chains of lipids which is a chain reaction consuming oxygen molecule [2,3]:

The truncation of the peroxidized acyl chain proceeds the intramolecular radical transfer of peroxy lipid radical instead of the intermolecular transfer (eq. (5)), or a Hock-type cleavage reaction of hydroperoxy lipid [2]. Therefore the lipid oxidation in this study is initiated by $\cdot\text{OH}$ generated from $\text{O}({}^3\text{P}_j)$ (eq. (1)) following eq. (3). Various kinds of radical and ionic species are possibly generated from $\text{O}({}^3\text{P}_j)$ in aqueous phase, but we focus on $\cdot\text{OH}$ because it is crucial for the oxidation of lipids at acyl chains [2,3]. The advantage of artificial lipid membrane system in this study is that the reactant is a pure lipid and well-characterized oxygen species were provided, thus we can reasonably assume that reaction proceeds ideally compared to other biological systems such as cells in culturing media.

We suppose that $\cdot\text{OH}$ is transported to SLB through the buffer solution by a flow in the solution induced by gas flow from the radical source, rather than by simple thermal diffusion. Previous studies showed that ROSs including $\cdot\text{OH}$ with significant amount for oxidative reaction circulate in an aqueous solution under the existence of gas flow [60–62], in spite of the short life time of $\cdot\text{OH}$, approximately 200 μs in an aqueous phase [27]. We emphasize that $\cdot\text{OH}$ works as the initiator of the chain reaction (eq. 3), and stoichiometric amount of $\cdot\text{OH}$ to lipid molecules is not necessary. We estimate the amount of lipid molecules desorbed from SLB on the basis of typical occupying area of one DOPC molecule in a bilayer, 0.64 nm [56]. Area fraction of 2% in a bimolecular membrane on a substrate ($8 \times 8 \text{ mm}^2$) corresponded to 4×10^{12} molecules = 6.6×10^{-12} mol. It may be an overestimation as the amount of oxidized lipid, because it is possible that some non-oxidized lipids are incorporated in desorbed micelles. Singlet oxygen $\text{O}_2({}^1\Delta_g)$ does not cause the oxidation of lipid directly in the buffer solution, but assists the lipid oxidation by supplying oxygen molecule to the lipid bilayer through the buffer solution for the chain reaction (eqs. (4) and (5)), which leads to the truncation of acyl chains. Inclusion of hydroperoxidized lipid, the product of the first step of the oxidation process in Fig. 6, into a lipid bilayer reduces the fluidity of membrane [41,63–65]. On the other hand truncated-chain lipids increase the fluidity of membrane [64–66]. Previously we reported the reduction of fluidity of DOPC-SLB by the DBD-based plasma irradiation before the generation pores using fluorescence recovery after photobleaching (FRAP) method [46]. Thus the pore formation in DOPC-SLB (Figs. 2, 4 and 5) is caused by the truncation of lipid, the second step of the lipid oxidation process (Fig. 6), rather than the hydroperoxidized lipid. Our previous studies also showed that H_2O_2 , which was also produced in this study by the recombination of $\cdot\text{OH}$ (eq. (2)), causes neither the pore formation or reduction of fluidity in DOPC-SLB [45,46].

The results in Fig. 4 and Table 1 show the time course of the pore formation, at the constant $\text{O}({}^3\text{P}_j)$ to $\text{O}_2({}^1\Delta_g)$ ratio, thus we reasonably assume the constant $\cdot\text{OH}$ to O_2 ratio in the buffer solution. Prior to the

nanopore formation, protrusions appeared as shown in Fig. 4a (180 s). We attribute the protrusion to the fragmented fatty aldehyde accumulated in the hydrophobic core of the lipid bilayer. Truncated-chain lipid, the other product of the fragmentation, bends its aldehyde-terminated shorter acyl chain toward the aqueous phase [64,65], and behaves like a detergent with a larger hydrophilic head group. As explained from the critical packing parameter [67], the truncated-chain lipids which have packing parameter less than 1 because of its relatively large hydrophilic head compared with hydrophobic tail, form micelle in aqueous phase [65]. Therefore the truncated-chain lipids desorb from the lipid bilayer after their concentration increases by the further irradiation of $\cdot\text{OH}$ and O_2 , leaving pores in the bilayer (Figs. 4b (240 s) and 4c (300 s)). Recent study by molecular dynamic simulation indicates that formation and desorption of "hydrophobic droplet" from a massively oxidized DOPC bilayer consisting of truncated-chain lipid and fragmented fatty aldehyde [68]. A lipid bilayer membrane is a flexible material, and the volume of the protrusions, corresponding to an area fraction of a few percent, is reasonably supplied by stretching the surrounding membrane slightly. Formation of defects or cracks exposing the hydrophobic region to an aqueous phase is energetically more unfavorable than stretching the bilayer membrane retaining the bilayer structure. On the other hand, the truncated-chain lipid stabilizes the edge of the pores forming a hemimicelle. It is reasonable that the pore edge gave clear contrast in the phase-shift images in Fig. 2c and Fig. 4b if the density of truncated-chain lipid is higher at the pore edge than the surrounding SLB still rich in non-oxidized lipid. Further incorporation of the truncated-chain lipid and the fatty aldehyde into SLB accelerates desorption of micelles and finally destructs the bilayer structure (Fig. 3c (420 s)). Previous study indicated that the threshold concentration of the deconstruction of bilayer structure is above 10% [69].

The results in Fig. 5 and Table 2 shows the dependence of nanopore formation on the irradiation distance d indicating the effect of the dose ratio between $\cdot\text{OH}$ and O_2 . The dose amount of $\cdot\text{OH}$, which is generated from $\text{O}(^3\text{P}_j)$ (eq. (1)) decreases with d while that of O_2 , resulting from $\text{O}_2(^1\Delta_g)$ (eq. (2)), increases. It means that the frequency of trigger reaction (eq. (3)) decreases with d , while O_2 for chain reaction (eqs. (4) and (5)) are provided sufficiently. As shown in Fig. 5, the number density of nanopore decreased with d . The smaller number of nanopores grew larger in Fig. 5b ($d = 16$ mm) than those in Fig. 5a ($d = 12$ mm). Figure 5c, and also Fig. 2a, shows that $\cdot\text{OH}$ is requisite for the formation of nanopore, and providing O_2 only is not sufficient.

We summarize the mechanism of the pore formation caused by the plasma-generated neutral oxygen species in Fig. 7:

- (1) $\text{O}(^3\text{P}_j)$ and $\text{O}_2(^1\Delta_g)$ from gas phase turns to $\cdot\text{OH}$ and O_2 , respectively, in the buffer solution.
- (2) $\cdot\text{OH}$ and O_2 oxidize lipid on its unsaturated bond: $\cdot\text{OH}$ initiates the reaction by producing a lipid radical, and O_2 propagates the generation of lipid hydroperoxide followed by fragmentation to truncated-chain lipid and fatty aldehyde (Fig. 6).
- (3) Fatty aldehyde is accumulated in the hydrophobic core of the lipid bilayer forming a protrusion. Positive curvature of the protrusion is assisted by the truncated-chain lipid.
- (4) After the concentration of the truncated-chain lipid in a lipid bilayer increases, truncated-chain lipids form a micelle and desorb to the aqueous phase accompanying with fatty aldehyde.

(5) A part of the truncated-chain lipid remaining in the lipid bilayer stabilizes the edge of a pore in the lipid bilayer forming hemi-micelle.

We propose these processes on the basis of the previous experimental and theoretical studies as discussed above. It is reasonable that the diameter of pore or area fraction did not show simple linear dependence on the dose of $O(^3P_j)$, which provides $\cdot OH$, because stoichiometric $\cdot OH$ is not necessary for the oxidation of lipid as we discussed above.

The significant difference of the plasma-induced reaction from reactions induced by physiological ROSs is that the rapid increase in the density of ROS. The radical source in this study effectively provides ROSs on the order of 10^{19} cm^{-3} is supplied in a few minutes by the radical source in this study [27]. Rapid and local accumulation of the products of lipid oxidation, such as protrusions of fatty aldehyde (Fig. 4a) and truncated-chain lipid at the nanopore edge (Fig. 2c and Fig. 4b), are necessary for the pore formation, because lipid molecules diffuse laterally in a bilayer. We note that pores in a lipid bilayer is energetically unfavorable even though the pore edge is capped with a hemimicelle, and the nanopores stably existed because we used the SLB system [34]. The life time of nanoscale pores is short in free-standing membranes like liposomes or in living cells, but anyway a channel through a lipid bilayer opens. H_2O_2 is transported into cells by some kinds of aquaporins, water channel proteins [70], and induces apoptosis or hinders the growth of living cells [71–73]. Plasma-generated H_2O_2 (eq. (2)) does not affect the structure and fluidity of a lipid bilayer membrane [45,46], but the plasma-generated nanopores open an additional pathway for the transportation of H_2O_2 . It is also known that oxidized lipids act as mediators stimulating various physiological cascades [2,3]. The morphological change of DOPC-SLB by the plasma-induced lipid oxidation in this study provides information for the fundamental understanding of the effects of atmospheric pressure plasma to cell membranes.

Conclusion

Neutral oxygen species ($O(^3P_j)$ and $O_2(^1\Delta_g)$), which were generated by atmospheric pressure plasma, caused the formation of the protrusion and nanopores in the supported lipid bilayer of phosphatidylcholine in a buffer solution. AFM topographies revealed that the protrusions appeared at low dose amount of $O(^3P_j)$ and the nanopores appeared at high dose. We attributed the morphological change to the accumulation of the products of lipid oxidation, truncated-chain lipid and fatty aldehyde: the truncated-chain lipid forms a micelle including fatty aldehyde, desorbs to aqueous phase leaving a nanopore, and also stabilizes the pore edge by capping with a hemimicelle.

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Table 1. Dependence on irradiation time ($d = 8$ mm): Summary of the irradiation time, dose amount and the results obtained from the AFM topographies Fig. 2b and Fig. 4.

Irradiation time (s)		180	240	300	420
Dose amount (10^{18} cm^{-3})*	$\text{O}(^3\text{P}_j)$	17.0	22.7	28.3	39.7
	$\text{O}_2(^1\Delta_g)$	25.5	33.9	42.4	59.4
Diameter of pore (nm)		16.6 ± 3.7	18.7 ± 5.5	20.8 ± 3.9	SLB was deconstructed
Area fraction of pore (%)		0.93	1.93	1.39	
Density of protrusion (μm^{-2})		22.3	Not found	1.3	

* Calculated on the basis of ref. [27].

Table 2. Dependence on irradiation distance (irradiation time was 420 s): Summary of the irradiation distance, dose amount and the results obtained from the AFM topographies in Fig. 5.

Irradiation distance (mm)		12	16	24
Dose amount (10^{18} cm^{-3})*	$\text{O}(^3\text{P}_j)$	12.5	3.96	1.25
	$\text{O}_2(^1\Delta_g)$	63.8	68.4	73.5
Diameter of pore (nm)		15.6 ± 2.3	46.0 ± 6.5	Not found
Area fraction of pore (%)		1.92	1.76	
Density of protrusion (μm^{-2})		Not found	Not found	0.6

* Calculated on the basis of ref. [27].

Figure Captions

Fig. 1. Schematic of the experimental setup: neutral oxygen radicals were irradiated to a sample cell containing a buffer solution and DOPC-SLB on a SiO₂/Si substrate at the irradiation distance (d).

Fig. 2. AFM images ($1.0 \times 1.0 \mu\text{m}^2$) of DOPC-SLB without and with the irradiation of neutral oxygen radicals: (a) AFM topography. O₂ + Ar gas was dosed without plasma generation; (b) AFM topography. plasma-generated neutral oxygen species were irradiated of DOPC-SLB through the buffer solution at $d = 8$ mm for 300 s. Cross section profiles at the white lines are also shown; (c) Phase-shift image simultaneously obtained with (b). The inset is the magnified image of the region indicated by the dotted square ($180 \times 180 \text{ nm}^2$).

Fig. 3. Fluorescence microscope images of DOPC-SLB: (a) before the irradiation of neutral oxygen species; (b) after irradiation at $d = 8$ mm for 300 s; (c) after irradiation at $d = 8$ mm for 420 s.

Fig. 4. AFM topographies of DOPC-SLB irradiated of the neutral oxygen species for various irradiation time at a fixed irradiation distance $d = 8$ mm: (a) 180 s. Cross section profile at the white line is also shown; (b) 240 s. The lower image is the simultaneously obtained phase-shift image at the region indicated by the dotted square ($150 \times 150 \text{ nm}^2$); (c) 300 s.

Fig. 5. AFM topographies of DOPC-SLB irradiated by the neutral oxygen radicals at various irradiation distance d for 420 s: (a) $d = 12$ mm; (b) $d = 16$ mm; (c) $d = 20$ mm.

Fig. 6. Two-step oxidation process of phosphatidylcholine molecule at its unsaturated acyl chains. First step: hydroperoxidation at double bond. The head group and the other acyl chain are omitted for the sake of simplicity. Second step: truncation of acyl chains resulting in formation of fragmented fatty aldehyde.

Fig. 7. Scheme of the morphological change of a lipid bilayer membrane caused by the irradiation of plasma-generated active neutral oxygen species.

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Figure 1

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