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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.

1. 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], cellar 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 gates controlling the transportation of materials and information. Everything needs to pass through a cell membrane when it enters into a cell and causes 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

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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 a 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 μm 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, $O(^3P_j)$ and $O_2(^1\Delta_g)$ of quantitatively evaluated amounts to SLB existing in a buffer solution [21,25–27]. After the irradiation of $O(^3P_j)$ and $O_2(^1\Delta_g)$, 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.

2. Experimental

2.1. Irradiation of neutral oxygen radicals to supported lipid bilayer

Fig. 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

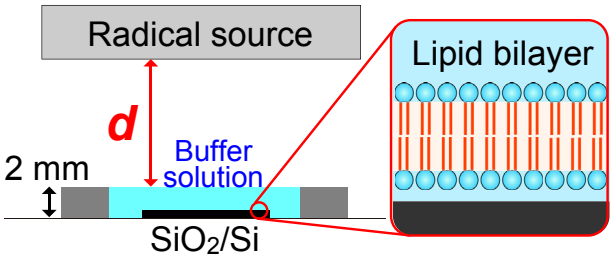

Fig. 1. Schematic of the experimental setup: neutral oxygen radicals were irradiated to a sample cell containing a buffer solution and DOPC-SLB on a SiO_2/Si substrate at the irradiation distance (d).

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 × 16 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 $O_2/(Ar + O_2)$ 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 AFM in the same room, and started the observation.

2.2. 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 phosphocholine 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 SiO_2/Si substrates by the vesicle fusion method [34]. A thermally oxidized SiO_2/Si substrate (8 × 8 mm^2, 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].

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