

# Mechanism of lipid peroxidation of liposomes by cold atmospheric pressure plasma jet irradiation

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Liposome lipid peroxidation induced by cold atmospheric pressure plasma jet (CAPPJ) irradiation was investigated. The formation of thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation final products, as a function of irradiation was observed. Lipid radicals, peroxidation reaction intermediates generated by CAPPJ irradiation, were confirmed by increased NBD-pen fluorescence intensity. Additionally, lipid peroxidation products, liposomal phosphatidylcholine (PC) isomers, were analyzed by LC-MS/MS. Products specific to singlet oxygen ( $^1O_2$ ) oxidation, 16:0/10-hydroperoxy-8E,12Z-octadecanoic acid (10-8E,12Z-HpODE) PC and 16:0/12-9E,13E-HpODE PC, were not detected, but radical oxidation specific products 16:0/13-9E,11E-HpODE PC and 16:0/9-10E,12E-HpODE PC were. This suggests that during CAPPJ irradiation, radicals, rather than  $^1O_2$ , are the primary reactive species of lipid peroxidation. This is also supported by the  $\beta$ -carotene quenching of  $^1O_2$  not suppressing TBARS and lipid radical generation. Also, neither TBARS formation nor lipid radical generation were suppressed by SOD, indicating that the superoxide radical ( $O_2^{\cdot-}$ ) is not responsible for the lipid peroxidation reaction. As the CAPPJ irradiation of water produces large quantities of hydroxyl radical ( $\cdot OH$ ) and  $\cdot OH$  scavengers decreased the amount of TBARS produced by CAPPJ irradiation, it is highly plausible that  $\cdot OH$  is the primary species involved in CAPPJ-induced liposome lipid peroxidation.

**Key Words:** cold atmospheric pressure plasma jet, liposome lipid peroxidation, TBARS, NBD-pen, LC-MS/MS

Plasma is a state of matter consisting of excited gas molecules, positively and negatively charged ions, free electrons, free radicals, and molecular fragments, emitting UV and visible light.<sup>(1,2)</sup> Plasma, with distinct characteristics setting it apart from ordinary neutral gases, is considered a fourth state of matter, in addition to solids, fluids, and gases. Plasma technology now enables plasma generation at near atmospheric pressure and room temperature, in a process referred to as cold atmospheric pressure plasma (CAP).

In addition to various physical and industrial applications, CAP is a promising tool for biomedical applications as it generates reactive species in a controlled manner. Plasma use in medicine has developed over the last decade, with applications including cancer cell apoptosis induction, wound healing, and skin disease damage regeneration.<sup>(3-5)</sup> Although the mechanisms behind plasma medicine's mode of action remain unclear, the most widely accepted theory assumes the action of oxidative stress, caused by CAP generated reactive oxygen species (ROS).

Lipids are essential cell membrane components maintaining cellular structure and influencing cell function. However, lipids

are vulnerable to ROS, with lipid oxidation implicated in various pathological conditions. Several human diseases are thought to involve lipid peroxidation, including atherosclerosis,<sup>(6)</sup> cancer,<sup>(7,8)</sup> diabetes,<sup>(9)</sup> and even neurodegenerative diseases<sup>(10)</sup> such as Alzheimer's<sup>(11)</sup> and Parkinson's disease.<sup>(12)</sup> Lipid peroxidation is a process in which free radical species, such as oxyl radicals, peroxy radicals, and hydroxyl radicals, remove electrons from lipids to produce reactive intermediates that undergo further reactions. Products of lipid peroxidation chain reactions display high biological activity,<sup>(13)</sup> destroying DNA, proteins, and enzyme activity, as well as activating cell death pathways.<sup>(14)</sup>

CAP induces ROS formation, generating species including hydroxyl radicals ( $\cdot OH$ ), superoxide anion radicals ( $O_2^{\cdot-}$ ), and reactive nitrogen species (RNS), such as nitric oxide ( $\cdot NO$ ) and peroxynitrite ( $ONOO^{\cdot}$ ).<sup>(15-18)</sup> Previously, we reported the generation of ROS such as  $\cdot OH$ ,  $\cdot H$ ,  $H_2O_2$ , and  $^1O_2$  in water by cold atmospheric pressure plasma jet (CAPPJ) irradiation.<sup>(19,20)</sup> These reactive species can cause lipid peroxidation. In fact, it was reported that when *E. coli* was irradiated with CAP, lipids were peroxidized and  $^1O_2$  and  $H_2O_2$  are released into the culture medium and cells.<sup>(21)</sup> However, the identity of the primary responsible species for lipid peroxidation remains unclear.

As lipid peroxidation is likely a fundamental component of plasma medicine treatments, we attempted identification of the major active species responsible for CAPPJ irradiation induced liposome lipid peroxidation. During experimental evaluation of these effects, the inherent antioxidant systems of cells and cell membranes may greatly complicate experimental results. Therefore, phosphatidylcholine (PC) liposomes were used. This simple system contains only lipids, avoids potential issues related to cell antioxidant systems, better mimics the cell membrane aspects, and simplifies the interpretation of direct CAP reaction results.

## Materials and Methods

**Chemicals.** Egg yolk PC was obtained from Nippon Oil & Fats Co. Ltd. 2,2,6-trimethyl-4-(4-nitrobenzo [1,2,5] oxadiazol-7-ylamino)-6-pentylpiperidine-1-oxyl (NBD-Pen) was purchased from COSMO Bio. Co. Ltd. (Tokyo, Japan). Xanthine oxidase was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), and superoxide dismutase (SOD) from bovine erythrocyte was obtained from Sigma-Aldrich Co. (St. Louis, MO). Other reagents were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). All reagents were of the highest grade available and used without further purification.

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**Preparation of liposomes.** The liposome (large unilamellar vesicles) samples were prepared as follows. Briefly, into a round bottom flask was added 3.8 ml of chloroform containing egg yolk PC (10 mg/ml) with the solvent subsequently removed by rotary evaporation. The residue was then dispersed in diethyl ether and KCl-Tris-HCl buffer (10 mM, pH 7.4) was added. The solution was sonicated, and the diethyl ether partially removed by rotary evaporation. The residue was then mixed using a vortex mixer, and diethyl ether further removed using a rotary evaporator producing the liposomes. To prepare sample suspensions, the resultant liposome suspension was diluted 50 times with KCl-Tris-HCl buffer.

**CAPPJ irradiation of liposomes.** CAPPJ was generated using a plasma head (TPN-20; NU global, Nagoya, Japan) and an electric regulating unit (PN-110TPG; NU global) with helium as the carrier gas. The helium gas flow rate (3 L/min) was regulated by a mass flow controller (CUBE GM2; Fcon Co., Ltd., Kochi, Japan). The 1-ml sample suspension was placed in a glass container with 2-cm inner diameter and 2-cm height and irradiated with CAPPJ at a distance of 1.5 cm between the sample surface and plasma jet tip.

**Measurement of 2-thiobarbituric acid-reactive substances (TBARS).** The quantity of TBARS was measured to evaluate lipid peroxidation. Trichloroacetic acid (15%), thiobarbituric acid (TBA, 0.375%), and butylated hydroxytoluene (0.04%) were dissolved in hydrochloric acid aqueous solution (0.25 mol/L). The CAPPJ-irradiated liposome sample was added to the above mixed solution, heated for 15 min at 95°C, and then cooled to room temperature. TBARS were measured at 532 nm using a UV-VIS spectrophotometer (UV-mini 1240; SHIMADZU, Kyoto, Japan).

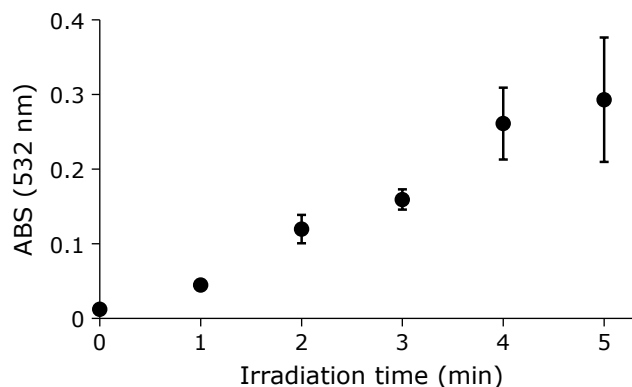
**Measurement of lipid radicals.** Lipid radicals generated by CAPPJ irradiation were detected using the NBD-pen fluorescent probe. NBD-pen is a fluorescence probe reported to primarily react with lipid radicals and minimally with ROS.<sup>(22)</sup> To 12.5 µl of the 2 mM NBD-pen solution was added 1 ml of the liposome sample suspension and, after mixing, was irradiated with CAPPJ. Then, 1.5 ml of KCl-Tris-HCl buffer was added, and the fluorescence intensity (ex 470 nm, em 530 nm) measured using a spectrofluorophotometer (RF-5300PC; SHIMADZU).

**Analysis of lipid peroxidation isomers by LC-MS/MS method.** To identify the major active species responsible for lipid peroxidation, PC hydroperoxide isomers were analyzed according to a previous report with minor modifications. Total lipids were extracted from liposomes using the Folch method, and the PC fraction purified via solid-phase extraction.<sup>(23)</sup> The obtained PC fractions were then analyzed using a 4000QTRAP system equipped with an Exion LC (SCIEX, Tokyo, Japan). PC hydroperoxide isomers were separated using a COSMOSIL column 5C18-MS-II (5 µm, 2.0 × 150 mm; Nacalai Tesque, Kyoto, Japan) with a binary gradient composed of solvent A (methanol) and solvent B (water). The gradient profile was as follows: 0 to 20 min, 90 to 100% B linear; 20 to 30 min, 100% B; 30 to 30.1 min, 100 to 90% B linear. A flow rate of 0.2 ml/min was used, and a column temperature of 40°C. Eluted column fractions were then mixed with 2 mM sodium acetate methanol solution (0.01 ml/min) to form the sodium adduct. PC hydroperoxide isomers were detected using multiple reaction monitoring.<sup>(24)</sup>

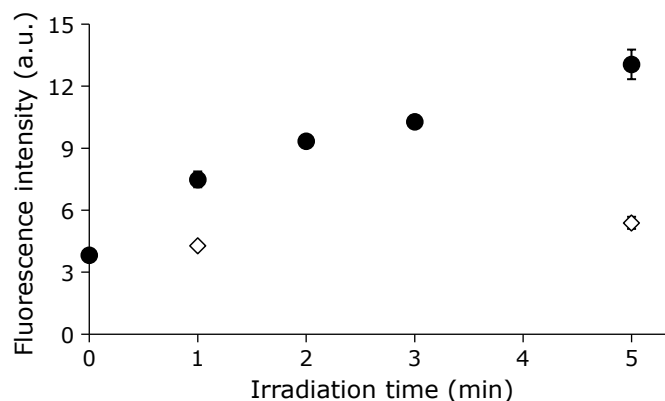
**Statistical analysis.** The statistical analysis of mean values was carried out using Welch's *t* test. Differences with a *p* value <0.05 was considered statistically significant.

## Results

**Formation of TBARS by CAPPJ irradiation of liposomes.** Liposomal suspensions were subjected to CAPPJ irradiation, and the products of lipid peroxidation measured using the TBA method. Lipid peroxidation products react with TBA forming TBARS, indicated by an absorption peak around 532 nm. With



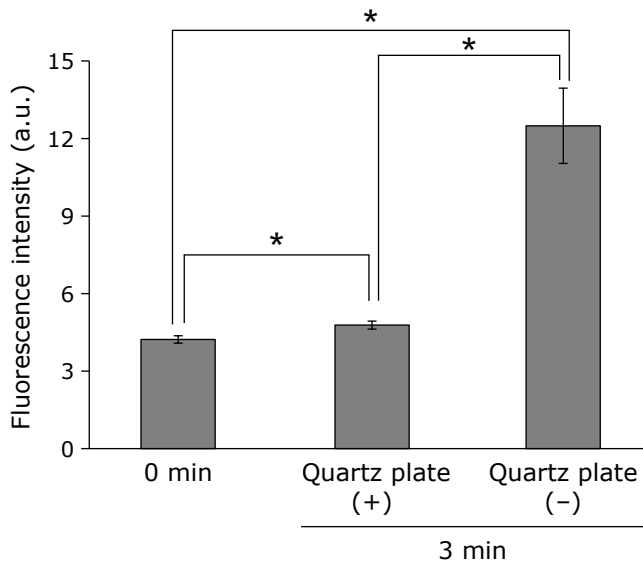
**Fig. 1.** Time-dependent TBARS formation by CAPPJ irradiation of liposome suspension. The liposome suspension (1 ml) was CAPPJ irradiated at a distance of 1.5 cm. The helium gas flow rate was 3 L/min. After irradiation, TBARS formation was evaluated by measuring the absorbance at 532 nm. Error bar shows SD (*n* = 3).



**Fig. 2.** Lipid radical production by CAPPJ irradiation of liposome suspension. The liposome suspension (1 ml) containing NBD-pen, a fluorescent probe, was CAPPJ irradiated at a distance of 1.5 cm. The helium gas flow rate was 3 L/min. Sample fluorescence intensity was measured (ex 470 nm, em 530 nm) immediately after irradiation. Closed circles show the CAPPJ irradiated sample fluorescence intensity. Open diamonds show the fluorescence intensity without CAPPJ irradiation (Only helium gas used). Error bar shows SD (*n* = 3).

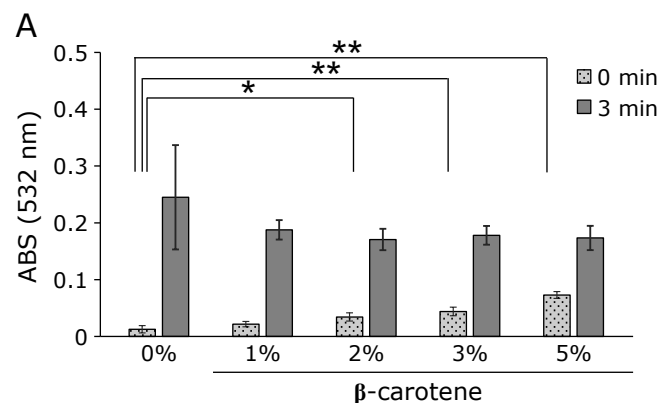
increased CAPPJ irradiation time, there is a concurrent increase in absorbance at 532 nm of the irradiated liposome reaction mixture, indicating an increase in TBARS concentration (Fig. 1).

**Formation of lipid radicals by CAPPJ irradiation of liposomes.** When liposomes premixed with NBD-pen were irradiated with CAPPJ, fluorescence induced by the reaction of NBD-pen with lipid radicals was detected. A time-dependent increase in fluorescence intensity was observed during CAPPJ irradiation (Fig. 2). No fluorescence intensity increase was observed when an NBD-pen solution without liposomes was irradiated (data not shown). These findings confirm the formation of lipid radicals during CAPPJ irradiation of the liposomal suspension. When only helium gas was blown onto the sample, no fluorescence intensity increase was observed (Fig. 2). Furthermore, no fluorescence intensity increase was observed when plasma gas flow was blocked by covering the sample container with a thin transparent quartz plate (Fig. 3). This confirmed that the observed reaction is not due to the light emitted by CAP, but is derived from the active species in the CAP gas.



**Fig. 3.** Requirement for the CAP gas flow to hit the sample for CAPPJ induced lipid radical production. The liposome suspension (1 ml) containing NBD-pen fluorescent probe was CAPPJ irradiated at a distance of 1.5 cm for 3 min. The sample was irradiated with a thin quartz plate placed on top of the glass container, allowing only UV and visible light to reach the sample while blocking gas flow [quartz plate (+)]. For comparison, fluorescence intensity without the thin quartz plate was measured [quartz plate (-)]. The helium gas flow rate was 3 L/min. Fluorescence was measured immediately after irradiation. Error bar shows SD ( $n = 3$ ). \* represents  $p < 0.05$ .

**Effects of  $\beta$ -carotene ( $\beta$ -car) on the formation of TBARS and lipid radicals by CAPPJ irradiation of liposomes.** Next, the ROS species involved in the CAPPJ irradiation induced liposome lipid peroxidation was investigated. First, the singlet oxygen ( $^1O_2$ ) scavenger  $\beta$ -car was incorporated in the liposomal membranes. Liposomes containing  $\beta$ -car were irradiated with CAPPJ with TBARS formation measured at 532 nm. As shown in Fig. 4A, the increase in TBARS by CAPPJ irradiation was slightly diminished with increasing  $\beta$ -car concentration, but complete inhibition was not observed, even for the 5%  $\beta$ -car sample. The absorbance at 532 nm at 0 min (no CAPPJ treatment) was slightly elevated with increasing  $\beta$ -car amounts, presumably due

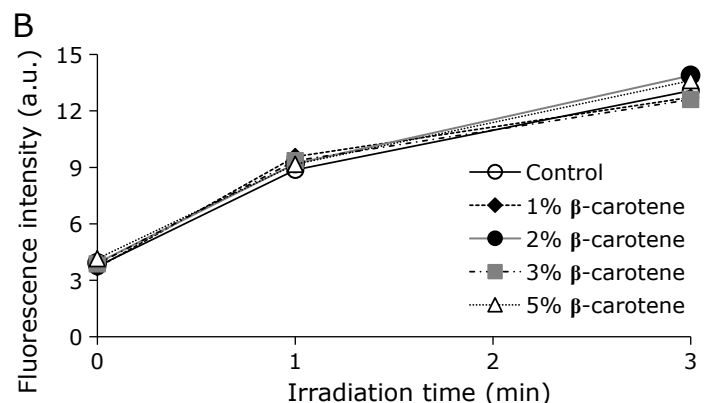


to  $\beta$ -car absorbance. Upon CAPPJ irradiation of liposomes containing 1–5%  $\beta$ -Car for 1 min to 3 min, no lipid radical formation suppression was observed (Fig. 4B).

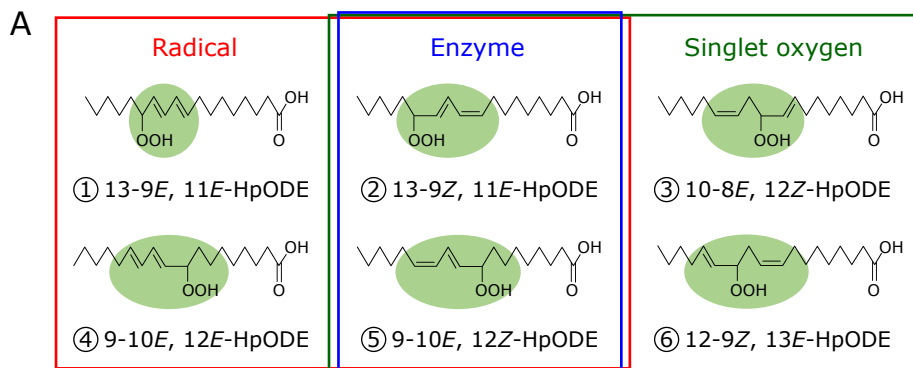
**Isomers of lipid peroxides produced by CAPPJ irradiation.** Lipid peroxidation results in the formation of different isomeric structures, which are produced by the lipid peroxidation mechanism. It is reported that 13-hydroperoxy-9E,11E-octadecadienoic acid (13-9E,11E-HpODE, ① in Fig. 5A), 13-9Z,11E-HpODE (② in Fig. 5A), 9-10E,12E-HpODE (④ in Fig. 5A) and 9-10E,12Z-HpODE (⑤ in Fig. 5A) are formed during the radical-induced lipid peroxidation of linoleic acid.<sup>(25,26)</sup> Upon  $^1O_2$ -induced peroxidation, 13-9Z,11E-HpODE (②), 9-10E,12Z-HpODE (⑤), 10-8E,12Z-HpODE (③ in Fig. 5A), and 12-9Z,13E-HpODE (⑥ in Fig. 5A) are formed.<sup>(25,27)</sup> Therefore, 16:0/13-9E,11E-HpODE PC (①) and 16:0/9-10E,12E-HpODE PC (④) are specifically products of radical-induced PC peroxidation, while 16:0/10-8E,12Z-HpODE PC (③) and 16:0/12-9Z,13E-HpODE PC (⑥) are products of  $^1O_2$ -induced PC peroxidation. As shown in Fig. 5B, CAPPJ irradiation of liposomes for 1 min formed large quantities of ① + ② (intensity  $1.1 \times 10^5$ ) at retention time of 9.73 min. Small peaks of ④ and ⑤ were also detected. A similar pattern but larger chromatogram intensity was obtained by evaluation of the liposomes subjected to CAPPJ irradiation for 2 min. Products of  $^1O_2$ -induced peroxidation, ③ and ⑥, could not be detected even after irradiation for 2 min. This shows clearly that the major CAPPJ irradiation induced peroxidation pathway is radical dependent.

**Effects of SOD on liposome peroxidation by CAPPJ irradiation.** The above results show that  $^1O_2$  is unlikely to contribute to liposome lipid peroxidation, indicating that radical ROSs are involved in CAPPJ irradiation induced lipid peroxidation. As several radical species, such as  $O_2^{\cdot-}$  and  $\cdot OH$ , are produced by CAPPJ irradiation of water, the involvement of these ROS in lipid peroxidation were investigated. To examine the possibility of lipid peroxidation by  $O_2^{\cdot-}$  produced by CAPPJ irradiation, TBARS formation and lipid radical formation in the presence of SOD was measured. As shown in Fig. 6, CAPPJ irradiation resulted in an absorbance increase, indicating TBARS formation and the continued generation of lipid radicals, despite the addition of SOD.

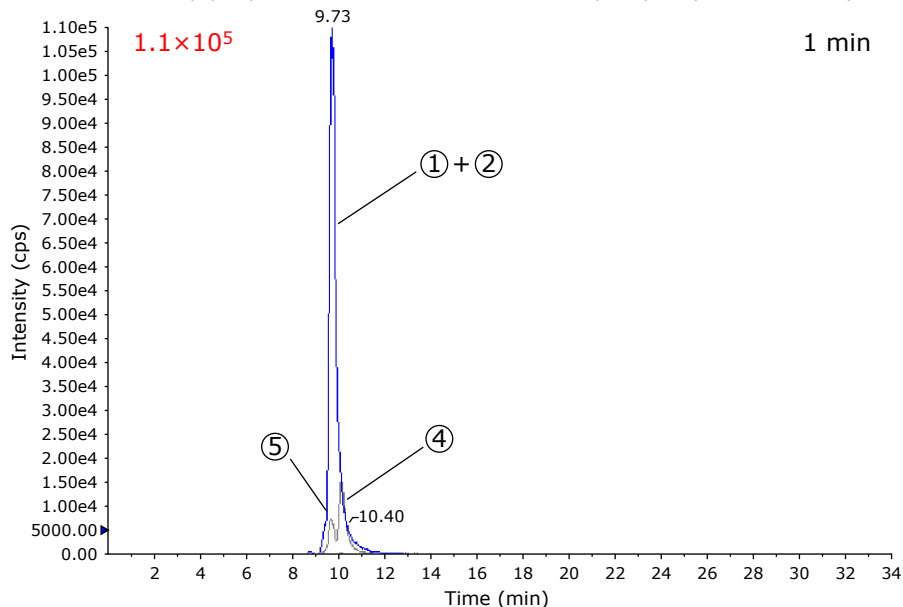
**Effects of chemically generated  $O_2^{\cdot-}$  on liposome peroxidation.** To confirm that  $O_2^{\cdot-}$  does not contribute to liposome lipid peroxidation, the hypoxanthine-xanthine oxidase (Hyp-XOD) system to generate  $O_2^{\cdot-}$  was used. Upon combining the liposome suspension and Hyp-XOD reaction solution, no increase in absorption indicative of an increase in TBARS was observed



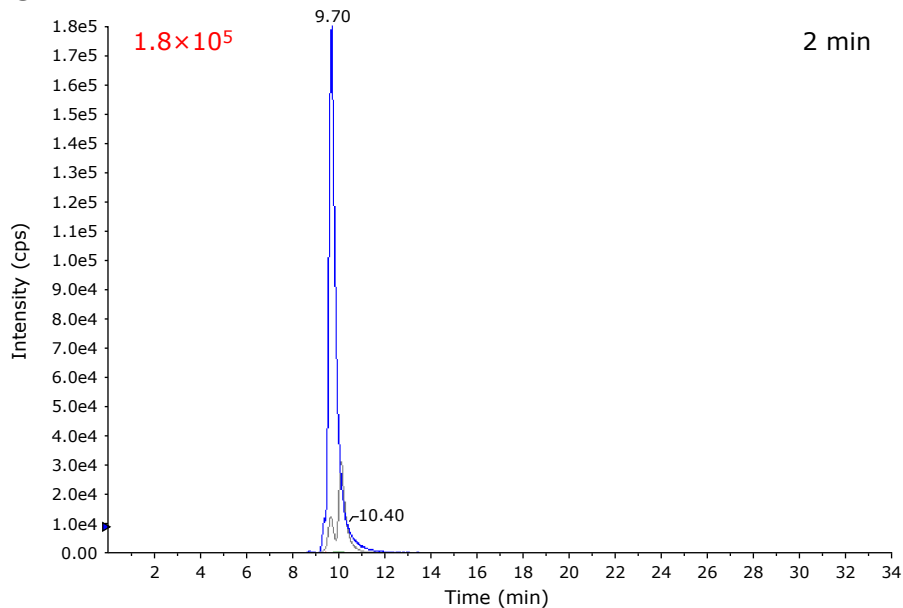
**Fig. 4.** Effect of  $\beta$ -car on lipid peroxidation by CAPPJ irradiation. (A) Liposomes containing various concentrations of  $\beta$ -car were CAPPJ irradiated for 3 min and TBARS formation measured by the absorbance at 532 nm. Error bar shows SD ( $n = 3$ ). (B) Liposomes containing various concentrations of  $\beta$ -car were mixed with the NBD-pen fluorescent probe and samples irradiated with CAPPJ for 1 or 3 min. Fluorescence intensity was measured immediately after irradiation. Error bar shows SD ( $n = 3$ ). \* represents  $p < 0.05$  and \*\* represents  $p < 0.01$ .



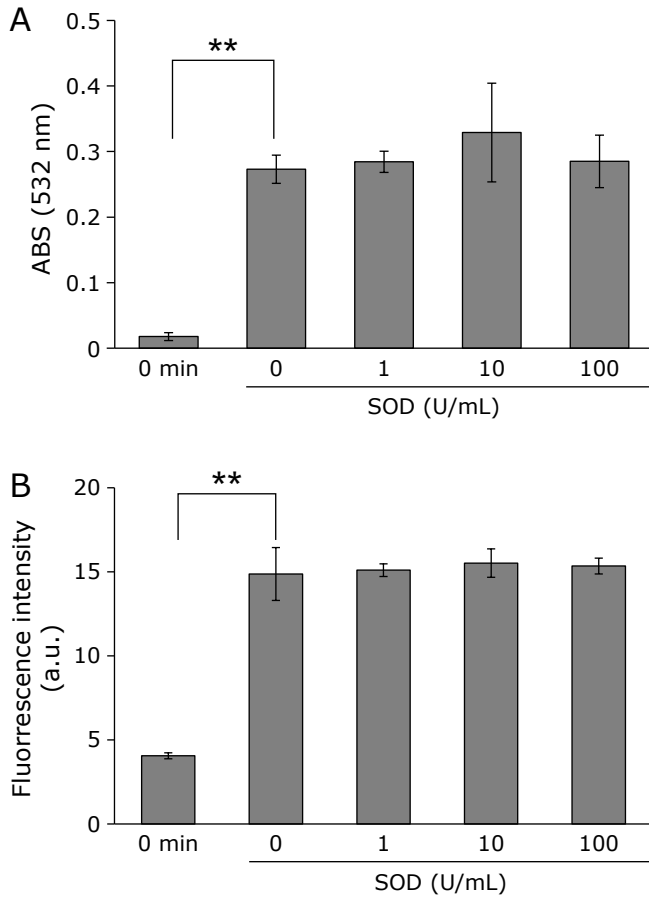
**B** ■ XIC of +MRM (4 pairs): 812.500541.500 Da ID: 13ZE from Sample 3 (1 min) of DataSET 1will (Turbo Spray) Max. 1.1e5 cps.



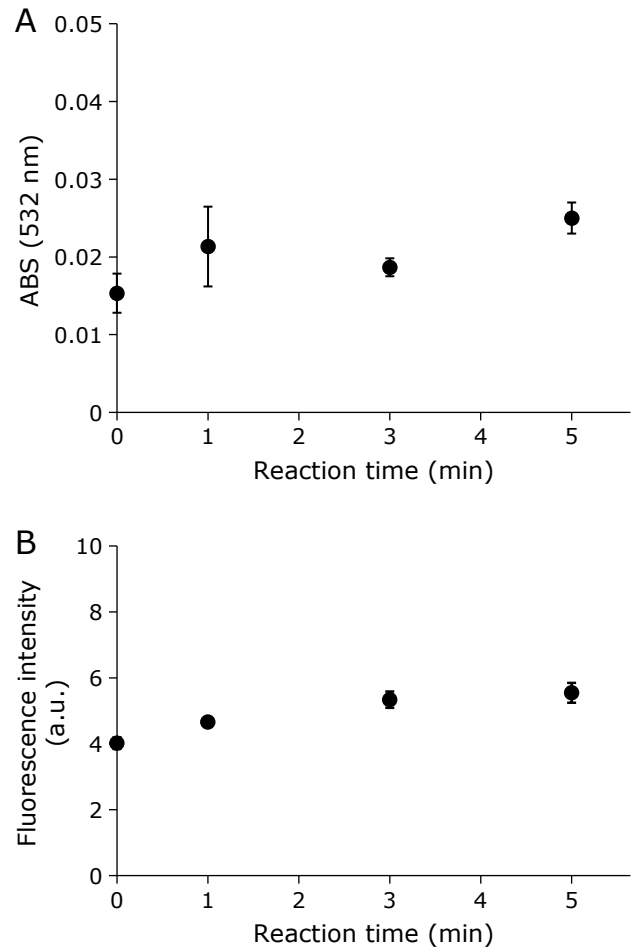
**C** ■ XIC of +MRM (4 pairs): 812.500541.500 Da ID: 13ZE from Sample 4 (2 min) of DataSET 1will (Turbo Spray) Max. 1.8e5 cps.



**Fig. 5.** Hydroperoxide isomers produced by CAPPJ irradiation induced lipid peroxidation. (A) General oxidation mechanisms of lipids and isomer structure. (B) LC-MS/MS chromatogram of lipid peroxides produced by 1 min of CAPPJ exposure. (C) LC-MS/MS chromatogram of the lipid peroxides produced by 2 min of CAPPJ exposure.



**Fig. 6.** Lipid peroxidation by CAPPJ irradiation in the presence of SOD. SOD of various concentrations was added to the liposome suspension and TBARS (A) with the measured fluorescence intensity (B) of samples after 3 min of CAPPJ irradiation. Error bar shows SD ( $n = 3$ ). \*\* represents  $p < 0.01$ .



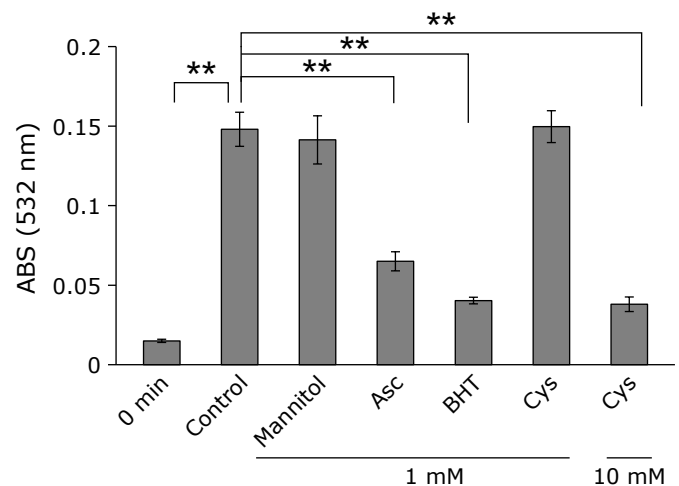
**Fig. 7.** Effect of chemically generated  $\text{O}_2^{\cdot-}$  on liposome lipid peroxidation. The liposome suspension combined with the  $\text{O}_2^{\cdot-}$  generating system (Hyp-XOD). (A) TBARS was measured after reaction. (B) NBD-pen was also added to the liposome suspension, and fluorescence intensity measured after reaction. Error bar shows SD ( $n = 3$ ).

(Fig. 7A). Although slight increase in the NBD-pen fluorescence was observed (Fig. 7B), the change does not indicate the lipid radical formation, because similar slight increase was also observed in the control experiment of Fig. 2 (containing no liposomes). Thus, as lipid peroxidation was not observed using  $\text{O}_2^{\cdot-}$  chemically generated outside the liposome membrane,  $\text{O}_2^{\cdot-}$  is not involved in CAPPJ irradiation induced lipid peroxidation.

**Effects of  $\cdot\text{OH}$  on liposome peroxidation by CAPPJ irradiation.** Next, the possibility that  $\cdot\text{OH}$  induced liposome lipid peroxidation was investigated using  $\cdot\text{OH}$  scavengers of various reaction rates. As shown in Fig. 8, most of  $\cdot\text{OH}$  scavengers suppressed the formation of TBARS. No effect was observed by mannitol probably due to its smaller rate constant of the reaction with  $\cdot\text{OH}$  in addition to its low solubility in membrane lipid phase.

## Discussion

Previous studies reported CAP irradiation results in an increase in TBARS,<sup>(28)</sup> but the detailed mechanisms of CAP irradiation induced lipid peroxidation remain unclear. In the present study, the CAP irradiation induced lipid peroxidation of liposomes was investigated by measuring lipid radicals, reaction intermediates, and TBARS, end products. As shown in Fig. 1 and 2, CAPPJ irradiation of liposomes was confirmed to increase both TBARS and lipid radicals.



**Fig. 8.** Effect of  $\cdot\text{OH}$  scavenger on lipid peroxidation by CAPPJ irradiation. CAPPJ was irradiated to the liposome suspension containing a  $\cdot\text{OH}$  scavenger such as mannitol, ascorbic acid (Asc), butylhydroxytoluene (BHT), or cysteine (Cys) for 3 min and TBARS was measured. Error bar shows SD ( $n = 3$ ). \*\* represents  $p < 0.01$ .



Lipids are peroxidized by (1) enzymatic oxidation by enzymes such as lipoxygenase, (2) autoxidation by radicals, and (3) photo-oxidation mediated by  $^1\text{O}_2$  with each oxidation process producing characteristic isomer products.<sup>(25–27)</sup> In the peroxidation of PC containing a linoleic acid fatty acyl chain, radical oxidation produces 16:0/9-10E,12Z-HpODE PC, 16:0/9-10E,12E-HpODE PC, 16:0/13-9Z,11E-HpODE PC and 16:0/13-9E,11E-HpODE PC. Oxidation with  $^1\text{O}_2$  produces 16:0/9-10E,12Z-HpODE PC, 16:0/10-8E,12Z-HpODE PC, 16:0/12-9Z,13E-HpODE PC, and 16:0/13-9Z,11E-HpODE PC. Therefore, the isomeric structures of lipid peroxidation products provide information elucidating lipid peroxidation pathways. Positive mode MS/MS analysis with alkali metal ions is reported for hydroperoxide isomer analysis, achieving high sensitivity and selectivity.<sup>(23,29,30)</sup> In the present study, the HpODE isomers produced by CAPPJ irradiation were analyzed using a similar method. As shown in Fig. 5, 13-HpODE and 9-HpODE was detected in the CAPPJ-irradiated sample. Furthermore,  $\beta$ -car, a  $^1\text{O}_2$  scavenger, did not inhibit CAPPJ irradiation induced formation of lipid-derived radicals (Fig. 4). These findings indicate that CAPPJ irradiation induced lipid peroxidation is caused by radicals rather than  $^1\text{O}_2$ .

CAPPJ irradiation of water generates numerous ROSs, and we previously reported the CAPPJ irradiation induced generation of  $\cdot\text{OH}$ ,  $^1\text{O}_2$ ,  $\text{H}_2\text{O}_2$ , and  $\text{O}_2^{\cdot-}$ <sup>(19,20)</sup> under conditions used in this study (helium gas flow rate was 3 L/min, and the distance from sample surface to plasma jet tip was 1.5 cm). Next, whether the CAPPJ irradiation generated  $\text{O}_2^{\cdot-}$  was involved in liposome lipid peroxidation was evaluated. Upon liposome irradiation by CAPPJ in the presence of SOD, no reduction in the production of lipid TBARS or lipid radicals was observed (Fig. 6). Additionally, when the Hyp-XOD system was used to chemically generate  $\text{O}_2^{\cdot-}$ , the liposomes showed no increase in lipid radical or TBARS formation (Fig. 7), supporting the findings that  $\text{O}_2^{\cdot-}$  does not initiate lipid peroxidation.<sup>(31)</sup>

In this study, the involvement of CAPPJ irradiation generated  $\cdot\text{NO}$  and  $\text{ONOO}^-$  in liposome lipid peroxidation was not investigated. As CAP irradiation induced nitrogen oxides ( $\text{NO}_x$ ) formation is reported,<sup>(15,32)</sup> their involvement in lipid peroxidation cannot be ruled out. However,  $\cdot\text{NO}$  is insufficiently reactive to extract a bis-allylic hydrogen from an unsaturated fatty acid,<sup>(33)</sup> instead potentially acting as an antioxidant, preventing lipid chain reactions by reacting with lipid radicals.<sup>(34,35)</sup> Another nitrogenous radical, formed by the reaction of  $\cdot\text{NO}$  and  $\text{O}_2^{\cdot-}$ , is  $\text{ONOO}^-$ . Upon addition of SOD, which reduces  $\text{O}_2^{\cdot-}$  thus limiting  $\text{ONOO}^-$  formation, no CAPPJ irradiation induced lipid peroxidation inhibition was observed (Fig. 6). This finding suggests that  $\text{ONOO}^-$  involvement in lipid peroxidation during CAPPJ irradiation is unlikely.

$\cdot\text{OH}$  scavengers decreased the amount of TBARS produced by CAPPJ irradiation (Fig. 8). We previously reported that CAPPJ irradiation of water generates at least 270  $\mu\text{M}$   $\cdot\text{OH}$  and about 17  $\mu\text{M}$   $^1\text{O}_2$  in 1 min under helium gas flow rates of 5 L/min.<sup>(19,20)</sup> Thus, the amount of  $\cdot\text{OH}$  generated by CAPPJ irradiation of water is 15 times higher than that of  $^1\text{O}_2$ . Furthermore, we found that the amount of  $\text{NO}_2$  generated by 1-min irradiation of CAPPJ

to water is around several  $\mu\text{M}$  (unpublished). Also,  $\cdot\text{OH}$  is very reactive towards all biological molecules, usually initiating free-radical chain reactions.<sup>(36)</sup> Considering all together, we can say that  $\cdot\text{OH}$  is the primary species driving lipid peroxidation during CAPPJ irradiation.

In conclusion, this study's findings eliminate  $^1\text{O}_2$  and  $\text{O}_2^{\cdot-}$  as species responsible for lipid peroxidation under CAPPJ irradiation. Furthermore, as  $\cdot\text{NO}$  and  $\text{ONOO}^-$  are also unlikely to be responsible for lipid peroxidation and  $\cdot\text{OH}$  scavengers decreased the amount of TBARS produced by CAPPJ irradiation,  $\cdot\text{OH}$  may be the primary responsible species for liposome lipid peroxidation during CAPPJ irradiation.

## Author Contributions

TT contributed to the study design, data acquisition, and manuscript drafting. KS collected data. SK and KN performed LC-MS/MS experiments. KT and AO contributed to critical revision of the manuscript. KA supervised the study, data interpretation and critical revision of the manuscript.

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## Abbreviations

$\beta$ -car	$\beta$ -carotene
CAP	cold atmospheric pressure plasma
CAPPJ	cold atmospheric pressure plasma jet
HpODE	hydroperoxyl octadecadienoic acid
Hyp-XOD	hypoxanthine-xanthine oxidase
NBD-Pen	2,2,6-trimethyl-4-(4-nitrobenzo [1,2,5] oxadiazol-7-ylamino)-6-pentylpiperidine-1-oxyl
PC	phosphatidylcholine
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TBARS	2-thiobarbituric acid-reactive substances

## Conflict of Interest

We received no financial support or other benefits from commercial sources for the work reported in the manuscript. None of the authors have financial interests that could create a potential conflict of interest or the appearance of a conflict of interest with regard to this work.

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