Traces of isotopic reactive species produced from a non-thermal plasma jet in bio-molecules

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Abstract
Heavy water (D₂O) is introduced into a non-thermal plasma jet (NTPJ) device to generate deuterium monoxide (OD) radicals instead of hydroxyl (OH) radicals. An NTPJ generated from a vapor mixture of N₂/H₂O and N₂/D₂O is applied to a cell membrane component and its effects are analyzed by means of ¹H NMR, GC-FID and TOF-SIMS spectroscopies. The results show that OH and OD radical species induce similar levels of oxidative breakage of lipid molecules. In addition, the ²H NMR spectra show that deuteriums are incorporated into the lipid oxidative products. In order to trace these effects in vivo, E. coli bacteria are treated with an NTPJ and analyzed using NanoSIMS. Deuterium is observed in both the cytoplasm and membrane, which are colocalized well with nitrogen and phosphorus atoms. The high colocalization of D atoms inside E. coli provides the first direct and visual evidence of the role of OD radicals, which may be utilized to visualize OH radical interactions inside cells.

1. Introduction

Recently, the non-thermal plasma jet (NTPJ) has been widely extended to applications in biological and medical devices [1–3]. Among various radical species, such as OH, O₃, H₂O₂, NO, and ONOO, the amount of OH radical and H₂O₂ generated from a NTPJ are an important parameter when attempting to induce biological responses [4–13]. A group in Germany explored that H₂O₂ had the strongest influence on plasma-treated media [6], and a Japanese group introduced OH radicals as an important factor in their plasma treatment method [7]. Simulations showed the oxidation of DNAs and lipids in a cell by OH radicals and the detailed information on their exact interaction mechanisms [8–10]. However, few experimental clues have been introduced due to the extremely short life time of OH radicals.

Therefore, we investigate the possibility of the use of an isotope as a new method for the detection of OH radical interactions with biomolecules. OD radicals instead of OH radicals are generated through a plasma jet and their incorporation in a lipid molecule is detected using NMR, GC-FID and TOF-SIMS spectrosopies. The high intensity of a deuterium atom in a specific position of a lipid molecule is detected by ²H NMR spectra. In addition, E. coli are treated with a deuterated NTPJ and the location of deuterium is determined by means of NanoSIMS. The NanoSIMS image shows that deuterium is incorporated in both the cytoplasm and membrane, which are colocalized well with nitrogen and phosphorus atoms in E.coli. This is the first direct and visual evidence of OH radical effects on cell components comparing to other previous reports using NanoSIMS [13–15]. The new method involving the use of OD radicals instead of OH radicals through a plasma jet can be used to elucidate the inter-cellular mechanisms of OH radicals.

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2. Experimental

2.1. NTPJ with N\textsubscript{2} and N\textsubscript{2}/H\textsubscript{2}O and N\textsubscript{2}/D\textsubscript{2}O gases

Figure 1 shows the schematics of the NTPJ device with porous alumina. Plasma is generated in the porous alumina between the electrodes, which are separated by a gap of 1 mm [16]. The percentage of the distilled water or heavy water vapor in the gas mixtures is controlled by a bubbling system with a mass flow controller (GMC 1200, Atovec). The flowing volume of gas in the NTPJ device is maintained at 1 SLM during all of the experiments. High voltage power is supplied to the NTPJ by a commercial transformer, resulting in an output frequency of 10 kHz and output voltage of 3 kV. The electrical power is measured with an oscilloscope (TDS2002C, Tektronix) and an AC current probe (P6021, Tektronix), and the optical emission spectra of the discharge in the region of 200–1000 nm is recorded by a spectrometer (HR4000, Ocean optics) with a resolution of 0.7 nm.

2.2. Measurement of the pH, ion concentration and radical concentrations

An amount of 500 μl of de-ionized (DI) water in a 24-well plate is placed 6 mm below the outer electrode and treated by a NTPJ with gases of N\textsubscript{2}/H\textsubscript{2}O (1.16%) or N\textsubscript{2}/D\textsubscript{2}O (0.75%) for 1, 3, and 5 min. Immediately after the plasma treatment, the pH and ion concentration of each liquid are measured using a pH meter (Eutech Instruments, Singapore) and an ion chromatograph (ICS-3000, Dionex, USA), respectively. The liquid is filtered with a 0.2 μm syringe filter before the ion chromatography measurement. OH radical generation in the liquid is evaluated using terephthalic acid (TA, Sigma-Aldrich), which reacts with OH radicals to generate 2-hydroxyterephthalic acid (HTA, Sigma-Aldrich) with stable fluorescence. 10 mM of a TA solution is made in a 7.5 mM NaOH aqueous solution. The concentrations of hydrogen peroxide and nitric oxide are determined with a Quantichrom peroxide assay kit (DIOX-250, BioAssay Systems) and a Quantichrom nitric oxide assay kit (D2NO-100, BioAssay Systems), respectively.
2.3. Plasma treatment and lipid sample preparation

Oleic acid (O1008, Sigma) is dissolved in CHCl3 to a final concentration of 25 mg/ml. 5 µl of the solution is loaded on a 10×10 mm² silicon wafer which is cleaned in a piranha solution (H2SO4: H2O2 = 4:1 (v:v)) and dried at 80°C overnight. The oleic acid thin film is dried under N2 gas flow to remove the solvent fully. The oleic acid thin film is located 6 mm away from the outer electrode and treated with a NTPJ for 5 min with N2/H2O (1.16%) or N2/D2O (0.75%). For the TOF-SIMS analysis, the oleic acid thin film is directly analyzed, and for the liquid NMR and GC-FID analyses, the plasma-treated oleic acid is recovered in a CHCl3 and CH3OH mixture (4:1 (v:v)).

2.4. NMR analysis

For the ¹H NMR measurement, the plasma-treated liquid samples are dried and recovered with CDCl3, and the ¹H-NMR spectra are recorded by a Bruker AVANCE 600 MHz spectrometer using a 5 mm TXI cryoprobe. For the ²H-NMR analysis, the samples are dried and recovered with CHCl3 and the spectra are recorded at 92.1 MHz. The NMR spectra are assigned with reference to the NMR lipid database.

2.5. GC–FID analysis

For the GC–FID analysis, the plasma-treated liquid samples are dried and recovered with a methylation mixture consisting of methan, benzene, 2,2-dimethoxypropane and H2SO4 at volume ratios of 39, 20, 5, and 2. Heptanes are added to the samples and the samples are digested at 80°C for 2hr. Transmethylation of the lipids takes place in a single phase. After cooling at room temperature two phases are formed; the upper one contains the fatty acid methyl esters (FAMES) ready for the GC–FID analysis [17]. The length of the fatty acids is analyzed by gas chromatography (Agilent 7890A, Agilent).

2.6. TOF-SIMS analysis

TOF-SIMS experiments are performed with a Bi⁺ primary ion beam with an intensity of 1 pA and a density of 1.50×1013 ions cm⁻² (TOFSIMS 5, ION-TOF GmbH, Germany). The pressure in the chamber is less than 10⁻⁹ mbar. For an oleic acid analysis, negative ions from areas in size 50×50 µm² are acquired in the high current bunched mode for 60 s. The spectra were acquired three times at different locations of each sample. The spectra of the mass-to-charge ratios (m/z (amu)) are analyzed with SurfaceLab 6.1 software.

2.7. Preparation of bacteria for NanoSIMS

Escherichia coli (ATCC 11775) are cultured in Luria-Bertani (LB) culture media (244610, BD Difco) until they reach the logarithmic growth phase. The suspension of E. coli is diluted into 5×10⁴ cells ml⁻¹ and 0.5 ml of this solution is treated with the plasma jet in a 24-well plate (30024, SPL). The distance between the outer electrode and the solution is maintained 6 mm during the exposure. The plasma-treated E. coli is transferred onto LB agar and covered with a 1% low-melting agar solution (15510-019, Invitrogen) to avoid any loss of bacteria during the washing steps. A bacteria-containing agar block is fixed with Karnovsky’s fixative for 6 h, post-fixed with 1% osmium tetroxide for 1 h, dehydrated with an ethanol series, and then embedded in epoxy resin (18010, Eponate 12 kit, Ted Pella, Inc.). Once the resin is polymerized, the bacteria-containing region is cut by ultramicrotomy to a thickness of 100 nm (MTX Ultracut, UCT). The thin sections are placed on a formvar-coated Cu grid for TEM observation or placed on alumina holder for the NanoSIMS analysis.

2.8. NanoSIMS analysis

NanoSIMS imaging is performed using the Cameca Nano-SIMS 50 instrument at the Korea Basic Science Institute (KBSI). Primary Cs⁺ ions are used as the primary ion source with the current of 0.4 pA and an impact energy of 16 keV. The ion beam is focused to a ~100 nm diameter spot size with a raster size of 10×10 µm² over the sample areas. The images were constructed with five replicate scans at a resolution of 512×512 pixels with a dwell time of 1 ms/pixel. The secondary total ions, ¹⁴N⁺, ³¹P⁻, and ²H⁻ are collected in the combined analysis mode at a mass resolution of ca. 6000 (M/ΔM), which is enough to resolve the potential molecular interference. Prior to the data collection step, each sample is exposed to pre-sputtering with a high Cs⁺ current in order to remove surface contamination and to ensure equilibrium during the sputtering process. Data are analyzed using the L’IMAGE® software developed by KBSI. The NanoSIMS images are analyzed to determine the colocalization of deuterium with other atoms such as nitrogen or phosphorus in E.coli. The value obtained from the equation of \[ \sqrt{ \frac{\Delta - \Delta_{\text{min}}}{\Delta_{\text{max}} - \Delta_{\text{min}}} } \times \frac{Y - Y_{\text{min}}}{Y_{\text{max}} - Y_{\text{min}}} \] is used for the colocalization of two ions [18].
3. Results and discussion

3.1. Physical properties of NTPJ with N2 and N2/H2O and N2/D2O gases

The addition of water vapor into N2 gas can change the discharge voltages as well as other physical and chemical parameters of plasma. At first, the length of the plasma plume is measured while increasing the ratio of the water vapor. Figure 1(b) shows the change in the length of the plasma plume of the N2 and N2/H2O and N2/D2O gases with the same input power. The addition of water vapor results in the shortening of the plume length; however, the length becomes longer again when the vapor concentration exceeds a certain value. The effects of H2O and D2O vapor on the plume length show similar tendencies as shown in the graph, though the effective amount of vapor differs in the two cases. The plume length with only nitrogen is 4.37 mm, which is reduced to 3.25 mm and 3.38 mm with an addition of 1.16% H2O vapor and 0.75% D2O vapor in the N2 plasma jet, respectively. The output power is also similarly reduced from 4.7 W to 2.8 W with an addition of 1.16% H2O vapor and to 2.5 W with an addition of 0.75% D2O vapor.

Secondly, we analyze the radical species generated from the NTPJ with the addition of water vapor. Figure 1(c) shows the optical emission spectra of NTPJs with N2, N2/H2O (1.16%) and N2/D2O (0.75%) gases in the region of 200–500 nm. As shown in the figure, the emission spectrum of the pure nitrogen plasma contains NO radicals and a N2 second-positive system (SPS) only. With the addition of water vapor, the OH related spectra become more dominant in both the H2O and the D2O cases. The expanded spectra in the range of 305–315 nm show the slight difference between the OH and the OD radicals in figure 1(d). The discharge with water vapor produces significantly enhanced UV radiation stemming from the transitions of the OH band at 307.1 nm and 309.6 nm and of the OD difference between the OH and the OD radicals in...
arrows]) appear to decrease significantly, as the double bond is known to be vulnerable to oxidative stress [22, 23]. In addition, there are new peaks at 2.1 ppm (marked with the star ▲), 1.60–1.90 (marked with the star ★) and 9.69 ppm (marked with the star ☆), which can be assigned as α-hydrogens near the carbonyl group, −CH2− with changed environments and aldehyde −CHO, respectively. These peak shifts may come from the formation of the carbonyl group (==O) through radical reactions to the double bond or allylic position, which are similar to lipid oxidation occurring in biological systems; however, new peaks for −OH or hydrogens near the hydroxyl group (generally between 3 ~ 4 ppm) are not observed in our experiments. The proton number of each position is summarized in figure 3(e) after normalization with the number of protons at the methyl end. Although it is not identical, the structural changes of oleic acid by both the N2/H2O and N2/D2O plasma treatments are similar. This result is evidence of the similar roles of OH and OD radical species in lipid peroxidation.

To understand in more detail the structural changes of plasma-treated oleic acid, GC–FID and TOF-SIMS analyses are performed. The GC–FID analysis provides the length of the fatty acid chains remaining after the plasma treatment. The peak area percentages of the compositions are shown in figure 4(a). The standard hydrocarbon chains from C4 are utilized to find the peak position. The peaks between each standard fatty acid peak are grouped such that they are close to the standard peak in an effort to determine the carbon chain length distribution of the remaining molecules. As expected from the 1H NMR data, the double bond of most oleic acids (C18:1) is broken to become stearic acids (C18:0) and subsequently the chains are broken and become shorter. Heptanoic acid (C7:0) is formed predominantly and hydrocarbon chains from C8 to C16 are generated similarly. This result implies the highly reactive potential of plasma, which can react with hydrogen at any position along the carbon chain as well as with hydrogen near a double bond. One end of the chain would consist of an aldehyde group which appears at 9.69 ppm in 1H NMR spectrum. The reduced solubility of oleic acid in shorter. Heptanoic acid

![Figure 2. Changes of the pH and the compositions of DI water after a NTPJ treatment with N2/H2O and N2/D2O feeding gases: (a) pH, (b) anionic ion concentration, (c) cationic ion concentration, (d) NO3 concentration, (e) OH concentration, (f) H2O2 in DI water after a NTPJ treatment for 1, 3 and 5 min.](image-url)
Figure 3. $^1$H NMR spectral changes of oleic acid after a treatment with NTPJ in a dry state: (a) molecular structure of oleic acid (C18:1, cis-9) and (b) its $^1$H NMR spectrum [1: CH$_3$ at 0.88 ppm, 2: CH$_2$ at 1.25 ppm, 3: CH$_3$:CH$_2$:CO at 1.60 ppm, 4: CH$_2$:CH=CH at 2.02 ppm, 5: CH$_2$:COO$^-$ at 2.30 ppm, 6: CH=CH at 5.30 ppm], (c)–(d) $^1$H NMR spectra of oleic acid after a plasma treatment with (c) N$_2$/H$_2$O and (d) N$_2$/D$_2$O feeding gases. Proton number variations are marked with stars and arrows. (e) The table shows the number of protons for each peak.

Figure 4. Mass spectroscopy of oleic acid after a treatment with NTPJ: (a) GC–FID analysis of the composition of oleic acid, (b) diagram of the molecular mass of chain-broken oleic acid, (c) TOF-SIMS spectra in the mass range of 40–300 m/z acquired in the negative mode of an oleic acid control (gas flow), and (d)–(e) TOF-SIMS spectra of NTPJ-treated oleic acid with N$_2$/H$_2$O and N$_2$/D$_2$O feeding gases for 5 min.
both the N$_2$/H$_2$O and the N$_2$/D$_2$O NTPJ treatment induce the breakage of C=C double bonds and the breakage of carbon chains similarly in the oleic acid molecule due to the highly reactive plasma induced radicals.

3.4. Trace of isotopic reactive species produced from non-thermal plasma jet in oleic acid
The isotopic D atom coming from the NTPJ can be detected in high-precision mass spectroscopy and by means of a $^2$H NMR spectra analysis. Figure 5(a) shows that the TOF-SIMS spectra ranged from 2.01 to 2.02 m/z and 17.99 to 18.02 m/z, indicating a slight mass difference between D$^-$ and H$_2^-$ or H$_2$O$^-$ and OD$^-$ molecules, respectively. Greater incorporation of OD ions by nearly tenfold is found in the N$_2$/D$_2$O treated samples as compared to the others. A much greater amount of D$^-$ atoms is found in the N$_2$/D$_2$O treated samples. Although these peaks show only fractional information, we can confirm that the deuterium-containing molecules are not only in the form of D$_2$O from which they originate. The $^2$H NMR spectra can give more information about these molecules. Figure 5(b) shows the overlap of the $^1$H NMR and $^2$H NMR spectra, as displayed by the solid line and the dotted line, respectively. Except for the solvent peak, only one $^2$H NMR peak at 2.14 ppm is observed in the N$_2$/D$_2$O-treated sample. This peak can be attributed to methyl ketone, RCOCH$_3$, where the protons are displaced with deuterium. Despite the fact that it does not precisely match the $^1$H NMR peak at 2.08 ppm, this type of slight shift may be attributed to the physical differences between a proton and a deuterium. Our NTPJ treatment may induce the creation of methyl ketones mainly in the oleic acid molecule among the general products of lipid oxidation including aldehydes, acids and ketones [20]. Thus, these results assure us that the tracing of the $^2$H atom or deuterated molecules makes it possible to trace radicals from an NTPJ directly in target molecules.

Figure 5. Identification of deuterium in NTPJ-treated oleic acid: (a) TOF-SIMS data in mass ranges of 2.01–2.02 m/z and 17.99–18.02 m/z, and (b) $^2$H NMR peak at a chemical shift of 2.14 ppm (φ). The data show the D atom intercalation in oleic acid.
3.5. Colocalization of an isotopic D atom with cellular components in *E. coli*

As the next tracing target, we choose a live bacterium, *Escherichia coli* (*E. coli*). After the N2/D2O plasma treatment, we visualize the molecular images of *E. coli* using NanoSIMS. Figure 6 shows the NanoSIMS images of 12C14N−, 31P−, and 12CD− molecules in bacteria exposed to N2/D2O plasma inside water. The 12C14N− ions provide the bacterial features, as they mainly come from the proteins. The 31P− ions are mainly located at the cell membrane whose dominant molecules are phospholipids. Occasionally there are very strong 31P− ion signals inside the bacteria, which may come from the DNA whose backbone is full of phosphorus. 12CD− ions are found in high amounts inside the bacteria, confirming that the D atoms from the plasma are incorporated into the cellular molecules. Here, the control is bacteria exposed to a N2/D2O gas flow without discharge for 10 min. The other two D− molecular images from bacteria treated for 5 and 10 min with plasma show a time-dependent increase in the ratio of D atoms to H atoms (CD/CH) inside the bacteria.

For a clear comparison of the images of D− and the other two molecules, the colocalization between 12C14N− and 12CD− and 31P− and D− molecules is visualized. Images are normalized and multiplied by each other as expressed in the experimental section. In general, there is a strong correlation between C14N and CD, indicating much interaction between the radicals and the intracellular proteins. The correlation between 31P and CD is not as strong as that of CN and CD. They are overlapped generally in the cell membrane; however, there is occasional strong colocalization between the 31P and D atoms in the bacteria treated with plasma for 10 min (arrow). Their positions in the cytoplasm imply that they are from the nucleic acids. This is an important result supporting the direct interaction of radicals from an NTPJ with DNA inside cells with a long time exposure.

4. Conclusion

In summary, we directly visualize evidence of OH radical interaction with biological molecules by utilizing OD radicals instead of OH radicals. We confirm that OD radicals generated by means of a N2/D2O discharge have physical or chemical properties similar to those of OH radicals generated via a N2/H2O discharge. OH and OD radicals induce similar chemical changes in DI water and similar oxidative breakage in lipid molecules. Due to its isotopic property, the direct interaction between OD radicals and lipid molecules is visually apparent in their chemical products. Most interestingly, D atoms are found inside bacteria in different positions depending on the plasma exposure times. Proteins appear to be vulnerable to OD radicals; however, a longer exposure time induces very strong interaction between OD radicals and DNA in the cytoplasm. This technique is shown to be very promising in an analysis of the interaction mechanism between plasma-generated OD (or OH) radicals and cell components.
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