Gas-liquid interfacial plasmas producing reactive species for cell membrane permeabilization

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Non-equilibrium atmospheric-pressure plasmas (APPs) in a liquid or in contact with a liquid are called gas-liquid interfacial atmospheric-pressure plasmas (GLI-APPs). GLI-APPs have attracted much attention as a novel technology that provides new physical and chemical effects on the surface of the liquid, resulting in the likelihood of exotic reactions in the liquid. Several applications of GLI-APPs have been developed in materials science including nanoparticle synthesis, surface treatment of nanomaterials, and the decomposition of persistent substances, as well as life science applications in medicine, agriculture and biology.

There are two types of GLI-APPs. In one type, the discharge plasma is generated using a liquid electrode: the APP is generated between a powered electrode in the gas phase region and a grounded electrode in the liquid phase region, namely the liquid works as the electrode. Liquid-electrode plasmas include in-liquid plasmas, where the powered electrode is also immersed in the liquid phase region. The plasma is mainly generated in gas bubbles formed in the liquid using an arc, streamer, pulsed direct current (DC) glow, or microwave discharge immersed in a liquid, such as water, organic solvent, liquid nitrogen or supercritical carbon dioxide.

In the other type of GLI-APPs, the discharge plasma is generated between metal electrodes in the gas phase region, and then irradiated onto the liquid. One example is the atmospheric pressure plasma jet (APPJ), which is widely used for applications in both materials and life sciences because it is easy to generate and can be used to gently irradiate various kinds of materials. Irradiating liquids with the APPJ (GLI-APPJ) conduces to a large number of reactions caused by plasma-induced stimuli, such as electric fields, ultraviolet (UV) light, reactive species, temperature and shock waves. Many studies on applications of GLI-APPJ to plasma medicine have reported exciting results using the plasma-induced stimuli, including the selective killing of cancer cells, blood coagulation for minimally invasive surgery, wound healing and tissue regeneration, and cell membrane permeabilization.
Given previous reports that hydroxyl radical (·OH) plays an important role in mediating the biological effects of plasma\(^{(43,44)}\) and that it is produced in both the gas and liquid phase regions, in this paper we focus on ·OH produced by GLI-APPJ and investigate its effect on cell-membrane permeability.

**Atmospheric Pressure Plasma Jet in Contact with Liquid**

Researchers in the plasma life science field make and use various kinds of APPJs that differ in electrode configuration, excitation frequency (pulsed DC,\(^{(45)}\) LF (~kHz),\(^{(46)}\) RF (~MHz),\(^{(47,48)}\) microwaves (~GHz)\(^{(49)}\) and working gas (He, Ar, O\(_2\), N\(_2\), H\(_2\)O). The choice of electrode, frequency, and working gas dictates the electron density and temperature, and the gas temperature of the generated plasma in the gas phase region, resulting in changes in the density of the excited particles such as metastable He\(^*\) and Ar\(^*\), and various kinds of reactive species such as ·OH, H\(_2\)O\(_2\), HO\(_2\) and NO\(_2\).

When these excited particles and reactive species in the gas phase region contact liquid, other reactive species are produced at the interface between the plasma and liquid. These newly-produced reactive species in the liquid phase region likely affect the activity of cells in the liquid, but these reactive species have a limited life-span and gradually disappear with time. Therefore, the distance between the plasma-liquid interface and a cell is important for effective control of cell activity. Consequently, to clarify the behavior of the reactive species produced by the GLI-APPJ, the reactive species should be measured in both the gas and liquid phase regions, and the temporal and spatial distributions of the reactive species should also be analyzed.

A schematic illustration of a typical experimental setup for investigating the effects of reactive species produced by the GLI-APPJ on cell activities\(^{(41)}\) is shown in Fig. 2A. The APPJ apparatus consists of a quartz tube (6-mm inner diameter and 10-mm outer diameter) with two electrodes. The powered electrode is a 1.5-mm diameter tungsten rod, and the grounded electrode is an aluminum foil on the outer surface of the quartz tube. He gas is used as the source gas and its flow rate \(f\) through the dielectric tube is regulated by a mass flow controller (MFC). Turning on the high-voltage \((V_{pp})\) power supply (which has a frequency of 8–10 kHz) generates He-APP that flows from the nozzle of a glass tube (2-mm inner diameter), irradiating the cell-suspended solution in chamber slides kept on a hot plate (37°C). The parameter \(L_{df}\) is defined as the distance between the bottom edge of the powered electrode and the surface of the solution in the chamber slides. Typical conditions of operation are \(V_{pp} = 5–10\) kV, \(f = 1–3\) L/min, and \(L_{df} = 45–60\) mm.

**Reactive Species in the Gas Phase Region**

Strong emission of visible He-APP is observed between the powered electrode and the surface of the solution (Fig. 2B) and the strength of the emission, i.e., the plasma density, can be changed by controlling the distance \(L_{df}\).\(^{(41)}\) The distance between the surface of the solution and the discharge electrode significantly influences various physical and chemical characteristics such as plasma length, shape, color, voltage, current, and the composition of the plasma-produced reactive species.\(^{(50–52)}\)

The current apparatus uses only He as the source gas to generate the APP, yet various reactive oxygen and nitrogen species (RONS) are produced in the gas phase region because ambient air is mixed in the He-AP. The density of RONS in the He-APP (gas phase region) has been evaluated using several measurement methods such as optical emission spectroscopy (OES)\(^{(53,54)}\) Fourier transform infrared spectroscopy (FT-IR),\(^{(55)}\) and laser-induced fluorescence (LIF).\(^{(56,57)}\) Here, we focus on OH radicals as a typical reactive species and use ·OH laser-induced fluorescence (OH-LIF)\(^{(58,59)}\) to measure the density and temperature of the plasma-produced ·OH in the APPJ with high spatial resolution. A wavelength-tunable dye laser outputs a 566 nm beam diameter of 4 mm whose frequency doubled beam corresponding with ·OH(A-X) transition at 283 nm. The 283 nm UV beam is shaped into a 1 mm × 11 mm beam sheet using lenses after beam energy tuning with a polarizing beam splitter and a half wave plate. The typical operational beam energy is up to 0.5 mJ/pulse and the estimated mean beam intensity is approximately \(1 \times 10^6\) W/cm\(^2\). An ICCD camera is used to collect the ·OH fluorescence through a UV camera lens, and a narrow band pass filter centered at 309.5 nm with a full width at half maximum (FWHM) of 9 nm is used to obtain the two-dimensional distribution of ·OH fluorescence in the

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**Fig. 2.** (A) Schematic illustration of the experimental setup for investigating the membrane permeability of adherent cells exposed to APPJ and the two-dimensional distribution of OH radicals in the gas phase using the LIF technique. (B) Typical photographs of APPJ irradiation and (C) two-dimensional distribution of OH radicals in the gas phase region.
The OH fluorescence images are normalized by Rayleigh scattering and corrected for fluorescence yield and Boltzmann factor with measured quenching rate of OH and two rotational lines of P(2) and Q(5), thus Fig. 2C indicates two-dimensional OH density distribution. In the horizontal direction, the density of OH has a peak at the geometrical axis. In the axial direction, the OH density remains high especially near the nozzle exit and in the vicinity of the solution surface, which shows that OH does not decay along with helium flow (11 mm from the exit). Therefore, the GLI-APPJ can supply a large amount of OH in solution and the maximum distribution of OH is in the center of the gas phase region.

**Reactive Species in the Liquid Phase Region**

The reactive species produced in the gas phase region are irradiated onto the liquid phase region, thus generating new reactive species. These new reactive species react with each other depending on their respective reaction rate and change into other chemical species within a certain time scale, which we define as life-span. We classify the reactive species based on their life-span into long-lived (life-span on the order of hours or more), short-lived (life-span on the order of minutes), or extremely-short-lived (life-span on the order of seconds or less). Long-lived reactive species can be measured by ion chromatography and optical absorption of the plasma irradiated solution, whereas short-lived and extremely-short-lived reactive species are measured primarily using the chemical probe method, where the short-lived species react with specific chemicals and are changed into relatively long-lived chemical species which are then analyzed by techniques such as photoluminescence (63, 64) and electron spin resonance (ESR) (65–67).

**Long-lived reactive species.** The long-lived reactive species in the plasma irradiated solution are analyzed by an optical absorption method (Fig. 3A). The absorbance spectrum of the plasma irradiated buffer solution (5 mM NaHCO₃aq) between 230 and 300 nm is fitted with synthesized spectra generated by linear combination of the absorption spectra of H₂O₂, NO₃⁻, NO₂⁻, and NO₂⁻ (Fig. 3B). The concentrations of these reactive species produced in the plasma irradiated solution are estimated from the least-square-error fitting of the synthetic spectrum to the measured absorption spectrum (Fig. 3C). The error bars plotted in Fig. 3C are the 99% confidence intervals of the fitting coefficients in the synthetic spectrum. The estimated concentrations of the reactive species are obtained as a function of the plasma irradiation time tᵢ.

**Short-lived reactive species.** The chemical probe method is used to observe the existence of short-lived and extremely-short-lived reactive species in the liquid phase region (Fig. 4A).

The total production of OH radicals as extremely-short-lived reactive species in the liquid phase (OH₅) after plasma irradiation is detected using terephthalic acid (TA). (60) Plasma-produced OH₅ can convert terephthalate anion (produced from TA) to 2-hydroxyterephthalate ion (HTA), a highly fluorescent material. HTA is optically excited by 310-nm UV light and emits strong fluorescence around 425 nm. The total production of OH₅ can be estimated from the fluorescence intensity due to the linear relationship between the fluorescence intensity around 425 nm and the concentration of HTA (C₇HTA). A solution of 2 mM TA was prepared, warmed to 37°C, and exposed to APPJ for tᵢ, then the fluorescence of the solution was measured. C₇HTA is analyzed as a function of tᵢ (Fig. 4B); the inset shows the fluorescence spectra of HTA solution after APPJ irradiation for different tᵢ. C₇HTA, an indicator of total OH₅ production, increased linearly with increasing tᵢ.

**Spatial distribution of short-lived reactive species.** HTA generated from TA by OH₅ is observed throughout the solution region, but actually OH₅ is produced and exists primarily at the surface of the plasma-irradiated solution; this is especially the case for extremely-short-lived reactive species such as OH₅. To evaluate the amount of extremely-short-lived reactive species produced at the plasma-liquid interface can reach the bottom of the solution, which is where the living cells in the sample are located, we measure the distribution of the short-lived reactive species at the bottom of the chamber slides for different solution thicknesses.

A gel reagent containing TA is used to measure the distribution of plasma-produced OH₅ (Fig. 5A). This gel reagent is a modification of a potassium iodide (KI)-starch gel reagent based on the well-known iodine-starch reaction typically used to measure the two-dimensional concentration distribution of reactive oxygen species. Photographs (6 mm × 6 mm area) of the gel reagent after plasma irradiation for tᵢ = 5 s are shown in Fig. 5B. Fluorescence profiles along the X-axis are obtained for solution thickness h = 0.5, 0.7, and 1.0 mm (Fig. 5C). The fluorescence of the gel reagent reflects the concentration of OH₅ and its vertical distribution decreases with increasing solution thickness. (41) Therefore, a relatively large amount of OH₅ is produced at the surface of the solution, but the OH₅ immediately disappears through the solution within 1 mm.

Short-lived reactive species other than OH, including various reactive oxygen and nitrogen species, are produced in plasma-irradiated solutions and behave as reactive chemical oxidants. The distributions of plasma-produced oxidizing species in solution are detected using a gel reagent containing KI-starch, as mentioned above. A 0.5% agarose/water solution containing 0.3% potassium iodide and 0.5% starch is prepared and then gels in the...
chamber slide. The solution is poured onto the gel reagent at room temperature with its thickness \( h \) controlled and then exposed to APPJ for \( t_i \). The obtained image of the gel reagent is then analyzed. Photographs (6 mm \( \times \) 6 mm area) of the gel reagent after plasma irradiation for \( t_i = 5 \) s are shown in Fig. 6A. The absorbance profiles along the X-axis are obtained for solution thickness \( h = 0.5, 0.7 \), and 1.0 mm (Fig. 6B). The absorbance of the gel reagent indicates the concentration of the oxidizing reactive species and its vertical distribution decreases with increasing solution thickness. In addition, the oxidizing reactive species are found to be horizontally distributed in a doughnut shape,\(^{41}\) as previously reported by Kawasaki et al.\(^{68,69}\) However, as shown in Fig. 5B and C, the horizontal distribution of \('\text{OH}_{\text{aq}}'\) is not doughnut shaped and clearly differs from that of other oxidizing species. Based on these results, \('\text{OH}_{\text{aq}}'\) in the liquid phase region is produced mainly in the central region of the plasma-solution interface, and this corresponds to the distribution of \('\text{OH}'\) in the gas phase region. In contrast, the predominant oxidizing species in solution detected in a doughnut-shaped distribution could be produced by reactive species other than \('\text{OH}'\) and these reactive species are generated upon mixing ambient air in the gas phase region of the He-APP.

**Cell Membrane Permeabilization by Gas-Liquid Interfacial Atmospheric Pressure Plasmas**

Cell membrane permeabilization is an important technique in molecular biology and medical procedures such as cancer pharmacotherapy,\(^{70}\) gene therapy,\(^{71}\) RNA-mediated suppression of gene...
expression,\(^{(75)}\) and the creation of induced pluripotent stem (iPS) cells.\(^{(76)}\) However, electroporation,\(^{(76)}\) a conventional method for permeabilizing cells, is problematic in terms of the low survival fraction, which reduces the efficacy of minimally invasive \textit{in vivo} treatments. Recently, it was reported that genes can be efficiently introduced into cells using APP and that the method enables spatially selective membrane permeabilization.\(^{(36-38)}\) Furthermore, the use of fluorescent dyes such as YOYO-1 and LIVE/DEAD Stain indicated that APP irradiation enhances the permeability of the cell membrane without killing the cell.\(^{(39-42)}\) To understand the underlying mechanism of cell membrane permeabilization, we have investigated the effects of plasma-produced reactive species in the liquid phase region on cell activity. These plasma-produced reactive species reportedly play key roles in many medical applications of GLI-APPJ. We also investigated the relationship between plasma-induced cell membrane permeabilization and the spatial distributions of reactive species in the liquid phase region.

In this experiment, we use the non-membrane-permeable fluorescent dye YOYO-1 (Y3601, Molecular Probes) as the delivery material to investigate the effect of plasma irradiation on cell membrane permeability. The green fluorescence of YOYO-1 increases 1,000-fold when the dye is transferred into cells and binds double-stranded DNA (dsDNA) in the nucleus, allowing transfected cells to be easily identified.\(^{(75,76)}\) In addition, YOYO-1 exhibits strong fluorescence within 0.5 s of binding to dsDNA, enabling rapid analysis of the fluorescence intensity of the cells and enumeration of the transfected cells as soon as the sample is irradiated with plasma.

Two types of plasma irradiation systems were fabricated to identify the reactive species produced by GLI-APPJ predominantly responsible for enhancing cell membrane permeability. One is a direct plasma irradiation (DPI) system (Fig. 7A), where a 100-µl sample of solution containing floating cells and YOYO-1 is directly irradiated with APPJ for a specified irradiation time \(t_1\). The other is an indirect plasma irradiation (IPI) system (Fig. 7B), where a 50-µl sample of solution containing 10 µM YOYO-1 is irradiated with plasma for a specified irradiation time \(t_1\); this plasma-irradiated solution is placed on ice for a defined period of time \(t_2\), then dripped into 50 µl of solution containing floating cells \((6 \times 10^5 \text{ cells/ml})\). In each case, the cell solution is subsequently incubated on ice for 15 min. LIVE/DEAD Stain (L10120, Molecular Probes), a cell viability assay reagent, is dripped into the cell solution, which is then incubated at room temperature for 20 min and observed through a confocal laser scanning microscope. Because YOYO-1 (green) and LIVE/DEAD (red) stain emit different fluorescence wavelengths, we could observe their respective fluorescence signals using optical filters.

We define the transfection efficiency \(\eta\) as the ratio of the number of transfected surviving cells to the total number of cells counted in the bright-field image. Cell viability is defined simply as the ratio of the number of surviving cells to the total number of cells counted.

Most of the reactive species produced in the plasma-irradiated solution are assumed to be short-lived and to dissipate rapidly with time. To investigate the effects of these transiently produced short-lived reactive species on cell membrane permeabilization, we measure the transfection efficiency \(\eta\) (%) of YOYO-1 as a function of the retention time \(t_r\) at \(t_r = 5\) s under DPI and IPI. The effect of the plasma-irradiated solution on transfection efficiency (i.e., enhancement of membrane permeability) decreases with increasing \(t_r\) and finally becomes constant (Fig. 8A). The effect observed beyond 30 min is due to long-lived reactive species such as H\(_2\)O\(_2\), whereas the effect observed within the first several minutes is due to short-lived reactive species. In addition, the difference in transfection efficiency between DPI and IPI is likely due to direct effects such as extremely-short-lived reactive

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**Fig. 7.** Schematic illustration of the two types of plasma irradiation systems. (A) Direct plasma irradiation (DPI) and (B) indirect plasma irradiation (IPI).

**Fig. 8.** (A) Transfection efficiency \(\eta\) (%) of YOYO-1 as a function of the retention time \(t_r\) at \(t_r = 5\) s under direct plasma irradiation (DPI) and indirect plasma irradiation (IPI). (B) Signal intensity of CYPMP-OH (open diamond and circles) and CYPMP-O\(_2^-\) (filled diamond and circles) adducts as a function of the retention time \(t_r\) at \(t_r = 300\) s under DPI and IPI. Each plot was obtained in a single experiment.
species. Thus, factors that enhance cell membrane permeability can be classified as direct irradiation effects associated with extremely-short-lived species, and indirect irradiation effects due to short-lived and long-lived reactive species.

To identify these direct and indirect irradiation effects, we detect 'OH and superoxide anion radicals $O_2^-$ in plasma-irradiated water by ESR. The half-life of $O_2^-$ is several tens of minutes in plasma-irradiated water at 4°C and is therefore a strong candidate as the short-lived key-product. The generation of $O_2^-$ in water by APPJ irradiation is detected by ESR using 5-(2,2-dimethyl-1,3-propanediol-1-propanol)-5-methyl-1-pyrroline N-oxide (DMPO) as spin-trap because the reaction rate of DMPO with $O_2^-$ is higher than that of the more commonly used 5,5-dimethyl N-oxide pyrrole (DMPO). Moreover, the ESR spectra of CYPMPO-OH and CYPMPO-O$_2^-$ adducts are clearly separated and readily identifiable. For DPI experiments, 500-µl of 5 mM CYPMPO/ultrapure water on ice is directly irradiated with plasma for $t_i$. For IPI experiments, 490-µl of ultrapure water on ice is irradiated with plasma for $t_i$, put on ice for retention time $t_r$, and then dripped into 10-µl of 250 mM CYPMPO/dimethyl sulfoxide (DMSO). In each case, ESR measurements are performed immediately after sample preparation.

The signal intensity of the ESR spectra of CYPMPO-OH (open diamond and circles) and CYPMPO-O$_2^-$ (filled diamond and circles) adducts are measured as a function of the retention time $t_r$ at $t_i = 300$ s under DPI or IPI (Fig. 8B). In the case of DPI, both CYPMPO-OH and CYPMPO-O$_2^-$ adducts are detected, whereas for IPI, CYPMPO-OH adducts derived from extremely-short-lived reactive species (‘OH) are not detected but CYPMPO-O$_2^-$ adducts are detected for $t_r = 1$ s. Moreover, the signal due to CYPMPO-O$_2^-$ adducts decreases with increasing $t_r$, indicating that the life-span of $O_2^-$ in plasma-irradiated water on ice is of the order of minutes or longer.

Taking account into the differences in the solutions used to obtain the results shown in Fig. 8A and B [Fig. 8A: PBS containing living cells and YOYO-1, Fig. 8B: water], the life-span of $O_2^-$ in PBS containing living cells and YOYO-1 is expected to be shorter than that in water, making $O_2^-$ a strong candidate as the short-lived reactive species that plays a critical role in IPI-induced cell membrane permeabilization. Furthermore, since CYPMPO-OH adducts are detected only in the case of DPI, the enhanced transfection efficiency observed using DPI is likely due to ‘OH as the extremely-short-lived reactive species.

Effects of Reactive Species Transport on Cell Membrane Permeabilization

Although the DPI method is effective for cell membrane permeabilization, it is difficult to understand the effects of the solution surrounding the floating cells because the distance between the cells and the surface of the solution, where the extremely-short-lived reactive species are produced, is uncontrollable. Therefore, we attempted to apply the DPI method to adherent cells instead of floating cells, where the distance between the adherent cells and the surface of the solution can be precisely controlled, thus allowing clarification of the effects on cell membrane permeability of the solution between the adherent cells and the plasma.

A typical DPI experiment ($t_i = 2$ s, $V_{pp} = 5.9$ kV, $h = 0.5$ mm) is shown in Fig. 9A. YOYO-1 fluorescence and bright field images of the cells after DPI in the peripheral region (no APPJ irradiation) are shown in Fig. 9B and in the central region (with APPJ irradiation) in Fig. 9C. YOYO-1 fluorescence is clearly observed in the central region but there is almost no fluorescence in the peripheral region. The estimated transfection efficiency in the APPJ irradiation area is more than 80%.

These results indicate that reactive species produced in the solution help the improvement in cell membrane permeability. If long-lived reactive species are produced in solution by the APPJ, there may isotropically diffuse and the affected area should become larger. Since the transfection efficiency is increased only in the central region (the APPJ irradiation area), the improvement in cell membrane permeability results primarily from the extremely-short-lived reactive species, which are rapidly inactivated and thus cannot affect the cells in the peripheral region. These results indicate that cell activities are mainly enhanced by the transiently produced reactive species which have strong effects compared with long-lived reactive species such as $H_2O_2$.

The thickness of the solution layer ($h$) is expected to affect cell membrane permeabilization due to the short life-span of the reactive species and thus we investigate the effects of the solution thickness (controlled by the volume of the solution added). A solution of 5 µM YOYO-1/HEPES-buffered saline (HBS; containing 150 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 5.6 mM D-glucose and 10 mM HEPES [pH adjusted to 7.4 with NaOH]) is prepared and kept at 37°C. The cell solution is exposed to APPJ for a plasma irradiation time $t_i$ and is incubated at 37°C for 30 min, then fluorescence images of the cells are obtained using a fluorescence microscope (CKX41, Olympus). We calculate the integral of the number of pixels above a pre-determined threshold value of the fluorescence using ImageJ and define this value as an indicator of cell membrane permeabilization.

Fluorescence (YOYO-1) images after APPJ irradiation are observed with a solution layer of various thicknesses $h$ (Fig. 10A). Plasma-induced cell membrane permeabilization is prominently observed as the solution layer thickness decreases and the transfer area increases in the horizontal direction. In addition, the amount of intracellular YOYO-1 (YOYO-1 transfer) is calculated as a function of $h$ (Fig. 10B). These data show that YOYO-1 is transferred into cells over a larger area as the solution thickness decreases.

As shown in Fig. 10A and C, ‘OH$_aq$ is widely distributed. The FWHM of a fluorescence peak from ‘OH$_aq$ is approximately 3 mm for $h = 0.5$ mm, which drastically decreases as the solution thickness increases. At $h = 0.5$ mm, YOYO-1 is transferred into cells over an area of approximately 3 mm in diameter but the transfer area decreases as the solution thickness increases. These data suggest that ‘OH$_aq$ may be the dominant factor in plasma-induced cell membrane permeabilization.
The reactive species which affect cell activity are produced at the plasma-liquid interface. Computer simulations suggest that the reactive oxygen and nitrogen species in the gas phase region are produced by the diffusion of ambient air into excited-species rich He-APP including metastable helium He* or air plasma, and the gas-phase OH radicals are mainly produced by the dissociation of H$_2$O near the plasma-liquid interface\(^{27,78}\) (Fig. 11).

A relatively large amount of gas-phase OH is present along the central axis, where the He concentration is highest, and in the vicinity of the liquid surface, where the density of evaporated water is highest. This suggests that OH production depends on metastable helium He* and evaporated water. Since OH and other oxidizing species in the gas phase region are believed to be produced from evaporated water and oxygen in ambient air, the maximum concentration of OH\(_{aq}\) and oxidizing species in the liquid phase region is likely to be in the center and annular sections, respectively (Fig. 5 and 6). The concentration of plasma-produced OH\(_{aq}\) is higher in the center in the horizontal direction and decreases with solution thickness (Fig. 5), consistent with the distribution of YOYO-1 fluorescence, suggesting that OH\(_{aq}\) may be the dominant factor in plasma-induced cell membrane permeabilization.

OH\(_{aq}\) is a precursor of other short- and long-lived reactive species, such as O$_2^\cdot$, H$_2$O$_2$ and HNO$_2$\(^{79,80}\) and these species can also alter the cell membrane structure.\(^{91,82}\) Although the dominant factor in plasma-induced cell membrane permeabilization is likely to be OH\(_{aq}\) in the liquid phase region, OH\(_{aq}\) and other short-lived reactive species derived from OH\(_{aq}\) are candidates for causing the observed enhancement of cell membrane permeability.

These results provide insights that enhance our current understanding of the mechanism of cell membrane permeabilization by GLI-APPJ and could thus facilitate improvements in APPJ technology for cell membrane permeabilization (e.g., for introducing drugs or genes into cells).

**Conclusion**

In summary, our aim was to identify the dominant factors in plasma-induced cell membrane permeabilization resulting from exposure to a gas-liquid interfacial atmospheric-pressure plasma jet (GLI-APPJ). We therefore observed the distribution of APPJ-produced reactive species in the gas and liquid phase regions, focusing primarily on OH radicals, and evaluated the effects of these reactive species on cell activity.

Plasma-produced OH\(_{aq}\) in the liquid phase region is distributed horizontally, with a higher concentration in the center resulting from the center-peaked OH distribution in the gas phase region. In contrast, the plasma-produced oxidizing species in solution are distributed horizontally in a doughnut shape, and most of these species are not OH\(_{aq}\). Plasma-produced OH\(_{aq}\) decays and cell membrane permeabilization decreases in solution; the diffusion length of OH\(_{aq}\) is on the order of several hundred micrometers, suggesting that the production of OH\(_{aq}\) is a dominant factor in cell membrane permeabilization. However, not only OH\(_{aq}\) but also other short-lived and long-lived reactive species originating from OH\(_{aq}\) are candidates responsible for the observed enhancement of cell membrane permeability, as clarified by the indirect plasma irradiation experiment. A more detailed investigation of the effects of various plasma-produced reactive species on cell activity is required.

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**Conflict of Interest**

No potential conflicts of interest were disclosed.

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