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Quantifying the concentration and penetration depth of long-lived RONS in plasma-activated water by UV absorption spectroscopy

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ABSTRACT
Reactive oxygen and reactive nitrogen species (RONS) are believed to play a key role in biomedical applications, which means that RONS must reach the target tissue to produce a therapeutic effect. Existing methods (electron spin spectrometry and microplate reading) to determine the RONS concentration are not suitable for experimental real-time measurements because they require adding an indicating reagent to the plasma-treated medium, which may alter the chemical composition of the medium. In this paper, we propose a method to measure the long-lived RONS concentration in plasma-activated water (PAW) by using UV absorption spectroscopy. Based on an analysis and fit of the absorption spectra of standard solutions (H2O2, NaNO2, and NaNO3), we propose a detailed fitting procedure that allows us to calculate the concentrations of simplex H2O2, NO2−, and NO3−. The results show that the pH and the cross reactivity between RONS in PAW correlate strongly with the absorption spectra. To confirm the accuracy of the calculations, we also use a microplate reader and add chemical reagents to measure the concentrations of H2O2, NO2−, and NO3−. The results show that the concentrations calculated by the proposed fitting method are relatively accurate and that the error range is acceptable. Additionally, the time-dependent diffusion of RONS in PAW is measured and analyzed at different depths in the PAW. This fitting approach constitutes a nonintrusive approach to measure RONS at different depths in PAW.

I. INTRODUCTION
Reactive oxygen and reactive nitrogen species (RONS) such as OH, O2−, H2O2, NO2−, NO3−, ONOO−, etc. are generated by non-thermal plasmas at atmospheric pressures and are believed to play a key role in biomedical applications, such as disinfection, wound healing, and cancer therapy.1–3 Generally, the use of plasma to treat tissue in the context of plasma medicine often involves a thin intervening liquid layer on top of the tissue.4–6 The RONS produced in the gas phase first solvate into the liquid and then react in the liquid layer prior to reaching the tissue,7–9 sometimes even generating secondary RONS within the liquid before reaching the surface of the underlying tissue.8–10 Although numerous works have studied how RONS affect plasma-activated water (PAW) when cell inactivation is induced,4–8,11–20 the actual physical mechanism of the inactivation remains unclear, and one of the most important reasons for this situation is that precisely determining the constituents and production of RONS in solution form a critical bottleneck because of the lack of effective technical methods to measure the spatial and temporal RONS distribution.21–24

Existing methods (microplate reader and electron spin resonance spectrometry) to determine the concentration of
RONS in the liquid phase have certain limitations: First, they only detect a single species in the liquid. Second, they cannot measure the time-dependent RONS concentration in solution. Third, they require the addition of specific chemical reagents (such as chromogenic agent, capture agent, scavengers, etc.) to the solution, which may destroy the chemical pathways and the balance of reaction dynamics and thereby modify the composition of the original solution. In addition, the selectivity of these methods has to be considered because some scavengers interact with several species. Therefore, to overcome the disadvantages of existing techniques, some scavengers interact with several species. To measure the time-dependent RONS concentration in solution, we therefore separate the total absorbance of RONS to obtain the single absorption lines of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ from the total absorbance of RONS. An automated program fits the UV spectra to more rapidly process larger datasets and to accurately measure the concentrations of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ in PAW, which allows the concentration of long-lived aqueous reactive species to be measured. However, how the pH and the cross reactivity in PAW affect the absorption has yet to be addressed, which motivates us to propose an optimized method based on UV spectroscopy to quantify the production and penetration depth of long-lived RONS in PAW. This work thus considers how the pH and cross reactivity affect the UV absorption and proposes a fitting method based on UV absorption that gives the concentration of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ in PAW. The time-dependent diffusion of RONS in PAW is also measured and analyzed.

II. EXPERIMENTAL METHODS

A. UV absorption spectroscopy

A conventional double-beam UV-Vis spectrophotometer (Shimadzu U-1800) was used to detect the absorption spectra of RONS in PAW. The samples were contained in a quartz cuvette (100–QS, Hellma Analytics) and had a standard optical path of 10 mm. The instrumental accuracy was 0.001, and the transmission spectra were recorded from 190 to 900 nm. Oh et al. confirmed that the absorption spectra of RONS extends below 300 nm (into the UV) and that no absorption occurs in the visible and near infrared (400–900 nm). The spectral resolution was 0.2 nm and the scan speed was 120 nm/min. The RONS concentration was determined from absorbance (Abs) spectra by using the Beer–Lambert law:

$$\text{Abs}(\lambda) = e \ell c,$$

where $e$ is the molar absorptivity of the chemical species at a certain wavelength $\lambda$, $\ell$ is the optical path length, and $c$ is the RONS concentration. However, the use of Eq. (1) would lead to large errors in the calculated concentration of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ because the absorption lines of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ overlap each other between 190 and 230 nm. We therefore separate the total absorbance of RONS to obtain the single absorption lines of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$, following which we calculate the corresponding concentration by using Eq. (1). In this way, the calculated value is more accurate than what can be obtained by using only the total absorbance lines.

B. Measuring RONS concentrations

The present experiments were all done at 25 °C because the absorption spectra are sensitive to the ambient temperature. Standard solutions of H$_2$O$_2$, NaNO$_2$, and NaNO$_3$ were diluted to different concentrations and used to measure the absorbance. A surface dielectric barrier discharge system was used to generate a surface plasma in air, and deionized water in a petri dish was placed underneath the surface plasma to confirm the accuracy of the RONS concentration in PAW as detected by UV-Vis spectroscopy. In addition, the concentration of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ was also measured by using a microplate reader (Thermo Scientific Varioskan® Flash Reader) and adding chemical reagents.

To measure H$_2$O$_2$, Amplex® Red reagent was added to the PAW to react with H$_2$O$_2$ in a 1:1 stoichiometry and produce the red-fluorescent oxidation product. This product may be excited at $\lambda = 550$ nm and emits at $\lambda = 595$ nm. To detect NO$_2^-$, the Griess reagent was added to the PAW, and the absorbance was measured at $\lambda = 540$ nm. Next, the nitrate reductase enzyme and Griess reagent were added to the PAW to measure the concentrations of nitrate and nitrite at the same wavelength. Finally, the NO$_3^-$ concentration was obtained by subtracting the NO$_2^-$ concentration from the total concentration of NO$_2^-$ and NO$_3^-$ (a similar method is discussed in our previous work).
standard solutions for H$_2$O$_2$, NaNO$_2$, and NaNO$_3$, respectively, under different concentrations. The resultant UV absorption spectra are shown in Figs. 1(a)–1(c), respectively. Figure 1 gives the following results: First, each standard solution has its specific maximum–absorption wavelength: that for H$_2$O$_2$ may be below 190 nm, that for NaNO$_2$ is at about 203 nm, and that for NaNO$_3$ is at about 210 nm. Second, the absorbance at the given wavelength increases with increasing concentration of the standard solution, and the absorbance of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ is almost linear in the concentration of standard solution, which is consistent with the experimental results reported in Ref. 32. Third, H$_2$O$_2$ may contribute to the broad spectra from 190 to 280 nm in Fig. 1, whereas NO$_2^-$ and NO$_3^-$ contribute only to the broad peak at 190–250 nm, so only H$_2$O$_2$ contributes to the absorbance from 250 to 280 nm. This is very important because the absorbance in the range 250–280 nm helps us to establish a quantitative relationship between absorbance and H$_2$O$_2$ concentration and thereby to determine the concentration of H$_2$O$_2$ in mixed RONS in PAW.

However, the spectra of standard solutions in Fig. 1 have different y-axis scales and use different standard concentrations of the relevant species, which may lead to confusion about the actual contributions to the overall spectrum. Therefore, the insets show expanded views from 235 to 260 nm. Note that the contributions of NO$_2^-$ and NO$_3^-$ are prominent above 250 nm when the concentrations of NO$_2^-$ and NO$_3^-$ exceed about 0.5 and 1 mM, respectively. Thus, in these experiments, we focus only on low concentrations of RONS (less than 0.5 mM), because, in plasma biomedical applications, organisms (such as myeloma cancer cells) would be killed when exposed to tens of mM of RONS. Moreover, during the plasma–liquid interactions, the concentrations of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ beyond the mM level are quantified from the first derivative of the absorption spectra.35

**B. Dependence of pH and hybrid species on RONS profiles**

A solution exposed to a plasma jet or surface-discharge plasma becomes acidic, with a pH that depends on the treatment time. The formation of H$^+$ may strongly affect the rates of generation and consumption of aqueous reactive species, such as OH, NO$_2^-$, and ONOO$^-$ . In other words, it may affect the absorbance spectra. In this section, we study how pH affects the absorption lines by measuring standard solutions of H$_2$O$_2$, NaNO$_2$, and NaNO$_3$. The absorption spectra of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ are shown in Fig. 2 for pH from 4 to 7. The influence of pH on the absorption spectra of H$_2$O$_2$ and NO$_2^-$ varies, but pH does not affect the absorption spectra of NO$_3^-$. For NO$_2^-$, the pH mainly influences the characteristic peak. Above 220 nm (in the tail of the absorption), the pH has no effect on the absorption spectra for NO$_2^-$ and NO$_3^-$. However, the opposite is true for H$_2$O$_2$. These results allow us to develop a fitting procedure to determine the concentrations of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$. In PAW, a variety of species may mix and react with each other to generate acidic conditions. For instance, H$_2$O$_2$ can react with NO$_2^-$ to form NO$_3^-$ to a certain extent due to the oxidation of H$_2$O$_2$ and the presence of H$^+$. Therefore, to study how mixing the solutions affects the absorption spectra, we mixed H$_2$O$_2$ with NaNO$_2$, H$_2$O$_2$ with NaNO$_3$, and NaNO$_2$ with NaNO$_3$ and measured their absorption spectra. Figure 3 shows the spectra of the mixed solutions with the fitted spectra superposed for the absorbance of the two-type single reactive species shown in Fig. 1. The results show that the measured spectra between 200 and 220 nm fall slightly below the fitted spectra for the H$_2$O$_2$+NaNO$_3$ and NaNO$_3$+NaNO$_2$ mixed solutions. A larger discrepancy occurs between the measured result and the fit from 200 to 220 nm for the H$_2$O$_2$+NaNO$_2$ mixed solution, which may be attributed to the cross reactivity between H$_2$O$_2$ and NaNO$_2$ that occurs in the
solution and that reduces the measured result.\textsuperscript{39,40} However, the measured spectra are very close to the fitted spectra from 220 to 250 nm for the three mixed solutions. Another important phenomenon is that the absorbance in Fig. 3(c) goes to zero above 250 nm, whereas the absorbance remains nonzero at 250 nm in Figs. 3(a) and 3(b) and only goes to zero at 260 or 270 nm. This result fully illustrates that only H$_2$O$_2$ contributes to the absorbance above 250 nm.

In addition, H$_2$O$_2$+NaNO$_2$+NaNO$_3$ mixed solutions were also prepared with different concentrations, and their absorption spectra are shown in Fig. 4. Evidence of the cross reactions in the mixed solutions appears between 200 and 220 nm, which slightly shifts the absorption peak and changes the shape of the absorption band. However, this change has only a small effect on the absorption peak and little effect on the tail. Therefore, to reduce the impact and improve the accuracy of the results, we use the spectral tail to analyze and calculate the RONS concentration. Furthermore, the spectra in Fig. 4 are consistent with the spectra in PAW obtained by spectrophotometer (see Fig. 8), which also confirms that H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ are the main species contributing to the absorbance between 190 and 300 nm.

C. Absolute concentration of H$_2$O$_2$ in PAW

An urgent and crucial question is how to calculate the concentrations of H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ in PAW by using
a spectrophotometer instead of by using chemical probes. The fundamental goal of this work is to resolve this problem. Based on the results shown in Figs. 1–4, we conclude that only H$_2$O$_2$ contributes to the absorption spectrum above 250 nm, so the main solution is that the H$_2$O$_2$ absorbance is separated from total absorbance, with the remaining absorbance being due to NO$_2^-$ and NO$_3^-$. Based on the results shown in Fig. 1(a), the fitting functions are obtained from the relationship between the known H$_2$O$_2$ concentration and absorbance at 250, 252.5, and 255 nm, respectively. These fitting functions are shown in Fig. 5. All fitting functions are almost linear at 250, 252.5, and 255 nm, respectively, and are given as follows:

\[ A_{250} = \varepsilon_{250} \Lambda (H_2O_2) \lambda = 7.247 \times 10^{-4} \times n(H_2O_2) \quad \text{at} \ 250 \ \text{nm}, \]  
\[ A_{252.5} = \varepsilon_{252.5} \Lambda (H_2O_2) \lambda = 6.214 \times 10^{-4} \times n(H_2O_2) \quad \text{at} \ 252.5 \ \text{nm}, \]  
\[ A_{255} = \varepsilon_{255} \Lambda (H_2O_2) \lambda = 5.315 \times 10^{-4} \times n(H_2O_2) \quad \text{at} \ 255 \ \text{nm}, \]  

where \( n(H_2O_2) \) denotes the concentration of H$_2$O$_2$ in solution, \( A_{250}, A_{252.5}, \) and \( A_{255} \) are the absorbances at 250, 252.5, and 255 nm, respectively, and \( \lambda = 1 \) cm is the optical path length. Given the absorbance from mixed solutions or from PAW at 250, 252.5, and 255 nm, we obtain the quantitative concentration of H$_2$O$_2$ by choosing an appropriate wavelength. Thus, the formula for calculating the H$_2$O$_2$ concentration is

\[ n(H_2O_2) = \left[ A_{250}/7.247 \times 10^{-4} \right] \text{or} \left[ A_{252.5}/6.214 \times 10^{-4} \right] \text{or} \left[ A_{255}/5.315 \times 10^{-4} \right]. \]  

D. Absolute concentrations of NO$_3^-$ and NO$_2^-$ in PAW

Based on the conclusions from Figs. 2–4, when the pH of a solution changes in Fig. 2 or when many species are mixed together in solution, as in Figs. 3 and 4, the actual measured absorption spectra and the fitted absorption spectra show a certain discrepancy between 200 and 220 nm. This result may be due to the pH affecting the absorbance between 200 and 220 nm because the ionization of NO$_2^-$ and H$_2$O$_2$ significantly influences the absorbance with increasing H$^+$ concentrations. The influence of hybrid species is due to physical and chemical reactions, such as the oxidation of H$_2$O$_2$, and to intermolecular forces.

However, in the tail of the absorption spectra (beyond 220 nm) for NO$_2^-$ and NO$_3^-$, the UV absorption spectra are basically the same in Figs. 2–4, so the reasons above motivate us to choose any wavelength greater than 220 nm to fit the relationship between concentration and absorbance. A unique advantage of this approach is that the pH and hybrid species do not affect the absorbance because of the similarity of the tails of the absorption spectra, which effectively eliminates any error and does not affect the result for the concentration of NO$_2^-$ and NO$_3^-$. The concentrations of NO$_3^-$ and NO$_2^-$ are obtained based on the known concentration of H$_2$O$_2$ obtained by using Eq. (5).

At a certain wavelength, the total absorbance of the solution is a superposition of absorptivity multiplied by the concentration. The resulting formula is

\[ A_A = \alpha n(NO_3^-) + \beta n(NO_2^-) + \epsilon n(H_2O_2). \]
To more quickly and intuitively obtain the concentration of each RONS, we introduce a matrix to facilitate the calculation by an automated curve-fitting program. The absorbance at a given wavelength is defined as the product between the matrix of coefficients and a vector of concentrations:

\[
\begin{bmatrix}
A_{\lambda_1} \\
A_{\lambda_2}
\end{bmatrix}
/ l =
\begin{bmatrix}
\alpha(\lambda_1) & \beta(\lambda_1) & \varepsilon(\lambda_1) \\
\alpha(\lambda_2) & \beta(\lambda_2) & \varepsilon(\lambda_2)
\end{bmatrix}
\begin{bmatrix}
(n(NO_3^-)) \\
(n(NO_2^-)) \\
(n(H_2O_2))
\end{bmatrix}.
\]  
(7)

To determine the concentration of the substance, the matrix can be transformed as follows:

\[
\begin{bmatrix}
(n(NO_3^-)) \\
(n(NO_2^-)) \\
(n(H_2O_2))
\end{bmatrix} =
\begin{bmatrix}
\alpha(\lambda_1) & \beta(\lambda_1) & \varepsilon(\lambda_1) \\
\alpha(\lambda_2) & \beta(\lambda_2) & \varepsilon(\lambda_2)
\end{bmatrix}^{-1}
\begin{bmatrix}
A_{\lambda_1} \\
A_{\lambda_2}
\end{bmatrix}
/ l,
\]  
(8)

where \( \alpha, \beta, \) and \( \varepsilon \) are the molar absorptivity coefficients and can be obtained by fitting the concentration and absorbance.

**FIG. 6.** Absorbance of (a) H$_2$O$_2$, (b) NO$_2^-$, and (c) NO$_3^-$ versus concentration at 230 and 235 nm.
functions of a single particle at a specified wavelength. \( A_\lambda \) in the vector is the absorption at wavelength \( \lambda \).

Furthermore, to fit the relationship between concentration and absorbance and obtain the molar absorptivity coefficient, we select at random two absorption wavelengths (230 and 235 nm) within the wavelength range 220–250 nm. The relationships between concentration and absorbance for H\(_2\)O\(_2\), NO\(_2^-\), and NO\(_3^-\) are shown in Figs. 6(a)–6(c), respectively. These results show that the absorbance of H\(_2\)O\(_2\), NO\(_2^-\), and NO\(_3^-\) is linear in concentration. Note that, at 230 and 235 nm, the PAW absorbance measured by UV-Vis spectrophotometer is the sum of three species: H\(_2\)O\(_2\), NO\(_2^-\), and NO\(_3^-\). Thus, according to Fig. 6, the two formulas for calculating the absorbance based on Eq. (8) are

\[
\begin{align*}
0.0020 \times n(H_2O_2) + 0.00228 \times n(NO_2^-) + 0.0085 \times n(NO_3^-) &= A_{230} \text{ at } 230\text{nm,} \\
0.0015 \times n(H_2O_2) + 0.0113 \times n(NO_2^-) + 0.0032 \times n(NO_3^-) &= A_{235} \text{ at } 235\text{nm,}
\end{align*}
\]

where \( n(H_2O_2) \), \( n(NO_2^-) \), and \( n(NO_3^-) \) are the concentrations of H\(_2\)O\(_2\), NO\(_2^-\), and NO\(_3^-\), respectively, in units of mg/L. The coefficients \( A_{230} \) and \( A_{235} \) are the absorbance at 230 and 235 nm, respectively. Putting the known values of \( n(H_2O_2) \) from Eq. (5) into Eqs. (9) and (10) gives \( n(NO_3^-) \) and \( n(NO_2^-) \), respectively. Equations (5), (9), and (10) are thus proposed for determining the RONS concentration in PAW with the help of UV absorption spectroscopy. This approach allows the concentration of H\(_2\)O\(_2\), NO\(_2^-\), and NO\(_3^-\) to be calculated given the total absorption spectra for PAW.

### E. Verifying the accuracy of RONS concentrations

To verify the accuracy of the results obtained from Eqs. (5), (9), and (10), we compare the results of experiments wherein various plasma treatment times, applied voltages, and plasma-water distances are used. The goal is to calculate the concentration of H\(_2\)O\(_2\), NO\(_2^-\), and NO\(_3^-\) in solution by using Eqs. (5), (9), and (10) and comparing the results with those obtained by using a microplate reader and adding chemical reagents. Figure 7 shows a flow chart describing the experimental procedure, and a detailed presentation of the surface discharge generator used in the experiment is available in Ref. 31. The surface generator was used to produce a stable room-temperature surface air plasma, which was then used to treat deionized water in a petri dish under a given parametrization. A volume of 2 mL of the treated deionized water was put into a quartz cuvette to measure its absorption spectrum by using the UV-Vis spectrophotometer. All UV absorption spectra were recorded and processed by using a PC equipped with software that implemented the requisite formulas. A volume of 2 mL of treated deionized water was also distributed over 96-well plates to be measured by microplate reader with added chemical reagents.

Figure 8 shows the PAW UV absorbance for various plasma-treatment times, applied voltages, and plasma-water distances. The absorption spectrum is quite similar to that for the H\(_2\)O\(_2\)+NaNO\(_2\)+NaNO\(_3\) mixed solutions (see Fig. 4), which confirms that the reactive species H\(_2\)O\(_2\), NO\(_3^-\), and NO\(_2^-\) are the main long-lived chemical constituents in PAW. In addition, the absorbances grow almost linearly with increasing plasma-treatment time, applied voltage, and plasma-water distance, which indicates that the generation and diffusion rates of the reactive species produced by the surface-discharge plasma are very stable for transporting into water. Furthermore, the absorption spectra are similar but not identical. Upon closer inspection, the central peaks blueshift slightly upon increasing the plasma exposure, applied voltage, or plasma-water distance. These differences in peak profiles reveal a change in the RONS concentration and in the ratio of the RONS dose in PAW.

Figure 9 compares the calculated results and the results measured by using the microplate reader and shows that the two results are fairly consistent. The discrepancies between the measured and calculated results are attributed to several causes. The first cause is the error coefficient when fitting the curve, which is due to the limited accuracy of the spectrophotometer. Second, the pH and cross reactivity when mixing multiple species affects the fits, and this effect cannot be completely eliminated. Third, the microplate reader measures a product produced by a series of chemical reactions with a reagent, and incomplete chemical reactions also lead
to errors. Fourth, the ambient temperature and humidity also affect the absorption spectra and the characteristics of the surface-discharge plasma.

However, the errors caused by these four factors fall within an acceptable range. The accuracy of the results for the concentration of H₂O₂, NO₃⁻, and NO₂⁻ is reasonable compared with the known concentrations of the standard solutions. Thus, the formulas used to fit the UV absorption are suitable for calculating the concentration of H₂O₂, NO₃⁻, and NO₂⁻ in PAW. In addition, the dose of RONS delivered into solution is easy to control quantitatively by adjusting the plasma exposure time, applied voltage, and plasma-water distances.

F. Time- and depth-resolved RONS concentrations

Because the time-dependent concentration of RONS in PAW is quite significant for biomedical applications, we measured the temporally and spatially resolved concentration of RONS. We measured the real-time, depth-resolved UV absorption spectra (Ocean Optics, USB 2000+) to analyze the liquid phase and investigate temporal variations during direct plasma treatment of deionized water. Because the UV-Vis spectrophotometer used in the work described above allowed us to determine and fix the measurement point through a quartz cuvette, we used this instrument instead of the UV-Vis spectrophotometer for making real-time measurements.

Figure 10(a) shows a schematic diagram of a measurement system that used a surface-discharge plasma to directly treat deionized water in a quartz cuvette. Details of the measurement and recording process can be found in our previous report. Figures 10(b) and 10(c) show the PAW absorption spectra as measured by UV-Vis and UV absorption spectroscopy and for various plasma-treatment times and applied voltages. The absorption spectra are exactly the same above 225 nm, which indicates that Eqs. (5), (9), and (10) may be used with these spectra to calculate the concentration of H₂O₂, NO₃⁻, and NO₂⁻ in PAW. This is another reason we choose the wavelengths greater than 220 nm to fit the spectra. The difference in the PAW absorption spectra below 220 nm in Figs. 10(b) and 10(c) is attributed to the inability of the UV absorption spectrometer to measure absorbance at these short wavelengths, and the accuracy of the absorption spectrum near 200 nm is degraded by absorbance in the cuvette.

The experimental setup depicted in Fig. 10 allowed us to monitor the RONS concentration in real-time and at different depths. The total absorbance is positively correlated with the total concentration of RONS, and the total absorbance spectrum reflects the total concentration of RONS, so we measured the total absorbance to determine the total concentrations of RONS. The total absorbance (range: 200–300 nm) is plotted in Fig. 11 as a function of plasma-treatment time and for different depths (5, 10, 15, and 20 mm) along the vertical axis. The total absorbance is defined as

\[
\text{Total absorbance} = \int_{200\text{nm}}^{300\text{nm}} \text{ABS}(\lambda) d\lambda
\]

\[
\approx \text{Total RONS concentration.} \quad (II)
\]

Figure 11 shows that a delay time of approximately 90 s precedes the appearance of RONS in PAW at a depth of 5 mm whereas essentially no RONS exists at 20 mm depth, which indicates that this delay time may depend on the penetration depth. At greater depths, a lower total absorbance, which corresponds to a smaller concentration of RONS, can be detected in the PAW. For the measurement of RONS at a depth of 5 mm, after the ~90 s delay time, the upstream RONS reaches the measurement point at 5 mm by diffusion, so the total
FIG. 9. Calculated concentrations of $\text{H}_2\text{O}_2$, $\text{NO}_3^-$, and $\text{NO}_2^-$ and concentrations measured by using the microplate reader for various (a) plasma-treatment times, (b) applied voltages, and (c) plasma-water distances.
absorbance gradually increases with treatment time. When the plasma is turned off after 5 min, so that the RONS are no longer generated, the total absorbance continues to rise due to the RONS accumulated upstream of the 5 mm measurement point. At about 6 min, the total absorbance at 5 mm begins to decrease, which indicates a reduced RONS concentration at 5 mm and a continued downward diffusion of RONS in PAW. The same phenomenon is also detected at the other measurement depths. However, no downward diffusion of RONS occurs at 15 mm, so the concentration approaches zero at 20 mm. Finally, note that the delay time depends on the penetration depth and the rate at which RONS is produced by the plasma source.

Next, we use Eqs. (5), (9), and (10) to calculate the absorption spectra at 5, 10, and 15 mm (the RONS concentration at 20 mm is too small to analyze) and obtain the RONS concentration as a function of plasma–treatment time at the different depths (see Fig. 12). The concentrations of $\text{H}_2\text{O}_2$, $\text{NO}_3^-$, and $\text{NO}_2^-$ follow dynamics similar to that of the total absorbance shown in Fig. 11. At 5 mm, after approximately 100 s delay, the concentration of $\text{NO}_2^-$ and $\text{NO}_3^-$ begins to increase rapidly and, after 220 s, the concentration of $\text{H}_2\text{O}_2$...
begins to increase rapidly. At 100 s after the plasma is turned off, the concentration of RONS begins to decrease, the generation rate of \( \text{H}_2\text{O}_2 \) becomes the slowest, and the generation rate of \( \text{NO}_2^- \) drops the fastest. At 10 and 15 mm, the concentration of RONS also increases gradually due to diffusion of the upstream RONS.

The time-dependent concentration of RONS in PAW is very important for biomedical applications. For example, to eradicate cells in tumors, the RONS needs to be generated throughout the tissue fluid so that they can penetrate cell membranes and react with intracellular components.\(^{41,42}\) Thus, the diffusion process of RONS and the spatial and temporal distribution of RONS concentration at different times and depths must be understood in detail. In addition, the RONS dose must be controlled at the target depth so that the RONS can eliminate germs without affecting normal tissues.\(^{43}\) This method to monitor and control the concentration of \( \text{H}_2\text{O}_2 \), \( \text{NO}_3^- \), and \( \text{NO}_2^- \) should aid the development of new models for predicting and analyzing plasma interactions with water and provide an in-depth commentary of implications that other is still exploring. We expect that the proposed method will provide new insights into the spatial and temporal distribution of RONS in PAW, it facilitates the measurement of long-lived reactive species. Further research should allow bio-related liquids other than deionized water to be used, such as normal saline, phosphate buffered saline, etc., which would have a big impact on plasma biomedical applications.

**IV. CONCLUSIONS**

This paper proposes an optics-based method to accurately measure the concentration of long-lived reactive species (\( \text{H}_2\text{O}_2 \), \( \text{NO}_3^- \), and \( \text{NO}_2^- \)) in PAW without adding any chemical reagents and provides the corresponding formulas for calculating these concentrations. This approach constitutes a simple and useful way to determine the concentration of RONS by overcoming the problem of experimental error caused by the different pH values that occur upon mixing \( \text{H}_2\text{O}_2 \), \( \text{NO}_3^- \), and \( \text{NO}_2^- \) in water. Although this method is presently suitable only for measurements in PAW, it facilitates the measurement of long-lived reactive species.

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