# Investigation of the Presence of OH Radicals in Electrolyzed NaCl Solution by Electron Spin Resonance Spectroscopy

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In the anode side of a two-chamber electrolyzer, electrolysis of a NaCl solution generates acidic electrolyzed oxidizing (EO) water, which exhibits bactericidal effects against a large number of pathogens. This study was undertaken to investigate whether OH radical species are present in EO water or are formed when EO water reacts with iron ions. Electron spin resonance spectroscopy (ESR) coupled with the spin trapping technique was used for the detection of free radicals. Samples of EO water were collected at 0.5, 1, 2, 3, and 5 min of electrolysis and immediately mixed with the spin trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The 5,5-dimethyl-2-hydroxypyrrolidine-*N*-oxyl (DMPO-OH) spin adduct, characteristic of OH radicals, was not observed. Starting with 2-min electrolysis, a seven-line spectrum characteristic of 5,5-dimethyl-2-pyrrolidone-*N*-oxyl (DMPOX) was formed. The reactions of EO water with $Fe^{3+}$ and $Fe^{2+}$ in the presence of DMPO yielded the spin adduct DMPO-OH. However, the addition of OH radical scavengers (ethanol and methanol) did not generate the characteristic DMPO-alkyl spin adducts. This indicated that the DMPO-OH spectrum was due to a nucleophilic addition of water to DMPO and not to trapping of OH radicals.

## INTRODUCTION

Electrolysis of a NaCl solution in an instrument in which the anode and cathode are separated by an ion-permeable membrane generates two forms of electrolyzed water. Acidic oxidizing water (pH 2.3–2.7, oxidation–reduction potential [ORP] 1050–1100 mV) is generated at the anode side, while alkaline reducing water (pH 10–11.5, ORP −800 to −900 mV) is generated at the cathode side. In the past decade, acidic electrolyzed oxidizing (EO) water has generated much interest, being tested in a large number of applications. A few examples include treatment of peritonitis and intraperitoneal abscess (*1*), cleaning and disinfection of dialyzers (*2*), sterilization of denture base (*3*), and cleaning the surface of semiconductors (*4*). EO water has been also reported to accelerate the healing of cutaneous wounds in rats (*5*) and to reduce the microbial contamination of porcine pancreas collected from slaughterhouse for islet xenotransplantation (*6*). Recently, EO water has been introduced into food safety as a means to disinfect fresh produce (*7*, *8*), poultry (*9*), seeds used for sprouting (*10*, *11*), and cutting boards (*12*, *13*).

To date, the apparent bactericidal effect of EO water has not been fully understood. The free available chlorine, the low pH,

and the high ORP of EO water have been reported to participate in its antimicrobial effect. It is of interest to investigate whether free radical species are present in EO water or are formed when EO water reacts with iron ions, which could be generated by an iron-containing organic material.

Electron spin resonance spectroscopy (ESR) is used to identify free radicals. A free radical is a paramagnetic species containing an unpaired electron, which exerts a magnetic moment that is detected by ESR. Hydroxyl (•OH) and super-oxide ($O_2^-$) radicals are highly reactive free radicals with a lifetime of less than 20 s (short-lived radicals) and hence their direct detection by ESR would be difficult to achieve. To overcome this difficulty, spin trap agents are used (*14*). The spin trapping agent reacts with a specific free radical to produce a more stable radical or spin adduct, which is detected by ESR. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) is a nitrone spin trap frequently used for detecting hydroxyl and superoxide radicals in biological systems (*15*).

The objectives of this study were (i) to determine whether oxygen-centered free radical species are present in EO water and (ii) to determine whether OH radicals are formed in the reaction of EO water with iron ions.

## MATERIALS AND METHODS

**Reagents.** 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), sodium phosphate dibasic, sodium phosphate monobasic, ethyl alcohol, methyl alcohol, ferric chloride, and ferrous ammonium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Mannitol was purchased

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from Fisher Scientific (Fair Lawn, NJ), and hydrochloric acid was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). DMPO was purified with activated charcoal (Matheson Coleman & Bell, Norwood, OH) by stirring for 1 h with a magnetic stirrer VWR model 320, set at speed 6, followed by filtration with no. 2 Whatman paper (Whatman International Ltd., Maidstone, England). Sodium phosphate buffer ($Na_2HPO_4-NaH_2PO_4$, pH 7) was purified with Chelex (Sigma) by stirring for 8 h, followed by filtration with no. 2 Whatman paper.

**Preparation of Electrolyzed Water.** Two types of electrolyzed water generators were used in this study. EO (HEW) water was prepared using a continuous generator Hoshizaki ROX 20TA-U (Hoshizaki Electric Co. Ltd., Japan) at 10 V and 14.4 ± 0.4 A. Deionized water and a 13.6% (w/v) NaCl solution were simultaneously pumped into the generator. EO (SOW) water was prepared using a batch-type Super Water Mini generator JED-007 (Altex Janix, Kanagawa, Japan) from 0.05% (w/v) NaCl solution; a 10-min electrolysis time was used.

**Reaction of EO Water with DMPO.** Samples of EO water (0.9 mL) were collected at 0.5, 1, 2, 3, and 5 min of electrolysis and immediately mixed with DMPO (0.1 mL, 100 mM). ESR spectra were recorded within 5 min.

**ESR Measurements.** ESR measurements were carried out on a Bruker EMX-X band (9 GHz) spectrometer with the following parameters: modulation frequency 10 kHz, modulation amplitude 1 G, microwave power 6 mW, time constant 40 ms, temperature 293.2 K, receiver gain 5.02e+005, scan rate 40 s, and number of scans 5.

**Dissolved Oxygen, pH, and ORP Measurements.** Dissolved oxygen was measured using an YSI model 95 dissolved oxygen meter (YSI Incorporated, Yellow Springs, OH). The pH and ORP were determined using a digital ion analyzer (Orion Research Inc., Beverly, MA), equipped with pH and ORP electrodes (ACCUMET®, Denver Instrument Co, Denver, CO).

## RESULTS AND DISCUSSION

DMPO is a nitrone spin trap largely used for trapping OH radicals with the generation of a more stable free radical, DMPO-OH, which is easily detected by ESR spectroscopy. DMPO has a $\beta$-hydrogen and yields adduct with hyperfine splitting due to both the $\beta$-hydrogen and the nitroxide nitrogen (*14*).

As a control, OH radicals were produced in the presence of DMPO through the Fenton reaction (eq 1) between ferrous ion and $H_2O_2$ (**Figure 1A**).

Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad (1)$$
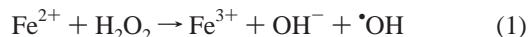
A four-line spectrum (hyperfine splitting constants $a_H = a_N = 14.9$ G) was obtained and assigned to 5,5-dimethyl-2-hydroxy-pyrrolidine-*N*-oxyl (DMPO-OH) spin adduct being similar with the DMPO-OH spectrum previously reported in the literature (*14*).

**Reaction of EO Water with DMPO.** To determine whether OH radicals were present in electrolyzed water, samples of EO water were collected during electrolysis and immediately mixed with DMPO (**Figure 2**). The DMPO-OH spectrum characteristic of OH radicals was not observed. Starting with 2 min of electrolysis, a seven-line spectrum was obtained (**Figure 2C,D**). This spectrum ($a_H = 4.0$ G, $a_N = 7.25$ G) was previously (*16*) assigned to 5,5-dimethyl-2-pyrrolidone-*N*-oxyl (DMPOX) and reported to result from the oxidation of DMPO by HOCl. No spectra (interfering ESR signals) were observed from a solution of 100 mM DMPO or a solution of 0.05% NaCl in the presence of 100 mM DMPO.

The formation of OH radicals was also tested in a neutral pH environment (spectra not shown). A seven-line spectrum was obtained and assigned to DMPOX, being similar with the
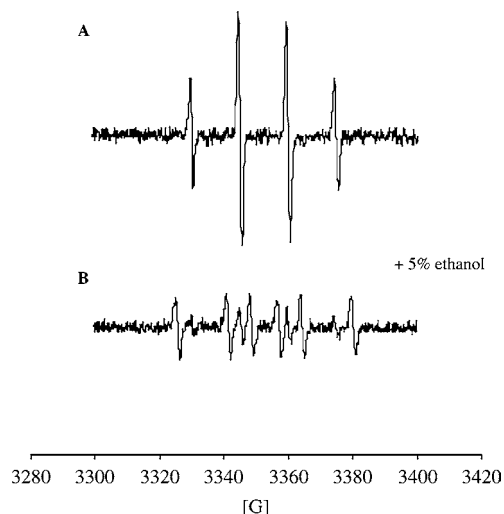


**Figure 1.** ESR spectra observed from the reaction of (**A**) 0.25 mM $Fe^{2+}$ + 20 $\mu$M $H_2O_2$ (Fenton reaction) and (**B**) 0.25 mM $Fe^{2+}$ + 20 $\mu$M $H_2O_2$ + 5% ethanol. Both reactions were carried out in 100 mM phosphate buffer in the presence of 100 mM DMPO. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.
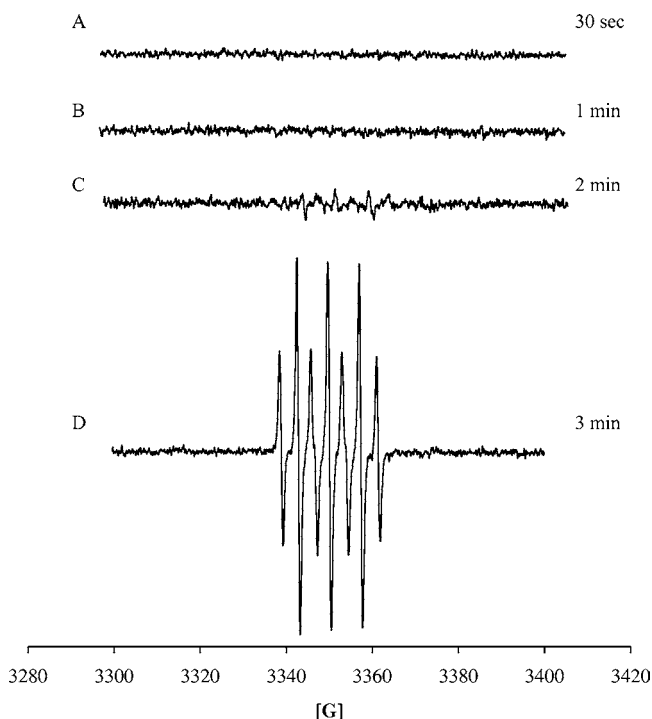


**Figure 2.** ESR spectra observed from the reaction of EO (SOW) water and DMPO (200 mM). EO (water) was collected during electrolysis at (**A**) 30 s, (**B**) 1 min, (**C**) 2 min, and (**D**) 3 min of electrolysis. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

DMPOX spectrum reported previously (*16*). Previous studies (*16, 19*) have shown that HOCl oxidizes DMPO with the formation of DMPOX.

**Reaction of EO Water with Iron Ions in the Presence of DMPO.** It is of interest to determine whether OH radicals are formed in the reaction of EO water with iron ions, which could be generated by an iron-containing organic material. In a previous study by Yahagi et al. (*5*), addition of $Fe^{3+}$ to
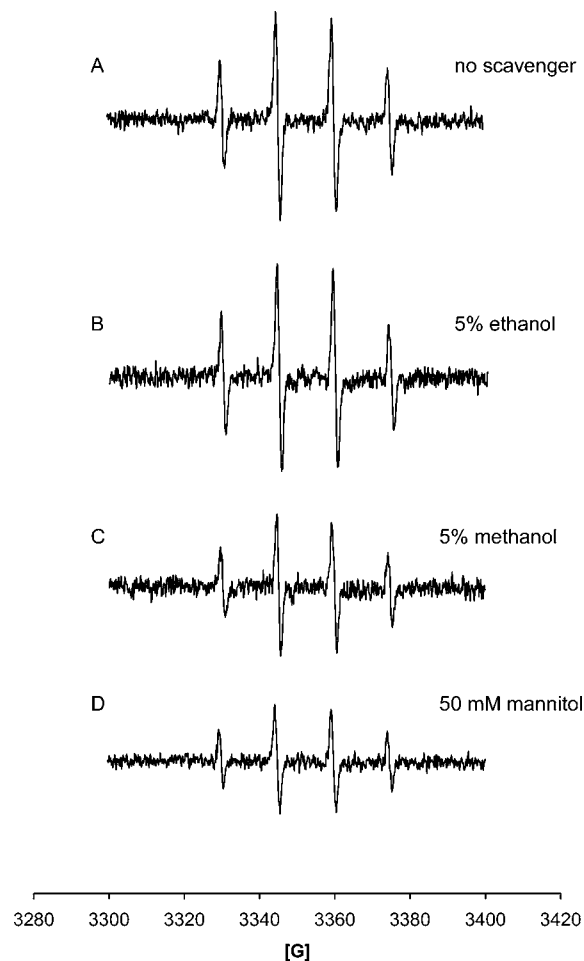
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**Figure 3.** ESR spectra observed from the reaction of EO (SOW) water with 0.25 mM $Fe^{3+}$ in the presence of the following scavengers: (**A**) no scavenger; (**B**) 5% ethanol; (**C**) 5% methanol; (**D**) 50 mM mannitol. All spectra were recorded in the presence of 100 mM DMPO. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.



**Figure 4.** ESR spectra observed from the reaction of EO (SOW) water with 0.25 mM $Fe^{2+}$ in the presence of the following scavengers: (**A**) no scavenger; (**B**) 5% ethanol; (**C**) 5% methanol; (**D**) 50 mM mannitol. All spectra were recorded in the presence of 100 mM DMPO. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.



**Figure 5.** ESR spectrum observed from EO (SOW) water and 0.25 mM $Fe^{3+}$. DMPO (100 mM) was added at 5 min after EO (SOW) was mixed with $Fe^{3+}$. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

electrolyzed oxidizing water was reported to generate a DMPO-OH adduct, which was assigned to trapping of OH radicals. However, no confirmation study was further done. In our study, a similar DMPO-OH spectrum was obtained in the reaction of both EO (SOW) water (**Figure 3A**) and EO (HEW) water (data not shown) with $Fe^{3+}$.

It is well established that hydroxyl radicals are generated through the redox reaction between ferrous ion and hydrogen peroxide. DMPO reacts with OH with the formation of DMPO-OH with a $k_{DMPO+\cdot OH}$ of $3.4 \times 10^9$ $M^{-1}$ $s^{-1}$ (*14*). However, in a different system, the generation of OH radicals should be confirmed by the addition of OH radical scavengers. If DMPO-OH was generated through the reaction of DMPO with ·OH, addition of OH radical scavengers should reduce the DMPO-OH signal and the characteristic DMPO-alkyl spin adducts should be formed.

In this study, ethanol, methanol, and mannitol were used as OH radical scavengers. Ethanol and methanol react with ·OH to form α-hydroxy ethyl radical and methyl radical, respectively, which react with DMPO yielding the characteristic DMPO-alkyl spin adducts (*14, 17*). Mannitol is known to scavenge the OH radicals (*18*).

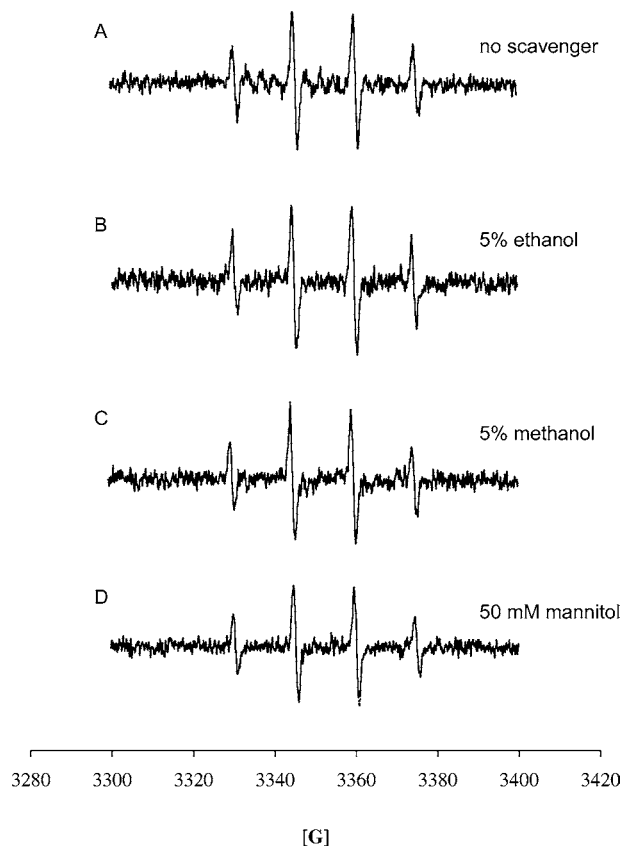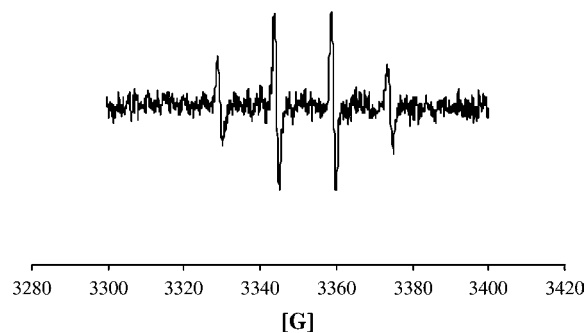The addition of OH radical scavengers to EO water did not yield DMPO-alkyl spin adducts (**Figures 3** and **4**). On the contrary, in the Fenton reaction control, the addition of ethanol inhibited the DMPO-OH signal and a new signal, characteristic of DMPO-CHOH-CH$_3$ was formed (**Figure 1B**). Moreover, when EO water was incubated with $Fe^{3+}$ for 5 min (i.e., much longer than any OH radical would persist), the DMPO-OH signal was still observed when DMPO was added to the incubation mixture (**Figure 5**). This observation provided further evidence that the DMPO-OH signal was not due to trapping of OH radicals. When acidic water (HCl solution, pH 2.2) was added to 0.25 mM $Fe^{3+}$ in the presence of DMPO (100 mM), a DMPO-OH spectrum was obtained through the nucleophilic

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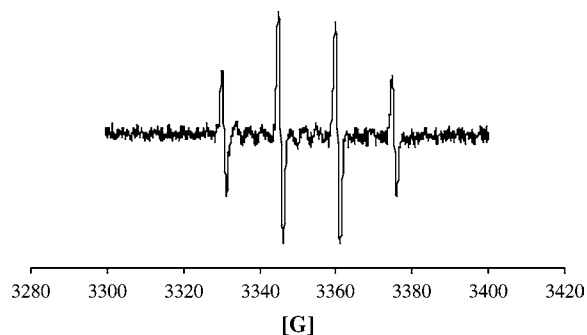
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**Figure 6.** ESR spectra observed from HCl solution (pH 2.2) + 0.25 mM $Fe^{3+}$ in the presence of 100 mM DMPO. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

addition of water to DMPO (**Figure 6**). Taken together, these observations indicated that the DMPO-OH spectrum observed in the reaction of EO water with iron ions in the presence of DMPO was derived from a nucleophilic addition of water to DMPO and not from trapping of OH radicals.

DMPO is used as a spin trap for oxygen-centered free radicals, such as $\bullet$OH and $O_2^-$. However, it has been reported that in the presence of iron ions, the DMPO-OH spectrum may be generated through a nucleophilic attack of water to DMPO (*20*). It has been proposed that $Fe^{3+}$ withdraws an electron from the double bond of DMPO, allowing nucleophiles to attack the positively polarized double bond of DMPO.
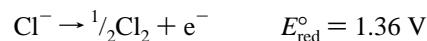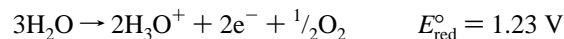
Proposed reaction (*20*):
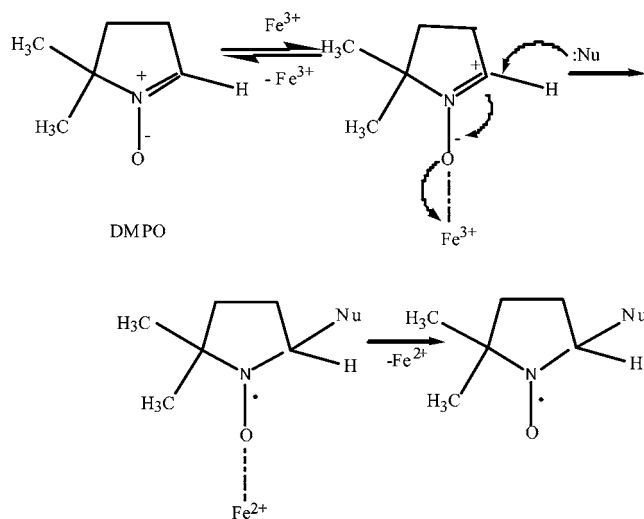


**Oxidizing Species in EO Water.** Hypochlorous acid has been reported to contribute to the high oxidizing potential of EO water (*21*). In our study, EO (SOW and HEW) water had a high ORP (+1063 and 1060 mV, respectively), a low pH (2.4 and 2.6, respectively), and a high level of dissolved oxygen (27 and 20 mg/L, respectively). The high ORP of EO water might also be due to the high level of dissolved oxygen, which was produced during electrolysis and dissolved as fine gas bubbles in EO water. In acidic solution, oxygen can act as a weak oxidizing agent. In a solution of pH 0, oxygen has a reduction potential of 1.229 V (*22*).

$$O_2 + 4H_3O^+ + 4e^- \rightarrow 6H_2O \qquad E^\circ_{red} = 1.229 \text{ V}$$
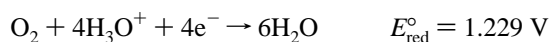
The anode and cathode reactions at the electrolysis of an aqueous

solution of NaCl are as follows:

Anode:

$$3H_2O \rightarrow 2H_3O^+ + 2e^- + {}^1/_2O_2 \qquad E^\circ_{red} = 1.23 \text{ V}$$

$$Cl^- \rightarrow {}^1/_2Cl_2 + e^- \qquad E^\circ_{red} = 1.36 \text{ V}$$

Side reaction:

$$Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^-$$

Cathode:

$$2H_3O^+ + 2e^- \rightarrow H_2 + 2H_2O \qquad E^\circ_{red} = 0 \text{ V}$$

$$Na^+ + 1e^- \rightarrow Na \qquad E^\circ_{red} = -2.71 \text{ V}$$

Side reaction:

$$Na + H_2O \rightarrow NaOH + {}^1/_2H_2$$
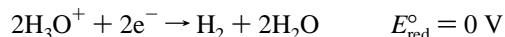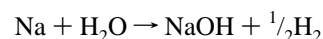
where $E^\circ_{red}$ represents the reduction potential in standard conditions (1 atm, 25 °C, and 1.0 M) for the half-cell reactions in the electrolysis of NaCl and water (*22*). The reactions that are favored to occur during electrolysis, and hence the outcomes of electrolysis, depend on several factors including the voltage applied between electrodes and the concentration of the electrolyte.

The molarity of the NaCl solution used to prepare the EO (SOW) water was 8.55 mM. For this concentration, the reduction potentials of the sodium and chloride half-cell reactions were calculated using the Nernst equation as $E(Na^+/Na) = -2.83$ V and $E(Cl_2/Cl^-) = 1.48$ V. This indicates that in ideal conditions of electrolysis (no energy is lost for the resistivity of the bulk electrolyte), $\Delta E = E_{cathode} - E_{anode} = -4.31$ V. The negative sign indicates that the anode and cathode reactions are not spontaneous and require external energy to occur.

Decomposition of $O_3$ or $H_2O_2$ could yield OH radicals. Ozone is a strong oxidant, and it is reduced to water and oxygen in an acidic aqueous solution (*22*), being characterized by a high reduction potential:

$$O_3 + 2H_3O^+ + 2e^- \rightarrow O_2 + 3H_2O \qquad E^\circ_{red} = 2.07 \text{ V}$$
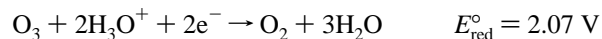
The electrolytic formation of ozone would therefore require a high voltage, the presence of a catalyst, or both.

$H_2O_2$ is unlikely to be formed in the anode chamber during the electrolysis of a NaCl solution. In an acid solution, $H_2O_2$ is thermodynamically unstable and will spontaneously disproportionate (*22*) and decomposes in the presence of chlorine species (*23*). Hydrogen peroxide can be electrolytically made from aqueous solutions of $H_2SO_4$, through the peroxidisulfuric acid ($H_2S_2O_8$) intermediary (*24*).

The results of this study indicate that the DMPO-OH spectrum generated as a result of the addition of $Fe^{3+}$ or $Fe^{2+}$ to EO water in the presence of DMPO was not formed through the free radical mechanism but through the nucleophilic addition of water to DMPO.

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