

5,5-Dimethyl-2-pyrrolidone-*N*-oxyl Formation in Electron Spin Resonance Studies of Electrolyzed NaCl Solution Using 5,5-Dimethyl-1-pyrroline-*N*-oxide as a Spin Trapping Agent

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Electrolyzed oxidizing (EO) water has recently generated much interest as a disinfectant in the food industry. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) is a spin trapping agent widely used in the electron spin resonance (ESR) characterization of oxygen-centered free radicals. The reaction between electrolyzed water, collected from the anode side of a two-chamber electrolyzer, and DMPO was investigated by ESR spectroscopy. Addition of DMPO to EO water generated an ESR spectrum identical to that of 5,5-dimethyl-2-pyrrolidone-*N*-oxyl (DMPOX), suggesting that a compound from EO water oxidized DMPO with the formation of DMPOX. To further investigate the electrolytically generated compound that oxidized DMPO, aqueous solutions of different sodium salts (sodium chloride, sodium citrate, and sodium iodide) with similar conductivities were electrolyzed. The DMPOX signal was not detected in the electrolyzed sodium citrate sample, suggesting that DMPOX formation in the electrolyzed NaCl sample might be due to an electrolytically generated chlorine species. A low DMPOX signal was also observed from the electrolyzed NaI sample, suggesting that a similar species obtained through the electrolysis of I⁻ can also oxidize DMPO. Hypochlorous acid is proposed to oxidize the spin trap DMPO with the formation of DMPOX. In a neutral pH environment, electrolyzed water also oxidized DMPO to DMPOX. This is consistent with the DMPOX formation in the reaction of chlorine water (containing HOCl and Cl₂) or sodium hypochlorite with DMPO.

KEYWORDS: Electrolyzed water; electron spin resonance spectroscopy; DMPO; DMPOX

INTRODUCTION

In recent years, electrolyzed oxidizing (EO) water has generated much interest as a disinfectant in the food industry (1, 2, 4–9). EO water is prepared through the electrolysis of a NaCl solution in the anode side of an instrument in which the anode and cathode are separated by an ion-permeable membrane. EO water is characterized by a low pH (ranging from 2.3 to 2.7) and high oxidation–reduction potential (ORP) (ranging from 1050 to 1100 mV) and exhibits strong bactericidal effect against a large number of Gram-positive and Gram-negative pathogenic bacteria. In antimicrobial suspension tests, the water collected at the anode side of a two-chamber electrolyzer has been reported to have strong bactericidal effects against *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enteritidis*, *Campylobacter jejuni*, *Bacillus cereus* (1, 2), *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Burkholderia cepacia*, *Serratia marcescens*, and *Pseudomonas aeruginosa* (3). EO water has been also tested as a means to disinfect fresh produce (4, 5), poultry (6), seeds used for sprouting (7, 8), and cutting boards (2, 9).

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) is a spin trapping agent frequently used in the electron spin resonance (ESR) characterization of oxygen-centered free radicals in biological systems (10). Free radicals are paramagnetic species containing an unpaired electron, which exerts a magnetic moment that is detected by ESR spectroscopy. In our previous study (11), the investigation of free radicals in EO water by ESR spectroscopy coupled with the spin trapping technique led to the detection of a seven-line spectrum, which has not been previously reported in the literature for the EO water system. Electrolysis of a NaCl solution generates, in the anode side of a two-chamber electrolyzer, acid-oxidizing water, which contains HOCl, Cl₂, and dissolved oxygen, and it is characterized by a low pH and a high ORP. In the present study, the effect of hypochlorous acid on the spin trapping agent was examined to determine the effect of this compound on the chemistry of the system.

This study reports on the formation of 5,5-dimethyl-2-pyrrolidone-*N*-oxyl (DMPOX) in the reaction of EO water with DMPO and investigates the formation of chlorine dioxide in EO water.

MATERIALS AND METHODS

Reagents. Chlorine water and sodium chloride were purchased from VWR International (West Chester, PA). Titanium trichloride solution (TiCl₃, 20%) and sodium iodide were purchased from EM Science

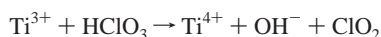
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(Cherry Hill, NJ). Potassium chlorate (KClO₃) and sodium citrate were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Clorox (sodium hypochlorite, 6%) was purchased from Clorox Corporation (Oakland, CA). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), ethanol, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma-Aldrich Co. (St. Louis, MO). 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). To remove any trace metal impurities such as iron, the sodium phosphate buffer 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 JED-007 Super Water Mini generator (Altex Janix, Kanagawa, Japan) from 0.05% (w/v) NaCl solution (conductivity 1.18 mS); a 10-min electrolysis time was used. The batch-type JED-007 generator was also used to electrolyze solutions of sodium citrate and sodium iodide with conductivities of 1.16 and 1.17 mS, respectively.

Preparation of ClO₂ radical. Chlorine dioxide was prepared as described by Ozawa et al. (12), by combining an acid solution of Ti³⁺ (2 mM) with KClO₃ (30 mM):



DMPO (91 mM) was added to the reaction mixture, and the ESR spectrum was recorded within 5 min.

Reaction of EO Water and Chlorine Water with DMPO. In acidic pH environment, EO water (0.9 mL) and DMPO (0.1 mL, 100 mM) were mixed, and the ESR spectrum was recorded within 5 min. In a neutral pH environment, EO water (0.45 mL) and Chelexed sodium phosphate buffer (0.45 mL, 90 mM, pH 7.2) were mixed with DMPO (0.1 mL, 100 mM), and the ESR spectrum was recorded within 5 min. Chlorine water (100 mg/L) and sodium hypochlorite (100 mg/L) were mixed with DMPO (100 mM), and the ESR spectra were recorded within 5 min. All concentrations are final.

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.

UV Scan. UV absorption scan of EO water was recorded from 200 to 400 nm with the use of a UV 2100PC UV-vis scanning spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD).

RESULTS

Reaction between EO Water and DMPO in an Acidic pH Environment. In acidic pH environment, EO (HEW) water reacted with DMPO (100 mM) with the formation of a seven-line spectrum (Figure 1A,B). This spectrum consists of a nitrogen triplet with each of the lines split into triplets in a 1:2:1 pattern. This ESR spectrum ($a_{\text{H}} = 4.0$ G and $a_{\text{N}} = 7.25$ G) is similar to that of 5,5-dimethyl-2-pyrrolidone-*N*-oxyl (DMPOX) reported previously (12), suggesting that DMPOX was formed in the reaction of EO water and DMPO.

Reaction between EO Water and DMPO in a Neutral pH Environment. The DMPOX spectrum was also obtained when the pH of the EO water was adjusted to 7.2 with sodium phosphate buffer (Figure 2). An aqueous DMPO solution (100 mM) and a solution of 0.05% (w/v) NaCl in the presence of

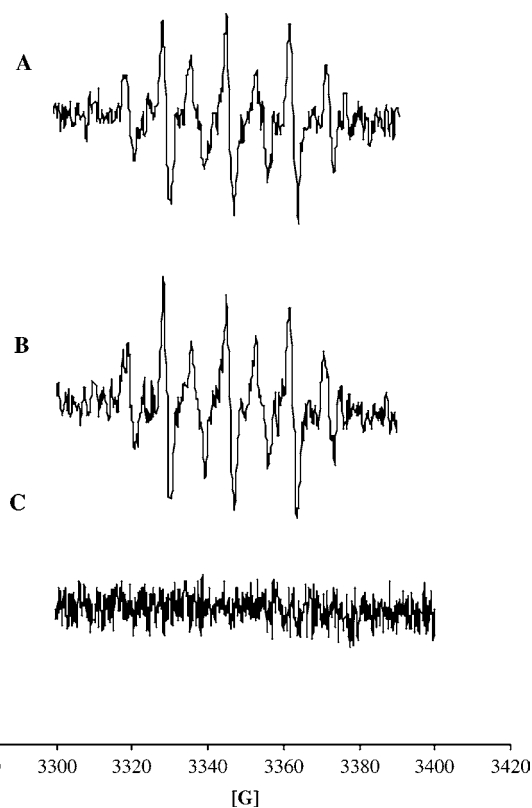


Figure 1. ESR spectra observed from EO (HEW) water (A) immediately after collection in the presence of DMPO (100 mM), (B) at 18 h after collection in the presence of DMPO (100 mM), and (C) immediately after collection, in the absence of DMPO. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

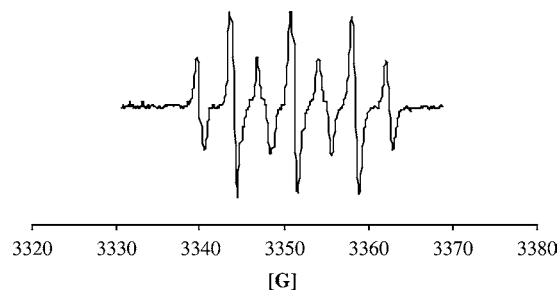


Figure 2. ESR spectra observed from the reaction between EO (SOW) water and DMPO (100 mM) in sodium phosphate buffer (pH 7.2). ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5; receiver gain, 5.00e+006.

100 mM DMPO showed no interfering ESR signals (spectra not included).

Electrolysis of Different Sodium Salts. To further investigate the involvement of the anion species in the electrolytic generation of a compound that oxidizes DMPO, aqueous solutions of different sodium salts with similar conductivity were electrolyzed (Figure 3). The DMPOX signal was not present in the electrolyzed sodium citrate sample, suggesting that the DMPOX formation in the electrolyzed NaCl solution might be due to a halogen-derived electrolytic species. A low DMPOX signal was also observed from the electrolyzed NaI solution suggesting that a similar species obtained through the electrolysis of iodide ion (I⁻) oxidizes DMPO.

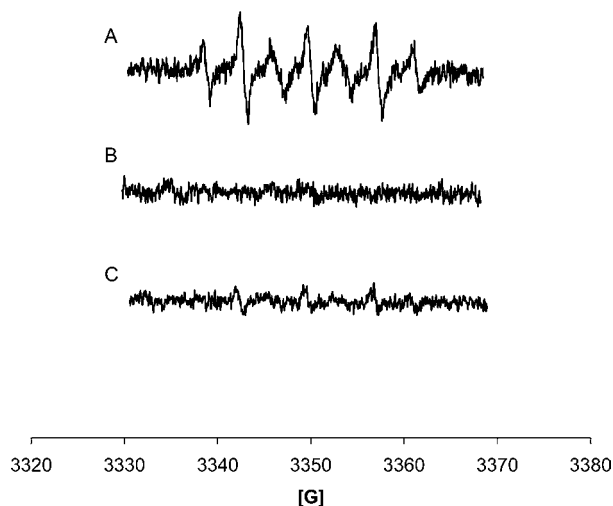


Figure 3. ESR spectra observed from the electrolysis of a solution of (A) sodium chloride, (B) sodium citrate, and (C) sodium iodide. All spectra were recorded in the presence of 100 mM DMPO. Electrolysis was performed using the batch type JED-007 electrolysis unit. The salt solutions had similar initial conductivities with values between 1.16 and 1.18 mS.

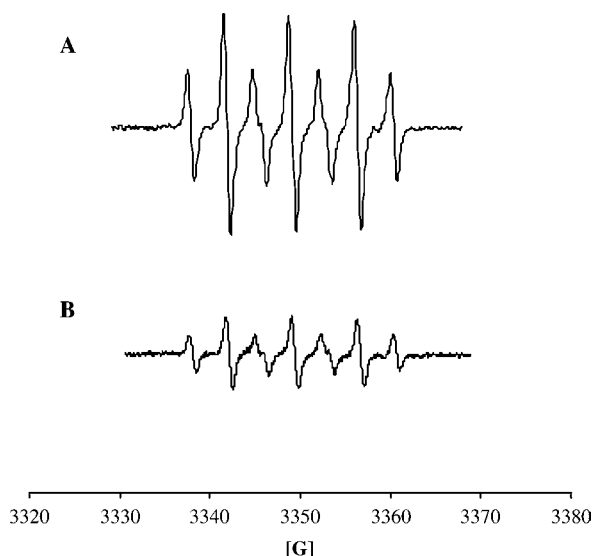


Figure 4. ESR spectra observed from (A) chlorine water (100 mg/L) and (B) sodium hypochlorite (100 mg/L) in the presence of 100 mM DMPO. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

A DMPOX spectrum was also observed in the reaction of chlorine water (pH 2.7) and sodium hypochlorite (pH 9.5) with DMPO (**Figure 4**). The electrolyzed water collected from the cathodic side (containing NaOH and dissolved hydrogen) did not yield an ESR spectrum in the presence of DMPO (data not shown).

An additional chlorine species was further investigated in the generation of DMPOX. Chlorine dioxide (ClO_2) is a relatively stable free radical, with a lifetime of more than 2 h, that has been shown to oxidize DMPO with the formation of DMPOX (*12*) (with the hyperfine splitting constants of $a_N = 0.72$ mT and $a_H = 0.41$ mT). As a control, chlorine dioxide was generated by the redox reaction of Ti^{3+} with KClO_3 (**Figure 5**). Chlorine dioxide absorbs UV light with an absorption maximum at 360 nm and has a molar absorptivity of $1100 \text{ M}^{-1} \text{ cm}^{-1}$ (*13*). UV scan of EO water did not indicate the presence of ClO_2 (**Figure**

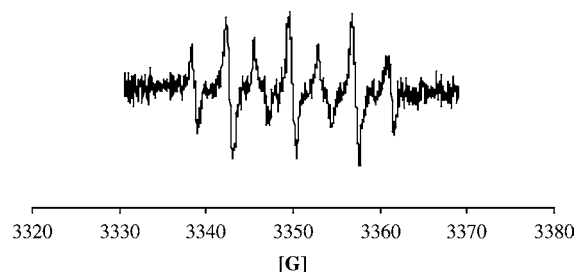


Figure 5. ESR spectrum observed from the reaction of 30 mM KClO_3 with 2 mM TiCl_3 in the presence of 91 mM DMPO. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

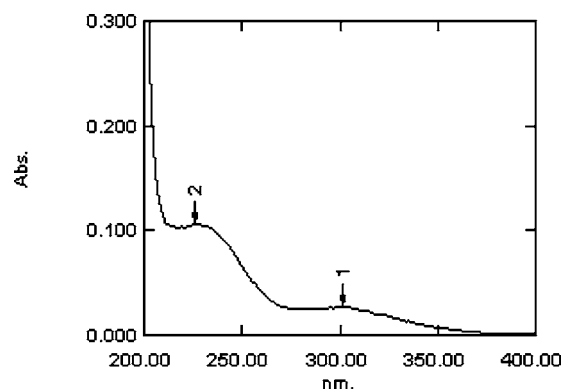


Figure 6. UV absorption scan of EO (HEW) water in a 1 cm path length. Deionized water was used as a reference. EO (HEW) refers to acidic electrolyzed oxidizing water obtained with the Hoshizaki electrolysis unit.

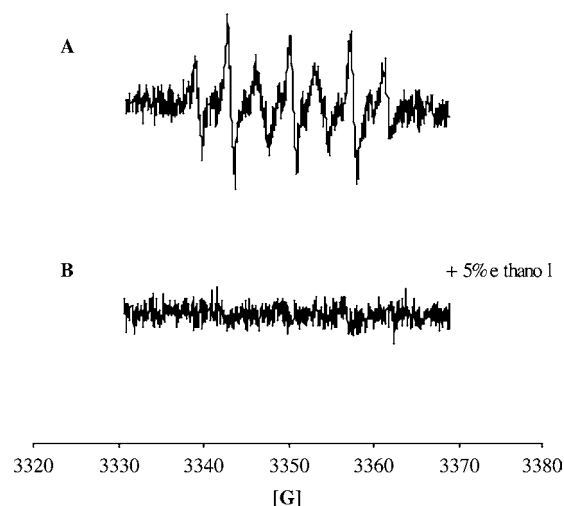


Figure 7. ESR spectra observed from (A) EO (SOW) water and (B) EO (SOW) water and 5% ethanol in the presence of 100 mM DMPO immediately after electrolysis. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

6). Two peaks, at 230 nm (assigned to HOCl) and 300 nm (assigned to ClO^-), were observed. Addition of ethanol (5%) prevented or reduced the oxidation of DMPO by EO water (**Figures 7 and 8**).

DISCUSSION

DMPO is a nitron spin trap widely used in the detection of oxygen-centered free radicals in biological systems. DMPO traps

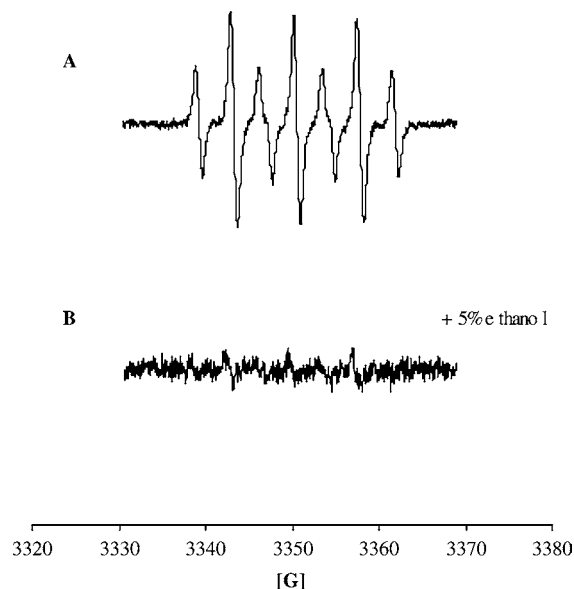


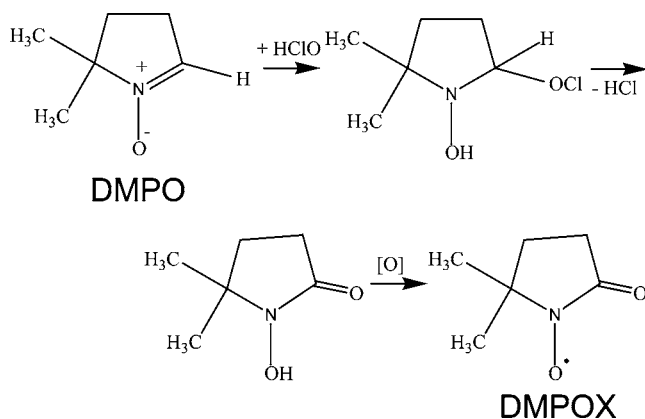
Figure 8. ESR spectra observed from (A) EO (HEW) water and (B) EO (HEW) water and 5% ethanol in the presence of 100 mM DMPO immediately after electrolysis. ESR parameters were as follows: modulation frequency, 10 kHz; modulation amplitude, 1 G; microwave power, 6 mW; time constant, 40 ms; scan rate, 40 s; number of scans, 5.

hydroxyl and superoxide radicals with the formation of nitron spin adducts, which are more stable and can be detected by ESR spectroscopy (14). However, the chemistry between DMPO and electrolyzed oxidizing water is not known. When DMPO was added to EO (HEW or SOW) water, a seven-line ESR spectrum was obtained. This ESR spectrum is identical to that of DMPOX, suggesting that a compound from EO water oxidized DMPO with the formation of DMPOX. DMPOX is a relatively stable free radical obtained through the oxidation of DMPO by a variety of compounds, including HOCl (15) and ClO₂ (12).

Hypochlorous acid is generated in the anode chamber by the hydrolysis of chlorine:



Electrochemically generated hypochlorous acid is considered to oxidize the spin trap DMPO with the formation of DMPOX. This is in agreement with Bernofsky et al. (15) who proposed the formation of DMPOX in the reaction of HOCl and DMPO:



In a neutral pH environment, anode electrolyzed water oxidized DMPO with the formation of DMPOX. This is consistent with

the DMPOX formation in the reaction of chlorine water (containing HOCl) or sodium hypochlorite with DMPO (Figure 4).

The possibility that the oxidation of DMPO was due to chlorine dioxide was also investigated. This free radical, known for its antimicrobial effect has a lifetime of more than 2 h and exhibits an ESR spectrum both by direct spectroscopy and with the use of spin trapping agents (DMPO, 3,3,5,5-Tetramethyl-1-pyrroline-*N*-oxide (M₄PO), and *N*-*t*-butyl- α -nitron (PBN)) (12). Direct ESR spectroscopy of aqueous solutions of ClO₂ gives a quartet signal with a line intensity ratio 1:1:1:1 and a hyperfine splitting of 1.85 mT centered at $g = 2.0106$ (12). Direct ESR spectroscopy of EO water did not indicate the presence of ClO₂ (Figure 1C). Moreover, UV scan of EO water did not show the presence of ClO₂ (Figure 6). Although ClO₂ radical was not detected in the tested EO water samples, the formation of ClO₂ is considered possible in the anode-electrolyzed water obtained through the electrolysis at a higher voltage. ClO₂ can be formed in the reaction of Cl₂ and ozone, or in the decomposition of ClO_x⁻ ($x = 2, 3$) in an acid environment. Ozone could be theoretically formed if a high voltage is applied between electrodes, a catalyst for ozone formation is used, or both.

In conclusion, the reaction of EO water with DMPO under both acid and neutral conditions yields the spin adduct DMPOX through the oxidation of DMPO by the chlorine species, hypochlorous acid, and hypochlorite ion.

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