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The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water

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Abstract

The Nernst equations between the oxidation–reduction potential (ORP), the concentration of hypochlorous acid and chlorine and the value of pH in electrolyzed oxidizing water (EOW) were developed in three parts, which were in agreement in the measured values. The role of ORP in EOW for killing *Escherichia coli* O157:H7 was studied. The inactivation effect of EOW on *E. coli* O157:H7 was also studied by spectroscopy measurements, and the inactivation mechanism was proposed.

Keywords: Oxidation–reduction potential (ORP); Electrolyzed oxidizing water (EOW); Inactivation effect; Inactivation mechanism

1. Introduction

Electrolyzed oxidizing water (EOW) has been reported to have strong bactericidal effects on many pathogenic bacteria, such as *Escherichia coli* O157:H7 (Kim, Hung, & Brackett, 2000, 2001), *Listeria monocytogenes* (Kim, Hung, Brackett, & Lin, 2003), *Bacillus cereus* (Buck, van Iersel, Oetting, & Hung, 2002; Iwasawa, Nakamura, Nakamura, & Murai, 1993; Shimizu & Hurusawa, 1992), and *salmonella* species (Fabrizio, Sharma, Demirci, & Cutter, 2002). In addition, it could disinfect *hepatitis B* virus and *human immunodeficiency* virus (Morita et al., 2000) and reduce germinations of many fungal species (Buck et al., 2002). EOW can also be used in agriculture for sterilization of fruits and vegetables (Al-Haq, Seo, Oshita, & Kawagoe, 2001), food materials and food processing materials (Al-Haq, Seo, Oshita, & Kawagoe, 2002; Izumi, 1999; Kim, 2003; Venkitanarayanan, Ezeike, Hung, & Doyle, 1999).

The major advantages of using EOW for inactivation of bacteria are less adverse environmental impacts and without the difficulties of transporting and storing potentially hazardous chemicals (Nakagawara et al., 1998). The preparation of EOW, often involves reactions in a cell containing the anode and cathode electrodes, separated by a membrane, and through which the dilute salt solution passes. Two types of water are generated: an electrolyzed basic solution (pH > 11 and ORP < −800 mV) produced from the cathode side and an electrolyzed acid solution (pH < 2.7 and ORP > 1100 mV) produced from the anode side, which was "EOW" in this study. The tap water was purified by SPMDs (Huckins et al., 2002) and reverse osmosis membrane, respectively.

In general, EOW is the solution: pH < 2.7, ORP > 1100 mV and free chlorine concentration of 10–80 ppm (Anonymous, 1997; Venkitanarayanan et al., 1999).

Although many studies showed that EOW could efficiently kill pathogens, there were few reports on the effects of ORP on the inactivation in the EOW. The primary aim of this study is to study the inactivation mechanism of ORP in EOW.

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2. Experimental

2.1. Preparation of electrolyzed oxidizing water (EOW)

EOW was prepared on an electrolyzed water generator S-3 (Sai'ai Corp., Guangzhou, China). The tap water was purified by a SPMDs and a reverse osmosis membrane (Fig. 1). The current density of anode electrode was about 0.4 A cm^{-2}. The salt concentration was about 0.2% in the electrolyzed chamber. The EOW (pH 2.5, ORP 1150 mV and free chlorine concentration 60 mg L^{-1}) was collected, otherwise mentioned. The values of ORP online performances were measured once every 5 s (included pH, ORP and free chlorine concentration). The values of pH and ORP of the EOW were measured on a pH/ORP meter (PHS-2C, Leici@, Shanghai, China) with a pHS-2A-type pH probe (Leici@, Shanghai, China) and an E-201-C-type ORP probe (Weiye@, Shanghai, China). The free chlorine concentration (or residue chlorine) was determined by iodometric method (American Public Health Association, 1975). The concentrations of hypochlorous acid and chlorine in Nernst equation were determined by the UV–Vis absorbance, which was performed in our previous literature (Liao, Zhou, & Xiao, 2005).

2.2. Preparation of E. coli O157:H7 culture

Nanopure water carbonate buffer pipette tips and all glassware used during the procedure were autoclaved at 121 °C for 60 min to avoid culture concentration. Five strains of *E. coli* O157:H7 (CAI, raw groud beef isolate; E0019, calf feces isolate; E09, beef isolate; 932 human feces isolate; F500, human feces isolate) were used here. Cultures were supplied three times to tryptic soy both (Microscopic Institute, Guangzhou, China), supplemented with 50 μg mL^{-1} nalidixic acid (A.R.) and 0.1% dextrose (A.R.) at 37 °C for 24 h. Nalidixic acid was used to minimize the growth of microorganisms other than *E. coli* O157:H7 in the enumeration media. The cultures of *E. coli* O157:H7 were centrifuged for 10 min (1500*g*, 24 °C) in a clinical centrifuge (PVP 6, Hongkong, China). The supernatant was decanted and the pellets were resuspended in 5 mL of 0.1% peptone water, before centrifuging again at 1500*g* for 15 min at 24 °C and then resuspended in 5 mL of 0.1% peptone water. Equal volume (2 mL) of five cultures was mixed to give an inoculum containing approximately 10log10 CFU mL^{-1} of *E. coli* O157:H7 procedures for inoculation. The cell inoculum *E. coli* O157:H7 (10log10 CFU mL^{-1}) was added to 100 mL EOW or modified EOW at 24 °C. After the 30 s treatment, the solution (1 mL) was serially diluted in sterile 0.1% peptone water and then was desposited on the tryptic soy agar. Inocula were all distributed on the agars. The petri-dishes were incubated for 2 h at 37 °C. Each sample was also inoculated into the tryptic soy broth to detect the presence of the number of survivors that would not be detected. Three independent replicate trials were conducted every time.

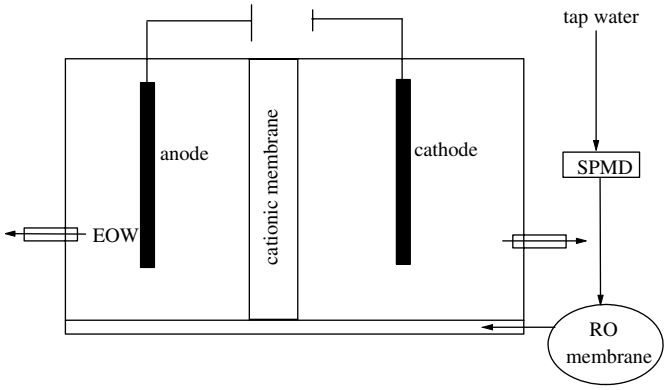

Fig. 1. The scheme of water-electrolyzing, which consisted of two chambers separated by a cationic membrane. Anode and cathode were installed in each chamber. The tap water was purified by SPMD and reverse osmosis (RO) membrane, respectively.

2.3. Electron microscopy and fluorescent measurements

Transmission electron microscopy (TEM) was observed on a Philips CM 300 UT/FEG microscope with a Shottky field emission gun operated at 80 kV and with an objective lens with a very short spherical aberration coefficient (0.65 nm). The survivors of *E. coli* O157:H7 were observed by American 3Y microscope spectrometer, which consisted of a 3Y (MSP UV–Vis 2000) spectrometer and a Germany Leica DMR-X microscope and a Nikon 1200 F camera with objective lens (Leica Germany 566501/50×) and ocular lens (Leica Germany 5.7808 and 5.7804). The percentage in *E. coli* O157:H7 population was determined as: %reduction = [initial count (CFU mL^{-1}) – final count (CFU mL^{-1})]/initial count (CFU mL^{-1}) × 100.

2.4. Integrity of cell membranes

E. coli O157:H7 membrane integrity was examined by the determination of the release of the material absorbing at 260 nm (A_{260}) (Chen & Cooper, 2002). *E. coli* O157:H7 cultures were harvested, washed and resuspended in 0.5% NaCl solution. The final suspension was adjusted to the absorbance at 420 nm (A_{420}) of 0.7. The *E. coli* O157:H7 solution (1.5 mL) was mixed with 1.5 mL of each *E. coli* O157:H7 suspension, and the absorbance of release materials at 260 nm or 420 nm was performed as in our previous literature (Liao et al., 2005)

2.5. Outer membrane permeabilization

Outer membrane (OM) permeabilization activity of *E. coli* O157:H7 was determined in the previous report (Loh, Grant, & Hancock, 1984; Ibrahim, Sugimoto, & Aoki, 2000). *E. coli* O157:H7 cultures were harvested, washed and resuspended in 0.5% NaCl solution. The final

suspension was adjusted to obtain an A_{420} of 1.0. The *E. coli* O157:H7 solution (1 mL)or distilled water was mixed with 20 μL of 1 mM 1-*N*-phenylnaphthylamine.

2.6. Inner membrane permeabilization

Inner membrane (IM) permeabilization was determined by measuring the release of cytoplasmic galactosidase from *E. coli* O157:H7 on the substrate of *O*-nitrophenyl-β-D-galactoside (Sudarshan, Hoover, & Knorr, 1992). Logarithmic-phase *E. coli* O157:H7 in the nutrient broth containing 2% lactose were harvested, washed and resuspended in 0.5% NaCl solution. The final suspension was adjusted to the volume of 1.2–1.5 mL, then was mixed with a suspension (1.5 mL) and a 30 mM *O*-nitrophenyl-β-D-galactoside acetate solution (150 μL).

2.7. Statistical analysis

All experiments were performed three times. The data from the independent replicate trials were pooled and the mean value and standard deviation were determined (Steel & Torrie, 1980). Data were analyzed using the general linear model procedures (Statistical Analysis System, 1985). A number 1 was assigned to log 10 CFU mL^{-1} value for statistical analysis purpose when either no bacteria were detected on the tryptic soy agar or the presence of bacteria was detected only by an enrichment procedure.

3. Results

3.1. The generation of ORP in EOW

The ORP of EOW was generally measured in the bulk of EOW, and had only an ORP value (Buck et al., 2002; Kim et al., 2000). To study the changing processes of ORP in EOW, the values of ORP were measured once every 5 s at room temperature (25 °C). The relations between ORP and electrolyzed time (keeping the different tap water flow rate, NaCl concentration and electrolyzed current density) are shown in Fig. 2. Three curves in Fig. 2A, B and C had identical features, containing curves 1, 2 and 3. The ORP values of curve 1 increased with the electrolyzed time to their maximal; those of curve 2 decreased with the electrolyzed time and then remained unchanged and those of curve 3 decreased with the electrolyzed time.

For curve 1, ORP values increased till their maxima. It is assigned to the chlorine evolution in water (Eq. (1)). Suggesting that after the electrolysis began, there is a reaction on the anode: $2Cl^- - 2e = Cl_2\uparrow$, and the chlorines reacted with water to get hypochlorous acid ($K_d = 4.0 \times 10^{-4}$). The corresponding ORP can be described by the Nernst equation:

$$2HClO + H^+ + e = 1/2Cl_2 + H_2O \quad (E^0_{ORP} = 1.63\ V) \tag{1}$$

$$E_{ORP} = E^0_{ORP} + 0.0592 \ln([HClO][H^+]/[Cl_2]) \tag{2}$$

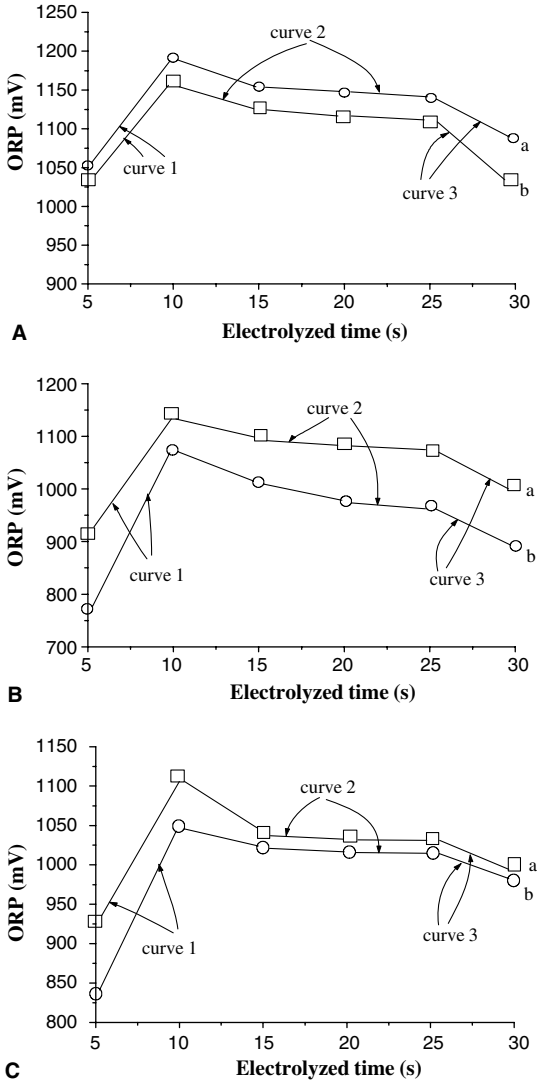

Fig. 2. The relationships of oxidation–reduction potential (ORP) dependent on electrolyzed time: (A) at different water flow rates (mL s^{-1}) a, 10 and b, 15; (B) at different NaCl concentrations (g L^{-1}) a, 0.2 and b, 0.15 and (C) at different current densities (A cm^{-2}) a, 0.4 and b, 0.3.

With increasing of hypochlorous acid in water, the ORP values (E_{ORP}) of the solution gradually increased (Eq. (1)), when chlorine was saturated in the solution, the concentration of hypochlorous acid reached its maximum (its decomposition can be neglected during the short time), leading E_{ORP} to attain its maximal value (Eq. (1)) (as curve 1 in Fig. 2). It was noted that, because of the values of ΔpH in the range of ±0.5 in course, it was reasonable that the effect of pH was neglectable.

For curve 2, the ORP values (E_{ORP}) gradually decreased and then remained unchanged (Fig. 2). It is assigned to the formation of hypochlorous acid. The corresponding Nernst equation was given:

$$HClO + H^+ + 2e = Cl^- + H_2O \quad (E^0_{ORP} = 1.56\ V) \tag{3}$$

$$E_{ORP} = E^0_{ORP} + 0.0296 \ln([HClO][H^+]) \tag{4}$$

When hypochlorous acid dominated over chlorine in water, the ORP values slightly decreased because of both different E^0_{ORP}. In addition, when hypochlorous acid concentration was maximal owing to its decomposition, the ORP values remained unchanged (Fig. 2). The neglect of pH was due to the same reasons as above.

For curve 3, because of the unsteadiness of hypochlorous acid (it easily ionized and decomposed, $pK_a = 7.5$), the ORP values gradually decreased (Eq. (4)). Many previous literatures also reported that the ORP values easily decreased in the storage of EOW (Al-Haq et al., 2001; Izumi, 1999; Kim et al., 2003; Nakagawara et al., 1998; Venkitanarayanan et al., 1999).

3.2. Inactivation of E. coli O157:H7

To study the effects of ORP in EOW on the inactivation of *E. coli* O157:H7, EOW 1 (pH 2.5; free chlorine concentration 50 mg L^{-1} and ORP 1150 mV) was original electrolyzed water, EOWs 2 and 3 were modified EOW 1 by adding iron, giving rise to the decrease of ORP and without affecting the pH value and residual chlorine concentration by addition of iron (Kim, Hung, Brackett, & Frank, 2001; Swaddle, 1997), and both were kept at 1105 and 800 mV, respectively. The inactivation results are shown in Fig. 3. The live *E. coli* O157:H7 had the fluorescence (Gilad, Clariel, Ronit, & Ervin, 2005). Therefore, 95%, 80% and 48% population of *E. coli* O157:H7 were killed within 10 s for EOWs 1, 2 and 3, respectively (Fig. 3), suggesting that higher ORP means higher efficiency of inactivation of *E. coli* O157:H7, which was in agreement with the previous report (Kim et al., 2001).

3.3. Electron microscopy shapes of E. coli O157:H7

The live *E. coli* O157:H7 cell had regular shape and membrane (outer and inner) (Fig. 4A). After treatment with EOW, the outer membrane was destroyed within 4 s (Fig. 4B), and the inner membrane was also damaged within 7 s (Fig. 4C).

3.4. UV–Vis absorption measurement

To further verify the inactivation of *E. coli* O157:H7, UV–Vis absorption measurements were performed. It was reasonable that if the membrane of *E. coli* O157:H7 was

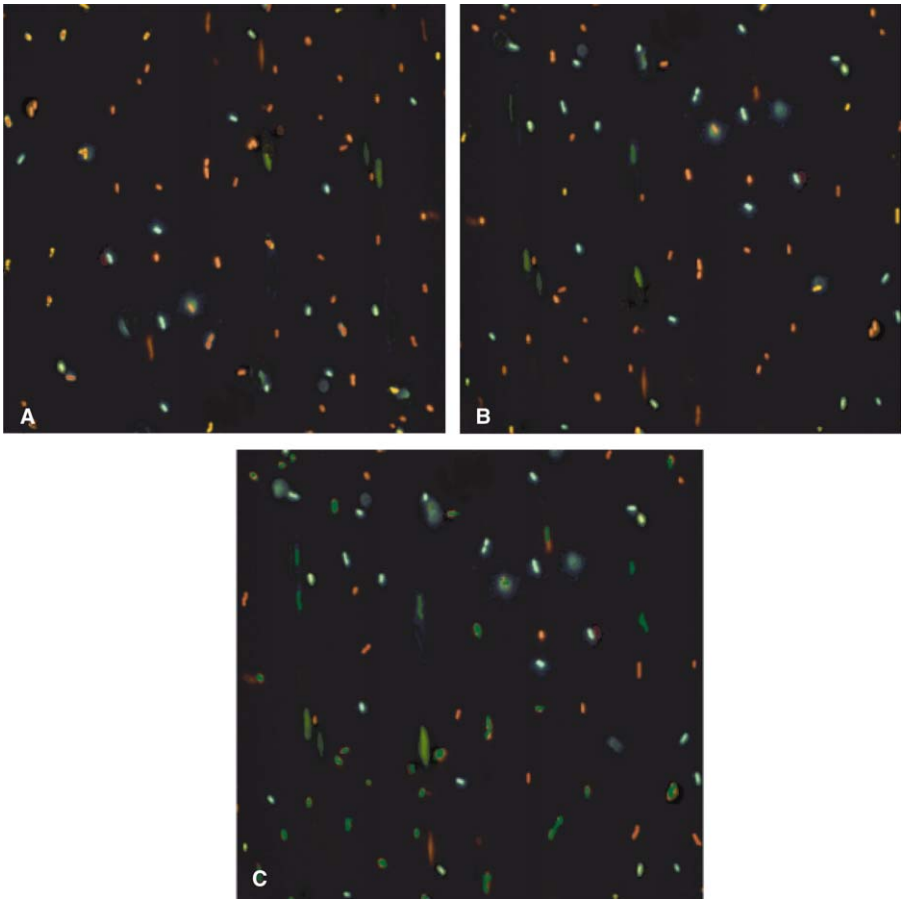

Fig. 3. Fluorescence microscopy of *E. coli* O157: H7, cells with integrity membranes showed green fluorescence, and those with damaged membranes exhibited orange-red fluorescence after treated within 10 s by electrolyzed oxidizing water (EOW) pH 2.5 and free chlorine concentration 50 mg L^{-1} for different oxidation–reduction potentials (ORP) (mV): (A) 1150; (B) 1105 and (C) 800.

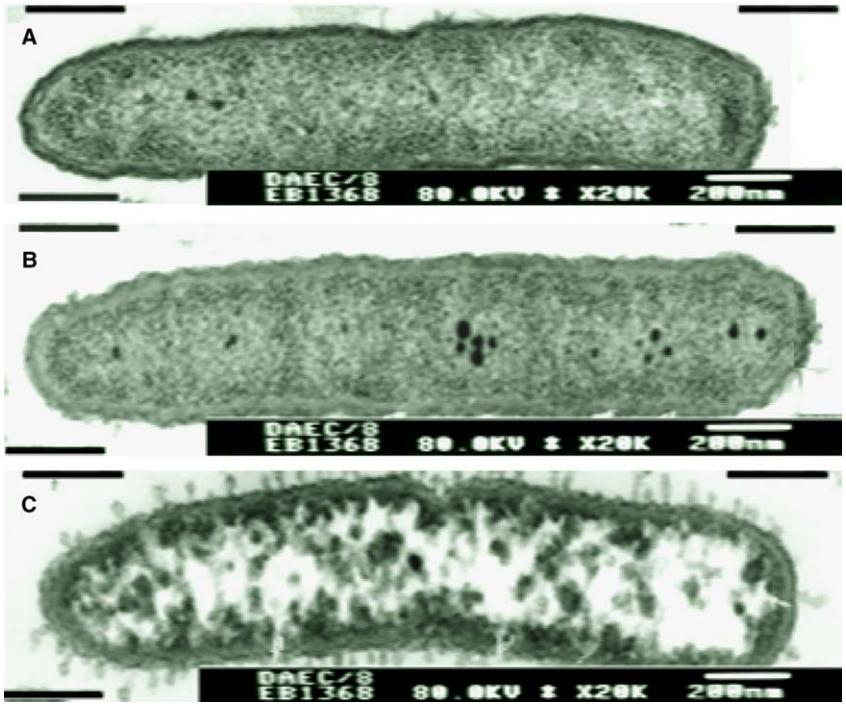

Fig. 4. TEM of *E. coli*: (A) without treatment by electrolyzed oxidizing water; (B) treated by electrolyzed oxidizing water within 4 s and (C) treated by electrolyzed oxidizing water within 7 s.

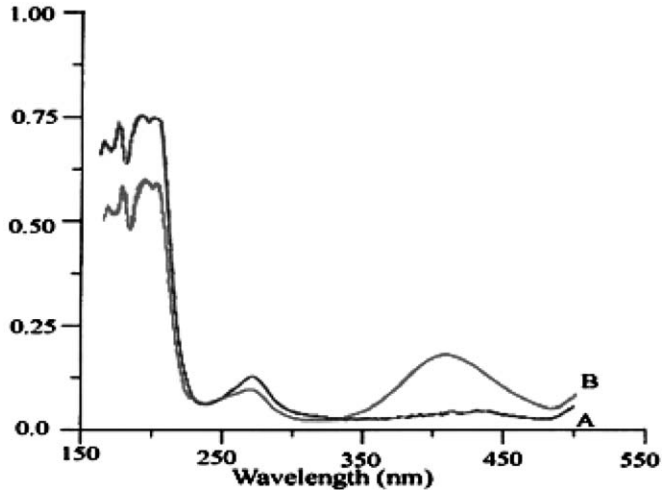

Fig. 5. UV–Vis absorbance spectra of *E. coli*: (A) treated with electrolyzed oxidizing water within 4 s and (B) treated with electrolyzed oxidizing water within 7 s.

damaged, there were the releases of intracellular components in *E. coli* O157:H7 cell within 4 s, as shown in Fig. 5A, the absorbance at 260 nm was due to the damage of outer membrane (Chen & Cooper, 2002), this was in good agreement with the result of fluorescent experiment. Within 7 s, as shown in Fig. 5B, the absorbance or A_{420} nm increased, suggesting that the inner membranes were damaged, it was due to the releases of cytoplasmic β-galactosidase from *E. coli* O157:H7 cells.(Liu, Du, Wang, & Sun, 2004; Chen & Cooper, 2002), this was also in agreement with the results of the above fluorescent experiment.

4. Discussion

In order to verify the proposed generation mechanism of ORP in EOW above, it was necessary to calculate some ORP data in the curves 1, 2 and 3 according to Eqs. (2) and (4). In an attempt to distinguish hypochlorous acid with hypochlorite ions in the water (iodometric method was not suitable), UV–Vis absorbance measurement was performed: the former had λ_{233} and the latter had λ_{290} (Aoki & Munemorl, 1983), and chlorine had λ_{229} (Aoki & Munemorl, 1983). Based on Table 1, the predicted values of ORP (from Eqs. (2) and (4)) were near to those measured (from Fig. 2) in curves 1, 2 and 3. The deviation was due to the fact that the measured concentrations of hypochlorous acid and chlorine were total concentrations, and the concentration in Eqs. (2) and (4) was the respective equilibrium concentrations and also due to the fact that the value of pH was regarded to remain unchanged. Therefore, the proposed Nernst equations of curves 1, 2 and 3 were reasonable.

ORP in EOW could damage OM and IM of *E. coli* O157:H7 based on the spectroscopic results, giving rise to the release of intracellular component till its inactivation. We proposed that the inactivation mechanism could be due to the perturbation of half-reaction:

$$\mathrm{GSSG} + 2\mathrm{H}^+ + 2\mathrm{e} = 2\mathrm{GSH} \quad (E^0 = -260\,\mathrm{mV}) \tag{5}$$

Cellular redox signaling (also termed 'redox cell signaling') contributed to the control of cell development, differentiation, growth, death and adaptation, and has been different in the various physiological and pathological

Table 1
Comparison of oxidation–reduction potential (ORP) between predicted values and measured values

Electrolyzed conditions[a]		Measured ORP (mV) at different times (s)						Predicted ORP (mV) at different times (s)[b]					
		5	10	15	20	25	30	5[c]	10[c]	15[d]	20[d]	25[d]	30[d]
Water flow rate (mL s^{-1})	10.0	105	120	115	114	114	112	105	121	116	116	116	104
		0	0	1	9	8	4	9	2	8	3	0	8
	15.0	103	115	112	112	112	104	103	115	113	113	113	103
		2	2	6	3	3	8	3	0	0	4	3	0
NaCl concentration (g L^{-1})	0.15	783	105	101	101	100	914	776	106	101	101	100	926
			8	3	0	9			0	0	5	0	
	0.20	917	102	110	110	110	104	920	103	112	112	111	102
			7	7	6	5	9		0	1	0	8	8
Current density (A cm^{-2})	0.30	836	104	103	103	103	989	844	105	104	104	103	993
			9	1	0	0			2	4	0	9	
	0.40	935	112	104	104	104	100	937	112	105	105	105	101
			0	9	8	8	1		8	5	0	0	4

[a] Other experimental conditions remain identical.
[b] Using the corresponding Nernst equation; the concentrations of hypochlorous acid, hypochlorite ion and chlorine were measured with UV–Vis absorbance; temperature kept at 25 °C.
[c] Using Eq. (2) in the text, pH 3.2.
[d] Using Eq. (4) in the text, pH 2.5.

processes (Levonen et al., 2001). A redox reaction was one that involved in the transfer of electrons: redox centers became reduced (gain electrons) or oxidized (lose electrons). The typical redox reaction involved in cell signaling was the breaking or formation of a protein disulfide bridge, converting cysteine into cystine (R–S– + R–S– = R–S–S–R + 2e) (Pomposiello & Demple, 2001; Zheng, 1998). The redox state of the glutathione disulfide-glutathione couple (GSSG/2GSH) could serve as an important indicator of *E. coli* O157:H7 redox environment. Although there were many redox couples in *E. coli* O157:H7 cells that work together to maintain the redox environment; the GSSG/2GSH couple was the most important redox couple. The moderately and severely oxidizing environments could result in apoptosis and necrosis, respectively. It was reasonable because apoptosis required the energy to carry out the organized program of a cell in the formation of ATP (Liu, Kim, Yang, Jemmerson, & Wang, 1996; Richter, Schweizer, Cossarizza, & Franceschi, 1996). A severely oxidative stress would deplete the energy stores and damage the machinery that produced the energy needed to implement the structural changes associated with apoptosis. In addition, the oxidative stress lead to a more oxidizing (positive) cell redox potential, resulting in the formation of disulfide bridges and consequent changes in the protein structure and function.

Based on the generation reasons of ORP in EOW, Eq. (3) was the primary reaction, in which $E^0 = 1560$ mV. Eq. (5) had $E^0 = -260$ mV (Alberty & Goldberg, 1993), thus, both Eqs. (3) and (5) easily constituted a presumable full cell reaction on theory, that is:

$$2HClO + 2GSH = GSSG + H^+ + H_2O + Cl^- \quad (6)$$

Therefore, it was possible that high ORP values in EOW lead to the oxidation of GSH, and destroyed the structures and functions of *E. coli* O157:H7. In addition, the redox environment was not suitable to the existence of *E. coli* O157:H7, because it had a redox potential of −280 mV (Brookes & Darley-Usmar, 2002). This was also reported in other literatures, for example, apoptosis in various cells was induced by exposure to low levels of H_2O_2; at higher levels necrosis was induced (Buttke & Sandstrom, 1994; Lee & Shacter, 1997). In leukemia cells, 9–30 mM H_2O_2 induced apoptosis; concentrations of 100 mM result in necrosis (Wagner, Buettner, Oberley, Darby, & Burns, 2000). It was found that apoptosis was related to a change in reduction potential of +72 mV in HL-60 cells (i.e., E increased from -239 ± 6 to -167 ± 9 mV) or a change of +186 mV in HT29 cells (Cai & Jones, 1998). Likely, it was possible that ΔE_{ORP} of Eq. (6) was more than +100 mV, resulting in the inactivation of *E. coli* O157:H7.

5. Conclusion

Compared with modified EOW, the role of ORP in EOW was studied. The results of electron microscopy and fluorescent measurements showed that ORP could damage the outer and inner membranes of *E. coli* O157:H7, thus leading to the inactivation of *E. coli* O157:H7. The inactivation mechanism was proposed that: ORP could affect and damage the redox state of GSSG/2GSH first, and penetrated the outer and inner membranes of *E. coli* O157:H7, then resulted in the necrosis of *E. coli* O157:H7.

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