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Locally Enhanced Electric Field Treatment (LEEFT) Promotes the Performance of Ozonation for Bacteria Inactivation by Disrupting the Cell Membrane

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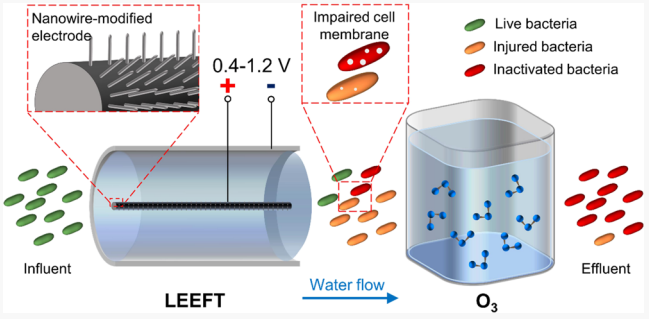
ABSTRACT: The adoption of ozonation for water disinfection is hindered by its high ozone demand and the resulting high cost. Electric field treatment inactivates bacteria by physically disrupting the integrity of the cell membrane. Assisted by nanowire-modified electrodes, locally enhanced electric field treatment (LEEFT) reduces the required voltage to several volts to induce sufficient electric field strength for efficient bacteria inactivation. In this study, the LEEFT is applied as a pretreatment of ozonation for bacteria inactivation. Our results show that a low-voltage (<0.4 V) LEEFT has no obvious effect on the following ozonation, but a higher-voltage (0.6−1.2 V) LEEFT significantly enhances the ozone inactivation. After the LEEFT, a large number of viable cells with impaired cell membranes are observed, shown by both selective plate count and staining methods. The mechanism inducing the enhancement is explained by the initially reparable pores generated by LEEFT that cannot recover in the subsequent ozonation and the greater intracellular diffusion of ozone after the membrane disruption induced by LEEFT. The application of LEEFT as a pretreatment process is beneficial to reduce the ozone dosage and disinfection by-product formation with a broader inactivation spectrum, which facilitates the application of ozonation in primary water disinfection.



1. INTRODUCTION

Disinfection is a water treatment process designed to inactivate waterborne pathogens and enhance overall public health.[1] Multiple techniques have been developed and employed in industrial applications, including chemical disinfectants (e.g., chlorine, chloramines, chlorine dioxide, and ozone (O_3)) and physical processes (e.g., UV, membrane filtration, and pulsed electric field treatment).[1−3] With the increase of the public awareness on water security and the understanding of disinfection techniques, expectations on the next-generation disinfection techniques have been evoked: effective inactivation of a broad spectrum of pathogens should be pursued with minimum use of chemicals and low impact on the environment (especially the production of by-products).[4] The future techniques should also be robust during natural disasters, versatile to be adopted in different scales (e.g., point-of-use), and provide residual antimicrobial power if water distribution systems are required.[5] Common strategies to realize the above goals are to improve the existing techniques, develop alternative disinfection methods, and apply multibarrier approaches.[4]

O_3 has been widely studied to inactivate pathogens in water because of its strong oxidation power and the nontoxic product (oxygen).[1,6,7] Multiple studies have revealed the mechanisms of O_3 disinfection, which primarily targets the cell membrane.[8−14] O_3 reacts with the membrane glycoproteins and glycolipids, affects the membrane-bonded enzymes, oxidizes the double bonds, and thus destroys the membrane functions.[8] The membrane permeability increases, which leads to the leakage of intracellular substances. Subsequently, O_3 can further react with the leaked macromolecules such as protein, DNA, and enzymes.[12] O_3 also attacks the cell wall and causes cell lysis.[14]

Ozonation has been combined with other processes for water disinfection. The combined disinfection can be operated as a simultaneous process (to use more than one disinfectant at the same time, denoted as A/B, where A and B are two independent processes) or a sequential process (to use one disinfectant after another, denoted as A-B, which means the B process is followed by the A process). The combination of ozonation with other processes is mostly motivated by three reasons. First, some pathogenic microorganisms cannot be inactivated effectively by a single step of disinfection. For

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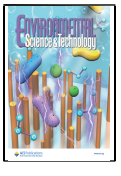

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example, poor disinfection performance has been observed on common chlorination to treat protozoa (e.g., *Cryptosporidium parvum*) and bacterial spores (e.g., *Bacillus subtilis*).[15−17] The involvement of O_3 has demonstrated the capability to inactivate a broad spectrum of pathogens with elevated efficiency.[17,18] Second, in the water distribution system, residual antimicrobial power (secondary disinfection) should be maintained, which cannot be provided by some well-established technologies.[1] This is due to the unstable nature and short lifetime of O_3 in water.[18−21] To tackle this problem, the combinations of O_3 with chlorine or chloramines have been studied, where chlorine or chloramines serve as secondary disinfectants.[18,19] Third, the combined disinfection of particular sets of disinfectants has shown a synergistic effect (in an A/B process) or enhanced performance (for B in an A-B process), achieving a higher level of inactivation than the sum of levels achieved by the individual disinfectant (A and B).[15,22−24] Overall, this can be beneficial to the reduction of operation cost, treatment time, chemical dosage, energy consumption, and/or disinfection by-product (DBP) formation.[16,20,25] The studies of O_3-related combined disinfection in the recent 20 years are summarized in Table S1.

Researchers including the authors' group have been developing a novel nanomaterial-enabled water disinfection technology, locally enhanced electric field treatment (LEEFT).[26,27] Electrodes with vertically grown conductive nanowires are used in LEEFT disinfection.[28] When a voltage is applied between the two electrodes, the electric field strength is enhanced dramatically near the nanowire tips due to the lightning-rod effect.[26,29] Such high electric field strength is able to induce irreversible electroporation on the cell membrane and thus inactivate pathogens.[30] As a refined form of the pulsed electric field treatment (PEFT), the LEEFT is considered a physical process that generates no DBPs in theory. Meanwhile, LEEFT reduces the voltage for effective inactivation from several kilovolts to only several volts, which dramatically reduces the energy consumption and equipment complexity.[28,29]

As both LEEFT and ozonation processes primarily target the membrane structures of bacterial cells, it is intriguing to study the combined disinfection with these two processes. It is hypothesized that in addition to directly inactivating bacteria by lethal irreversible electroporation, LEEFT induces sublethal damage on the cell membrane, making bacterial cells easier to be inactivated by ozonation. In this study, the LEEFT is thus applied as a pretreatment of ozonation (LEEFT-O_3) to inactivate bacteria in water. The LEEFT-O_3 process demonstrates higher inactivation efficiency than the sum of each individual process, against both Gram-positive (G+) and Gram-negative (G−) bacteria. The sublethal population with cell membrane damage is quantified by selective plate count and flow cytometry, and the probable mechanisms to cause the enhanced ozonation are proposed.

2. MATERIALS AND METHODS

2.1. Bacterial Culturing and Water Sample Preparation. Two strains of model bacteria, *Escherichia coli* (ATCC 10798, Gram negative) and *Staphylococcus epidermidis* (ATCC 14990, Gram positive), were used. *E. coli* and *S. epidermidis* were incubated in LB and nutrient broth (BD Difco), respectively, for 12−18 h at 35 °C on a shaker (200 rpm). After the incubation, the bacteria were in the stationary phase with a concentration of ∼10^9 CFU/mL. To prepare the bacterial samples for disinfection experiments, the bacterial culture was centrifuged at 5000 rpm for 5 min, the supernatant was removed, and then the remaining bacterial pellet was resuspended using deionized (DI) water (18.2 MΩ/cm; Thermo Scientific Barnstead Nanopure). This washing procedure was repeated three times. Subsequently, the washed bacteria suspension was diluted using DI water with a 1:100 ratio to obtain bacterial water samples with an initial concentration of ∼10^7 CFU/mL.

2.2. LEEFT Disinfection Device. A tubular coaxial-electrode device was constructed to conduct the LEEFT disinfection (Figure S1).[31,32] It consisted of a copper (Cu) wire center electrode, a coaxial cylindrical outer electrode made of Cu, and a plexiglass holder (McMaster-Carr). The length of the device was 12.7 cm. The diameter of the outer and inner electrode was 0.95 cm and 76 μm, respectively. The Cu wire center electrode was modified with polydopamine-coated copper oxide nanowires (PDA-CuONWs) through procedures reported previously (see details in Text S1.1 and the morphology in Figure S2).[33]

2.3. LEEFT Disinfection Procedures. The LEEFT disinfection was conducted by allowing the bacterial water samples through the LEEFT device with a fixed flow rate (4 mL/min, corresponding to a 2.5 min hydraulic retention time (HRT)). A direct current (DC) voltage was applied between the two electrodes using a Keithley 2400 SourceMeter. The applied voltage for LEEFT was 1.0 V by default but varied from 0 to 1.2 V to study the effect of LEEFT intensity. The electric current monitored simultaneously using the SourceMeter was lower than 100 μA under all conditions tested. The increase of temperature in the effluent was not detected throughout the experiments. The treated water samples (10 mL) were collected in 15 mL centrifuge tubes for the following O_3 treatment.

2.4. Ozone Disinfection Procedures. Gaseous O_3 was generated using a corona discharge generator (A2Z ozone generator, MP-1000) and bubbled in a chilled (ice bathing) flask of DI water to make the predissolved stock solution.[34] The dissolved O_3 concentration was determined by the indigo colorimetric method (Standard Method 4500 O_3 of the American Public Health Association).[35] For the O_3 disinfection, a certain amount of O_3 was injected into the water samples followed by gentle shaking of the centrifuge tube. After reacting for 2 min, 1 mL of sodium thiosulfate ($Na_2S_2O_3$, 1 mol/L; Sigma-Aldrich) was added to quench the residual O_3 and terminate the reaction.[34] The range of the ozone dosage (0.02−0.12 mg/L) was determined by the preliminary results (Figures S3−S5 and Text S2). The principle of selecting the ozone dosage was that the ozonation generated moderate inactivation of the bacteria so that the effect of LEEFT pretreatment could be easily observed. When the effect of LEEFT intensity (i.e., applied voltage) was studied, the O_3 dosage was fixed at 0.08 mg/L.

2.5. Quantification of Bacterial Concentration. The bacterial concentration was measured by the standard plate count following the standard method (9215 heterotrophic plate count) of the American Public Health Association with a detailed description in Text S1.2.[36] The quantification of sublethal population after treatment was performed using the selective plate count.[37] Specifically, the selective plates were prepared by adding 2% sodium chloride (NaCl; Sigma-Aldrich) to the regular agar solution before being autoclaved. The reason for selecting 2% was that such salt concentration

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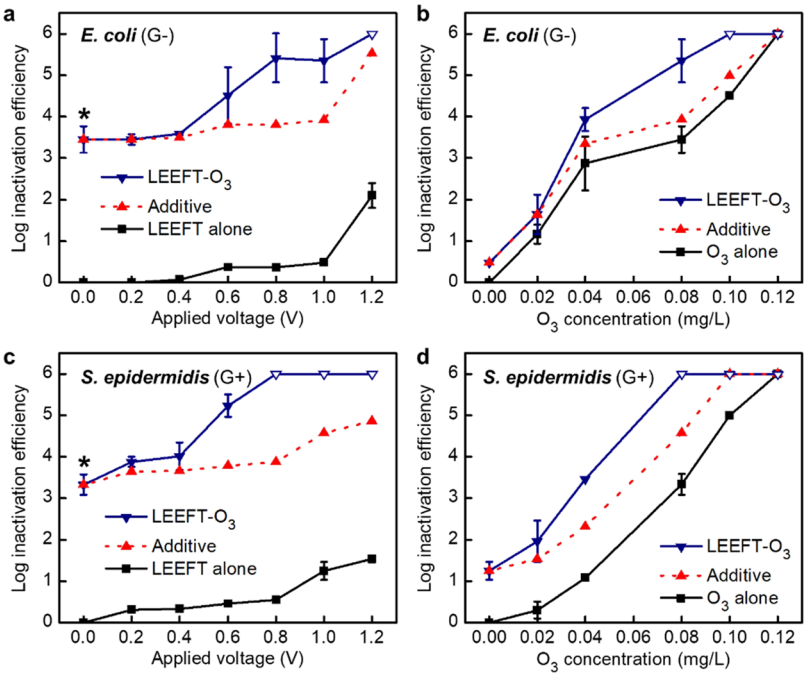

Figure 1. Enhanced disinfection effect using the LEEFT-O_3 process. (a, b) Log inactivation efficiency of *E. coli* (a) with a constant O_3 concentration (0.08 mg/L) and various applied voltages of LEEFT and (b) with a constant applied voltage (1.0 V) in LEEFT and various initial O_3 concentration. (c, d) Log inactivation efficiency of *S. epidermidis* (c) with a constant initial O_3 concentration (0.08 mg/L) and various applied voltages of LEEFT and (d) with a constant applied voltage (1.0 V) in LEEFT and various O_3 concentration. For the log inactivation efficiency, a solid symbol (e.g., solid inverted triangle) indicates the exact value, while a hollow symbol (e.g., hollow inverted triangle) indicates an inactivation efficiency of >6-log (the detection limit), and no bacteria colony grew on the agar plates. The symbol "*" indicates the control experiments of the treatment by O_3 only (0.08 mg/L dosage).

did not affect the growth of untreated bacteria and, at the same time, distinguished the injured cells (Figure S6). The log inactivation efficiency (η_1) was determined by the influent and effluent bacterial concentrations (c_{in} and c_{eff}, respectively) using regular agar plates ($\eta_1 = -\log_{10}(c_{eff}/c_{in})$). The sum of inactivation and injury efficiency (η_{sum}) was determined using the selective agar plates ($\eta_{sum} = -\log_{10}(c_{eff\text{-}s}/c_{in\text{-}s})$, where the $c_{eff\text{-}s}$ and $c_{in\text{-}s}$ are the effluent and influent bacterial concentration quantified by the selective agar plates). Thus, the log injury efficiency (η_2) was calculated by subtracting the log inactivation efficiency from the summed efficiency ($\eta_2 = \eta_{sum}\text{-}\eta_1$).

2.6. Flow Cytometry Analysis. The bacterial water samples were analyzed by the flow cytometry cooperated with fluorescent staining (SYTO 9 and propidium iodide (PI)). This method is for the detection of the sublethal bacterial population with the damaged cell membrane that allows the penetration of the PI dye. A detailed description of the experiments can be found in Text S1.3.

3. RESULTS AND DISCUSSION

3.1. Disinfection Performance of the LEEFT-O_3 Process. The inactivation efficiency of bacteria during disinfection with the LEEFT-O_3 process was compared with that of each individual treatment as a function of applied voltage (as the LEEFT intensity) and O_3 dosage (as ozonation intensity) (Figure 1a–d). Less than 1-log inactivation of *E. coli* was observed when the applied voltage was under 1 V, while a significant efficiency increase to ~2-log was achieved with 1.2 V (Figure 1a, black line). The inactivation efficiency elevated after the O_3 treatment (Figure 1a, blue line). The red dashed line in Figure 1a indicates the direct sum of log inactivation efficiencies of LEEFT and ozonation. If the blue line (experimental results of LEEFT-O_3) approximately overlaps with the red line, it means that LEEFT does not affect the subsequent O_3 disinfection. On the other hand, if the blue line significantly deviates upward from the red line, an enhanced ozonation performance is found, and the value of the offset indicates the extent of the enhancement. The log inactivation efficiency of LEEFT and ozone is addible based on the fact that the inactivation efficiency of ozone is independent of the initial bacteria concentration (see detailed discussion in Text S3 and Figure S7). As shown in Figure 1a, the LEEFT pretreatment poses almost no effect on the subsequent ozonation at lower voltages (0.2 and 0.4 V). Nevertheless, the LEEFT enhances the ozonation when the applied voltage is higher (0.6–1.2 V). Notably, an increase of ~1.5-log inactivation was achieved with 0.8 V. According to previous LEEFT studies, an applied voltage of less than 1 V cannot contribute to satisfying disinfection (>4-log) and thus is considered not applicable for LEEFT.[29,33] Nevertheless, according to the findings in this study, a voltage higher than 0.4 V is effective for pretreatment, boosting the disinfection efficiency of the following ozonation.

The effect of LEEFT pretreatment (1.0 V) on ozonation with different O_3 dosages (0–0.12 mg/L) was further studied. As shown in Figure 1b (black line), the inactivation efficiency increases with O_3 dosage when it is applied alone, which agrees with previous studies.[9,15] The LEEFT pretreatment demonstrates a notable enhancement effect as the increment of the inactivation efficiency between the direct sum of LEEFT and ozonation (red dashed line) and the observed experimental results of LEEFT-O_3 (blue line). For example, the LEEFT-O_3 process with a 0.08 mg/L O_3 dosage achieved a >5-log inactivation, which is ~1-log higher than the direct sum and

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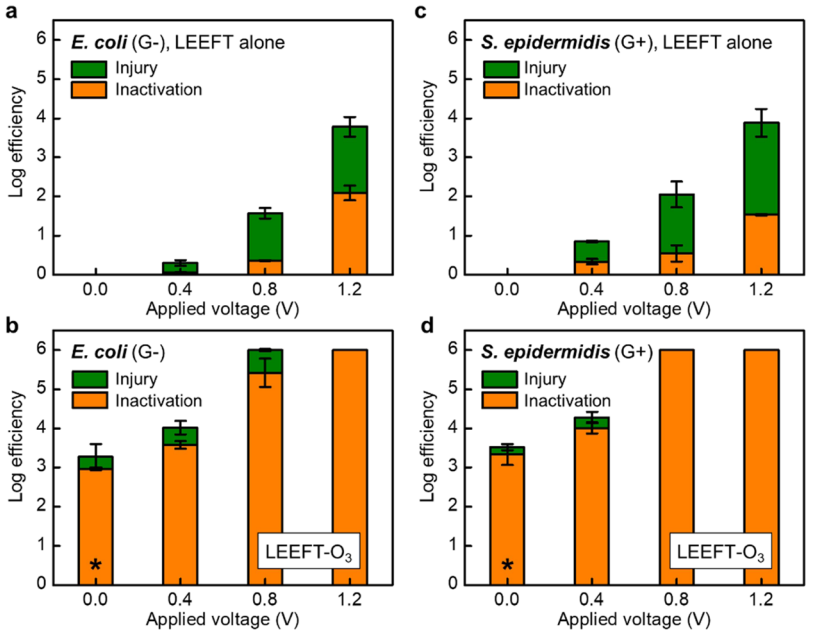

Figure 2. Characterization of cell membrane damage after LEEFT alone and LEEFT-O_3 by selective plate count. (a, b) Injury (indicating the injured-but-not-inactivated population) and inactivation (indicating the inactivated population) efficiency of *E. coli* after (a) LEEFT alone and (b) LEEFT-O_3. (c, d) Injury (indicating the injured-but-not-inactivated population) and inactivation (indicating the inactivated population) efficiency of *S. epidermidis* after (c) LEEFT alone and (d) LEEFT-O_3. The upper detection limit of the inactivation and injury efficiencies is 6-log. The symbol "*" in the figures indicates the control experiments of the treatment by O_3 only (0.08 mg/L dosage).

~1.5-log higher than the efficiency of the stand-alone ozonation.

The observation of the enhancement effect is hindered in the higher inactivation efficiency zone due to the maximum detection limit of the bacterial concentration in this study. For example, in Figure 1b, when the O_3 dosage is 0.12 mg/L, the three lines (blue, red, and black) overlap at the inactivation efficiency of 6-log. This does not mean no enhancement effect. Instead, the LEEFT-O_3 process is expected to achieve more than 6-log inactivation of *E. coli* but just cannot be examined with the current experimental setup. This limitation applies to all the results obtained from the plate count method in this study (Figures 1a–d and 2a–d).

The G+ model bacteria *S. epidermidis* behave similarly during the LEEFT-O_3 process. The noticeable enhanced inactivation by O_3 treatment was found when LEEFT was used as the pretreatment (Figure 1c,d). Compared with *E. coli*, *S. epidermidis* showed less vulnerability to O_3 in this study, which agreed with several previous studies.[14,38] As shown in Figure 1d, the disinfection efficiency of *S. epidermidis* is lower than that of *E. coli* when a low dosage of O_3 (e.g., 0.02 mg/L) is applied. Nevertheless, *S. epidermidis* cells were more sensitive to the LEEFT. For example, when LEEFT was used alone, the inactivation efficiency of *S. epidermidis* was higher than that of *E. coli* (Figure 1c). In addition, the enhanced inactivation of *S. epidermidis* commenced with a lower LEEFT voltage of 0.4 V compared with the 0.6 V for *E. coli* (Figure 1c). Complete inactivation (>6-log and no live bacteria detectable in the effluent) was achieved with as low as 0.8 V LEEFT voltage and 0.08 mg/L O_3 treatment.

3.2. Characterization of Bacterial Membrane Damage by Selective Plate Count. Exposure to disinfection processes can result in the sublethal injury of bacterial cells.[39,40] The sublethal bacterial cells are usually more vulnerable to the subsequent disinfectants, observed in the use of chlorine, UV, and hydrogen peroxide (H_2O_2).[41] The injury may occur on the cell membrane, nucleic acids, proteins, or enzymes, which is determined by the inactivation mechanisms.[42] The cell membrane damage was hypothesized as a critical reason causing the enhancement effect of LEEFT as a pretreatment for O_3 disinfection. Thus, the selective plate count was applied in this study to quantify the sublethal bacterial population with membrane damage after LEEFT and LEEFT-O_3. Adding NaCl to the regular agar medium has been used to quantify the sublethal population caused by membrane damage because only the bacterial cells whose cytoplasmic membrane remains intact can endure the salinity gradient and multiply.[42−44] Different NaCl concentrations (typically 1−10% w/v) have been used in previous studies.[39,43,45,46] In fact, when higher levels of NaCl are used, the less severely injured bacterial cells will be inhibited to grow, which thus results in a higher sublethal percentage.[39,42] Therefore, there is no optimal value for the NaCl concentration, and 2% w/v was used in this study.

Bacterial water samples treated by LEEFT of different applied voltages (0, 0.4, 0.8, and 1.2 V) with or without the subsequent ozonation (0.08 mg/L) were analyzed by the selective plate count. The total injured population after LEEFT or LEEFT-O_3 was distinguished into the "inactivated" (indicated by "log inactivation efficiency") and "injured-but-not-inactivated" (denoted as "injury" and indicated by "log injury efficiency") categories. The latter was determined by the plate-counting difference between the regular and selective medium plates.[47] As shown in Figure 2a, the log injury efficiency of *E. coli* subjected to the LEEFT increases with the applied voltage. For example, at 1.2 V, the LEEFT causes damage to more than 4-log of treated bacteria, ~2-log of which are inactivated. The sublethal population is very limited after the ozonation alone and increases with the assistance of the LEEFT pretreatment (Figure 2b). Notably, the additional inactivation caused by the enhancement effect may not be fully

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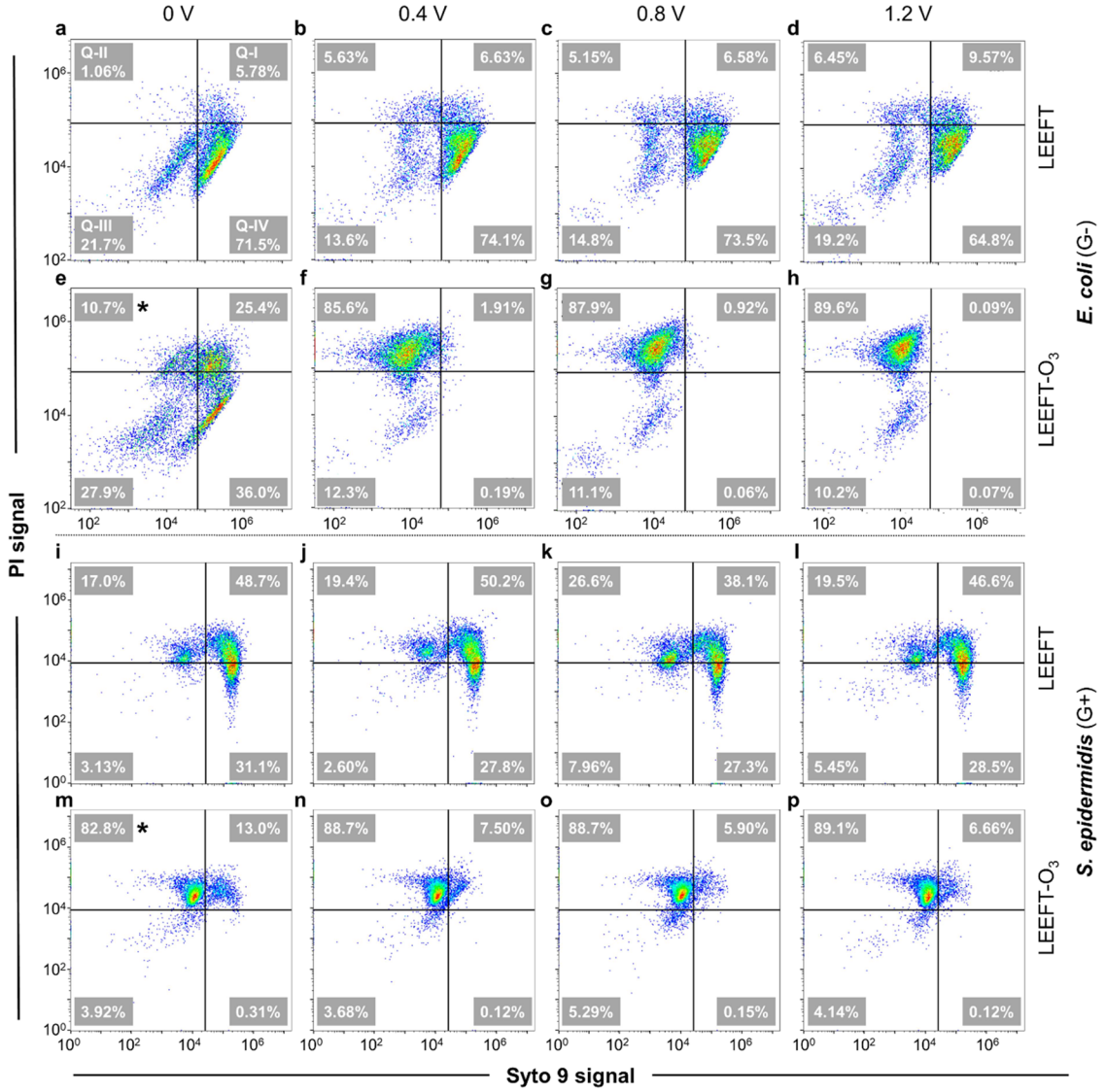

Figure 3. Characterization of cell membrane damage after LEEFT alone and LEEFT-O_3 by flow cytometry with a four-quadrant diagram. The y-axis and x-axis indicate the signal of PI and SYTO 9, respectively. (a–d) *E. coli* population after LEEFT with various applied voltages (0, 0.4, 0.8, and 1.2 V). (e–h) *E. coli* population after LEEFT-O_3 in which the applied voltages are 0, 0.4, 0.8, and 1.2 V, respectively. (i–l) *S. epidermidis* population after LEEFT with various applied voltages (0, 0.4, 0.8, and 1.2 V). (m–p) *S. epidermidis* population after LEEFT-O_3 in which the applied voltages are 0, 0.4, 0.8, and 1.2 V, respectively. The symbol "*" in the figures indicates the control experiments of the treatment by O_3 only (0.08 mg/L dosage).

revealed by the "detected" log injury efficiency. For example, when the applied voltage is 0.8 V, the inactivation and injury efficiencies after the LEEFT are 0.37-log and 1.21-log, respectively. The inactivation efficiency after ozonation is 5.41-log, which contains 2.97-log caused by ozonation alone and 1.58-log (1.58 = 0.37 + 1.21) induced by LEEFT. However, there is still a 0.86-log gap (0.86 = 5.41 − 2.97 − 1.58) of the disinfection efficiency. A possible reason is the incomplete quantification of the injured population. A higher injured population (or injury efficiency) is expected when a higher salt concentration is used (Figure S6).

The increased injury efficiency after the LEEFT was also observed with G+ bacteria, *S. epidermidis* (Figure 2c). The injured *S. epidermidis* population caused by LEEFT is higher than that of *E. coli*, which could explain the greater sensitivity of LEEFT against *S. epidermidis*. Effective inactivation of *S. epidermidis* was achieved by the subsequent ozonation (Figure 2d).

3.3. Characterization of Bacterial Membrane Damage by Flow Cytometry. Flow cytometry with fluorescent staining using SYTO 9 and PI provides the physiological information of the entire bacterial population as a supplement of the plate count method.[28,48] Whether the PI-stained bacterial cells are dead is still controversial, but PI staining is widely regarded as an indicator of severe membrane damage.[49,50] The characterization of the sublethal population by flow cytometry after LEEFT and LEEFT-O_3 is shown by a four-quadrant diagram in Figure 3 (Figure 3a–h for *E. coli* and 3i–p for *S. epidermidis*). Quadrants I (Q-I), II (Q-II), and IV (Q-IV) represent the injured, inactivated, and live bacterial cells, respectively, while quadrant III (Q-III) stands for the impurities and cell debris that are not stained by either SYTO 9 or PI.[51] As shown in Figure 3a–d, the Q-I percentage

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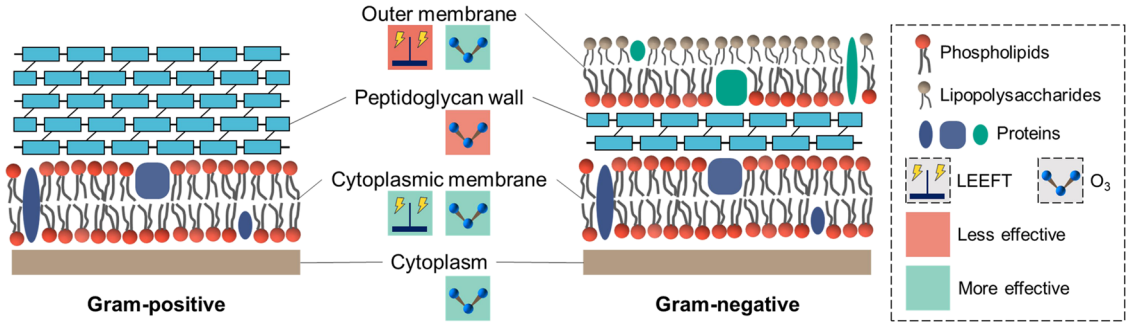

Figure 4. Illustration summarizing the disinfection mechanisms of LEEFT and ozonation against G+ and G− bacteria. The envelope of G+ bacteria consists of a very thick layer of peptidoglycan wall and an inner cytoplasmic membrane made of phospholipids. In G− bacteria, the peptidoglycan wall is much thinner but is covered by an outer membrane, which is made of lipopolysaccharides on the external leaflet and phospholipids on the internal leaflet.

increases and the dense population in Q-IV slightly shifts upward. This result indicates the increase of the sublethal group of *E. coli* with the increase in applied voltages (0, 0.4, 0.8, and 1.2 V corresponding to Figure 3a−d, respectively) after LEEFT. The subsequent ozonation dramatically damaged the bacterial cell membrane, as evidenced by the high population stained by PI (Q-II) (Figure 3e−h). With the increase of applied voltages (0 to 1.2 V), the Q-II population increases (Figure 3e−h), which reflects the more substantial enhancement effect observed previously (Figure 1a). A similar increase in the injured population (Q-I) was observed on *S. epidermidis* subjected to LEEFT (Figure 3i−l corresponding to the applied voltages of 0, 0.4, 0.8, and 1.2 V, respectively). Nevertheless, as shown in Figure 3i−l, the injured population (Q-I) of *S. epidermidis* accounts for a much higher percentage compared with that of *E. coli* (Figure 3a−d). This phenomenon was also in line with the higher inactivation and injury efficiency observed by the culture-based methods (Section 3.2). After the ozonation, a majority population of *S. epidermidis* was stained by PI. (Figure 3m−p). With stronger LEEFT (0−1.2 V), the percentage of the debris (Q-III) increases (Figure 3a−d,i−l for *E. coli* and *S .epidermidis*, respectively), which may be caused by the leakage of intracellular substances induced by the reversible and irreversible electroporation. The debris analysis after ozonation is more complex since O_3 can react with both the bacterial cell membrane and also the intercellular substances.

The debate of whether to use the plate count method or flow cytometry to quantify the bacterial cells has been in place for a long time.[52,53] In this study, the plate count method demonstrates higher resolution and more distinguishable results than the flow cytometry method. With the assistance of selective media, the inactivation and injury efficiency can be quantified on a log scale. On the other hand, results in flow cytometry are typically presented in percentage with a worse resolution. For example, ozonation (0.08 mg/L) could already inactivate ∼3-log of bacteria, which leads to a high but hard-to-distinguish percentage of PI-stained population (Q-II, 82.8−89.6%, Figure 3e−h,m−p). Due to its nonselective oxidation, O_3 can react with both the bacteria and the leaked organic matters (e.g., DNA and proteins), which further increases the component complexity of the system and the difficulty of data analysis. The high resolution of the selective medium technique is fairly important since the last surviving cells are critical to quantify the efficiency of water disinfection. Therefore, the plate count method is more suitable for the quantification of the sublethal population in the current LEEFT-O_3 disinfection process.

3.4. Mechanism Interpretation of the Enhanced O_3 Disinfection Assisted by the LEEFT Pretreatment. As shown in our results, the population of bacteria with the damaged membrane increases after the LEEFT. The damaged membrane is evident by selective plate count and flow cytometry. The inability of bacteria to survive in selective media is considered to be a result from structural (primary) and metabolic (secondary) injuries of the bacterial cell membrane.[41,54] The uptake of PI indicates the formation of water channels formed on the cell membrane, which allows the exchange of large and small molecules.[8,28,55,56] The permeabilized cell membrane of the treated bacteria is also indicated by the leakage of nucleic acids out of the bacterial cells after the LEEFT (Text S1.4, Figures S8a,b for *E. coli* and *S. epidermidis*, respectively). According to the above results, the enhancement effect of LEEFT on O_3 disinfection is likely to be caused by the membrane damage and could be inferred by two mechanisms.

First, both reversible electroporation and irreversible electroporation occur during the LEEFT disinfection.[57] For a stand-alone LEEFT process, only the irreversible electroporation contributes to the disinfection.[28,33] In contrast, the bacteria subjected to reversible electroporation would repair the sublethal injures, especially in a favorable environment (nutrient-rich agar plate with optimal growth temperature during quantification).[58,59] In the sequential LEEFT-O_3 process, the transient electroporated pores on the cell membrane formed during the LEEFT may not be able to reseal during the subsequent ozonation.[60] Thus, the inactivation of the repairable cells by ozonation could contribute to the enhanced disinfection performance.

Another explanation roots from the increased membrane permeability of the electroporated bacterial cells.[58] In conventional ozonation, O_3 interacts with the surface components of the cell membrane before oxidizing the intracellular substances.[9,10,61] When O_3 is able to diffuse into the cells, the bacteria are already killed because of the cell membrane damage. During the LEEFT-O_3 process, the increased permeability by LEEFT could facilitate the diffusion of extracellular substances into the cells, including O_3.[57,59,62] After penetrating the cell membrane, O_3 could attack the cytoplasmic constituents, including nucleic acids, plasmids, and enzymes.[14] This thus provides an additional disinfection mechanism compared with the stand-alone ozonation, which

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could reduce the O_3 dosage to achieve a similar level of disinfection.

3.5. Different Inactivation Sensitivity of LEEFT and O_3 against G+ and G− Bacteria. In addition to the enhancement effect of LEEFT on O_3 disinfection, another important observation in this study is the different sensitivities of G+ and G− bacteria subjected to LEEFT or ozonation. G− bacteria (e.g., *E. coli*) are more vulnerable to O_3 treatment, while G+ bacteria (e.g., *S. epidermidis*) are more vulnerable to LEEFT. This could be explained by the different envelope structures of G+ and G− bacteria illustrated in Figure 4. During electroporation, aqueous pores only form in the bilayer structures.[57,59,62,63] The additional outer membrane of G− bacteria is more resistant to electroporation because of the reduced mobility of the lipopolysaccharide molecules.[58,63−65] This could explain the observation that the G+ bacteria are more susceptible than the G− bacteria in LEEFT disinfection. On the other hand, O_3 is a nonselective oxidant that reacts with all components on the cell envelope.[8,9,14] The presence of a rigid and much thicker peptidoglycan wall of G+ bacteria could provide extra protection for the cell membrane against O_3 and make it less effective.[58,66,67] Even though the individual method of the LEEFT or ozonation might be insufficient to inactivate certain pathogens, the LEEFT-O_3 disinfection has a more universal disinfection effect against both G+ and G− bacteria.

3.6. Environmental Implications. Ozonation is a well-established process for water disinfection. The practical adoption is primarily hindered by the high cost. The oxidation by O_3 is nonselective, which means that O_3 reacts with not only the pathogens but also the various types of natural organic matters (NOMs) in natural water.[11] Meanwhile, O_3 is not stable at room temperature (O_3 half-life = 2−20 min at room temperature), which then loses the antimicrobial power.[68] Both facts lead to the requirement of a higher O_3 dosage to achieve the disinfection goal, which thus results in a higher cost. The pretreatment by LEEFT has been shown to reduce the O_3 dosage while maintaining a satisfactory disinfection performance, which could relieve the problem of the high cost. The formation of DBPs has been another obstacle of ozonation.[69] Bromate (BrO_3^-), one of the O_3-associated DBPs, is regulated at a maximum contaminant level (MCL) = 0.010 mg/L by the U.S. Environmental Protection Agency.[70] During continuous ozonation, bromate forms in the existence of bromide (Br^-) in the water sources.[11,69,71,72] The reduction of the O_3 dosage with the assistance of LEEFT will reduce the formation of bromate (see Figure S9, detailed methods in Text S1.5, and additional discussion in Text S4) and maybe other DBPs as well.

The development of the LEEFT technology is also critical to the implementation of the LEEFT-O_3 process. Specifically, a major concern is the robustness of the LEEFT electrodes during long-term application. In addition, the effectiveness of the LEEFT pretreatment for different pathogens and in complex water matrixes should also be investigated in the future. For example, the higher conductivity caused by the existence of natural ions leads to a higher current due to the reduction of the system resistance. The higher current accelerates the electrochemical reactions and thus the corrosion of the nanowire-modified electrodes.[27] The NOM has also been found to accumulate around the nanowires and thus hinders the cells from reaching the nanowire tips.[73] Thus, it is worthwhile to investigate the influence of different inorganic ions and organic molecules on the LEEFT-O_3 process. The effect of other water quality characteristics (e.g., pH and alkalinity) should also be studied comprehensively in the future. Nevertheless, in this study, the observed minimum voltage of LEEFT to induce an enhanced ozonation performance (0.4−0.6 V) is found to be lower than that to conduct stand-alone disinfection (>1 V). In this sense, the LEEFT can be powered by more types of energy sources with a relatively low DC voltage output (e.g., photovoltaic systems and microbial fuel cells without being connected in series). This will promote the application of the LEEFT-O_3 process to fit in different water treatment processes and applications. Meanwhile, the lower applied voltage also reduces the requirement of the electrodes in term of its electrochemical robustness.

In summary, the sequential application of LEEFT and O_3 treatment was rationally designed for water disinfection. An enhanced inactivation of ozonation by the LEEFT was observed on both G+ and G− bacteria. The increased bacterial population with impaired membrane after LEEFT was found to play an important role in the enhancement effect. The enhancement effect of disinfection was inferred in two aspects, where O_3 could inhibit the reseal of reversible pores induced by LEEFT and LEEFT enhanced the membrane permeability and facilitated O_3 diffusion into the bacterial cells. Compared with the conventional ozonation process, the LEEFT-O_3 process demonstrates a more universal disinfection against both G+ and G− bacteria. Meanwhile, the LEEFT pretreatment effectively reduces the O_3 dosage, which thus will reduce the overall cost and DBP formation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c03968.

Supplementary experimental materials and methods (Text S1); the preliminary results of the inactivation of bacteria with LEEFT or O_3 independently (Text S2); the justification of the addable effect of LEEFT-O_3 disinfection (Text S3); results and discussion on the bromate formation during the LEEFT-O_3 process (Text S4); supplementary and preliminary experimental results (Figures S1-S9); the literature review of O_3-related combined disinfection in the recent 20 years (Table S1); and supplementary references (PDF)

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Notes

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