Inactivation of Bacteria in an Aqueous Environment by a Direct-Current, Cold-Atmospheric-Pressure Air Plasma Microjet

Fuxiang Liu, Peng Sun, Na Bai, Ye Tian, Haixia Zhou, Shicheng Wei, Yanheng Zhou, Jue Zhang,* Weidong Zhu,* Kurt Becker, Jing Fang

A direct-current, cold-atmospheric-pressure air plasma microjet (PMJ) sustained in a quasi-steady gas cavity in a liquid medium is used to inactivate *Staphylococcus aureus* (*S. aureus*) suspended in the liquid. The temperature and the pH value of the liquid change to steady-state values of about 40 °C and 3.0–4.5, respectively, after 10 min of plasma treatment. The decrease in the pH is attributed to the reaction of NO_x produced in the air plasma with water at the gas–liquid interface. The concentrations of NO_3^- and NO_2^- are measured to be 37 $mg \cdot L^{-1}$ and 21 $mg \cdot L^{-1}$, respectively, after a 20 min of plasma treatment. Effective inactivation of *S. aureus* is found to start after the pH values decreases to about 4.5. This is attributed to the high oxidizing potential of the perhydroxyl radical ($HOO^{\bullet}$) on the fatty acid in the cell membranes of the microorganisms in the liquid.

F. Liu, N. Bai, Y. Tian, H. Zhou
West China College of Stomatology, Sichuan University, Chengdu, China
F. Liu, P. Sun, N. Bai, Y. Tian, H. Zhou, S. Wei, Y. Zhou, J. Zhang, J. Fang
Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China
E-mail: zhangjue365@yahoo.com.cn
S. Wei, Y. Zhou
School of Stomatology, Peking University, Beijing, China
W. Zhu
Department of Applied Science and Technology, Saint Peter's College, New Jersey, USA
E-mail: wzhu@spc.edu
K. Becker
Department of Physics, Polytechnic Institute of New York University, New York, USA

Introduction

Over 100 different types of bacteria, protozoa, and viruses exist in contaminated water. These microorganisms are responsible for various serious illnesses such as kidney failure and degenerative heart disease. These contaminations are usually treated by chemical methods. Municipal water treatment plants mainly use chlorine to treat these contaminations. Ozone, as a substitute of chlorine, starts to gain popularity in recent years due to potential health and environmental hazards of chlorine. More recently, attempts have been made to inactivate bacteria in water with non-thermal plasmas (e.g., pulsed streamer discharge plasma, gliding arc discharge plasma, etc.) because of the simplicity and efficiency of plasma-based methods. Discharges in and in contact with liquids generate intense UV radiation, shock

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waves and reactive radicals (OH, atomic oxygen, hydrogen peroxide, etc.), which are believed to be effective agents to inactivate/convert many forms of biological and chemical matter.[1] Recently, Sakiyama and coworkers achieved a 99.5% inactivation rate of *Escherichia coli* (*E. coli*) in 180 s by microplasma-initiated electrolysis in a normal saline solution.[2] Woedtke and coworkers observed a 7-log reduction of *E. coli* and *Staphylococcus aureus* (*S. aureus*) in a physiological saline solution (0.85%) following treatment of the solution by a surface dielectric barrier discharge (DBD).[3]

Here we report the results of studies of the inactivation of bacteria (*Staphylococcus aureus* as a model) in an aqueous environment by an atmospheric-pressure non-thermal air plasma microjet (PMJ). We monitored the temperature and pH change of the liquid as well as NO_2^-, NO_3^-, H_2O_2 concentration in the liquid as a result of the PMJ exposure.

Experimental Part

The device used in this study is comprised of two metal tubes as electrodes separated by a ceramic tube. The two metal electrodes are separated from each other by ≈0.5 mm. The openings in the two electrodes are ≈0.8 mm in diameter and the depth of the exit opening is approximately 1 mm. A more detailed description of the device can be found in the literature.[4] The high-voltage electrode is completely embedded in the device and powered by a DC negative-polarity high-voltage power supply (Matsusada AU5R120). The outer electrode is grounded for safety considerations. A ballast resistor of 5 kΩ and a current monitoring resistor of 100 Ω are connected in series in the circuit between the powered electrode and the DC power supply. Compressed air is used as the working gas at a flow rate of approximately 2–3 slm. The discharge sustaining voltage ranges from 400 to 600 V with an operating current of 20–35 mA.

When the exit nozzle of the plasma device was immersed in water, a quasi-steady gas cavity inside the water sustained the PMJ. Figure 1 shows a schematic diagram of the experiment as well as a photo of the PMJ sustained in water. No gas cavity is clearly visible due to the long exposure time of the picture. Three kinds of aqueous media were used in this study: (1) 20 mL sterile water; (2) 19 mL sterile water with 1 mL Luria–Bertani (LB) culture media; (3) 19 mL sterile water with 1 mL bacteria suspension (with LB culture media). All solutions were contained in a 50 mL beaker.

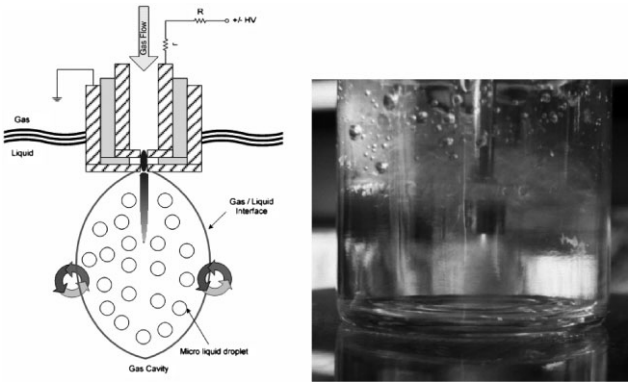

Figure 1. Plasma treatment: (a) a schematic diagram and (b) a picture of the PMJ sustained in a quasi-steady gas cavity in water.

S. aureus (gram positive) was used as a model in the inactivation experiments. The bacteria, purchased from China General Microbiological Culture Collection Center (CGMCC number 1.2465), were cultured in a LB liquid for 12–18 h until they reached the logarithmic growth phase. The suspension of bacteria in their vegetative state was injected into the water and the solution was subsequently treated with the PMJ for various periods of time. After plasma treatment for a prescribed period of time, 150 μL of the suspension was removed and spread more or less uniformly on an LB agar culture medium in a standard Petri dish (90 mm in diameter), which was then sealed and incubated at 37 °C for about 21 h for colony forming unit (CFU) counts.

The concentrations of nitrate anions (NO_3^-) and nitrite anions (NO_2^-) in sterile water were measured with a high-performance liquid chromatography, HPLC (Dionex ICS-2500 equipped with an ED50 electrochemical detector and a DIONEX ASRS 4-mm suppressor module). Samples (25 μL) were injected into the system for analysis. The pH values of the three liquid media were evaluated with a Microprocessor pH-meter (HANNA pH213 Instruments, USA).

All experiments were repeated five times (unless stated otherwise) to obtain a sense of the reproducibility of the results and to assign error bars to reported data.

Results and Discussions

Inactivation of *S. Aureus*

The CFU count of *S. aureus* in the Petri dish following PMJ treatment for various times ranging from 0 to 20 min were evaluated. In the first 10 min of PMJ treatment, no obvious decrease of the number of CFUs was observed. After 12 to 14 min treatment, a reduction on the bacterial colonies became apparent. After a 16 min treatment, no CFUs were observed in the Petri dish, which indicates a complete inactivation of *S. aureus* in water.

Scanning electron microscope pictures of *S. aureus* were taken before and after 20 min of PMJ treatment (Figure 2).

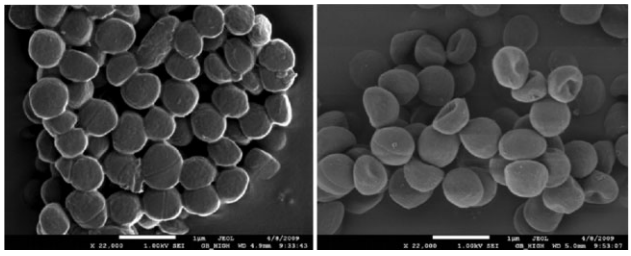

Figure 2. Scanning electron microscope pictures of *S. aureus* (a) before PMJ treatment in water and (b) after 20 min of PMJ treatment (the experiment was done with an initial pH value of 7.5).

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As presented, the bacteria underwent a transition from initially smooth surfaces to surfaces with a single-dip after the PMJ treatment. This was not observed in the negative control samples where only gas was introduced into the liquid without a plasma. The change of the morphology of the cell wall is considered detrimental for the survival of the bacteria.

Liquid Property Evaluation

The plasma-liquid system presented in this paper represents a highly complex environment. The basic understanding of the system as well as the associated physical and chemical processes is very limited. However, in an effort to further investigate the mechanism of *S. aureus* inactivation, experiments determining the average liquid temperature and acidity were conducted to evaluate the change of the properties of the liquid during the PMJ treatment.

The temperature in the active plasma region of the PMJ was evaluated via optical emission spectroscopy (by fitting experimental data of the rotational (0, 0) band of the second positive N_2 system to modeling data) and was found to range from 400 to 2000 K[5–7] The temperature at the nozzle surface in the present case was measured to be around 350 K in air at an operating current of 30 mA and an air flow of 3 slm. The temperature depends strongly on the operating current and the air flow as well as on the operating gas (i.e., molecular gas vs. atomic rare gas). However, when submersed in a liquid, the continuous flow of room-temperature air, the mixing of the air above the liquid surface, and the liquid itself serve as cooling agents in the system. The time evolution of the overall liquid temperature is shown in Figure 3. The temperatures of the three liquid media increased with the PMJ treatment

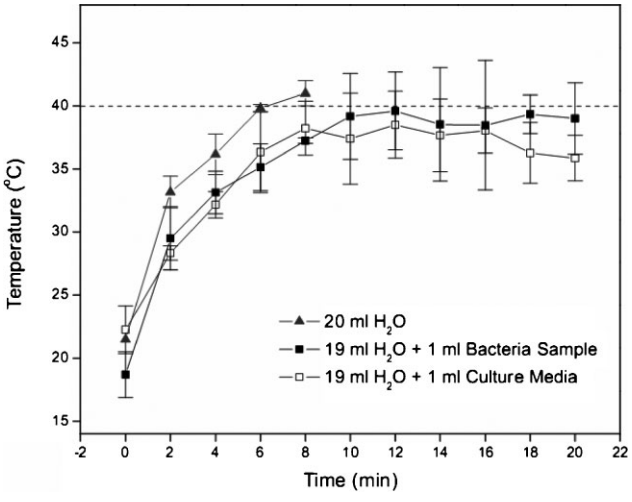

Figure 3. Temperature of thee liquid media (see insert in figure) for different PMJ treatment times.

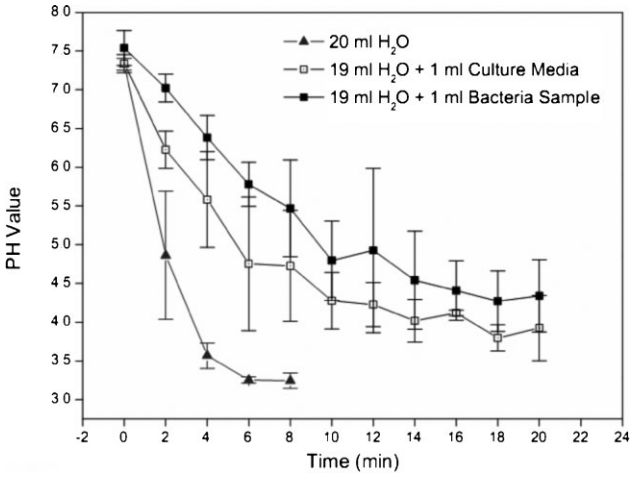

Figure 4. pH values of three liquid media (see insert in figure) for different PMJ treatment time.

time and reached equilibrium at approximately 313 K (or 40 °C) after 10 min of plasma exposure. This temperature is not sufficient for the inactivation of *S. aureus* in a liquid via purely thermal effects.

The pH value of the liquids was monitored with a microprocessor pH meter at different PMJ treatment time. The results are shown in Figure 4. All three liquid media became acidic after exposure to the PMJ. It is interesting to note that the pH value for sterile water decreased to approximately 3.2 in about 6 min, while it took longer for the pH value to stabilize at 4.5 and 4.2 in sterile water with LB culture and in sterile water with bacteria suspension, respectively. Laroussi and coworkers,[8] using a low-frequency DBD in air, observed a similar immediate and pronounced pH decrease in deionized water and a slightly delayed and less pronounced pH decrease in an Alga-Gro medium. The difference was attributed by these authors to the buffering capacity of the culture media and the bacteria sample in the liquid.

We always observed an increase in the acidity of the liquid due to air plasma action.[9] This is attributed to the multistep reaction of plasma-generated reactive species, including NO_x, O, O_3, with water at the gas–water interface (the quasi-steady gas cavity surface as well as on the surfaces of micro droplets of liquid inside the gas cavity). The concentrations of NO_2^- and NO_3^- in sterile water were monitored with HPLC and the result is shown in Figure 5 (experiments were repeated three times). The concentrations of NO_2^- and NO_3^- increase steadily from 0 $mg \cdot L^{-1}$ and 0.7 $mg \cdot L^{-1}$ to about 37 $mg \cdot L^{-1}$ and 21 $mg \cdot L^{-1}$, respectively, over 20 min of PMJ treatment. It is important to note that these experiments were usually conducted after all other experiments were completed (typically in the span of 2–3 h). A potential source of systematic error might be due to the fact that NO_2^- tends to be oxidized to NO_3^- in a liquid over time.

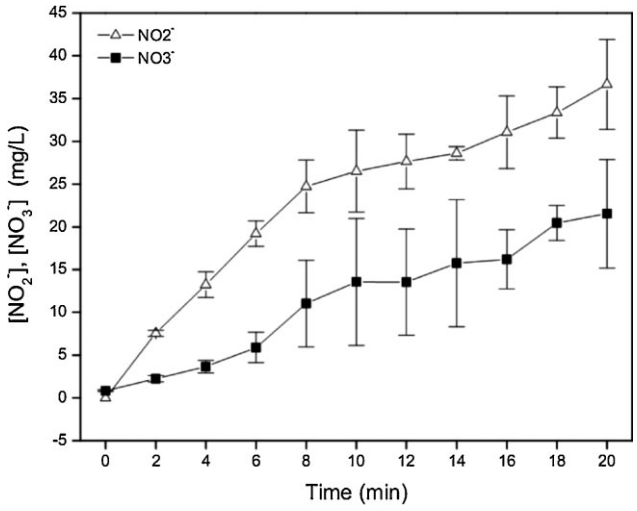

Figure 5. The concentration of NO_2^- and NO_3^- in sterile water after PMJ treatment.

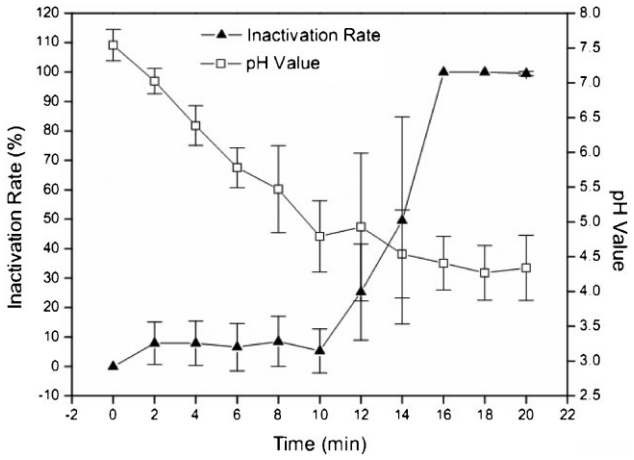

Figure 6. Inactivation rate of *S. aureus* treated with a PMJ in water plotted together with the change in the pH value of the liquid as a function of plasma exposure.

Optical emission spectroscopy was used to evaluate the existence of nitric oxide in the system. The emission spectrum is dominated by N_2 lines in the UV-A and UV-B regions due to the high concentration of nitrogen in air. Intense emissions from NO are also observed in the UV-C region from 215 to 280 nm. However, we cannot exclude the presence of some copper emissions in this region because of the use of copper as electrode material. Near-infrared emission specta show emissions from O (777.2 nm).

Inactivation of Bacteria with Preset pH Value and Temperature of the Liquid

The inactivation rate of the bacteria is defined as 100% minus the percent ratio of CFU counts of plasma treated sample to that of the control one. Inactivation rates at different PMJ treatment times are plotted together with the pH change in sterile water with bacteria suspension in Figure 6. It is interesting to note that during the first 10 min, the pH value decreased from 7.5 to approximately 4.5, while the inactivation rate of *S. aureus* stayed below 10%. From 10 to 16 min of plasma exposure, the pH value was steady at about 4.5 and a fast increase in the inactivation rate was observed, reaching 100% after 16 min.

One important question remains: is a liquid with a low pH value (and no plasma action) sufficient to inhibit the growth of *S. aureus* or does the plasma play a critical role? To address this concern, experiments were performed at a preset pH value of 4.2 (HNO_3 was used to adjust the initial pH of the liquid) and at room temperature for up to 20 min. The inactivation rates all remained below 10% after 20 min. An experiment at a preset pH value of 4.2 and a constant temperature of 40 °C (to simulate the heating of the liquid by the PMJ) showed approximately the same inactivation rates. Therefore, we conclude that simply lowering the pH value does not inactivate or inhibit the growth of *S. aureus* in water.

Further experimental results with a preset pH value of 4.5 in the presence of the PMJ are shown in Figure 7. It is not surprising to see that the inactivation rate starts to increase immediately when the PMJ treatment starts. In approximately 6 min, all *S. aureus* in the liquid are inactivated. A slight change in the pH value from 4.5 to approximately 3.7 is observed.

Inactivation Mechanism

Due to the strong electric field between the closely spaced electrodes, the electron energy distribution in the plasma is highly non-Maxwellian with a relatively large concentration of high-energy electrons.[10,11] These high energy electrons are required for the single- or multiple-step ionization and excitation of species in the air in or around

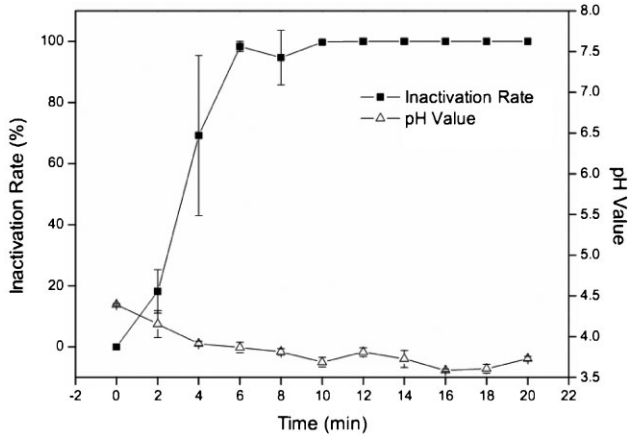

Figure 7. Inactivation rate of *S. aureus* in water with pH preset at 4.5 at room temperature with PMJ treatment.

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aqueous media. Of all the plasma-activated species that are injected into the liquid, superoxide radical ($O_2^{-\bullet}$) and its direct conjugate, the perhydroxyl radical ($HOO^\bullet$) are believed to play an important role in the initiation of the oxidation of the fatty acid in the cell membrane, thus leading to the inactivation of microorganism. Aikens and Dix[12] showed that it is $HOO^\bullet$ rather than $O_2^{-\bullet}$ that initiates the fatty acid peroxidation. The acid dissociation constant (pKa) of $HOO^\bullet$ was found to be about 4.88 in aqueous solutions[13,14] (Equation (1)).

$$HOO^\bullet \overset{pKa}{\longleftrightarrow} H^+ + O_2^{-\bullet} \quad (1)$$

$HOO^\bullet$ is in a pH-dependent equilibrium with $O_2^{-\bullet}$ with the ratio between $[O_2^{-\bullet}]$ and $[HOO^\bullet]$ calculated by

$$\frac{[O_2^{-\bullet}]}{[HOO^\bullet]} = 10^{pH-pKa} \quad (2)$$

When the pH value is lowered to a critical value (in our case, ≈4.5), the balance of Equation (1) starts to shift towards the left. This result is consistent with the observation by Hamaguchi and coworkers[15] in the treatment of *E. coli* in aqueous solution by a low-frequency helium plasma jet, where a pH threshold of 4.7 was required for effective inactivation. $HOO^\bullet$ is a selective oxidizer for organic molecules. The high oxidizing power of the $HOO^\bullet$[16] and the low oxidation potential of $O_2^{-\bullet}$[17] rapidly reduce hydroperoxyl to hydrogen peroxide (Equation (3)).

$$HOO^\bullet + O_2^{-\bullet} + H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

H_2O_2 in deionized water treated by a PMJ was evaluated with a HACH hydrogen peroxide test kit (Model HYP-1; HACH Company, Loveland, Colorado, USA) and showed a concentration of about 80 $mg \cdot L^{-1}$ after 15 min of plasma treatment.[18] To evaluate the germicidal effect of H_2O_2, we added H_2O_2 into aqueous medium (3). The H_2O_2 concentrations were 80 $mg \cdot L^{-1}$, 400 $mg \cdot L^{-1}$, and 800 $mg \cdot L^{-1}$, respectively. Experiments were repeated three times and the results are shown in Figure 8. Only when the H_2O_2 concentration in water reached 800 $mg \cdot L^{-1}$, did we observe an average inactivation rate of 80% for *S. aureus*. Therefore, H_2O_2 produced by the PMJ in water is not believed to directly participate in the inactivation process of bacteria in the current system.

The hydroxyl radical ($HO^\bullet$) is a strong oxidizer with any organic molecule. This may limit the diffusion of these radicals from the site of generation and thus their direct role in the complex environments of cells and tissue.[19] Other factors to consider include ions and UV radiation. Although a significant ion current from the PMJ operating in air (≈1 mA) was detected at a distance of 1 mm from the exit nozzle,[20] the inactivation potential of charged particles is probably negligible, because the ions tend to neutralize in the liquid before reaching the cell surface. UV emissions from the PMJ may stimulate the dissociation of water locally and enhance the $OH^\bullet$ production. However, we estimate that the UV intensity in the current situation is not high enough to kill bacteria directly by breaking the double-helix of the DNA. Further spectroscopic studies of the UV irradiation in the liquid are under way.

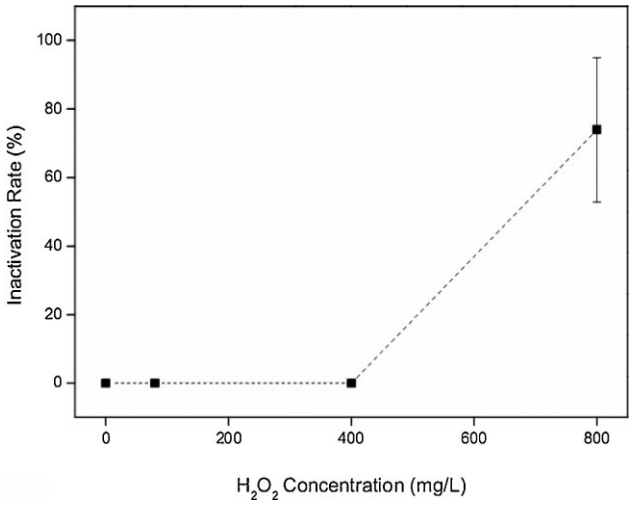

Figure 8. Inactivation of *S. aureus* in water by H_2O_2 at different H_2O_2 concentrations.

Conclusion

We demonstrated that a direct-current, atmospheric-pressure cold air PMJ sustained in a quasi-steady gas cavity in a liquid can effectively inactivate *S. aureus* in 20 min. During the plasma exposure, the temperature of the liquid increased to about 40 °C within 6–8 min, while the pH value decreased to about 4.5 in the liquid with the *S. aureus* suspension. A rapid change in the inactivation rate from 10% to 100% was observed, when the pH value decreased to ≈4.5. The inactivation is attributed to a change in the acidity of the liquid in conjunction with the direct interaction of plasma-activated species with the cells, particularly the hydroperoxyl radical ($HOO^\bullet$), which initiates the peroxidation of the fatty acid in the cell membrane. This ultimately causes the inactivation of the bacteria in an aqueous environment. Unlike other approaches to inactivate bacteria in a liquid, the approach described here employs a comparatively simple setup, direct current and compressed air, and does not require the presence of electrolysis for the ignition/sustainment of the plasma, or the addition of chemicals to preset the pH value of the liquid.

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