Liquid-phase electrical discharge plasmas with a silver electrode for inactivation of a pure culture of *Escherichia coli* in water

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Abstract

This study investigates the possible application of direct-in-liquid electrical discharge plasmas for inactivation of *Escherichia coli* in water. Aqueous suspensions of pure cultures of *Escherichia coli* (ATCC 700891) were treated in a reactor that featured point-to-plate electrode configuration. The effects of the high voltage electrode material, plasma discharge frequency, and electrode polarity on inactivation rates were investigated. While it has been observed that all three selected parameters influence the effectiveness of the treatment, the choice of the high voltage electrode material appears to play the most important role. Silver is the most effective electrode material resulting in 6.5 log reduction in 60 s. To develop an understanding of the mechanistic action of silver on *Escherichia coli* and allow the further development of this technology for water disinfection purposes, control experiments with nanoparticle and ionic silver with and without plasma were performed.

Key words: disinfection, electroporation, *Escherichia coli*, plasma, silver, water

1. Introduction

Harmful microbes are a major threat to human health and the environment. Even today, despite advances in the development of sanitation methods, waterborne disease outbreaks continue to occur (Hilborn et al., 2013; Hlavsa et al., 2014). Chlorination and ultraviolet (UV) radiation are the most commonly used disinfection processes. Although low-cost, chlorination is potentially unsafe because of chemical byproduct formation. UV radiation may be constrained by water transmittance, and is energy intensive (Davis & Tchobanoglous; Sommer, Cabaj, Pribil, & Haider, 1997). Other alternatives include ozone, advanced oxidation processes including electrical plasma, and pulsed electric field. Although many are still in the exploratory or developmental stages, these techniques seem to have the potential to ensure the safety and quality of water (Martínez-Huitle & Brillas, 2008; Moreau, Orange, & Feuilleley, 2008; Son et al., 2014; Zhang, Wang, Wu, Guan, & Jia, 2006).

Plasmas formed by direct-in-liquid electrical discharges have been shown to inactivate aqueous suspensions of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enterica*, *Microcystis aeruginosa*, *Pseudomonas putida*, *Bacillus subtilis*, and others without a significant increase in temperature during the treatment (Abou-Ghazala, Katsuki, Schoenbach, Dobbs, & Moreira, 2002; Locke, Sato, Sunka, Hoffmann, & Chang, 2006; Parvulescu, Magureanu, & Lukes, 2012). Inactivation mechanisms of liquid plasmas have been broadly attributed to chemical (hydrogen peroxide and oxidative radical species such as OH and O), physical (shockwaves and UV), and electrical effects. The relative importance of these individual contributions to the overall inactivation effect will depend on a number of parameters including input energy, duration of the plasma pulse, electrode polarity and material, electrical conductivity, and ionic composition of water (Edebo, Holme, & Selin, 1969; L. Zhu, He, Gao, Tan, Yue, & Chang, 2014; Zhu, Zhang,

Jia, & Yang, 2009). Before the technology can be optimized and scaled-up, it is important to determine the parameters that influence the treatment process.

This study investigates the effectiveness of direct-in-liquid electrical discharge plasma process for inactivation of *Escherichia coli* in water as a function of high voltage electrode material, discharge frequency, and electrode polarity. The novelty of the study lies in using silver, a well-known antimicrobial agent, as the high voltage electrode material, and investigating its mechanistic role in the inactivation process. *Escherichia coli* was chosen as a model microorganism for two reasons. Firstly, *Escherichia coli* is one of the most studied pathogenic bacteria. Secondly, while most strains of the bacterium *Escherichia coli* are harmless and live in the intestines of healthy humans and animals, some of them like *E. coli* O157:H7 and other enterohaemorrhagic strains of *Escherichia coli* are still an emerging cause of waterborne illness (World Health Organization, 2004).

2. Materials and Methods

2.1 Experimental Setup

Experiments were conducted in a 300 mL glass vessel using a point-to-plane electrode arrangement. Both the high voltage (HV) electrode (nickel-chromium (NiCr) wire, $d=0.80$ mm or silver (Ag) wire, $d=1.0$ mm) and the grounded electrode (stainless steel plate, $d=4.75$ cm) were immersed in water and separated by a distance of 0.5 cm (for negative polarity) and 2.0 cm (for positive polarity), as shown in Fig. 1. Because negative polarity discharges generally require higher voltages to be produced than positive polarity discharges, the strength of the electric field for the negative polarity discharge was increased by decreasing the electrode gap distance (the electric field strength is inversely proportional to the electrode gap distance and directly proportional to the applied voltage). The applied voltage for both positive and negative polarity

discharge was the same. Electrical discharges were created by charging a load capacitor to 17 kV with a high voltage DC power supply and then discharging the stored charge into the plasma reactor via a rotating spark gap. The frequency of the discharge is determined by the rotation speed of the spark gap, and was varied between 95 Hz and 250 Hz, depending on the experiment. The electrical circuit diagram is shown in Fig. 2. The voltage and current in the plasma reactor were measured and recorded using a Tektronix P6015A high voltage probe and a Tektronix P6021 current probe connected to a Tektronix TDS 3032C oscilloscope. Unless stated otherwise, all the experiments were performed with positive polarity discharges. Examples of the voltage and current waveforms for both positive and negative discharges are shown in Fig. 3.

Optical emission spectroscopy (OES) measurements were performed by placing an optical fiber connected to an Avaspec Multichannel Fiber Optic Emission Spectrometer (Avantes, AvaSpec-ULS2048) outside of the reactor perpendicular to the plasma (Fig. 1). The data were collected and analyzed using Avasoftware 7.6 software (Avantes) with an integration time of 1600 ms.

Figure 1. Schematic diagram of the plasma reactor.

Figure 2. Electrical circuit diagram.

(a)

(b)

Figure 3. Voltage and current waveforms for: (a) negative and (b) positive polarity discharges.

2.2 Bacteria Preparation and Plating

Escherichia coli (ATCC 700891) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Stock cultures of microorganisms were stored in a nutrient broth (Becton, Dickinson and Company (BD), Franklin Lakes, New Jersey, USA) at 4°C. Before each experiment, bacteria were inoculated into 10 ml tubes containing sterilized Luria-Bertani Miller (LB) broth (BD, USA) and grown by incubation overnight at 37°C. After the cultivation, the bacterial cells were harvested by centrifugation (Beckman Coulter, Indianapolis IN, USA) at 2400 *g* for 10 min at room temperature. The cell pellet was washed thrice and re-suspended in a sterilized 0.01 M NaCl solution. Bacterial quantification was completed by standard plate counting (SP) method. Twenty microliter aliquots of 10-fold serial dilutions were plated on LB agar in triplicate and incubated for 24 h at 37°C prior to counting. Plates with 30-300 colonies were counted and bacterial population were reported as log colony forming units per mL (log CFU/mL).

2.3 Experimental Methods

Aqueous suspensions of bacteria were prepared by adding 10 mL of 0.01 M NaNO₃ and 10 mL of re-suspended bacterial cells in 200 mL of deionized water. The initial solution conductivity was 100 µS/cm.

Experiments were conducted in a semi-batch mode using a peristaltic pump at 1000 mL/min to ensure complete mixing of the solution. Unless stated differently, aqueous samples were withdrawn from the reactor every 30 s and analyzed immediately using the SP technique described in Section 2.2. The initial temperature of the solution in the reactor was 22°C. For all experiments, the solution temperature after the treatment was between 23 and 26°C. Conductivity

and pH were measured after each experiment using a portable pH/conductivity meter (Orion 4 star, Thermo Scientific). The concentration of silver ions (Ag^+) in the solution was determined by the flow injection analysis technique (Özel, Liu, Alkasir, & Andreescu, 2014). All experiments were run in triplicate and reported values represent the mean value of three different experiments. Statistical analyses were performed with SigmaPlot 12.3 (Systat Software GmbH, Erkrath, Germany). Two sample t-test with a significance level of 0.05 was applied.

2.4 Scanning Electron Microscopy

The plasma-treated aqueous solution was centrifuged and supernatant discarded in order to obtain dense cell pellets for scanning electron microscopy (SEM). The SEM sample preparation followed the protocol described by (Ziuzina, Patil, Cullen, Keener, & Bourke, 2013). Cell pellets were fixed for 2 h by 2.5% glutaraldehyde in sodium cacodylate buffer (SCB) in an ice bath, then washed three times with SCB followed by fixation in 1% osmium tetroxide at 4°C for 2 h. The fixed cells were washed three times with SCB, and then three times with deionized water. Dehydration of samples was achieved by washing with increasing concentrations of ethyl alcohol (50, 70, 80, 90, 99.5%, Sigma Aldrich, 200 proof, molecular biology grade). In last step, all samples were freeze-dried (Dura Stop/Dura Dry Freeze Dryer, model#TDS2C0B50B0), then fixed on carbon tape and sputter-coated with gold particles for 2 min. Images were taken by JEOL (JSM-7400F, Field Emission Electron Microscopy, US) at 1 kV.

3. Results

3.1 Electrode Material

Fig. 4 compares inactivation of *Escherichia coli* in an aqueous suspension using nickel-chromium and silver high voltage electrodes. Liquid plasma discharge using a silver HV electrode resulted

in ~6.5 log reduction in 60 s compared to <1 log reduction using a NiCr electrode for the same treatment time. The effect of the HV electrode material on inactivation of *Escherichia coli* was statistically significant ($P = <0.001$).

Figure 4. The effect of the HV electrode material on inactivation of *Escherichia coli* at 120 Hz discharge frequency. Insert: early treatment times showing the effect of Ag HV electrode.

3.2 Discharge Frequency and Electrode Polarity

Table 1 reports the time required for 5 log reduction of *Escherichia coli* suspensions at different discharge frequencies using silver as the HV anode. In the frequency range investigated, there is an optimum discharge frequency for the *Escherichia coli* inactivation of 120 Hz. Discharge power, also reported in Table 1, was calculated from Equation 1:

$$P = \frac{1}{2} \times C \times V^2 \times f \quad (1)$$

where C (=0.75 nF) is the capacitance of the load capacitor, V (=17 kV) is the applied voltage, and f (Hz) is the discharge frequency. Because P is directly proportional to f , discharge power increases with an increase in frequency. However, according to the results, there is no obvious correlation between the discharge power and the inactivation time.

Table 1. The effect of the discharge frequency on inactivation of *Escherichia coli*. The high voltage electrode is a silver anode (+).

Frequency (Hz)	Power (W)	Time for 5 log reduction (s)
95	21.1	300
105	23.4	120
120	26.7	60
140	31.1	90
170	37.8	< 5 log*
250	55.6	< 5 log*

*< 5 log reduction achieved in 300 s

Plasma discharge polarity had a significant effect ($P = <0.001$) on bacterial decimation rates, as shown in Fig. 5. For negative polarity plasmas at 120 Hz, 5 log reduction was achieved within 30 s, compared to 60 s with a positive polarity discharge at the identical frequency.

Figure 5. The effect of plasma polarity on inactivation of *Escherichia coli* (HV electrode material is silver).

3.3 Mechanistic action of silver on *Escherichia coli*: effect of nanoparticle and ionic silver

Due to the effectiveness of the silver electrode during the discharge, experiments were conducted to elucidate the mechanism(s) by which bacterial inactivation rates were increased relative to the NiCr electrode material.

Erosion of the high voltage electrode during a direct-in-liquid electrical discharge has been shown to release both ions and metal particles in the bulk solution (Potocký, Saito, & Takai, 2009). The phenomenon is believed to result from a series of events initiated by current heating leading to electrode material melting (Goryachev, Ufimtsev, & Khodakovskii, 1997; Holzer & Locke, 2008; Lukeš, et al., 2006). In this study, the release of silver particles from the electrode was confirmed qualitatively by collecting and filtering the plasma-treated solution with a 0.5 μm filter and observing a layer of metallic silver deposited on the filter. The presence of silver in the plasma was confirmed by optical emission spectroscopy (Fig. 6). It must be noted that the 242

and 328 nm silver lines originate from excited silver particles that were either sputtered from the HV electrode or formed by the reduction of the silver ions released from the electrode. The concentration profile of Ag^+ in the bulk liquid released by the silver electrode for positive and negative polarity plasmas is shown in Fig. 7.

Figure 6. Optical emission spectrum of plasma recorded with the silver high voltage electrode. Insert: wavelengths showing emissions from excited Ag.

Figure 7. Concentration of Ag^+ in the bulk liquid produced during the treatment at different electrode polarities at the frequency of 120 Hz.

Both silver nanoparticles (AgNPs) and silver ions (Ag^+) have well-known antibacterial and antiviral properties against a wide range of microorganisms including *Escherichia coli* (Morones, et al., 2005; Sondi & Salopek-Sondi, 2004). To investigate effect of released silver on inactivation of *Escherichia coli*, experiments with AgNPs and solution of AgNO_3 salt in the absence and presence of plasma were performed.

Commercial AgNPs (500 mg/L, size <100nm, surface area $5.0 \text{ m}^2/\text{g}$, 99.5% trace metals basis) were added to two separate solutions containing *Escherichia coli* (the differences among the initial concentrations shown in Fig. 8 are due to an experimental error). The 500 mg/L concentration of AgNPs was chosen arbitrarily since the concentration of the metallic silver released from the HV electrode has not been measured. One solution was exposed to in-liquid plasma discharge using NiCr electrode for up to five minutes. The other solution containing the same concentration of AgNPs was incubated for five minutes without the plasma (Fig. 8).

Despite the known toxicity of AgNPs to bacteria (AshaRani, Low Kah Mun, Hande, & Valiyaveetil, 2008; Kim et al., 2007), the inactivation rate was low compared to that in the presence of the plasma. Since the arbitrarily chosen concentration of AgNPs for this experiment of 500 mg/L is extremely high and in fact much higher than the measured bulk liquid concentration of the metal eroding from the HV plasma electrode (Kirkpatrick, 2004), another experiment with only 10 mg/L of AgNP was performed. As shown in Fig.8, this concentration of silver is not high enough to cause significant inactivation.

To explore potential Ag^+ ion toxicity effects, *Escherichia coli* was further exposed to 2 and 20 $\mu\text{g/L}$ solutions of AgNO_3 salt (Fig. 9). Selected concentrations for AgNO_3 are based on the concentration of the silver ions produced by the plasma treatment ($\sim 2 \mu\text{g/L}$, positive polarity, Fig. 7) and published papers (20 $\mu\text{g/L}$) (Feng, Wu, Chen, Cui, Kim, & Kim, 2000; Sondi et al., 2004). The treatment effects were comparable to those of AgNPs in the absence of the plasma and therefore could not explain the overall treatment effect observed when *Escherichia coli* was exposed to in-liquid plasmas using a silver electrode. Indeed, Fig.10 shows that the presence of either of the two concentrations of Ag^+ ions in the solution has a negligible effect on the treatment effectiveness when NiCr is used as the HV electrode material. Almost identical inactivation curve is reported in Fig.4 for the plasma treatment of bacterial suspension containing only NaNO_3 . Control (no plasma) experiments conducted with 12 mg/L and higher concentrations of Ag^+ revealed significant inactivation even at $t=0$ min (data not shown).

Figure 8. The comparison of inactivation effect of two different concentrations of AgNPs with (HV electrode material is NiCr, positive polarity, 120 Hz) and without electrical discharge plasma.

Figure 9. Control experiments with the addition of AgNO₃ salt. Electrical discharge plasma is turned off. The solution conductivity is 100 µS/cm.

Figure 10. Plasma treatment of *Escherichia coli* in the presence of 2 and 20 µg/L AgNO₃. HV electrode is NiCr (positive polarity). The discharge frequency is 120 Hz.

SEM analysis was utilized to visualize the effect of the plasma treatment on the morphology of the bacteria. Figure 11 shows SEM images of untreated and plasma-treated *Escherichia coli*. Prior to the treatment, images confirmed the presence of healthy cells, whereas after the liquid plasma discharge treatment, a large portion of bacterial cells was found to have small holes similar to those observed during electroporation (Liu et al., 2014; Martínez-Huitle et al., 2008; Pliquett, Joshi, Sridhara, & Schoenbach, 2007).

(a)

(b)

Figure 11. SEM images of (a) untreated and (b) plasma treated (treatment time is 12 min and the HV electrode is silver) *Escherichia coli*.

4. Discussion

Despite significant efforts to understand the mechanism(s) by which aqueous plasmas inactivate microorganisms, the best of the hypotheses are still limited to the “combined outcome of chemical, physical and electrical effects”. Due to the obvious complexity of the subject, the focus of this study was not on elucidating the mechanism of inactivation but in using experimental findings to offer an explanation for the superior performance of the silver compared to the NiCr HV electrode material. The following inactivation mechanism is thus proposed for plasma discharges in liquid with silver HV electrode, and does not constitute the general mechanism for cases with other conditions or other HV electrode materials.

The Reactive Oxidative Species (ROS) produced directly by the plasma such as OH and HO₂ radicals are expected to play a very limited role in the inactivation of the suspended bacteria due to their short lifetimes and diffusion lengths (Sato, Ohgiyama, & Clements, 1996). However, even though these species may react with the membrane of cells that are very near to the plasma

channel, this oxidative inactivation pathway is most likely insignificant due to bulk liquid transport limitations. Also, the intensity of the UV light produced by low power discharges such as those used in this study is too low to have any effect on the inactivation (Lukes, Clupek, Babicky, & Sunka, 2008). Fundamental mechanistic experiments that were performed in our laboratory as a part of another study confirm these hypotheses and further suggest that at solution electrical conductivities $< 200 \mu\text{S}/\text{cm}$ the single most significant inactivation mechanism is the electric field-induced electroporation which was also demonstrated in this study.

Electroporation alone could potentially explain why pulsed electrical discharges in water that use a non-catalytic HV electrode material such as NiCr inactivate *Escherichia coli*. However, the use of silver results in additional effects that lead to significantly higher inactivation rates. The Ag electrode releases both silver ions and silver nanoparticles into the surrounding solution. Control plasma experiments conducted with Ag^+ at the concentration that is released after a 3-minute plasma treatment suggest that these ions alone cannot explain the effects observed when silver is used as the HV electrode. Notably, control experiments conducted without the plasma with concentrations of Ag^+ that are ~ 0.2 and ~ 11 times higher than those released by the silver electrode are consistent with the known antimicrobial properties of silver (Jung, Koo, Kim, Shin, Kim, & Park, 2008; Pandian, Deepak, Kalishwaralal, Viswanathan, & Gurunathan, 2010). Although our control experiments suggest that Ag^+ are not responsible for the superior performance of the silver HV electrode, literature reports that a continuous release of Ag^+ ions exhibits higher antimicrobial effects than adding a specific amount of Ag^+ at the beginning of the experiment (Liau, Read, Pugh, Furr, & Russell, 1997) and that electrically generated Ag^+ has better antimicrobial properties than other silver-based compounds (Berger, Spadaro, Bierman, Chapin, & Becker, 1976; Berger, Spadaro, Chapin, & Becker, 1976).

In the absence of the plasma, AgNPs exhibited a negligible effect on the inactivation. However, when the plasma was turned on, the extent of the inactivation strongly depended on the concentration of the commercially added particles, as suggested by literature (Sondi et al., 2004). Due to the inability to measure the concentration of the particles emitted by the silver HV electrode, two extreme concentrations of AgNPs were used: 12 mg/L and 500 mg/L. Since the concentration of the silver particles released into the bulk liquid during the plasma treatment most likely lies between these two extremes, we believe that AgNPs and not silver ions are responsible for the superior performance of the silver HV electrode. It must also be mentioned that the size of the silver particles emitted by the plasma electrode is most likely significantly smaller than 100 nm which is the size of the commercial AgNPs used in these experiments. The shape and the size of AgNPs are known to have a significant effect on the inactivation effectiveness (Agnihotri, Mukherji, & Mukherji, 2014).

The mechanism of deactivation of *Escherichia coli* by AgNPs is not completely clear although studies suggest that AgNPs increase the permeability of the cell wall by attaching to it, resulting in cell death. Also, the penetration of AgNPs inside the bacteria and their interaction with sulfur and phosphorus compounds such as DNA has been observed (Morones et al., 2005). After AgNPs react with intracellular materials, DNA loses its replication ability and cellular proteins are inactivated. Direct-in-liquid discharge plasma treatment facilitates the penetration of the nanoparticles by electroporating the cell wall which amplifies the bactericidal effect of AgNPs.

In our results, we showed how at certain frequencies during negative discharge using silver HV electrode bacterial cells are inactivated faster than for the positive polarity treatment. Again, the possible pathway of inactivation originates from the assumption that the cell membrane is physically affected by short pulses of electric field, which allows AgNPs (and Ag⁺ ions) to enter

freely into the cytoplasm of *Escherichia coli* cells. Because negative polarity discharges had a higher voltage gradient (smaller electrode gap) compared to the positive polarity, this enhanced electroporation featured an observably higher rate of electrode erosion, which increased the presence of Ag in the solution.

The complicated relationship between discharge frequency and inactivation rate is thought to be due to the pulsed electric field. The amount of time between subsequent pulses (inversely proportional to discharge frequency) influences the number and size distribution of the pores formed via electroporation (Garner et al., 2007; Joshi & Schoenbach, 2002; Weaver & Chizmadzhev, 1996), which would in turn affect the amount of Ag ions and AgNPs as well as the size of AgNPs that are able to enter into the cell interior. Though it could not be confirmed that this particular phenomenon accounted for the non-linear nature of the frequency-inactivation rate relationship, it was accepted as the most likely explanation.

5. Conclusions

In this study, we have shown that liquid discharge plasma is an effective method for inactivation of *Escherichia coli*. Liquid discharges with negative polarity achieve much higher inactivation rates than with positive polarity. Discharge frequency plays a complex role in the inactivation, where the inactivation rate decreases at higher frequencies despite the higher input power. Direct-in-liquid discharge plasma treatment electroporates the cell wall. The superior performance of the silver high voltage electrode compared to NiCr electrode is due to the penetration of the silver nanoparticles released into the solution from electrode erosion into the cell interior.

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