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In vitro antimicrobial effects and mechanisms of direct current air-liquid discharge plasma on planktonic *Staphylococcus aureus* and *Escherichia coli* in liquids

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ABSTRACT

The direct inactivation effects of an atmospheric pressure direct current (DC) air plasma against planktonic *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) in aqueous solution are investigated in vitro. Upon plasma treatment, extensively analyses on cell culturability, metabolic capacity, membrane integrity, surface morphology, cellular proteins, nucleic acids and intracellular reactive oxygen species (ROS) for both bacterial species were carried out and significant antimicrobial effects observed. Compared with the cellular culturability, a sub-lethal viable but non-culturable (VBNC) state was induced while more *S. aureus* entered this state than *E. coli*. Damaged bacterial outer structures were observed and the total concentrations of cellular protein and nucleic acid decreased for both bacteria after plasma treatment. The plasma-induced aqueous reactive species (RS) and intracellular ROS might produce detrimental effects to the bacteria, while *S. aureus* was less susceptible to the discharge after a 20-min exposure compared to *E. coli*.

1. Introduction

Atmospheric pressure plasmas have attracted lots of attentions in the past decades because of several urgent applications such as plasma medicine [1–4]. Plasma deactivation of microorganisms is increasingly being accepted as a potential cure of microbe-associated wounds and diseases. The anti-bacterial efficacy of atmospheric-pressure low-temperature plasmas (APLTP) on a wide range of Gram-positive and -negative pathogenic microorganisms have been studied and proven leading to effective bacteria-killing approaches and reduced application of antibiotics in infection control [5–16]. Moisan et al. studied the inactivation effects and kinetics of different discharges (dielectric barrier discharge, microwave discharge) and working gases (air, Ar, O_2, CO_2, and N_2/O_2, Ar/O_2 mixtures) on various bacterial species [17] and Ehlbeck et al. reviewed the plasma efficiency on about 20 types of Gram-positive and -negative aerobic and anaerobic bacteria. Inactivation was achieved with different plasma sources such as dielectric barrier discharge (DBD), plasma jet, corona discharges, and microwave discharge and plasma exposure deactivates the microbes effectively leading to significantly reduced bacteria population [18]. Daeschlein et al. reported the decontaminative effect of an Ar/O_2 atmospheric pressure plasma jet (APPJ) against various types of common pathogenic microbes accounting for chronic or acute wounds in vitro, including *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Candida albicans*, etc. and the effectiveness nearly reached that of antiseptics [19].

As the practical applications of plasma on wound healing as well as bacteria inactivation generally happen where the plasma interacts with liquids such as exudative tissue fluid or medium, plasma-driven microbiocidal effects in the aqueous environment have been investigated. Scholtz et al. observed complete sterilization of *E. coli* within 2 min and *S. epidermidis* within 4–5 min in aqueous suspensions following the plasma action but a time of 30 min was required to fully inactivate *C. albicans* [20]. Shen et al. investigated the inactivation effect of a gas-liquid phase atmospheric-pressure Ar plasma on *S. aureus* and >2.0-log cell reduction was observed after treatment for 40 min [21]. The plasma-treated liquid still retained the antimicrobial capacity arising from acidity and H_2O_2. Zhang et al. studied He DBD gas-liquid phase plasma driven inactivation on *S. aureus* and found near 90% of the bacteria population to be deactivated after exposure for 8 min [22].

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Plasma-generated and induced formation of large quantities of localized reactive oxygen species (ROS) in the gas and liquid phases including hydroxyl radicals (•OH), atomic oxygen (O), ozone (O_3), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) could destroy the bacterial structure and functions by various pathways [23–26]. The contribution of multiple reactive nitrogen species (RNS) should also be considered, including nitric oxide (NO) and various derivatives after interaction with water such as nitrites (NO_2^-), nitrates (NO_3^-) and peroxinitrites ($ONOO^-$). Specifically, plasma-driven reactive species (RS) play pivotal roles in biocidal processes by affecting the cell wall components, structure and functions of phospholipid bilayer, structure of cellular proteins and nucleic acids, gene expressions, protein synthesis, enzyme activities, etc. via physical and biochemical mechanisms [27–31]. The respective and synergistic effects of a wide spectrum of plasma-triggered aqueous RS on multiple bacteria have been reported [32–37]. Dobrynin et al. investigated the significance of ions and humidity in different gas mixtures and found that O_2 and H_2O played important roles in generating ROS and enhancing consequent deactivation efficiency [38]. Effects of ROS and RNS in the plasma-treated liquids were discussed by Oehmigen et al. and led to inactivation of microbes by the surface DBD plasma and the NO_3^- and NO_2^- species contribute to the increasing aqueous acidification [39]. Lukes et al. found that plasma at the gas-liquid interface and in water could induce formation of various types of RS accounting for the subsequent chemical and biological effects [24,40]. Zhang et al. discovered efficient deactivation by the plasma-induced short-lived RS and that the long-lived RS accounted for the continuous residual bactericidal effects [22].

The topic of this research is of great importance and very timely because the structural and functional changes of microbes after interacting with the plasma-triggered primary and secondary RS in the aqueous solution are crucial to plasma medicine and hygiene. In this work, the cellular functional and structural changes such as the bacterial culturability, metabolic capacity, membrane structure, surface morphology, and total concentration of cellular proteins, nucleic acid, and ROS upon plasma treatment are systematically analysed to reveal the the direct effects of atmospheric pressure air plasma on *S. aureus* and *E. coli* in the aqueous solution which are representative Gram-positive and -negative pathogenic microbes [41]. Besides showing variation in the inactivation effects of plasmas on bacteria, by combining extensively multiple cell diagnostics it provides some of the fundamental mechanisms of plasma-driven antimicrobial effects on above two types of bacterial cells.

2. Materials and methods

2.1. Plasma source

As shown in Fig. 1, the air plasma is generated in the gas and gas-liquid interphase between the high voltage electrodes (a 105 fine copper rods array) and bacterial suspension as described in Ref. [29]. A 10 kV DC voltage drives the plasma source and two ballast resistors R_1 and R_2 (both 25 MΩ) restricted the discharge current in preventing arc and streamer keeping the discharge in the corona regime. The gas temperature of the plasma was assumed equivalent to T_{rot} (the rotational temperature) and was acquired through comparing the 368-381 nm N_2 (C,B) band simulation result with the measured one by obtaining a good fit with suitable T_{rot} and T_{vib} (the vibrational temperature) by the Specair software. The plasma gas temperature is about 300 K and could be directly and safely touched by human skin (Fig. 1).

2.2. Chemical measurements in plasma treated deionized water

The concentrations of some representative long-lived reactive species in the plasma-treated dH_2O including hydrogen peroxide (H_2O_2), ozone (O_3), nitrite (NO_2^-) and nitrate (NO_3^-) were measured on a spectrophotometer (PhotoLab 6100, WTW, Germany) with 4 relevant test kits: 18,789, 00607, 00609, and 09713 according to manufacturer's instructions, respectively [21,28]. The pH value of the plasma-treated dH_2O was measured with a pH-meter (PHS-3C, Leici, China).

2.3. Preparation of bacteria

The single *E. coli* ATCC-25922 or *S. aureus* NCTC-8325 colony was picked and overnight incubated at 310 K and 225 rpm orbital agitation in fresh sterile Luria-Bertani (LB) or tryptic soy broth (TSB) medium. The specific cell quantity of growth for both kinds of bacteria was measured by the standard cell counting method using optical microscope with hemocytometer to approximately 1×10^7 mL^{-1}, thus calibrated by counting the number of the CFUs (colony forming units) grown on the LB or TSB agar plates, respectively.

2.4. Plasma exposure

4 mL of the overnight cultured bacteria were placed in a sterile petri dish. The plasma was generated between the electrode array tip and

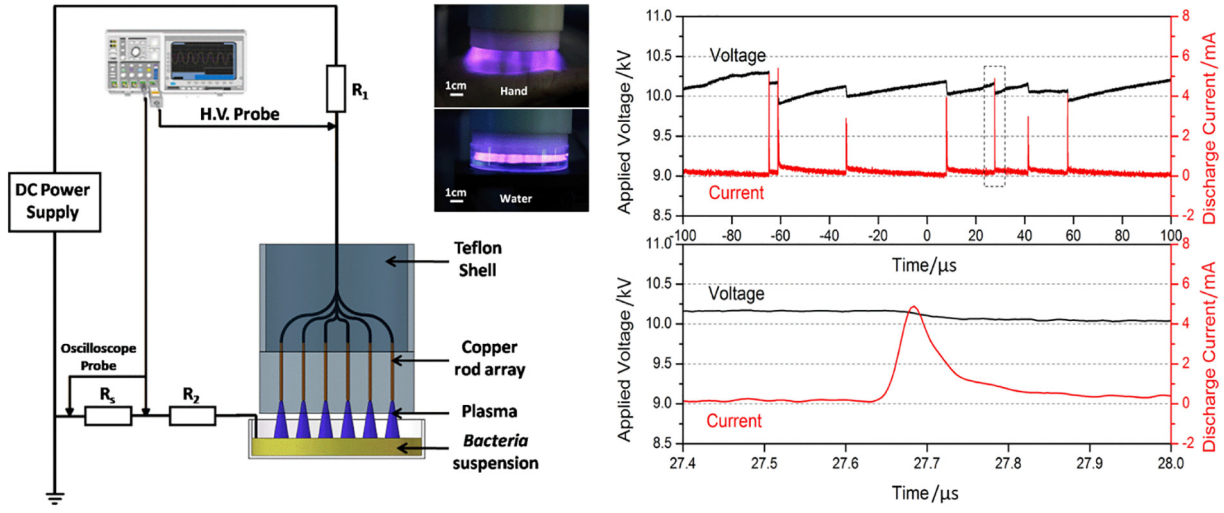

Fig. 1. Schematic and photographs (Left) and voltage and current waveforms (Right) of the atmospheric-pressure air-liquid discharge plasma. (Bottom axis: Time/μs; Left axis: Applied voltage/kV; Right axis: Discharge current/mA).