Fluorescence detection of damaged cell membrane of bacteria by air transient spark discharge

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Transient spark (TS) discharge plasma generated in ambient air was applied to liquid solutions of planktonic *E. coli* and *S. aureus* bacteria. A fluorometric screening method using Propidium iodide (PI) dye for evaluation of plasma-induced cell membrane damages is proposed. The effect of TS treatment was tested on both exponential and stationary growth phase bacteria. The highest 65% of *E. coli* population with damaged membranes was detected after 15 min treatment in exponential growth phase. Treatment in stationary growth phase caused membrane damages maximum in 20% of the *E. coli* for 20 min plasma treatment. Despite this, 15 min treatment was enough for total inactivation of *E. coli* (7 logs reduction) in both growth phases. On the contrary, *S. aureus* inactivation did not exceed 3 logs after TS discharge treatment in stationary phase. Moreover, up to 20 min treatment of *S. aureus* did not cause membrane damages.

Introduction

The bactericidal activity of cold atmospheric plasma (CAP) along with their fungicidal, sporicidal, and virucidal effects is responsible for decontamination, sterilization, tissue regeneration, antitumor activity, etc. [1]. For safe implementation of CAP technologies, the understanding of plasma-bacteria interaction is crucial. To elucidate the mechanisms underlying the bactericidal efficiency, the detailed examination of the damaged cellular components (cell membrane, DNA, proteins) is required.

Extracellular reactive oxygen and nitrogen species (RONS) generated in gaseous and liquid phases are the main triggers of multiple plasma-cell pathways. Cell membrane destruction is the initial mechanism for bactericidal effect of plasma since it causes the intracellular damages of proteins and DNAs [2]. Commonly used methods for the cell membrane integrity determination (fluorescence microscopy, flow cytometry, and fluorimetry) are costly and often also non-suitable for analyses of plasma-treated samples due to cross-reactivity and pH-dependence of fluorescent probes. Here, we present a specific fluorometric method for a rapid evaluation of cell membrane integrity using propidium iodide (PI) [3] as a fluorescent probe designed for CAP-treated bacterial suspensions.

Materials and methods

Transient spark (TS) discharge is a DC-driven self-pulsing repetitive streamer to spark transition discharge [4]. It was operated in ambient atmospheric pressure air in a contact with

a liquid circulated by a peristaltic pump repetitively through a discharge zone. All results were obtained at the constant applied voltage ~14 kV and current pulse frequency ~2 kHz. TS discharge was applied to planktonic suspensions of *S. aureus* CCM 3953 and *E. coli* CCM 3954 bacteria. CAP-induced cell membrane integrity was evaluated in different liquids (0.85% saline and Luria-Bertani (LB) culture media) and in different bacterial growth phases (stationary and exponential), and correlated with bactericidal effect. CAP-induced membrane damages were evaluated by fluorimetry with propidium iodide (PI) dye. In parallel classical cultivation tests with colony forming unit (CFU) enumeration method was used for estimation of bactericidal effect.

Fluorescent dye Propidium iodide (PI)

Propidium iodide (PI) is a hydrophobic cationic dye. For both eukaryotic and prokaryotic cells PI is excluded by the cells that have their plasma membrane integrity preserved, but it stains DNA in the cells that have damaged membranes [5-6]. PI is known to exhibit a fluorescence enhancement upon intercalation with DNA (Fig. 1). Here, PI was employed to assess the membrane integrity of bacteria directly after the plasma treatment.

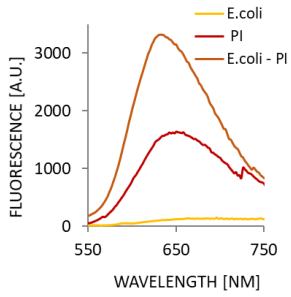

Fig. 1. Fluorescence spectra of *E. coli* – PI.

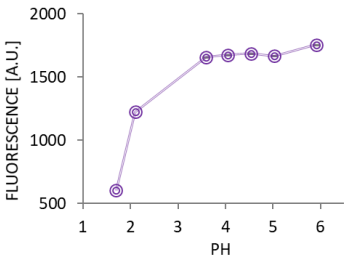

Fig. 2. Effect of the pH on fluorescence intensity of PI in saline.

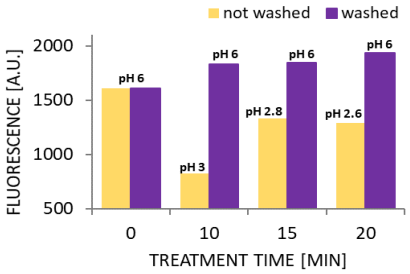

Fig. 3. Fluorescence intensity of *E. coli* stained by PI immediately after plasma treatment in saline (acidic pH) or with added washing step (pH 6.0).

The maximum excitation and emission wavelengths were determined as 535 nm and 636-652 nm, respectively and were thus chosen for later measurements. To find the optimal conditions for analysis the influence of the pH value and the PI concentration on the fluorescence signal have been studied. Since plasma treatment changes the acidity of the liquid in most cases, the fluorescent signal of the PI in saline solution at different pH levels regulated by HCl was measured. PI was shown to be dependent on the pH value (Fig. 2). Fluorescence intensity of PI decreased with decreasing pH of saline. Accordingly, washing step of bacterial suspension after plasma treatment was added to exclude the influence of pH

on PI fluorescence intensity (Fig. 3). Different concentrations of PI were used for staining *E. coli* for calibration using isopropanol to kill bacteria. The highest fluorescence intensity was measured for 1.5 mM PI (Fig. 4).

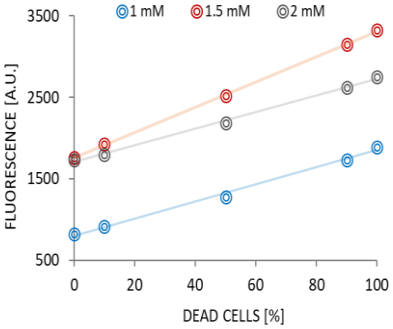

Fig. 4. Effect of PI concentration on staining of *E. coli* in saline. pH 6.0, t = 24°C.

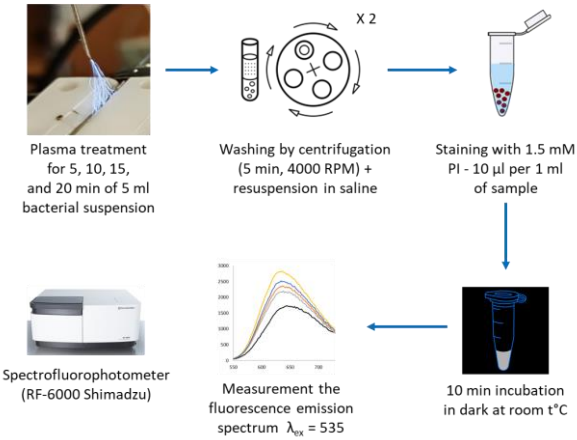

Fig. 5. Schematic summary of the PI staining method.

Based on the results (Fig. 2-4) the PI staining method was proposed (Fig. 5). Specifically, 10 µl of 1.5 mM PI stock solution was mixed with 1 ml sample (washed twice and resuspended in sterile saline to avoid a pH dependent effect) thoroughly, followed by an incubation in dark for 10 min. The fluorescence intensity was evaluated with λ_{ex} = 535 nm and λ_{em} = 636-652 nm by spectrofluorophotometer (*Shimadzu RF-6000*). The percentage of cell membrane damage was calculated using the calibration curve based on fluorescence intensity as a result of the PI method.

Results and Discussion

Membrane damages of *E. coli* and *S. aureus* treated by TS discharge plasma in LB culture media were not detected. Likewise, bactericidal effect for both bacteria was insignificant and did not exceed 0.1 log reduction (data are not shown). Thus, results obtained during plasma treatment of bacterial suspensions only in physiological saline solution (0.85% NaCl) are presented and discussed.

After 20 min plasma treatment of *E. coli* in stationary phase up to 20% of cells with damaged membranes were detected. For *E. coli* in exponential phase 35 and 65% of cells were found with damaged membranes after 5 and 15 min (Fig. 6). Plasma treatment for 5 and 10 min inactivated 2.17 and 3.18 log of *E. coli* in stationary phase, and 4.05 and 5.45 log inactivation in exponential phase, respectively (Fig. 7).

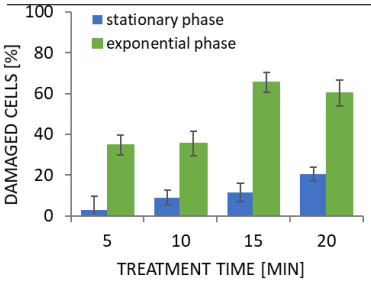

Fig. 6. The number of *E. coli* with damaged cell membranes treated by TS discharge in saline in stationary and exponential growth phase.

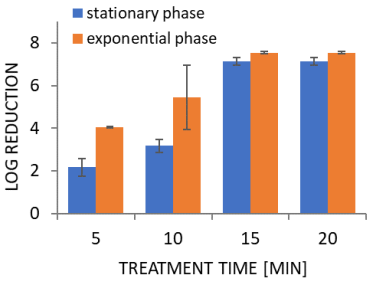

Fig. 7. Bactericidal effect on *E. coli* treated by TS discharge in saline in stationary and exponential growth phase.

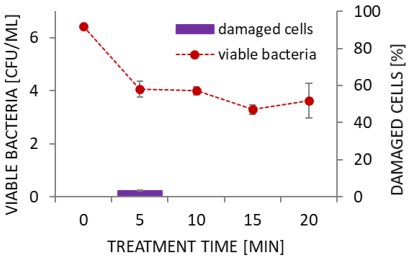

Fig. 8. Bactericidal effect compared to membrane damage in stationary growth phase for *S. aureus* treated by TS discharge in saline.

Although, plasma treatment for 15 min showed a complete log reduction of *E. coli*, not all bacteria were found with damaged membranes (Fig. 6). Supposedly, it corresponds to killing mechanism without damaging membrane, or the state of viable but non-culturable (VBNC) bacteria. Increasing number of *E. coli* with damaged membranes correlated with faster inactivation rate in exponential phase (Fig. 7).

Membranes of *S. aureus* were detected intact after TS discharge treatment up to 20 min. Bactericidal effect reached 2.36, 2.42, 3.13, and 2.79 log reduction in 5, 10, 15, and 20 min treatment (Fig. 8). On the contrary to Gram-negative *E. coli*, a mechanism for inactivating Gram-positive *S. aureus* without damaging the integrity of the membrane is proposed.

Conclusion

PI method was developed into an optimal sensitive analytical tool for identification of cell membrane integrity caused by CAP. This novel available screening technique may contribute to a comprehensive understanding of plasma-bacteria interaction for biomedical applications.

Acknowledgment

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