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Use of Green Fluorescent Protein for Rapid Assessment of the Bactericidal Activity under Cold Plasma Irradiation

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ABSTRACT: Bacterial inactivation by nonthermal or cold plasma is affected by many factors, yet the mechanisms have not been thoroughly clarified. Developing effective methods for rapid evaluation of bactericidal activity of cold plasma is thus crucial for not only optimizing the experimental conditions for the best sterilization effect, but also revealing the underlying mechanisms. Although conventional methods such as determination of colony-forming units are still the standard method for assessment of bacterial inactivation efficiency, they are time-consuming and cannot provide real-time measurement. In this work, green fluorescent protein (GFP) was utilized as the indicator for monitoring the bactericidal activity of cold plasma irradiation. GFP-expressing recombinant *Escherichia coli* was exposed to discharge plasma, and the intracellular fluorescence signal was detected with a flow cytometer. We found that the bacterial GFP fluorescence intensity decreased exponentially with plasma exposure time, and the result was in good accordance with the inactivation curve obtained by measuring colony-forming units. As such, this work demonstrates that GFP is useful for high-throughput screening assay for antimicrobial activity of nonthermal plasma irradiation.

KEY WORDS: bacterial inactivation, fluorescence, green fluorescence protein (GFP), discharge plasma, flow cytometer

I. INTRODUCTION

Since M. Laroussi reported the capability of plasma-induced bacterial inactivation in 1996, many researchers have been engaged in the investigation of application and development of nonthermal plasma technique for killing various microbes including bacteria, virus, and fungi, both in solution and on surfaces.[1,2] It has been reported that many factors such as nonthermal plasma–induced active agents (including UV radiation, charged particles, reactive species, heat, and electric fields) and experimental conditions (such as treatment surface, humidity) can affect bacterial inactivation efficiency.[3] Evaluating the bacterial inactivation effect or bactericidal efficiency normally requires monitoring the cells by counting colony-forming units (CFU) or determining turbidity. In the CFU method, bacteria are serially diluted in solution, and then a certain amount of bacterial

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suspension is removed and spread on a plate. After a suitable incubation period (commonly overnight or 24 hours) at a fixed temperature, bacterial colonies are counted. In the turbidity approach, a certain amount of bacterial suspension is diluted with liquid culture medium and then cultivated at a certain temperature. At certain times (total 12 hours or longer), the turbidity, which reflects the number of cells in solution, is measured with a photometer or spectrometer. Although these approaches have been proven valid and useful, they are generally time-consuming and cannot provide real-time measurement. Therefore, it is still necessary to develop more effective methods for rapid evaluation of the bactericidal effect induced by nonthermal plasma under varied plasma discharge conditions.

Recently, fluorescence based assays using extracellular dyes that can stain both viable and nonviable cells have been developed and used for rapid assessment of viability of microorganisms.[4,5] Green fluorescent protein (GFP), an intrinsically fluorescent protein in organisms, can be employed for such purposes. GFP was first purified from jellyfish, *Aequorea victoria*, and it exhibits bright green fluorescence upon exposure to blue to ultraviolet light, requiring no cofactors or exogenous substrates.[6] It is a compact monomeric globular protein with high resistance to heat, alkaline pH, and chemical agents.[7] Since the GFP gene was cloned from *A. victoria*[8], it has been expressed in numerous organisms, such as bacteria,[9] yeast,[10] fungi,[11] plants,[12] and animals.[13] GFP has been successfully applied in varied biosensors for protein localization, cellular Ca^{2+} levels, pH, redox state, cell cycle, and temperature.[14–16] GFP accumulating in cells during growth can be measured with fluorescence-based techniques, and the fluorescence intensity of GFP is proportional to the number of cells that express it.[14] Based on this property, researchers have used GFP as an indicator of antimicrobial susceptibility in prokaryotic and eukaryotic organisms.[9,17–22]

Enlightened by those previous studies, in this work we made use of GFP fluorescence intensity in GFP-expressing recombinant *Escherichia coli* (*E. coli*) to evaluate the bactericidal ability of nonthermal plasma, attempting to establish a new assay method based on GFP fluorescence recording to assess the plasma-induced bactericidal effect.

II. MATERIALS AND METHODS

We constructed the *E. coli* strain with GFP expression used in this study by transforming pUC18-lac-GFP (the plasmid that *GFP* gene inserted downstream of *lac* promoter) into competent cells (namely, *E. coli* DH5α, with genotype as *F-φ80d lacZΔM15 Δ[lacZYA-argF] U169 end A1 recA1 hsdR17 [rk-,mk+] supE44λ- thi-1 gyrA96 relA1 phoA*).[23] The transformed *E. coli* was cultured in 100 mL Luria-Bertani (LB) culture media and incubated with shaking at 37°C overnight. The suspension of *E. coli* was then diluted with distilled water to a concentration of 10^6 CFU mL^{-1}. Then, 1 mL of the bacterial suspension was placed into a centrifugal tube for plasma treatment. The construction of the experimental setup of nonthermal plasma discharge is shown in Figure 1. Briefly, a needle-like anode made of stainless steel was placed 2–3 mm above the bacteria suspension. The upper end of the anode was connected to an AC power supply. The output voltage

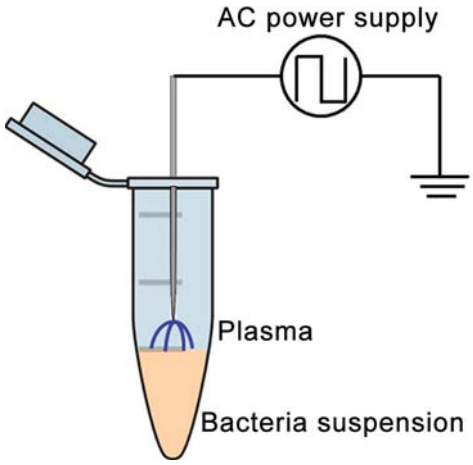

FIG. 1: Schematic diagram of the cold plasma irradiation system.

was about 9.6 kV, and the frequency was 10 kHz. Immediately after the plasma treatment, the intracellular GFP fluorescence intensity was monitored using a flow cytometer (BD FACSCalibur, BD Biosciences, USA) in the FL1 channel. Additionally, the treated bacterial suspension was diluted with distilled water, and 100 μL of the diluted bacterial suspension was plated on a 9-cm Petri dish. Every sample was plated in triplicate.

III. RESULTS AND DISCUSSION

The fluorescence spectrum in the FL1 channel of the *E. coli* strain is the spectrum of GFP. The impact of discharge plasma on the GFP spectra is shown in Figure 2(a). A bright GFP signal was observed for the transformed *E. coli* without plasma treatment. With the increase of plasma irradiation time, the fluorescence peak shifted from right to left, indicating the bleaching of intracellular fluorescence upon plasma irradiation. After plasma irradiation for 120 sec or longer, the GFP signal in the bacteria almost completely disappeared. The mean bacterial GFP fluorescence was calculated from 20,000 cells per sample and plotted against plasma irradiation time (Fig. 2b). As shown in the figure, the plasma caused rapid loss of GFP fluorescence in the transformed *E. coli* in a plasma treatment time-dependent manner. Fluorescence level decreased 71% after 60 sec treatment, and then decreased more slowly to reach a plateau at 89% loss after 120 sec of plasma discharge treatment. After irradiation longer than 120 sec, the fluorescence level was almost unchanged.

To achieve the rapid and quantitative analysis of bacteria inactivation caused by plasma based on the recorded GFP fluorescent signal, the survival fraction (SF) of transgenic *E. coli* can be calculated according to the following equation:

$$\mathrm{SF} = (F - F_d)/(F_0 - F_d) \tag{1}$$

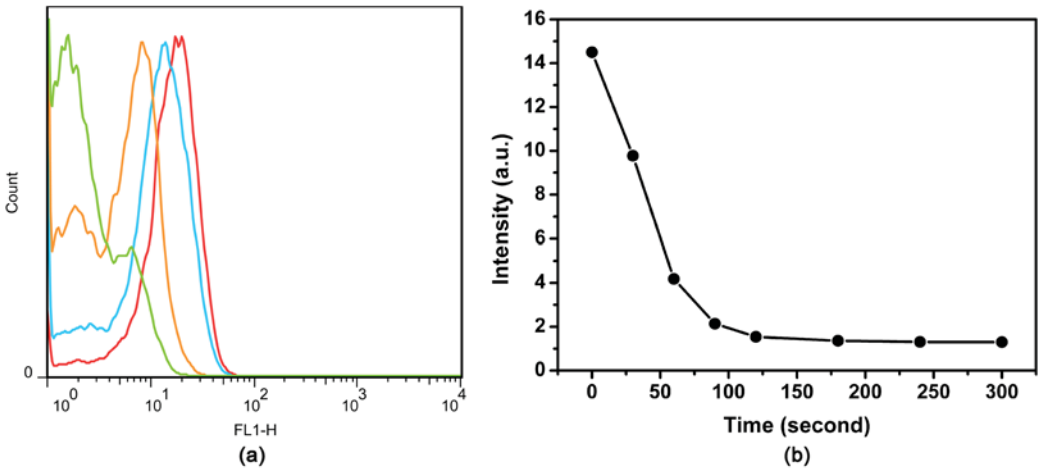

FIG. 2: (a) GFP fluorescence in transgenic *E. coli* after plasma treatment for 0 (red), 30 (blue), 60 (orange), and 90 sec (green) (from right to left). (b) Dependence of GFP fluorescence intensity on plasma exposure time.

where F represents the mean GFP fluorescent intensity after plasma treatment for a certain period. F_d represents the mean background GFP fluorescent intensity in the bacteria that were dead, and F_0 represents the mean GFP fluorescent intensity in the bacteria that were alive prior to plasma treatment. In this work, F_d was measured by flow cytometer in GFP-expressing *E. coli* after plasma treatment for 300 sec. According to equation (1), SF was plotted against plasma exposure time; the result is shown in Figure 3. The figure shows that SF decreases exponentially with plasma exposure time. The SF can be fitted according to the equation as follows:

$$\mathrm{SF} = 1.06468 \times \exp(-t/46.55394) \tag{2}$$

where t represents the plasma exposure time.

To evaluate the effectiveness and accuracy of the GFP-based bactericidal assay, we compared the fluorescence data with the CFU results and established the correlation between the two methods. Herein, the bacterial suspension after plasma treatment was also tested with the CFU measurement, and the results are shown in Figure 3. Similarly, the number of viable cells decreased exponentially with plasma treatment time. The equation for SF from the CFU measurements is obtained as follows:

$$\mathrm{SF} = 1.00005 \times \exp(-t/46.55394) \tag{3}$$

Clearly, the loss of GFP fluorescence is correlated with a reduction in the number of viable cells as obtained by the CFU measurements, and the two fitted curves are in agreement.

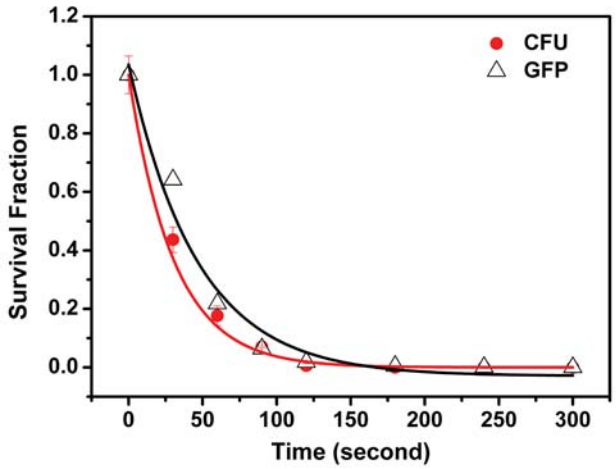

FIG. 3: Comparison of the inactivation kinetics obtained from CFU (circle) and GFP fluorescence (triangle) measurements in GFP-transformed *E. coli* after plasma treatment for different times.

The reason for the decrease of GFP fluorescence intensity with plasma discharge time is ascribed to the plasma-produced reactive oxygen species (ROS), which can damage GFP and thus quench the fluorescence.[24] At the early stage of the discharge, ROS production was relatively low, and less ROS penetrated the cell membrane. During this time, the plasma irradiation did not affect intracellular GFP. With longer plasma treatment, more ROS was produced and penetrated into the bacteria, which eventually killed the bacteria. In addition, bacteria themselves could also produce intracellular ROS via metabolic processes (such as respiration), which induced oxidative stress.[25] Regardless of the sources of the ROS, they could affect the bacteria and inactivate GFP.

In conclusion, our results have unambiguously shown the usefulness of intracellular GFP fluorescence signal detected with flow cytometer as a tool for monitoring plasma-induced bactericidal effects in transgenic *E. coli*. This technique can provide a rapid, real-time quantification of dead bacteria over plasma exposure time. In addition, it does not require additional preparation, and so it can be used to perform high-throughput screening of antimicrobial activity of cold plasma treatment. This may greatly facilitate studies to elucidate the mechanism of the nonthermal plasma bactericidal activity, which we are currently conducting in parallel.

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