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Eradication of marine biofilms by atmospheric pressure non-thermal plasma: A potential approach to control biofouling?

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ABSTRACT

Although the antimicrobial activity of atmospheric pressure non-thermal plasmas, including its capacity to eradicate microbial biofilms, has been gaining an ever increasing interest for different medical applications, its potential utilisation in the control of biofouling and biodeterioration has, to date, received no attention. In this study, the ability of atmospheric pressure plasma to eradicate biofilms of four biofouling bacterial species, frequently encountered in marine environments, was investigated. Biofilms were grown on both polystyrene and stainless steel surfaces before being exposed to the plasma source. Viability and biomass of biofilms were evaluated using colony count method and differential Live/Dead fluorescence staining followed by confocal laser scanning microscopy. Rapid and complete eradication of all biofilms under study was achieved after plasma exposures ranging from 60 to 120 s. Confocal microscopy examination showed that plasma treatment has mediated not only cell killing but also varying degrees of physical removal of biofilms. Further investigation and tailored development of atmospheric pressure non-thermal plasma sources for this particular application could provide an additional powerful and effective weapon in the current anti-biofouling armamentarium.

1. Introduction

Biofouling is the undesired accumulation of living organisms on surfaces in contact with liquids (Flemming, 2002; Whelan and Regan, 2006). Biofouling is considered a major problem in many industries causing the loss of billions of dollars every year and increasing the pressure on several resources, by increasing running costs, fuel consumption (Chambers et al., 2006; Whelan and Regan, 2006) and greenhouse gas emissions, in addition to impeding the performance of a staggeringly diverse range of affected equipment (Whelan and Regan, 2006). Biofouling develops by the formation of a conditioning layer followed by the adhesion of bacterial cells which grow into a sessile form of bacterial growth known as biofilm (Whelan and Regan, 2006). This stage of the complex process of biofiouling is often referred to as "micro-fouling" which in turn facilitates further attachment of larger organisms ranging from macroalgae up to various species of invertebrates in a process referred to as "macro-fouling".

Since formation of biofilms is an essential stage in the biofouling process, their prevention and/or eradication may have an important role in the control of biofouling. Biofilms are characterised by the production of extracellular matrix which significantly enhances their mechanical stability and their tolerance to biocides, making them difficult to eliminate (Ceri et al., 1999; Flemming, 2002). Different anti-biofouling techniques that target biofilms are available in current practice, such as mechanical cleaning, use of biocides and the use of antimicrobial paints and coatings (Vladkova, 2009). Unfortunately, most of these techniques have substantial drawbacks related to their cost, reduced efficacy and/or compatibility (Whelan and Regan, 2006), as well as their negative environmental impacts (Evans et al., 1995), which necessitate development of new methods that circumvent these limitations.

Atmospheric pressure non-thermal plasma (APNTP) has been under intensive research in the evolving field of 'plasma medicine' for a range of different medical applications, including its development as an emerging antimicrobial tool (Laroussi, 2005; Fridman et al., 2008; Kong et al., 2009). Interestingly, APNTP has been reported to have an excellent activity in the eradication of several clinically significant microbial biofilms (Vleugels et al., 2005; Abramzon et al., 2006; Sladek et al., 2007; Akishev et al., 2008; Joshi et al., 2010; Koban et al., 2010; Alkawareek et al., 2012a,b).

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However, to date, no investigations have been conducted in relation to the potential utilisation of this approach in the context of biofouling control, where it could offer great promise as a result of its many favourable characteristics, including rapid biofilm inactivation, low capital and operational cost, utilisation of virtually non-toxic gases and absence of environmentally harmful residues, in addition to its high design flexibility and output tuning ability to suit different applications in different settings.

In this study, the anti-biofilm activity of atmospheric pressure non-thermal plasma has been evaluated against a range of biofouling-associated marine biofilms grown on both polystyrene and stainless steel surfaces. Standard colony count and Live/Dead differential fluorescence staining followed by confocal laser scanning microscopy were used to assess both biofilm cell killing and physical removal efficacies of APNTP.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The following microbial strains were used in this study: *Pseudomonas fluorescens* DSM-304, *Staphylococcus saprophyticus* DSM-4852, *Vibrio alginolyticus* DSM-2171, *and Vibrio proteolyticus* DSM-30189. All microbial strains used in this study were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and stored at −20 °C in Microbank vials (Pro-Lab Diagnostics, Cheshire, UK). Lysogeny broth (LB) was used as a growth medium for *P. fluorescens* whereas nutrient broth supplemented with 2.5% sodium chloride (NB+2.5%NaCl) was used for the rest of the bacterial strains. Before testing, all bacterial strains were subcultured at 30 °C with orbital shaking at 120 rpm.

2.2. Plasma source

The plasma source used in this study, presented in Fig. 1, was previously described in Alkawareek et al. (2012a,2012b). Briefly, the plasma jet consists of a quartz tube with inner and outer diameters of 4 mm and 6 mm, respectively. Two copper electrodes (2 mm wide) encircle the tube with inter-electrode distance of 25 mm. For this study, the output of a high voltage pulse source (Haiden PHK-2k), operating at repetition frequency of 20 kHz, and voltage amplitude of 6 kV, was applied to the downstream electrode, which is 10 mm from the end of the plasma tube. The upstream electrode was grounded. The plasma jet was operated with 2 Standard Litres per Minute (SLM) helium and 0.5% oxygen admixture. The plasma source generates an intense core plasma between the two electrodes and a luminous plume which, under the operating conditions discussed here, extends up to several centimetres beyond the exit of the tube. Importantly, the zone of production of biocidal agents (or kill zone) has been previously shown to extend well beyond the focused visible plume (Alkawareek et al., 2012a,b).

2.3. Biofilm growth and treatment conditions

For biofilm survival curve experiments, biofilms of each bacterial strain were grown on the peg lid of the Calgary Biofilm Device (CBD) (Innovotech Inc., Alberta, Canada). An overnight culture of each bacterial strain was adjusted to an optical density (OD_{550}) equivalent to 1×10^7 cfu ml^{-1} in the appropriate growth medium. One hundred and fifty microliters of the standardised bacterial suspension was added to each well of Calgary Biofilm Device which was then incubated at 30 °C for 48 h in a humidified compartment with an orbital shaking at 120 rpm. The bacterial inoculum was replaced by fresh growth medium after the first 24 h of incubation.

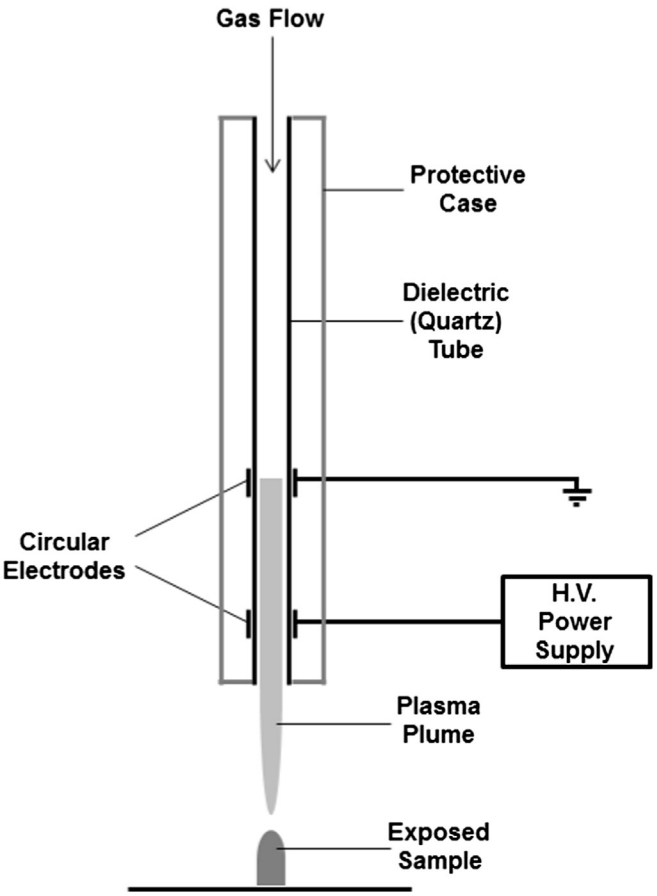

Fig. 1. A longitudinal section diagram of the plasma source utilised in this study.

After growing the biofilms for 48 h, individual pegs were broken off the CBD with sterile pliers and rinsed with 200 μl of phosphate buffered saline (PBS), to remove any planktonic or loosely adhered bacteria, before being exposed to the plasma jet for up to 4 min with a distance of 10 mm between the end of plasma jet tube and top of the peg. After plasma exposure, the pegs were placed in the wells of a 96-well microtitre plate containing 200 μl PBS in each well, and sonicated for 10 min. After sonication, the pegs were discarded and the resultant bacterial suspensions were used to determine the number of surviving bacterial cells by colony count method. All experiments were performed in triplicate.

For confocal microscopy examination, *V. alginolyticus* and *V. proteolyticus* biofilms were grown on stainless steel coupons (10 mm diameter) placed at the bottom of a 24-well plate with 1 ml of the standardised bacterial suspension (1×10^7 cfu ml^{-1}) added to each well. Biofilms were grown for 3 days at the conditions mentioned above. At the end of the biofilm growth period, a rinsing step was carried out using 0.9% NaCl solution before exposing the biofilm-bearing coupons to the plasma jet for 0, 60, and 240 s at a distance of 10 mm from the end of the plasma jet tube.

2.4. Confocal scanning laser microscopy

Following exposure to the plasma jet, biofilms grown on the stainless steel coupons were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes), and examined by confocal laser scanning microscope (Leica TCS SP2 Confocal Microscope, Leica Microsystems, UK).

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