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Mechanistic insights into response of *Staphylococcus aureus* to bioelectric effect on polypyrrole/chitosan film

Jieyu Zhang [a], Koon Gee Neoh [a, b, *], Xuefeng Hu [b], En-Tang Kang [a, b]

[a] NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Kent Ridge 117576, Singapore
[b] Department of Chemical and Biomolecular Engineering, National University of Singapore, Kent Ridge 117576, Singapore

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ABSTRACT

Treatment of biofilm-related infections in orthopedics remains a serious clinical challenge. It is known that an electric current can significantly enhance the potency of some antibiotics against biofilms (bioelectric effect) but the uncertainty of the mechanisms and the electrolytic cell-like system used in previous studies limit its applications. Herein, the behavior of *Staphylococcus aureus* (*S. aureus*) on an electrically conductive polypyrrole/chitosan film upon passage of a direct current (DC) through the film was investigated in the absence and presence of gentamicin. The killing efficacy of the bacteria within the biofilm by gentamicin was greatly enhanced by the DC treatment. From an analysis of the gene expression by the biofilm bacteria after treatment with gentamicin, DC and their combination, it is postulated that the promotion of bacterial autolysis by DC treatment is responsible for the enhanced susceptibility of biofilm *S. aureus* to gentamicin. This postulate is supported by an increase in the amount of extracellular deoxyribonucleic acid and adenosine triphosphate, and the appearance of disrupted bacterial cells in the biofilm after DC treatment. These findings provide a new insight into the interaction between DC and bacteria, and offer potential benefits for the treatment of infections in orthopedics.

1. Introduction

Implant-associated infection is a serious problem in orthopedics since it not only results in implant failure, but it also puts the patient at risk of systemic infections or even death [1]. The formation of bacterial biofilm is usually a causative factor in implant-associated infections. Once a mature biofilm is established, conventional antibiotic-based therapies are not efficacious. Under such circumstances, surgical replacement of the infected implant would be the only choice, which results in severe patient distress and huge costs [2]. Thus, development of effective strategies to combat implant-associated infections is urgently needed. In 1992, it was discovered that an electric current can enhance the efficacy of some antibiotics such as tobramycin and ciprofloxacin against biofilms formed by certain types of bacteria [3,4]. This effect, termed the bioelectric effect, may offer a solution to eradicate the implant-related infections, but uncertainties in the optimal electrical parameters and antibiotic types as well as the mechanism limit its application [5,6].

Most studies on the bioelectric effect employed two electrodes immersed in an electrolyte (usually the bacterial culture medium) with passage of current between them, similar to an electrolytic cell [7–9]. In such systems, the electric current was usually in the order of mA, and reactive oxygen species as well as metal ions (when metal electrodes were used) were generated due to electrochemical reactions on the electrode surface [10,11]. These species are harmful to the human body, which may also limit the potential biomedical applications of bioelectric effect using this electrical configuration [12].

In our previous study, it was found that osteoblast functions were enhanced on an electrically conductive polypyrrole/chitosan (PPY/chitosan) film when a direct current (DC) was applied along the film [13]. This is a different electrical configuration from the electrolytic cell-like system mentioned above. In our system, electrolytic effects should be minimized because a major portion of the current passed through the film rather than the medium. In addition, the PPY/chitosan film is flexible, non-cytotoxic and biodegradable [13], which is beneficial for potential biomedical applications since sites prone to infections can be covered by the flexible film, and surgery for its subsequent removal is not necessary due to its biodegradability. Thus, if the above-mentioned bioelectric effect can be implemented in this PPY/chitosan film-

* Corresponding author. Blk E5, 4 Engineering Drive 4, #02-34, Singapore 117576, Singapore. Tel.: +65 65162176; fax: +65 67791936.
E-mail address: chenkg@nus.edu.sg (K.G. Neoh).

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based system, it can potentially be highly beneficial for the dual purpose of combating infections and promoting osteoblast functions in implant-associated applications.

To verify this hypothesis, the behavior of *Staphylococcus aureus* (*S. aureus*) inoculated on the PPY/chitosan film when subjected to DC, gentamicin and their combination was investigated. *S. aureus* was chosen as the target bacteria because it is the main pathogen for implant-related infections [1]. The choice of gentamicin is based on the fact that it is frequently used in orthopedic surgery (e.g. supplemented in bone cement) to prevent implant-related infections due to its heat stability and wide-spectrum antibiotic activity [14]. To the best of our knowledge, investigations of possible bioelectric effect involving *S. aureus* and gentamicin have not been reported in literature. In particular, the effects of DC on biofilm bacteria at the genetic level have not been studied so far. Thus, in this work, the expression of selected genes by the biofilm bacteria after DC treatment was investigated. These target genes have been shown to influence *S. aureus* biofilm formation via different pathways [15–17], and thus their expression patterns would help to elucidate the mechanisms of the interaction between DC and bacteria. Extracellular deoxyribonucleic acid (eDNA) released in the biofilm, extracellular adenosine triphosphate (eATP) level and bacterial cell morphology were also assessed with the aim of furthering the current understanding of the bioelectric effect.

2. Materials and methods

2.1. Materials

PPY/chitosan film was prepared as per our previous study [13], and the details are given in the Supplementary Information. The thickness of the PPY/chitosan film was 0.15 ± 0.01 mm and its conductivity was 0.05 ± 0.008 S/cm. The film was biodegradable, with ~10% loss of its original weight after 10 weeks of incubation in 10 μg/mL lysozyme solution at 37 °C [13]. *S. aureus* 25,923 was obtained from American Type Culture Collection (Manassas, VA), and gentamicin sulfate were purchased from Sigma–Aldrich Chemical Co. St. Louis, MO. All other chemicals, if not specified, were purchased from Sigma–Aldrich Chemical Co.

2.2. Susceptibility of planktonic and biofilm S. aureus to gentamicin

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of gentamicin for planktonic *S. aureus* were determined by the broth microdilution method as recommended by Clinical Laboratory Standards Institute [18]. The details of MIC and MBC determination are given in the Supplementary Information.

For biofilm-related experiments, *S. aureus* biofilm was first formed on PPY/chitosan film as follows: A sterilized PPY/chitosan film (80 mm × 10 mm) was mounted in a custom-built bacterial culture setup as described in the Supplementary Information (Fig. S1a). The setup comprised a flat polycarbonate bottom plate and a top polycarbonate plate with 6 wells of 64 mm in diameter, and the film was mounted between them. 200 μL of bacterial suspension (1×10^6 cells/mL) in tryptic soy broth (TSB) was added to each well. After incubation at 37 °C for 24 h, the bacterial suspension was removed and the biofilm was washed three times with phosphate buffered saline (PBS, pH 7.4). The preformed biofilms were used for subsequent experiments.

For investigation of the susceptibility of biofilm *S. aureus* to gentamicin, the preformed biofilm was exposed to 200 μL of TSB supplemented with gentamicin at MBC for 2 days. The TSB with gentamicin was refreshed after 24 h. The treated biofilms were then washed three times with PBS, and viewed using confocal laser scanning microscopy (CLSM: Nikon, Ti-E microscope with A1 confocal system, Tokyo, Japan). The bacterial count was quantified by the spread plate method. The morphology of bacteria in the unwashed biofilms was observed using field emission scanning electron microscopy (FESEM: JEOL JSM-6700, Tokyo, Japan). To investigate the effect of gentamicin dosage on biofilm disruption, the preformed biofilm on PPY/chitosan film was exposed to 200 μL of TSB supplemented with gentamicin at different concentrations (from MBC to 128 times MBC) for 2 days at 37 °C. After that, the broth was removed, and the biofilm remaining on the film was washed three times with PBS. The number of viable bacteria was quantified by the spread plate method.

2.3. Effect of DC on S. aureus adhesion

DC was applied to the custom-built bacterial culture setup (shown in Fig. S1a) via two alligator clips attached to the two opposite ends of the PPY/chitosan film (which were covered with copper tape to ensure good electrical contact) and then connected to a current source (Keithley Instruments Inc., Model 6221, Boston, MA) as shown schematically in Fig. S1b. With the PPY/chitosan film mounted in the setup, 200 μL of bacterial suspension in PBS (1×10^8 cells/mL) was added to each well and incubated at 37 °C. Different DC current (50–250 μA) was then applied to the films for 4 h. After that, the bacterial suspension was removed by aspiration, and the films were washed three times with PBS. The adherent bacteria on the films were observed using scanning electron microscopy (SEM: JEOL JSM-5600LV, Tokyo, Japan) and fluorescence microscopy (Nikon, Eclipse Ti–U, Tokyo, Japan), and quantified by the spread plate method.

2.4. Effect of DC on preformed S. aureus biofilm

S. aureus biofilms were first formed on PPY/chitosan films after 24 h incubation in TSB as described above. The preformed biofilms were incubated in TSB and subjected to 200 μA of DC for 4 h per day. DC was first applied 32 h after *S. aureus* were seeded on the films as illustrated in Fig. S1c. The TSB was refreshed after incubation of the preformed biofilm for 24 h, and the experiment was conducted for 2 days. The biofilms were then washed three times with PBS, and observed using CLSM and the bacterial count was quantified by the spread plate method. For observation of the morphology of the biofilm bacteria using FESEM, the unwashed biofilm was used.

2.5. Combined effect of DC and gentamicin on preformed S. aureus biofilm

S. aureus biofilms were first formed on PPY/chitosan films as described above. The preformed biofilms were then incubated in TSB supplemented with gentamicin at MBC and also subjected to 200 μA DC for 4 h per day. DC was applied as described above. The TSB with gentamicin was refreshed after incubation of the preformed biofilm for 24 h and the experiment was conducted for 2 days (Fig. S1c). The biofilms were then washed three times with PBS, and observed using CLSM. The bacterial count was quantified by the spread plate method. For observation of the morphology of the biofilm bacteria using FESEM, the unwashed biofilm was used.

2.6. Characterization of adherent bacteria and biofilm

For SEM or FESEM observation, the bacteria on the substrates were fixed with 4% (v/v) glutaraldehyde for 30 min and then dehydrated with serial ethanol (25, 50, 75 and 100% for 10 min each) at room temperature. After drying in air, the substrates were coated with platinum.

For fluorescence microscopy and CLSM observation, the bacteria were stained with the LIVE/DEAD® Baclight™ bacterial viability kit (Molecular Probes L13152, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol before imaging was carried out.

Quantification of adherent bacteria was conducted using the spread plate method according to a previously published report [19]. Briefly, the adherent bacteria were harvested by sonication for 7 min, and then resuspended in PBS. The bacterial suspension was then serially diluted, and 100 μL of each dilution was spread onto a growth agar plate for determination of the viable cell number after incubation overnight at 37 °C. The number of adherent bacterial cells was expressed as cells per cm^2 of substrate surface.

2.7. Gene expression

The expression of autolysis and cell–cell interaction regulator genes by biofilm *S. aureus* was analyzed by real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). After treating the preformed biofilm with gentamicin at MBC, DC, or their combination, the medium was removed. The biofilm was subjected to three rounds of washing with PBS, and the biofilm bacteria on the PPY/chitosan films were then suspended in PBS via 7 min of sonication, followed by centrifugation and resuspension in 200 μL of diethylpyrocarbonate-treated water. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with an additional treatment step with DNase I (Sigma–Aldrich, St. Louis, MO) to eliminate residual genomic DNA. The concentration of total RNA was measured with the Quant-iT™ RNA assay kit (Invitrogen, Carlsbad, CA), and a solution containing 1 ng of total RNA was then added to each PCR mixture prepared with an iScript™ One-Step RT-PCR kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Real-time qRT-PCR was conducted on the Bio-Rad iQ™5 multicolor real-time qRT-PCR detection system. Dissociation curves were used to confirm the amplification specificity, and expression levels of target genes were normalized with respect to that of the reference gene, 16S rRNA. The primers used for qRT-PCR were synthesized commercially (1st Base Pte. Ltd., Singapore) and are shown in Table S1.

2.8. Quantification of eDNA

Quantification of eDNA was carried out following the method reported previously with minor modification [20]. Briefly, after treating the preformed biofilm with gentamicin at MBC, DC, or their combination, the medium was gently removed by aspiration using a pipette, and the remaining unwashed biofilms on the PPY/chitosan films were resuspended in 0.9% (w/v) NaCl solution, vortexed for 1 min and treated with proteinase K (5 μg/mL) for 30 min at 37 °C. The bacterial solution was then filtered through a 0.2 μm filter (Sartorius, Göttingen, Germany), and the elute