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Highly sensitive label-free dual sensor array for rapid detection of wound bacteria

Roya Sheybani, Anita Shukla*

School of Engineering, Center for Biomedical Engineering, Institute for Molecular and Nanoscale Innovation, Brown University, Providence, RI 02912, United States

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ABSTRACT

Wound infections are a critical healthcare concern worldwide. Rapid and effective antibiotic treatments that can mitigate infection severity and prevent the spread of antibiotic resistance are contingent upon timely infection detection. In this work, dual electrochemical pH and cell-attachment sensor arrays were developed for the real-time spatial and temporal monitoring of potential wound infections. Biocompatible polymeric device coatings were integrated to stabilize the sensors and promote bacteria attachment while preventing non-specific cell and protein fouling. High sensitivity (bacteria concentration of 10^2 colony forming units (CFU)/mL and −88.1 ± 6.3 mV/pH over a pH range of 1–13) and stability over 14 days were achieved without the addition of biological recognition elements. The dual sensor array was demonstrated to successfully monitor the growth of both gram-positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) over time through lag and log growth phases and following antibiotic administration and in simulated shallow wounds conditions. The versatile fabrication methods utilized in sensor development, superior sensitivity, prolonged stability, and lack of non-specific sensor fouling may enable long-term *in situ* sensor array operation in low resource settings.

1. Introduction

Bacterial infection is among the leading causes of death worldwide (Spellberg et al., 2008). Wound infections, in particular, complicate the treatment of injured patients in over 2.4 million cases per year in the United States alone, costing over 35 billion USD (Boucher and Corey, 2008; Scott II, 2009). Rapid and effective antimicrobial treatments can help mitigate the severity of these infections, prevent the development of antibiotic resistant superbugs (Spellberg et al., 2013; Taubes, 2008), and preclude the formation of bacterial biofilms, which are significantly more difficult to treat than planktonic bacteria (An and Friedman, 1998). Timely microbial detection is critical for the rapid initiation of these therapies (Cars, 1989). Conventional methods of bacterial detection (e.g., polymerase chain reaction (PCR), culture and colony counting methods, and immunology-based methods), while sensitive (detecting down to approximately 10 colony forming units (CFU) of bacteria (Lazcka et al., 2007)), are highly time-consuming (requiring hours to days), and are unsuitable for point-of-care applications where *in situ* monitoring of the infection is required (Lazcka et al., 2007; Sin et al., 2014).

Biosensors are miniaturized devices that combine a biological sensing element and a transducer (Blum and Coulet, 1991), and have the potential to provide equally reliable results as conventional instruments in much shorter times. Previous attempts at developing infection detection biosensors (Dutta et al., 2005; Heflin et al., 2012; Lillehoj et al., 2014; Mannoor et al., 2012; Varshney and Li, 2009; Ward et al., 2014; Wu et al., 2014), however, have suffered from a number of limitations including the inability to operate *in situ* (Rackus et al., 2015; Yoo and Lee, 2016), lengthy detection times (Pejcic et al., 2006), lack of sensitivity, use of cytotoxic materials or materials that are unstable *in vivo* (Dargaville et al., 2013; Glinel et al., 2012), and a need for large external equipment yielding them infeasible for wearable operation (Sin et al., 2014; Weingarten et al., 2010). These limitations are in part due to the complexity of biosensor design for operation in biological fluids, as sensor sensitivity and specificity may be compromised by low analyte concentrations and non-specific biofouling of the sensor surface (Kricka, 1998). Bio-recognition elements (e.g., enzymes, immunoassays, and aptamers) while capable of providing superior sensitivity and selectivity (Dargaville et al., 2013; Varshney and Li, 2009), often require complicated fabrication methods to ensure reliable and repeatable operation (Pejcic et al., 2006; Varshney and Li, 2009), suffer from limited shelf-life and stability (Ronkainen et al., 2010), and

* Corresponding author.
E-mail address: anita_shukla@brown.edu (A. Shukla).

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may cause local toxicity and sensitization (Glinel et al., 2012). In this work, we sought to develop sensors for use in bacterial wound infections that are highly sensitive, but not subject to the limitations of molecular labels.

Monitoring a single aspect of a potentially infected wound is unlikely to accurately describe the complex wound environment (Kim et al., 2015). For example, wound pH, which is often used as a marker of infection, is known to fluctuate both during the healing process and in the presence of bacteria (Sharpe et al., 2013; Zhuk et al., 2014). As such, pH can only be used as a non-specific, although highly sensitive, indicator of infection and may allow for tracking of wound healing progression (Dargaville et al., 2013). Direct sensing of bacterial attachment can be used to describe specific bacteria content. Thus, together sensing pH and cell-attachment have the potential to accurately describe the status of the wound in real-time.

Here, we report the development of a highly sensitive, arrayed electrochemical sensor device comprised of multiple semi-selective label-free sensors to monitor both the local pH and attachment of bacterial cells across a potentially infected wound, providing a rapid indication of this infection. The use of complementary sensors allows for accurate assessment of wound infection while reducing sensor specificity requirements. Functional biocompatible polymeric coatings were integrated to enable superior sensor sensitivity and stability, compared to many existing sensor designs, while reducing non-specific biofouling. We implemented electrochemical sensors in our arrayed sensor device as they are low cost, highly sensitive, easily miniaturized through microfabrication, low power, relatively simple to instrument, and do not require molecular labels (Lazcka et al., 2007; Pejcic et al., 2006). Building upon existing work on interdigitated impedance biosensors for detection of bacterial cells (Dargaville et al., 2013; Rackus et al., 2015; Varshney and Li, 2009) and electrochemical pH sensors (Kakooei et al., 2013), we developed an advanced dual sensor array for the real-time, accurate assessment of potential wound infections, capable of *in situ* operation. Our sensors do not utilize biological recognition elements, and do not require the addition of external solutions, pre- or post-sample processing, or visual/optical access to the wound. Furthermore, the compact design of the sensor array and versatile fabrication methods allow for multiple arrays to ultimately be incorporated into a single flexible substrate (e.g., a bandage) to provide real-time spatial information about a wound infection, helping to optimize future *in vivo* antimicrobial treatments. In this work, we have demonstrated successful spatial and temporal monitoring of both gram-positive and gram-negative bacteria, before and after antibiotic administration, as well as sensor array operation in simulated wounds, as a proof-of-concept towards future use *in vivo*.

2. Materials and methods

Fabrication methods for each sensor, as well as experimental methods for the characterization of the pH sensor, are detailed in Supplementary Materials 1 and 2.1.

2.1. Experimental

Measurements for both the pH and cell-based sensors were carried out using a VERSASTAT4-200 potentiostat (Princeton Applied Research, Oak Ridge, TN).

2.1.1. Bacteria cell attachment-based sensor characterization in growth media

To assess electrochemical drift, sensors were placed in vials of 1× PBS. At various time points over the course of 24 h, 2-electrode potentiostatic impedance measurements were carried out. To monitor growth of bacteria, infected solutions of each of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, and *Streptococcus pyogenes* (prepared as detailed in Supplementary Materials 2.2) were added to separate vials of fresh broth solution along with a sensor. At various time points, impedance and absorbance measurements were carried out. In order to ensure the presence of sensors or electrochemical measurements does not affect bacterial growth, absorbance measurements were also carried out on two vials (one containing a sensor) that did not undergo electrochemical measurement.

To assess sensor sensitivity, sensors were placed in infected solutions with working concentrations of 10^{n} CFU/mL (with n=1–6) and after 5 min of incubation at 37 °C, impedance measurements were carried out. Each sensor's measurement was normalized to that same sensor's response in fresh broth solution in order to avoid cross-contamination of samples. Bacteria growth over time and sensitivity measurements were also carried out for chitosan-coated sensors in infected solutions of *S. aureus*.

2.1.2. Bacteria cell sensor response to antibiotic administration

S. aureus and *P. aeruginosa* infected solutions at 10^{5} CFU/mL were prepared. After 30 min of incubation, vancomycin hydrochloride hydrate (Sigma-Aldrich, St. Louis, MO) and carbenicillin disodium (Fisher Bioreagents, Pittsburgh, PA) were introduced at a final concentrations of 83 μg/mL and 74 μg/mL to *S. aureus* and *P. aeruginosa*, respectively. At each time point, bacteria concentration was measured along with impedance. Several hours after the addition of the antibiotic, the sensors were electrochemically cleaned within the same solution (i.e. ± 0.5 V applied across the sensor, for 10 s at each limit, 5 times). After 5 min, impedance measurements were performed. The effects of cleaning on the integrity of the chitosan coating was also assessed as described in Supplementary Materials 2.3.

2.1.3. Individual sensor operation in simulated wound fluid

First, pH sensors were placed in 5 mL of either 1× PBS or wound fluid (WF) solution (prepared as detailed in Supplementary Materials 2.4). For different pH values, sensors were allowed 5 min to reach equilibrium, before the open circuit potential between the sensing element and reference was measured and averaged over 60 s.

In order to measure the effects of WF on the bacterial cell sensors and the electrochemical measurement, one of each uncoated, chitosan-coated, Nafion®-coated, and chitosan-Nafion®-coated sensors were placed in WF without any bacteria and measurements were carried out periodically over approximately 5 h. Measurements were continued following the addition of infected solutions of *S. aureus* to either fresh 1× CMHB or WF.

2.1.4. Sensor array operation in vitro in simulated infected wound models

A simulated colonized wound model was prepared as previously described ((Bowler et al., 2012); see Supplementary Materials 2.5.1). A chitosan-Nafion®-coated bacterial cell sensor, along with a pH sensor were encased in agar infected with 10^{3} CFU/mL *S. aureus*. WF was added to hydrate the samples (25 mL). The two control groups examined were agar that was kept sterile throughout the experiment and agar which was non-sterile, and thus capable of serving as a growth medium for bacteria that was not intentionally introduced (considered non-pathogenic bacteria). At various time points, the bacterial cell sensors were electrochemically cleaned *in situ* and the response of the sensors was recorded as previously described. In order to assess the effects of gravitational sedimentation of bacteria on sensor response, the experiment was repeated with sensors placed face-down.

In order to assess spatial monitoring using multiple sensor arrays, we investigated the placement of multiple dual sensor arrays *in vitro* in a simulated wound infection model (detailed in Supplementary Materials 2.5.2). Two dual sensor arrays (each containing a chitosan-Nafion®-coated bacterial cell sensor and a pH sensor) were placed approximately 100 mm apart on agar. Invasive infection, hindering normal wound healing, has been shown to occur at bacteria concentra-