Highly Sensitive Label-Free Dual Sensor Array for Rapid Detection of Wound Bacteria

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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 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; Pejic 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 allows 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 $1\times$ PBS. At various time points over the course of 24 hours, 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 $83\ \mu\text{g/mL}$ and $74\ \mu\text{g/mL}$ 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 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 seconds.

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 hours. 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 concentrations of greater than 10^5 CFU/gram tissue (Robson et al. 1999). To mimic this infection condition, one array was encased in initially sterile agar while the other was flooded with infected molten agar containing either rapidly replicating *E. coli* (England 2013) or slower growing *S. pyogenes* at 10^5 CFU/mL. Once the agar solidified, excessive amounts of WF (45 mL) were added to hydrate the simulated wound (**Supplementary Materials Fig. 1a**). A second *S. pyogenes* wound model was prepared in which considerably less ($\sim <65\%$) WF was added representing a drier wound and the area of initial infection was reduced to a quarter of the plate (**Supplementary Materials Fig. 1b**).

3. Results and Discussion

In this work, we have developed a highly sensitive, arrayed electrochemical sensor device comprised of dual 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. We first characterized operation of each sensor individually. Sensor sensitivity at different pH values and bacteria strains, stability, and integrity of the polymer coatings for repeated operation over 14 days was assessed. Simulated wound fluid and shallow wound

environments were then conducted to assess sensor response in conditions mimicking *in vivo* use.

3.1. Sensor Design and Sensing Mechanism:

The sensor design and proposed detection method of each sensor in the dual sensor array is shown in **Fig. 1a**. The bacterial cell attachment based sensor is comprised of an interdigitated thin film gold (Au) electrode, to directly sense the adsorption of bacteria on the sensor, and an Au counter/reference electrode (**Supplementary Materials Fig. 2**). The interdigitated design optimizes the surface area available for sensing (as compared to a monopolar electrode structure) (Liu et al. 2014); interdigitated sensors have previously been implemented in bacterial biosensors (Varshney and Li 2009). In contrast to the typical use of carbon electrodes, which readily corrode (Cohen et al. 2013; Grigoriev et al. 2010), Au increases sensor stability, which is critical for operation in complex biological environments (Voskerician et al. 2004). Once in the test environment (e.g., wound), a small alternating current can be applied across the electrodes through a range of frequencies and impedance magnitude and phase data can be acquired. Under potentiostatic conditions, the two electrode system can be approximated by an electrical representation with a parallel contribution of the polarization (faradaic) resistance and double layer capacitance (as seen in **Fig. 1a**), in series with the solution resistance (Faulkner and Bard 2001). Attachment of bacteria to the sensor surface causes a shift in the measured impedance, most prominently seen at mid-range frequencies dominated by the double layer capacitance (a representative phase versus frequency Bode plot for *S. aureus* is shown in **Supplementary Materials Fig. 3**). Phase values at these frequencies can be correlated to bacteria concentration. It is important to note that these measurements are carried out under low power conditions ($< 100 \mu\text{W}$) and the excitation voltages required are within the “water window” (AC potential

amplitude of 10 mV RMS and zero DC potential versus open circuit). Under these conditions, only non-Faradaic (reversible) reactions occur, and there is no change in bulk solution chemical composition (Gutierrez 2012).

The pH sensor design utilizes metal oxide (e.g., iridium oxide)-coated thin film gold (Au) as the sensing element versus a silver/silver chloride (Ag/AgCl) reference electrode (**Supplementary Materials Fig. 2**). Changes in pH lead to absorption of hydrogen ions causing valency changes in the oxygen atoms of the metal oxide, resulting in a generated potential relative to the reference electrode (Ochoa et al. 2014). In contrast to typical fabrication of reference electrodes that place the reference electrode adjacent to the sensing electrode (Huang et al. 2011; Phair et al. 2011), we fabricated the reference electrode circularly surrounding the sensing electrode. We hypothesized that this reference placement would lead to improved sensor stability during lengthy measurements required for biological applications.

3.2. Individual Sensor Characterization:

Prior to examining sensor response in bacterial cultures, sensitivity, stability, and drift associated with the sensors was characterized.

3.2.1. pH Sensor Characterization:

The pH sensor response was calibrated over a wide range of pH values (1 - 13). As expected, electrochemical deposition of hydrated iridium oxide led to a super-Nernstian relationship between the measured potential and pH (Kakooei et al. 2013). An average sensitivity of -88.1 ± 6.3 mV/pH was obtained (**Supplementary Materials Fig. 4a**). A 5 min equilibration time was sufficient for sensor response to stabilize with less than 1% variation. Similar sensitivity values have previously been reported using other electrochemical pH sensor designs (Kakooei et al. 2013). However, operation of these sensors *in vivo* will not only require sensor

sensitivity, but also stability over long durations. Sensor sensitivity and stability have been shown to decrease significantly in biological environments ($>15\%$ over 8 days) (Kakooei et al. 2013), dependent on the metal oxide deposition and adhesion quality (Kakooei et al. 2013) and reference electrode stability (Huang et al. 2003). In addition to fabricating the reference electrode circularly around the sensing electrode, we cast a solid-state potassium chloride (KCl) electrolyte on the reference electrode to slow the rate of Ag/AgCl depletion (Huang and Huang 2002). Sensor response was monitored daily over 14 days (**Supplementary Materials Fig. 4b**), demonstrating an average sensitivity of -58.0 ± 7.1 mV/pH with less than $8.4 \pm 1.0\%$ variation in the measured potential. This long-term sensor reproducibility and minimal variation has not previously been reported for existing pH sensor designs (Kakooei et al. 2013). Without the electrolyte, an average sensitivity of -39.1 ± 11.6 mV/pH and approximately $16.6 \pm 2.5\%$ variation was noted, further supporting the need to stabilize the reference electrode.

3.2.2. Bacteria Cell Attachment-based Sensor Characterization in Growth Media:

Drift is often observed in impedance measurements due to changes in the adsorbed ionic intermediaries on the electrode surfaces, especially inherent to noble metals such as Au used in 2-electrode potentiostatic impedance measurements (Cote and Gill 1987; Kaisare et al. 2011). This drift can negatively impact the sensitivity of measurements in complex biological environments over time. Testing our cell attachment sensors, a minimal drift of 1.010 ± 0.004 with less than 1% variation after the first hour, was noted over a period of 24 hours in $1\times$ phosphate buffered saline (PBS). It is important to note that as drift in measurement is predominantly due to perturbation caused by the measurement excitation voltage (Kaisare et al. 2011), it is not expected to affect sensor operation during storage and between measurements.

Having confirmed the stability of the sensors, we examined the response of the bacteria cell sensor within bacteria inocula in growth media. We tested a range of bacteria commonly found in wounds (Bowler et al. 2001; Giacometti et al. 2000), including gram-positive *S. aureus* and *S. pyogenes*, and gram-negative *P. aeruginosa* and *E. coli*. We hypothesized that sensor response would be a function of bacteria size and shape parameters, as the bacteria augment the capacitive contribution to the circuit. Sensor response was calculated by normalizing the peak value of the measured Bode phase plot at mid-range frequencies ($10^1 - 10^3$ Hz) to the value measured for sensors placed in media in the absence of bacteria (**Supplementary Materials Fig.3**). The bacterial cell sensors were able to successfully detect growth of all four bacteria (above drift) within 30 minutes of inoculation, throughout the lag and log growth phases in real-time (**Fig. 1b**). The sensor response reached a plateau over time at bacterial concentrations of approximately 2×10^7 , 3×10^6 , 3.5×10^6 , and 8.5×10^4 CFU/mL for *S. aureus*, *P. aeruginosa*, *E. Coli*, and *S. pyogenes*, respectively. We determined that the plateau concentration depends on the size and projected area of the bacteria cells on the sensor surface (having a geometric surface area of approximately 19.6 mm^2), and represents complete occlusion of the sensing area by the bacteria. Once the plateau is reached, a gradual and slow change in sensor response is observed, representing the attachment of additional bacteria on the initial bacteria layer already attached to the sensor. Our experimental results confirm that the capacitive contribution of the attached bacterial cells to the measured impedance in the pre-plateau phase is directly proportional to the projected area of the bacteria and inversely proportional to the bacteria height (i.e., the separation distance between the electrode surface and the surrounding solution caused by bacterial adsorption to the sensor) as described in **Fig. 1a** with an R^2 value of 0.98 (**Fig. 1d**).

Supplementary Materials Table 1 provides details of the projected areas and heights utilized in this analysis based on reported bacteria dimensions (Jorgensen et al. 2015).

Sensor sensitivity, defined as the minimum bacteria concentration detectable by the sensor with the recorded response above the electrochemical drift, was also found to depend on bacteria size as expected. Sensors were able to reliably detect bacteria concentrations equal to or greater than 1×10^3 CFU/mL for *S. aureus* and 1×10^2 CFU/mL for *P. aeruginosa*, *E. coli* and *S. pyogenes* (**Fig. 1c**), which are larger than *S. aureus*. A linear relationship was observed between the normalized sensor response and the log of bacterial concentration ($R^2 = 0.9$). In order to further improve sensor sensitivity, we coated the sensor surface with chitosan, a biocompatible polysaccharide derived from chitin (Dutta et al. 2004). Based on studies by Mitik-Dineva., *et al.*, we hypothesized that electrostatic interactions between the positively charged chitosan polymer (Ilium 1998) and negatively charged bacteria would enhance bacteria adsorption at low bacteria concentrations (Mitik-Dineva et al. 2006). We observed an improvement in sensor sensitivity for *S. aureus* of 1×10^2 CFU/mL (**Supplementary Materials Fig. 5**). This minimum detectable concentration is 5 orders of magnitude lower than previously developed electrochemical bacterial sensors. To the best of our knowledge, similar sensitivity values have not yet been achieved for direct *in situ* measurement without the use of molecular labels or cytotoxic materials (Dargaville et al. 2013; Heflin et al. 2012; Lillehoj et al. 2014; Mannoor et al. 2012; Radke and Alocilja 2005; Varshney and Li 2009; Ward et al. 2014). High sensitivity can be attributed to the interdigitated sensor design, increasing the effective sensing area and limiting edge effects resulting from partial attachment of bacteria cells (Liu et al. 2014), and further improvement by incorporating the chitosan coating.

3.2.3. Bacteria Cell Sensor Response to Antibiotic Administration:

The cell attachment sensors were able to successfully monitor the effects of antibiotic administration on bacteria growth (**Fig. 2a, b**). Vancomycin, a potent bactericidal glycopeptide antibiotic was added to *S. aureus* in its log growth phase at a concentration (83 $\mu\text{g/mL}$) significantly greater than the known minimum inhibitory concentration (MIC) of vancomycin against *S. aureus* (1 – 2 $\mu\text{g/mL}$ (Hanberger et al. 1991)). Within 3 hours, a deviation from the expected sensor response for untreated *S. aureus* was observed, with the bacteria cell sensor response rapidly plateauing as bacterial death was observed. Debris from the dead bacteria occluded the sensor surface, leading to an unchanged sensor response. This debris was successfully removed through mild *in situ* electrochemical cleaning (i.e., ± 0.5 V applied across the sensor, for 10 seconds at each limit, repeated 5 times). Once the cleaning procedure was applied, an immediate zeroing of the sensor response was observed, indicating that no viable bacteria remained in the sample. We also used the cell attachment sensor to monitor the response of *P. aeruginosa* upon administering carbenicillin, a penicillin antibiotic, which is not typically used for treatment of infections caused by *P. aeruginosa*, due to its very high and broad MIC range against this bacteria (32- 128 $\mu\text{g/mL}$ (Chadwick 1969)). At a mid-MIC concentration of carbenicillin (74 $\mu\text{g/mL}$), we observed *P. aeruginosa* to grow as expected for untreated bacteria demonstrating that this carbenicillin concentration was ineffective in inhibiting the growth of our particular *P. aeruginosa* strain (PA 14). The sensor response was also found to plateau as expected. Applying the electrochemical cleaning process to these sensors, we found that the sensors could immediately continue to detect the presence of live bacteria and the bacteria were observed to be growing unhindered during the next 30 minutes of measurement following the cleaning (**Fig. 2b inset**). As expected (Gutierrez 2012), the cleaning method does not appear to affect the live bacteria and can be used to maximize temporal resolution in the

sensor response over lengthy measurement times (Otto et al. 2006). We also examined the effects of this cleaning process on the chitosan-coated sensors. Integrity of the chitosan coating was electrochemically assessed (Sheybani and Meng 2012) over 14 days (**Supplementary Materials Fig. 6**). No significant difference (one way analysis of variance (ANOVA); $\alpha = 0.05$) was observed in sensor coating for sensors subjected to daily cleaning compared to sensors stored in $1\times$ PBS.

3.3. Sensor Operation in Simulated Wound Infections:

3.3.1. Individual Sensor Operation in Simulated Wound Fluid:

In order to mimic *in vivo* operation, the effects of protein and mammalian cell fouling on sensor response were evaluated in simulated wound fluid (WF) and mammalian cell containing media. WF contains an abundance of concentrated proteins (Sharp 2013) as compared to the less complex, more diluted buffered bacteria culture medias. As previously observed with similar sensors (Sharpe et al. 2013), the pH sensor response was not affected in WF over a range of pH values (1-13) tested (**Fig. 3a**). For the bacterial cell attachment sensors, Nafion®, which is known to form gels which have small micrometer sized pores (large enough to allow bacteria cells to enter) (Zawodzinski et al. 1994) and has demonstrated resistance to proteins and mammalian cell biofouling (Pejcic et al. 2006; Wisniewski and Reichert 2000), was introduced in addition to chitosan, to further block access of large components (e.g., mammalian cells) to the sensor surface. For uncoated sensors, we observed a uniform shift in sensor response to bacterial growth in WF, correlated to the normalized response of the sensor in WF without any bacteria. Subtraction of the shift curve from the sensor response in bacteria infected solution, resulted in a response curve similar to that obtained for measurements in infected culture media (**Fig. 3b**), suggesting that interfering analytes in the WF lead to fouling of the uncoated cell

sensor electrode surface more rapidly than bacteria culture media. In fact, in the absence of bacteria, rapid fouling of the uncoated sensor surface by WF components (within 15 minutes; data not shown) led to a measured cell sensor response that increased over time. The uniform shift in sensor response in infected WF was not observed for chitosan-coated, Nafion®-coated, and chitosan-Nafion®-coated sensors (**Fig. 3b**), which displayed a similar response as sensors in infected bacteria growth media. In addition to decreasing protein adsorption, Nafion® acts as a solid polymer electrolyte, helping reduce electrochemical drift by stabilizing the counter/reference electrode (Pletcher and Saxton 2009). Antifouling properties of the chitosan-Nafion®-coated sensors in the presence of adherent mammalian cells (i.e., bone marrow-derived human mesenchymal stem cells) was further investigated (detailed in **Supplementary Materials 2.6**). Cells were introduced in suspension to coated and uncoated cell-attachment sensors. The coating completely prevented mammalian cell attachment (**Supplementary Materials Fig. 7**), while uncoated sensors were rendered non-functional due to rapid fouling within 15 minutes.

3.3.2. Sensor Array Operation in vitro in Simulated Infected Wound Models:

It is well recognized that the pH of infected wounds can fluctuate depending on the infectious microbes present. However, fluctuations in pH may not always be a definitive marker of infection, as these changes are also observed in normal wound healing (Schneider et al. 2007; Sharpe et al. 2013). Thus, pH alone is not capable of accurately diagnosing the presence of an infection. Having explored sensor operation individually, we tested the response of dual sensor arrays comprised of the pH and bacteria cell attachment sensors developed in this work in a simulated shallow wound infection model. Similar simulated wound infection models have previously been employed to study new devices, wound dressings, therapeutics, etc., *in vitro* (Bowler et al. 2012; Percival et al. 2007; Sharp 2013; Walker et al. 2011). As shown in **Fig. 4**,

the sensors were able to successfully detect the growth of pathogenic *S. aureus* in the model, along with non-pathogenic bacteria growing in the non-sterile control (**Fig 4a**), with a larger growth response measured for the infected sample (**Fig 4b**). The pH of the infected model wound increased considerably over time, from pH 8.0 to 10.7 (**Fig. 4c**). These results corroborate prior *in vivo* findings indicating that wound pH becomes more alkaline by *S. aureus* colonization (Schneider et al. 2007). While the cell attachment sensors required electrochemical cleaning to regain responsiveness over time, pH-sensor response continued to track infection progression. These results confirm that the response of each sensor on its own is not sufficient to confidently conclude the presence of infection. However, accurate assessment of the wound environment is possible when the results from the array are considered concurrently.

The effects of gravitational bacterial sedimentation on sensor response were assessed, by examining the dual sensor array placed face-down in the simulated wound. Similar trends to those in which the sensors are placed facing upward were observed, although the response was slightly slower with an approximate 30 min delay (**Supplementary Materials Fig. 8**).

In addition to temporal monitoring of an infected wound, spatial monitoring can help formulate individualized local treatments. We investigated the placement of multiple dual sensor arrays *in vitro* in a simulated wound infection in order to assess their spatial resolution (**Supplementary Materials Fig. 1**). As shown in **Fig. 5a**, fast spreading and growth of bacteria was observed for *E. coli*, along with an expected increase in environmental pH as a result of bacterial metabolism (Fig. 5b). A slower growth and spreading of bacteria to the second array was measured for the samples infected with *S. pyogenes*. Specifically, in the wound model with excessive WF (Fig. 5c), infection spreading to the sterile region was aided by the WF, while in the drier wound, a slower spreading through the hydrated agar was observed (Fig. 5e). For the

rapidly growing *E. coli*, the initially sterile array reached the infection concentration of 10^5 CFU/ML in less than 30 minutes for the model with excessive WF (Fig. 5a), whereas in the same model with *S. pyogenes*, an extra hour was required for this infection concentration to be reached (Fig. 5c). Furthermore, in the simulated *S. pyogenes* infection, the initially sterile array reached this concentration approximately three times faster in the wound model with excessive WF (Fig. 5c) as compared to the dry wound (Fig. 5e; 75 minutes versus 210 minutes, respectively). In *S. pyogenes* simulated wound infections, a gradual increase in the environmental acidity surrounding the initially sterile sensor array was also observed. This drop in pH can be attributed to lactic acid production by *S. pyogenes* (Jorgensen et al. 2015Jorgensen et al. 2015). The rate of acidification depends on bacteria concentration; pH surrounding the initially sterile array decreased approximately twice as rapidly in the wound model with excessive WF (Fig. 5d) compared to the drier wound (Fig. 5f). These results suggest that spreading of pathogenic species in different wound environments could be successfully monitored by using multiple dual sensors arrays. Therefore, in order to achieve spatial monitoring in large heterogeneous wounds, multiple sets of sensors could be integrated throughout the length of a smart wound bandage.

4. Conclusion

We have designed and characterized a highly sensitive electrochemical dual sensor array for the real-time monitoring of pH and bacteria cell attachment. The array utilizes unique sensor design elements and functional coatings that eliminate non-specific fouling to obtain superior sensitivity and stability. The sensors are capable of detecting five orders of magnitude lower bacteria concentrations than similar existing bacteria sensors and maintaining stability during long term *in situ* operation over 14 days. This sensitivity is achieved without the use of bio-recognition elements (e.g., enzymes, immunoassays, and aptamers) reducing fabrication

complexity, extending sensor shelf-life and stability for *in situ* operation in low resource settings. While electrochemical pH sensors with similar sensitivity have been previously reported, the use of complementary sensors allows increased selectivity and accurate infection detection in complex wound environments. The combination of the dual sensor array was observed to provide accurate, real-time temporal and spatial information *in vitro* in simulated wound infection models. The results of these studies are highly promising, warranting future testing in more complex *in vivo* wound environments, which may consist of polymicrobial environments.

The fabrication methods and materials presented here can be applied to flexible substrates and wireless data transfer can be integrated such that these sensors may be further developed on clinically-relevant materials (e.g., bandages, sutures, catheters, etc.) and used remotely. The sensor array could also be combined with drug delivery systems to allow for closed-loop operation. Finally, each of the sensors, individually or as an array, could readily be repurposed for use in other *in vitro* and *in vivo* infection conditions (e.g., drug efficacy screening, device-associated infection models, etc.), further contributing to the versatility of the sensors developed in this work.

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Figure 1. (a) Top: operation concept for dual electrochemical sensor array (array footprint approximately 20 mm $\times$ 14 mm); bottom: proposed detection method of the pH (left) and bacteria cell attachment (right) sensors. Attachment of bacteria on the cell sensor augments the capacitance, C , based on projected bacteria area, A , and height, d ; k is relative permittivity of the dielectric material and ϵ_0 is permittivity of space; (b) Bacteria cell sensor response following *S. aureus*, *P. aeruginosa*, *E. coli*, and *S. pyogenes* growth over time (bacteria concentration was monitored using the 0.5 McFarland turbidity standard (absorbance, or optical density, at 600 nm (for *S. aureus*, *P. aeruginosa* and *S. Pyogenes*) and 540 nm (for *E. coli*) $\approx 10^8$ CFU/mL)); (c) Relationship between cell attachment sensor response and bacteria concentration for the 4 bacterial strains tested. Sensitivity was defined as the minimum bacteria concentration detectable by the sensor above electrochemical drift (approximately 1.010); (d) Empirical relationship between sensor response and bacterial cell dimensions (inset shows the experimentally derived correlation).

Figure 2. Cell attachment sensor response following antibiotic administration to media infected with (a) *S. aureus* or (b) *P. aeruginosa* (Sensor response is normalized to starting bacteria concentration of 10^5 CFU/mL). Mild *in situ* electrochemical cleaning can remove dead bacteria debris from the sensor surface without affecting live cells in solution (inset: The cleaning method does not affect the live bacteria and the sensors immediately detect the continued growth of live bacteria at a rate similar to the expected growth trajectory of non-antibiotic-treated bacteria shown as dashed line).

Figure 3. Sensor operation in simulated wound fluid (WF): (a) pH sensors (inset equations show the relationship between measured potential and solution pH); (b) bacterial cell sensor response following *S. aureus* growth over time (uncoated —■— (*ANOVA, $p < 0.05$), chitosan-coated —●— (ANOVA, $p = 0.439$, $F(1,14) = 0.632$), Nafion®-coated —▲— (ANOVA, $p = 0.920$, $F(1,14) = 0.010$), and chitosan-Nafion®-coated —◆— (ANOVA, $p = 0.541$, $F(1,14) = 0.393$), in $1\times$ Cation adjusted Mueller Hinton Broth media and WF, respectively), inset: absorbance measurements over time.

Figure 4. Sensor array operation in a simulated wound infection model: (a) Photographs of the cell attachment and pH sensors encased in sterile and non-sterile agar controls and agar infected with *S. aureus* (scale bar = 5 mm). Bottom images show observed cloudiness after 25 hours of incubation as a result of bacterial colonization; (b) Bacterial cell sensor and (c) pH sensor response over time.

Figure 5. Spatial resolution obtained with multiple sensor arrays operating in simulated wound infection models: Bacteria cell sensor and pH sensor response over time for rapidly replicating *E. coli* in the wound model with excessive WF (a, b) or slower growing *S. pyogenes* infected samples in the wound model with excessive WF (c, d) and the comparatively drier wound model (e, f), respectively.