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Identification and characterisation of *Staphylococcus aureus* on low cost screen printed carbon electrodes using impedance spectroscopy

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ABSTRACT

Staphylococcus aureus infections are a cause of significant morbidity and mortality, in addition to representing a considerable economic burden. The aim of this study was to explore a low cost screen printed electrode as a sensor for the detection of *S. aureus* using impedance spectroscopy. *S. aureus* was incubated in chambers containing the electrodes and the results analysed using a novel normalisation approach. These results show that it is possible to detect the presence of *S. aureus* in LB media after 30 min incubation of a 1% growth culture, in addition to being able to see immediate cell concentration dependant changes in 0.9% NaCl. These observations imply that a number of electrochemical mechanisms cause a change in the impedance as a result of the presence of *S. aureus*, including adsorption to the electrode surface and the metabolism of the bacteria during growth. The study suggests that this detection approach would be useful in a number of clinical scenarios where *S. aureus* leads to difficult to treat infections.

1. Introduction

Staphylococcus aureus is a component of the skin's normal microflora for an estimated 30% of the population and can be a significant source of infection in immune compromised individuals (Tong et al., 2015). Prosthetic device infections, boils and serious infectious diseases such as meningitis, pneumonia and sepsis are among numerous conditions directly associated with this pathogen (Madigan et al., 2012). *S. aureus* is easily transferred between individuals both in the community and care settings due to its commensal presence with uncompromised hosts. Furthermore, it is of a growing concern due to its association with hospital acquired infections (HAI) and an increasing resistance to antibiotics (Tong et al., 2015). In Scottish paediatric hospitals, 6.1% of patients are affected by *S. aureus* infections (Reilly et al., 2012), and *S. aureus* is estimated to be the cause of around 16% of HAI in Europe. Furthermore, it has been found that over 40% of these *S. aureus* strains are methicillin resistant (ECDC, 2013; Sievert et al., 2013).

Gold standard identification of *S. aureus* is based upon growth on selective agar and is not optimised for rapid, point of care identification of the bacterium. In clinical practice, the turn-around time to identify the bacterial species responsible for an infection is 2–3 days due to the time required for growth on selective media (Bissonnette and Bergeron, 2015). Recent technologies, such as matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS), can rapidly identify species but are expensive and still require well-isolated colonies (Bourassa and Butler-Wu, 2015). Currently, molecular techniques, such as PCR and next generation sequencing, that rely on recognition elements for a specific pathogen often require specialist sample processing and can also be expensive to implement (Clarridge, 2004; Heaney et al., 2015). Several assays have been developed based upon these techniques in order to identify the presence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in clinical samples, with detection times of less than one hour (Corrigan et al., 2012; Hulme, 2017). Therefore, there is a recognised and growing need for low cost point of care devices that are capable of detecting pathogenic microorganisms in real time at low cost (O'Neill Report, 2016).

One approach to bacterial detection that is both cost effective to implement and capable of providing real-time information is electrochemical impedance spectroscopy (EIS). In EIS, the electrochemical impedance across an electrode-electrolyte interface is measured over a wide range of frequencies to elicit information about the properties of the interface. By looking for changes in the interface, it is possible to detect the presence and growth of bacteria without the need for surface recognition elements. A wide range of microorganisms have been characterised through this approach, including, *Escherichia coli,*

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Pseudomonas aeruginosa, Pseudomonas stutzeri Staphylococcus epidermidis and S. aureus (Bayoudh et al., 2008; van Duuren et al., 2017; Harris et al., 1987; Harris and Kell, 1985; Muñoz-Berbel et al., 2006; Paredes et al., 2014; Pires et al., 2013; Ward et al., 2014; Yang et al., 2004; Yardley et al., 2000). Six potential mechanisms have been identified that could affect the impedance measured in an electrochemical cell, as a result of microbial growth, and result in a unique signature for a particular species (Ward et al., 2014).

Additive printing processes, such as screen printing, are versatile and cost effective approaches to the production of printed electronics and sensors (Khan et al., 2015). In screen printing, ink is pushed through a mesh stencil onto a substrate, printing a specific design. This printing technique can be implemented at relatively low cost contrasted to microfabrication and scales well from prototyping to mass production. Therefore, it is widely used to mass produce low cost sensors, such as glucose test strips (Bruen et al., 2017).

In this paper, the impedance of *S. aureus* is explored in a range of media using disposable screen printed carbon electrodes, which could form the basis of a low cost infection sensor for use in a wide range of medically important situations where infections must be rapidly diagnosed. A normalisation approach is implemented (Connolly and Shedden, 2010). The aim is to use the normalised impedance parameter to identify a characteristic signature that can be used to detect a specific species of microorganism. The observed changes in the spectrum are then explored further in order to elucidate the underlying processes affecting the impedance.

2. Materials and methods

2.1. Media, buffers and microbial strains

LB media was used for the growth of stock cultures and impedance experiments by mixing 10 g of tryptone (Fisher Scientific), 5 g NaCl (Sigma Aldrich) and 5 g yeast extract (Fluka) with 1000 ml of w/v dH_2O. The media was then autoclaved at 121 °C for 20 min. Foetal Bovine Serum (Biosera) was heat inactivated at 80 °C for 10 min prior to use. *S. aureus* strains NCTC8325 and NCTC10788 from the National Culture Type Collection (Public Health England) were used as described. Inoculated media was placed in an orbital incubator overnight at 37 °C, 150 rpm prior to use.

2.2. Electrode printing and electrode assembly

Electrodes were screen printed in-house onto a Polyethylene Terephthalate (PET) substrate (Hi-Fi Films) using a solvent cure carbon ink (Henkel). After printing, electrodes were cured in a fan assisted box oven for 30 min at 150 °C. A dielectric ink (Gwent) was then printed to cover the electrode tracks and define the electrode area, and cured at 150 °C for 30 min. Printed electrodes were assembled into plates consisting of eight electrodes, with each electrode located at the bottom of a well. The plate was produced with a laser cut piece of acrylic (Fig. 1A). This was then mounted onto the PET substrate using Servisol silicone adhesive sealant (Servisol). Once the sealant had cured, the electrodes were conditioned in 0.9% NaCl, in a two electrode configuration by placing a potential of 2 V (vs OCP) across the working electrode for 3 min, followed by a potential of − 2 V (vs OCP) for a further three minutes (Wang et al., 1996). Prior to the start of the experiment, the assembly was sterilised in a 70% v/v solution of ethanol and filled with the appropriate media for the experiment. The electrode was then incubated overnight at 37 °C to allow background currents related to surface processes to decay (Bard and Faulkner, 2001). The electrodes are designed for single use, therefore at the end of the experiment, they were autoclaved and disposed of as waste.

2.3. Microbial impedance measurements

Impedance measurements were carried out using a Solartron SI1260 in a two electrode configuration, with a 200 mV_{rms} perturbation potential (vs OCP). Impedance measurements were performed from 1 MHz to 1 Hz. Due to the noise in the normalised impedance spectrum, data above 100 kHz has been omitted from the figures of the impedance spectra. The impedance data was normalised using the patented normalisation approach by Connolly and Shedden (2010):

$$IP_{norm} = \frac{IP_{t=n}}{IP_{t=0}}$$

Where IP is the impedance parameter of interest (i.e. reactance, resistance, impedance modulus or phase) and n represents the normalised time point. Normalisation was performed against a fresh aliquot of sterile media (LB, FBS or 0.9% NaCl) after overnight incubation of the electrode chambers in the media, (defined as $t = 0$).

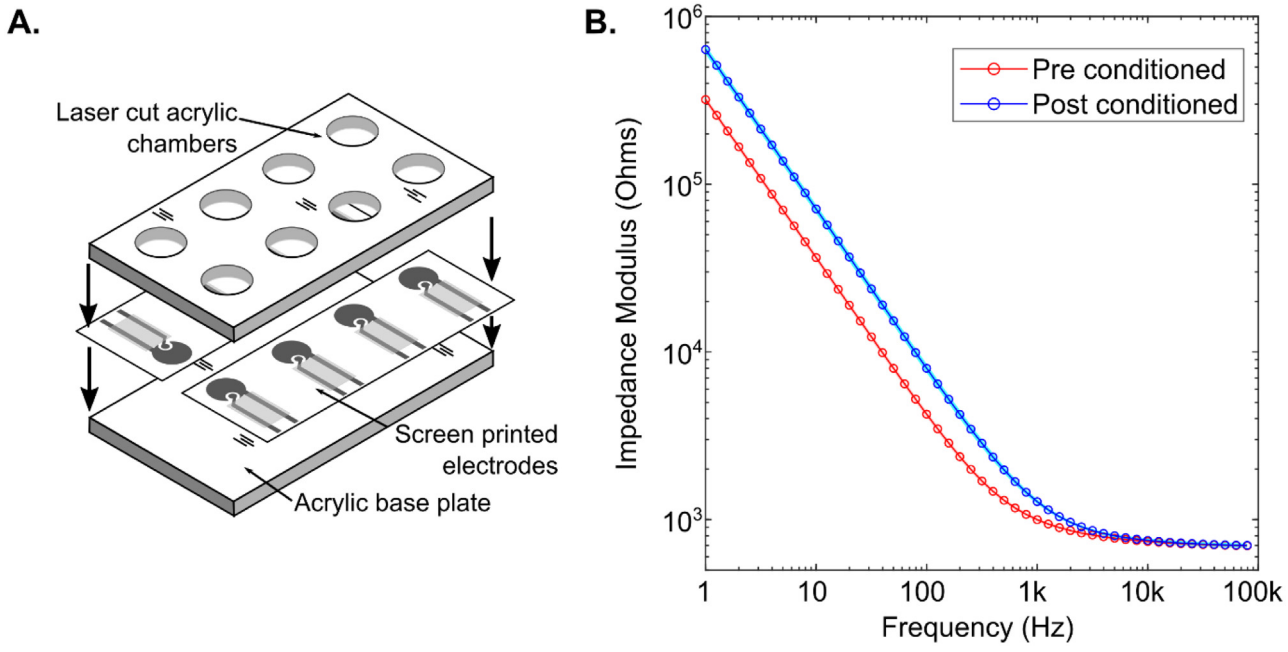


Fig. 1. (A) Schematic and photograph of the electrode chambers used in the experiment. (B) Baseline performance of the electrodes in 0.9% NaCl.

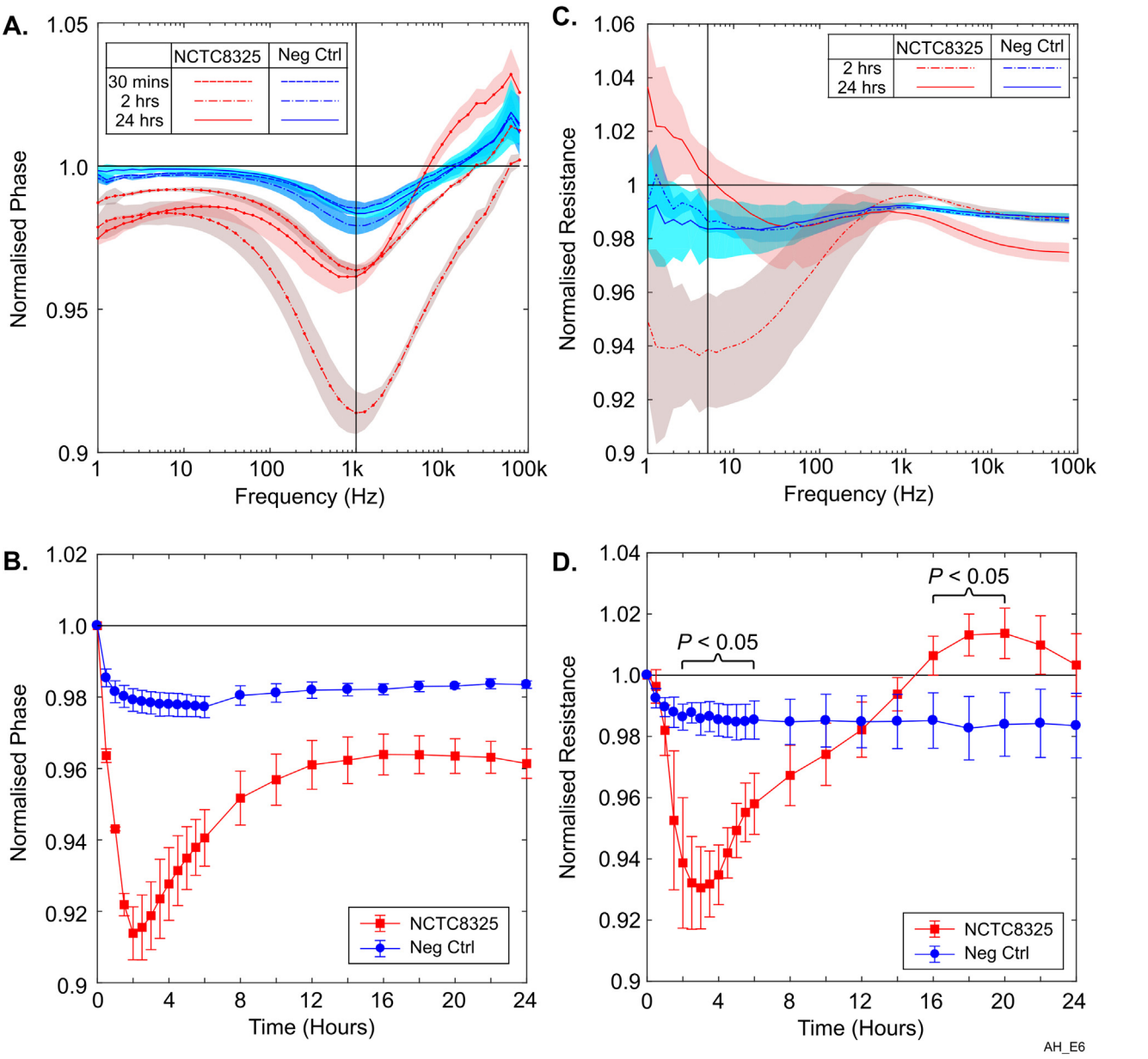


Fig. 2. (A) Normalised phase spectrum of NCTC8325 growth in LB media. The greatest change what observed at 2 h, after which the trough gradually returned to the same value seen at 30 min. $n = 3$, background shading represents +/− 1 SD. (B) Normalised phase at 1 kHz, showing all time points. Normalised phase measurements from 30 min onwards were significantly different from the negative control ($P < 0.007$, $n = 3$). Error bars represent +/− 1 SD.(C) A trough was apparent at 5 Hz, which disappeared at later timepoint, to be replaced by a shoulder (background shading represents (+/− 1 SD, $n = 3$). (D) Time based plot, showing the values of the normalised resistance where the 5 Hz trough was observed at 2 h. The trough was significantly different at 2–6 h and the shoulder was significantly different between 16 and 20 h. (Error bars represent +/−1 SD, $n = 3$).

2.4. Cell washing

Overnight LB culture media was centrifuged at 13,400 rpm for 5 min. The resulting supernatant was then removed and replaced with sterile 0.9% NaCl, then the cells were re-suspended and centrifuged for a further 5 min. The supernatant was removed and replaced with a second aliquot of fresh 0.9% NaCl. This was then diluted to the required concentration with additional sterile 0.9% NaCl, where the washed cells were treated as 100% concentration. To obtain a 128% concentration, a 200% concentration of washed cells was produced by replacing the supernatant with half the volume of sterile 0.9% NaCl after the second wash and diluted accordingly.

2.5. Colony counting

Colony counting was performed using the drop plate method (Herigstad et al., 2001). An aliquot of the culture was taken and used to create a series of ten-fold serial dilutions in LB media. 10 µl drops of each dilution were then plated onto LB agar plates and incubated for up to 18 h. Colonies were counted at the concentration where between 3 and 30 colonies per drop were present and used to estimate the starting cell concentration.

2.6. Statistical analyses

Impedance data was tested for normality using the Anderson-Darling test. As the Anderson-Darling test suggested no significant

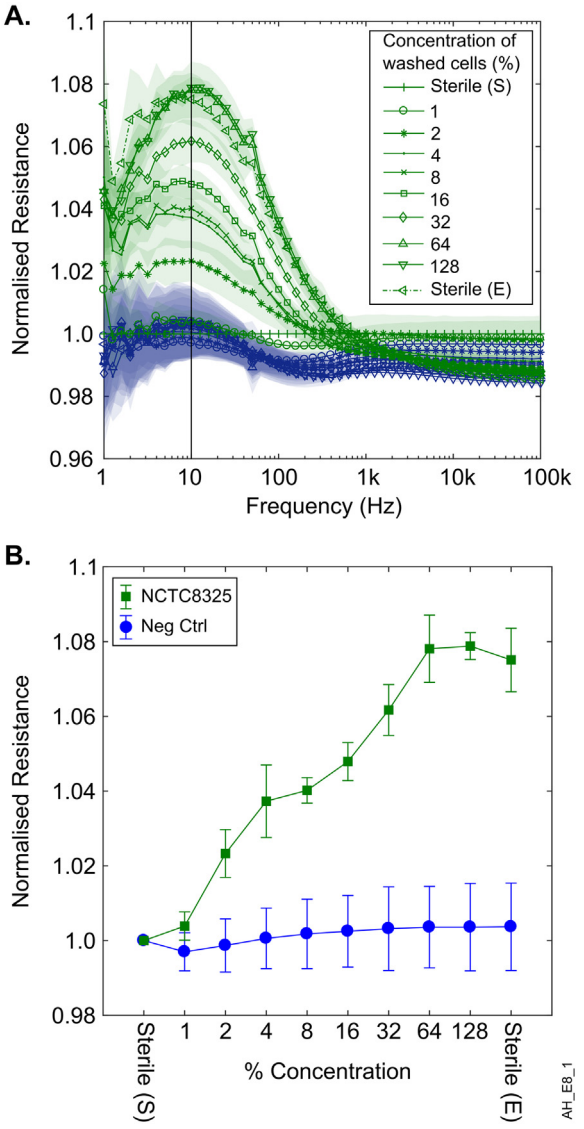


Fig. 3. Measurement of NCTC8325 in 0.9% NaCl. The cell concentration was incrementally doubled from a 1% starting concentration of 1.8×10^6 CFU/ml (range 1.2×10^6 to 2.5×10^6 CFU/ml, $n = 3$). (A) An increasing peak is visible in the chambers containing NCTC8325, which appears to be concentration dependant. Replacement of the 0.9% NaCl at the end of the experiment with a sterile aliquot (sterile (E)) has little impact upon this peak ($n = 3$, background shading represents +/− 1 SD). (B) Normalised resistance data at 10 Hz, showing the increase in normalised resistance as a function of concentration (Error bars represent +/− 1 SD, $n = 3$).

deviation from normality, a two-sample *t*-test was used to test the significance of any observed changes ($\alpha = 0.05$).

3. Results

3.1. Baseline impedance characteristics

Electrodes were assembled into the custom made chambers (Fig. 1A). Baseline impedance measurements were performed in 0.9% NaCl, before and after electrochemical conditioning of the electrodes was performed and show that the impedance decreases from low frequency to higher frequencies across the impedance spectrum (Fig. 1B). The electrochemical conditioning approach used led to a small reduction in the baseline impedance, in addition to an increase in the consistency between electrodes.

3.2. Impedance measurements of S. aureus growth in LB media

To investigate the impact of *S. aureus* growth in LB media, impedance measurements were carried out using *S. aureus* NCTC8325 was grown in LB media over a 24 h period, from a starting concentration of 1.8×10^6 CFU/ml (range 1.3×10^6 to 2.3×10^6 CFU/ml, $n = 3$). At the end of the experiment, the mean cell density had increased to 3.6×10^8 CFU/ml (range 2.9×10^8 to 4.6×10^8 CFU/ml, $n = 3$).

The normalisation approach highlighted distinct changes in the impedance spectra throughout time at around 1 kHz for the inoculated chambers, whereas no clear variation was observed in the chamber containing only sterile media (Fig. 2A and B). Low frequency changes were also seen in the normalised resistance (Fig. 2C and D), initially in the form of a trough at 5 Hz increasing in magnitude up to 2.5 h. Interestingly after four hours, the trough was replaced by a rise in the normalised resistance contrasted to the negative control.

3.3. Impedance measurements of S. aureus growth in FBS

Measurements performed in FBS, showed similar changes in the impedance spectrum. A comparable normalised phase trough to that observed in LB media was seen in FBS for both NCTC8325 and NCTC10788 strains of *S. aureus* (Fig. S1). This suggests that the mechanism resulting in a change in the impedance could also be seen in a chronic wound infected with *S. aureus*, given that wound exudate is a complex electrochemical environment similar to FCS. Furthermore, after 24 h of incubation in FBS, a normalised phase peak at higher frequencies had emerged, in addition to a normalised resistance peak between 100 Hz and 1 kHz. These changes were observed with two different strains of *S. aureus* NCTC8325 and NCTC10788.

3.4. Impedance measurements of S. aureus in 0.9% NaCl

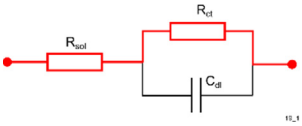
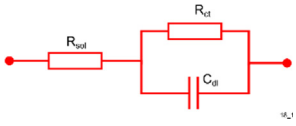
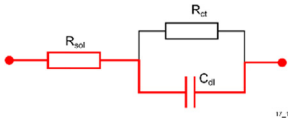
To explore the effect that incubation of *S. aureus* without any media had upon the impedance, an overnight culture of cells was washed in 0.9% NaCl, then a 1% overnight culture of cells was incubated in the electrode chambers at 37 °C for 2 h 30 min. The average starting cell density was found to be 7.7×10^5 CFU/ml and the ending density to be 6.0×10^5 CFU/ml, confirming a lack of growth within the 0.9% NaCl. A normalised resistance peak was observed in both negative control and NCTC8325 electrodes at low frequency (Fig. S2A), but this was not significantly different. In contrast, both the NCTC8325 electrode and negative control within the normalised phase showed very similar impedance signatures to those observed in sterile LB (Fig. S2B).

3.5. Impedance measurements at increasing cell density in 0.9% NaCl

In order to investigate the effect that an increasing cell density would have upon the impedance, a set of measurements was performed in 0.9% NaCl to determine whether any of the changes in impedance were related to the *S. aureus* cells to the exclusion of other factors. The cell concentration was incrementally doubled from 1% to 128%. The 1% culture had cell concentration of 1.8×10^6 CFU/ml (range 1.2×10^6 to 2.5×10^6 CFU/ml, $n = 3$). Impedance measurements were performed at each concentration and with a sterile aliquot of 0.9% NaCl at the start and end of the experiment. A separate colony count was performed at 128% and indicated at the cell density was 5.9×10^7 CFU/ml (range 3.6×10^7 CFU/ml to 9.3×10^7 CFU/ml, $n = 3$).

These measurements demonstrated that concentration dependant peaks occurred in the normalised resistance and normalised phase data. In the normalised resistance spectrum, a peak is present at 10 Hz (Fig. 3A). The peak increased in a concentration dependant manner and was significantly different above a concentration of 2% (Fig. 3B), which is equivalent to approximately 3.6×10^6 CFU/ml ($P < 0.02$, $n = 4$ for Neg Ctrl and $n = 3$ for NCTC8325). A concentration dependant peak

Table 1
Potential causes of the changes observed in the normalised phase and resistance data. The Randles equivalent circuit has been used to provide an indication of the likely current path, shown in red, at each frequency R_{ct} is the charge transfer resistance at the interface, C_{dl} is the double layer capacitance and R_s is the solution resistance. Further equivalent circuit modelling could be included in a future study to explore the impedance values of R_{ct}, R_s and C_{dl} further.

Measurement Media	Low Frequency < 100 Hz	Mid-frequency 100 Hz – 5 kHz	High Frequency > 5 kHz
Approximate current path			
Normalised Phase			
LB	No peaks or troughs observed.	Trough at 1 kHz, greatest after 2 h.	Peak shoulder present after 24 h.
FBS		Trough at 1 kHz, visible at 24 h.	Peak at 12.5 kHz, visible at 24 h.
0.9% NaCl		No trough observed.	Cell concentration dependant peak at 10 kHz.
Normalised Resistance			
LB	Trough visible at 5 Hz between 1 and 6 h post inoculation.	Slight peak visible at 1 kHz after 2 h, not significantly different from negative control.	No peaks or troughs observed.
FBS	No peaks or troughs observed.	Peak observed after 24 h at 200 Hz.	
0.9% NaCl	Concentration dependant peak at 10 Hz.	No peaks or troughs observed.	
Suspected cause of changes	Normalised resistance peak caused by increase in cell concentration. Normalised resistance trough caused by cell growth/metabolism related changes in rich growth media.	Normalised resistance peak and normalised phase trough caused by changes in media during growth and metabolism of *S. aureus*.	High frequency changes in normalised phase caused by a concentration of cells on electrode surface, possibly caused by the capacitive effects of microbial cell polarisation.

was also observed at approximately 10 kHz in the normalised phase data (Fig. S3).

4. Discussion

In this study, impedance spectroscopy was used to detect *S. aureus* by obtaining characteristic impedance signatures in LB, FBS and 0.9% NaCl. A number of electrochemical processes are believed to affect the impedance at different frequencies, as a consequence of the presence of *S. aureus*. For the purpose of this discussion, these have been divided into low frequency, mid-frequency and high frequency impedance changes (Table 1).

Within 30 min of a 1% inoculation into LB media, a trough formed at 1 kHz in the normalised phase data. These results were observed across several experiments performed by different researchers using the same screen printed electrodes, thus demonstrating the repeatability of the method. In previous studies, changes in the growth of micro-organisms at these frequencies have been related to the breakdown of nutrients within the media (Cady, 1975; Ur and Brown, 1975). The changes observed here after 30 min are unlikely to be related to biofilm formation and growth, because 30 min in an nutrient rich environment is insufficient time for a biofilm to form given that biofilm formation generally occurs in response to environmental stress (de la Fuente-Núñez et al., 2013).

To investigate whether changes in the impedance were related to growth and metabolism, the experiment was repeated in 0.9% NaCl to determine if the same trough was observed at 1 kHz. Under these conditions, it was found that the trough in the normalised phase did not occur, supporting the hypothesis that it is related to cell growth or metabolism. Electrochemically, the trough observed could be related to changes in the double layer capacitance. Specifically, the breakdown of nutrients and metabolites within the media would result in an increase in the number of ions present in the electrolyte. When these adsorb on the electrode surface, they would affect the charge density and therefore the impedance observed at intermediate frequencies, where the interplay between the double layer and the charge transfer resistance are dominant factors.

Measurements were performed with washed *S. aureus* cells in 0.9% NaCl, resulted in a concentration dependant change in the impedance at low frequency, suggesting that the presence of the *S. aureus* cells had an impact upon the impedance. Furthermore, the impedance signature did not return to its baseline value, when the final aliquot of 0.9% NaCl was removed from the electrode surface and replaced with a sterile aliquot. This suggests that *S. aureus* has a persisting effect on the electrode surface, which is not related to the breakdown of metabolites within the media. In previous studies, such low frequency changes have been attributed to the adhesion of bacteria or biofilm formation on the electrode surface (Bayoudh et al., 2008; Bonetto et al., 2014; Markx and Kell, 1990; Paredes et al., 2014, 2012; Settu et al., 2015; Yang et al., 2004). Given these experiments were performed with washed cells, it is likely that adsorption of cells on the electrode surface was the dominant factor here.

At higher frequencies, a peak was seen in the normalised phase in experiments performed in LB media and 0.9% NaCl. There are a number of explanations for this peak, which could be due to changes in the media caused by *S. aureus*, biofilm formation at the electrode surface and polarisation of the bacteria cells in the presence of an electric field (Markx and Kell, 1990; Paredes et al., 2012; Yardley et al., 2000). Caution should be exercised, however, as this peak could also be due to changes at the electrode interface caused by the media and not specific to the presence of bacteria (Settu et al., 2015).

In this study, the impedance spectrum has been analysed using a normalisation approach, and changes have been attributed to possible physical world effects using the Randles equivalent circuit. Using the results here as a basis, in a future study, equivalent circuit modelling could be used to gain a deeper understanding of values of the double layer capacitance, charge transfer resistance and solution resistance, based upon the measured real and imaginary impedance.

A key tenet of this work is that the sensors used should be low cost and disposable after use. The raw material costs for this study are estimated to be £0.18/electrode. This is based upon procurement of low quantities of the PET printing substrate and inks (both carbon and dielectric), excluding machine operating costs and setup costs. The cost of glucose testing strips, which are based upon a screen printed electrode with several modifications is between £0.14 to £0.32 per strip (British Medical Association, Royal Pharmaceutical Society of Great Britain, 2015). Therefore, at mass production scales, the manufacturing costs would be comparable.

5. Conclusions

This study demonstrates that *S. aureus* can be detected within 30 min from a starting concentration of 1.8×10^6 CFU/ml using a novel normalisation approach. This approach allows screen printed electrodes to be used without the need for any surface recognition elements and therefore a sensor can be produced at low cost. The electrodes could be of clinical benefit in a number of infection monitoring scenarios where there is a need to identify the presence of pathogenic bacteria, such as chronic wound infections, urinary tract infections and respiratory tract infections.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2018.03.048.

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