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Lab on a chip sensor for rapid detection and antibiotic resistance determination of *Staphylococcus aureus*†

Chathurika D. Abeyrathne,*[a,b] Duc H. Huynh,[a,b] Thomas W. Mcintire,[c] Thanh C. Nguyen,[a,b] Babak Nasr,[a,b] Daniela Zantomio,[d] Gursharan Chana,[a,e] Iain Abbott,[f] Peter Choong,[g] Mike Catton[f] and Efstratios Skafidas[a,b,e]

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The Gram-positive bacterium, *Staphylococcus aureus* (*S. aureus*), is a major pathogen responsible for a variety of infectious diseases ranging from cellulitis to more serious conditions such as septic arthritis and septicaemia. Timely treatment with appropriate antibiotic therapy is essential to ensure clinical defervescence and to prevent further complications such as infective endocarditis or organ impairment due to septic shock. To date, initial antibiotic choice is empirical, using a "best guess" of likely organism and sensitivity- an approach adopted due to the lack of rapid identification methods for bacteria. Current culture based methods take up to 5 days to identify the causative bacterial pathogen and its antibiotic sensitivity. This paper provides proof of concept for a biosensor, based on interdigitated electrodes, to detect the presence of *S. aureus* and ascertain its sensitivity to flucloxacillin rapidly (within 2 hours) in a cost effective manner. The proposed method is label-free and uses non-faradic measurements. This is the first study to successfully employ interdigitated electrodes for the rapid detection of antibiotic resistance. The method described has important potential outcomes of faster definitive antibiotic treatment and more rapid clinical response to treatment.

Introduction

S. aureus, a Gram-positive bacterium, has variable antibiotic sensitivity with two main subtypes: methicillin sensitive and methicillin resistant (MSSA and MRSA). It is a major pathogen that can cause bacteremia, skin and soft tissue infections, and more deep seated and difficult to treat osteoarticular infections and heart infections such as infective endocarditis.[1] *S. aureus* is the causative organism in 20–88.2% of skin and soft tissue infection cases, 16–34% of infective endocarditis cases and 18–73% of prosthetic joint infection cases.[1] In addition, the morbidity and mortality rate from these infective conditions is considerable at 29.8% and 23.3% in MRSA and MSSA infective endocarditis, respectively[2] and 38–86% in septicaemia for patients with *S. aureus* bacteremia.[3] A major factor in positive patient outcome is early detection and appropriate antibiotic treatment. Currently, culture techniques are required to diagnose bacterial infections and determine antibiotic sensitivity.[4,5] These techniques are labor intensive, require skilled personnel, take 24 hours to 5 days to provide results, and clinical sensitivity may be poor.[4,5] Due to the significant amount of time in ascertaining results for these traditional approaches, patients are routinely treated with empirical antibiotics to cover the most probable pathogens until culture results are available to guide more definitive treatment. This sub-optimal scenario can result in incorrect initial therapy leading to persistence of symptoms of septicaemia, organ compromise due to ongoing sepsis and increased mortality. As up to 12% of patients with sepsis die before microbiological results become available to the clinician, more rapid identification of the causative bacteria is likely to improve patient outcome.[6] Broader spectrum antibiotics are more likely to be prescribed empirically resulting in undesirable side effects, increased cost and higher rates of antibiotic resistance.[5] In addition, the development of

[a]Centre for Neural Engineering, The University of Melbourne, Carlton, VIC 3053, Australia. E-mail: chathurika.abeyrathne@unimelb.edu.au
[b]Department of Electrical and Electronic Engineering, Melbourne School of Engineering, The University of Melbourne, Victoria 3010, Australia
[c]Melbourne Medical School, The University of Melbourne, Victoria 3010, Australia
[d]Department of Haematology, Austin Health, Victoria 3084, Australia
[e]Department of Psychiatry, Royal Melbourne Hospital, The University of Melbourne, Victoria 3050, Australia
[f]Victorian Infectious Diseases Reference Laboratory, Doherty Institute, Victoria 3000, Australia
[g]Department of Surgery at St. Vincent's Hospital, University of Melbourne, Victoria 3000, Australia

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new classes of antibiotics and *S. aureus* vaccine[7] have stagnated over the last few decades, making strategies to reduce antibacterial resistance an important objective.

Reducing the time taken to identify the infective pathogen and its antibiotic sensitivities from days to hours is a promising solution to the issues outlined above. A number of commercial products utilizing real time polymerase chain reaction (PCR), a technique which amplifies microbial DNA to ascertain microbial species, have been developed to detect pathogens directly in whole blood samples and do reduce the turnaround time to 1–12 h.[5] However, while PCR provides a way to rapidly detect bacterial species, it still relies on further analysis to determine antibiotic sensitivity. In addition, these methods rely on low mutation microbial DNA rate in order to accurately maintain good prediction of susceptibility, are expensive, costing more than AUD$400 per test (€300),[5] and may not be applicable in resource poor environments.

Here, we develop an interdigitated electrode sensor for rapid detection of *S. aureus* and determination of antibiotic sensitivity. Our lab on a chip design offers distinct advantages of low fabrication cost, increased signal-to-noise ratio together with fast establishment of steady-state and low ohmic drop.[8] Our sensors measure the impedance change caused by binding of bacteria to antibodies directed against *S. aureus* immobilized on the surface of the sensor and the change in the conductivity of the medium caused by the growth of bacteria.[8] Although antibody-functionalized interdigitated electrodes have been previously investigated for the detection[9–13] or monitoring of bacterial growth[14–16] and the differentiation of live and (heat treated) dead bacterial cells,[17,18] there are important limitations that have been addressed in our method. Firstly, bacterial detection studies using antibody-functionalized non-conductive oxide (glass, Al_2O_3, SiO_2) on metal electrodes, have undertaken the negative control experiments using media lacking bacteria.[9–13] This negative control does not differentiate between the binding of bacteria to the antibodies and sensor surface (negative control without antibodies), and therefore lacks specificity. Secondly, in papers investigating the differentiation of dead and live bacteria, the samples are comprised of either live or dead cells killed post heat treatment (≈80 °C).[17] These measurements again are not representative of a bacterial sample responding to antibiotic, which will contain both live and dead cells.

In this paper, we have developed a biosensor using interdigitated electrodes, for rapid and label-free detection of *S. aureus* using sensors functionalized with anti-*S. aureus* antibody. In addition, the sensors described have the requisite surface hydrophilicity and hydrophobicity properties, uniform functionalization for optimal detection of *S. aureus* at concentrations consistent with animal models of sepsis. The negative control experiments are undertaken by investigating the binding of non-specific bacteria to anti-*S. aureus* antibody and binding of *S. aureus* to sensor surface that is not functionalized with antibody. To the best of our knowledge, the paper provides for the first time the rapid determination of the sensitivity of *S. aureus* to flucloxacillin using this methodology. The platform is highly affordable and easy to run as a point of care device, making it highly attractive for development into a routine clinical assay. The paper is organized as follows: in "Materials and methods" section, the fabrication of the sensors, bacterial cultivation, functionalization of sensors, bacterial adhesion on different surfaces, specificity and antibiotic sensitivity test are explained. The "Measurement set-up" section presents the electrical and florescence measurement set-up for the detection of bacteria. The results are discussed in "Results and discussion" section and "Conclusions" section provides the conclusion to the paper.

Materials and methods

Materials

3-Aminopropyltriethoxysilane (99% APTES), glutaraldehyde (25%), ethanolamine (99.5%) and goat serum were purchased from Sigma-Aldrich. Anti-*Staphylococcus aureus* antibody (ab37644) was purchased from Abcam and quality control ATCC bacterial strains were provided by the Victorian Infectious Diseases Reference Laboratory.

Fabrication of the interdigitated electrodes (IDE)

IDE's were designed as shown in Fig. 1(a) and fabricated using laser ablation lithography (SUSS SLP300). A layer of 5 nm of Cr and 100 nm Au were evaporated on glass microscopy slides using an electron beam evaporator (Intlvac Nanochrome II). The coated glass slides were patterned using the laser ablation system to obtain the IDE's. The frequency was set to 100 kHz and power to 76% for ablating the IDE's and 82% for removing the remaining metal layer. The gap between electrodes was 5 μm and the radius of inner and outer electrode was 10 μm and 50 μm respectively [Fig. 1(a) and (b)]. A layer of SiO_2 (25 nm) was evaporated on the sensors using the e-beam evaporator.

Bacterial cultivation

In the present study, *MSSA* (ATCC 25923), *MRSA* (ATCC 33591), *Staphylococcus epidermidis* (*S. epidermidis*: ATCC 12228), *Streptococcus pyogenes* (*S. pyogenes*: ATCC 19615) and *Escherichia coli* (*E. coli*: ATCC 25922) reference strains were utilized. The bacterial strains were cultured in Luria broth (LB) for 18 h at 37 °C. The optical density (OD) of the bacterial culture was measured at 600 nm wavelength for determination of bacterial growth in the stationary phase. They were then centrifuged at 3000 rpm (937 rcf) for 3 min and washed using 1 ml of phosphate buffered saline (PBS). The number of cells per ml was calculated using a haemocytometer.

Functionalization of the sensor and immobilization of antibody

The sensors were functionalized using the protocol described in ref. 19. Initially, the sensors were cleaned using acetone, isopropanol, Milli-Q water and dried using N_2 gas. The sensors were then dipped in 2% APTES in ethanol for 1hr and washed

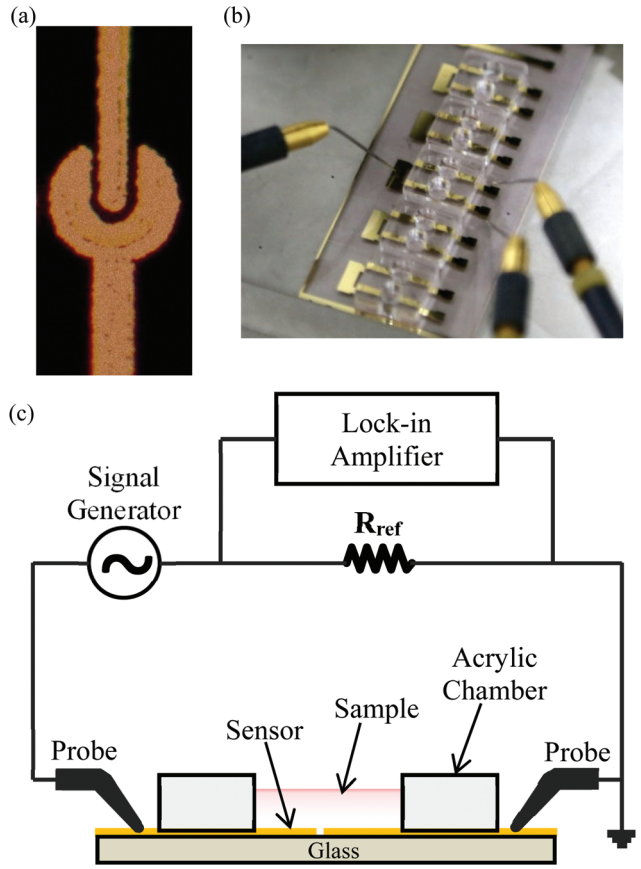

Fig. 1 (a) Fabricated IDE sensor using laser ablation lithography, where the gap between electrodes is 5 μm. (b) Array of IDE sensors. (c) The electrical measurement set-up. The sensor is excited by the signal generator and connected in series with a reference resistor. The lock-in-amplifier measures the voltage drop across the reference resistor.

3 times for 15 min using 100% ethanol, followed by 2.5% glutaraldehyde in water for 2 h. The sensors were then washed 3 times for 15 min each in Milli-Q water. The APTES and glutaraldehyde solutions were filtered using 0.22 μm filters. Then 10 μl of antibody (100 μg ml^{-1}) in PBS was incubated on the sensors for 30 min at room temperature and then overnight in a refrigerator (4 °C). Sensors were then washed 3 times for 15 min each with PBS, blocked for 1 h using 1% ethanolamine and 1% goat serum in PBS and following on washed using in PBS.

The sensors were incubated in 10 μl of bacteria in PBS for 1 h and they were then washed using PBS for 15 min to remove unbounded bacteria cells.

Bacteria adhesion on surfaces

To study the bacteria adhesion on different surfaces, we used both naïve glass slides and SiO_2 coated glass slides with and without plasma cleaning. The thickness of SiO_2 was 25 nm and the surfaces were plasma cleaned for 5 min. The protocol described in "Materials and methods" section was used to immobilize the antibody. The MSSA bacteria (10 μl) in stationary phase (10^9 cells per ml) were incubated for 1 hour in both positive and negative controls. The bacteria cells were counted using ImageJ software.

Specificity

The glass slides coated with a SiO_2 layer, without plasma cleaning were used for this study. The protocol described in "Materials and methods" section was used to functionalize and immobilize anti-*S. aureus* antibody. The negative controls were not incubated with the antibody. A 10 μl of each bacterium species (MSSA, MRSA, *S. epidermidis*, *S. pyogenes and E. coli*) in stationary phase (≈10^9 cells per ml) were incubated for 1 hour in both positive and negative controls. After washing the samples for 15 min, they were imaged using a microscope, and ImageJ software was used to count the bacterial cells.

Antibiotic sensitivity test: culture method

Different concentrations of flucloxacillin [0, 0.002, 0.1 and 5 mg ml^{-1}] were made in LB media. The antibiotic solution (20 μl) was added to the filter papers of size 1 mm × 1 mm and left for 3 h in a closed petri dish. Then 100 μl of MSSA bacteria solution, 10^4 and 10^9 cells per ml, was spread on horse blood agar plates, with filter papers placed in each quarter of the plate. The agar plates were then incubated at 37 °C for 18 h.

Measurement set-up

Electrical measurements

Electrical measurements were performed using the SR 830 lock-in-amplifier [Fig. 1(c)] at room temperature. The IDE sensor was connected in series with a reference resistor, R_{ref} = 1 kΩ. The sensor was excited by a sinusoidal signal at frequencies of 100 Hz, 1 kHz, 10 kHz and 100 kHz and an amplitude of V_{pp} = 25 mV. The lock-in amplifier technique measures the voltage drop across the reference resistor. The amplitude of the lock-in voltages was recorded and the magnitude of the impedance of the sensor was calculated from

$$|V_{out}/V_{in}| = |R_{ref}|/|R_{ref} + Z| \quad (1)$$

where V_{out} is the rms voltage drop across the reference resistor, V_{in} is the rms input voltage from the signal generator, R_{ref} is the reference resistor impedance and Z is the sensor's impedance.

Fluorescence measurements

For fluorescence measurements, 10 μl of primary antibody (2 μg ml^{-1} anti-*S. aureus* antibody-ab37644) was incubated for 1 h followed by 10 μl of secondary antibody (4 μg ml^{-1}) for 1 h. The sensors were washed using PBS for 15 min after each incubation step. The fluorescence measurements were carried out using an inverted fluorescent microscope (Zeiss, BioImaging Station). The reflected light illumination and the appropriate filter for Alexa Fluor-594 dye were selected. The exposure time

was set to 3 s and the ZEN imaging software was used to capture and analyze the images.

Results and discussion

This paper presents a low cost sensor for the rapid detection of bacteria and antibiotic sensitivity determination. The cost of fabricating a sensor using the method presented in this paper is AUD$0.25. The sensors can be functionalized and stored for later use as in ref. 20, 21. Then bacteria can be detected within an hour and antibiotic sensitivity can be determined within 2 hours.

Equivalent circuit of the sensor in growth media

The equivalent circuit of the sensor in aqueous solutions can be represented as shown in ref. 14, where the circuit consists of two double layer capacitors, a bulk medium resistor, and a dielectric capacitor. The double layer capacitance of electrodes represents the effect of ionic species near the surface of electrodes[22] and dominates the impedance in the 0.2 Hz to 1 kHz region.[14] The bulk medium resistance appears in the 1 kHz to 1 MHz frequency range[14] and represents ion transport across the bulk solution and the change in conductivity of the bulk medium.[14,22] The dielectric capacitance denotes the dielectric behavior of medium and dominates at frequencies greater than 1 MHz.[14]

Detection of MSSA bacteria and adhesion of surfaces

The detection of MSSA and adhesion of bacteria on different surfaces were analyzed using the method described in "Materials and methods" section. Fig. 2 shows the average number of bacteria cells attached on naïve glass slides and SiO_2 coated glass slides with different treatments. These results show the ability of differentiating positive from negative control samples is higher using the SiO_2 coated glass slides that were not plasma cleaned. Here, negative control was not incubated with the anti-*S. aureus* antibody. The transmission light images of the SiO_2 coated glass slides that were not plasma-cleaned show the binding of MSSA bacteria to antibodies [Fig. 3(a) and (b)].

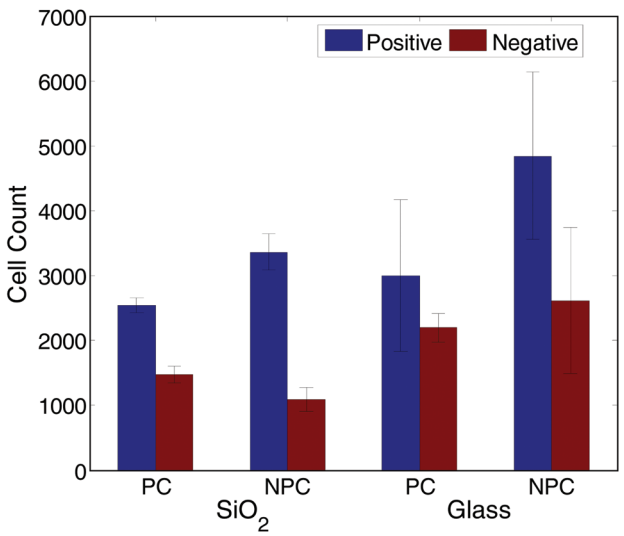

Fig. 2 MSSA bacterial cell count that were adhered on different surfaces. The negative control was not incubated with anti-*S. aureus* antibody. Here, PC is plasma cleaned and NPC is not plasma cleaned.

The electrical measurements were performed on the sensors that were fabricated on naïve glass slides and also sensors that were coated with a layer of SiO_2. These sensors were not plasma cleaned. Here, MSSA in the stationary phase, at concentration of 10^8–10^9 colony forming units per ml, were used as these concentrations are consistent with animal models of sepsis.[23,24] The voltage of the empty sensors with 10 μl of PBS were measured using the lock-in-amplifier. The sensors were then functionalized using the protocol mentioned in "Materials and methods" section and the stationary phase MSSA bacteria were incubated for 1 hour. After washing the sensors using PBS, the output voltage was measured in 10 μl of PBS and the magnitude of the voltage difference ($|\Delta V|$) was obtained for both positive and negative controls. The mean and error of $|\Delta V|$ were plotted for both controls at various frequencies [Fig. 3(a)]. The electrical impedance measurements of SiO_2 coated sensors at 100 Hz to 100 kHz were able to differentiate the positive samples from the negative control samples. Our results show that the maximum difference in voltage values is observed in the resistive region centered around 10–100 kHz in frequency. It follows that the impedance change, as a consequence of MSSA binding, is dominated by the change in conductivity in bulk medium.

However, the electrical measurements of sensors on a naïve glass surface was not able to differentiate positive and negative controls [Fig. 3(d)]. The reason for this is the double layer effect and polarization of electrodes due to the direct contact of electrodes with PBS. The fluorescence measurements confirmed the electrical reading and the binding of MSSA to anti-*S. aureus* antibody can be differentiated using the proposed method (Fig. 4).

Specificity

The specificity of anti-*S. aureus* antibodies was investigated using the method described in "Materials and methods" section. The number of cells bound to both positive and negative controls is shown in Fig. S1 (see ESI†). The difference in the cell count of positive and negative controls gives the number of cells bound to the antibody. The results show that both MSSA and MRSA strains of *S. aureus* and *S. epidermidis* bind to anti-*S. aureus* antibody. The MSSA strain binds more to anti-*S. aureus* antibody compared to MRSA and *S. epidermidis*. The binding of other bacteria strains, *S. pyogenes* and *E. coli*, to anti-*S. aureus* antibody is insignificant.

The electrical measurements were performed to study the specificity of anti-*S. aureus* antibodies in detecting MSSA bacteria using SiO_2 coated sensors. The antibody was immobilized in all the sensors with *E. coli* selected as the non- specific bacteria type to control for non-specificity. The voltage of the

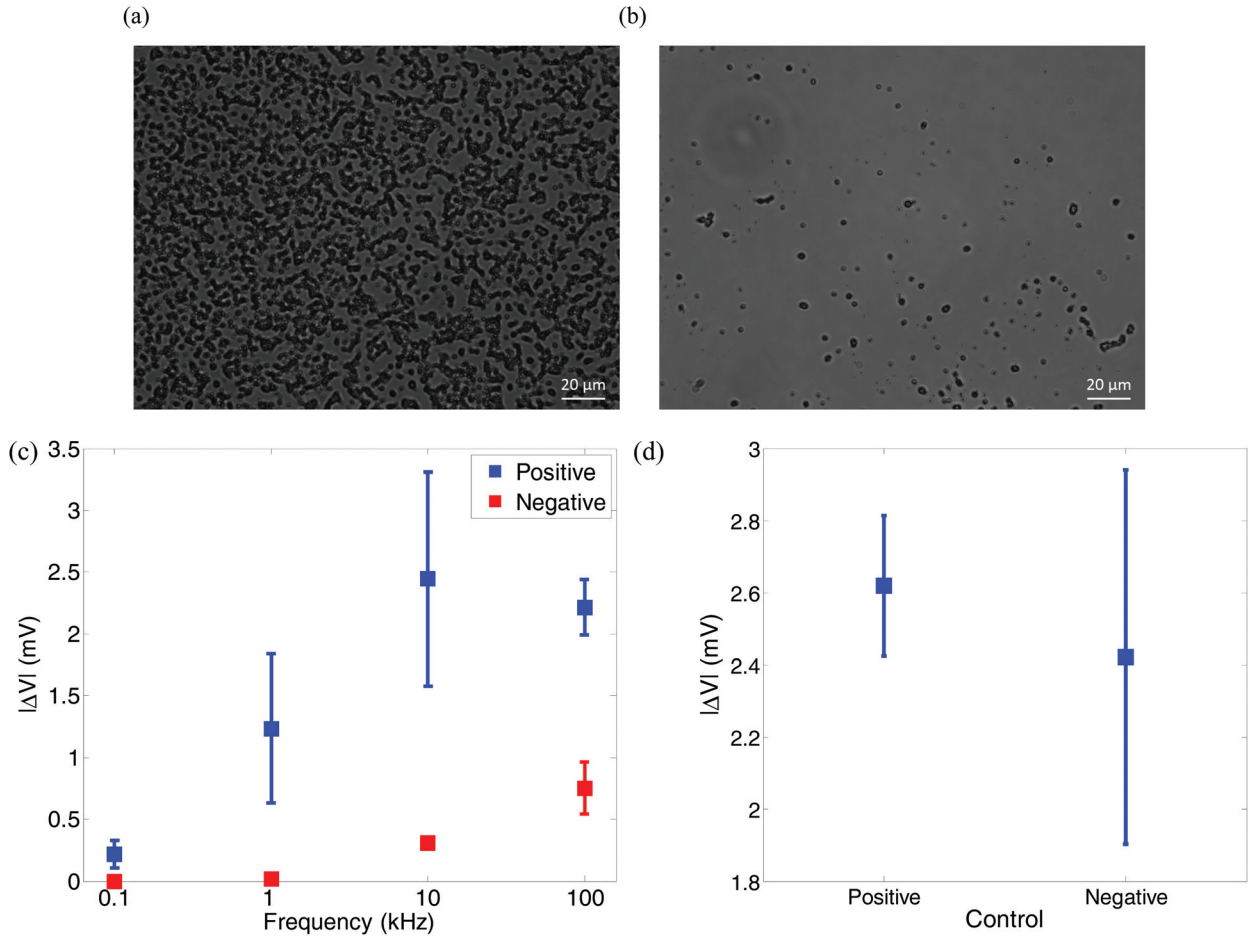

Fig. 3 Microscopic image of (a) positive and (b) negative controls. (c) The mean and error of change in voltage at 100 Hz, 1 kHz, 10 kHz and 100 kHz. The measurements were performed using the sensors that were fabricated on glass slides with a layer of SiO_2. (d) The mean and error of change in voltage at 100 kHz. The sensors were fabricated on glass slides and did not have a SiO_2 layer. Here, MSSA at stationary phase was used and the negative control was not incubated with anti-*S. aureus* antibody.

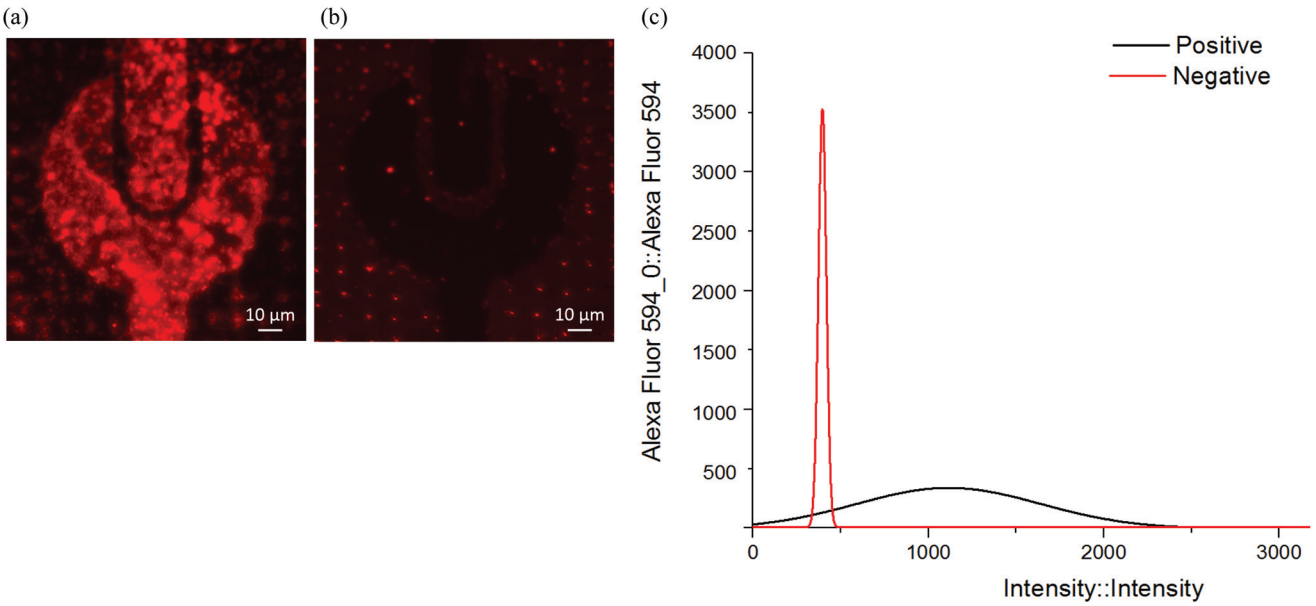

Fig. 4 Fluorescence microscopy images of (a) positive and (b) negative controls. (c) The fluorescence measurements of positive and negative control. Here, MSSA at stationary phase was used and the negative control was not incubated with anti-*S. aureus* antibody.

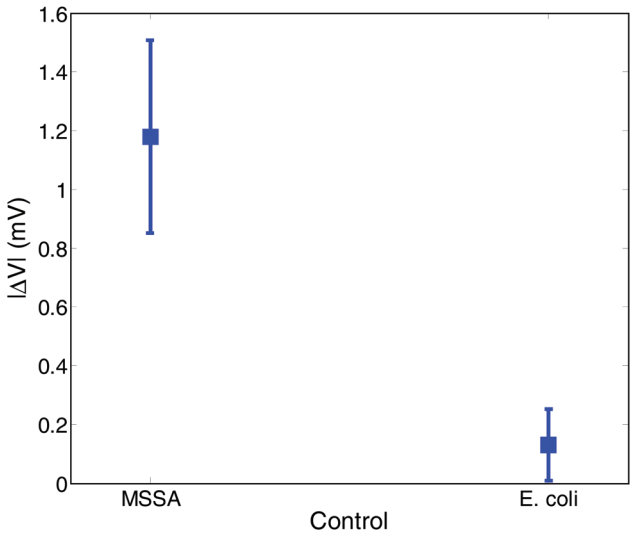

Fig. 5 The mean and error of change in voltage at 100 kHz. Here, the sensors were fabricated on glass slides with a SiO_2 layer and anti-*S. aureus* antibody were immobilized in both controls. MSSA and *E. coli* strains were incubated for 1 h.

empty sensors and after incubating stationary phase bacteria (MSSA and *E. coli*) were measured as explained in the previous section. Fig. 5 shows the magnitude of voltage difference ($|\Delta V|$) obtained for both MSSA and *E. coli* bacteria cells. The results demonstrate the specificity of our sensor in detecting *S. aureus* bacteria.

S. aureus growth in different media

The ability to ascertain antibiotic sensitivity depends on accurately measuring differential bacterial growth rates. Growth medium is a critical factor in maximizing bacterial growth. The ideal growth medium enhances bacterial growth without significantly contributing to the overall conductivity.[8] As bacterial colonies grow they produce metabolites and increase the conductivity of the medium by converting uncharged or weakly charged substances present in the growth medium (yeast and peptone) into highly charged substances (amino acids, aldehydes and ketones).[8] Different growth media such as water, PBS and Luria broth (LB) have been used for bacteria culturing. It is important to understand which media facilitates fastest bacterial growth. Fig. S2 (see ESI†) shows the bacterial cell count growth rates in different growth media. The overnight incubation of immobilized MSSA bacteria on glass slides showed that LB significantly aids the growth of the MSSA.

MSSA bacteria were then immobilized on the sensors, captured by the antibodies on the sensor surface, media was added and then incubated at 37 °C. Before measuring, the sensors were washed with PBS for 15 min. Changes in impedance for Water, PBS, 1 M NaCl and LB growth media at 100 kHz were measured and recorded at 1 h, 2 h, 3 h and 18 h. Fig. S3 (see ESI†) shows the change in the impedance compared to the empty sensors for various media. The impedance of MSSA in water at 100 kHz is large compared to that of other media due to the lower electrical conductivity of water. However, the impedance reduces as the population of Gram-positive bacteria decreases in water.[25] The growth of bacteria in NaCl was slower in the first few hours but then increased with significant changes in impedance observed after 18 h of incubation. PBS and LB both provided sufficient growth after an hour with LB providing the largest growth rate and corresponding change impedance compared to the other media.

Response to antibiotics

The antibiotic sensitivity test was carried out using the standard culture method as described in "Materials and methods" section. Fig. 6 shows the growth of MSSA in media with added flucloxacillin. The area around the filter paper indicates the sensitivity of bacteria growth to flucloxacillin concentration. The plates in Fig. 6(a) and (b) indicate that bacterial growth is inhibited for flucloxacillin concentrations above 0.1 mg ml^{-1}, however, for robust inhibition of MSSA at a concentration of 10^9 cells per ml a higher flucloxacillin concentration is required.

The impedance measurements can differentiate live and dead bacteria cells as live bacterial cells change the conductivity of the medium as consequence of their metabolic process.[8] The MSSA bacteria in media were placed in the wells of the sensors and incubated at 37 °C for 1 hour. Then LB and LB with antibiotic (flucloxacillin 300 mg ml^{-1} filtered using 0.2 μm filters) were added to the sensors with the attached bacteria and then incubated at 37 °C for 1 h, 2 h and 3 h. All sensors were washed for 15 min in PBS to remove unattached bacteria and antibiotic. Then a 10 μl of LB was added to the sensor, before impedance measurements. The measured impedance changes post application of antibiotic is shown in Fig. 6(c). The impedance measurements show significant differences between naïve sample and sample exposed to flucloxacillin measured at 100 kHz. Although there is some growth in bacteria in the presence of antibiotics with time, the growth is reduced compared to bacteria not exposed to the antibiotic. Thus, the proposed sensors detect the inhibition of growth, relative to a naïve sample, to establish bacterial antibiotic sensitivity.

Future work and directions

The results from this study provide a first step towards developing an integrated on-chip system for detection of *S. aureus* bacteria and assessment of antibiotic sensitivity of *S. aureus*. A next important step will be to investigate the detection of *S. aureus* in whole blood in clinical cases. The concept is shown in Fig. 7. Here, the sample to be tested is added to the sensing region (pre-functionalised area) by the users. The key difficulty in using the whole blood is the interference from the cells other than *S. aureus*. Thus, the sensor has a filter paper to remove red blood cells (6–8 μm in diameter) and segmented neutrophils (9–16 μm in diameter) in the blood sample. Most bacteria are generally 0.2–2 μm in diameter and therefore can filter through onto the chip. The unbound cells, including

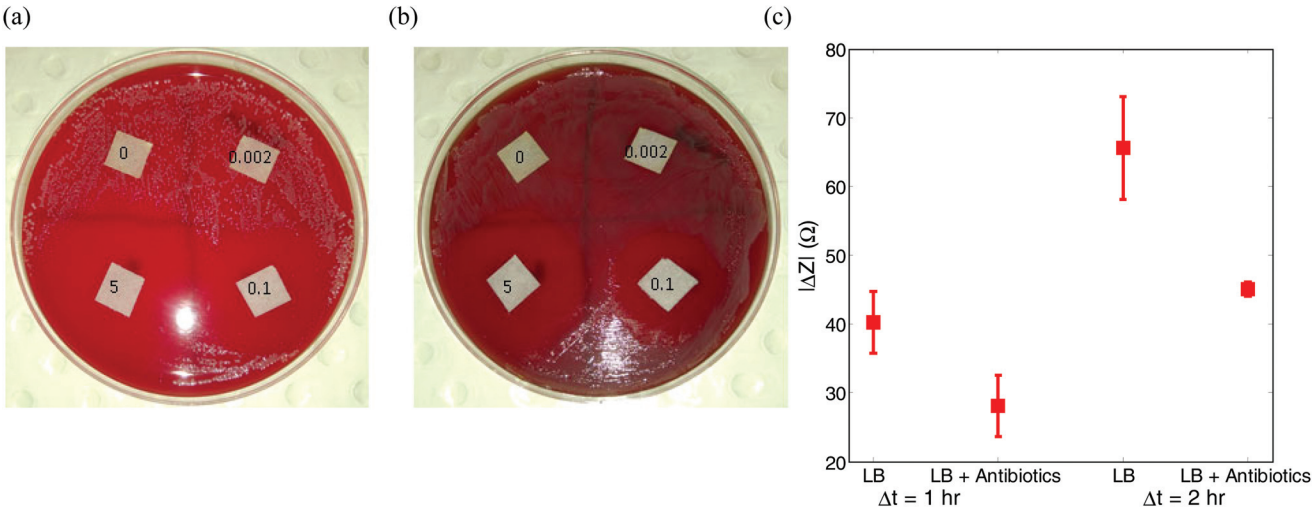

Fig. 6 Antibiotic sensitivity of MSSA to flucloxacillin at 0, 0.002, 0.1 and 5 mg ml^{-1}. Concentration of bacteria is (a) 10^4 cells per ml and (b) 10^9 cells per ml. (c) Changes in the impedance of the sensor due to growth of the bacteria in the presence and absence of flucloxacillin (300 mg ml^{-1}). Here, the concentration of MSSA is 10^9 cells per ml.

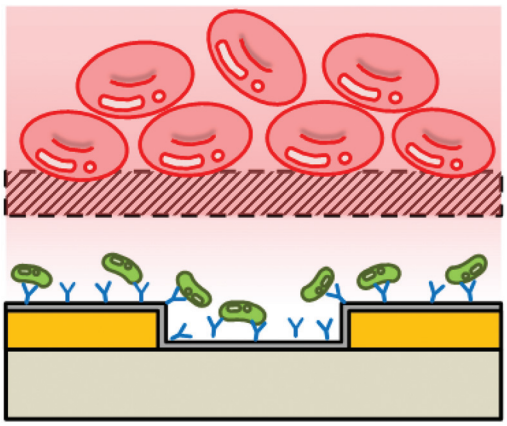

Fig. 7 The concept of the sensor for detection of bacteria in blood samples.

non-specific bacteria in the blood sample are then washed away after incubation of sample for 1 h. The detection of other bacteria requires specific antibodies against those bacteria. The key issue in detection of specific bacteria is the availability of commercial antibodies. To detect bacteria species accurately, synthetic antibodies could be utilized. The assessment of antibiotic resistance depends on the cell division of bacteria types. The response of bacteria to antibiotics can be detected within few hours for the bacteria with short generation times.

Conclusions

This paper proposes a biosensor that is based upon an antibody-functionalized, silicon dioxide passivated, interdigitated electrodes for the detection of *S. aureus*. The antibody-functionalized non-plasma-cleaned SiO_2 coated glass slides exhibited specific binding of *S. aureus* bacteria. Electrical measurements of the sensors conducted at 100 kHz were able to differentiate positive from negative control samples, illustrating the ability of detecting *S. aureus* in a sample within an hour. Electrical measurements of MSSA in LB illustrated change in measured sensor impedance with growth. The results illustrate that the proposed biosensor can be used to detect the sensitivity of MSSA to flucloxacillin within 2 hours. Taken together, our findings provide promising data for further developing of the sensor for use in whole blood as well as for the detection of other bacterial subtypes with corresponding assessment of antibiotic resistance. In doing so, such devices offer the potential to provide substantial savings in health care as well as greatly improved patient care and prognosis.

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