Analytical Methods

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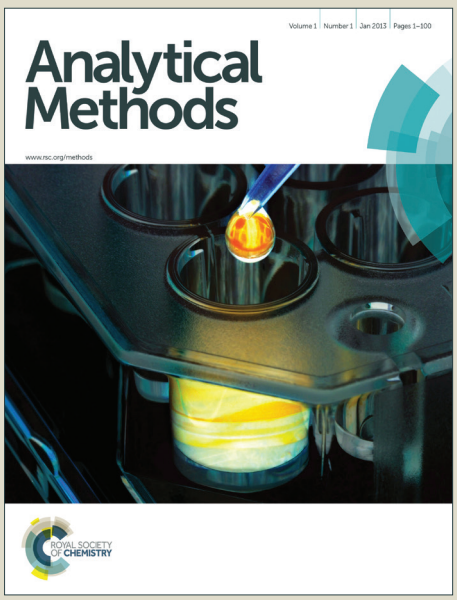

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Improved Monitoring of *P. aeruginosa* on Agar Plates

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Described is the fabrication of a disposable electrochemical assay that is integrated with standard King's A agar culture plates, for the selective and specific detection of *Pseudomonas aeruginosa*. Agar plates provide several advantages over liquid culture, including protecting the sensor from biofouling and faster identification in small sample volumes. Cultures of *P. aeruginosa*, starting from initial cell counts of 10^2 to 10^8 cells in 5 microliter volumes, were incubated at 23, 37, and 42 °C and monitored both visually and electrochemically. Square wave voltammetry scans confirmed the production of a redox species, pyocyanin, over time that was dependent on the initial load of cells. The pyocyanin easily diffuses through the agar to reach the electrode surface. Using this simple and cheap approach, positive identification of *P. aeruginosa* was achieved several hours faster via electrochemical detection compared to traditional visual analysis.

Introduction

Antibiotic resistance is an acute public health problem. With the rapid emergence of antibiotic resistant strains, mankind is currently at a tipping point in the fight against dangerous bacterial pathogens.[1] Resistance has increased in part due to the approaches physicians take when treating patients who show symptoms of infection, such as the over-prescribing of broad-spectrum antibiotics.[2, 3] Faster positive detection of dangerous pathogens in patient samples could lead to more targeted antibiotic regimens being prescribed, which slows the emergence of antibiotic resistance. The Infectious Diseases Society of America has created a list of six antibiotic resistant bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii, Pseudomonas aeruginosa*, and *Enterobacter* species (ESKAPE pathogens) that are responsible for the majority of healthcare-associated infections (HAIs).[4] Of these ESKAPE pathogens, we focus in this paper on the early detection of *Pseudomonas aeruginosa*, an opportunistic pathogen associated with lung infections and responsible for over 10% of all nosocomial infections.[5-9]

There are several effective routes for the identification of bacteria from patient samples. The most common approach is culturing bacteria from patient samples using agar containing selective growth medium that only allows certain bacteria to thrive. While a wide variety of agar media have been proposed and used for the specific and selective detection of microbes, the technique essentially has not changed since it was first proposed in 1881.[8] Agar plates can also be used with serial dilutions of antibiotics to determine the minimum inhibitory concentration (MIC) required to kill the bacteria. While cheap (about US$1/plate) this approach requires long incubation times (sometimes longer than 24 hours) to obtain a positive identification.[10] In some instances, a second form of identification is required after incubation.[11]

A more modern approach is the use of polymerase chain reaction (PCR) schemes to increase the amount of pathogenic DNA or RNA present in a sample, which is then used for rapid identification.[10, 12] Several authors have improved this approach by incorporating PCR into microfluidic systems, where the inherent small volumes allow trapping of individual cells and their subsequent genetic analysis.[13, 14] PCR-based approaches are much more selective and sensitive; however, they use expensive reagents and require specialized personnel or expensive instrumentation to handle the sample prep and data analysis.[14]

A simpler, intermediate alternative is the detection of secreted molecules produced by bacteria growing on agar plates. *P. aeruginosa* secretes several virulence factors that help promote infections within hosts.[15] Of these factors, the phenazine molecule pyocyanin (PYO) has great potential to be used as a diagnostic marker, as *P. aeruginosa* is the only species known to produce this particular molecule.[16] PYO detection is often performed via solvent extraction to remove the molecule of interest from cell cultures (liquid and agar) and then measured optically or electrochemically.[17] Alternatively, PYO production can be measured on selective growth media.[18] However, this sample preparation is not necessary as the supernatant from *P. aeruginosa* cultures can be directly placed onto electrochemical sensors and measured. Several groups have identified *P. aeruginosa* in liquid cultures by detecting pyocyanin electrochemically,[16, 19-22] but this format is not typically employed by pathologists for analysis.

Bellin *et al.* (2014) microfabricated an array of electrodes with an agar layer placed on top of it, to spatially monitor the change in PYO concentration gradients produced in a bacterial colony.[23] While truly novel, the array's complicated fabrication makes it difficult to implement. Furthermore, the thin agar film required to measure diffusing molecules was not thick enough to facilitate colony growth. This necessitated growing a bacterial colony on thicker agar and then transferring it to the microfabricated system for monitoring. While other researchers have shown cell monitoring using microfabricated impedance sensors beneath agar gels, the lack of specificity of this technique hinders its use for pathogen identification.[24]

This paper proposes a disposable screen-printed electrochemical system embedded within growth agar to continually detect the presence and concentration of PYO. We chose to use agar plates for several reasons. First, they provide a barrier between the electrode and the bacteria. This prevents bacteria from forming biofilms directly on top of the electrodes, potentially increasing the lifetime of the electrodes versus electrodes in liquid media, which are often exposed to the bacteria directly. Second, this approach uses small volumes for sample analysis. The results demonstrate *P. aeruginosa*

detection in 5 µL sample volumes. While the device used in this study employed large volumes of agar, future versions could utilize smaller amounts of agar with very small volumes of patient samples. Finally, agar plates containing selective medium are commonly used for identification of infecting pathogens in patient samples. Embedding electrodes in the solid medium allows technicians to continue using their preferred format.

Since PYO production is indicative of *P. aeruginosa* presence, the spatial resolution required in other reported sensors is unnecessary.[23] This allows the use of thicker agars promoting cell growth, and, in turn, simplifying the detection platform. To this end, embedded electrodes in King's A agar (used to grow *P. aeruginosa* and increase PYO production) were used to determine whether different bacterial loads of *P. aeruginosa* could be detected faster than with visual inspection.[18]

Materials and methods

Device Fabrication

Disposable electrodes were embedded into culture plates by creating a small slit on the side of the plate approximately 2 mm from the bottom. Disposable carbon electrodes with miniaturized silver chloride pellet references (Zensors T-100) were then inserted into the slits. The slits were sealed with silicone glue and allowed to cure overnight. Sterilized King's A agar (BD Diagnostics DF0449-17-0) was poured onto the plates, covering the electrodes with broth thicknesses ranging from 2.5 to 5.0 mm for diffusion measurements. Once the agar solidified, plates were stored at 4 °C until use. For experiments involving exact agar thickness, polydimethylsiloxane (PDMS) (Dow Corning 184 Sil. Elast. Kit 0.5kg, Ellsworth Adhesives) wells were used to contain the agar and position it over the electrodes.

Bacterial culture

Bacterial cultures of *P. aeruginosa* strain PA14 were grown overnight in 3 mL of trypticase soy broth (TSB, BD Diagnostics B11768), centrifuged at 10,000 rpm for 3 minutes, then reconstituted in fresh TSB. Cell counts of the reconstituted solution were

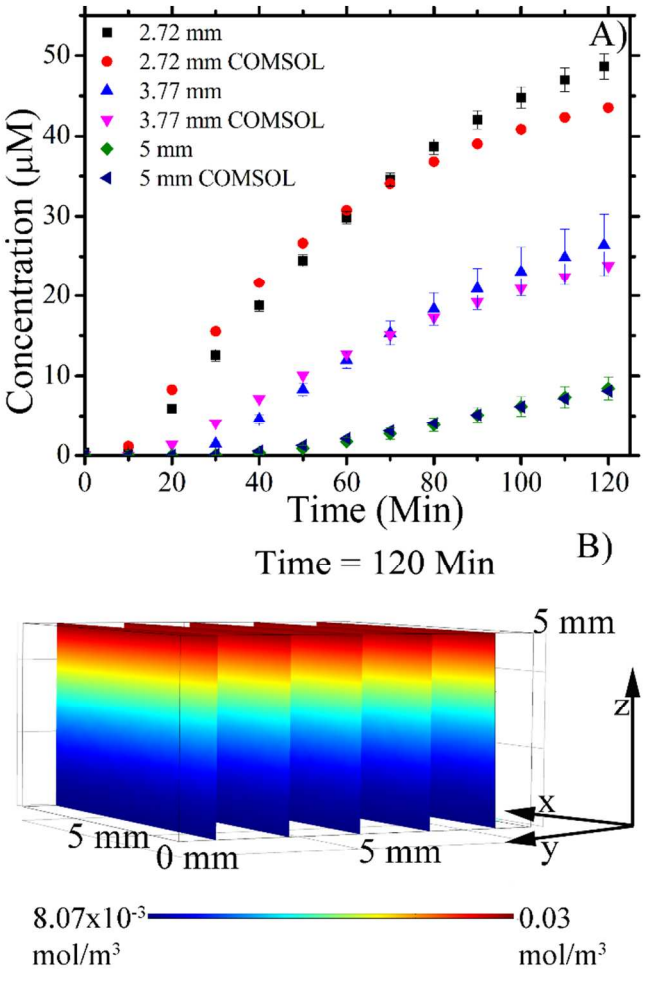

Fig. 1. A) Concentration over time of PYO, calculated from max peak currents of SWVs, as it diffuses through solid agar to the embedded electrode. 200 µL of a 100 µM PYO solution was pipetted onto 2.72, 3.77, and 5 mm agar slabs. Error bars are the standard error of the mean from 3 separate runs per concentration. Simulation data use a diffusion coefficient of 1.0 x 10^{-9} m^2/s and an proportionally diluted concentration for each thickness. B) COMSOL simulation of the concentration profile in a 5-mm-thick slab of agar for 28 µM PYO diffusing downward after 120 min.

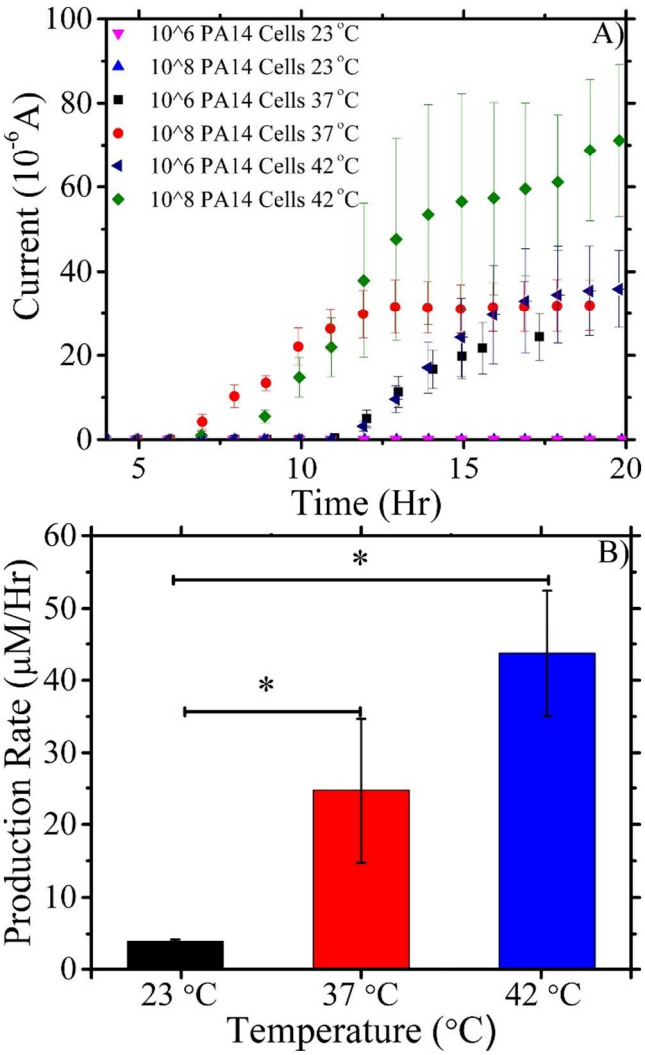

Fig. 2. A) Maximum peak currents versus time for different initial concentrations of PA14 grown on King's A Agar. Maximum currents were determined from SWVs recorded every hour using a frequency of 15 Hz and an amplitude voltage of 50 mV. B) Maximum average production rate of PYO per hour between temperatures. * $p < 0.05$.

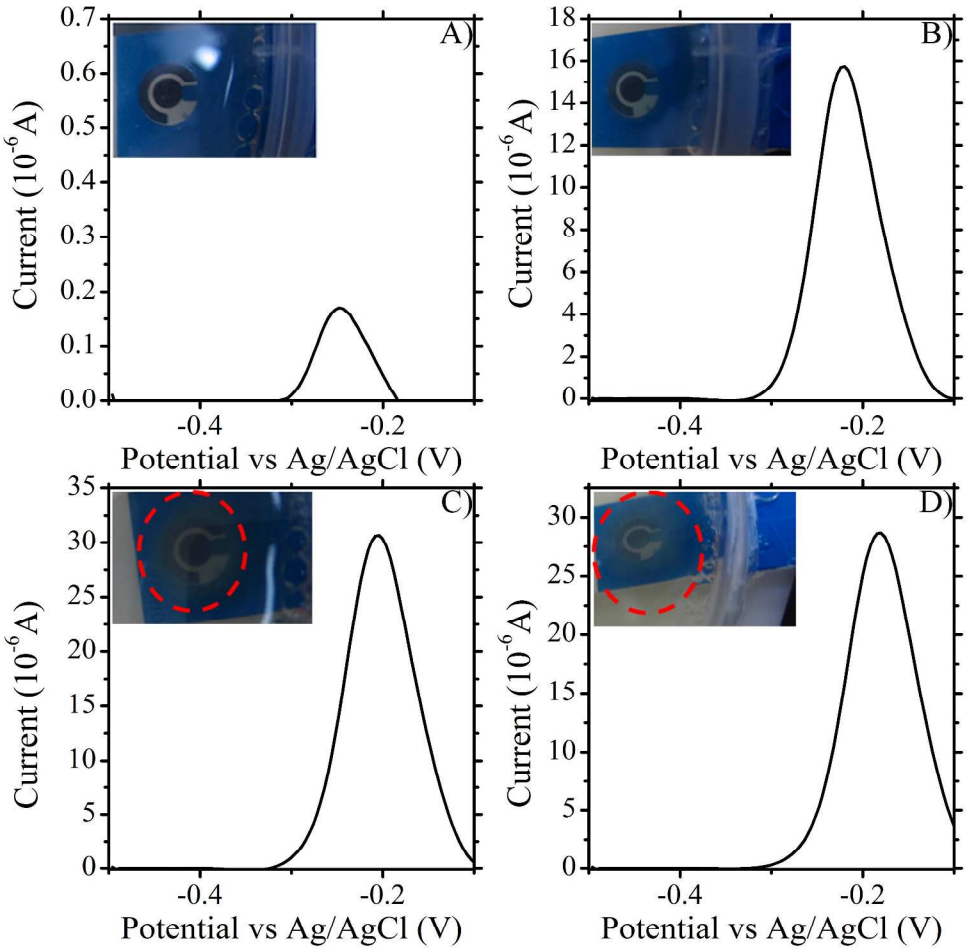

Fig. 3. Maximum peak currents versus time for an initial PA14 load of 10^8 cells grown on King's Agar after A) 6, B) 8, C) 14, and D) 19 h incubated at 37 °C. SWVs were taken every hour using a frequency of 15 Hz and an amplitude voltage of 50 mV. Circles in C) and D) highlight the appearance of visible bacterial colonies.

performed using an INCYTO C-chip disposable hemocytometer (DHC-N01). Stock solutions were diluted with TSB to desired approximate concentrations of cells. 5 µL drops were pipetted onto the prepared plates directly above the working electrode to produce bacterial loads of 10^2-10^8 *P. aeruginosa* cells and incubated at 23, 37, or 42 °C.

Electrochemical measurement and data analysis

Samples were removed from incubation and connected to a multipotentiostat (CHI 1040C) to perform voltammetric measurements. Square wave voltammetric (SWV) scans were obtained using a frequency of 15 Hz and amplitude voltage of 50 mV in the potential window from -0.5 to 0 volts. Voltammetric scans were analyzed using OriginPro 8.5 to obtain baselines and determine the maximum current produced by redox active molecules present on the plates. All experiments were performed in triplicate, unless otherwise noted. PYO (Cayman Chemicals 10009594) was dissolved in dimethyl sulfoxide (DMSO D128-1) to a concentration of approximately 5 mg/mL and used as stock. When not in use, PYO stock solution was stored at -20 °C and allowed to warm to room temperature before use. COMSOL Multiphysics 4.4 was used to model the three dimensional diffusion of dilute PYO concentrations through agar using the transport of dilute species module.

Scanning electron microscopy preparation

After testing, samples were prepared for scanning electron microscopy (SEM) imaging. First, the agar surrounding the disposable electrode was cut free. Both the agar and the electrode were fixed in a 2.5% glutaraldehyde (EMSDIASUM 16120) solution containing 0.1 M cacodylate buffer (EMSDIASUM 11654). After dehydrating in ethanol (Fisher BP2818-4 30-100%), the samples were further dried with hexamethyldisilizane (EMSDIASUM 16700), sputtered (Cressington Sputter Coater 208HR) with platinum or palladium metal (to provide a conductive surface) and then visually inspected using a field emission SEM (Hitachi S-4800).

Results and discussion

Modeling pyocyanin diffusion

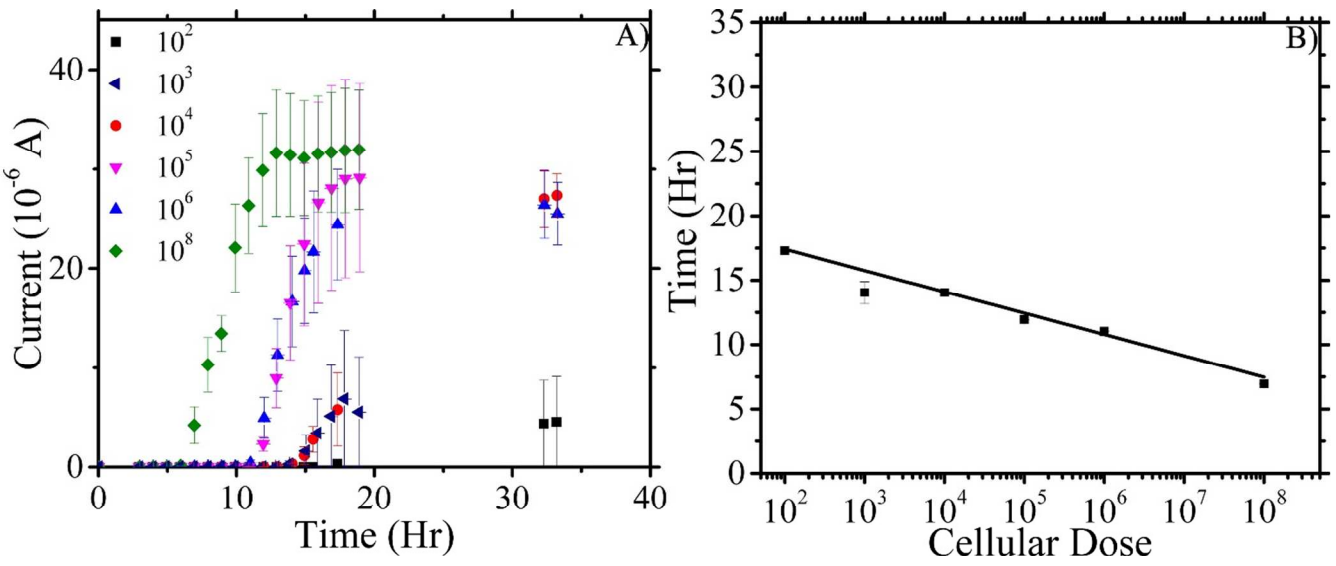

Fig. 4. A) Average maximum peak current for PA14 bacterial loads ranging from 10^2 to 10^8 cells. The error bars show one standard deviation from the mean current measured for 3 separate experiments at each starting load. B) Time to measure a significant current change for a given starting concentration of PA14 grown at 37 °C. Points were considered different if the value minus the standard error of the mean was greater than the previous current value plus the standard error of the mean for each individual experiment.

We performed electrochemical measurements to determine the rate at which molecules of PYO diffuse through agar. To do this, PDMS wells were plasma bonded to disposable electrodes and King's A agar was poured onto the wells at three different thicknesses. After solidification, 200 µL of 100 µM PYO was pipetted directly onto the agar. Square wave voltammetry measurements were recorded every minute, from -0.5 to 0 V at a frequency of 15 Hz and an amplitude voltage of 50 mV. The resulting peak current after baseline subtraction was used to determine the approximate PYO concentration (Calibration Curve shown in Fig. S1) and was plotted versus time (Fig. 1A). Samples were run in triplicate and the error bars are the standard error of the mean.

It was observed that the measured concentrations never reached the delivered initial concentration of PYO (Fig. 1A). Based on simulation data using a constant PYO concentration of 100 µM, the most likely explanation is that the water content in the solid agar diluted the PYO (Fig. S3). For the 5-mm-thick slab (approximately 500 µL), it is expected that at equilibrium the final concentration of PYO throughout the agar should be approximately one quarter of the initial value. A COMSOL simulation of PYO diffusion using a concentration of 28 µM matches the peak signal observed in our experimental results (Fig. 1A and B). Prior studies have shown that the diffusion coefficient of PYO in TSB is approximately 2.5×10^{-9} m^2/s.[16] Using a diffusion coefficient of 1.05×10^{-9} m^2/s combined with the dilution effect simulate results that follow a similar trend to the experimentally measured values (Fig. 1A). This suggests a reduction in the diffusion of PYO through the solid agar, which is in general agreement with values reported by other groups.[23] Importantly, these results show that PYO diffuses through the agar and that our embedded electrode system can measure the concentration of PYO at the surface of the electrode.

Electrochemically monitoring *P. aeruginosa* growth

After confirming that PYO readily diffuses through agar to reach a buried electrode, we investigated the specific detection of *P. aeruginosa*. Wild type strain PA14 was chosen for these tests due to its virulence and PYO production.[25] Cultures of PA14 were grown overnight in TSB, centrifuged, and reconstituted in fresh TSB. The bacterial concentration in the stock solution was then determined via cell counting. The stock solution was diluted to the desired concentration with TSB. We selected a 5 µL drop for the inoculation volume, as this amount of solution reliably maintained its shape upon inversion of the agar plate. Larger inoculation drops tended to spread around the plate when inverted. 5 µL drops of PA14 (containing either 10^6 or 10^8 cells) were pipetted onto agar plates above the working electrode and incubated at 23, 37, and 42 °C to determine how temperature affects the time for electrochemical detection of *P. aeruginosa*. The selected temperatures encompass the range that is typically used for culturing *P. aeruginosa*.[26]

The average peak current from square wave voltammetric scans of the samples from -0.5 to 0 volts after baseline subtraction was recorded and plotted versus time (Fig. 2A). The reported current is the average from 3 separate embedded electrode devices at that time point, with the error bars equal to the standard error of the mean. Readings were collected until the peak current reached a maximum plateau.

We observed a significant current change for the entire range of tested bacterial doses (Fig. 2A). As expected, the time to detect a measureable PYO signal decreased progressively for samples containing increasing numbers of cells. PYO has been shown to be regulated by quorum sensing, which requires a threshold concentration of bacteria to produce a response, supporting the observed bacterial dose correlation with time.[17]

Temperature influences both the growth rate of cellular organisms, as well as the production rate of metabolites.[27, 28] Fig. 2 shows that increasing temperature positively impacted PYO production. Bacteria grown at 23 °C showed little measurable PYO production compared to cells grown at higher temperatures for the same culturing period. Cells grown at 23 °C did eventually produce a measureable electrochemical signal at later time points (Fig. S4). Furthermore, cells cultured at higher temperatures had greater slopes indicating increased production rates of PYO (Fig. 2B and Fig. S5).

A significant benefit of the proposed system is the reduced amount of time needed for detection compared to standard visual

inspection. We observed an increase in electrical signal several hours before any colonies were visible on the plates (Fig. 3). Even after the formation of a visible colony, several additional hours of incubation were necessary before conclusive identification of *P. aeruginosa* was possible via the characteristic blue coloration associated with PYO production on King's A agar. The electrochemical detection of PYO in this potential window also allows for the selective detection of *P. aeruginosa* from other clinically relevant pathogens (Fig. S6-S8). The earlier time to detection, offered by this method, can allow faster administration of the appropriate antibiotic regimen, resulting in better treatment and curbing the emergence of antibiotic resistant strains.[2,3]

Subsequently embedded electrodes were loaded with PA14 ranging from approximately 10^2-10^8 cells cultured at 37 °C. Fig. 4A shows the mean peak current measured for 3 experiments at each starting cell load and the standard deviation. The time to detection for cellular load was determined as the point where the first measureable peak in the voltage range of -0.5 to 0 volts appeared. The time to detection was plotted versus the cell load (Fig. 4B) and a linear response was determined. This approach can potentially be used to determine whether a sample from a patient correlates to an infectious load.[29] More importantly, the positive identification of PA14 by electrochemical detection was achieved for all bacterial loads within 20 hours (Fig. 4). This finding is supported by Connell *et al*, who recently showed that PYO production could be measured from as few as 500 cells in a confined environment in less than 10 hours.[30]

Since PA14 is well known for its mobility, and agar is a porous medium, we investigated the possibility that the increased electrochemical signal was due to growth of cells directly on the surface of the electrodes.[31] After the completion of a test, the agar block containing the electrode was removed and prepared for imaging via scanning electron microscopy (SEM). If the cells were indeed growing on the surface of the electrode, then the resulting images of the electrodes, and the agar surface in contact with the electrode, would contain bacteria. The SEM images (Fig. S9 and S10) show that there was little to no growth on the surface of the working electrode or the agar in contact with the electrode surface. By comparison, images of the agar surface with the colony had large fields of PA14 confirming that the measured signal was due to the diffusion of PYO. This is an excellent alternative versus systems that rely on the removal of sample, which may change the growth conditions or require additional solvent extraction.[16, 19, 32]

Another prospective use for this system is examining the antibiotic susceptibility of *P. aeruginosa* via the electrochemical detection of PYO (Figure S10). Agar plates mixed with antibiotic cocktails of choice have been used for many years by clinicians to successfully create an antibiotic regimen for infected patients.[33] As shown in Fig. 2 and 3, the electrochemical detection of *P. aeruginosa* via PYO occurs much faster compared to colony formation. The proposed method could allow faster determination of the efficacy of applied antibiotics.

Conclusions

Screen-printed carbon electrodes embedded in King's A agar were used to electrochemically monitor the production of PYO by cultures of *P. aeruginosa* strain PA14. This method provided a detection platform specific to *P. aeruginosa* that costs about US$2.50 per device. We were able to determine the presence of *P. aeruginosa* electrochemically several hours faster than by visual inspection of plates. Further, sample preparation is exactly the same as what is currently done by health-care professionals and laboratories, which lowers a major barrier to adoption of the technology. Most importantly, the specificity of the detection decreases the chances of pathogen misidentification and eliminates the need for histological analysis.

Acknowledgements

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Notes and references

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Electronic Supplementary Information (ESI) available: ESI contains a calibration curve for PYO measured in TSB media, solid TSB agar, and solid Kings A agar; a plot of the measured maximum current vs time for the bacterial loads used in Fig. 4; curves from which the maximum PYO production rate in Fig. 2B was calculated; and scanning electron microscopy images of *P. aeruginosa* grown on King's A agar as well as the surface of the electrodes after cell growth. See
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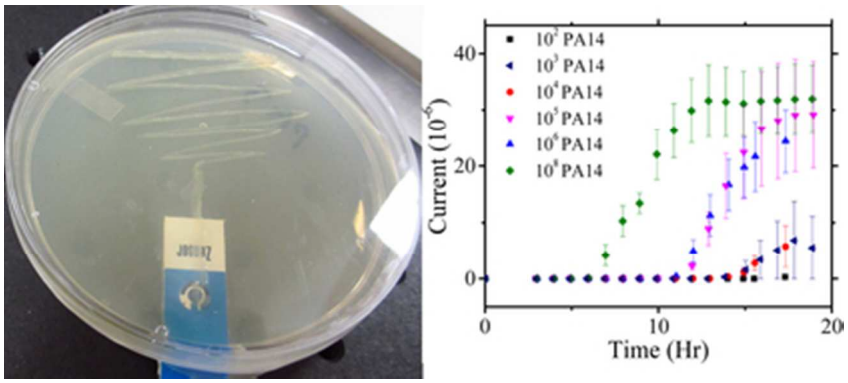
1. M. A. Fischbach and C. T. Walsh, *Science*, 2009, 325, 1089-1093.
2. R. A. Weinstein, *Emerging Infectious Diseases*, 2001, 7, 188-192.
3. S. Stone, R. Gonzales, J. Maselli and S. R. Lowenstein, *Annals of Emergency Medicine*, 2000, 36, 320-327.
4. H. W. Boucher, G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg and J. Bartlett, *Clinical Infectious Diseases*, 2009, 48, 1-12.
5. D. Banerjee and D. Stableforth, *Drugs*, 2000, 60, 1053-1064.
6. R. L. Gibson, J. L. Burns and B. W. Ramsey, *American Journal of Respiratory and Critical Care Medicine*, 2003, 168, 918-951.
7. J. Chastre and J. Fagon, *American Journal of Respiratory and Critical Care Medicine*, 2002, 165, 867-903.
8. A. Smith, History of the Agar Plate, http://www.labnews.co.uk/features/history-of-the-agar-plate/, Accessed 5/18, 2014.
9. J. B. Lyczak, C. L. Cannon and G. B. Pier, *Microbes and Infection*, 2000, 2, 1051-1060.
10. J. A. Jordan and M. B. Durso, *Journal of Molecular Diagnostics*, 2005, 7, 575-581.
11. J. A. Washington, ed. S. Baron, *Medical Microbiology*, Galveston (TX): University of Texas Medical Branch at Galveston, 4th edn., 1996.

12. F. Zucol, R. A. Ammann, C. Berger, C. Aebi, M. Altwegg, F. K. Niggli and D. Nadal, *Journal of Clinical Microbiology*, 2006, 44, 2750-2759.
13. J. W. Hong, V. Studer, G. Hang, W. F. Anderson and S. R. Quake, *Nature Biotechnology*, 2004, 22, 435-439.
14. E. A. Ottesen, J. W. Hong, S. R. Quake and J. R. Leadbetter, *Science*, 2006, 314, 1464-1467.
15. C. van Delden, in *Virulence and Gene Regulation*, ed. J.-L. Ramos, Springer US, 2004, DOI: 10.1007/978-1-4419-9084-6_1, ch. 1, pp. 3-45.
16. T. A. Webster, H. J. Sismaet, J. L. Conte, I. p. J. Chan and E. D. Goluch, *Biosensors and Bioelectronics*, 2014, 60, 265-270.
17. L. E. Dietrich, A. Price-Whelan, A. Petersen, M. Whiteley and D. K. Newman, *Molecular Microbiology*, 2006, 61, 1308-1321.
18. M. K. W. E. O. King, and D. E. Raney, *Journal of Laboratory and Clinical Medicine*, 1954, 44, 301-307.
19. D. Sharp, P. Gladstone, R. B. Smith, S. Forsythe and J. Davis, *Bioelectrochemistry*, 2010, 77, 114-119.
20. T. A. Webster and E. D. Goluch, *Lab on a chip*, 2012, 12, 5195-5201.
21. H. J. Sismaet, T. A. Webster and E. D. Goluch, *The Analyst*, 2014, 139, 4241-4246.
22. T. A. Webster, H. J. Sismaet and E. D. Goluch, *Nano LIFE*, 2013, 03, 1340011.
23. D. L. Bellin, H. Sakhtah, J. k. Rosenstein, P. M. Levine, J. Thimot, K. Emmett, L. E. Dietrich and K. L. Shepard, *Nature Communications*, 2014, 5.
24. A. Bajwa, S. Tan, R. Mehta and B. Bahreyni, *Sensors*, 2013, 13, 8188-8198.
25. J. He, R. L. Baldini, E. Deziel, M. Saucier, Q. Zhang, N. T. Liberati, D. Lee, J. Urbach, H. M. Goodman and L. G. Rahme, *Proceedings of the National Academy of Sciences*, 2004, 101, 2530-2535.
26. K. Todar, *Pseudomonas aeruginosa*, http://textbookofbacteriology.net/pseudomonas.html, Accessed 6/19/14, 2014.
27. K. Takahashi, S. Tereda, H. Ueda, F. Makishima and E. Suzuki, *Cytotechnology*, 1994, 15, 57-64.
28. M. B. Al-Fageeh, R. J. Marchant, M. J. Carden and C. M. Smales, *Biotechnology and Bioengineering*, 2006, 93, 829-835.
29. G. W. Lau, H. Ran, F. Kong, D. J. Hassett and D. Mavrodi, *Infection and Immunity*, 2004, 72, 4275-4278.
30 J. L. Connell, J. Kim, J. B. Shear, A. J. Bard, and M. Whiteley, *PNAS*, 2014, 111, 18255-18260.
31. T. S. Murray and B. I. Kazmierczak, *Journal of bacteriology*, 2006, 188, 6995-7004.
32. A. S. Downey, S. M. Da Silva, N. D. Olson, J. J. Filliben and J. B. Morrow, *Applied and Environmental Microbiology*, 2012, 78, 5872-5881.
33. J. M. Andrews, *Journal of Antimicrobial Chemotherapy*, 2001, 48, 5-16.



A disposable electrochemical assay that is integrated with standard culture plates for the selective and specific detection of Pseudomonas aeruginosa.
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