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ORIGINAL PAPER

Bacterial Sample Concentration and Culture Monitoring Using a PEG-Based Osmotic System with Inline Impedance and Voltammetry Measurements

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Abstract
Impedance measurements using graphite electrodes were used to detect the increase in culture medium conductivity due to bacteria growth in real time along with simultaneous voltammetric monitoring of pyocyanin concentration. Electrochemical methods were compared to conventional continuous monitoring of bacterial cultures using turbidity measurement at an optical density of 600 nm (OD600). A practical osmotic system was further designed for concentrating bacterial cultures during growth to enable earlier detection using the electrochemical methods. Bacterial cultures, starting from an initial culture density of 1×10^8 cells/mL, were grown inside a sealed cellulose ester dialysis membrane, while polyethylene glycol in LB medium was used as the draw solution outside the membrane to gradually concentrate the growing cultures. 0.7-mm-diameter graphite for mechanical pencils was utilized as working and counter electrodes with a platinum wire reference electrode for electrochemical measurements with and without the osmotic system. In the absence of forward osmosis, impedance measurements detected culture growth ~ 1 h faster than conventional OD600. Integrating osmosis showed a twofold decrease in the time to detect pyocyanin production as an indicator for bacterial growth. For impedance monitoring, removal of water by osmosis was conflated with the impedance decrease due to cell growth; however, the results show a promising ability to detect bacteria growth via an observed shift in osmotic impedance profile when bacteria are present in a sample. By monitoring the deviation in the impedance profile, a threefold improvement in detection time was achieved when compared to conventional OD600 measurements.

Keywords Osmotic concentration · *Pseudomonas aeruginosa* · Bacterial growth detection · Impedance electrochemical monitoring · Forward osmosis

1 Introduction

Rapid pathogen detection is critically important across various fields, such as clinical diagnostics, food industry, drinking water quality, and environmental monitoring [1–3]. In particular, the recent emergence of antibiotic-resistant strains of bacteria has further heightened the demand for rapid pathogen detection [4, 5]. The centers for disease control and prevention (CDC) reports that each year in the USA, at least 2 million people become infected with antibiotic-resistant strains and at least 23,000 people die each year as a direct result of these infections [6]. One of the highly antibiotic-resistant strains noted by the CDC is *Pseudomonas aeruginosa*, gram-negative bacteria that are the dominant cause of nosocomial infections and chronic lung disease [7, 8].

There are numerous technologies available to provide rapid *P. aeruginosa* identification but despite innovative advances in diagnostic methods, patients still routinely receive broad spectrum antibiotic therapies or ineffective antibiotic combinations because of the additional time required to conduct successful antimicrobial susceptibility testing (AST) [9–12]. Conventional AST usually takes 24–48 h after positive *P. aeruginosa* identification and is performed by utilizing either the disk diffusion, broth or agar dilution testing guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI) or the

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European Committee on Antimicrobial Susceptibility Testing (EUCAST) [13–15]. In general, standard AST is preferably performed by the manual broth dilution method in which positive cultures are incubated in growth medium with the addition of a combination of antibiotics in varying concentrations into 96 micro-well plates. The culture is maintained at 35–37 °C for a period of 16–20 h to observe visual culture turbidity within the micro-well plates [12, 14]. The lowest antibiotic concentration that leads to no visual growth within the incubation period is termed the minimum inhibitory concentration (MIC) and is used to quantitatively report resistant strains and antibiotic efficacy [16]. There are newer commercially available automated systems that can perform the broth dilution AST, including *P. aeruginosa* testing by utilizing more sensitive optical detection platforms that detect bacteria growth using absorbance at 600 nm wavelength in micro-well plates [17]. However, these systems are relatively expensive in comparison with the manual methods; and more importantly, they are also limited by the time required for culture growth. Therefore, enabling rapid culture growth with low-cost sensitive monitoring can decrease the time required to perform the manual and automated AST, which will ultimately improve disease management.

In this work, we evaluated the ability to rapidly grow and monitor *P. aeruginosa* by concentrating the bacteria while simultaneously monitoring growth kinetics using low-cost graphite electrodes with electrochemical detection. The inoculation culture density is proportional to the time to detect growth, and therefore, concentrating bacteria while it grows could be an advantageous method to shorten the time to detect growth [18]. Recent studies have shown that *P. aeruginosa* growth can be electrochemically monitored by detecting the concentration of pyocyanin, a redox active molecule that is expressed by the bacteria [19, 20]. However, only a few of these studies directly perform inline continuous monitoring in part because of electrode fouling during culture growth, while majority of studies analyze dead-end samples for single-use detection [21]. We therefore studied the ability to continuously monitor pyocyanin production using graphite electrodes as a secondary indicator of *P. aeruginosa* growth.

Furthermore, bacterial growth has also been historically detected using impedance measurements, particularly in the food industry [22]. The impedance-based approach, also known as impedance microbiology, uses metal electrodes to measure the change in the amount of electrolytes that are excreted by live bacteria into the culture medium as they grow [23]. This impedance-based bacteria growth profile is proportional to the amount of bacteria present and with optimal conditions, distinguishing between species can be performed by reviewing the bacteria growth kinetics [24, 25]. These two electrochemical methods were utilized to study the growth kinetics of *P. aeruginosa* during osmotic concentration. Since pyocyanin and culture conductivity both increase due to volume reduction, early detection of *P. aeruginosa* growth can be achieved using low-cost electrochemical methods.

2 Experimental

In all of the results shown, data points represent the mean value obtained from three independent experiments and error bars represent one standard deviation from the mean.

2.1 Chemicals and Apparatus

For osmotic experiments, 1-mL cellulose ester membrane containers with 100–500 Dalton molecular weight cutoff (MWCO) membranes were purchased from Spectrum Labs (F235061). Carbon electrodes were sourced locally from pencil graphite (Pentel HB 0.7 mm) with 700 µm diameter and 60 mm length and served as both the working and counter electrodes for electrochemical experiments. The pencil graphite was insulated by dipping in a hot glue gun, and the tip was re-covered by lightly grinding on fine sandpaper to provide an electrode surface area roughly 700 µm in diameter. Platinum wire (Fisher scientific, 43,014) with 300 µm diameter and 60 mm length was used as the reference electrode. A micro-sample osmotic system with inline monitoring was designed by placing hot glue onto two insulated pencil graphite electrodes along with one platinum wire. A 5 mm hole was drilled into the cap closure of the cellulose ester membrane compartment, and the electrodes were inserted into the membrane compartment and then sealed onto the cap with the hot glue gun. The fully assembled osmotic testing system is shown in Fig. 1. This system was used for osmotic sample concentration and osmotic culture growth experiments. At the start of the experiment, the cap with electrodes was removed, a 1 mL sample was syringe filled into the container, and then the cap with electrodes was placed back onto the membrane compartment. The membrane compartment was then placed into a 200-mL beaker that contained an osmotic gradient solution of 65% weight by volume (w/v) polyethylene glycol, molecular weight 1500 (PEG1500) in LB media. The PEG1500 is much larger in size than the 100–500 Dalton pores in the membrane, which limit the ability of PEG1500 to diffuse into the sample inside of the membrane. The temperature was controlled at 37 °C for all experiments by placing the beaker on a hot plate with mixing at 300 rpm.

Reagent-grade sodium chloride (CAS 7647-14-5), potassium chloride (CAS 7447-40-7), sodium phosphate dibasic (CAS 7784-85-6), and potassium phosphate monobasic (CAS 7778-77-0) chemicals, along with PEG1500 (CAS

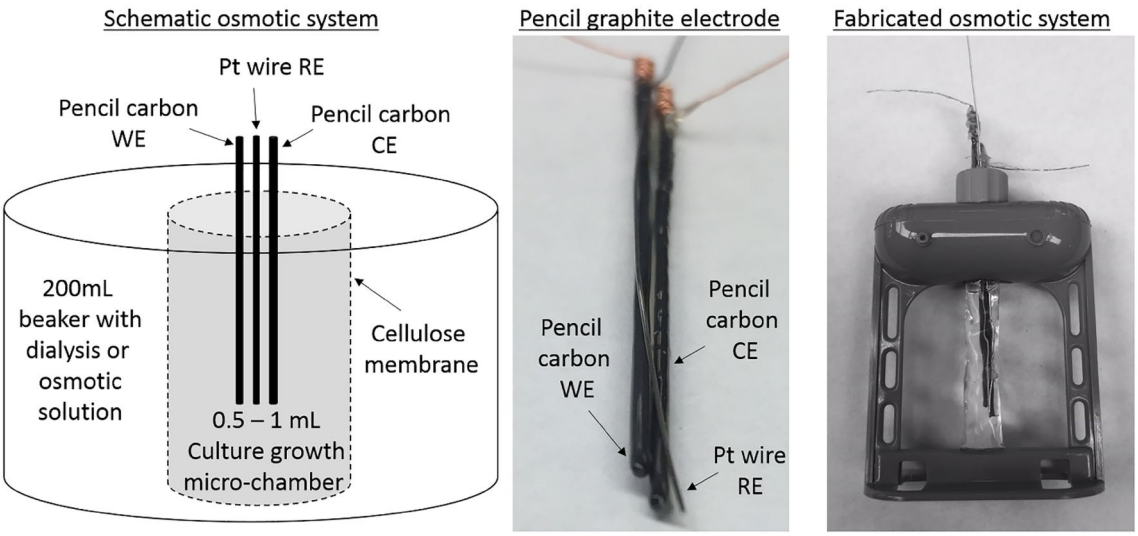

Fig. 1 Schematic and pictures of the osmotic testing system used for electrochemical monitoring of *P. aeruginosa* cultures using pencil graphite working (WE) and counter (CE) electrodes and platinum wire as the reference electrode (RE)

25322-68), were sourced from Fisher Scientific. Lysogeny broth (LB) growth medium (Becton, Dickinson and Company, 244620) was prepared at standard concentrations of 10 g/L tryptone, 10 g/L sodium chloride and 5 g/L yeast extract in deionized water. A 65% w/v solution of PEG1500 in LB media was prepared based on the solubility limit reported by the manufacturer (650 g/L). Pyocyanin (*N*-methylphenazin-1-one, CAS#85-66-5) was purchased in powder form from Cayman Chemicals (10009594) and dissolved in DI water to make a 500 µM stock solution. This stock was refrigerated and used to make dilutions.

2.2 Osmotic System Experimentation

Initial studies were carried out to measure osmotic water loss in the system using electrochemical impedance readings. The osmotic system was first sanitized by soaking in 10% ethanol for 10 min and then flushing and soaking in DI water prior to sample loading. LB media were used as the test solution, and 500–900 µL of LB was syringed into the membrane compartment. The electrodes were then inserted into the membrane compartment. The membrane compartment was then placed into a 200 mL bath of 65% w/v PEG1500 in LB media to provide the osmotic gradient outside the membrane. The osmotic solution was maintained at 37 °C using a hot plate with a stir bar spinning at 300 rpm. Impedance readings from the electrodes were recorded every 2 min on an electrochemical workstation (Zahner-Elektrik, IM6ex) in galvanostatic mode at a frequency of 25 kHz, 50 nA current setting, and an amplitude of 50 nA. Figure 2a shows the impedance profile in terms of the impedance signal change percent from the initial time point.

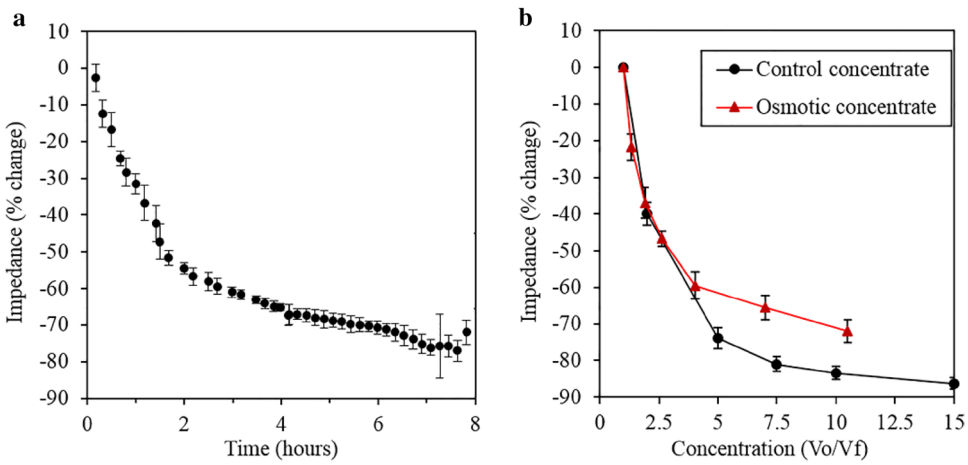

Fig. 2 **a** Impedance readings of LB media during osmotic concentration with 65% w/v PEG1500 in LB media outside of membrane. **b** Impedance readings calibrated to volume measurements in the osmotic system (osmotic concentrate) compared to impedance readings of control sample concentrates made without osmotic concentration (control concentrate)

The osmotic water loss from the LB medium in the compartment through the membrane was also measured by volume using a syringe. This allowed the impedance signal change to be correlated with the amount of water lost in the sample. Syringe data were collected every 10 min for a period of 180 min and compared to inline impedance data to perform the calibration shown in Fig. 2b. The amount of water lost was calculated as the ratio between the initial sample volume (V_o) and the sample volume recorded at the specified time (V_f). Since small ions have the ability to migrate across the membrane, the impedance signal change from osmotic concentration was compared to impedance measurements acquired from control sample concentrates prepared without osmotic concentration. Concentrated control LB samples were prepared by adding 1–20 times the specified concentration of chemicals, and subsequently, impedance readings were obtained using the fabricated three-electrode sensor. Figure 2b shows that the change in impedance deviates between the osmotic and control concentrate samples over time. The decrease in impedance for the osmotically concentrated LB is more gradual than for the known control concentrate LB, indicating that there is movement of ions across the membrane during osmotic concentration as expected.

2.3 Electrochemical Signal Amplification

Pyocyanin has been used as a biomarker for the electrochemical detection of *P. aeruginosa* having a redox potential at approximately − 200 mV against a Ag/AgCl reference electrode [26]. Square wave voltammetry (SWV) was used to detect pyocyanin from fluid samples using pencil graphite electrodes by scanning at a potential window from − 0.7 to 0.0 V using an amplitude set-point of 0.025 V, frequency of 15 Hz, and increment of 0.004 V. The use of a platinum wire reference electrode was found to yield similar faradaic peak position when compared to Ag/AgCl reference. A calibration was performed by spiking known pyocyanin concentrations into LB medium and measuring the faradaic peak response at the pyocyanin redox potential region. The first and second derivatives of the faradaic peak response were used to calculate half of the peak height at each tested pyocyanin concentrations. The value at half of the peak height is referred to as the magnitude peak current.

The pyocyanin concentration has been shown to increase during culture growth and has been used as an indication of the cell density [20, 27, 28]. To test whether the current generated by pyocyanin can increase simply due to the change in ions during osmotic concentration, we investigated the pyocyanin signal amplification profile from the osmotic system. A 900 µL sample containing 1 µM pyocyanin in LB medium was prepared and syringed into the membrane compartment. The electrodes were then inserted into the compartment, and a 200 mL volume of 65% w/v PEG1500 in LB media was used as the osmotic gradient placed outside the compartment. Electrochemical SWV scans were collected in triplicate at 10-min intervals. The faradaic peak increased as the sample was concentrated in the first hour of osmotic operation shown in the raw scans (Fig. 3a) and the time course study (Fig. 3b). A slight shift of the pyocyanin electrochemical peak in the positive direction was observed for concentrated samples (Fig. 3a). This is indicative of slight pH shifts triggered by changing ion concentrations and transport across the membrane during osmotic volume reduction.

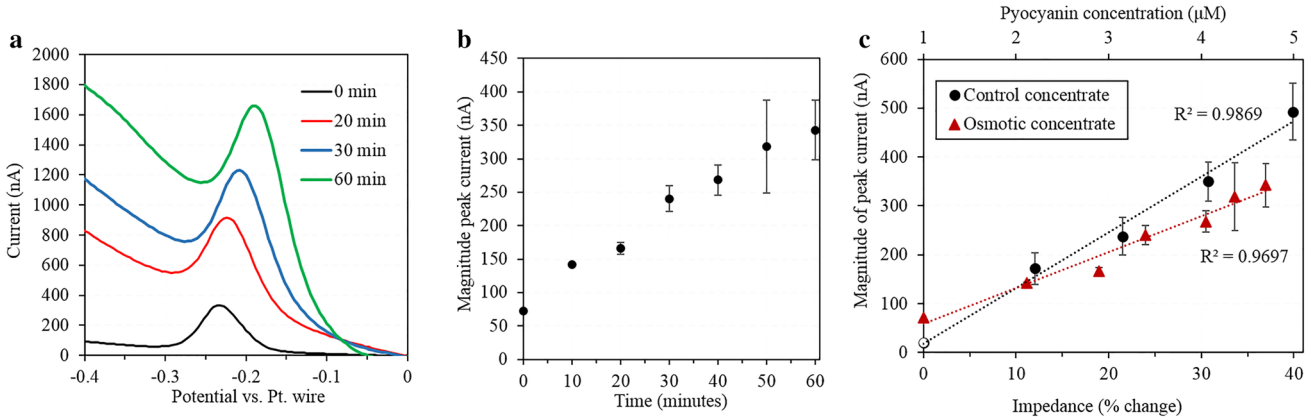

Fig. 3 **a** SWV scans showing time course osmotic concentrations of 1 µM pyocyanin prepared in LB media. **b** Time course data of analyzed magnitude of peak current from the SWV scans during osmotic concentration. **c** Comparison of impedance and SWV data for samples concentrated using osmotic system as compared to offline-prepared concentrates of pyocyanin in LB medium. The second *x*-axis shows the prepared pyocyanin concentrations for the control concentrate. It is used as a calibration standard to correlate peak currents in the osmotic concentrate to a pyocyanin concentration

In order to determine whether the correlation between measured current and pyocyanin concentration changed due to osmotic concentration, 5 µM pyocyanin in 5 times concentrated LB medium was prepared. The stock was then diluted with DI water to make 4 µM in 4×, 3 µM in 3×, 2 µM in 2×, and 1 µM in 1× concentrates of pyocyanin in LB (5 total samples). Impedance and SWV data were collected for these prepared concentrates, and these data are referred to as the control concentrate (Fig. 3c). For osmotic concentrate samples, an initial sample containing 1 µM pyocyanin in 1× composition LB was placed into the membrane compartment and exposed to an osmotic gradient for 1 h (similar to Fig. 3b). Impedance and SWV measurements were collected for the osmotic concentrate every 10 min (7 total samples). Both the control and osmotic concentrate samples were prepared in triplicate. The impedance and SWV results from the osmotic system were then compared to the control samples. There was minimal deviation in signal due to the osmotic system for the measured concentrations, as shown by the comparison of the test and control samples (Fig. 3c). The second *x*-axis in Fig. 3c indicates the pyocyanin concentration in the control samples and serves as a calibration standard in correlating peak currents to a pyocyanin concentration in the osmotic system. It is expected that the currents will deviate more with greater osmotic sample concentration, as the pyocyanin will also slowly diffuse out of the membrane compartment.

2.4 Culture Growth

Pseudomonas aeruginosa (strain PA14) cultures were initially incubated overnight at 37 °C with LB media in 15-mL glass tubes. After overnight growth, manual cell counts using disposable hemocytometers (Incyto, model C-Chip) were performed alongside optical density readings using a spectrophotometer (Molecular devices, Spectramax paradigm) to determine the appropriate number of cells to inoculate for growth kinetics experiments. A 900 µL LB sample containing an inoculum cell density of 1×10^8 CFU/mL was then prepared. Control cultures not exposed to forward osmosis were incubated in a 2-mL micro-centrifuge tube maintained at 37 °C. Test cultures were incubated into the osmotic system with LB media on the outside of the membrane. Cultures were also grown in the presence of an osmotic gradient solution outside the membrane. The osmotic gradient contained 65% w/v PEG1500 in LB. The electrode sensors described in Fig. 1 were utilized in the control and test cultures to simultaneously monitor impedance and SWV readings with staggered timing to ensure the impedance data acquisition did not interfere with voltammetric scanning using the same electrode. Impedance readings were collected every 2 min in galvanostatic mode at a 50 nA current setting, frequency of 25 kHz, and an amplitude of 50 nA. SWV scans were also performed every 2 min in the control and test cultures by scanning from −0.7 to 0.0 V using an amplitude set-point of 0.025 V and frequency of 15 Hz.

3 Results and Discussion

3.1 Culture Growth Using Electrochemical Monitoring

Electrochemical culture growth monitoring using impedance and voltammetry was compared to conventional growth monitoring using a spectrophotometer measuring absorbance at 600 nm wavelength (Molecular devices, Spectramax paradigm) for the controlled cultures that were not exposed to forward osmosis. Three trials of each method were conducted where *P. aeruginosa* cultures were inoculated at 1×10^8 cells/mL in LB media and monitored for 20 h. Comparable profiles were noted with the pencil graphite electrodes when using the decrease in impedance and SWV pyocyanin monitoring techniques as compared to conventional OD600 method (Fig. 4a). The decrease in impedance and conventional OD600 methods showed similar growth profiles with less variation as compared to growth based on pyocyanin detection. The time to detect growth for all methods was then quantified by selecting the time where three consecutive readings were above three standard deviations from the baseline reading. The impedance method resulted in detecting growth 1 h earlier than conventional OD600 measurements (Fig. 4b). The increase in ions produced by the bacteria during proliferation was also detected earlier than production of pyocyanin. To further improve the detection potential of electrochemical monitoring, growth using the osmotic system was subsequently studied.

3.2 Growth Monitoring in Semi-permeable Membrane

The use of a semi-permeable membrane for bacterial growth is advantageous in providing media replenishment to maintain a steady substrate concentration similar to perfusion, yielding optimal growth [29]. Nutrient transport across the membrane can improve growth, but it may also negatively impact monitoring of target molecules that can quickly diffuse across the membrane. Therefore, 1×10^8 cells/mL cultures were incubated in 1 mL LB media at 37 °C inside the membrane, while LB media was also maintained outside the membrane. Culture growth was monitored using impedance and voltammetry utilizing the three-electrode sensor inside the membrane compartment.

The impedance initially decreased during the first hour, but then, interestingly, the impedance stabilized and

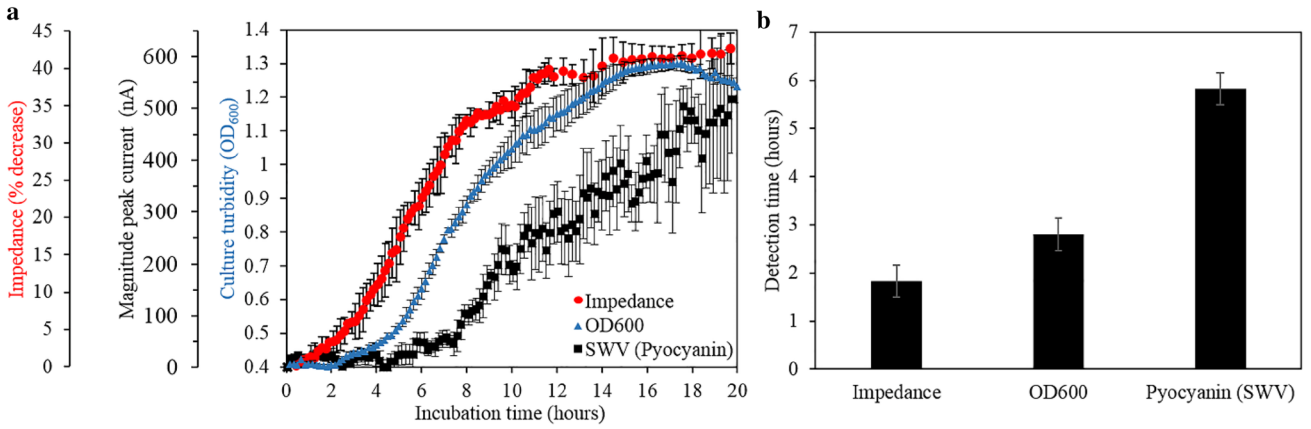

Fig. 4 **a** Monitoring of *P. aeruginosa* growth in LB medium at 37 °C using impedance, SWV pyocyanin detection, and OD600 methods. **b** Comparison of the time to detect *P. aeruginosa* growth using the three methods

started increasing at a steady rate during the next 5 h and eventually reached a new steady state, 12% higher impedance, 8 h after the start of the experiment (Fig. 5a). The membrane had a significant effect on the transport of ions and water during cell growth. It is expected that the initial decrease in impedance is the result of normal cellular processes. However, as the gradients in nutrient and ion concentrations built up across the membrane, small ions diffused out of the membrane compartment, while glucose, amino acids, and other non-ionic small molecules diffused into the compartment since they were being consumed by the bacterial cells. Eventually, the flux of ions across the membrane reached a steady state that correlated with the cellular growth rate. Supporting this explanation is the fact that the current increase generated by pyocyanin production shown in Fig. 5b has a similar shape and magnitude to the current profile shown in Fig. 4a. This similarity indicates that the cellular growth rate remains the same for cultures grown in this perfusion-based membrane system. Therefore, the observed steady state in impedance (ion concentration inside the membrane) is a result of steady-state ion replenishment across the membrane and indicates that ion transport across the membrane occurs more rapidly than the rate of ion production by the bacteria. These impedance results warrant further investigation; however, they are beyond the scope of this manuscript. In the case of pyocyanin production (Fig. 5b), the increase in the magnitude of peak current corresponds to bacterial growth, as more pyocyanin will be produced by the culture as it proliferates. The profile indicates that the bacteria produce pyocyanin at a faster rate than the rate of pyocyanin diffusion through the membrane.

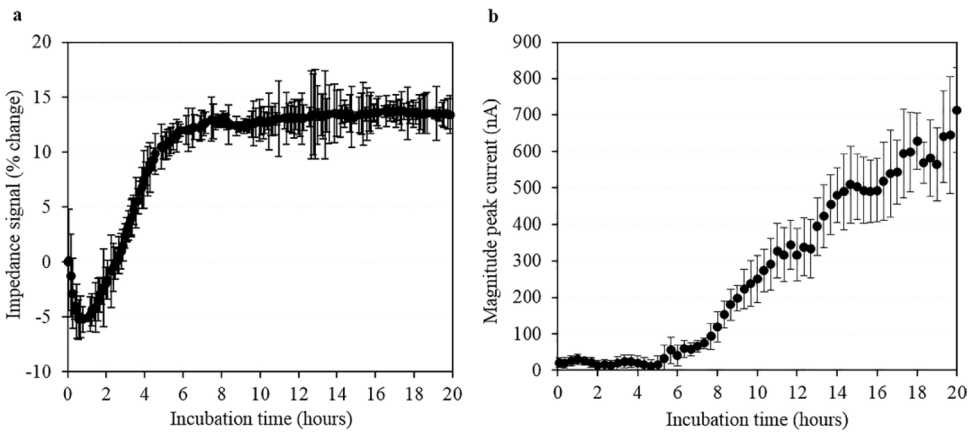

Fig. 5 **a** Impedance and **b** voltammetric monitoring of *P. aeruginosa* growth at 37 °C in the osmotic system with only LB medium outside the membrane compartment to maintain a perfusion-based system. No osmotic gradient was present at the beginning of the experiment, but cellular functions created gradients across the membrane. Typical growth profiles using pyocyanin (magnitude of peak current) were maintained, indicating that bacteria produce pyocyanin at a higher rate than diffusion of the molecule out through the membrane

3.3 Growth Monitoring in an Osmotic Gradient

The addition of an osmotic gradient in culture monitoring was then studied to determine whether it would impact the time to detect bacterial growth during concentration. The osmotic system was inoculated with 1×10^8 cells/mL in LB, while an osmotic gradient was applied outside the membrane using 65% w/v PEG1500 in LB medium maintained at 37 °C on a stirred hot plate. The cultures were monitored by impedance and SWV. These osmotic test cultures were compared to controlled cultures maintained in the absence of an osmotic system, grown in LB media at 37 °C in microcentrifuge tubes. For the control cultures, impedance and SWV measurements were also performed using the same pencil graphite electrodes (Fig. 1).

Monitoring the culture using SWV detection of pyocyanin showed significant improvement in the time to detect growth with the 65% w/v PEG1500 osmotic gradient (Fig. 6a) indicating that concentrating cultures during growth enabled more rapid detection. The cultures grown when LB media were used as an osmotic gradient showed marginal improvement in the SWV growth profile but did not yield a more rapid detection time. The time to detect growth for all methods was then quantified by selecting the time where three consecutive readings were above three standard deviations from the baseline reading. The SWV osmotic system detected growth in about 3.5 h, which is a twofold improvement in detecting growth by monitoring pyocyanin levels (Fig. 6b). This detection time is similar to conventional growth monitoring using OD600 that detected growth in 3 h (Fig. 4b).

Monitoring the osmotic test cultures using impedance showed a new approach for more rapid detection of culture growth. The rate of volume reduction in the 65% w/v PEG1500 osmotic test cultures slowed down in the presence of bacteria when compared to the rate of volume reduction in the absence of bacteria (Fig. 7a). The proposed mechanism is that the presence of bacteria increases the osmotic concentration inside the membrane therefore decreasing the osmotic gradient across the membrane. This impedance profile shift may

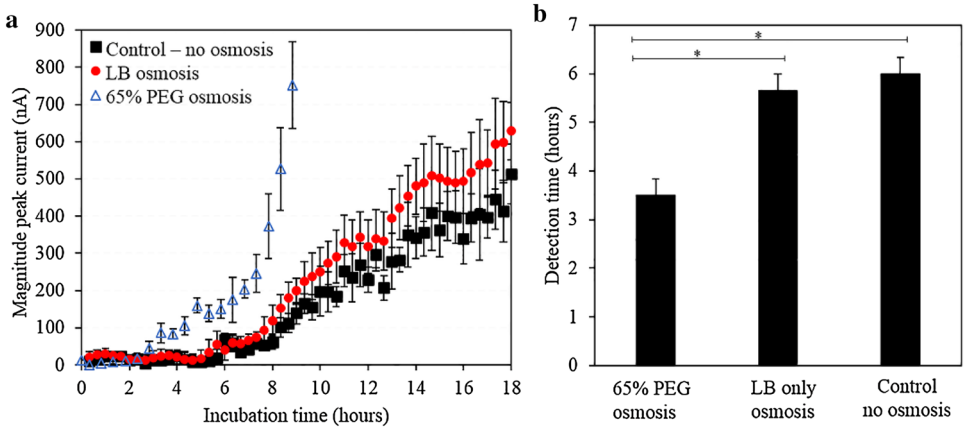

Fig. 6 **a** Pyocyanin detection for bacteria growth without osmotic system compared to growth using 65% w/v PEG1500 or LB media osmotic gradients. **b** Comparison of time to detect *P. aeruginosa* growth with and without osmotic system shows a twofold reduction when osmotic system is used. Statistical significance greater than 3 standard deviations from the average is indicated in the graph

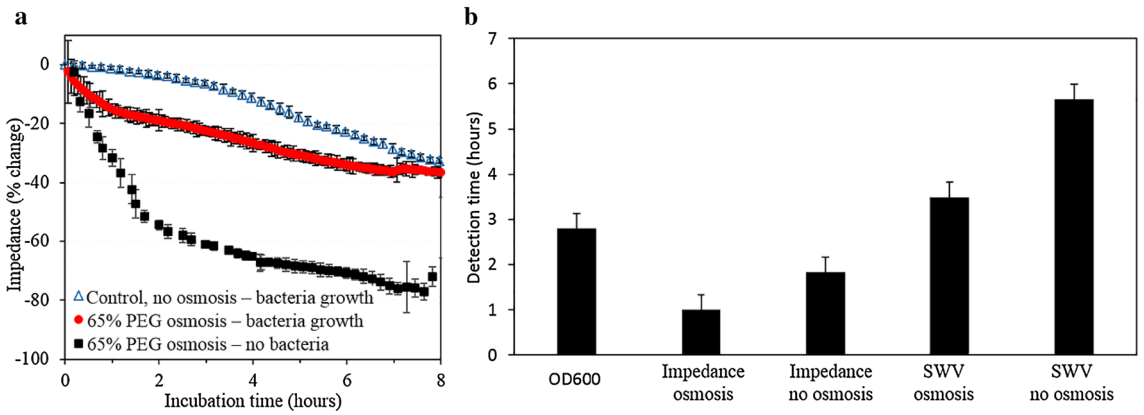

Fig. 7 **a** Comparison of impedance profiles in 65% w/v PEG1500 osmotic system with and without bacterial growth, compared to impedance profiles without the osmotic system. **b** Comparison of time to detect bacteria growth using impedance and SWV in the presence and absence of an osmotic system, compared to detection using conventional OD600

serve as an indicator of culture growth (Fig. 6a). The proposed detection time for this system was defined by performing an analysis of variance between the impedance decay with bacteria compared to impedance decay without bacteria. The detection time was defined as the time point at which the mean of the change in impedance data with bacteria was more than 3 standard deviations from the mean of the change in impedance data in the absence of bacteria. This detection time was found to be approximately 1 h as compared to 1.8 h when detecting growth using impedance data without an osmotic system (Fig. 7b). This new approach showed improved detection time when compared to pyocyanin SWV detection, and, more importantly, showed a threefold reduction in detection time compared to conventional detection using OD600 (Fig. 7b).

4 Conclusions

In summary, an osmotic system was designed to monitor bacterial growth using SWV and impedance electrochemical methods. *Pseudomonas aeruginosa* production of pyocyanin and ions was monitored using readily available pencil graphite as electrodes. Cultures were also successfully grown in the osmotic system containing 65% w/v PEG1500 to decrease the time to detect growth in conditions similar to antimicrobial susceptibility testing. The system led to a twofold improvement in the time to detect *P. aeruginosa* growth, providing similar growth detection times as conventional methods that use OD600. In addition, measuring impedance using the pencil graphite electrodes without an osmotic system detected culture growth roughly 1 h sooner in comparison with conventional OD600 measurements. Furthermore, in the presence of no cells, the osmotic system showed a substantial decrease in the impedance signal; but in the presence of cells grown in the osmotic system, the rate impedance signal drop slowed down. Therefore, growing bacteria using an osmotic system along with impedance monitoring shows promising ability to detect bacterial growth in roughly 1 h, which is a threefold reduction in detection time when comparing to detection using OD600. In general, the results from this work show that a simple osmotic system with electrochemical detection is a promising method for earlier detection of *P. aeruginosa* growth, with potential value in AST applications.

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