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<td>Buzid, Alyah; Reen, F. Jerry; Langsi, Victor K.; Ó Muimhneacháin, Eoin; O'Gara, Fergal; McGlacken, Gerard P.; Luong, John H. T.; Glennon, Jeremy D.</td>
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<tr>
<td>Publication date</td>
<td>2017-01-20</td>
</tr>
<tr>
<td>Type of publication</td>
<td>Article (peer-reviewed)</td>
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| Link to publisher's version | [http://dx.doi.org/10.1002/celc.201600590](http://dx.doi.org/10.1002/celc.201600590)  
Access to the full text of the published version may require a subscription. |
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| Embargo information | Access to this article is restricted until 12 months after publication by the request of the publisher. |
| Embargo lift date | 2018-01-20                                                                                                                                 |
| Item downloaded from | [http://hdl.handle.net/10468/3555](http://hdl.handle.net/10468/3555)  
Downloaded on 2019-03-10T17:04:08Z |
Accepted Article

Title: Direct and Rapid Electroanalysis of Pseudomonas aeruginosa Signaling Molecules in Bacterial Cultures and Cystic Fibrosis Sputum Samples via Surfactant-Assisted Membrane Disruption

Authors: Alyah Buzid, F. Jerry Reen, Victor K. Langsi, Eoin Ó Muimhneacháin, Fergal O’Gara, Gerard P. McGlacken, John H.T. Luong, and Jeremy D. Glennon

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: ChemElectroChem 10.1002/celc.201600590

Link to VoR: http://dx.doi.org/10.1002/celc.201600590
Direct and Rapid Electrochemical Detection of *Pseudomonas aeruginosa* Quorum Sensing Signaling Molecules in Bacterial Cultures and Cystic Fibrosis Sputum Samples through Cationic Surfactant-Assisted Membrane Disruption


**Abstract:** Rapid detection of pathogenic bacteria present in patient samples is of utmost importance for the clinical management of bacterial-induced diseases. Herein, we describe an efficient and direct electrochemical approach for the detection of 2-heptyl-3-hydroxy-4-quinolone (PQS), 2-heptyl-4-hydroxyquinoline (HHQ), and pyocyanin (PYO) as three molecular signatures of *Pseudomonas aeruginosa* (PA), a frequently infecting pathogen with high antibiotic resistance. The cationic surfactant hexadecyltrimethylammonium bromide (CTAB) enhances the effectiveness of an unmodified thin-film boron-doped diamond (BDD) electrode for the direct detection of PYO, HHQ, and PQS in bacterial cultures of PAO1 and PA14. Differential pulse voltammetry (DPV) is then used for monitoring the production of these microbial metabolites in bacterial cultures of PAO1 over 10 h without any sample pretreatment. A proposed mechanism for the interaction of CTAB with bacteria cells is examined by zeta (ζ) potential measurements. Furthermore, the detection method is successfully extended to a clinical fluid matrix and applied to PA spiked cystic fibrosis (CF) sputum samples.

**Introduction**

As a gram-negative pathogenic bacterium associated with hospital-acquired infections, particularly in patients with compromised immunity, *Pseudomonas aeruginosa* (PA) is one of the prime causes of morbidity and mortality in ~80% of patients with cystic fibrosis (CF).[1] This bacterium is also a common cause of pneumonia infections which is prevalent in intensive care units.[2] The infection is life-threatening, and the effectiveness of clinical management is limited by the ability of PA to form multicellular aggregates called biofilms, which act as a direct barrier to phagocytic cells and offers inherent antibiotic resistance.[3] PA produces a wide array of extracellular factors, which are critical for colonization and disease progression. Many of these factors are regulated in a cell density-dependent manner termed quorum sensing (QS). QS, an important cell-cell communication process, involves the production and sensing of small extracellular signaling molecules, enabling bacteria to monitor the population and respond to cell density and collectively control gene expression.[4] The QS framework of PA has two N-acyl homoserine lactone (AHL) regulatory circuits (LasIR and RhlIR) linked to the 2-alkyl-4(1H)-quinolone (AHQ) system to form a complex hierarchical network controlling gene expression.[5] The primary components of the AHQ signaling pathway are 2-heptyl-3-hydroxy-4-(1H)-quinolone (referred as the *Pseudomonas* Quinolone Signal, or PQS) and its biosynthetic precursor, 2-heptyl-4-hydroxyquinoline (HHQ).[6] One class of QS-controlled extracellular factor that PA utilizes to establish host infections are the phenazine compounds. Four main phenazines are produced by PA, and these are pyocyanin (1-hydroxy-N-methylphenazine, PYO), 1-phenazine-1-carboxamide (PCN), phenazine-1-carboxylic acid (PCA), and 5-methylphenazine-1-carboxylic acid (5-MCA).[7] PA is the only species recognized to produce PYO, unlike other phenazines which are produced by species such as *Pseudomonas fluorescens* and *Pseudomonas chlororaphis*.[8] PYO is considered as an important virulence and pro-inflammatory factor.[9] Also, it acts as a redox-active molecule and generates reactive oxygen species (Figure 1). PYO inactivates host proteases and is considered as a direct determinant of PA virulence. In keeping with the QS-regulation of virulence systems, induction of PYO is governed by PQS in PA.[10] The pKₐ values of PQS, HHQ, and PYO are present in Table 1.
Figure 1. The chemical structures of PQS, HQ, and PYO molecular signals.

Table 1. Estimated pK values of PQS, HQ, and PYO.[11]

<table>
<thead>
<tr>
<th>Analyte</th>
<th>pK_{a1} (NH)</th>
<th>pK_{a2} (OH)</th>
<th>pK_{a3} (OH)</th>
</tr>
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<tbody>
<tr>
<td>PQS</td>
<td>3.43</td>
<td>9.89</td>
<td>13.86</td>
</tr>
<tr>
<td>HQ</td>
<td>3.02</td>
<td>11.46</td>
<td>-</td>
</tr>
<tr>
<td>PYO</td>
<td>4.90</td>
<td>-</td>
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Most gram-negative bacteria produce bilayered membrane vesicles (MVs), consisting of an outer leaflet of lipopolysaccharides (LPS) and an inner leaflet of phospholipids (PL).[12] Such MVs serve as trafficking vehicles for a broad range of biologically relevant molecules, including PQS,[13] bacterial toxins, DNA, antibiotic resistance determinants, and antimicrobial compounds.[14] The bacterial LPS has three layers: lipid A, core oligosaccharide, and polysaccharide O-antigen. Lipid A molecules with phosphate and carbonyl groups in the outer membrane (OM) display negative charges. The bacterial OM is stabilized in vivo by a calcium salt bridge,[15] and provides an extra barrier which plays a crucial role in protecting the organism from antibiotics. PQS interacts strongly with LPS and is trafficked between cells via MVs; it is also required for MV formation.[16] Therefore, extraction may be necessary for the optimal analysis of PQS from bacterial cultures.[17] There is an emergence of antimicrobial agents to address bacterial infection through the interaction of cationic agents and the anionic bacterial OM by electrostatic interaction.[18] The PA of PQS in bacterial cultures of Staphylococcus aureus[20] has been used to ascertain the role of PQS as an antibiotic resistance determinant. The establishment of a simple method for the detection of PQS in bacterial cultures of PA (wild-type) without the prior requirement to extract the microbial metabolites from the culture is a fast and simple method that requires the addition of the cationic surfactant hexadecyltrimethylammonium bromide (CTAB) to the cell culture medium, and the effects of CTAB concentration, pH, and incubation time investigated. The mechanism of CTAB interaction with the bacterial cells was examined by zeta (ζ) potential measurement. The conditions for the analysis were then applied to monitor the production of signaling molecules in the bacterial PAO1 strain and to detect the signals in CF patient sputum spiked with the bacterial strain PA-PAO1 using the bare BDD electrode. The level of HQS and PQS released in pediatric CF patients is higher when compared to laboratory strains.[24] Single analyte detection has a significant limitation in clinical pathogen detection due to the genetic heterogeneity that exists among PA.[25] Therefore, the detection of PYO alone as described in Alatrakchi et al.[26] using disposable screen-printed gold electrodes and Webster et al.[28] may not be sufficient to ascertain the absence or presence of PA.

As a continuing effort of our research activities, this work unravels the direct detection of all three signaling molecules in CF sputum samples based on in situ cationic surfactant-assisted membrane disruption. CTAB, which removes the lipids of the cell membranes, thus obviating the time-consuming solvent and solid phase extraction step, is advocated for this purpose considering its low cost and availability. The BDD electrode is not subjected to any fouling during the analysis, a classical problem associated with screen-printed electrodes (SPE)[29, 30] or other carbon-based materials.
Results and Discussion

Differential Pulse Voltammetry (DPV) of Surfactant-Treated Bacterial Cultures

The DPV for PYO, PQS, and HHQ individually and for a standard mixture of PYO, PQS, and HHQ in the presence of CTAB are shown in Figure 2. There were two detection windows for both PYO and PQS whereas HHQ exhibited one single peak. For PYO, the first pronounced peak at the negative potential reflects the oxidation of PYO whereas the second peak at a high potential is responsible for the polymerization of this compound (Figure 2a). The negative peak of PYO has been overlapped by some endogenous compounds in biological fluids including human saliva. PQS also has two oxidation peaks, reflecting the presence of –NH in the phenol ring. The DPV for PYO, PQS, and HHQ in the absence of CTAB can be viewed in Figure S1. The main purpose of using CTAB here is to disrupt the cell, effecting the release of the signaling molecules. CTAB is hydrophobic and not electroactive, so it should not appreciably affect the electrochemical behavior of the BDD electrode (Figure S2). However, CTAB would not be expected to enhance the electrical performance, but rather increase the levels of the signal molecules presented to the electrode. The extraction of intracellular molecules from bacterial cells is tedious and prone to noticeable errors and also more challenging when only minute sample volumes (e.g., patient sputum) are available.

As discussed in the introduction section, the proposed mechanism of CTAB interaction with the PA-OM is primarily based on electrostatic and hydrophobic interactions, resulting in disruption of the cellular membrane (Scheme 1). This is similar to the effect described for surfactants on E. coli and S. aureus. Furthermore, this surfactant aids in the solubilization of PQS from its hydrophobic affinity for the lipid-rich membrane of the bacterial cells (Scheme S1).
Therefore, the effect of the cationic surfactant (CTAB) on the DPV of the bacterial culture PAO1 was evaluated (Figure 3). All measurements were performed in the presence of the bacteria. Varying CTAB concentrations (0, 0.5, 1.0, and 2.0 mM) were investigated while maintaining the pH and equilibration time at pH 7.0 and 5 min, respectively. Figure 3a clearly shows that PQS and HHQ were not detected in the absence of CTAB. The concentration of 1.0 mM and 2.0 mM CTAB provided sufficient cell membrane disruption to release and allow detection of PQS and HHQ from the lipid membrane. Both concentrations result in the disappearance of the oxidation peak of PYO at ~ +0.8 V. However, 1.0 mM provides better detectability and higher peak current of PQS and HHQ than 2.0 mM. It is worth noting that, while the addition of CTAB at these concentrations led to an increased lag phase in PAO1 growth, exponential phase, and comparable biomass were achieved (Figure S3a). However, in the longer term, following 4-day incubation, cell viability was significantly reduced in the presence of 2.0 mM CTAB (Figure S3b). Indeed concentration-dependent suppression of microbial growth by CTAB has previously been outlined in previous reports, both of fungal cells [38] and bacterial organisms [39-40]. Therefore, 1.0 mM was selected as the effective CTAB concentration. Different CTAB pH solutions (pH 6.0 - 8.0) were studied with the concentration and equilibration time constant at 1.0 mM and 5 min, respectively. At pH 7.0, an apparent peak separation of PYO and PQS was achieved in the potential range of +0.85 V to +1.15 V (Figure 3b). The varying of the equilibration time (0 - 30 min) of surfactants with bacterial culture was also studied. The higher peak intensities of the target analytes were obtained using 5 min as the equilibration time (Figure 3c). This is consistent with previous reports which described the adsorption of CTAB on the PA surface reaching equilibrium in less than 5 min [41]. No attempts were made to grow PA bacterial cultures in the absence of oxygen because anaerobic PA limits PQS production and subsequently limits all PQS controlled virulence factors [42]. Furthermore, it is time-consuming to remove oxygen from the sample with nitrogen bubbling (> 30 min), and this step is more problematical with minute sample volumes. The addition of an electron receptor also adds another step and such a compound might also be electroactive and interferes with the measurement of the biomarkers, a subject of future endeavors.

In order to show that the applicability of the method was not restricted only to PAO1, the developed approach was also applied to the PA14 strain. Under the chosen conditions, the DPV of bacterial cultures PAO1 and PA14 with and without CTAB treatment can be compared (Figure 4). The DPV shows that bacterial PA14 strain produced ~3 times more PYO and HHQ and ~2 times more PQS than the PA-PAO1 strain.

As also shown in Figure 4, PYO is oxidized at a negative potential (~0.14 V) whereas the second peak at +0.8 V is responsible for the polymerization of this oxidized compound. CTAB displays ionic and hydrophobic interactions with the oxidized PYO to form a stable complex. This complex becomes more resistant to oxidation/polymerization, resulting in a noticeable decrease or even disappearance of the PYO peak at +0.8 V.

![Scheme 1. Schematic diagram of membrane vesicles disruption by cationic surfactant (CTAB) on the PA.](image)

![Figure 3. DPV responses towards the effect of varying a) CTAB concentrations (0 - 2.0 mM) at pH 7.0 and equilibration at 5 min; b) CTAB pH (6.0 - 8.0) at 1.0 mM CTAB and equilibration at 5 min; and c) equilibration time (0 - 30 min) at 1.0 mM CTAB and pH 7.0. Bacterial PAO1 strain was grown for 9 h with the CD0.05 of 2.2:1. 50 mM acetate buffer, pH 5.0 consisting of 20% ACN was used as an electrolyte for the detection on the BDD electrode vs. Ag/AgCl.](image)
The zeta potential (ζ) provides further evidence for the selective binding of CTAB to PA cell membrane. Upon the addition of CTAB, the ζ potential for PA becomes less negative (Table 2), indicating that the active CTAB binding to the outer membrane of PA.[17]

Table 2. Zeta potential (ζ) of bacterial culture PAO1 before and after the addition of CTAB.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta potential (ζ, mV)</th>
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<tbody>
<tr>
<td>PAO1 wild-type</td>
<td>-29.8 ± 1.5</td>
</tr>
<tr>
<td>PAO1 wild-type + CTAB</td>
<td>-25.7 ± 1.4</td>
</tr>
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</table>

[a] Zeta potential of the PAO1 strains in the absence and presence of CTAB. Measurements were performed in triplicate at 30 °C, and the data were presented as the mean ± standard deviation (SD). The bacterial PAO1 strain was grown for 9 h with the OD_{600 nm} of 2.87.

The analytical parameters for the simultaneous determination of PYO, HHQ, and PQS containing 1.0 mM CTAB, pH 7.0 and an equilibration time of 5 min in LB media are presented in Table 3. The calibration curve of target analytes exhibited an excellent linearity ($R^2 \geq 0.995$) in the linear range of 5 - 50 μM. The limit of detection (LOD) was estimated from the regression line of the calibration curve in the LB media (n = 3). The LOD for PYO, HHQ, and PQS in the LB media was 2.06, 3.61, and 4.85 μM, respectively, whereas the LOD of PYO, HHQ, and PQS in the buffer solution was 1.74, 2.48, and 1.07 μM, respectively. The reproducibility of the BDD electrode for signaling molecules detection was studied. The BDD electrode was used for repeated detection (3 times) for 30 μM of PYO, HHQ, and PQS each. The relative standard deviation (R.S.D %) values of the potential were 1.76 %, 2.54 %, and 3.64 % for PYO, HHQ, and PQS, respectively, representing acceptable precision of the BDD electrode. The DPV traces associated with a standard curve can be seen in Figure S4. The resulting LOD values deserve a brief comment here since such values are highly dependent upon the electrolyte medium. Without CTAB, the BDD electrode vs. Ag/AgCl in 50 mM acetate buffer, pH 5.0 containing 20% ACN exhibits very low LOD values for PYO, HHQ, and PQS: 50 nM, 250 nM, and 250 nM, respectively as reported by Buzid et al.[29b] However, such corresponding values are only 0.15 μM, 0.62 μM, and 1.25 μM, when the analysis is performed in the CF sputum sample.[29b] The LOD values obtained for the above biomarkers are 2.06, 3.61, and 4.85 μM when the DPV measurement is performed in the LB medium with 1.0 mM CTAB (Table 3), all well within the physiologically relevant range reported for CF sputum.[27, 41] The rationale behind such differences in LOD was not understood, but the LB medium contains casein enzymic hydrolysate (10 g/L) and yeast extract (5 g/L), which might adsorb on the BDD electrode during the DPV measurement, resulting in higher LOD values for the biomarkers. The presence of CTAB in the electrolyte does not exhibit an appreciable effect on the electroanalysis of BDD as mentioned earlier.

Direct Monitoring of HHQ, PQS, and PYO Production in the Bacterial Strain PAO1 and Analysis of Clinical Samples

The rapid and early detection of PA and other pathogenic or contaminant organisms would be a significant advance for both clinical and industrial applications. Furthermore, direct detection without any pre-treatment step would also be advantageous in terms of total analysis time and reproducibility. Therefore, direct time-course analysis and matrix interference assays were performed to ascertain the applicability of the developed method. A time-course analysis of bacterial PAO1 strain carrying a pqsA-lacZ promoter fusion was performed for 10 h to monitor the real-time concentration profiles of HHQ, PQS and PYO from an early log phase into the stationary phase of growth. In addition to measuring the DPV response on the BDD electrode, the kinetics of pqsA promoter activity was measured in tandem, providing a comparator profile for signal production at the level of gene expression. Both HHQ and PQS are co-inducers of the PqsR transcriptional regulator that controls the expression of the pqsA-E biosynthetic operon.[42] As such, increased detection of these molecules should be preceded by a similar increase in pqsA-E gene expression via the pqsA promoter. Samples were taken from cultures at 1 h intervals from the mid-log phase and monitored as before (Figure 5). No measurement was taken between 0 to 4 h because the significant production of the three signaling molecules only emerged after 4 h into the experiment (between late log phase and entry into the stationary phase of growth), consistent with the established kinetics of AHQ signal.
production in PA\textsuperscript{[1a, 27a]} HHQ and PQS were initially identified at the highest concentration. As the cells entered the stationary phase, both PQS\textsuperscript{,}, and PYO become more abundant.\textsuperscript{[27a, 43]} Data was consistent with the parallel monitoring of pqsA promoter activity in these cells, with increased HHQ and PQS production occurring in tandem with a spike in pqsA promoter activity, signaling activation of the system in the cell culture (Figure S5).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear range (µM)</th>
<th>Linear regression equation (I: µA, C: µM)</th>
<th>Correlation coefficient (R²)</th>
<th>LOD (µM)\textsuperscript{[a]}</th>
<th>R.S.D % (n = 3)\textsuperscript{[b]}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYO</td>
<td>5 - 50</td>
<td>( I_{PYO} = 3.28 \times 10^{-9} C + 2.17 \times 10^{-9} )</td>
<td>0.995</td>
<td>2.06</td>
<td>1.76</td>
</tr>
<tr>
<td>HHQ</td>
<td>5 - 50</td>
<td>( I_{HHQ} = 1.76 \times 10^{-10} C + 2.32 \times 10^{-9} )</td>
<td>0.997</td>
<td>3.61</td>
<td>2.54</td>
</tr>
<tr>
<td>PQS</td>
<td>5 - 50</td>
<td>( I_{PQS} = 1.58 \times 10^{-10} C - 4.22 \times 10^{-9} )</td>
<td>0.982</td>
<td>4.85</td>
<td>3.64</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} LOD calculated as 3 x standard deviation \textsubscript{intercept/slope}. \textsuperscript{[b]} R.S.D (%) calculated from triplicates DPV measurements in the LB media for the potential at 30 µM each of PYO, HHQ, and PQS (n = 3).

Matrix interference is a vitally important consideration, and it is essential that detection of the analytes is achievable in clinical samples to which the system may be applied. CF sputum samples contain a significant amount of phospholipids, proteins, and DNA, which increase the viscosity of the sputum samples, in addition to serum transudates and exudates as well as dead leukocytes, bacteria, bacteria metabolites, and cellular debris.\textsuperscript{[49]} However, blank sputum samples from the lungs of pediatric patients were tested, and no oxidation peaks were obtained from the DPV of blank CF sputum samples consistent with the absence of PA. Sputum samples were then spiked with aliquots of 8 h cultures of PAO1, equilibrated for 20 min, and analyzed for the presence of all three analytes \( n = 3 \). In our previous work\textsuperscript{[29b]} the production of the signaling molecules PYO, HHQ, and PQS in growing the cultures and the sputum samples was performed following 11 days incubation. In order to measure the effect of CTAB on standardized samples, spiking the sputum samples with equilibrated amounts of PA provided a fast, standardized and straightforward approach. Notably, neither PQS nor HHQ were detected in the absence of CTAB in both the cultures and spiked sputum samples (Figure 6a). Upon the addition of CTAB, all three signals were identified in both the culture and the spiked sputum sample, emphasizing the effectiveness of CTAB in enhancing presentation of the signal molecules to the electrode, and indicating that this clinically relevant matrix does not interfere with the application of this direct detection procedure (Figure 6b).
**Experimental Section**

**Chemicals and Materials**

Sodium phosphate monobasic, acetic acid, sodium phosphate dibasic, sodium acetate anhydrous, ethanol, acetonitrile (ACN), CTAB, pyocyanin, β-mercaptoethanol, and 2-nitrophenyl β-D-galactopyranoside were all purchased from Sigma-Aldrich (Dublin, Ireland). Phosphate buffer solutions (50 mM, pH 6.0 - 8.0) were used to study the effect of pH on CTAB. An electrolyte of acetate buffer (50 mM, pH 5.0, with 20 % ACN) was utilized for the detection. ACN was used to prepare the stock solution of 2.0 mM PQS, HHQ, and PYO. All reagents were of the analytical grade, and all aqueous solutions were prepared with deionized water obtained from a water purification system (Millipore, Bedford, MA).

**Apparatus**

Electroanalysis was performed using a CHI1040A electrochemical workstation (CH Instrument, Austin, TX) with cyclic voltammetry (CV) and DPV. The electrochemical cell consists of three electrodes using the BDD (0.5 mm, thickness) as a working electrode with a diameter of 3 mm, 0.1 % doped boron (Windsor Scientific, Slough Berkshire, UK). An Ag/AgCl (3 M KCl) (BAS/ Analytical Instruments, West Lafayette, IN) serves as a reference electrode whereas a Pt wire is used as a counter electrode (Sigma-Aldrich, Dublin, Ireland).

**Synthesis of HHQ and PQS**

HHQ and PQS were synthesized as previously described by McGlacken *et al.*[10] HHQ and PQS were deemed analytically pure based on NMR analysis. All spectra were consistent with that previously published.[10] These synthesized compounds, in addition to standard PYO, have been used for control experiments (Figure 2, and Figure S1), calibration curve measurements (Table 3, and Figure S4).

**Electrode Preparation**

The BDD electrode was polished with wet papers (Nylon and MasterTex), followed by alumina slurries (0.3 µm and 0.05 µm, respectively) (Buehler, UK) until a mirror finish was obtained. After washing with deionized water, the electrode was sonicated in ethanol and deionized water for 5 and 10 min, respectively. Subsequently, the electrode was voltammetrically cleaned by CV between -1.0 and +2.0 V vs. Ag/AgCl (3 M KCl) at a scan rate of 100 mV s⁻¹ in a 50 mM acetic acid buffer (pH 5.0) until a steady CV profile was obtained.

**Direct Analysis and Monitoring the Production of PQS, HHQ, and PYO in Bacterial Strain PAO1 Cultures and Spiked Sputum**

In brief, overnight bacterial cultures of PAO1 and PA14 were transferred into a fresh Luria-Bertani (LB) broth (OD₆₀₀nm 0.05) using a modified version of the Fletcher protocol.[22b] The bacterial culture PAO1 was grown for 9 h, and aliquots diluted and equilibrated for 5 min with 1.0 mM CTAB prepared in 50 mM phosphate buffer, pH 7.0 (1.4 v/v, sample: surfactant). For a direct analysis in PA14, the bacterial culture was grown for 7 h and treated with 1.0 mM CTAB under the same conditions. For a time-course study, 0.5 mL bacterial PAO1 culture aliquots were taken at regular time intervals. The growth and promoter activity was measured by the OD₆₀₀nm and Miller Assay, respectively. All bacterial culture samples were treated with 1.0 mM CTAB prepared in 50 mM phosphate buffer, pH 7.0 (1.4 v/v, sample: CTAB) and equilibrated for 5 min. Then, the sample (0.2 mL) was diluted in the electrolyte 50 mM acetate buffer, pH 5.0 containing 20 % ACN (0.8 mL). Patient sputum samples were spiked with bacterial culture PAO1 (1:4, v/v, sputum sample: bacterial culture was grown for 9 h, and treated with 1.0 mM CTAB prepared in 50 mM phosphate buffer, pH 7.0 (1:4, v/v, sample: surfactant).

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Figure 6. DPV of a blank CF sputum sample, bacterial PAO1 strain, and a CF sputum sample mixed with bacterial culture PAO1. a) the bacterial strain, PAO1 and CF sputum samples were diluted in 50 mM phosphate buffer, pH 7.0 at a ratio of 1:4, respectively. b) the bacterial strain PAO1 and CF sputum samples were treated with 1.0 mM CTAB prepared in 50 mM phosphate buffer (pH 7.0) at a ratio of 1:4, respectively. Electrolyte: 50 mM acetate buffer, pH 5.0 consisting of 20 % ACN was used for the detection on the BDD electrode vs. Ag/AgCl.

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**Conclusion**

In brief, the unmodified BDD electrode was successfully utilized for a fast and direct voltammetric analysis of PYO, HHQ, and PQS in the bacterial cultures of strains PAO1 and PA14 using CTAB, without the requirement for liquid-liquid or solid-phase extraction. The method was applied to simultaneous monitoring of PYO, HHQ, and PQS production in PAO1 over 10 h. The time-course of the quorum sensing metabolites followed the established kinetics, with HHQ and PQS shown to be produced maximally prior to entry into the stationary phase of the bacterial growth curve. Additionally, the method revealed the expected kinetics of PYO toxin production, occurring after the initial induction of HHQ and PQS. Finally, the application of the developed method was successfully extended to CF sputum, showing applicability for direct detection of these PA signature signaling molecules in this clinical matrix.
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8 h), equilibrated for 20 min and then treated with CTAB as mentioned earlier.

Promoter Fusion Assays

Promoter fusions assays were performed using the pqsA-lacZ reporter plasmid pLP0996, which is routinely used to investigate promoter activity of the autoinducing pqsA-E PQS biosynthetic operon.\(^{43}\) Briefly, overnight cultures of PA01 and isogenic pqsA mutant strains containing the pqsA-lacZ promoter fusion were diluted to Ab\(_{\text{rdm}}\) 0.05 in 20 mL LB and grown at 37°C with shaking at 150 rpm. The β-galactosidase activity was measured over time as described by Miller.\(^{46}\)

Zeta Potential Measurements

The PAO1 wild-type was grown for 9 h, diluted with 1.0 mL CTAB (1:4, v/v, sample; CTAB), and equilibrated for 5 min. Then, the samples containing CTAB were diluted with deionized water (1:2, v/v) and kept on ice for zeta potential measurements. As a control (without CTAB), the PAO1 wild-type was grown for 9 h, diluted with 50 mM phosphate buffer, pH 7.0 (1:4, v/v, sample: phosphate buffer, pH 7.0) and equilibrated under the same conditions.

Growth and Viability Assays

PA01 pLP0996, PA01 pqsA pLP0996, and PA14 were grown overnight at 37°C with shaking in LB media. Cells were subsequently transferred into fresh LB media, starting OD\(_{600}\) 0.05, and treated with increasing concentrations of CTAB (0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, and 2 mM). After transfer to multi-well plates, growth was monitored static at 37°C (with shaking for 10 sec at 30 min intervals prior to measurement) over 25 h. In addition, CTAB treated cells were transferred into 1.5 mL tubes and incubated at 37°C for 4 days at which point serial dilutions on LB plates were performed to quantify the viable cell count. All experiments were performed using three independent biological replicates.

Data Analysis

All data were processed using Origin Pro 8.5.1 (OriginLab, USA). Each measurement was repeated in triplicate with the results presented as the mean ± SD. For the monitoring study of the bacterial PA-PAO1 strain, such complex DPV data were processed using the second derivative of the peak area.

Compliance with Ethical Standards

Sputum samples were collected from pediatric patients attending the CF clinic at Cork University Hospital, Ireland. Ethical approval was granted by the Clinical Research Ethics Committee (REC) for sputum collection, and samples were handled according to the approved guidelines. Written informed consent from all patients/guardians was obtained for acquisition and analysis outlined in this study.

Acknowledgments

This research was financially supported by SFI/EA Technology Innovation Development Award (TIDA) (SFI/12/TIDA/B2405). FOG acknowledges the grants awarded by the European Commission (FP7-PEOPLE-2013-ITN, 607786; FP7-KBBE-2012-6, CP-TP-312184; FP7-KBBE-2012-6, 311975; OCEAN 2011-2, 287589; Marie Curie 256596; EU-634486), Science Foundation Ireland (SSP-2C, 12/RC/2275; 13/TIDA/B2625; 12/TIDA/B2411; 12/TIDA/B2405; 14/TIDA/2438), the Department of Agriculture and Food (FIRM/RSF/CoFoRD; FIRM 08/RDC/629; FIRM 1/ F009/MabS; FIRM 13/F/516), the Irish Research Council for Science, Engineering and Technology (PD/2011/2414; GOI/PG/2014/647), the Health Research Board/Irish Thoracic Society (MRCG-2014-6), the Marine Institute (Beaufort award G2CRA 2007/082 and Teagasc (Walsh Fellowship 2013). JDG thanks the Science Foundation Ireland (08/1/RG/1412) for research funding of the Irish Separation Science Cluster (ISSC) under the Strategic Research Cluster Programme. GPM acknowledges support from the Science Foundation Ireland (SFI/12/IP/1315, SFI/09/RFP/CHS2353, and SSPC2 12/RC/2275), the Irish Research Council (GOIP/2013/336) and UCC for a Strategic Research Fund Ph.D. Studentship.

Keywords: cystic fibrosis • electrochemical detection • membrane disruption • Pseudomonas aeruginosa • surfactant

8. a) T. A. Webster, E. D. Goluch, Lab Chip 2012, 12, 5195-5201.
Membrane disruption of bacteria: The differential pulse voltammetry (DPV) is used to detect the Pseudomonas aeruginosa signaling molecules after membrane disruption by the cationic surfactant. The method is applied to detect 2-heptyl-3-hydroxy-4-quinolone (PQS), 2-heptyl-4-hydroxyquinoline (HHQ), and pyocyanin (PYO) in clinical sputum sample of cystic fibrosis (CF) spiked with bacterial culture PAO1 (see picture).