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Electrochemical Detection of Quorum Sensing Signaling Molecules by Dual Signal Confirmation at Microelectrode Arrays

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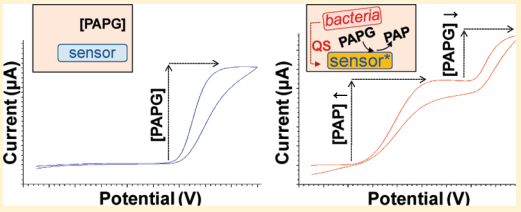
Supporting Information

ABSTRACT: N-Acyl homoserine lactones (AHLs) are produced by Gram-negative bacteria to regulate gene expression in a cell density dependent manner. For instance, expression of virulence factors by pathogens such as *Pseudomonas aeruginosa* is induced only when a threshold concentration of AHLs is reached, which indicates that the bacterial population is big enough to promote infection. In this study, the indicator strain *Agrobacterium tumefaciens* NTL4 (pZLR4), which carries a β-galactosidase (β-gal) reporter gene under the control of a quorum sensing promoter, was used to develop an electrochemical biosensor to detect AHLs using the model N-(3-oxo)-dodecanoyl-L-homoserine lactone (oxo-C12-HSL), an AHL previously detected in cystic fibrosis patients infected with *P. aeruginosa*. The substrate 4-aminophenyl β-D-galactopyranoside was used to detect β-gal activity by cyclic voltammetry. Furthermore, simultaneous monitoring of substrate consumption and *p*-aminophenol production by β-gal allowed on-chip result verification by dual-signal confirmation. The sensor exhibited high reproducibility and accurately detected oxo-C12-HSL in a low picomolar to low nanomolar range in spiked liquid cultures and artificial saliva, as well as AHLs naturally released by *P. aeruginosa* in culture supernatants. Moreover, detection took just 2 h, required no sample pretreatment or preconcentration steps, and was easier and faster than traditional methods.



Communication between bacteria in a cell-density-dependent manner, also known as quorum sensing (QS),[1] has attracted the interest of researchers in many fields during the past decade. QS allows bacteria to regulate the expression of certain genes, including the expression of virulence factors in pathogens such as *E. coli* O157:H7, *Vibrio cholerae*, *Erwinia*, *Pseudomonas aeruginosa*, or some *Staphylococcus*.[2−7] Although different QS circuits exist, the signaling molecules, also known as autoinducers, can be broadly grouped into two main categories: short peptides for Gram-positive bacteria and acyl homoserine lactones (AHLs) for Gram-negative bacteria. AHLs, which have been the most studied so far, have a conserved structure composed of a homoserine lactone ring and a lateral acyl chain that vary in length, saturation, and oxidation degree.[8] QS has been proposed as a potential target for both the diagnosis and treatment of infectious diseases.[9] Detection of QS autoinducers has been accomplished by high-performance liquid chromatography (HPLC) coupled with mass spectrometry[10] and the use of genetically engineered bacterial reporter strains in combination with thin layer chromatography.[11] However, these are mostly time-consuming and tedious approaches. Attempts to simplify detection have been described by coupling the use of reporter strains to colorimetric, bioluminescent, and fluorescent detection strategies.[12] These methods were often compatible with the study of culture supernatants and diluted physiological samples, such as saliva, with reported detection limits of 0.5−100 μM for AHLs, in assay times between 2 and 24 h.[13−16]

The objective of this work was to develop an electrochemical biosensor for the rapid, simple, and sensitive detection of AHLs. We used the model N-(3-oxo)-dodecanoyl-L-homoserine lactone (oxo-C12-HSL), an AHL produced by the opportunistic pathogen *P. aeruginosa*, and the genetically engineered reporter strain *Agrobacterium tumefaciens* NTL4,[17] which contains a *traG*::*lacZ* fusion gene that is expressed under the control of a promoter that responds to AHLs. Up to date, only one report exists for the electrochemical monitoring of QS.[18] That work was based on the straightforward detection of pyocyanin, which is redox-active on its own. Detection by square wave voltammetry using a carbon fiber electrode was efficient in both spiked samples and *P. aeruginosa* cultures in a concentration range between 1 and 100 μM. Nevertheless, only electroactive molecules are detectable by that strategy.

In this work, AHLs were indirectly detected by measuring β-galactosidase (β-gal) activity (AHL-induced) using 4-aminophenyl β-D-galactopyranoside (PAPG) as the substrate, which is hydrolyzed by the enzyme into *p*-aminophenol (PAP; Figure 1a). Cyclic voltammetry at gold microelectrode arrays provided detection limits down to 2 pM, in minute sample volumes (20 μL), after a 2 h assay and a few seconds of measurement. Furthermore, simultaneous monitoring of PAPG consumption and PAP production made possible on-chip result corroboration via dual-signal

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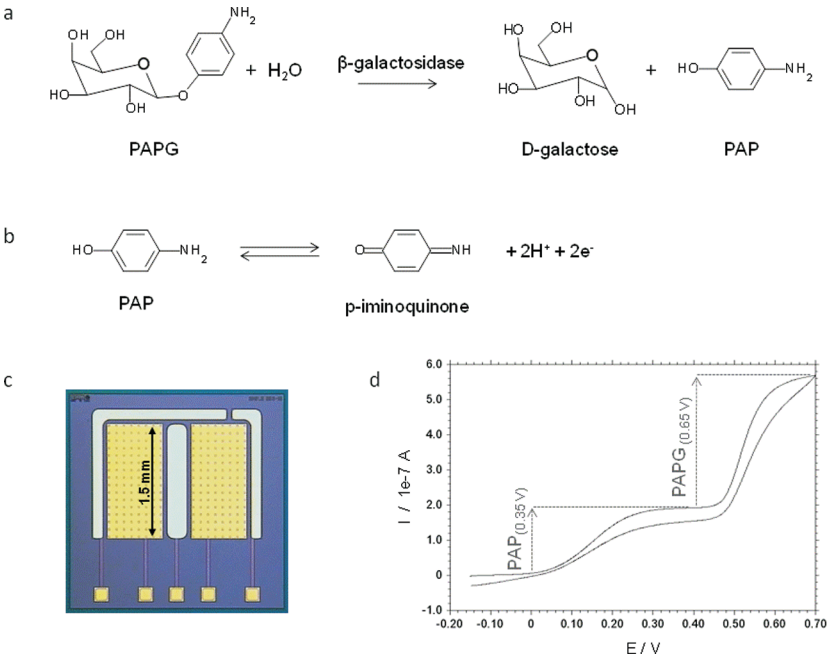

Figure 1. (a) Irreversible hydrolysis of PAPG into PAP catalyzed by β-gal. (b) Reversible electro-oxidation of PAP at an electrode surface. (c) Amplified picture of one of the devices used in this work. Each chip contains 258 gold microdisks arranged in a square lattice over two 1.5 × 0.7 mm neighbor surfaces (128 microdisks each), as well as platinum pseudoreference and auxiliary electrodes. (d) The graph indicates how current is measured in a scan in which an oxidation wave at 0.35 V indicates production of PAP, and the oxidation at 0.65 V implies remaining native PAPG.

confirmation. The performance of the biosensor was evaluated in both spiked liquid cultures and artificial saliva, as well as in *P. aeruginosa* culture supernatants. Our results demonstrate that the method reported here could be used as a rapid and highly sensitive alternative to traditional methods for AHL detection.

EXPERIMENTAL SECTION

Chemical Reagents and Biocomponents. Potassium chloride (KCl), potassium ferricyanide ($K_3[Fe(CN)_6]$), N-(3-oxo)-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL), 4-aminophenol (PAP), and 4-aminophenyl β-D-galactopyranoside (PAPG) were from Sigma-Aldrich. Sterile 0.22 μm filters were from Millipore. 3-oxo-C12-HSL was reconstituted in ethyl acetate (acidified with formic acid 0.5% v/v) to 20 mg mL^{-1}, aliquoted, and stored at −20 °C. Immediately before use, a stock aliquot was kept temperate, and serially diluted in ethyl acetate to obtain 1000× working concentrations.

Bacterial Strains and Culture Conditions. *A. tumefaciens* NTL4[17] was cultured in AB minimal medium (pH 7.0) supplemented with 0.5% mannitol[19] at 30 °C with continuous agitation. Gentamicin (30 μg mL^{-1}) was used to maintain the strain but not in the AHL detection bioassay. *P. aeruginosa* PA01 was grown in AB minimal medium supplemented with 0.5% glucose at 37 °C with continuous agitation.

Calibration of the AHL Bioassay. *A. tumefaciens* was grown to an OD of 0.4, and 2 mL aliquots were spiked with PAPG (0.25−0.5 mg mL^{-1}) and different concentrations of oxo-C12-HSL at a final 1:1000 volume ratio. The mixture was then agitated at 30 °C, followed by electrochemical monitoring.

Detection of AHLs in Artificial Saliva. Artificial saliva consisted of 0.6 g L^{-1} Na_2HPO_4, 0.4 g L^{-1} KCl, 0.4 g L^{-1} NaCl, 4 g L^{-1} mucin from maxillary glands, and 4 g L^{-1} urea dissolved in deionized water, adjusted to pH 7.2, sterilized by autoclaving and stored at 4 °C until used.[20] Artificial saliva was spiked with decreasing concentrations of oxo-C12-HSL, and 200 μL of each sample were inoculated into a 2 mL culture of *A. tumefaciens*, which was processed as stated above.

Detection of AHLs in *P. aeruginosa* Supernatant. *P. aeruginosa* was grown overnight in AB medium supplemented with 0.5% glucose. The culture was then centrifuged and filtered through 0.22 μm sterile filters to remove whole cells and cell debris. Aliquots of 2, 20, and 200 μL of the supernatant were tested.

Alternatively, the *P. aeruginosa* culture was centrifuged; the supernatant was discarded, and the bacteria were resuspended and serially diluted in fresh culture medium in order to obtain bacterial suspensions at final titers ranging from 5 × 10^0 to 5 × 10^7 CFU mL^{-1}. These suspensions were enriched for 5 h at 37 °C and agitated to allow production of AHLs. The suspensions were next filtered to remove cells and cell debris, and 200 μL aliquots of the supernatants were assayed.

Bacterial cells in the culture were enumerated by preparing a 10-fold dilution series in phosphate buffer saline, followed by plating 100 μL of the different dilutions onto tryptic soy agar. The plates were incubated overnight for 24 h. Plates with colonies in the range of 30−300 were counted, and the initial bacterial cell number was estimated.

Electrode Fabrication and Characterization. Gold microelectrode arrays were produced using standard photolithographic techniques. Briefly, a 1 mm thick thermal oxide layer was grown over silicon wafers. Wafers were metallized using titanium, nickel, and gold, 20, 20, and 150 nm thick, respectively, and patterned photolithographically in a wet etching step, followed by silicon oxide and silicon nitride deposition to provide electrical insulation of the devices. The final geometry of the arrays was defined by photolithography in a dry step using reactive ion etching. Finally, the wafers were diced, wire bonded, and encapsulated onto printed circuit boards. Each device contains 258 gold

microdisks of 10 μm radius each, separated 100 μm from their nearest neighbors and arranged in a square lattice over two 1.5 × 0.7 mm adjacent surfaces. Each chip encloses also platinum pseudoreference and auxiliary electrodes (Figure 1).

Before their utilization, the chips were washed with ethanol, isopropanol, and water, dried under a nitrogen flow, and electrochemically activated using an Autolab PG12 potentiostat (Eco-Chemie, The Netherlands). This consisted in series of potential pulses (10 s each) from 0 to −2 V in 0.1 mM KCl, using a glassy carbon rod and an Ag/AgCl (3 M KCl) as auxiliary and reference electrodes. The electrodes were then characterized by cyclic voltammetry in ferricyanide.

Electrochemical Detection of β-gal Activity. β-gal activity was detected by cyclic voltammetry (CV). CV registered between −0.15 and +0.45 V vs the chip Pt pseudoreference allowed electrochemical measurement of the enzyme product PAP. Alternatively, CV between −0.15 and +0.75 V vs Pt made possible simultaneous monitoring of both enzyme substrate and product (PAPG and PAP, respectively). All CV measurements were done at a scan rate of 50 mV sec^{-1} using a CHI750C multipotentiostat (CH Instruments, Inc., Austin, TX, USA).

Statistical Analysis. Data shown are the average from at least three independent measurements (using different chips each). The lower limit of detection (LOD) was calculated as the signal registered for the blanks plus three times their standard deviation. The assay variability was calculated for each enzyme concentration in terms of coefficient of variation as follows: [%CV = (SD/mean) × 100], where "mean" is the average of at least three values independently obtained and "SD" is the standard deviation of these values.

RESULTS AND DISCUSSION

Electrochemical Detection of 3-oxo-C12-HSL-Induced β-gal Activity in the Sensor Strain. Optimization of Assay Conditions. The reported biosensor uses a genetically engineered *Agrobacterium tumefaciens* reporter strain (NTL4)[17] that, in the presence of AHLs, produces the enzyme β-gal. Electrochemical detection is based on the fact that β-gal hydrolyzes PAPG, a synthetic analogue of the enzyme natural substrate lactose, into PAP (Figure 1a), which is electroactive and can be detected electrochemically.[21] Interestingly, PAPG is also electroactive, an attribute that, to the best of our knowledge, has not been exploited for biosensing purposes before. In view of this, we studied if PAP and PAPG could generate distinguishable oxidation and reduction waves. This would allow the simultaneous monitoring of PAPG consumption and PAP production by β-gal, presumably being more informative than detection of only PAP production.

Electrochemical Detection of PAP and PAPG. Electrochemical detection was done using gold microelectrode arrays with integrated pseudoreference and auxiliary Pt electrodes (Figure 1c). By definition, microelectrode arrays consist of a high number of single microelectrodes which are wired in parallel. Interestingly, when measurement is extended for short times (in our case, measurement takes just few seconds), each individual microelectrode acts as a diffusionally independent unit. For this reason, a microelectrode array shows mass transfer rates significantly higher than those expected for a single electrode with surface area equivalent to the sum of the areas of all the microdisks in the array. Accordingly, the signals generated by a microelectrode array are also larger than those produced by a single electrode of equivalent area.[22,23] In addition, using microelectrodes allows operation in extremely small volumes, which generates minimal waste and disposal of reagents.[24] These features have been reported to provide reduced detection limits and assay times during biosensor development.[24]

The electrochemistry of PAP entails a reversible two-electron transfer reaction (Figure 1b). Interestingly, PAP is oxidized at mildly positive potentials and causes nearly undetectable levels of electrode fouling, which allows electrode reutilization.[25,26] Under the current experimental conditions, the CVs obtained for 20 μL of PBS spiked with different concentrations of PAP showed an oxidation wave around 0.35 V vs Pt, which height was proportional to PAP concentration (Figure S-1, Supporting Information). These microelectrodes exhibited an LOD of 10 pM for PAP, which is significantly lower than the LOD reported by Laczka for the chronoamperometric detection of PAP using also microelectrode arrays, but external reference and auxiliary macroelectrodes in 5−10 mL of solution (4 μM).[25] The assay variability varied between 0.9 and 5.5% CV for all the concentrations of PAP. PAPG, on the other hand, oxidizes at higher potentials than PAP (0.65 V vs Pt; Figure 1d) and generates no detectable peaks at 0.35 V vs Pt.

Electrochemical Detection of 3-oxo-C12-HSL Using the Sensor Strain. Next, we confirmed if the measurement setup described allowed detection of the β-gal produced by the sensor strain in the presence of synthetic 3-oxo-C12-HSL. The effect of increasing concentrations of enzyme substrate in detectability was also studied.

A. tumefaciens was grown to an OD of 0.4 and was next inoculated with 0.05−0.5 mg mL^{-1} of PAPG plus 5 μM 3-oxo-C12-HSL. Negative controls inoculated with *A. tumefaciens* and PAPG but not 3-oxo-C12-HSL, as well as negative controls inoculated with PAPG and 3-oxo-C12-HSL but no *A. tumefaciens*, were performed in parallel. After shaking for 1, 2, or 5 h at 30 °C, aliquots of the different tubes were recovered and studied by CV either directly or after filtration to remove cells and cell debris.

CVs recorded between 0 and +0.45 V vs Pt allowed monitoring of PAP production, which translated into increase in current at 0.35 V proportional to β-gal activity. On the other hand, CVs registered between 0 and +0.75 V vs Pt allowed the simultaneous study of PAP production and PAPG consumption by the enzyme (Figure S-2, Supporting Information). No differences in electrode response were detected between filtered and not-filtered samples and, within the short measurement times (few seconds), no evidence of bacteria or medium adsorption onto the electrodes were observed. Hence, sample filtration was not necessary for optimal electrode performance.

After 1 h of incubation, PAP production was undetectable for all the concentrations of PAPG tested. On the contrary, after 2 and 5 h of incubation, production of PAP was detectable in all the samples of *A. tumefaciens* inoculated with 3-oxo-C12-HSL, but in none of the negative controls (Figure S-3, Supporting Information). Simultaneously, the current registered at 0.65 V vs Pt for these samples decreased by 21−100% compared to the negative controls, which was consistent with PAPG consumption by β-gal (Figure S-3b, Supporting Information). Hence, simultaneous monitoring of PAP production and PAPG consumption was feasible. Furthermore, in the presence of β-gal, this generated two synchronized signals of opposite trend (i.e., increase in current at 0.35 V concomitant to decrease in signal at 0.65 V vs Pt), which resulted in on-chip dual-signal confirmation. On top

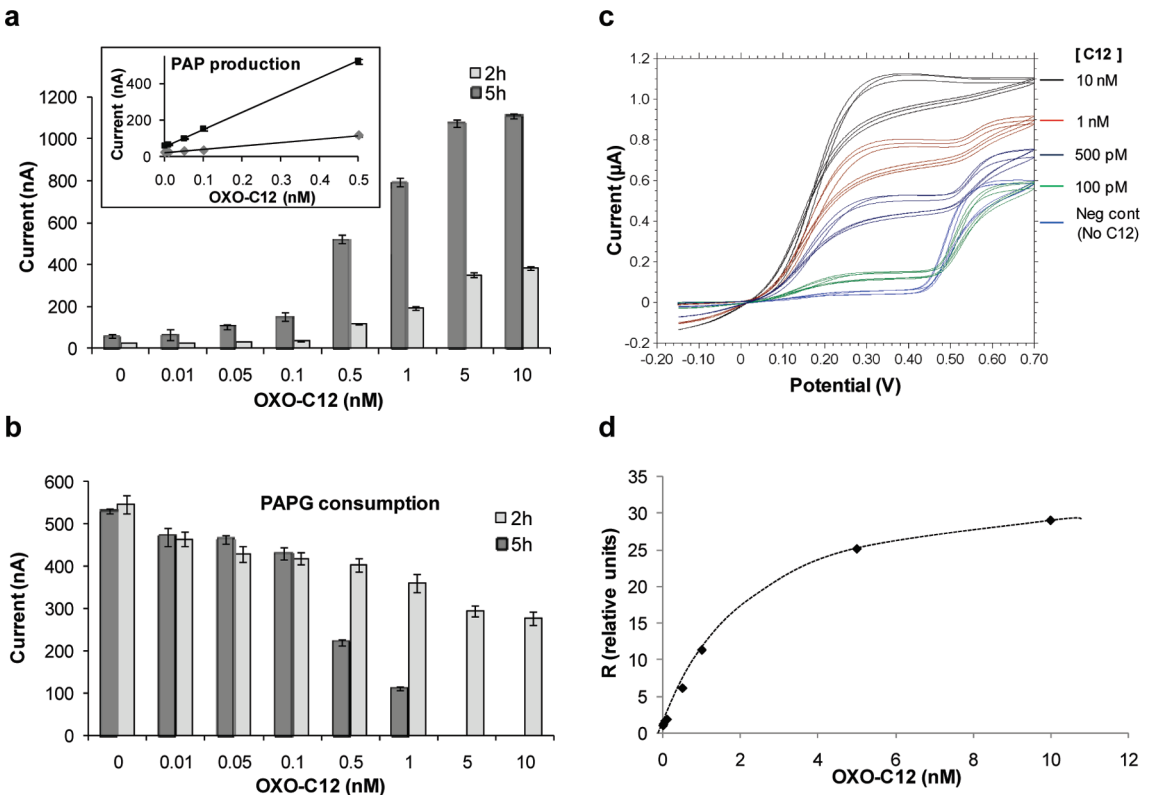

Figure 2. Detection of 3-oxo-C12-HSL by simultaneous monitoring of PAP production (a) and PAPG consumption (b) after incubation of the sensor strain with 0.25 mg mL^{-1} PAPG for 2 (light bars) and 5 h (dark bars). (c) Example of the CVs obtained (from −0.15 to +0.7 V, scan rate 50 mV sec^{-1} vs on-chip Pt pseudoreference) after incubation for 5 h in the presence of 0.5 mg mL^{-1} PAPG and increasing concentrations of 3-oxo-C12-HSL. Three independent replicas (using different microelectrodes) are shown. As 3-oxo-C12-HSL increases, current registered at 0.35 V, corresponding to 3-oxo-C12-HSL-induced PAP production, rises. Simultaneously, the difference in current measured between 0.35 and 0.65 V vs Pt, due to unconsumed PAPG, decreases. (d) Plot of the dual-signal compensation ratio (R) calculated from data in (a) and (b) versus concentration of 3-oxo-C12-HSL.

of this, dual-signal monitoring made possible signal normalization between days or experiments (for example, to compensate slight differences in PAPG concentration or sensor strain titer).

Data Analysis by Dual-Signal Compensation. As described above, in the presence of 3-oxo-C12-HSL, the sensor strain produces β-gal. This translates into an increase in the signal attributed to PAP production and concomitant decrease of the signal due to PAPG. Data normalization was next undertaken in an attempt to simplify data analysis but still integrate the two types of signals obtained in a single parameter. The best results were achieved by calculating the following ratio:

$$R = (S_{iPAP}/S_{iPAPG})/(B_{iPAP}/B_{iPAPG})$$

where R is the dual-signal ratio, S_{iPAP} and S_{iPAPG} are the currents registered at 0.35 and 0.65 V vs Pt (Figure 1d) for a sample S, and B_{iPAP} and B_{iPAPG} are the currents registered at 0.35 and 0.65 V vs Pt for the blanks, B, where B is the average of at least 3 independent blanks. In this context, R should be close to 1.00 for the blanks and for samples not induced with 3-oxo-C12-HSL, in which lack of β-gal activity results in neither PAPG consumption nor PAP production. R tends to infinite and cannot be calculated in samples in which PAPG has been completely consumed ($S_{iPAPG} = 0$). Finally, R is between 1.00 and infinite for samples in which β-gal production has been induced due to the presence of 3-oxo-C12-HSL. Accordingly, it was established that a sample was positive for 3-oxo-C12-HSL presence when it showed simultaneous production of PAP and consumption of PAPG and thus fitted with the three following statements: (i) $(S_{iPAP}/\mathrm{LOD}_{iPAP}) > 1$, which means that PAP production is above LOD_{iPAP} where $\mathrm{LOD}_{iPAP} = (B_{iPAP} + 3\mathrm{SD})$ and corresponds to the averaged current registered at 0.35 V vs Pt for at least 3 independent blanks, plus 3 times their standard deviation. (ii) $(S_{iPAPG}/\mathrm{LOD}_{iPAPG}) < 1$, which means that PAPG consume is below LOD_{iPAP} where $\mathrm{LOD}_{iPAP} = (B_{iPAPG} - 3\mathrm{SD})$ and corresponds to the averaged current registered at 0.65 V vs Pt for at least 3 independent blanks, after subtracting 3 times their standard deviation. (iii) $R_S > 1.12$, which implies that R_S is above LOD_R calculated as the averaged R of the blanks plus three times their standard deviation.

In addition, samples that fitted two of the previous statements exhibited increased PAP/PAPG ratios but could not be considered "positives" according to the previously described premises. Nevertheless, all these samples would be statistically positive if only PAP production or only PAPG consumption (depending on the concentration of PAPG used, which determines which of the two reactions is more sensitive, as it will be discussed later in the text) were monitored. Accordingly, they were labeled as suspected or faint positives that should be reconfirmed.

Detection of 3-oxo-C12-HSL in Spiked Culture Medium Samples. In the experiments that followed, the *A. tumefaciens* sensor strain was spiked in parallel with decreasing concentrations of 3-oxo-C12-HSL in order to determine the assay LOD. Figure 2a,b and Table S-1 (Supporting Information) summarize the currents registered for different concentrations of 3-oxo-C12-HSL

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in the presence of 0.25 mg mL^{-1} PAPG, and Figure 2c shows the scans registered after 5 h of incubation. As anticipated, PAP production and PAPG consumption were directly proportional to the amount of 3-oxo-C12-HSL present in the medium. When *R* was calculated as described above, it was equal to 1 ± 0.04 in all the blanks and it was >1.12 for the 3-oxo-C12-HSL-induced samples. Moreover, *R* was proportional to the amount of 3-oxo-C12-HSL initially inoculated over the whole concentration range studied (Figure 2d).

In general, longer incubations produced improved detectability. For example, LODs decreased from 25 to 3.6 pM 3-oxo-C12-HSL for incubations of 2 and 5 h, respectively, when monitoring PAP production and from 22.5 to 9.8 pM 3-oxo-C12-HSL when monitoring PAPG consumption. However, extended assay times also evidenced complete substrate consumption and signal saturation for the highest concentrations of 3-oxo-C12-HSL tested. The assay variability (% CV) was below 5% for most of the concentrations assayed. If the assay LOD was alternatively calculated from the plot of *R* (*R*-LOD), values of 22 and 3 pM, equivalent to 0.44 and 0.06 nmol of 3-oxo-C12-HSL in the 20 μL of sample assayed, were obtained for 2 and 5 h incubations, respectively. These numbers were in good agreement with the LODs obtained separately for PAP production and PAPG consumption.

Using higher concentrations of PAPG (0.5 mg mL^{-1}) resulted in enhanced signals only in the 5 h assay and just a slight improvement in the LOD calculated for PAP production (2 pM for both 2 and 5 h incubations). On the other hand, the differences in current at 0.65 V due to PAPG consumption were less informative, with LODs above 300 nM in all cases. Hence, using higher PAPG concentration provided shorter assay times and slightly better LODs calculated from PAP production but at a cost of lower result reliability because the dual signal compensation ratio was not applicable under these experimental conditions.

Independently of this, the LODs obtained in this work are lower than those previously reported. For instance, LODs ranging between 0.28 and 9.3 pmol have been reported for the lactone ring present in the AHL molecule by HPLC coupled with mass spectrometry.[10]

Detection of 3-oxo-C12-HSL in Spiked Artificial Saliva. The performance of the biosensor was next evaluated in a more complex sample matrix. With this purpose, artificial saliva samples were spiked with decreasing concentrations of oxo-C12-HSL. A 200 μL aliquot of each sample was then inoculated into 2 mL of *A. tumefaciens* supplemented with 0.5 mg mL^{-1} of PAPG. The data recorded after 2 and 5 h incubations are summarized in Figure 3 and detailed in Table S-2, Supporting Information. In the case of detection of PAP production, the LOD increased slightly (8 pM) compared to the previous calibration experiment at an incubation time of 2 h and did not ameliorate after extended incubation. In the case of PAPG consumption, higher LODs where achieved: 0.7 and 0.1 nM after 2 and 5 h incubations, respectively. This could be attributed to the increased viscosity of saliva samples, which presumably affected negatively molecule diffusion and/or enzyme performance. The nonspecific adsorption of mucins, present in the artificial saliva at relatively high concentration, onto the gold electrodes could also negatively affect the measurement to a certain extent.[27]

The *R*-LODs for detection in spiked artificial saliva were of 14 and 10 pM 3-oxo-C12-HSL after 2 and 5 h incubations, respectively. As before, these numbers were of the same order of magnitude than the LODs calculated from the values of PAP production. In addition, these LODs fit the AHL concentration range reported by some clinical studies. For instance, Erikson et al. detected 3-oxo-C12-HSL between 1 and 22 nM in the sputum of 78% of cystic fibrosis patients.[28] Furthermore, the authors estimated that 20−40% of the molecules had been lost along the complex extraction procedure, so the real concentrations in the samples could be higher.

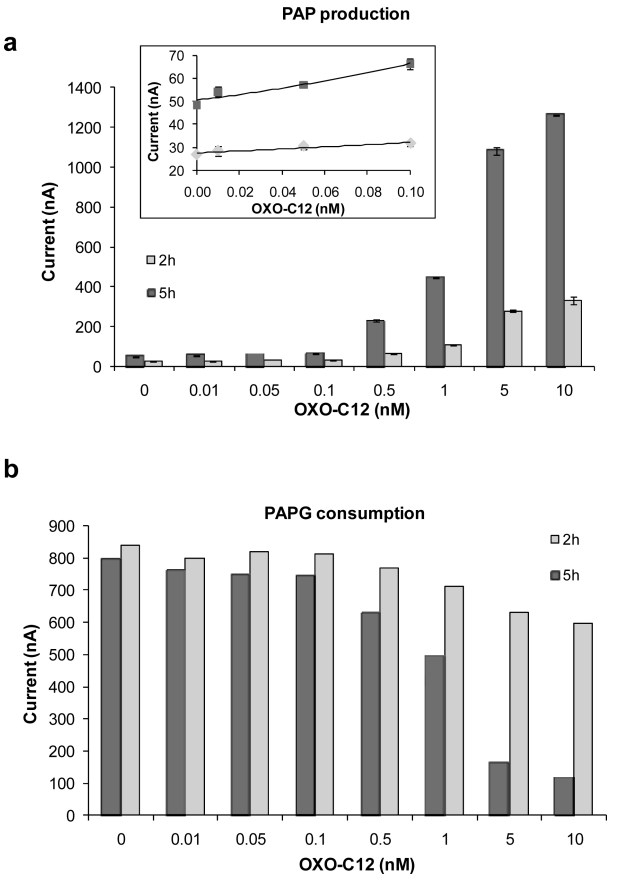

Figure 3. Detection of oxo-C12-HSL in spiked artificial saliva. Artificial saliva samples were spiked with decreasing concentrations of oxo-C12-HSL, and 200 μL of each concentration were inoculated in 2 mL of the sensor strain. The mixtures were then incubated at 30 °C for 2 and 5 h in the presence of PAPG. CV were obtained from −0.15 to +0.7 V, at scan rate of 50 mV s^{-1} vs on-chip Pt pseudoreference, after depositing 20 μL drops of each sample on top of the electrodes. Averages of at least three independent replicas (using different microelectrodes) are shown. (a) As 3-oxo-C12-HSL increases, current registered at 0.35 V, corresponding to 3-oxo-C12-HSL-induced PAP production, rises. The inset shows the amplification of the signals registered in the lowest concentration range studied. (b) Simultaneously, the current increase measured between 0.35 and 0.65 V vs Pt, due to unconsumed PAPG, decreases.

Electrochemical Detection of AHLs Produced by *Pseudomonas*. The developed biosensor was finally applied to detection of the QS response in bacterial cultures. The aim was to confirm if the method developed could detect the autoinducer within the concentration range produced by a bacterial population and if other components in such a complex medium (either present in the culture media or produced by the microorganisms) interfered with detection.

Quantization of 3-oxo-C12-HSL in Pseudomonas Overnight Cultures. We started by studying overnight cultures. After growing overnight, a bacteria culture is in the stationary growth

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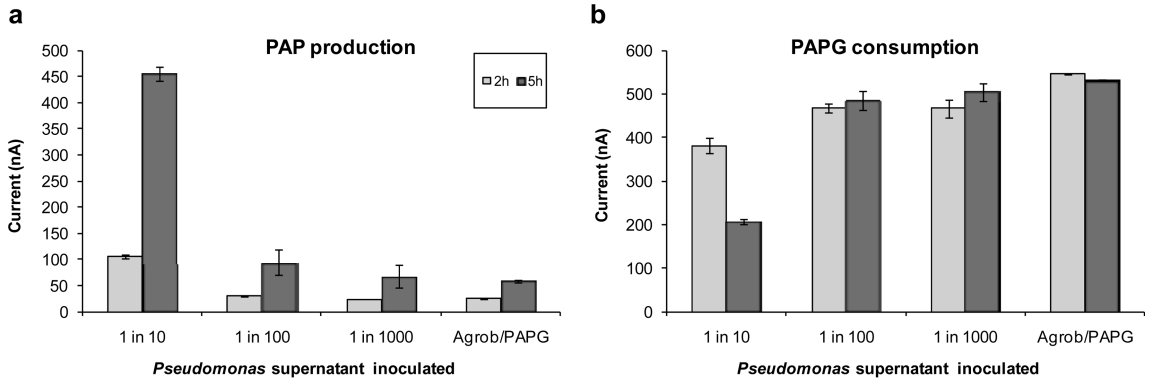

c

2h

Dil 1 in	S_{iPAP} Average	S_{iPAP} Desvest	S_{iPAP} %CV	S_{iPAPG} Average	S_{iPAPG} Desvest	S_{iPAPG} %CV	R N	R N>1.12	S_{iPAP} / LOD_{iPAP} N	S_{iPAP} / LOD_{iPAP} N > 1	S_{iPAPG} / LOD_{iPAP} N	S_{iPAPG} / LOD_{iPAP} N < 1	C12 (pM)
10	106.3	3.3	3.1	381.7	17.3	4.5	5.88	•	3.81	•	0.79	•	++ (452)
100	31.1	1.8	5.8	467.4	9.5	2.0	1.40	•	1.11	•	0.97	•	++ (41)
1000	24.3	0.2	0.9	467.0	20.4	4.4	1.10		0.87		0.97	•	- -

5h

Dil 1 in	S_{iPAP} Average	S_{iPAP} Desvest	S_{iPAP} %CV	S_{PAPG} Average	S_{PAPG} Desvest	S_{PAPG} %CV	R N	R N>1.12	S_{iPAP} / LOD_{iPAP} N	S_{iPAP} / LOD_{iPAP} N > 1	S_{iPAPG} / LOD_{iPAP} N	S_{iPAPG} / LOD_{iPAP} N < 1	C12 (pM)
10	455.7	9.6	2.1	205.6	5.9	2.9	20.06	•	7.53	•	0.40	•	++ (478)
100	93.7	1.9	2.0	485.3	21.5	4.4	1.75	•	1.55	•	0.95	•	++ (38)
1000	67.9	1.1	1.6	504.3	20.4	4.0	1.22	•	1.12	•	0.98	•	++ (4)

Figure 4. Detection of 3-oxo-C12-HSL produced by *Pseudomonas* in overnight culture supernatants. Following filtration, 2, 20, or 200 μL of culture supernatant were inoculated in 2 mL of sensor strain containing PAPG 0.25 mg mL^{-1}. Electrochemical monitoring of PAP production (a) and PAPG consumption (b) were performed after 2 (light bars) and 5 h (dark bars) incubations. (c) Example of the dual signal confirmation analysis, which shows the averaged values obtained from 3 independent replicates for the different statistical parameters as described in the text. *R*: dual-signal ratio; S_{iPAP} and S_{iPAPG}: currents registered for samples at 0.35 V and 0.65 vs Pt; LOD_{iPAP}: averaged current registered at 0.35 V vs Pt for 3 independent blanks, plus 3 times their standard deviation; LOD_{iPAP}: averaged current registered at 0.65 V vs Pt for 3 independent blanks, after subtracting 3 times their standard deviation; (++) indicates that a sample fits the three parameters defined for dual-signal compensation and is positive for 3-oxo-C12-HSL; (+-) indicates that a sample fits two of these three parameters, is thus suspected to contain 3-oxo-C12-HSL and should be additionally studied; and (--) indicates that a sample is negative for 3-oxo-C12-HSL.

phase, and the maximal bacterial population that the culture medium can sustain has been reached. Because 3-oxo-C12-HSL is produced in a cell density dependent manner, for those specific growing conditions, the autoinducer's concentration should be at its maximum in an overnight culture.

Pseudomonas was grown overnight, centrifuged, and filtered in order to remove cells and cell debris. The supernatants were then inoculated in the sensor strain at final dilution factors of 1:10, 1:100, and 1:1000, together with a negative control which consisted of culture medium with no bacteria. These solutions were incubated for 2 and 5 h in the presence of 0.25 mg mL^{-1} PAPG.

Table S-3 (Supporting Information) summarizes the signals registered for each sample, as well as the three parameters used to determine if the sample presented 3-oxo-C12-HSL. Three replicates of each sample were measured in parallel using different chips, which averaged values are illustrated in Figure 4.

3-oxo-C12-HSL produced by *Pseudomonas* in the overnight culture was detected in both the 1:10 and 1:100 inoculations. Even if longer sensor incubations generated higher signals and slightly lower LODs, better signal coincidence between 1:10 and 1:100 inoculations were observed after 2 h. The concentration of 3-oxo-C12-HSL in the culture was next estimated by interpolation of the oxidation currents registered for each sample in a calibration plot. After correction for the dilution factor, the signals registered for the 1:10 samples were equivalent to 3-oxo-C12-HSL around 4.5 nM (2 h) and 4.8 nM (5 h) in the ON. If the 1:100 samples were considered instead, concentrations were between 3.7−4.6 nM (2 h) and 3.6−4.1 nM (5 h). Therefore, the results obtained for the different dilutions were consistent. These values are significantly lower than the numbers reported by Pearson (1 μM after ethyl acetate extraction of not filtered *P. aeruginosa* cultures, including any autoinducer molecules present inside the cells)[29] but of the same order of magnitude than those obtained by Charlton in *Pseudomonas* biofilm effluents (14 ± 3 nM).[30]

Only one of the replicates corresponding to the 1:1000 inoculations generated a faint positive after 2 h. After 5 h of incubation, 1 of the 3 replicates was positive for 3-oxo-C12-HSL and the other 2 could be considered faint positives. Hence, while long incubations were more useful for qualitative detection of extremely low concentrations of 3-oxo-C12-HSL, short assay times provided more accurate quantization.

Detection of 3-oxo-C12-HSL Production by Different Titers of P. aeruginosa. Finally, the sensor was used to study production of 3-oxo-C12-HSL by *Pseudomonas* at different titers. With this aim, *Pseudomonas* was grown to 5×10^7 CFU mL^{-1}. The culture was centrifuged; the supernatant was discarded, and the bacteria were serially diluted in fresh culture medium. The tubes, containing 5×10^0 to 5×10^7 CFU mL^{-1}, plus negative controls with no bacteria, were then agitated at 37 °C for 5 h. These pre-enriched cultures were next filtered, and 200 μL of the supernatants were

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inoculated into 2 mL of the sensor strain with 0.5 mg mL^{-1} PAPG. Following 2 and 5 h incubations, electrochemical measurements were carried out as previously described (Table S-4, Supporting Information).

As anticipated, the measurements obtained on *Pseudomonas* 5×10^7 CFU mL^{-1} immediately after reconstitution in fresh medium, thus depleted of any 3-oxo-C12-HSL previously released by the cells, induced no β-gal activity in the sensor. After 5 h of pre-enrichment, only *Pseudomonas* reaching the stationary phase (5×10^7 CFU mL^{-1}) had generated enough 3-oxo-C12-HSL as to induce consistent responses of the sensor, equivalent to 5−5.5 nM. The following concentration of *Pseudomonas*, 5×10^6 CFU mL^{-1}, generated faint signals after 2 h of assay and were positive for 3-oxo-C12-HSL after 5 h of incubation, with concentrations of 3-oxo-C12-HSL between 0.04 and 0.23 nM. Hence, a difference in bacteria population of just an order of magnitude results in 3-oxo-C12-HSL production 100 times lower. A concentration of 5×10^5 CFU mL^{-1} generated faint positives after both 2 and 5 h incubations. The rest of the samples were negative. Remarkably, the titers reported for *P. aeruginosa* in the sputum of cystic fibrosis patients range between 10^7 and 10^8 CFU mL^{-1} in most cases.[28] Therefore, the LOD obtained in this work would be good enough to detect the presence of AHL in these patients.

CONCLUSIONS

We have described the first electrochemical biosensor reported to date for the fast, simple, and sensitive detection of QS autoinducers. The assay is based on the use of an indicator strain, *Agrobacterium tumefaciens* NTL4 (pZLR4), which produces β-gal in response to the presence of QS autoinducers, coupled to detection of β-gal activity by CV using microelectrode arrays. Using PAPG as the substrate for β-gal allowed simultaneous monitoring of PAPG consumption and PAP production by the enzyme and made possible on-chip dual-signal confirmation. The biosensor provided efficient quantization of oxo-C12-HSL in a low pM to low nM concentration range, with LODs down to 2 and 14 pM in liquid cultures and in artificial saliva, % CV below 8% for all the concentrations and conditions tested, in a 2 h assay time, and in the absence of sample pretreatment or preconcentration steps. The bioassay efficiency has been additionally proved by detecting oxo-C12-HSL released by *P. aeruginosa* in culture supernatants, which allowed easier and faster detection compared to traditional methods, with minimal sample manipulation by the user.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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