Electrochemical Sensing Methodology for Antibiogram Assays

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We report an electrochemical method of determining antibiotic susceptibility by detecting the electrochemical oxidation peak signature of the hydrolyzed *fluorescein* ester. Conventionally, antibiotic susceptibility studies are conducted using optical techniques. However, an electrochemical approach used in conjunction with the popular antibiogram assay offers an avenue to move away from these expensive and time consuming colorimetric techniques. Tests were conducted using a conventional three-electrode system in samples of *E.coli* incubated with *fluorescein diacetate*, with and without the antibiotic Penicillin and Streptomycin. A peak/no-peak signature observed in a voltammetric scan indicated the presence/absence of *fluorescein* in the sample, suggesting that the antibiotic may/may not be considered effective. In the future, this electrochemical approach could be miniaturized onto portable electronic systems such as point-of-care antibiogram apparatus for rapid antibiotic tests.
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Antibiotic susceptibility tests are important as a non-invasive method of detecting cell viability by determining which antibiotic would be best suited in treating a bacterial infection. The determination of cell viability itself is important in a broad range of studies including tissue culture and cytotoxicity studies, detecting the growth of bacteria in freshwater and food, and antibiogram assays.[1–4] Generally, antibiogram assays are conducted using colorimetric techniques by adding a dye to a test sample of cells. The dye is cleaved by intracellular enzymes of viable cells, reduced to a fluorescent compound and detected visually by its color change. The amount of dye produced is quantified using microplate spectrophotometery.[5] However recently, atomic-force microscope cantilevers have been used by measuring the resonant vibrations of the cantilevers caused by bacterial metabolism.[6] Fluorescent dyes have also been used in combination with flow cytometry to determine cell viability.[7] Several modern techniques such as scanning electrochemical microscopy (SECM) have also demonstrated exceptional sensitivity in determining cell viability by measuring O_2 reduction by living cells in the sample.[8] However, these techniques are all quite expensive, require costly instrumentation and do not lend themselves to economical and portable test platforms. Automation of colorimetric techniques also requires costly optical detection instruments. Thus, it would be beneficial to move toward a portable, low-cost electrochemical detection system that does not rely on the subjective visual-based determination and gives more confidence in the detection. In the past, electrochemical pathogen detection techniques have been reported, specifically, involving the electrochemical signature of *fluorescein* binding to DNA/RNA.[9] However, the electrochemical signature of *fluorescein* has not been applied to antibiotic susceptibility studies in antibiogram assays. In this paper, we present an electrochemical method of conducting antibiogram assays. We have observed an oxidation peak signature associated with hydrolyzed product of the non-fluorescent compound *fluorescein diacetate* which is created by the free and membrane bound enzymes of live bacterial cells.[3,10] This result and procedure has been applied to a model antibiogram test.

Fluorescein diacetate hydrolysis to *fluorescein* is a popular technique applied to colorimetric assays in cell viability tests. The *fluorescein diacetate* (a *fluorescein* ester) is converted to *fluorescein* by living cells in the sample through cellular esterases as illustrated in Figure 1a.[11] The characteristic green glow of the chemical *fluorescein*

can otherwise be detected visually or read by spectrofluorimeters.[11] However, the phenolic sites of the *fluorescein* molecule (f-Ph-OH), also allow for its electrochemical detection. Figure 1b illustrates the interaction of these phenolic sites of *fluorescein* with the chemically modified working electrode surface. The reaction taking place is shown in equation 1.

$$\mathrm{f-Ph-OH \rightarrow f-Ph-O\cdot + H^+ + e^-} \qquad [1]$$
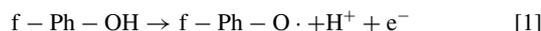
It is these electro-reduced phenolic sites of the *fluorescein* molecule that produce a peak signature in an electrochemical scan. In an antibiogram assay, the cells would be added to a quantity of the antibiotic and *fluorescein diacetate*. This solution would then be tested with an electrochemical voltammetric scan to check for the presence of the characteristic peak of *fluorescein*. A peak produced in this scan would correspond to the presence of live cells, meaning that the antibiotic is ineffective for the cells. Conversely, if no peak is produced in the scan, then the antibiotic can be administered to the patient.

Experimental

Experiments were carried out using a three-electrode system by applying voltammetric measurements to the working electrode immersed in the sample and detecting the anodic oxidation current-peak signature produced due to the *fluorescein* in the sample.

Reagent and sample preparation.— Electrochemical tests were carried out on four sets of solutions with Phosphate Buffered Saline as the supporting electrolyte: *fluorescein*, *fluorescein diacetate* incubated with live *E. coli*, plain *fluorescein* diacetate as a control, and *fluorescein diacetate* incubated with live *E. coli* and Penicillin and Streptomycin. The results of the different samples were compared to check for the presence of *fluorescein* in the sample. An oxidative peak signature in an antibiotic susceptibility test indicated that the antibiotic was ineffective in killing the live cells. The working electrode was chemically modified by depositing Graphitized Mesoporous Carbon (GMC; 50 nm and 99.955% purity) on the electrode surface. Penicillin and Streptomycin (Pen Strep, 10,000 units/mL) was purchased from Lonza; GMC, *fluorescein diacetate* and *fluorescein* were purchased from Sigma Aldrich; and all reagents were used without further purification. The supporting electrolyte was 1X Phosphate Buffered Saline (PBS; 8% NaCl, 0.2% KCl, 1.44% Na_2HPO_4, 0.24% KH_2PO_4) of
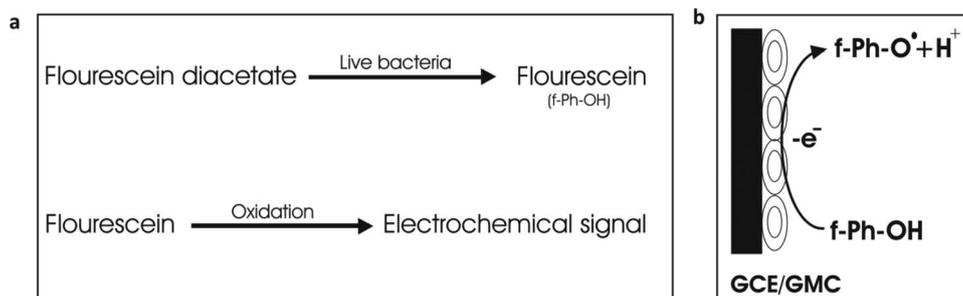
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Figure 1. (a) Illustration of the hydrolysis of *fluorescein diacetate* to *fluorescein* by cellular esterases. (b) Schematic representation of the reaction of the Glassy Carbon Electrode, modified with Graphitized Mesoporous Carbon, with *fluorescein*.

pH 7.0. *E. coli* (DH5α) was used as a standard organism in our tests. Stock solutions of *fluorescein* and *fluorescein diacetate* were prepared in acetone. The first set of solutions was prepared by adding 50 ppm (final concentration) of *fluorescein diacetate* to *E. coli* cells that were pelleted and brought up in PBS to 1 OD (600 nm), and incubated for 2 hours at 37°C. The second set of *fluorescein* test solutions was prepared by serial dilutions in PBS to 10 and 15 ppm concentrations. The third set of *fluorescein diacetate* test solutions was prepared with serial dilutions in PBS to 50 ppm. The fourth set of solutions was identical to the first set but with the addition of 1000X Pen Strep.

Instruments.— The CHI 1200B electrochemical workstation was used for all voltammetric measurements with the conventional three-electrode system.[12] A Glassy Carbon electrode (GCE) of 0.0707 cm^2 geometrical surface area with its chemically modified electrode (CME) was used as a working electrode and Platinum wire as a counter electrode. Ag/AgCl with 1M KCL was used as a reference electrode for all experiments in this work.

Setup and procedure.— To clean the electrode surface, the working electrode was mechanically polished with the bio-analytical system (BAS, USA) polishing kit and cleaned with acetone followed by double deionized water. Cyclic voltammetry (CV) was then performed on

the working electrode in PBS in a potential window of −0.2 to 1.2 V with a scan rate of 50 mV/s. Next, 1 mg GMC was dispersed in 500 μL of ethanol and sonicated for five minutes. 10 μL of this solution was drop-casted on the GCE surface and air dried at room temperature (28°C) for 2 minutes. This electrode was then electrochemically pretreated by subjecting it to one cycle of CV in PBS in a potential window of −0.5 V to 0.5 V at a scan rate of 50 mV/s. This CME was then used as a working electrode for our experiments. The three electrodes were immersed in the 4 mL working volume of the solution and linear sweep voltammetry (LSV) was performed in a potential window of 0.2 to 0.8 V at a scan rate of 50 mV/s. The same test was repeated for the four sets of solutions.

Results and Discussion

Experimental results.— Figure 2a presents the electrochemical responses for 10 and 15 ppm *fluorescein*. This shows a characteristic electrochemical peak current of 0.606 μA occurring at a potential of 0.620 V for 10 ppm, and 0.882 μA at 0.617 V for 15 ppm concentrations of *fluorescein* samples. Figure 2b shows an overlay of the electrochemical responses of *fluorescein diacetate*, 0.1 OD *E. coli* in *fluorescein diacetate* incubated for 2 hours at 37°C, and an identical set incubated with 1000X Pen Strep for the same duration and
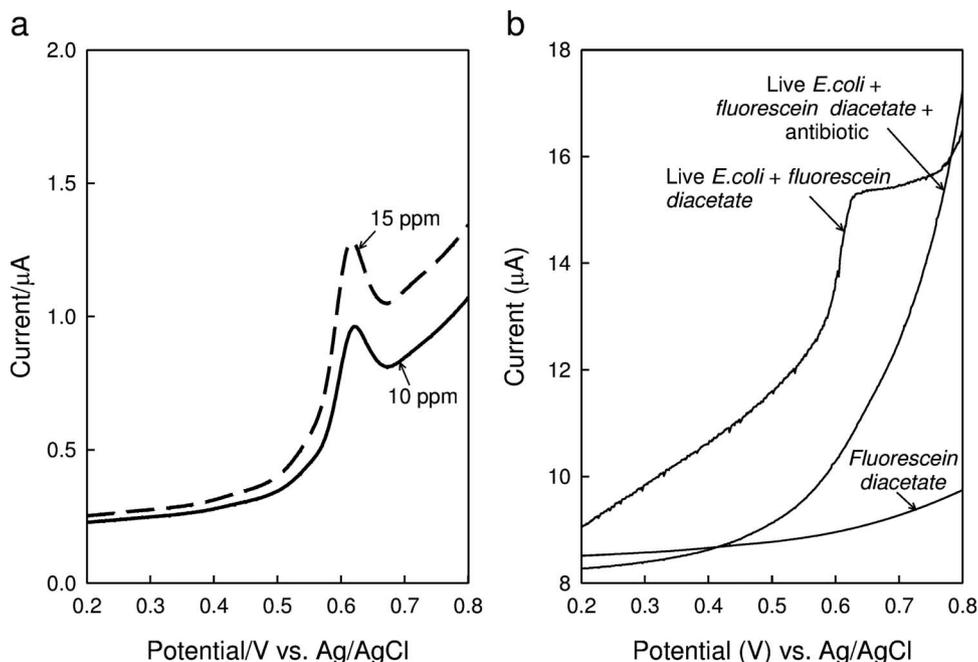

Figure 2. (a) Electrochemical response of *fluorescein* for 10 and 15 ppm concentrations. (b) Electrochemical response for 0.1 (OD) live *E. coli* in 50 ppm of *fluorescein diacetate* incubated at 37°C for 2 hours, an identical batch incubated at 37°C for 2 hours with Penicillin and Streptomycin control *fluorescein diacetate* and the control *fluorescein diacetate*.

temperature. Pure *fluorescein diacteate* is our control sample and it does not exhibit any characteristic electrochemical peaks. The electrochemical response of the sample containing live *E. coli* and *fluorescein diacetate* has a clear anodic oxidative peak current of 1.754 μA at 0.627 V, whereas the sample of *E. coli* incubated with *fluorescein diacetate* and Pen Strep does not show any characteristic peak.

Discussion.— The *fluorescein* molecule has a phenolic group present in its chemical structure. During an electrochemical voltammetric test, the oxidation of this phenolic group results in a peak signature as phenoxy radicals are formed.[12–14] This phenomena best describes the peak signatures observed in both the pure *fluorescein* sample (Figure 2a), and the sample of *fluorescein diacetate* hydrolyzed by *E. coli* to *fluorescein* (Figure 2b).

For the antibiotic susceptibility test, we found that no characteristic peak is observed for a sample of *E.coli* incubated with *fluorescein diacetate* and Pen Strep. Since the antibiotic is successful in killing the live cells, the *fluorescein* ester remains unhydrolyzed. Thus, in an electrochemical scan, we do not observe the characteristic peak of the *fluorescein* molecule. This signature of *fluorescein* can thus be used as an electrochemical alternative in antibiotic susceptibility tests. While a commercial electrochemical workstation was used in this preliminary study, in the future this approach could be miniaturized onto portable electronic systems such as a handheld potentiostat. This enables antibiogram assays to be brought to the electronic instrumentation domain.

Conclusions

In conclusion, we have shown an electrochemical technique for conducting antibiotic susceptibility tests by detecting the peak signature of hydrolyzed *fluorescein diacetate*. The phenolic groups present in the *fluorescein*, act as an electroactive molecule which forms phenoxy radicals and results in the current-peak characteristics observed in electrochemical voltammetric scans. In an antibiotic susceptibility study, a peak signature observed in a test would suggest that the antibiotic is ineffective in killing the cells. Conversely, if no peak is observed, then the antibiotic may be considered effective. This offers an electrochemical alternative to the optical colorimetric approach used in antibiogram assays. Moving to an electrochemical domain would allow the development of portable and economical systems for antibiogram devices and antibiotic susceptibility experiments. An electrochemical system could also be designed to be operated by the layman without requiring user knowledge or expertise in electrochemistry. Such systems would also be able to interface with electronic mobile devices and offer diagnostic capability to rural and remote locations.

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