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ELECTROCHEMICAL DETECTION OF HYDROLYZED FLUORESCEIN DIACETATE FOR CELL VIABILITY TESTS

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INTRODUCTION

Optical techniques play an important role in cell viability assays. Several modern techniques such as atomic-force microscope cantilevers[1] and spectrophotometry[2,3] have demonstrated the capability to determine cell-viability with exceptional sensitivity; however these techniques are quite expensive and do not lend themselves to economical and portable test platforms. Even automating simple colorimetric techniques requires fairly expensive optical detection systems. Pathogen detection using electrochemical techniques, in particular, using the electrochemical signature of *fluorescein* binding to DNA/RNA have been reported[4], but the electrochemical signature of *fluorescein* has not been adapted directly to cell viability tests. Here we show an economical and electronic method of determining cell viability by electrochemically detecting the peak signature of the hydrolyzed product of the non-fluorescent compound *fluorescein diacetate*[5]. We have observed a distinct electrochemical signature; an oxidation peak associated with *fluorescein*, the product of *fluorescein* ester hydrolyzed by cellular esterases and efflexed out of only live bacterial cells[2].

Fluorescein diacetate hydrolysis is one of the popular colorimetric assays in cell viability tests. The *fluorescein* ester is cleaved by the bacteria and converted to *fluorescein* through the process of cellular esterases[6]. *Fluorescein* has a characteristic green glow, which can be either visually observed or read by spectrofluorimeters[6]. The *fluorescein* acts as an electroactive molecule owing to the presence of the phenolic sites of the *fluorescein* (f–Ph–OH) and has a peak signature created by its electro-reduced species as illustrated in figure 1. This phenomenon allows the electrochemical detection of *fluorescein* and the observation and experimental results are presented in this paper.

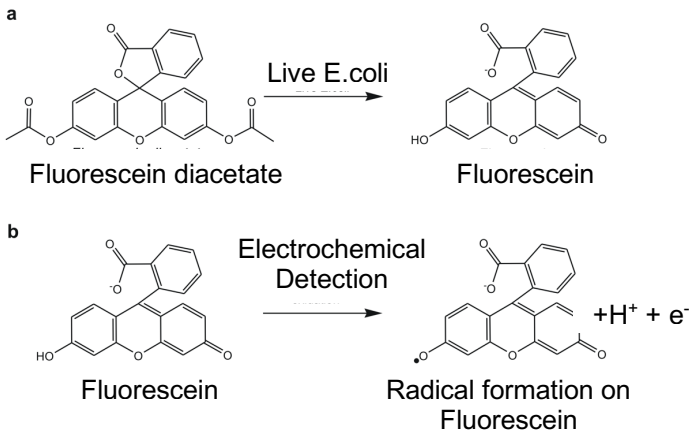


Figure 1: (a) Illustration of the structure of *fluorescein diacetate* and its hydrolysis to *fluorescein* by esterases. (b) Electrochemical oxidation of *fluorescein*.

EXPERIMENTAL

Experiments were conducted using a 3-electrode system by applying a voltage sweep (voltammetric measurement) to the working electrode presented to the sample and detecting the anodic oxidation current-peak signature produced due to the hydrolyzed species of *fluorescein diacetate*.

Apparatus, Materials and Reagents

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 dead *E. coli*. The oxidative peak currents for the samples were compared to confirm the definitive detection of *fluorescein* produced due to the hydrolysis of *fluorescein diacetate* by live *E. coli*. All voltammetric measurements were carried out using the CHI 1200B electrochemical workstation. The three electrode system consists of a Glassy Carbon electrode (GCE) of 0.0707 cm^2 geometrical surface area and its chemically modified electrode (CME) as a working electrode and Platinum wire as a counter electrode. For all our experiments reported in this work a Ag/AgCl with 1M KCl was used as a reference electrode. *E. coli* (DH5α) was used as a model organism in our tests. Chemical modification of the working electrode was performed by depositing Graphitized Mesoporous Carbon (GMC; 50 nm and 99.95% purity) on the electrode surface. GMC, *fluorescein diacetate* and *fluorescein* were purchased from Sigma Aldrich and were used without further purification. 1X Phosphate Buffered Saline (PBS; 8% NaCl, 0.2% KCl, 1.44% Na_2HPO_4, 0.24% KH_2PO_4) of pH 7.0 was used as a supporting electrolyte.

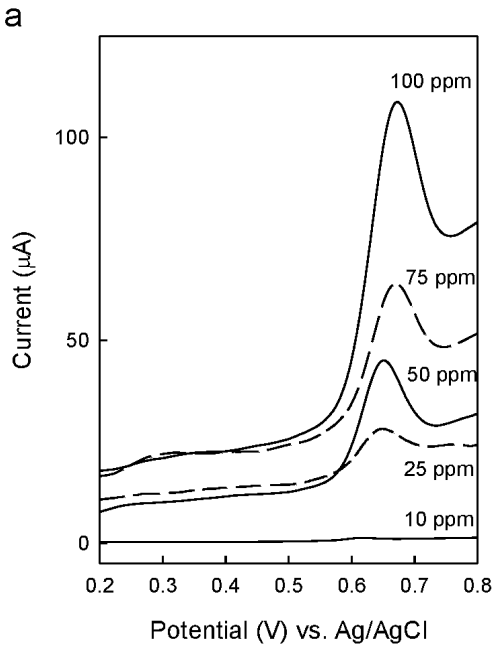
METHODS

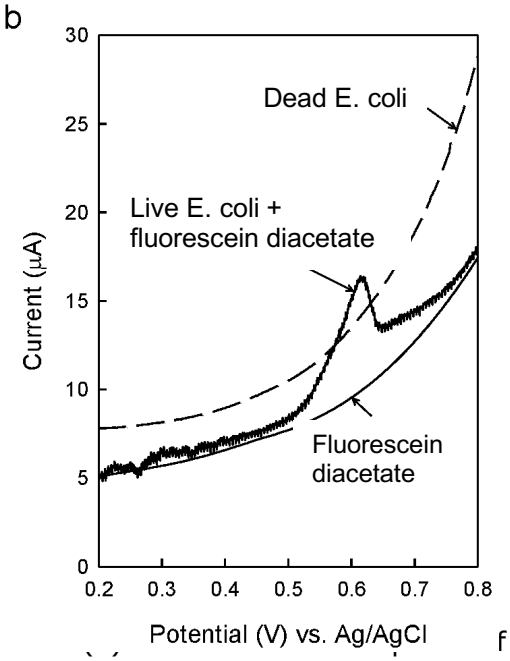
Reagent and Sample Preparation

A working volume of 4mL was used for all our electrochemical characterization. The 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 37ºC. The second set of *fluorescein* test solutions was prepared by serial dilutions in PBS to 10, 25, 50, 75 and 100 ppm concentrations. The third set of *fluorescein diacetate* test solutions was prepared with serial dilutions in PBS to 50 ppm. The dead *E.coli* sample was prepared in PBS and brought to 1 OD, incubated for 2 hours and then killed by heating it to 100ºC for 10 minutes.

Procedure

Prior to the surface modification of the working electrode, the surface was mechanically polished using the bio-analytical system (BAS, USA) polishing kit and cleaned with acetone followed by double deionized water. This electrode was then subjected to 10 cycles of cyclic voltammetry (CV) in a potential window of -0.2 to 0.8V in PBS with a scan rate of 50mV/s. Subsequently, 1 mg GMC was dispersed in 500µL of ethanol and sonicated for five minutes. 10µL of this solution was drop-casted on the surface of the bare pretreated GCE and air dried at room temperature (28ºC) for 2 minutes. This electrode was then pretreated electrochemically by performing one cycle of CV in PBS in a potential window of -0.5V to 0.5V at a scan rate of 50 mV s^{-1}. This modified electrode was then used as a working electrode for our experiments.





Figu f
fluorescein for 10, 25, 50, 75 and 100 ppm concentrations. (b) Electrochemical response for dead *E. coli*, 1 (OD) live *E. coli* in 50 ppm of *fluorescein diacetate* incubated at 37°C for 2 hours, and the control *fluorescein diacetate*.

The experiment was conducted using the conventional 3-electrode electrochemical setup[9]. The electrodes were immersed in the 4mL working volume of the solution and linear sweep voltammetry (LSV) was performed in a potential window of 0.2 to 0.8V at a scan rate of 50mV/s. The same test was repeated for the four sets of solutions.

RESULTS

Figure 2 presents the results of our experiment. Figure 2(a) presents the electrochemical responses for 10, 25, 50, 75 and 100 ppm *fluorescein.* This shows a characteristic electrochemical peak current of 0.606μA occurring at a potential of 0.620V for 10 ppm, 28.00μA at 0.642V for 25 ppm, 44.89μA at 0.648V for 50 ppm, 63.72μA at 0.665V and 108.32μA at 0.668VV for 100 ppm concentrations of *fluorescein* samples. Figure 2 (b) shows an overlay of the electrochemical responses of *fluorescein diacetate,* 1 OD 600nm *E. coli* in *fluorescein diacetate* incubated for 2 hours and 1 OD dead *E. coli*. 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 4.37μA at 0.614V, whereas the response of dead *E. coli* does not show any electrochemical signature peaks.

DISCUSSION

We hypothesize that the peak signature observed in figure 2(b) for the sample of *E. coli* incubated with *fluorescein diacetate* is due to the phenolic group in fluorescein, which readily undergoes electro-oxidation with the formation of phenoxy radicals[7,8,9] during the voltammetric scan as given in equation 1 below (see figure 1b also). Thus offering a definitive signature of hydrolyzed *fluorescein diacetate.*

$$\text{f-Ph-OH} \rightarrow \text{f-Ph-O}\cdot + H^+ + e^- \quad (1)$$

In summary, we have demonstrated a useful technique to electrochemically detect bacterial cell viability using the cellular hydrolysis of *fluorescein diacetate*. The *fluorescein* thus produced acts as an electroactive molecule owing to the presence of the phenolic groups sites creating a characteristic oxidative peak signature in electrochemical scans. This offers a reliable electrochemical method for the popular bacterial viability tests giving an avenue to move away from the more involved and subjective colorimetric detection protocols. Further, electrochemical detection techniques will enable the development of new portable and potentially economical electronic systems for rapid cell viability tests and antibiogram devices for antibiotic susceptibility experiments. Such systems would be capable of easily interfacing with mobile devices such as cell phones and tablet computers and offer rapid diagnostic capability to remote locations.

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