Sensing and Bio-Sensing Research 13 (2017) 109–114

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

Development of electrochemical inhibition biosensor based on bacteria for detection of environmental pollutants☆

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ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form 20 October 2016
Accepted 21 October 2016

Keywords:
Inhibition biosensor
Escherichia coli
Shewanella oneidensis
Optical measurements
Electrochemical measurements
Cyclic voltammogram

ABSTRACT

The main aim of this work is to develop a simple inhibition electrochemical sensor array for detection of heavy metals using bacteria. A series of electrical measurements (cyclic voltammograms) were carried out on samples of two types of bacteria, namely *Escherichia coli* and *Shewanella oneidensis* along with optical measurements (fluorescence microscopy, optical density, and flow cytometry) for comparison purposes. As a first step, a correlation between DC electrical conductivity and bacteria concentration in solution was established. The study of the effect of heavy metal ions (Hg^{2+}) on DC electrical characteristics of bacteria revealed a possibility of pattern recognition of inhibition agents. Electrical properties of bacteria in solution were compared to those for immobilized bacteria.

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1. Introduction

Heavy metal pollution is a problem associated with areas of intensive industry. Zinc, copper, and lead are three of the most common heavy metals released from road travel. Lead concentrations, however, have been decreasing consistently since leaded gasoline was discontinued [1]. The existing high-tech methods of their detection are usually expensive and laboratory based. This work is a part of ongoing research targeting the development of novel, simple, and cost effective methods for monitoring environmental pollutants, particularly pesticides, petrochemicals and heavy metals being common contaminants of water resources. It is known that microorganisms are very sensitive to heavy metals [2,3]. The use of microorganisms for assessment of general toxicity of aqueous environment was reported previously [4]. Identification of the types of pollutants in the environment and the evaluation of their concentration is much more difficult task which is impossible to solve using a single inhibition type of sensor. However, the sensor array approaches utilising several types of bacteria being inhibited differently by different types of pollutants could solve the above problem. Electrochemical measurements were successfully used for studying electrical properties of cells deposited on screen printed gold electrodes and showed great prospects of using such cell-based sensors for detection of various pollutants [5,6]. Our previous experiments went further and expanded this idea to more complex bio-objects such as bacteria. Using two types of bacteria, *Escherichia coli* and *Deinococcus radiodurans*, we confirmed the principles of inhibition sensor array which was capable of differentiation between radionuclide and heavy metal pollutants [2,7]. In this work, we used simple electrochemical measurements for establishing the correlation between conductivity of liquid bacteria samples and immobilized bacteria, and studying the effect of heavy metal ions (Hg^{2+}) on them. In addition to *E. coli* bacteria, we used another type of bacteria, *Shewanella oneidensis* known by its high resistance to heavy metals. The main focus of this work is on electrical characterisation of both free bacteria in solution and bacteria immobilized on the surface of metal electrodes.

2. Experimental methodology

2.1. Bacteria sample preparation

Two types of bacteria were selected for this work: *Escherichia coli* (*E. coli*), which is quite sensitive to different environmental pollutants, and *Shewanella oneidensis* (*S. oneidensis*) known by its extreme resistance to heavy metals. LB (Luria-Bertain) broth was used as a medium [8] for both bacterial cell cultures. Both types of bacteria and respective growth media were acquired from Sigma-Aldrich Co. Other chemicals, i.e. $HgCl_2$ salt and poly L-lysine (PLl) were also purchased from Sigma-Aldrich Co. Cultivation of bacteria was performed in several stages.

☆ 26th Anniversary World Congress on Biosensors 2016
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http://dx.doi.org/10.1016/j.sbsr.2016.10.007
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The first step was to cultivate a specific strain of bacteria in Petri dish containing solid broth agar, in order to use it as a bacteria source in future. In the second stage, one colony of bacteria was added into a sterile flask containing 50 ml of liquid broth. Finally, the flask containing the bacterial culture was placed inside shaking incubator operating at 150 rpm shaking speed. The incubation temperatures were 30 °C for *S. oneidensis* and 37 °C for *E. coli*. Bacteria start growing after 16 h for *E. coli* and 24 h for *S. oneidensis*.

2.2. Experimental procedures

To study the inhibition effect of heavy metals on the above bacteria, $HgCl_2$ salt (from Sigma-Aldrich) was selected. Solutions in different concentrations (0.1, 1, 10, 100 mM) of $HgCl_2$ were prepared by multiple dilution of 1 M stock solution of $HgCl_2$ in deionised water. Bacteria samples were mixed with $HgCl_2$ solutions in 1:1 ratio and kept incubated for 2 h.

The effect of heavy metal on the bacterial density was examined and analyzed using three different optical experimental techniques: fluorescence microscopy, UV–visible spectrophotometry, and flow cytometry. GALLIOS flow cytometer BECKMAN-COULTERPN A75199AA instrument was used for counting the percentage of live and dead bacteria after colouring bacteria samples with L7012 Live/Dead Bacterial Viability Kit. Fluorescence microscopy measurements were performed using Olympus-BX60 instrument. In this study, bacterial samples were also stained using L7012 Live/Dead (L/D) BacLight Bacterial Viability Kit [9], which is a mixture of (SYTO-9) green fluorescence nucleic acid stain and the red fluorescence nucleic acid stain propidium iodide. The cultivated bacteria density and changes in the live bacteria counts after exposure to pollution were monitored using optical density photometer (6715 UV/Vis Spectrophotometer JENWAY OD600). The electrochemical measurements, i.e. cyclic voltammograms (CV), were carried using gold screen printed three-electrode assemblies and DropSens microSTAT200 potentiostat. The same CV measurements were carried on both types of bacteria immobilized on the surface of gold electrodes via poly L-lysine (PLl) [10]. For that purpose, the surface of gold was modified in a 1:1000 mixture of PLl (1 mg/ml) and deionised water for 1 h at 37 °C. Then bacteria were immobilized by dropping stock solutions of either *E. coli* or *S. oneidensis* in LB broth on the modified electrodes, keeping it there for 1 h, then washing out non-bound bacteria with phosphate buffer solution (PBS). The CV measurements of the electrodes with freshly immobilized bacteria were carried out in PBS both before and after treatment with $HgCl_2$ in different concentrations.

3. Experimental results and discussion

3.1. Optical measurements

The numbers of live and dead bacteria were determined with fluorescence microscopy and OD600 similarly to that described in [7]. Live and dead bacteria appeared as green and red dots, respectively, on fluorescence microscopy images in Figs. 1 and 2 for *E. coli* and *S. oneidensis*, respectively.

It is clear, that the exposure to $HgCl_2$ reduces the number of live (green) bacteria and increases the dead ones (red), while *S. oneidensis* bacteria are much less affected.

Similar and even more pronounced pattern was observed in flow cytometry experiments where bacteria were stained with the same L7012 Live/Dead Bacterial Viability Kit and appeared on the graphs in Fig. 3 as blue (live) and orange (dead) dots. The increase in the dead *E. coli* bacteria count after exposure to $HgCl_2$ salt is visually apparent. Image analysis of Fig. 3A(b) yields the percentage of live *E. coli* 43.88% and 56.12% for dead ones.

In addition to that, dead *E. coli* bacteria appear mostly in bottom-left quadrant of the graph in Fig. 3A(b) indicating the increase in the bacteria size most-likely due to rapture of cell membranes. Contrary, *S. oneidensis* bacteria were affected much less, the percentages of live and dead bacteria after exposure to 1 M $HgCl_2$ solution were 83.36% and 16.64%, respectively. Again, dead bacteria appeared slightly enlarged since they were shifted to the bottom-left in Fig. 3B(b).

The result of optical density measurements of both bacteria samples and the effect of their treatment with $HgCl_2$ salt of different concentrations are shown in Table 1. The bacteria density was assessed (and presented as absorbance) by losses of light intensity in the middle of visible range (600 nm) as a result of light scattering on bacteria. The reduction in optical density upon increasing the $HgCl_2$ concentration is much more pronounced for *E. coli* than that for *S. oneidensis.*

Among the three optical methods used, flow cytometry appeared to be the most reliable and not affected by different motility of *E. coli* and *S. oneidensis.* It is known that dead *E. coli* bacteria are not motile and tend to sediment which may affect the results of static fluorescent microscopy and optical density measurements. Nevertheless, the results of optical testing of bacteria samples provided a background for further study using much simpler electrochemical method.

3.2. Electrochemical measurements on liquid bacteria samples

The electrochemical measurements of bacterial samples were carried out using DropSens potentiostat and screen printed gold electrodes. Potential was recorded against Ag/AgCl reference electrode. Typical cyclic voltammograms for *E. coli* and *S. oneidensis* of different concentrations (i.e. dilutions with LB broth) are shown in Fig. 4.

Generally, the CV graphs in Fig. 4 are almost featureless in the selected voltage range from −0.5 V to +0.5 V which was chosen deliberately in order to avoid electrochemical reactions on the electrodes. Slight increase in the cathode current at −0.2 V indicates the beginning of hydrogen reduction.

The values of cathode current at −0.5 V appear to decrease with the increase in bacteria concentration (or dilution ratio 1:10, 1:5, 1:2, 1:1) with the largest current shown for clear LB broth and the lowest for

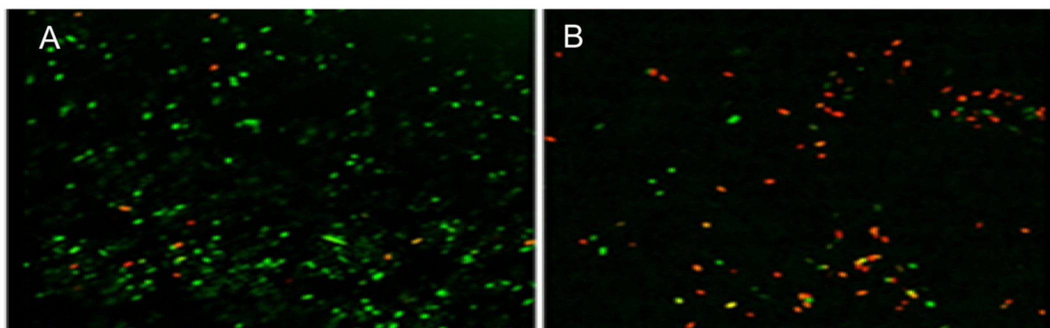

Fig. 1. Fluorescence microscopy images of *E. coli* before (A) and after (B) treatment with $HgCl_2$ salt (1 M).

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