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

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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 (Hg2+) 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 (Hg2+) 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-Bertani) 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. HgCl2 salt and poly-L-lysine (PLL) were also purchased from Sigma-Aldrich Co. Cultivation of bacteria was performed in several stages.
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 dilutions 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-COULTER 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 measurements. Nevertheless, the results tend to sediment which may affect the results of static cultivation. 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 fluorescence 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 highest current shown for clear LB broth and the lowest for...
bacteria stock solution. That means that bacteria adsorbed on the surface of gold electrodes act as insulating layer reducing the current. These results are very important since they establish a correlation between the values of cathode current and bacteria concentration in solution.

The next step was to study the effect of Hg\(^{2+}\) ions on CV characteristics of both S. oneidensis and E. coli bacteria. A series of CV measurements were carried out on liquid samples of bacteria stock solution in LB broth which was mixed in 1:1 ratio with different concentrations of HgCl\(_2\) and kept for 2 h prior to CV measurements. The results of these measurements in Fig. 5 show substantial increase in cathode current (I\(_c\)) for E. coli samples (Fig. 5A) upon increasing the concentration of HgCl\(_2\) while this effect is much less pronounced for S. oneidensis samples (Fig. 5B). In addition to that, the oxidation and reduction peaks appeared on CV curves for S. oneidensis samples in Fig. 5A which are definitely related to electrochemical reactions associated with the presence of HgCl\(_2\). The anodic peak increases with the increase in HgCl\(_2\) concentration. However, such electrochemical reactions do not appear on E. coli samples in Fig. 5B. It is known that heavy metal salts act as co-factor for S. oneidensis bacteria growth [11], so the oxidation peak at about 0.2 V on Fig. 5A is most-likely related to the interaction of HgCl\(_2\) with S. oneidensis.

When analysing the effect of heavy metal salts on CV characteristics of liquid bacteria samples the effect of extra Hg\(^{2+}\) and Cl\(^-\) ions on conductivity of liquid medium has to be taken into account. In order to find out the true effect of heavy metal ions on bacteria, the values of cathode current (I\(_c\)) of bacteria samples has to be normalized by the reference current I\(_{ref}\) of LB broth diluted 1:1 with particular concentration of HgCl\(_2\). The values of relative changes of cathode current \(\Delta I_c/I_{ref}\) at \(-0.5\) V of both S. oneidensis and E. coli bacteria are plotted in Fig. 6 against the concentration of HgCl\(_2\).

As one can see the effects of HgCl\(_2\) on S. oneidensis and E. coli are completely different: \(\Delta I_c/I_{ref}\) goes up with the increase in HgCl\(_2\) concentration for E. coli which means that E. coli bacteria inhibited by Hg\(^{2+}\) ions becoming less electrically resisting, while \(\Delta I_c/I_{ref}\) is almost flat at low concentrations of HgCl\(_2\) and slightly increases at high concentration of 1 M. This means that S. oneidensis are practically not affected by HgCl\(_2\) low concentrations but inhibited at high concentrations. This is a very promising result showing a possibility of pattern recognition of heavy metals using the two bacteria.

### 3.3. Electrochemical measurements on immobilized bacteria

The results given in the previous section are important as a further step towards the development of bacteria-based inhibition sensor array, but it is still far away from real sensor development. Dealing with liquid bacteria samples is not the way forward because of natural variations of bacteria concentration even in laboratory samples not to mention “real” samples taken for analysis. The problem of having a reliable reference for such measurements is a very difficult one. It would be much more useful for real sensor development to use bacteria immobilized on the electrode surface.

In this work, both selected bacteria, e.g. S. oneidensis and E. coli, were immobilized on the surface of gold electrodes via poly l-lysine as described in more detail in Section 2.2. Fig. 7 shows a series of CV measurements carried out in PBS on gold electrodes with S. oneidensis and E. coli immobilized from their respective solutions of different concentrations in LB broth (stock solution and 1:2, 1:5, 1:10 dilutions with LB broth were used). Both graphs show characteristic oxidation and reduction peaks associated with electrochemical reactions in PBS. It is clear from

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>HgCl(_2) concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
</tr>
<tr>
<td>E. coli</td>
<td>0.813</td>
</tr>
<tr>
<td>S. oneidensis</td>
<td>0.827</td>
</tr>
</tbody>
</table>
As one can see, the effect is similar to that observed on liquid bacteria samples. The values of $\Delta I_c/I_c$ for E. coli increase steadily with the increase in HgCl$_2$ concentration, while S. oneidensis bacteria are practically unaffected by HgCl$_2$ in a wide concentrations range apart from a slight increase in $\Delta I_c/I_c$ at 0.1 mM. High concentration of HgCl$_2$, however, causes the damage to S. oneidensis bacteria cell membrane, and they sharply become more conductive.

4. Conclusions and future work

The effect of heavy metals (Hg in this case) on two types of bacteria, e.g. E. coli and S. oneidensis was studied using three different optical techniques: fluorescent microscopy and flow cytometry which yields directly the ratio of live/dead bacteria, stained, respectively, with “green” and “red” fluorescent dyes as well as optical density measurements at 600 nm. All three optical methods are capable of detecting the effect of heavy metals on the above bacteria, though the flow cytometry is much more reliable. The results obtained were encouraging, however the use of expensive and bulky optical instrumentation is not the way forward for portable and cost-effective sensor development.

Simple electrochemical tests, e.g. cyclic voltammograms, either on gold electrodes immersed into liquid bacteria samples or (even better)
on gold electrodes with immobilized bacteria appeared to be very successful. The values of cathode current was found to correlate with bacteria concentration and thus with the concentration of HgCl$_2$ salt acting as inhibitor for bacteria. The effect of HgCl$_2$ on the two bacteria used was different; *E. coli* is strongly inhibited by HgCl$_2$, while *S. oneidensis* is practically unaffected in a wide concentration range of HgCl$_2$. The latter fact opened possibility of exploiting the principles of pattern recognition for identification of pollutants.

This work proved the concept of a novel, simple and cost effective electrochemical bacteria-based sensor and sensor array for preliminary assessment of the presents of pollutants in water. Future work which is currently underway will focus on extending the range of pollutants to pesticides and petrochemicals as well as adding another type of bacteria to the sensor array.

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