Amperometric Immunosensors for screening of Polycyclic Aromatic Hydrocarbons in water

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Abstract. An amperometric immunosensor with low limit detection was developed for the screening of polycyclic aromatic hydrocarbons (PAHs) in water. The system was based on detecting the specific substance using an immunological reaction by measuring the chemical responses to specific antibodies. An integrated biochip with a three electrode system was fabricated. Gold was used as the working electrode with platinum was used as the counter electrode. A modified Ag/AgCl reference electrode was employed to enhance the stability of the immunosensors. Indirect competition enzyme-linked immunosorbent assay (ELISA) was carried out within the electrode using alkaline phosphatase (AP) as the labelled-enzyme. The system shows acceptable reproducibility and good stability. The immunosensor exhibited a wide linear response to PAHs. A limit of detection for this sensor was in the range of 1 to 10 ng ml⁻¹ in aqueous sample.

1. Introduction
The technique of immunoassay has been widely used in the field of clinical science. Recent developments however have seen its application extended to areas such as food science, health screening, determination of pollutants in environment and for illicit drug detection.

In comparison with other immunoassay detection methods, electrochemical enzyme immunoassay offers the advantages of sensitivity and low detection limit [1]. Moreover, this technique introduced the miniaturized device that allow for simple and low cost instrumentation with only small sample volume required [2]. There are a number of transducers that have been used in electrochemical detection including amperometric, potentiometric and conductometric. These are all widely used in the measurement of pollution in water.

Amperometric detection is based on the measurement of current when a potential is applied to the working and reference electrodes of the system [3]. The species of interest is either oxidised or reduced at the working electrode causing a transfer of electrons which generates a measureable current. Amperometric probes are highly suitable when oxidase or dehydrogenase enzymes are employed to generate electrooxidizable peroxide or NADH species [4].
immunosorbent assay (ELISA). ELISA is based on the ability of antibodies to bind specifically to
target antigens for monitoring of PAHs. In ELISA, immobilization and incubation steps are applied to
bind the reagents to the surface of the electrodes [5]. The binding of target antigens to antibody within
the substrate will lead to enzymatic interaction with the production of a current signal.

2. Experimental

2.1 Fabrication of biochips

The fabrication of biochips was carried out at the Central Fabrication Facility in the Tyndall National
Institute. The electrodes were fabricated on a 100 mm pyrex wafer using e-beam lithographic
techniques for electrode patterning. The wafer underwent lift-off processes where the gold (Au)
working electrode was deposited first, followed by the platinum (Pt) counter electrode and the final
stage was the silver (Ag) reference electrode. To coat the Ag/Cl layer onto the reference electrode, the
wafer was dipped in 50 mM ferric chloride solution for 50 seconds [6]. The metal lift-off process was
carried out by immersing the electrodes in hot acetone, isopropanol and finally rinsing under running
denized water. The electrodes were packaged using flip-chip technology where epoxy resin was used
to attach the electrodes to the printed-circuit board (PCB) [7]. Figure 1 shows the interface for
connecting the biochip with the electrochemical instrument of PalmSens potentiostat (Palm
Instruments BV, Netherlands).

2.2 Competition Assay

The assay for amperometric detection for biochip followed the ELISA format. BSA-Pyrene coating
conjugate was synthesized by coupling the adipic acid dihydrazide with bovine serum albumin (BSA),
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) and 1-pyrene butyric acid [8].
Immobilization of antibodies was carried out directly on the electrode surface to enhance signal for
maximum sensitivity. 5 µl of sample was required to completely cover the working area of the
biochip. Three replicates were carried out for each concentration of the antigen (n=3). The
immobilized biochip for each antibody was allowed to incubate for one hour at 37°C. The inhibition
ELISA was carried out with the use of alkaline phosphatase as labelled-enzyme. Primary monoclonal
4D5 (α-pyrene) antibody was allowed to compete with a known concentration of PAHs. The enzyme
used specifically converts the substrate (para-aminophenyl phosphate) into a detectable signal. The
pAPP is dephosphorylated to form p-aminophenol (pAP) by reaction with enzyme, which is then
oxidised at 0.3 V to form the imino-quinone, thus two electrons are generated (figure 2). This
substrate was chosen rather than other common substrates as the product of para-aminophenol can be
oxidized at much less positive potential than the nitrophenol, an enzymatic product from nitrophenyl phosphate which would usually be used as an AP substrate for spectrophotometric detection [9].

Figure 2. Competition assay format with electrochemical reaction occurs at the surface of gold electrode when 0.3 V potential is applied (primary antibody; analyte; and AP-labelled antibody).

3. Results and Discussions

3.1 Scanning Electron Microscopy (SEM)

The morphology of the electrodes surface was examined by using SEM to study the stability of the electrodes. The surface of the reference electrode was covered with an AgCl layer. Different sizes of AgCl grain could be seen on the surface but very few pores are observed. The AgCl layer with the fewest pores functioned as a stable and durable Ag/AgCl reference electrode [10]. Smooth and clear surfaces were obtained for the platinum electrode and gold electrode.

Figure 3. SEM photographs of the surfaces of (a) Ag/AgCl reference electrode, (b) gold working electrode and (c) platinum counter electrode.

3.2 Electrochemical Detection

Investigation of the stability of the reference electrode was carried out by placing the reference electrode in 1M KCl for 1 hour. The potential of the integrated Ag/AgCl reference electrode was measured against the commercial reference electrode (figure 4). The potential shifted about 20 mV after 15 minutes of operation and continued stable for 1 hour. This is acceptable when compared to the 13 mV shifted potential for glucose sensors reported by Se-Ik et al [11]. Kinetic study was tested to
determine the concentration of pAPP substrate that would yield a signal of 95 % of $I_{\text{max}}$. Figure 5 represents the Michaelis-Menten plot for different concentrations of pAPP in reaction with AP labelled. The $K_m$ for the pAPP substrates was 1.7 mg/ml and the corresponding concentration of substrate at 95 % was 2.4 mg/ml (figure 5).

Figure 4. Potentiometric detection of on-chip Ag/AgCl versus commercial reference electrode in 1M KCl.

Figure 5. Michaelis-Menten plot for different concentrations of the substrate pAPP.

The amperometric detection of the Ag-Ab immobilized biochip was measured by detecting the amount of the antibodies that react with the benzo[a]pyrene. The detection time was set to 60 seconds where pAPP was injected at 40 seconds when the solution reached equilibrium. The scan rate applied was 20 mV s$^{-1}$. The observed trends are illustrated in figure 6.

Figure 6. Benzo[a]pyrene indirect competition for 4D5 monoclonal antibodies.

The limit of detection (LOD) for this sensor was found to be 5.6 ng ml$^{-1}$ with an IC$_{50}$ value of 9.82 ng ml$^{-1}$. This sensor allowed detection in a linear range from 6 to 120 ng ml$^{-1}$. The $R^2$ value of 0.9589 was lower than that reported by other investigators but is still within acceptable limits [12]. The future studies will be performed in order to enhance the sensitivity of the sensor by modifying the surface of the gold working electrode.

4. Conclusions
A portable amperometric immunosensor based on an integrated three electrode system was developed. This system provides fast response times, on-field measurement and requires only small volumes of sample. Further investigation will focus on modification of surface electrodes to enhance the binding
of antigen to antibodies thus improving the sensitivity of the sensor towards PAHs. Analysis on real sample water will be performed using this immunosensor.

References
[1] Diaz-Gonzalez M, Gonzalez-Garcia M B and Costa-Garcia A 2005 *Electroanalysis* 17 1901
[2] Wang J 2002 *TrAC Trends in Analytical Chemistry* 21 226
[3] Monroe D 1990 *Critical Reviews in Clinical Laboratory Sciences* 28 1
[4] Wang J 2000 *Analytical Electrochemistry* (New York, John Wiley & Sons)
[5] Moore E J, Kreuzer M P, Pravda M and Guilbault G G 2004 *Electroanalysis* 16 1653
[6] Polk B J, Stelzenmuller A, Mijares G, MacCrehan W and Gaitan M 2006 *Sensors and Actuators B: Chemical* 114 239
[7] Moore E, Rawley O, Wood T and Galvin P 2009 *Sensors and Actuators B: Chemical* 139 187
[8] Li K, Chen R, Zhao B, Liu M, Karu A E, Roberts V A and Li Q X. *Analytical Chemistry* 71 302
[9] Duan C and Meyerhoff M E 1994 *Analytical Chemistry* 66 1369
[10] Kim H R, Kim Y D, Kim K I, Shim J H, Nam H and Kang B K 2004 *Sensors and Actuators B: Chemical* 97 348
[11] Se-Ik P, Sang Beom J, Sejin P, Hee Chan K and Kim S J *Sensors Journal IEEE* 3 267
[12] Fahnrich K A, Pravda M and Guilbault G G 2003 *Biosensors and Bioelectronics* 18 73