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Design and characterisation of a thin-film electrode array with shared reference/counter electrodes for electrochemical detection

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ABSTRACT

In the current study, a novel electrode array and integrated microfluidics have been designed and characterised in order to create a sensor chip which is not only easy, rapid and cheaper to produce but also have a smaller imprint and good electrochemical sensing properties. The current study includes the assessment of the effects of an Au quasi-reference electrode and the use of shared reference/counter electrodes for the array, in order to obtain a small array that can be produced using a fine metal mask. In the study, it is found that when Au is used as the quasi-reference electrode, the arrays with shared reference and counter electrodes result in faster electron transfer kinetics and prevent the potential change with respect to scan rate, and hence is advantageous with respect to conventional electrodes. In addition, the resulting novel electrode array has been shown to result in higher current density (10.52 $\mu A/cm^2$; HRP detection assay) and measured diffusion coefficient (14.40×10^{-12} cm^2/s; calculated from the data of cyclic voltammetry with 1 mM potassium ferricyanide) with respect to conventional electrodes tested in the study. Using the new electrode arrays, the detection limits obtained from horse radish peroxidase (HRP) and bisphenol A assays were 12.5 ng/ml (2.84×10^{-10} M) and 10 ng/ml (44×10^{-9} M), respectively. Performing the HRP detection assay in a flow injection system using array integrated microfluidics provided 25 times lower detection limit (11.36×10^{-12} M), although Ti has been used as electrode material instead of Au. In short, incorporation of this new electrode array to lab-on-a-chip or MEMs (micro-electro mechanic systems) technologies may pave the way for easy to use automated biosensing devices that could be used for a variety of applications from diagnostics to environmental monitoring, and studies will continue to move forward in this direction.

1. Introduction

Biosensors have been envisioned to play a significant analytical role in diagnostics, bioprocesses, quality assurance in agriculture and food industries, environmental monitoring and homeland security. All of the mentioned sectors have considerable market size. One example is the global food safety testing market by contaminants which is estimated to grow at an annual growth rate of 10.46% to $2.5 billion in 2015 (MarketPublishers, 2011). Another important application area is in vitro diagnostics (IVD) with a yearly worldwide market of $42 billion in 2007 (KaloramaInformation, 2008). Therefore, further research to develop new biosensor devices for food safety and diagnostics has a great socio-economic significance. The detection methodology of biosensors is quite varying and ranges from optical (e.g., fluorescence detection, SPR, interferometry, optical grating) and piezoelectric to electrochemical (e.g., amperometric, impedimetric, voltammetric) transducers (Becker and Cooper, 2011; Cooper, 2002; Hintsche et al., 1994; Homola, 2006; Sheikh et al., 2008; Turner, 2000). High sensitivity, selectivity, rapid analysis, the ability to operate in turbid solutions and the possibility of miniaturisation enabled electrochemical biosensors to became the most widely used biosensors (Shah and Wilkins, 2003) (Newman and Turner, 2005). The design of an electrochemical biosensor involves careful consideration of many parameters such as electrode design, sensor surface chemistry, recognition element immobilisation on electrode surface, assay conditions and enzyme/mediator selection (Borgmann et al., 2011).

Despite the numerous advantages of electrochemical sensors and many years of scientists' efforts, important hurdles in their development still remain. A key issue that needs to be addressed is the development of electrode probes that can be fabricated into useful arrays for multiplex detection (Drummond et al., 2003). Electrochemical measurements usually involve a three electrode system that consists of a working, counter and reference electrode.

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Traditional electrochemical measurements involve the use of Ag/AgCl as the reference electrode. This functions as a redox electrode for the reaction between Ag metal and its salt, AgCl. This reference electrode is usually formed by a glass tube containing a silver wire that is coated with a thin layer of silver chloride (Czaban and Rechnitz 1976). Although this reference electrode provides fast electrode kinetics, it is not practical when portable, smaller and single use electrochemical biosensors are required. Thus, a new type of electrochemical chip that comprises planar electrodes in the form of either screen printed electrodes (SPEs) or thin film electrodes has gained widespread use. SPEs are printed using inks (carbon or metals together with a polymer matrix) on plastic or ceramic substrates (Hart and Wring, 1994) and thin film electrodes are deposited on glass or silicon wafers by means of evaporation or sputtering methods. Thin film technologies enable the manufacturing of electrodes with high precision and resolution. If micron sized electrodes need to be produced a photolithography process is required (Wu et al., 1993); otherwise a fine metal mask is used to create the electrode patterns on substrates (Zhou et al., 2003). By a sequential process, different metal inks can be printed on SPE; although carbon and gold inks are the most widely used working or counter electrode materials, as pseudo-reference electrodes, Ag/AgCl ink is widely used. However, in the case of thin film electrodes, it is not possible to evaporate or sputter Ag/AgCl to form a reference electrode. Therefore, for thin film electrodes, as an alternative, application of quasi-reference electrodes in the form of Au or Pt has gained some use. While some studies exist that investigate the usability of Ag/AgCl pseudo electrodes, the studies that examine the effects of quasi-reference electrode on electrochemical measurements are rather limited (Kasem and Jones, 2008).

A number of studies have been performed to assess the electrochemical properties of microelectrodes with different geometries and sizes (Guo and Lindner, 2009; Kurita et al., 2000). From these studies it was observed that, although microelectrodes do have several advantages over macro-electrodes (Stett et al., 2003), such as higher current density, smaller sensor footprint, and higher diffusion coefficient, the most obvious disadvantage includes their higher impedance due to interfacial capacitance, which results in very low currents (within or below nano-ampere range) (Ordeig et al., 2008) and their need for expensive and time consuming fabrication involving photolithography (Fiaccabrino and Koudelka-Hep, 1998). Especially if single use sensors are considered for end user applications such as diagnostics, food or environmental testing, cheaper and less time consuming production procedures need to be considered. For this reason, in the current study a novel electrode array has been designed and characterised in order to create a sensor chip which is not only easy, rapid and cheaper to produce but also has a smaller imprint and good electrochemical sensing properties. The current study includes the assessment of the effects of an Au quasi-reference electrode and the use of shared reference/counter electrodes for the array. In addition, this study investigates the electrochemical performance of new electrode arrays by means of an enzyme (horse radish peroxidase, HRP) and bisphenol A detection assays.

2. Materials and instrumentation

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) tablets, mercaptoethanol, mercaptoundecanoic acid (MUDA), ethanolamine, spectrophotometric grade ethanol, horse radish peroxidase (HRP), TMB ready to use reagent (containing H_2O_2), bisphenol A and potassium ferricyanide were purchased from Sigma-Aldrich (Poole, UK). Potassium chloride (KCl) was purchased from Fisher Scientific (Loughborough, UK). Oxygen free argon was purchased from Habas (İstanbul, Turkey). Ultrapure water (18 MΩ/cm) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan).

3. Methods

3.1. Transducer fabrication

Three electrode arrays were designed. **Design 1** consists of eight Au electrodes of different sizes (diameter sizes: 1.5, 2, 3, and 4 mm) and each has its own Au counter and quasi-reference electrode. **Design 2** consists of eight Au electrodes of different sizes (diameter sizes: 1.5, 2, 3, and 4 mm) and all share the same Au counter and quasi-reference electrodes. **Design 3** consists of eight Au electrodes of 1.5 mm diameter and all share the same Au counter and quasi-reference electrodes. An electron beam evaporator device was used to deposit Ti and Au metals on the glass slides. The designs of the electrodes were formed on the glass slides by means of Fine Metal Masks made of a laser cut patterned stainless steel. Before the application of Au (200 nm), a 20 nm Ti layer is applied on to the fine glass slides as an intermediary adhesive layer to increase the adhesion between Au and glass slide. A flow cell was designed and fabricated using PMMA to be used for the electrode arrays.

3.2. Electrochemical analysis

Cyclic voltammetry measurements were performed with a Dropsens MicroStat 8000 Electrochemical Analyzer with the general purpose electrochemical software Dropview 1.4 (Dropsens, Astuias, Spain). The electrochemical analyzer and the purpose built shielded cables enable simultaneous electrochemical measurements of eight electrodes. Cyclic voltammetry (CV) tests were performed using 1 mM potassium ferricyanide solution in 1 M KCl.

3.3. Assay

Bisphenol A detection assay was performed by injecting bisphenol A (in PBS) at varying concentrations on to the plasma cleaned bare Au electrode array. After bisphenol A injection, the flow stopped and 0.5 V potential has been applied to the electrode arrays for 60 s and the current vs. time plot has been obtained. The current value at the 60th second of the test has been recorded as sensor response. For the horse radish peroxidase (HRP) assays, initially plasma cleaned bare Au electrode arrays were immersed in ethanolic solution of 2 mM mercaptoundecanoic acid (80%) and 2 mM mercaptoethanol (20%) mixture for overnight. Later the electrode arrays were rinsed with ethanol and water. After drying with nitrogen stream, the arrays were stored at $+4\,^{\circ}C$ till use. Enzyme assays were performed by mixing HRP and TMB reagent, then injecting on to the flow cell containing electrode arrays (Fig. 1). The chronoamperometric responses obtained at -0.1 V potential at 60 s of the measurements were used as assay response.

Three data points were used to obtain the mean and standard deviation of the results. The limit of detection (LOD) was calculated as the signal obtained from the assays that is equivalent to three times the standard deviation of the signals obtained from the blank standards.

4. Results and discussion

4.1. Cyclic voltammetry

To investigate the electrochemical behaviour of the designed electrode arrays, a cyclic voltammetry technique has been utilised

and voltammograms were recorded for a model electroactive species, potassium ferricyanide. Typical voltammograms for the three designs of electrode arrays are presented in Fig. 2. Au thin film electrodes with Au quasi-reference electrodes had shown oxidation and reduction peaks for potassium ferricyanide similar to those generated when Ag/AgCl was used as a reference electrode (Fig. 2(A)). The **design 3** electrode array consists of eight working electrodes with shared counter and reference electrodes. For the array to be potentially used as a multianalyte detection sensor, each electrode within the array has to result in the same electrochemical response irrespective of their position in the array. Therefore, as can be seen from Fig. 2(C), all eight electrodes within the same array displayed the same voltammogram, indicating the suitability of the array for multiplexed measurements.

4.2. Scan rate dependence of peak current

The observed peak current on the forward potential scan of the cyclic voltammetry measurements is given for the case of reversible electron transfer by the Randles–Sevcik equation (at 25 °C)

$$I_p = 0.4463nFAC\left(\frac{nF\nu D}{RT}\right)^{1/2} \quad (1)$$

where I_p is peak current, n is number of electrons transferred in the redox event, A is electrode area, F is Faraday constant, D is diffusion coefficient, C is concentration, ν is scan rate, and $T=298$ K.

The Randles–Sevcik equation defines a linear correlation between the peak current and the square root of the scan rate for a reversible electron transfer (Wang, 2006). Thus, plots of I_p and $\nu^{1/2}$ are a very convenient tool to characterise the electrochemical reversibility of a given redox system using a selected electrode system. The irreversible reactions result in slow reaction kinetics which means the equilibria are not reached rapidly with respect to the voltage scan rate, and this causes the shift in the maximum current as the voltage scan rate is changed. Three parameters are generally derived from cyclic voltammetry measurements in order to characterise a reversible process:

- the peak potential separation $\Delta E_p = E_{pc} - E_{pa} = 59/n$ mV at all scan rates at 25 °C;
- the peak current ratio is $I_{pa}/I_{pc} = 1$ at all scan rates;
- the peak current function $I_p/\nu^{1/2}$ is independent of ν.

The cyclic voltammograms obtained from the use of three different electrode array designs have been analysed to assess the effect of electrode design and the reversibility of the reaction on the electrode surface in light of these three parameters. The peak potential separation of **designs 1**, **2** and **3** electrodes was calculated to be 91 mV, 56 mV and 87 mV, respectively. For all the designs I_{pa} and I_{pc} ratio was found as 1, as expected from a reversible reaction. For all the electrode array designs, the increase of cyclic voltammetry scan rate resulted in an increase of peak current obtained from the measurment, as expected from a reversible reaction. However, when the scan rate was increased, a shift in the peak potential was observed for **design 1** (Fig. 3(A)). No shift in peak potential was observed for other designs (Fig. 3).

The electrode array designs have also been compared in terms of measured diffusion coefficient that has been calculated using Eq. 1. As seen from Table 1, electrode arrays of **design 3** with its 1.5 mm diameter electrodes resulted in the highest measured diffusion coefficient (14.40×10^{-12} cm^2/s), indicating that this design can be preferred over others for electrochemical detection.

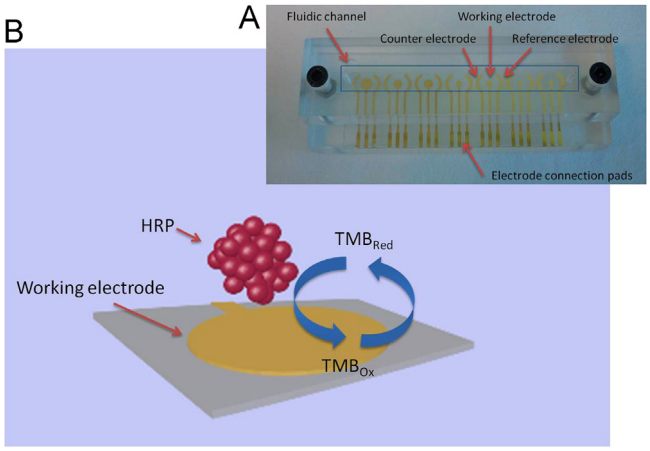

Fig. 1. (A) **Design 1** electrode array consists of eight Au electrodes of different sizes (diameter sizes: 1.5, 2, 3, 4 mm) and each has its own Au counter and quasi-reference electrode. (B) Enzyme assays were performed by mixing HRP and TMB reagent, and then injecting to the flow cell containing electrode arrays.

4.3. Au quasi-reference electrode

In the current study, a shorter/quicker and a lower-cost protocol has been utilised for electrode array fabrication by using the Fine Mask Method instead of photolithography. In addition to this, a new electrode array has been designed in which shared reference and counter electrodes have been used, minimising the size of the sensor. This has eliminated the main disadvantage of

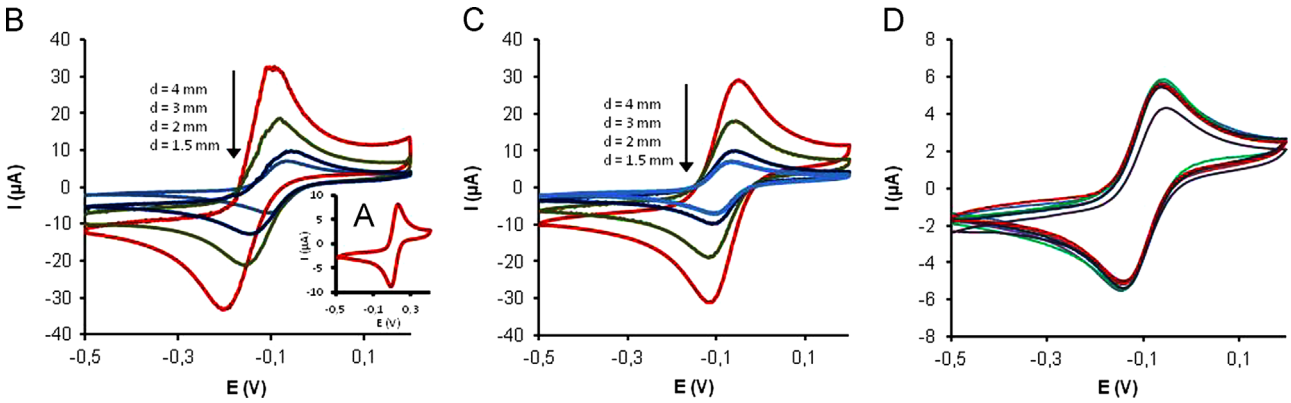

Fig. 2. Cyclic voltammetry (*CV*) has been performed with 1 mM $K_4[Fe(CN)_6]$/KCl at 100 mV/s scan rate, using (A) conventional screen printed Au electrode with Ag/AgCl pseudo-reference electrode, (B) **design 1** and (C) **design 2** arrays containing eight Au electrodes with diameters 1.5, 2, 3 and 4 mm; and (D) **design 3** array containing eight Au electrodes with diameter 1.5 mm.

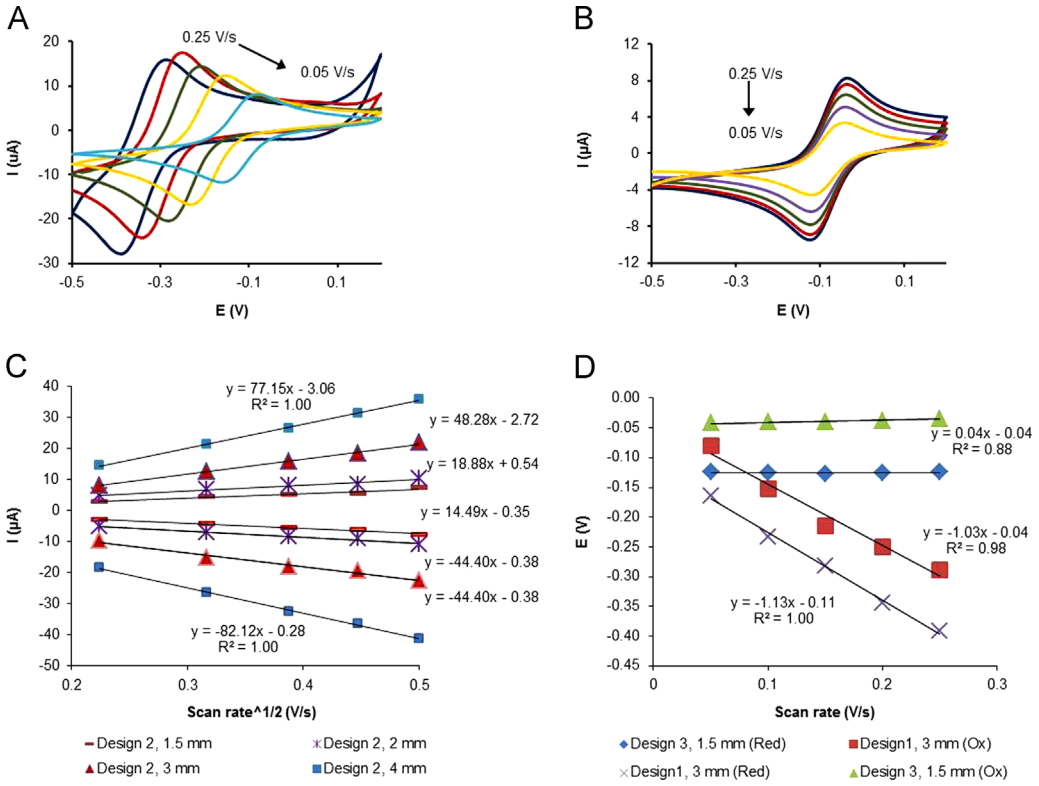

Fig. 3. The *CV* of **design 1** ($d=3$ mm) (A) and **design 3** ($d=1.5$ mm), (B) arrays at varying scan rates: 50, 100, 150, 200, 250 mV/s (inner to outer *CV* traces, respectively). The results of cyclic voltammograms at varying scan rates have been used to obtain scan rate$^{1/2}$ vs. oxidation/reduction current (C), and scan rate vs. oxidation/reduction potential (D).

Table 1
The measured diffusion coefficient for three electrode array designs.

Electrode type	Measured diffusion coefficient (cm^2/s)
Design 2 – 1.5 mm	9.30×10^{-12}
Design 3 – 1.5 mm	$\mathbf{14.40 \times 10^{-12}}$
Design 1 – 2 mm	6.90×10^{-12}
Design 2 – 2 mm	5.00×10^{-12}
Design 1 – 3 mm	3.05×10^{-12}
Design 2 – 3 mm	6.45×10^{-12}
Design 2 – 4 mm	5.21×10^{-12}

larger macroelectrodes. The important factors for this miniaturisation are the effect of shared reference and counter electrodes on the electrochemical response obtained from the sensors and the use of an Au quasi-reference electrode. The experiments show that measured diffusion coefficient is higher when smaller electrodes are utilised. Here the significance is that different designs (individual [**design 1**] and shared electrode design [**design 2**]) behaved similarly in terms of measured diffusion coefficient. The arrays with shared reference and counter electrodes (**designs 2** and **3**) have been found more advantageous with respect to **design 1** electrode arrays, since **designs 2** and **3** do not cause a potential peak shift when the scan rate is increased. Although we are not sure about the reasons of this result, one interpretation could be the use of Au quasi-reference electrodes for testing in **design 1**, as suggested by Prakash and colleagues (Prakash et al. 2008). However, as we change the electrode array to '**design 2** or **3**' with shared Au quasi-reference electrode, this problem disappears. This indicates that use of shared Au quasi-reference electrode provides better potential control during the reaction between the working and reference electrodes. From these results we may conclude that, when Au is used as quasi-reference electrode, the arrays with shared reference and counter electrodes (**design 2**) result in faster electron transfer kinetics and hence can be preferred with respect to **design 1** (more conventional electrodes) to be used as electrochemical sensors. Within **design 2** array, electrodes with 1.5 mm diameter have shown the highest current density and measured diffusion coefficient, which provided the basis for **design 3** electrodes. **Design 3** array provides a smaller sensor chip imprint that would be particularly useful when a flow cell is designed for sensing which allows the implementation of microfluidics for sample and reagent transportation to the sensor surface for automated, easy to use biosensing applications.

4.4. Assay performance

4.4.1. Bisphenol A detection

Bisphenol A (a potential endocrine disrupter) has been widely used as a major component in the production of polycarbonate and epoxy resins that are used to manufacture plastic food/water containers and cans. However, there is a risk of contamination of food due to the migration of bisphenol A from packaging to food and beverages. As a result, under EU regulations the maximum allowed concentration of bisphenol A in drinking water is 500 ng/ml (Commission of the European Communities, 1980). The current bisphenol A detection involves the use of gas chromatography (GC) and HPLC techniques. Both of these techniques are expensive, time consuming, require an expert user and a laboratory environment. An electrochemical sensor, on the contrary, may provide a quick, cheap and portable method for bisphenol A testing (Portaccio et al., 2010; Ruana et al., 1993). Amperometric detection is based on measuring the current generated by the oxidation or reduction of an electroactive species at a working electrode while the potential is kept constant. As bisphenol A is an electroactive substance, it is possible to detect its presence by means of amperometric measurements (Hiroi et al., 1999). Therefore, to assess the performance of electrode arrays developed and to

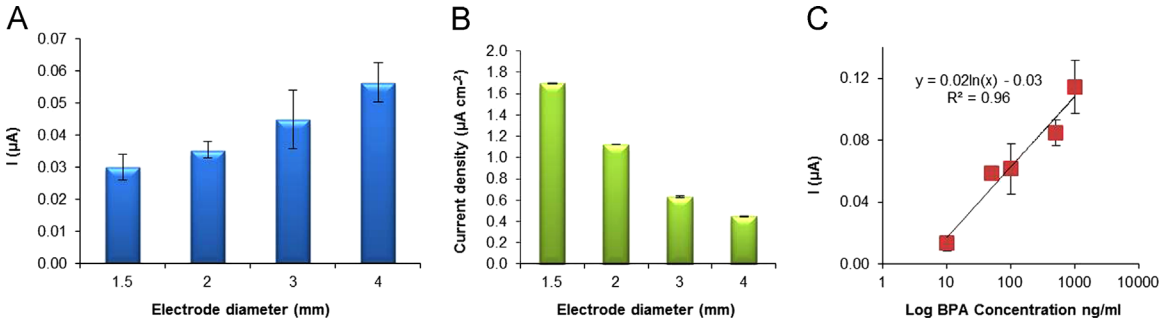

Fig. 4. Bisphenol A detection has been performed with chronoamperometry technique (0.5 V) using **design 2** (A and B) and **design 3** (C) electrode arrays and the current vs. BPA concentration obtained from the tests was shown in figures.

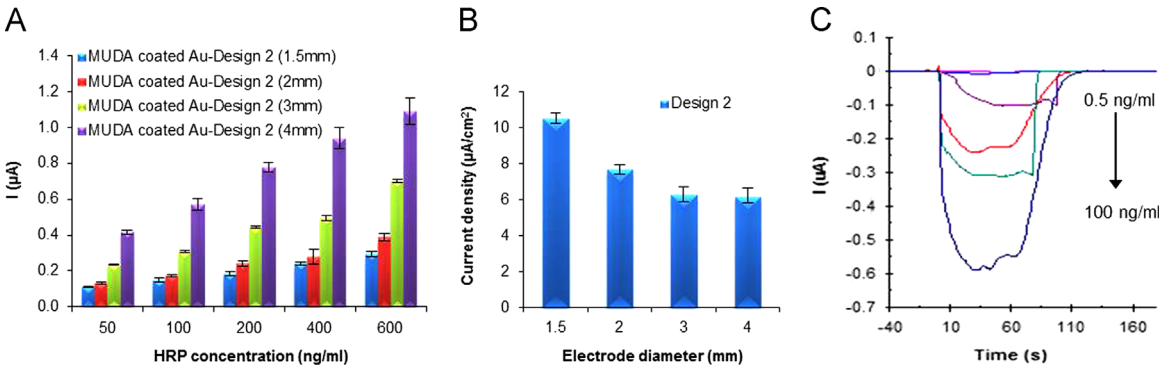

Fig. 5. (A) Enzyme assays were performed by mixing horse radish peroxidase (HRP) and TMB reagent, and then injecting to the MUDA coated electrodes in a flow cell for chronoamperometry measurement. The current measurements were taken after 60 s of the reaction at −0.1 V potential. (B) The current response obtained from the chronoamperometry results after the addition of 200 ng/ml HRP and TMB reagent was used to assess the current density on different sized electrodes. (C) The HRP assay was repeated using fluidics integrated **design 3** sensor chips with Ti electrodes and amperometric measurement was taken in real time during 200 μl/min flow rate.

investigate their potential use for bisphenol A testing, an assay has been performed using the **designs 2** and **3** electrodes.

Initally, 500 ng/ml bisphenol A has been injected to the **design 2** electrodes and the resulting current at 0.5 V on different sized electrodes was obtained (Fig. 4(A)). Later, the current response obtained from the chronoamperometry results was used to calculate the current density on different sized electrodes. As seen from Fig. 4(B), 2 mm, 3mm and 4 mm diameter sized electrodes showed 34%, 63% and 74% lower current density with respect to 1.5 mm diameter sized electrodes. In order to create a calibration curve for bisphenol A detection, varying concentrations of bisphenol A were injected to **design 3** sensor arrays and chronoamperometric measurements were taken. As can be seen from Fig. 4(C), the chronoamperometric test resulted in a detection limit of 10 ng/ml bisphenol A (44×10^{-9} M) with a linear range between 10 and 1000 ng/ml. This is well below the acceptable threshold level of bisphenol A in drinking water. This result supports the capability of the **design 3** electrode array as a convenient tool for bisphenol A testing in water samples, and hence further studies will continue to build a novel sensing device based on this array for commercial use.

4.4.2. HRP detection assay

To assess the amperometric performance of the electrodes designed, an enzyme assay has been performed by the injection of HRP and its substrate TMB reagent to the MUDA coated electrodes in a flow cell. The Au surface of the electrodes was coated with a self assembled monolayer (MUDA) to prevent the adsorbance of HRP on the electrode array surface and also to mimick a recognition element (antibody, DNA, etc.) immobilised on the electrode, in other words an electrode with a reduced electrochemically active area. Higher current was obtained from the chronoamperometric measurements on larger electrodes irrespective of the electrode array design (Fig. 5(A)). The current response obtained from the chronoamperometry results after the addition of 200 ng/ml HRP and TMB reagent was used to assess the current density on different sized electrodes. As seen from Fig. 5(B), 2 mm diameter sized electrodes showed 27% lower current density, 3 and 4 mm diameter sized electrodes showed ~40% lower current density with respect to 1.5 mm diameter sized electrodes. The detection limit of HRP assay performed with **design 3** electrode arrays has been 12.5 ng/ml (2.84×10^{-10} M) (data not shown). This result also confirmed that the newly designed electrode array (**design 3**) with Au quasi-reference electrode and shared reference/counter electrodes can be used as a convenient tool for amperometric measurements.

4.4.3. HRP detection assay with flow injection system

Towards the realisation of an electrochemical device for use at the point of care, it is essential to miniaturise the electrode array and integrate it with a microfluidic system. Therefore based on the **design 3** electrodes, a new electrode array has been fabricated that consists of 1 mm diameter electrodes and was formed by the evaporation of Ti on a silicon dioxide wafer. Ti was tested as an alternative electrode material as it is cheaper than Au. Also a sensor casette made of PMMA was designed and fabricated, that created a fluidic channel on the electrodes. PBS was used as running buffer in between the injections of HRP and its substrate TMB reagent. After testing different flow rates, 200 μl/min was chosen for the assay. As seen from Fig. 5(C), performing the assay in fluidics environment enabled the detection of HRP down to 0.5 ng/ml (11.36×10^{-12} M) (25 times lower than the assay performed in static), although this time Ti instead of Au has been used as the electrode material. The performance of the Ti electrode array in a fluidic assay also outperformed in terms of current

density (50.96 μA/mm^2), five times higher than the assays when performed using **design 3** electrodes in static mode. A similar study has been performed by Fanjul-Bolado and colleagues where they have used a flow injection system and carbon SPE for the detection for HRP detection (Fanjul-Bolado et al. 2005). The detection limit achieved after 15 min of HRP and TMB incubation was 2×10^{-14} M of HRP. In another study, the HRP detection has been achieved down to 4.8×10^{-11} M, only after 30 min incubation with enzyme substrate (Fanjul-Bolado et al. 2004). While the HRP and substrate have been incubated for 15 min or 30 min in the studies discussed above, in the current study the picomolar determination of HRP has been achieved without prior incubation of HRP and substrate, indicating the superior performance of the electrode array used. These results prove the performance of both the electrode arrays designed and the clear advantage of the flow injection assay format.

5. Conclusion

In the study, a new electrode array has been fabricated and its performance has been compared to a more conventional electrode design. The electrode array, **design 3**, consists of eight working electrodes (1.5 mm diameter) with shared Au quasi-reference electrode and shared counter electrodes. The proposed novel electrode array is easy to fabricate, has a small imprint allowing fluidic system integration, enables multiplexed measurements and performs well in terms of electrochemical detection as shown through HRP and bisphenol A detection assays. Therefore, it could be used as a convenient tool to fabricate portable, convenient, fast, sensitive, low cost and automated sensing devices for a variety of applications including diagnostics, environmental monitoring and food quality control. Our further work involves the use of new electrode arrays for different applications and also the integration of electrode array and its fluidics cassette to an electrochemical analyser to obtain an automated biosensor device.

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