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Electrochemical biosensor microarray functionalized by means of biomolecule friendly photolithography

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ABSTRACT

Microfabrication permits the incorporation of dense electrode arrays in microsystems and small volume diagnostic devices. However, the specific functionalization of arbitrary shape electrodes with different biomolecules remains a challenging issue. In the present work, the problem of fabricating closely spaced microelectrodes (20 μm sensor diameter and 20 μm-spaced interdigitated electrodes array) that can be modified selectively in order to create multi-analyte sensor arrays is addressed by employing a biomolecule friendly photolithographic procedure for the sequential immobilization of different biomolecules onto separated electrodes of the same array. The concept was demonstrated with selective detection of oligonucleotides for breast cancer gene mutation detection, the hormone T4 detected with specific antibodies and sarcosine and glucose detected with specific enzymes immobilized in two-analyte arrays in order to assure that the method is compatible with all the types of biorecognition molecules used in biosensors. Electrochemical techniques were used in this array, because of the low cost, high sensitivity and easy miniaturisation of these transducers. Although the array was composed of only two sets of electrodes, the results demonstrate that the method proposed is generic and could be used for patterning of electrochemical multi-analyte biosensors at even higher resolution.

1. Introduction

Demand for multi-analyte sensing devices has been increasing for the last years due to their potential applications in biomedicine, biotechnology, industry and environmental analysis (Albers et al., 2003; Brian et al., 1997; Maestre et al., 2005; Mir et al., 2008; Jia et al., 2004; Wittstock, 2002; Brecht, 2005; Mir and Katakis, 2008; Wilson and Nie, 2006; Zen et al., 2002; Cho et al., 2002; Taitt et al., 2002). When compared to the single analyte assays, these multi-analyte devices allow simplification of the analysis procedure, decrease of the testing time and cost and improvement of the test efficiency.

In order to perform multi-analyte assays, various approaches have been proposed. Among them, the fabrication of microelectrodes on a suitable substrate and the immobilization of different biofunctional molecules on these microelectrodes appear attractive. Microelectrodes with complex and arbitrary shapes and sizes can be patterned on wafers with excellent reproducibility using modern photolithography techniques, which permit the production of transducer arrays with very small dimensions. One of the most prominent examples of microelectrodes designs is an interdigitated microelectrode array. Interdigitated electrode (IDE) arrays have been used as highly sensitive detectors because of their inherent features, such as larger currents, high sensitivity, and rapid current rise to a steady state (Padeste et al., 2004; Cohen and Kunz, 2000; Postlethwaite et al., 1996; Radke and Alocilja, 2005; Hintsche et al., 1994; Zhu et al., 1994; Min and Baeumner, 2004; Jin et al., 2001; Zhang et al., 2000; Wang and Chen, 1994; Sandison et al., 2002; Pearce et al., 2005). IDE array consists of many parallel bands of electrodes, each separated by a small insulating gap.

In multi-analyte biosensing devices, the biomolecule patterning step on electrodes separated by few micrometers appears especially challenging. A good spatial control during biomolecule deposition step is strictly necessary; each biomolecule has to be precisely deposited on the surface of the relevant sensor without losing its biofunctionality and avoiding mixing that can deteriorate the biosensor specificity.

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One of the main problems in arrays with short gaps between electrodes is the crosstalk between them. Crosstalk occurs when there are freely diffusing species detectable at adjacent microelectrodes (Frebel et al., 1997; Strike et al., 1995; Smistrup et al., 2005; Yu and Wilson, 2000). In order to avoid this problem, different approaches have been developed like using interference-suppressing biomolecule layers (Kojima et al., 2003), electrolysis of the interference-causing compound at band electrodes placed between the individual sensors (Quinto et al., 2001) and blocking agents (Yu et al., 2006).

In the present work, different biorecognition elements were patterned to perform a DNA sensor, an immunosensor and an enzymatic sensor on IDE arrays by biomolecule friendly photolithography (Douvas et al., 2001, 2002; Petrou et al., 2007; Diakoumakos et al., 2002). The photoresist used is based on a methacrylate copolymer that allows processing at mild conditions. It is synthesized from four methacrylate monomers using free radical polymerization (Diakoumakos et al., 2002), and a photoacid generator sensitive at $\lambda > 300$ nm. This photoresist was processed under conditions that do not cause intolerable denaturation of the immobilized biomolecules. In previous publications, this photoresist polymer was tested with immobilized proteins labelled with fluorophores (Douvas et al., 2001, 2002; Petrou et al., 2007; Diakoumakos et al., 2002). In this work, we go a step forward in order to test this biocompatible photolithographic technique with all the different biorecognition molecules used in biosensors and detecting targets for real applications. In these experiments, electrochemistry was chosen for the array detection, due to the high sensitivity, low cost and easy miniaturisation of this technique.

The biosensor selective response and the crosstalk of the modified IDEs were tested chronoamperometrically by injecting the specific substrate. It is demonstrated that an amperometric, interference and crosstalk free, dual electrode biosensor can be used indifferently for DNA, enzyme and antigen detection in an array format. The integrated device showed high specificity and sensitivity, possibility of multi-biorecognition, absence of crosstalk and complete suppression of electroactive interferences. Since current densities remain high along with the absence of crosstalk, the possibility of creating a sensor that is competitive with current market technology is envisioned.

2. Experimental

2.1. Materials

The AZ 5214 photoresist and the tetramethyl ammonium hydroxide (TMAH) based AZ MIF726 developer was purchased from Clariant and the etching solution for the chromium (UTE-1) was supplied by Cyantek. The biocompatible photoresist was synthesized as reported previously (Diakoumakos et al., 2002). NaCl and H_2O_2 were purchased from Panreac and NaH_2PO_4 from Aldrich, whereas anti-digoxigenin-horseradish peroxidase (anti-digoxigenin-HRP) and anti-digoxigenin-alkaline phosphatase labelled antibody (anti-digoxigenin-ALP) were purchased from Roche. Rabbit anti-T4 antiserum (anti-T4) was purchased by OEM Concepts Inc. Bovine IgG-T4 conjugate (BIgG-T4) was prepared following a previously published procedure (Georgiou and Christofidis, 1996). T4 standard solutions (0–230 ng mL^{-1}) were prepared in T4-free human serum (Scantibodies, San Diego, CA). Glucose oxidase (GOx) (200 U mg^{-1} from *Aspergillus niger*) and sarcosine oxidase (SOx) (20 U mg^{-1} from recombinant *E. coli*) were acquired from Biozyme. Glucose, sarcosine, polystyrene sulphonate (PSS), polyvinyl pyridine (PVP), thioctic acid, *N*-succinimidyl ester dithio dithiopropionic acid (DTSP), 3-mercapto-1-propane-sulfonic acid (MPSA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC), sodium salt citrate buffer (SSC), trizma hydrochloride (Tris–HCl), polyethylene glycol sorbitan monolaurate (Tween 20), ethylendiaminetetraacetic acid (EDTA), *N*-Succinimidyl-S-acetylthioacetate (SATA), p-aminophenyl phosphate, 2-mercaptoethanol, goat anti-rabbit IgG antibody HRP labelled, SA HRP labelled, thyroxine (T4) and the oligonucleotides were purchased from Sigma. The redox copolymer was synthesized as reported in the literature (Gregg and Heller, 1991).

2.2. Methods

2.2.1. Fabrication details

The patterning of the gold IDE array consists of 50 microelectrodes with a width of 20 μm and a distance between electrodes of 20 μm and it was carried out with photolithographic techniques. All the details of this procedure are described in Supplementary information.

In the patterning of the IDE array with biomolecules, all the process steps after the biomolecules deposition were carried out below 50 °C in dilute aqueous base solution of TMAH (concentration smaller than 0.26×10^{-3} N) and by exposure at wavelengths above 300 nm, so as to avoid denaturation of the already attached biomolecules.

The bio-photoresist was spin-coated on the IDE array wafer at 3000 rpm and then baked on a hot plate at 120 °C for 5 min and exposed on the same mask-aligner with a dose of 160 mJ cm^{-2}. The wafer was post-exposure baked at 50 °C for 3 min and developed in TMAH 1.04×10^{-3} N for 3 min. With this procedure the electrodes contained in the first pad were opened and the first type of biomolecules was attached to the opened electrodes. After the biomolecule attachment the exposure, post-exposure bake and development steps were repeated in order to open the electrodes connected to the second pad. After the attachment of the second biomolecule to these electrodes, the wafer was flood exposed in mask-aligner using a glass as a filter in order to cut-off all the wavelengths below ~300 nm. Then, the wafer was thermally treated at 50 °C for 5 min and developed in 2.6×10^{-3} N TMAH for 5 min. With this procedure all the remaining photoresist film was stripped away, leaving the gold electrodes ready to detect the analytes of interest. The figure below shows the basic steps followed for the photolithographic patterning of two biomolecules on the IDE array (Fig. 1).

2.2.2. DNA patterning on the electrodes

2.2.2.1. Bienzyme-oligonucleotide patterning detection. Following the procedure described above the bio-photoresist was removed from the top of the first set of electrodes while the other set remained protected. A thiol-oligo–HRP conjugate was formed by mixing 0.2 μg mL^{-1} of thiol-oligo-digoxigenin and 1:2000 of anti-digoxigenin-HRP antibody and was incubated for 1 h at 37 °C. 20 μL of this solution was mixed with 10 μL of 2 mg mL^{-1} redox copolymer and the mixture was placed on the IDE surface. The electrodes were incubated 2 h for the formation of dative binding between thiol-group in the oligonucleotides–HRP conjugate and the gold surface. The wafer was washed and the unmodified surface was blocked with 1 mM mercaptoethanol for 1 h at room temperature. The second set of electrodes was UV exposed as described above. On the second set of electrodes, a thiol-oligo–ALP conjugate and redox polymer were immobilized following the same procedure as with the first electrode set. After each biomolecule immobilization, the array was measured amperometrically with a bi-potentiostat.

The electrochemical cell is a drop of 100 μL of 50 mM HEPES buffer pH 7.5, 200 mM NaCl (HEPES buffer) on the IDE, which is in contact with the reference (Ag/AgCl) and counter electrode (Pt). A voltage of −100 mV was applied in the first set of electrodes where

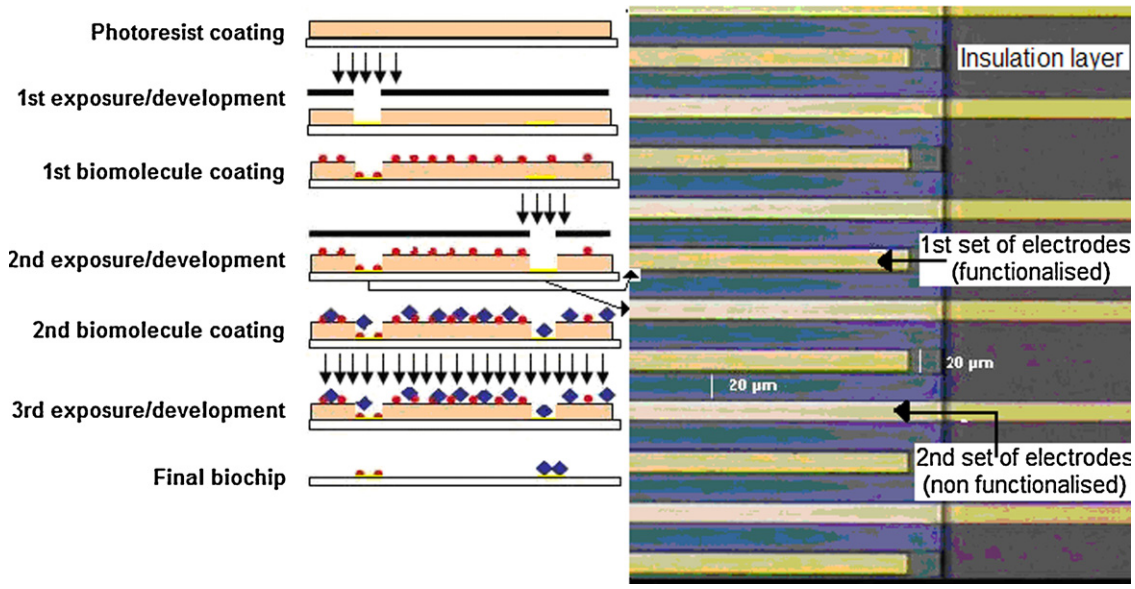

Fig. 1. Bio-photolithographic process for biomolecules patterning on the electrode array. On the right, gold IDE array detail; each set of electrodes consists of 50 microelectrodes with a width of 20 μm and a distance between electrodes of 20 μm.

the oligonucleotide–HRP conjugate was immobilized and +300 mV in the second set of electrode where the oligonucleotide-ALP conjugate was attached. Both substrates, hydrogen peroxide for HRP detection and p-aminophenyl phosphate for ALP detection were added (Fig. 2). In addition, the mentioned potential values applied to the electrodes were mutually exchanged between the electrodes in order to detect the non-specific adsorption and crosstalk.

2.2.2.2. Amperometric detection of BRCA1 gene mutations. On the first set of electrodes (uncovered after exposure and development of the bio-photoresist), a solution of 40 μg osmium copolymer, 5 μg poly(ethylene glycol) (PEG) and 2 μg streptavidin (SA) was placed for 1 h at room temperature. The wafer was washed and incubated with a 1 μM solution of biotinylated probe corresponding to mutant-type sequence in 50 mM phosphate buffer, pH 7, 150 mM NaCl (PBS buffer), for 30 min at room temperature and then the unreacted SA molecules on the surface were blocked with a 1 μM biotin solution in PBS buffer for 30 min at room temperature. The second set of electrodes was then uncovered and the same mixture of copolymer, PEG and SA was placed on top of it and then incubated with a 1 μM solution of biotinylated probe corresponding to complementary type sequence and finally blocked in the same manner as with the first set of electrodes. The electrodes were then incubated with a 1 μM solution of biotinylated target oligonucleotide in HEPES buffer for 30 min at room temperature and then 0.25 μg mL^{-1} SA–HRP in HEPES buffer was left to react for 30 min at room temperature. After each step, the arrays were washed with the buffer used in the previous step

In order to measure the non-specific adsorption of the SA–HRP label on the sensor platform in the absence of target a control experiment was performed in electrodes modified with the wild-type sequence oligonucleotide without the addition of target oligonucleotide.

The arrays were measured amperometrically in the same manner as described in the previous section.

2.2.3. Enzymes patterning on the electrodes

In order to have a higher enzyme activity, stable immobilization of the enzymes and better analytical response, different methods were tested for the immobilization of GOx and SOx. In the first

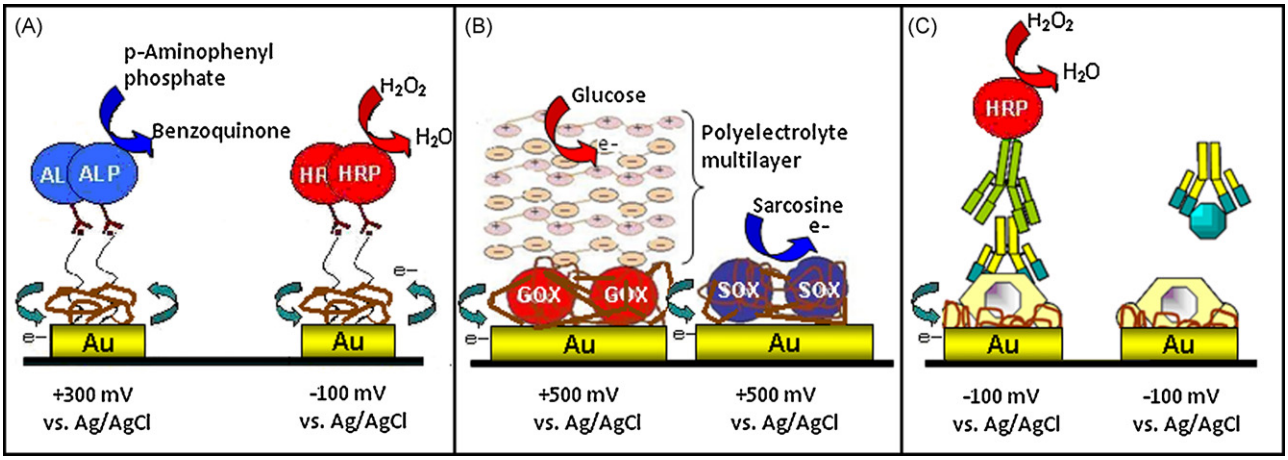

Fig. 2. Schematic representation of the three configurations constructed on the IDE; DNA with different enzyme labels, ALP and HRP immobilized on each electrode (A), sarcosine and glucose enzymatic sensors, using a polyelectrolyte multilayers blocking on the first set of electrodes in order to avoid the non-specific adsorption from the second enzyme (B) and competitive immunoassay for T4 detection (C).

method, both enzymes were modified by introducing thiol groups on the enzyme molecules using SATA. Then the electrodes were incubated with a mixture containing 30 μg of the thiolated GOx and 30 μg redox polymer for 2 h at room temperature using gold-thiol chemistry. In the second method, self-assembled monolayers (SAM) were formed by modifying the electrode with 0.1 M thioctic acid dissolved in 1:1 ethanol and water for 1 h. Then the thioctic acid monolayer was activated with 0.6 mg EDC for 30 min followed by the covalent interaction of the mixture containing 30 μg of GOx and 30 μg redox polymer for 2 h. In the third method, SAMs were formed on the gold electrode by incubating with 6 μL of 0.25 M MPSA and then linked covalently with the mixture containing 30 μg of GOx and 30 μg redox polymer for 2 h. In the fourth method, an enzyme polymer mixture (30 μg of GOx and 30 μg redox polymer for 2 h) was immobilized by covalent linkage on a 20 mM DTSP SAM formed for 1 h on gold electrode. Finally, in the fifth method, enzymes were immobilized by direct adsorption of a mixture containing 30 μg of GOx and 30 μg redox polymer for 2 h on the gold electrode. All the above experiments were also repeated on the second set of electrodes by substituting the GOx with the second enzyme, SOx.

The polyelectrolyte multilayers blocking on the first set of electrodes were deposited by sequential immersion of the electrode modified with GOx in PSS and PVP polyelectrolyte solutions. Fig. 2 shows the bienzyme patterning (GOx, SOx) of the interdigitated array using photolithography. Initially, the first IDE was exposed to remove the bio-photoresist and incubated with 40 μL mixture containing 200 U mL^{-1} of GOx and 0.5 mg of redox polymer for 2 h. At the end of this period, the electrode was carefully rinsed and self-assembled by alternate layers of oppositely charged polyelectrolytes. Cyclic voltammogram of IDE array was received in the presence of PBS buffer in order to characterize the redox polymer adsorption. Before surface modification on the second IDE, the protective layer of the bio-photoresist on the gold patterned surfaces was removed by UV exposure. The modification of the second set of electrodes was done by incubation with 40 μL of mixture containing 200 U mL^{-1} of SOx and 0.5 mg of redox polymer for 2 h.

Amperometric measurements of the modified electrodes were performed at +500 mV *vs.* Ag/AgCl in unstirred PBS buffer. Several additions of the substrate were made, in order to check the substrate specific response from the modified IDE array. Initially the response to glucose was checked to make sure that the enzyme GOx was functional even after the self-deposition of polyelectrolyte multilayers and then, the response of the IDE array to sarcosine was checked. Finally, simultaneous response to sarcosine and glucose was monitored in the same cell by first injecting sarcosine followed by glucose for crosstalk assessment.

2.2.4. Amperometric immunoassay on the patterned electrodes

The hormone T4 was amperometrically detected on the IDE microarray through a competitive assay employing a bIgG-T4 conjugate as solid-phase reagent. The first set of electrodes was used to detect the analyte in standard solution prepared in T4-free human serum of interest whereas in the second set of electrodes the response obtained in absence of analyte was determined using the zero standard. After removing the photoresist film from the first set of electrodes, a mixture of 40 μg osmium copolymer, 2 μg PEG and a 5 μg mL^{-1} bIgG-T4 solution was incubated on the electrode. The wafer was washed and blocked with a 1% (w/v) BSA solution in PBS buffer for 1 h at room temperature. After that, the electrode was incubated with a mixture of 1 volume of zero standard and five volumes of anti-T4 antibody diluted 1/5000 (v/v) in assay buffer (50 nm Tris–HCl, pH 7.8, 0.15 NaCl, 0.5% (w/v) BSA, 0.5% (w/v) Thimerosal, 0.05% (w/v) NaN_3). The photoresist film was removed from the second set of electrodes and was incubated with the same mixture of redox polymer, PEG and bIgG-T4 as in the first set of electrodes and blocked in the same manner. After that, it was incubated with a mixture of one volume of T4 standard (104 ng mL^{-1}) and five volumes of anti-T4 antibody diluted 1/5000 (v/v) in the assay buffer. The antibody–antigen interaction detection was carried out on both set of electrodes through incubation with 0.18 μg mL^{-1} of anti-rabbit-HRP (Fig. 2).

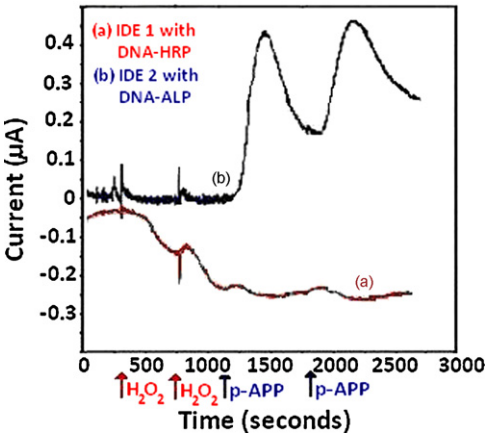

Fig. 3. Amperometric response of IDE array (area of 0.1 cm^2) where the first set of electrodes has been modified with oligonucleotide–HRP conjugate ($n = 2$; RSD = 0.006) (a) and the second set with oligonucleotide-ALP conjugate ($n = 2$; RSD = 0.04) (b) during sequential injection of the enzyme substrates (arrows).

The arrays were measured amperometrically using a bipotentiostat. The electrochemical cell consisted of a drop of 80 μL of 50 mM citrate buffer, pH 5.5, 200 mM NaCl on the working electrode, where reference (Ag/AgCl) and counter electrodes (Pt) were in contact with the drop. A 0 mV potential was applied in both set of electrodes and 1 μL of H_2O_2 in a final concentration of 100 mM was added into the cell to detect the HRP label.

3. Results and discussion

3.1. Amperometric detection of DNA patterned by biocompatible photolithography

3.1.1. Bienzyme-oligonucleotide patterning detection

During the photolithographic patterning process on the two electrode-sets array, there is a risk that the second biomolecule will be non-specifically adsorbed on the first set of electrodes. Therefore, before the detection of DNA hybridization, the non-specific adsorption of the second oligonucleotide on the first set of electrodes was tested. For this purpose, two different redox enzymes were used as DNA labels, HRP and ALP (Fig. 2).

Measurement of the enzymes response in each electrode for both substrates was carried out by using either each one separately, or a mixture of the two substrates. Fig. 3 shows the response obtained after the addition of the two substrates on the bienzyme DNA patterned array.

Two additions of 2.5 mM of H_2O_2 were made onto the electrochemical cell and, as it is shown in Fig. 3a, after which only the electrode where the oligonucleotides–HRP conjugate was immobilized responded. After the first and second injection, a reduction current of 84 and 75 nA was obtained respectively, current measured when a new baseline is reached 300 s after the injection. This slow response is as a consequence of the lack of stirring and therefore longer time is required for the substrate diffusion to the electrode surface. When 0.3 mM of p-aminophenyl phosphate was added into the cell, a current of 20 nA was recorded in the first set of electrodes just after the injection. However it returned

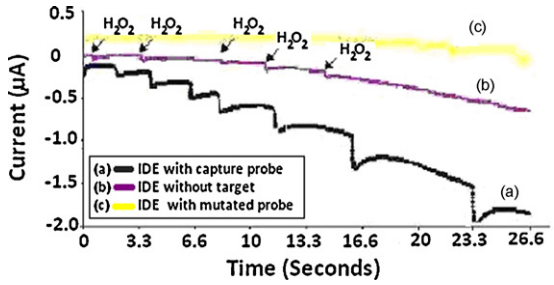

Fig. 4. Amperometric detection of hybridization reactions on IDE with (a) a complementary to target capture probe ($n = 3$; RSD = 0.5) or (c) a non-complementary to target capture probe ($n = 3$; RSD = 0.08) and (b) a complementary to target without the addition of target ($n = 3$; RSD = 0.1).

rapidly to the initial base line, bringing no response. While an oxidation response was measured for the oligonucleotide-ALP modified electrode, which reached a value of around 171 nA after a second injection (Fig. 3b).

With a mixture of both substrates into the cell, the potential values applied to the electrodes were mutually exchanged between the electrodes. The lack of response from both electrodes shows that there is not non-specific adsorption of the DNA-enzymes on the neighbouring electrodes and no crosstalk between electrodes.

Therefore, we can conclude that following the proposed immobilization/blocking procedure neither detectable crosstalk nor non-specific adsorption of the second oligonucleotide–enzyme conjugate occurred.

3.1.2. Amperometric detection of BRCA1 gene mutation

Once the non-specific adsorption of oligonucleotides was ruled out, the detection of hybridization reactions was investigated. Amperometric detection of oligonucleotide hybridization corresponding to a deleterious mutation in breast cancer 1 (BRCA1) gene was carried out. In the same microarray, a mutant-type sequence and a wild-type sequence capture probe were immobilized on different electrodes by biocompatible photolithography. To check the non-specific adsorption of the SA–HRP conjugate onto the electrode surface in absence of hybridization reaction another array was modified with the wild-type sequence and the response after incubation with the SA–HRP conjugate was recorded without hybridization.

Fig. 4 shows the amperometric results obtained from the hybridization reaction onto the patterned arrays. In the first array, a probe corresponding to the wild-type sequence of mutation in BRCA1 gene was immobilized in the first electrode, and a probe corresponding to the mutant-type sequence of this mutation was immobilized on the second electrode. The response of these two electrodes after incubation with a target oligonucleotide complementary to the wild sequence probe that was labelled with biotin for subsequence reaction with SA–HRP, was recorded and it is presented in Fig. 4 (plots a and c, respectively). This interaction was detected amperometrically with the addition of H_2O_2. The signal obtained from the set of electrodes modified with complementary probe was higher, 1.8 μA for 1.6 M H_2O_2. On the other hand, in the set of electrodes where the mutant probe was immobilized, a response of 0.05 μA was obtained for the same concentration of substrate, which is related with the non-specific hybridization of a mutated sequence.

In another array complementary capture probe was immobilized (Fig. 4b), and incubated with buffer instead of the target oligonucleotide, in order to measure the non-specific adsorption of the enzyme label. In this case, a value of 0.4 μA was determined.

3.2. Amperometric detection of enzymes patterned by biocompatible photolithography

Different strategies to immobilize enzymes on gold electrode surfaces were tested and compared in order to obtain a higher enzyme activity, stable immobilization and better analytical response on the sensor surface. The amperometric responses obtained with the different immobilization strategies from each immobilized enzyme, GOx and SOx are summarised in a table presented in Supplementary information.

Among the strategies tested, direct adsorption of the enzymes provided both a stable response from both enzymes and higher current values. Thus, direct adsorption procedure was chosen for the patterning of the IDE array with enzymes. GOx with redox polymer were adsorbed on the first IDE and SOx with the same redox polymer were patterned on the second set of electrodes.

Cyclic voltammogram obtained from the first IDE modified with GOx and redox polymer followed by polyelectrolyte multilayers and before exposing the bio-photoresist on the second IDE to UV light shown a well-defined redox wave characteristic of the surface confined redox couple from the first IDE and a complete absence of redox behaviour from the second IDE (figure in Supplementary information). This clearly demonstrates that redox polymer along with the GOx was immobilized successfully and the absence of redox behaviour indicates that the bio-photoresist polymer on the second IDE was intact and acted as protection layer of the second IDE against non-specific response of the redox polymer and GOx mixture. Cyclic voltammogram after immobilization of the SOx and redox polymer presented a quasi-reversible behaviour characteristic of the redox polymer.

One of the main problems encountered during enzymes immobilization on the photolithographically patterned array was the non-specific adsorption of the enzyme immobilized on the second IDE, which comes in contact with both IDE and may be adsorbed also on the first unprotected IDE. Therefore, different reagents were tested for blocking the non-specific response from the second enzyme. The use of self-deposited multilayers prepared after the first and prior to the second enzyme immobilization by sequential deposition of different polyelectrolytes (PSS and PVP) was chosen as the most effective method (Narváez et al., 2000). The idea behind the polyelectrolyte adsorption step is to make sure that the second enzyme, if deposited on top of the first electrode, will be far enough, so that it does not produce any non-specific response (Narváez et al., 2000). After four polyelectrolyte layers sarcosine signal was completely avoided, however about 50% of the initial GOx sensor signal was blocked.

Chronoamperometric detection of both enzymes response (figure presented in Supplementary information), demonstrated the immobilization of the redox polymer with the enzymes, without loss of the enzyme functionality. Both glucose and sarcosine sensors were characterized independently by taking the amperometric measurements from both sets of electrodes with the addition of their respective substrates. A maximum current of 1.20 μA cm^{-2} with 20 mM sarcosine was obtained from the second set of electrodes, however this current was not stable and decreased until a stable current of 0.8 μA cm^{-2}. On the other hand, no current was recorded from the first IDE where GOx and redox polymer was immobilized. An oxidation current from the first IDE with increasing glucose concentrations was observed, while the response from the second IDE was completely absent. Anodic currents were plotted against the glucose concentration and there was a current response maximum of around 3.23 μA cm^{-2} with 60 mM glucose. This demonstrates that the GOx has retained part of its activity even after blocking with the polyelectrolyte multilayer on the first IDE.

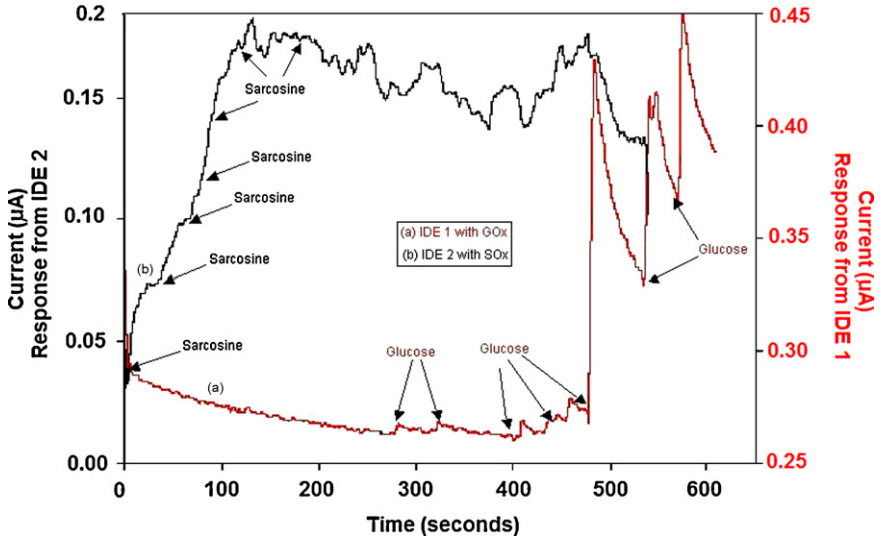

Fig. 5. The signal independence of the (b) sarcosine ($n = 3$; RSD = 0.005) and (a) glucose sensors ($n = 3$; RSD = 0.01) within the IDE microarray at +500 mV *vs.* Ag/AgCl in PBS buffer during successive injection of sarcosine and glucose.

Amperometric response was measured from both electrodes by injecting sarcosine and glucose sequentially one after the other within the same buffer, in order to see whether there was non-specific response (Fig. 5). Initially the experiment started with the addition of sarcosine and after some injections glucose was added into the cell. The response was measured initially with sarcosine and it was observed an increase in the oxidation current from the second IDE where SOX and redox polymer were immobilized. A maximum current of 1.5 $\mu A\,cm^{-2}$ at a concentration of 10 mM of sarcosine was detected and reached a steady state with no increase in the current for further addition of sarcosine, while in the first IDE for the same injections did not increase the response. When glucose was injected there was an increase in the oxidation current response maximum of around 3.8 $\mu A\,cm^{-2}$ at 40 mM glucose from the first IDE while no response was detected from the second set of electrodes. These results demonstrate that non-specific response was completely absent on both electrodes and that the response obtained from both IDE was substrate specific.

Even at higher substrate concentrations there was not response due to the substrate of the other enzyme, indicating that the non-specific adsorption, which results in crosstalk, did not occur and therefore the proposed electrode modification procedure can be used for the fabrication of multi-analyte sensors. This also proves that polyelectrolyte multi-layering step has proven to be successful in preventing the non-specific response from the second enzyme. There might two reasons behind this result. Since the last polyelectrolyte used in these experiments to form the polyelectrolyte multilayers is PVP, which possess dense positive charges, the SOx and redox polymer, which also possess excess positive charge, might be electrostatically repelled, so that non-specific immobilization is avoided. The other reason might be that the multilayer forms an insulating layer and even though there is non-specific adsorption of the SOx and redox polymer, the response might be completely prevented by the insulating properties of polyelectrolyte multilayer.

3.3. Amperometric T4 immunoassay on the patterned electrodes

Amperometric detection of T4 was performed through a competitive immunoassay. For this purpose a bIgG-T4 conjugate was immobilized on the electrode surface. A mixture of target (T4) standard solution in human serum and anti-T4 antibody was incubated on the modified electrode. By increasing the target concentration

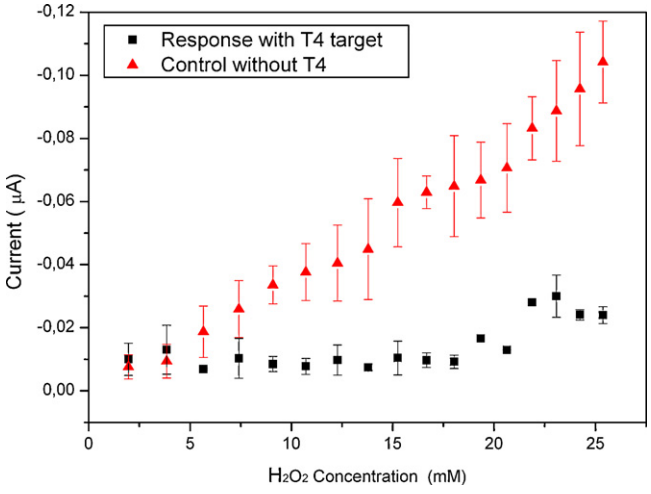

Fig. 6. Amperometric detection of T4 through a competition assay on IDE microarrays. (a) represent the response from the set of electrodes assayed using either a 104 ng mL^{-1} T4 standard solution or (b) the zero T4 standard. ($n = 2$).

in the standard solution, less amount of free anti-T4 antibody was available in the electrochemical cell in order to interact with the immobilized bIgG-T4, and therefore, lower signal was detected as compared to the absence of analyte (zero standard) (Fig. 2).

Fig. 6 represents the amperometric response from two sets of electrodes, the first corresponding to zero standard and the second one incubated with the target molecule (T4, 104 ng mL^{-1}). A concentration of 25 mM of H_2O_2, yielded a current of 110 nA on the first set of electrodes and a non-specific current of 20 nA on the second set of electrodes where the target was incubated. These results demonstrate the feasibility of this competitive assay, although an improvement in the sensor sensitivity could be achieved, reducing the non-specific signal. These results suggest that this competitive assay can detect T4 in concentrations in the order of part per billion.

4. Conclusions

We can conclude that biocompatible photolithography is a successful technique for the patterning of different kind of biomolecules on dense electrode arrays. DNA, enzymes and antibodies were patterned with this technique and detected

electrochemically on a 20 μm bandwidth interdigitated array. A BRCA1 gene mutation, which is related with predisposition to breast/ovarian cancer, was detected amperometrically; a current of 1.4 μA was obtained after hybridization with the complementary probe while only 0.05 μA was detected after hybridization with the non-complementary probe. Two enzymes, GOx and SOx, were also patterned with this technique on different sets of electrodes on the IDE microarray. When a mixture of both substrates was introduced into the electrochemical cell a separate response was obtained for each enzyme. However, a polyelectrolyte multi-layering was required to avoid crosstalk between the two enzymes. Finally, the hormone T4 was detected on the IDE microarray patterned with biocompatible photolithography. A competitive immunoassay was employed in order to detect this analyte. A current of 70 nA was detected in the electrode assayed with the zero standard and seven times less current was obtained from the electrode where the analyte was incubated, showing the high specificity of the system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.02.012.

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