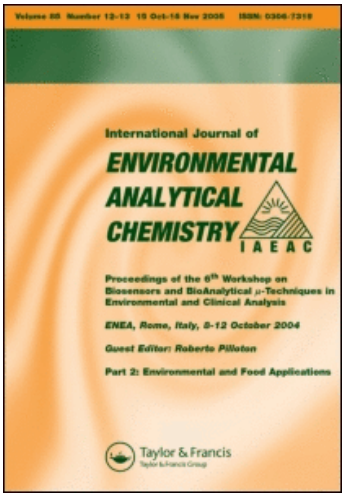

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Strategy for the development of sensor platforms for multi-analysis

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STRATEGY FOR THE DEVELOPMENT OF SENSOR PLATFORMS FOR MULTI-ANALYSIS

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Colloidal gold biofunctionalised nanomodules were synthesised and characterised by fluorescence and colorimetric measurements. These biofunctionalised nanomodules were selectively electrodeposited on electrode arrays. When the biomolecule used was an oligonucleotide probe, the gold conjugates were hybridised with complementary and mutated sequences, and the hybridisation efficiency was measured by amperometry. Significant differences in current density between complementary and mutated target sequences were achieved. Horseradish peroxidase-colloidal gold was also selectively electrodeposited on screen-printed electrodes providing proof-of-concept for a construction method of enzyme multi-sensors. Oligonucleotide and enzyme arrays were thus demonstrated, providing a generic platform for use in multi-analysis.

Keywords: Electrodeposition; Array; Colloidal gold; Oligonucleotide; Horseradish peroxidase (HRP); Biorecognition nanomodule; Hybridisation

INTRODUCTION

Microarraying techniques are facilitating the development of multi-sensing devices. These microarrays enable scientists to detect the presence of several target analytes quickly, inexpensively and simultaneously. Although most of the microarrays are focused on diagnosis or clinical applications, environmental applications could also benefit from these multi-sensing tools. For example, DNA arrays can be applied to toxicology studies (to find correlations between toxic responses in toxic agents and changes in the genetic profiles of the subjects exposed to such agents), to environmental analysis (since such toxic agents can be present in water, food and soils), and to biowarfare combat (identifying toxic biological agents) [1,2]. Apart from DNA arrays, enzyme arrays can be used for environmental applications like detection of pesticides [3]. Antibody-based arrays are also used for environmental multi-analysis [4].

There are a variety of arraying methods currently developed and used, each having different advantages and resolution capabilities [5–12]. This article describes a new arraying technique based on the site-addressed immobilisation of biorecognition

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nanomodules on photolithographically defined electrodes. These biorecognition nanomodules are created by conjugation of the sensing biomolecule to colloidal gold nanoparticles through electrostatic interactions, passive adsorption or dative bonds between the gold and sulfhydryl groups. After the conjugation, the biorecognition modules are selectively electrodeposited on predetermined sites of an array. Several reports refer to the use of colloidal gold to immobilise enzymes on sensors by electrodeposition. Crumbliss *et al*. conjugated glucose oxidase (GOx), HRP and xanthine oxidase to colloidal gold and electrodeposited these conjugates onto platinum or glassy carbon by applying +1.6 V (vs. Ag/AgCl) for 2 h [13]. These enzyme electrodes gave an electrochemical response to the corresponding enzyme substrates in the presence of ferrocene mediators. With this study, they demonstrated the utility of colloidal gold as biocompatible matrix suitable for the fabrication of enzyme electrodes. Yabuki and Mizutani also conjugated GOx to colloidal gold and deposited the conjugate onto glassy carbon, gold and platinum following the same procedure [14]. None of these previous articles mentions the possibility to selectively electrodeposit the bionanomodules with micrometric or sub-micrometric resolution. The present work demonstrates the directed deposition with photolithographic resolution as a viable approach to arraying. Although the work has been specifically focused on oligonucleotide arrays, the approach is generic, and different biorecognition nanomodules can be used, as demonstrated by preliminary HRP-colloidal gold electrodepositions. The selective deposition by electrochemical methods offers the advantages of high probe density and low cost arrays. Furthermore, as the overall strategy presented here is an integrated electrochemical approach (uses electrochemical methods for both arraying and measurement), manufacturing costs can be minimised for miniaturised and portable multi-sensor arrays for *in situ* and real-time monitoring.

EXPERIMENTAL

Materials

In the experiment, 20 nm (21.7 ± 2.0 nm) diameter colloidal gold and HRP-10 nm colloidal gold conjugate (9.3 ± 0.5 nm) were obtained from Sigma (St. Louis, MO). The original concentrations were 4.9×10^{11} and 3.2×10^{13} particles/mL for 20 nm colloidal gold and HRP-10 nm colloidal gold conjugate, respectively. Unconjugated gold colloids contained about 0.01% $HAuCl_4$ suspended in 0.01% tannic acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate and 0.02% sodium azide as preservative. HRP-colloidal gold conjugate was a suspension in 50% glycerol with 0.15 M NaCl, 0.01 M 2-morpholinoethanesulfonic acid (MES), pH 6.5, 0.25% bovine serum albumin (BSA). Oligonucleotide-colloidal gold nanomodules were synthesised by conjugation of thiol-modified oligonucleotides to gold nanocolloids and characterised by fluorescence and colorimetric techniques [15,16]. The oligonucleotides used were from Eurogentec (Seraing, Belgium). Table I shows the sequences and modifications of the oligonucleotides as well as their purpose in the experiments. Polyoxyethylenesorbitan monolaurate (Tween 20), BSA, streptavidin-HRP and 3,3′,5,5′-tetramethyl-benzidine (TMB) liquid substrate system for ELISA (enzyme-linked immunosorbent assay) (containing TMB and H_2O_2 in an slightly acidic buffer) were also purchased from Sigma (St. Louis, MO). Antidigoxigenin-HRP Fab fragments (antidig-HRP) were obtained

TABLE I Oligonucleotides used as probes and targets

Abbreviation	*Sequence*	*Purpose*
PROBE	3′ digoxigenin-ACTTAACCGAGTCGACCGA-SH 5′	Conjugate with antidig to detect the selective electrodeposition
COMP	3′ TCGGTCGACTCGGTTAAGT-biotin 5′	Observe the effect of four mutations with respect to probe in the hybridisation step
4-MUT	3′ TCGGTGGGCTCGGGTGAGT-biotin 5′	

from Roche (Basel, Switzerland). Interdigitated gold electrodes (IDEs; 5 μm) were from ABTECH Scientific Inc. (Richmond, VA) and 1.5-mm-diameter glassy carbon electrodes were from Cypress Systems (St. Louis, MO).

Instrumentation

Electrodepositions and chronoamperometries were performed using an AUTOLAB PGSTAT10 potentiostat (Utrecht, The Netherlands) in a conventional three-electrode cell, with Ag/AgCl as reference electrode and Pt as counter-electrode or in a two-electrode system (for the screen-printed electrodes), with screen-printed Ag/AgCl as the reference and counter-electrode. Absorbance values were measured with a Molecular Devices 340PC 96-well plate reader (Sunnyvale, CA). A Nikon E600FN microscope in transmittance mode and equipped with a Sony charge-coupled device (CCD) camera (Tokyo, Japan) was used for light microscopy. All solutions were made from distilled water purified through a Milli-Q water system.

Oligonucleotide Recognition Nanomodule Electrodeposition

First, the IDEs were cleaned with 'piranha's solution' (70% H_2SO_4 : 30% H_2O_2) and cyclic voltammetry (CV) (10 scans between −0.3 and +1.7 V (vs. Ag/AgCl) at 0.5 V/s in 0.33 M H_2SO_4). Afterwards, the IDEs were immersed in a three-electrode cell containing the PROBE-colloidal gold conjugate suspension (1.8×10^{12} particles/mL in 10 mM phosphate buffer, 0.1 M NaCl, pH 7.0), and +1.6 V (vs. Ag/AgCl) was applied at one of the two sets for 2 h. The set at which no potential was applied was used as a control to measure non-specific adsorption. The deposition was characterised by light microscopy.

The PROBE-colloidal gold conjugate (7.7×10^{11} particles/mL in 10 mM phosphate buffer, 0.1 M NaCl, pH 7.0) was also electrodeposited on glassy carbon electrodes by applying +1.1 V (vs. Ag/AgCl) for 2 min, +0.8 V (vs. Ag/AgCl) for 5 min, and +0.6 V (vs. Ag/AgCl) for 10 min. No potential was applied at the control. The electrodeposition was detected by CV at 0.05 V/s in 0.1 M H_2SO_4.

The PROBE-colloidal gold conjugate was electrochemically deposited on screen-printed electrodes (Fig. 1a). The electrodes were first blocked with BSA. After rinsing, a 0.5 μL drop of PROBE-colloidal gold conjugate (1.3×10^{12} particles/mL in 0.1 M tris-HCl buffer, 0.1 M KCl, pH 8.0, 1% BSA) was placed on the screen-printed electrode and +1.2 V (vs. Ag/AgCl) was applied for 2 min. No potential was applied at the control. The electrodes were then rinsed and incubated in antidig-HRP.

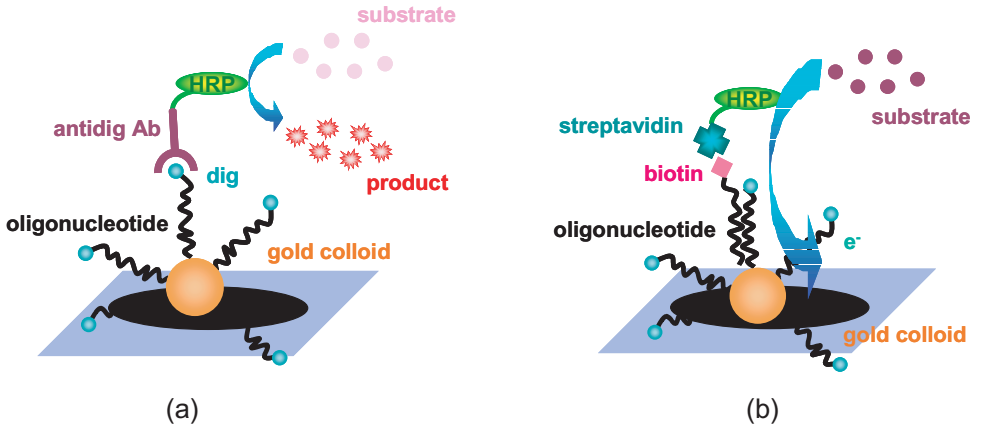

FIGURE 1 (a) Electrodeposited PROBE-colloidal gold on a carbon screen-printed electrode. The PROBE-colloidal gold is electrodeposited on the carbon screen-printed electrode; the antidig-HRP recognises the digoxigenin of the electrodeposited PROBE-colloidal gold; the HRP label reacts with its substrate and the mediator to give an electrochemical signal (schematically: $H_2O_2 + HRP_{red} \rightarrow H_2O + HRP_{ox}$; $HRP_{ox} + Os^{II} \rightarrow HRP_{red} + Os^{III}$; $Os^{III} + e^- \rightarrow Os^{II}$). (b) Electrodeposited PROBE-colloidal gold on a carbon screen-printed electrode and subsequent hybridisation. The PROBE-colloidal gold is electrodeposited on the carbon screen-printed electrode; the oligonucleotide of the PROBE-colloidal gold hybridises with COMP or 4-MUT; the streptavidin-HRP recognises the biotin; the HRP label reacts with its substrate and the mediator to give an electrochemical signal (schematically: $H_2O_2 + HRP_{red} \rightarrow H_2O + HRP_{ox}$; $HRP_{ox} + Os^{II} \rightarrow HRP_{red} + Os^{III}$; $Os^{III} + e^- \rightarrow Os^{II}$).

For the electrochemical detection, 1 μL of H_2O_2 (1 : 500 dilution in 50 mM acetate buffer, 0.15 M NaCl, pH 5.1) and 1 μL of [Os(2,2′-bipyridine)$_2$Cl(4-(aminomethyl)pyridine)]Cl (0.2 mM in water) were mixed and placed on the working electrode. After 2 min incubation, −0.1 V (vs. Ag/AgCl) was applied for 1 min and the current values at different times were measured.

Functionality of Electroarrayed Biorecognition Nanomodules

The PROBE-colloidal gold conjugate was electrochemically deposited on carbon screen-printed electrodes as in the previous section, and hybridisation was detected electrochemically (Fig. 1b). No potential was applied at the control. For the hybridisation step, after the conjugate incubation the electrodes were introduced in 70 μL of a solution of complementary oligonucleotide (COMP) or oligonucleotide with four-point mutations (4-MUT) (5 μg/mL in 10 mM tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.3× saline-sodium citrate (SSC), 2× Denhardt's solution, pH 8.0, corresponding to 4.7×10^{14} oligonucleotide molecules/mL) for 1 h at 55°C with stirring, and rinsed with water and with 0.1 mM tris-HCl buffer, 0.1% Tween 20, to observe the ability of the electrodeposited conjugates to distinguish 4-MUT in 19-base oligonucleotides (Table I). The electrodes were then incubated in 50 μL of a streptavidin-HRP solution (0.4 μg/mL in 0.1 M tris-HCl buffer, 0.1 M KCl, pH 8.0, 1% BSA) for 1 h at 37°C with stirring, and rinsed with water and with 0.1 mM tris-HCl buffer, 0.1% Tween 20. After incubation in streptavidin-HRP, hybridisation from electrodeposited conjugates was detected electrochemically following the same detection system as for the antidig-HRP label.

HRP-Nanomodule Arraying

A 2 μL drop of HRP-colloidal gold conjugate suspension (3.2×10^{13} particles/mL in 50% glycerol with 0.15 M NaCl, 0.01% MES, pH 6.5, 0.25% BSA) was placed on screen-printed electrodes, and +0.8 V (vs. Ag/AgCl) was applied for 1 or 5 min. No potential was applied at the control electrode. After rinsing, the electrodes were incubated in commercial TMB liquid substrate for 1 h at 37°C with stirring, and the reaction was stopped with H_2SO_4. The colorimetric response at 450 nm was monitored with a 96-well plate reader.

RESULTS AND DISCUSSION

Oligonucleotide Recognition Nanomodule Electrodeposition

The PROBE-colloidal gold conjugates were successfully deposited on the set of the IDEs at which a potential had been applied. Figure 2 shows the light-reflectance change due to the electrodeposition of the conjugates. The lines marked by the arrows correspond to the electrodes, and the other lines correspond to the gaps between the electrodes. Whilst the lines corresponding to the set at which no potential has been applied remain at the same color (lighter) than before the electrodeposition (*results not shown*), the lines associated with the set at which +1.0 V (vs. Ag/AgCl) has been applied for 30 min show a different color (darker). As both the electrodes and the gap between them are 5 μm in width, the photograph demonstrates the resolution of the arraying technique, which in this case is 5 μm and in general is limited only by the photolithographic resolution technique used to pattern the electrode array.

The PROBE-colloidal gold deposition on glassy carbon electrodes was detected electrochemically. Electrodes at which +1.1 V (vs. Ag/AgCl) had been applied for 2 min showed a marked increase in electroactive surface area reflected in the peak intensity increase in the oxidation peak at +1.6 V (vs. Ag/AgCl) and the reduction peak at +1.3 V (vs. Ag/AgCl) (Fig. 3). The electrode at which no potential had been applied showed significantly lower oxidation and reduction peaks. Comparing the surface coverage, the latter peaks represented 26% of non-directed deposition, as compared with the specific electrodeposition. Lower potentials for higher times,

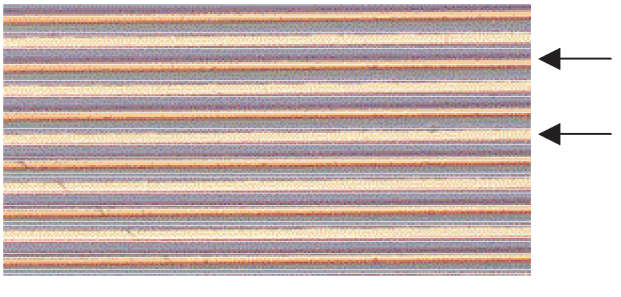

FIGURE 2 Gold IDE (5 μm width electrodes and gaps) under light microscopy after immersion in a three-electrode cell containing a PROBE-colloidal gold suspension and application of +1.6 V (vs. Ag/AgCl) for 2 h at one of the sets (darker line, arrow above). No potential was applied at the control set (lighter line, arrow below).

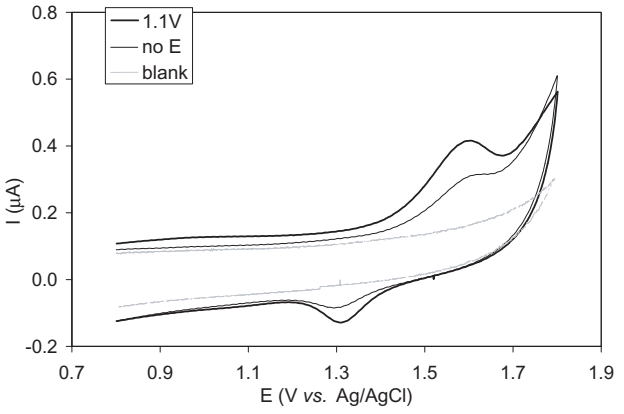

FIGURE 3 CV at 0.05 mV/s in 0.1 M H_2SO_4 of 1.5-mm-diameter glassy carbon electrodes after immersion of the electrode in a three-electrode cell containing a PROBE-colloidal gold suspension and application of +1.1 V (vs. Ag/AgCl) for 2 min (black line). No potential was applied at the control electrode (thick grey line). The thin grey line shows the CV of the electrode before immersion and potential application.

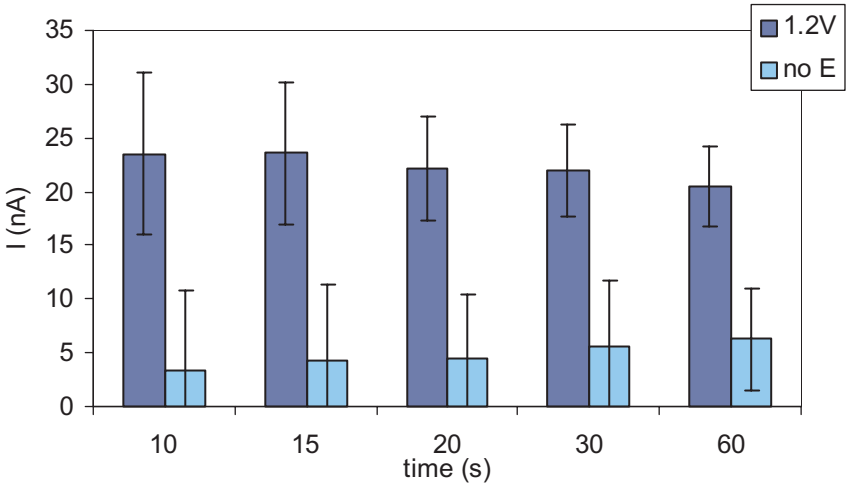

FIGURE 4 Currents obtained from the enzymatic reaction of the antidig-HRP label with osmium complex due to the electrodeposition or non-specific adsorption of PROBE-colloidal gold on carbon screen-printed electrodes (experimental details are given in the Experimental section). The amperometric response shown is background-subtracted.

+0.8 V (vs. Ag/AgCl) for 5 min and +0.6 V (vs. Ag/AgCl) for 10 min, resulted in higher non-specific adsorption.

Figure 4 shows the background-subtracted electrochemical signal obtained from an electrode, as shown in Fig. 1(a), at different measurement times to optimise the reading time in terms of signal/background ratio. Although the intensity values from electrodes where electrodeposition was attempted are very low (20–24 nA), it is possible to see that the values from the electrodes where non-specific adsorption might have occurred are considerably lower. Despite the large error bars, it appears that at shorter reading times, the ratio of currents is improved. At a reading time of 10 s, the current arising

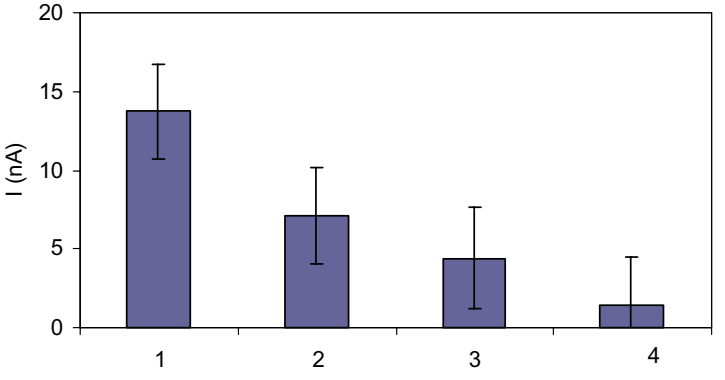

FIGURE 5 Currents obtained from the enzymatic reaction of the streptavidin-HRP label with osmium complex due to the hybridisation of electrodeposited or non-specifically adsorbed PROBE-colloidal gold on carbon screen-printed electrodes (experimental details are given in the Experimental section). The amperometric response shown is background-subtracted. (1) Electrodeposition with PROBE-colloidal gold and hybridisation with COMP; (2) exposition to PROBE-colloidal gold and hybridisation with COMP; (3) electrodeposition with PROBE-colloidal gold and hybridisation with 4-MUT; and (4) exposition to PROBE-colloidal gold and hybridisation with 4-MUT.

from non-specific adsorption is only 14% of the current arising from electrodes patterned with electrodeposition. Although this statement is clear from the trend of data, the large error bars do not allow us to be entirely certain of the real improvement that shorter reading times could have.

Functionality of Electroarrayed Biorecognition Nanomodules

Figure 5 shows the background-subtracted amperometric responses at 10 s obtained from electrodes on which electrodeposition was attempted and compares them with those of electrodes that were exposed to PROBE-colloidal gold conjugates. All electrodes were incubated for 1 h at 55°C with COMP or 4-MUT. The background currents were around 5 nA. As expected, the current intensity from the hybridisation of the PROBE-colloidal gold conjugate with COMP is the highest, with only 32% of this current resulting from the hybridisation of 4-MUT. However, a 52% response is observed, resulting from the non-specific adsorption of colloidal bionanomodules on electrodes (columns 2 and 4). It can be seen that there is selective electrodeposition of the PROBE-colloidal gold conjugate on carbon screen-printed electrodes; this PROBE-colloidal gold conjugate can be hybridised; and the response to hybridisation can be monitored electrochemically. Moreover, the electrochemical DNA sensor can differentiate 4-MUT in a 19-mer oligonucleotide. Electrochemical amplification systems are being investigated in order to increase the current arising from the hybridisation detection, which will provide the possibility of further miniaturisation of the system.

HRP-Bionanomodule Arraying

Figure 6 shows the absorbance values resulting from the incubation of TMB for 1 h at 37°C with differently modified electrodes. It can be seen that the absorbance values are higher when the electrodeposition was performed for 5 min instead of 1 min (compare

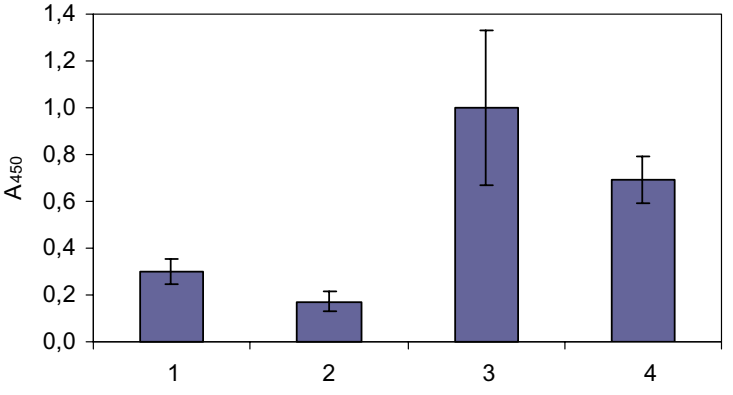

FIGURE 6 Absorbance at 450 nm obtained from the colorimetric reaction of the electrodeposited or non-specifically adsorbed HRP-colloidal gold with TMB on carbon screen-printed electrodes (experimental details are given in the Experimental section). (1) Electrodeposition for 1 min; (2) exposition for 1 min; (3) electrodeposition for 5 min; (4) exposition for 5 min.

columns 1 and 3). However, at the same time, the non-specific adsorption also increases with time (compare columns 2 and 4). Still, whereas at 5 min the absorbance resulting from the non-specifically adsorbed HRP modules is 69% of the directed one, at 1 min this non-specific adsorption is reduced to 58%. At short times, the difference between electrodeposition and non-specific or passive adsorption is higher. With this experiment, it has been demonstrated that the directed electrodeposition of biorecognition nanomodules is feasible not only with oligonucleotide nanomodules but also with enzymatic nanomodules.

CONCLUSIONS

Selective deposition of biorecognition nanomodules on different electrode surfaces has been demonstrated by several techniques. We have shown: (1) selective electrodeposition of oligonucleotides on an IDE with a spatial resolution of 5 μm; (2) differences between electrodeposition and non-specific adsorption observable after 1 min of electrodeposition, which indicates that the technique allows fast arraying processes; (3) that there is less than 14% non-selective deposition within sites; and (4) that, based on these principles, facile amperometric discrimination of four mutations in a 19-mer oligonucleotide is possible. The selective electrodeposition of oligonucleotide and enzyme nanomodules has been demonstrated to be an approach suitable as an arraying technique in terms of feasibility, resolution and arraying time. The ability to address the biorecognition probes to specific locations confers the possibility to create a microarray suitable for multi-analysis, as the preliminary experiments of this work have shown. The approach is generic, i.e. any biomolecule can be conjugated to colloidal gold, and any biorecognition nanomodule can be electrodeposited, thus opening up the possibility of using the strategy in genomic, proteomic and enzymatic platforms. The technique is relevant to environmental analysis because, with the additional demonstration for antibodies (work in progress), multi-sensors can be produced. In terms of commercial feasibility, generic multi-sensors are the only possibility for the

development of environmental analysis biodetection devices because environmental analysis is characterised by multi-sensing needs with small (as compared with diagnostics) markets for each particular analyte.

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