Analyst

Cite this: *Analyst*, 2011, **136**, 1810

www.rsc.org/analyst

COMMUNICATION

Aptasensor based on the selective electrodeposition of protein-linked gold nanoparticles on screen-printed electrodes

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Received 25th January 2011, Accepted 23rd February 2011
DOI: 10.1039/c1an15070g

The electrodeposition of DNA–gold nanoparticles previously developed in our group has been used as starting point for the electrodeposition of proteins attached to gold nanoparticles. We have performed a proof of principle by developing a methodology based on the electrodeposition of proteins bound to gold nanoparticles on screen-printed gold microelectrodes using, in a first approach, horseradish peroxide-conjugated gold nanoparticles (gold–HRP). The electrodeposition was achieved at a current positive potential of 800 mV *vs.* Ag/AgCl and the functionality of the electrodeposited HRP-particles was tested by electrochemical reduction of H_2O_2. Furthermore, we used this proof of concept in an aptasensor application to detect *Leishmania infantum* KMP-11. Hence, we have demonstrated not only the functionality of the electrodeposition of proteins bound to gold nanoparticles, but also the utility of the method with the aim of developing a real biosensor containing multiple enzymes or proteins in a multimodular device.

1. Introduction

The fundamental principles of miniaturised and parallelised microspot ligand-binding assays were described more than twenty years ago by Ekins and co-workers, when they explained why microspot assays are more sensitive than any other ligand-binding assay.[1] Since then, the high sensitivity and enormous potential of microspot technology have already been demonstrated by the advances in biochips technologies which have triggered the capability of analyzing nucleic acids and proteins for novel applications in biological and medical research.

The current concept of generating miniaturized devices for protein detection is usually based on printing small amounts (fmole range in pL volume) of numerous individual antibodies (<500 antibodies per array) with the desired specificities in discrete positions (<200 μm sized spots) on an ordered pattern onto a solid support: a microarray. The protein microarray technology is used to study expression profiling of complex proteomes, having direct bearing on numerous applications. The first generation of protein microarrays has already demonstrated its potential, generating detailed protein expression profiles and paving the way for new discoveries within the field of proteomics in biomedical research. The process of designing highly miniaturized, high-density and high-performing antibody microarray set-ups have, however, proven to be challenging.[2] The fast advance in protein array technology is directly related with the recent developing of the chemical surfaces, capture reagents, detection methods and arraying devices.

Immobilization of proteins on the sensor surface has strong consequences on the quality of analyses since it influences not only the efficiency of its attachment, but also the degree of non-specific binding and the accessibility to its targets. Moreover, the protein layer directly affects the reproducibility, selectivity and the resolution of the device. The immobilization process should allow the correct orientation and accessibility of the bioreceptor to the target molecule for the correct recognition. Proteins can be immobilized using non-covalent interactions with hydrophobic (nitrocellulose, polystyrene) or positively charged (poly-lysine, aminosilane) surfaces. These materials are well known from established protein assays such as ELISA or western blotting. A non-covalent attachment of protein might be too weak to prevent the loss of capture molecules during the assay procedure. So far, the covalent attachment of proteins on microarrays is usually performed using a variety of chemically activated surfaces (*e.g.* aldehyde, epoxy, active esters) or specific bimolecular interactions (*e.g.* streptavidin–biotin, his-tag–nickel-chelates). Three major supports are currently available: glass slides, membranes, and plates. Glass slides are suitable for high throughput and have low background for fluorescence detection. The major problem with glass slides is the poor protein-binding capacity. To overcome this problem, glass surfaces can be treated with aminosilane, poly-lysine, or aldehyde for surface activation.[3] Many companies and academic labs are developing different sophisticated surface chemistries for protein arrays applications.

The aim of this study is to present a novel application of a methodology previously developed by our group.[4] Combining the usage of gold–protein complex[5] and the electrodeposition of affinity modules, we have developed a method in which enzyme-linked gold nanoparticles (horseradish peroxidase—HRP) are electrodeposited on selected positions of a four gold working screen-printed

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microelectrode array. Furthermore, the detection of the enzyme activity was used to check the electrodeposition of the complexes by using hydrogen peroxide to give an electrochemical reaction.[6] Furthermore, taking advantage of this procedure, we have developed an electrochemical aptasensor for the detection of *L. infantum* KMP-11 (LiKMP-11) in which a DNA aptamer population that specifically binds LiKMP-11 has been used as the recognition element.[7] We believe that our results open the possibility to use new methods to design and fabricate larger protein arrays and, in addition, to use aptamers as biorecognition elements.[8–10]

2. Materials and methods

Colloidal gold sols were prepared as described previously.[11] Briefly, a solution of aqueous sodium citrate was added to a boiling, rapidly stirred solution of gold(III) chloride ($HAuCl_4$) and the solution was refluxed for 30 min. The final concentrations (w/v) were 0.01% $HAuCl_4$ and 0.03% sodium citrate. The particle medium size was estimated by transmission electronic microscopy (TEM) as approximately 18 nm. The screen-printed electrochemical sensor with four working electrodes (AC81) was purchased from BVT Technologies (Czech Republic). The sensor was formed on a corundum ceramic base. On to this surface, four working electrodes were applied with a diameter of 1.00 ± 0.05 mm (0.785 mm^2). Electrodes were made of pure gold. At the end of the sensor, there was a contact connected with the active part by the silver conducting path which was covered by a dielectric protection layer.

The HRP conjugation to gold nanoparticles was carried out as describe elsewhere.[12,13] Briefly, 250 μL of colloidal gold was added to 25 μL of HRP at concentrations ranging from 0.1 to 6 mg mL^{-1} and incubated for 5 minutes. Then 250 μL of 1 M NaCl, the minimal concentration required to keep the gold complexes stable, were added. Finally, to form the complexes, 100 μL of 2.4 mg mL^{-1} HRP in 0.1 M pH 7.5 phosphate buffer were added to 1 mL of 30 nM gold nanoparticles solution and allowed to react at room temperature for 30 minutes. The unbound HRP was removed by centrifugation and, once reacted, the suspension was washed for three times with 10 mM phosphate buffer, pH 7, 0.1 M NaCl (THC) and stored stable for at least three months. A final characterization was performed by means of measuring the plasmon resonant peak at 523 nm. The labeling of recombinant LiKMP-11 with gold particles was performed following the standard protocol developed by Moreno *et al.*[7] The minimal amount of LiKMP-11 to be added to the gold solution was found to be 150 ng μL^{-1}. Typically, 250 μL of the gold suspension was used to attach 40 μg (3.6 nmol) of LiKMP-11. Excess reagents were then removed twice by centrifugation for 30 min at 14 000 rpm and resuspended with SCHMK buffer (110 mM NaCl, 1 mM $MgCl_2$, 20mM HEPES, pH 7.0, 1 mM $CaCl_2$, and 5 mM KCl). LiKMP-11–colloidal gold complexes were stored at 4 °C.

The quality control of the gold–HRP complexes was carried out through their immobilization on an ELISA 96-well microtiter plate. This immobilization was performed by drying up 50 μL of modified nanoparticles for 1 h at 37 °C. After washing twice with THC buffer, a colorimetric reaction was developed after incubation for 15 min at 37 °C with ABTS®.

All the electrochemical measurements were carried out in a multipontentiostat PGSTAT10 from EcoChemie B.V. with up to six channels. The electrodeposition of the gold–HRP complexes was performed in a 1 mL electrochemical cell. As reference electrode, an Ag/AgCl KCl from Bioanalytical Systems was used and a platinum wire as the counter electrode.

The electrodeposition procedure of both, HRP and KMP-11–gold complexes, was carried out at 800 mV *vs.* Ag/AgCl positive potential on two working electrodes whereas the other two working electrodes were left in open circuit. When BSA–gold complexes were used as a negative control, they were electrodeposited subsequently in those electrodes left in open circuit. Once finished, the chips were washed twice with THC.

LiKMP-11 detection was performed by incubating the prepared electrodes in 50 μL of the digoxigenin-labelled aptamer population (0.1 pmole μL^{-1}) in SELEX buffer (110 mM NaCl, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.0, 1 mM $CaCl_2$, 5 mM KCl, 0.05% Tween 20 and 0.01% BSA) for 1 h at 37 °C. Afterwards, they were thoroughly washed with SELEX buffer and, finally, the Au colloid-modified electrode was incubated with 150 units μL^{-1} anti-digoxigenin antibody labelled with horseradish peroxidase in SELEX buffer.

The displacement assay was carried out as follows. Once the LiKMP-11–gold complexes were electrodeposited and incubated with the digoxigenin-labeled aptamer population, the screen-printed gold electrodes were incubated with the reaction buffer containing 50 μL of LiKMP11 at different concentrations ranging from 0 to 0.5 mg mL^{-1}. After two washes with selection buffer, the electrodes were incubated with 150 units μL^{-1} antidigoxigenin antibody labelled with horseradish peroxidase in the same buffer. The electrochemical measurement took place in a 5 mL electrochemical cell in phosphate buffer 0.1 M pH 7 at 0 V *vs.* Ag/AgCl with increasing amounts of hydrogen peroxide and 50 mM of catechol.

3. Results

The reaction scheme of the process is shown in Fig. 1. First of all, the functionalized gold nanoparticles were prepared according to previously reported methodologies.[14] A final concentration of 2.4 mg mL^{-1} of HRP was used in order to achieve the best stability of the complexes.

The electrodeposition of the gold–HRP complexes was achieved at a positive constant potential of 800 mV *vs.* Ag/AgCl following the conditions already achieved in our lab.[4] The particles were routinely electrodeposited in two of the four working electrodes, while the other two were left in open circuit in order to evaluate the non-specific adsorption of gold–HRP complexes on the working electrodes. Alternatively, BSA–gold complexes were electrodeposited to act as a negative control. The best way to characterize not only the electrodeposition procedure but also the integrity and functionality of the gold complexes after the immobilization is the enzymatic reaction corresponding to the HRP. Therefore, once electrodeposited, electrochemical measurements were recorded at 0 V *vs.* Ag/AgCl with increasing amounts of hydrogen peroxide and 50 mM of catechol (Fig. 1A).

Fig. 2A shows the response values obtained in a typical experiment, when adding successive quantities of hydrogen peroxide to the electrochemical cell. It is clearly noted that the electrodeposited channels 1 and 4 give a reduction signal of 300 nA compared with the relatively low non-specific signal in channels 2 and 3. As additional controls, gold–BSA particles were prepared and electrodeposited in channels 2 and 3 with the aim to demonstrate the setup capability to selectively electrodeposit dissimilar particles (Fig. 2B). All values are

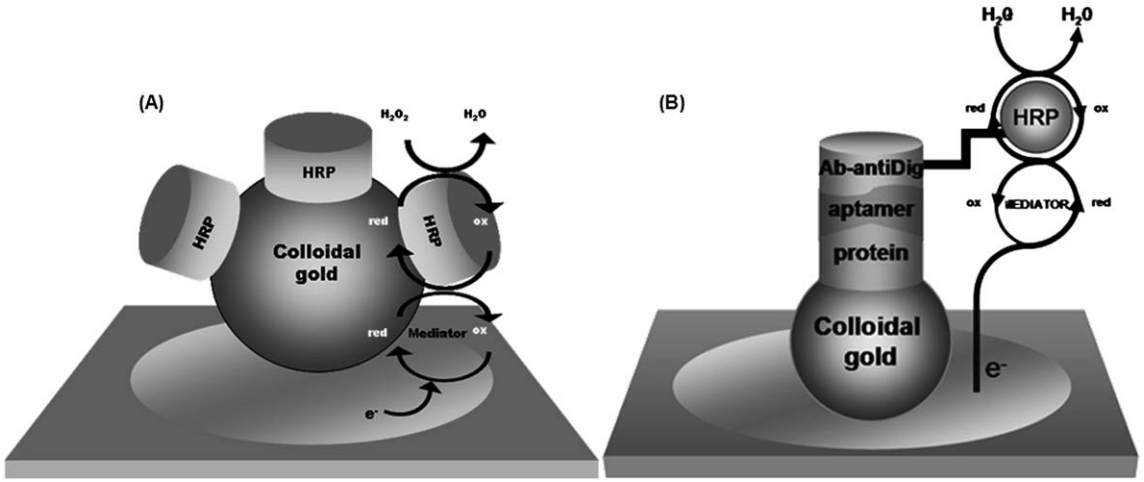

Fig. 1 (A) Reaction scheme of the HRP–gold electrodeposition procedure and its straightforward detection reaction. (B) Scheme of the LiKMP-11 aptasensor proposed.

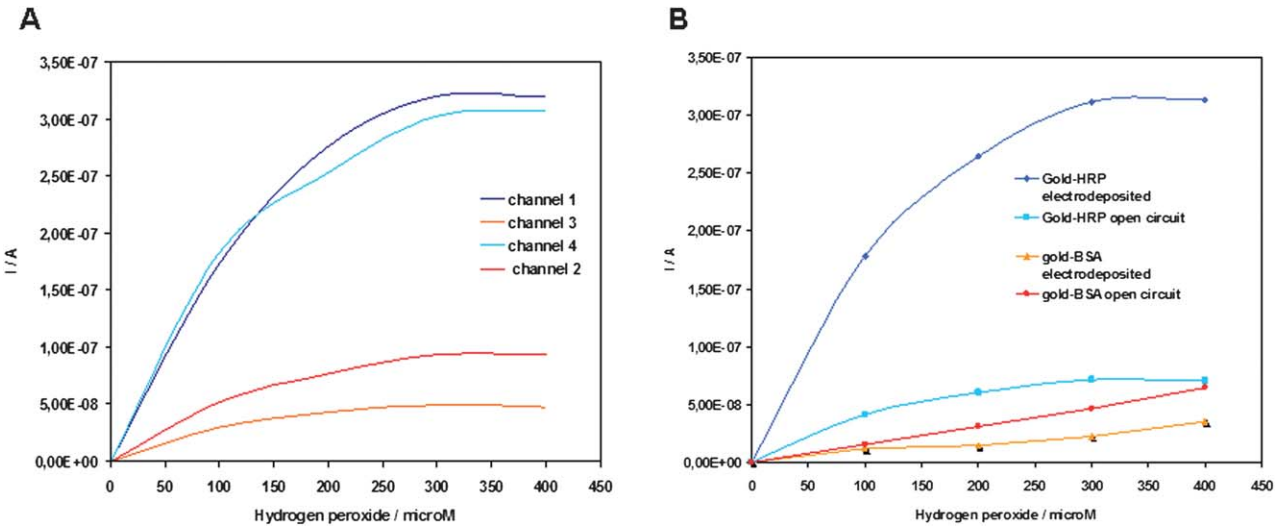

Fig. 2 Amperometric response of the hydrogen peroxide reduction by the HRP. (A) HRP was electrodeposited on two working electrodes, channels 1 and 4 while the channels 2 and 3 were left in open circuit during the electrodeposition procedure in order to test the non-specific adsorption of the gold–HRP particles. (B) Comparison with the electrodeposition of gold–BSA particles as well as the open circuit channels incubated with both kinds of particles.

the average of three measurements with a standard deviation of less than 3% (error bars are not shown).

Additionally, the electrodeposition conditions (pH ranges, time of electrodeposition and NaCl concentration) were tested in order to improve the deposition results. In Fig. 3 are shown the most effective results of the HRP performance given the established conditions mentioned above.

In order to test whether this electrodeposition methodology could be used with other different protein–gold particles complexes, LiKMP-11 attached to gold nanoparticles and BSA–gold particles were used. In this case, we used digoxigenin-labelled aptamers that specifically recognize LiKMP-11 and the electrochemical reaction was developed using an anti-digoxigenin antibody labelled with horseradish peroxidase at 0 V *vs.* Ag/AgCl and 400 μM of hydrogen peroxide and 50 mM of catechol. The reaction scheme is shown in Fig. 1B. The results shown in Fig. 4 show that a reduction signal of 65 nA was obtained at the working electrodes with LiKMP-11-nanoparticles complexes electrodeposited. Whereas a net intensity current of 9 nA was achieved at the negative control working electrodes. These results confirmed that LiKMP-11 had been successfully electrodeposited onto the electrode surface and that the aptamers population was able to recognize it.

Finally, in order to evaluate the response of the biosensor to the LiKMP-11 concentration, a displacement assay was carried out. The microelectrodes, once electrodeposited and incubated with digoxigenin-labelled aptamer population, were incubated with different concentrations of LiKMP-11 (0, 0.05, 0.25 and 0.5 mg mL^{-1}) for 1 h. After two washes with SELEX buffer, the electrodes were incubated with 150 units μL^{-1} anti-digoxigenin antibody labelled with horseradish peroxidase in SELEX buffer and the H_2O_2-electroreduction currents were then measured. Fig. 5 shows the normalized relation R/R_0 response with respect to the intensity of current in the absence of

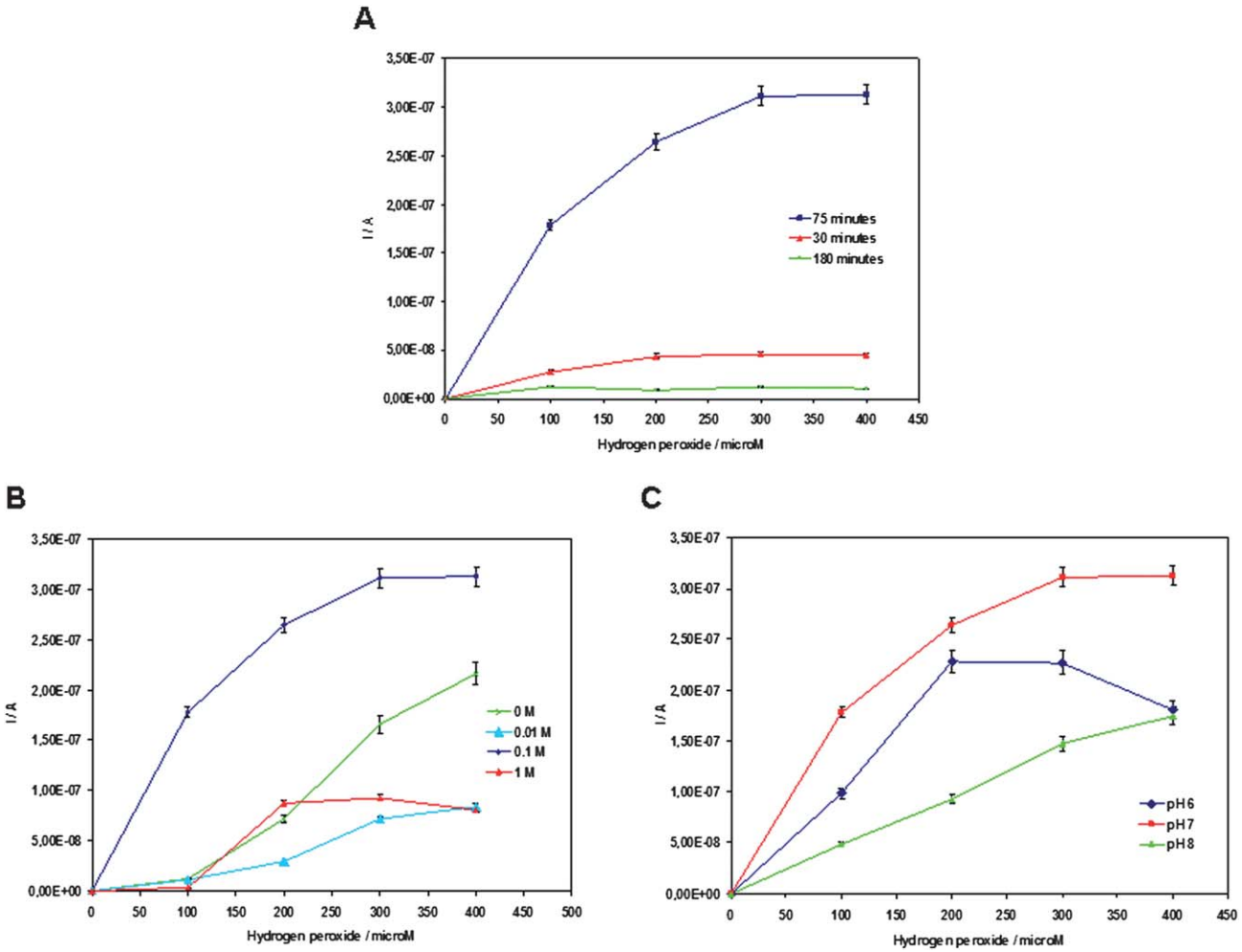

Fig. 3 Amperometric response of the hydrogen peroxide reduction by the HRP–gold complexes electrodeposited. All the experiments were run in phosphate buffer adjusting the time of electrodeposition, the NaCl concentration or pH, alternatively. (A) Electrodeposition time comparison in the range 30, 75 and 180 minutes. (B) NaCl concentration range in the electrodeposition procedure from 1 M to 0 M. (C) Electrodeposition in a pH range. Data correspond to the average of three independent measurements.

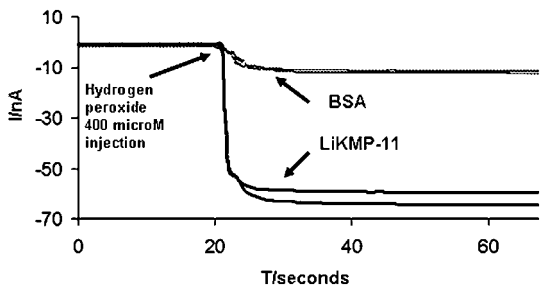

Fig. 4 Amperogram of the KMP11 and BSA recognition by the digoxigenin labelled aptamer population. Current intensity of electrodes with gold–KMP11 and gold–BSA electrodeposited. The values correspond to the average of 4 assays. H_2O_2 concentration was 400 μM.

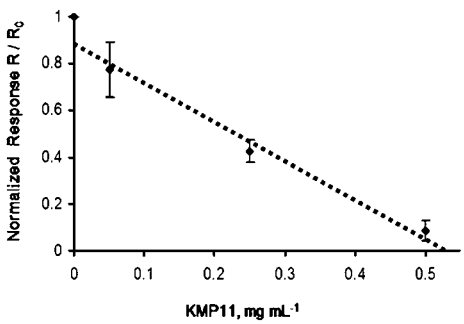

Fig. 5 Competition assay showing the calibration curve corresponding to the amperometric responses of the KMP11 concentrations. Each response (R) is normalized against response obtained without KMP11 (R_0: total union in the absence of the protein target). The values correspond to the average of 4 assays. H_2O_2 concentration was 400 μM.

LiKMP-11 *versus* the concentration of LiKMP-11 tested and a relationship between the intensity of current and the concentration of LiKMP-11 was obtained. The results clearly show that current decreased linearly with the target protein concentration and that when the analyte is absent, aptamer is available for binding to the electrode surface, giving a maximum amperometric signal. A limit of detection (0.9 of normalized response) of 0.025 mg mL^{-1} was achieved.

4. Discussion

Herein, we report the electrodeposition of HRP-linked gold nanoparticles as a novel approach for the electrodeposition of protein–gold nanoparticles complexes. Additionally, a biosensor for LiKMP-11 was designed as a direct application of the protein–gold complexes electrodeposition using an aptamer population as the recognition element.

Recently, it has been reported that the gold nanoparticles could not only steadily attach HRP, but also efficiently retain their bioactivity with a fast electrocatalytic response to the reduction of H_2O_2.[15,16] This fact supported the idea of using HRP–gold nanoparticles to test the electrodeposition procedure of protein–gold on gold microarray positions. The gold–protein complexes fabrication is widely used because the interaction of gold nanoparticles with protein molecules is very strong although is not covalent.[14,17] The electrodeposition procedure itself is similar to the one we used with DNA-capped gold nanoparticles.[4] In fact, we intended to test slightly different electrodeposition conditions although we did not improve the final result. Evidence of electrodeposition of gold–HRP was indirectly tested with the electrochemical readout. Reduction signals of 300 nA are in good agreement with previous results using similar conditions for the HRP.[6,18,19] Gold surface of the nanoparticles has high surface energy and, therefore, they are very active. The small size of the colloidal gold particles (approximately 18 nm) gives the protein molecules more freedom in orientation and so increases the possibility that the prosthetic group of the HRP is closer to the metal particle surface. This makes the distance for electron transfer between the protein and the metal particles shorter and, therefore, the charge transfer is easier.

It should be pointed out that it was very important to achieve a multiple immobilization of two gold complexes species, HRP and BSA. Therefore, we could design, produce and immobilize different protein–gold complexes in larger microelectrode arrays. In contrast with other reported methods,[18,20–22] our deposition technology is stepwise avoiding further modifications between each protein deposition and adsorption procedures based on the immobilization of the receptor *via* absorption.[23] Moreover, the attachment of the protein target to gold nanoparticles prior to its electrodeposition allows its application in array format. By contrast, other reported methods[24] are functional with only one protein immobilization and, thus, not being applicable to array fabrication.

Finally, we used the aptamer population previously reported by our group[7] for a direct application and, hence, proof of concept of a biosensor assembly using the electrodeposition procedure. Aptamers are being extensively used in the biosensor field[9,25–28] and we have taken advantage of their properties in terms of stability and selectivity. LiKMP-11–gold complexes electrodeposited in two of the working electrodes were detected using an aptamer population as the recognition element. We also managed to obtain a calibration curve of the biosensor through a competition assay in which free LiKMP-11 was able to displace the bound aptamer population. As a consequence, a linear relationship between free LiKMP-11 concentration and the intensity of the electrochemical reduction current was achieved. Although the limit of the detection should be improved, these results open new ways of research in order to enhance the protein deposition in larger microarrays. The feasibility of the biosensor configuration to recognize and specifically report the presence of proteins in solution demonstrates the suitability of this technology for the construction of integrated systems which is expected to be of general applicability to affinity biosensors.

Although the detection limits are obviously high and, therefore, need some improvement, the main aim of this paper is to confirm the electrodeposition procedures as a real alternative to current methods of array fabrication. Easy and cheap for the chip. Future experiments are leading us to the electrodeposition of many different proteins in larger microelectrode arrays and their recognition by antibodies and/or aptamers for direct multi-biosensing purposes.

5. Conclusions

In this paper, we report the proof of concept procedure for the selective immobilization of protein functionalized gold nanoparticles on arrayed screen-printed gold electrodes. The gold complexes were selectively electrodeposited on specific positions of an arrayed gold microelectrode. Although direct evidence of the electrodeposition was not possible due to the intrinsic characteristics of the gold electrode, indirect evidence was achieved using HRP gold nanoparticles electrodeposition and hydrogen peroxide reduction by means of the HRP reaction using catechol as an electron transfer agent. Net intensity current of about 300 nA at a final concentration of 400 μM of hydrogen peroxide and 50 mM of catechol was obtained. In addition, we report an electrochemical biosensor based on aptamers that can recognize and specifically report the presence of *Leishmania infantum* KMP-11. The target protein was conjugated with gold nanoparticles and the complexes electrodeposited on gold screen-printed electrodes. Using this method, we have been able to detect 25 μg mL^{-1} (2.27 μM) of LiKMP-11.

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