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INVESTIGATION OF IMMOBILIZING ANTIGENS ON GOLD SURFACE BY POTENTIOMETRIC MEASUREMENTS AND FLUORESCENCE MICROSCOPIC

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Abstract. *An antigen modified gold electrode based on a self-assembled lipoic acid monolayer has been developed. The contact angle measurement and cyclic voltammetry confirm the formation of a dense self-assembled monolayer on gold from 100 mM lipoic acid in ethanol. The electrochemical behavior shows a stable activity in a range of potential from -0.2 V to 0.65 V vs. Ag/AgCl. By applying a potential of 1 V vs. Ag/AgCl the monolayer is completely removed by oxidative desorption and a clean gold surface re-established. This allows for an easy renewal of the gold surface and recycling of modified immunosensor chips. This lipoic acid monolayer was covalently functionalized with a small molecule antigen synthesized from progesterone-3-(O-carboxymethyl)oxime and 4,7,10-trioxa-1,13 tridecanediamine to form a well ordered, low unspecific binding, antigen layer for an antibody-antigen interaction study.The effectiveness of antigen - antibody binding reaction was demonstrated by fluorescence imaging using a fluorescence-labeled anti-progesterone antibody as the binding partner.*

Keywords: antigen immobilization, electrochemical immunosensor, lipoic acid monolayer.

I. INTRODUCTION

Self-assembled monolayers are a convenient method for immobilization of biological compounds because they are reproducibly formed on gold surfaces utilizing the strong interaction between thiols, disulfides and gold [1]. Many studies paid attention to sulfide and disulfide moiety compounds, with lipoic acid being one of the most useful compounds for gold surface functionalization [2, 3]. Lipoic acid was widely used because its alkyl chain length and disulfide attachment

to gold surface results in the enhanced stability of the monolayer [4, 5]. The functionalization of the self-assembled monolayer after activation with a mixture of dicyclohexyl carbodiimide and N-hydroxysuccinimide is a well established method for the further functionalization of biosensors [6].

To achieve an appropriate diagnostic result, various biosensors require an efficient immobilization method to yield stable antigen modified layers and avoid unspecific binding with specific targets. Many electrochemical immunosensors were developed for real-time and low concentration measurements [7]. Such electrochemical immunosensors enabling direct detection without labeling are usually prepared by immobilizing various kinds of antibodies or antigens on surfaces based on self-assembled monolayers. Many immobilization methods have been used to bind antibodies onto gold surface electrodes [8]. However, when antibodies are immobilized on a solid surface their binding activity is usually inferior to the one of soluble antibodies because of partial denaturation of the proteins and steric hindrance of the molecules in solid phases compared with molecules in solution. Therefore, several studies were conducted on the immobilization of antigens. However these studies were only concerned with enzyme linked immunosorbent assay [9].

There are many methods for characterizing the properties of self-assembled monolayers [10]. Although electrochemical immunosensors can reach low detection limits, the electrical and, even more, the electrochemical properties of self-assembled monolayers have not been the subject of many studies. Mendes et al (2004) studied the characterisation of self-assembled thiol monolayers on gold surfaces by electrochemical impedance spectroscopy [11]. Despite previous studies on the topic, it is not yet well understood how the applied potential affects the self-assembled monolayers' properties. In general, monolayers are known to be unstable under the effect of applied potentials because of their reductive and oxidative desorption [11].

Our work focussed on the electrochemical properties of lipoic acid monolayers as a function of the applied potential. The aim of this research is to select a suitable potential for lipoic acid monolayers by using cyclic voltammetry and characterize the monolayer formation with contact angle measurements. The activity of a small molecule antigen covalently immobilized on lipoic acid monolayers is tested with progesterone-3-(O-carboxymethyl)oxime ("P4-3-CMO") as the antigen and a monoclonal antibody directed against progesterone (Hybrotec clone BF12). For ease of detection, the antibody was conjugated with a far-red fluorescence dye, FEW-S2314 which has the same excitation and emission maxima as the popular dye Cy5 (GE Healthcare).

II. EXPERIMENTAL

II.1. Material and reagents

Dicyclohexyl carbodiimide (DCC, 99%, Aldrich D80002, Mr 206.33), the N-hydroxysuccinimide (NHS, 97%, Fluka 56480, Mr 115.09) and $(\pm) - \alpha$-lipoic acid (99%, Sigma T5625, Mr 206.33) stock solutions were prepared with absolute ethanol (99.8%, Sigma-Aldrich 34852, Mr 46.07). 4,7,10-trioxa-1,13 tridecanediamine(97%, Aldrich 369519, Mr 220.31, density 1.005g/mL) and progesterone-3-(O-carboxymethyl)oxime (Sigma P3277, Mr 387.51) were purchased from Sigma Aldrich. Tween 20 (for molecular biology, A4974,0250) and PBS dry salt powder (Dulbecco's 1x, A0964,9100) were purchased from Panreac Applichem (Germany). A far red fluorescence dye "S2314" (S2314-mono-NHS-ester, order no. S 2314, excitation max: 648 nm, emission max: 663 nm), was purchased from FEW Chemicals (Germany). A monoclonal anti-progesterone

antibody (clone BF12) was provided by Hybrotec GmbH (Germany). Phosphate buffered saline (PBS) was prepared by dissolving 9.55g of the dry salt mixture (Panreac Applichem, A0964, 9100, 1x Dulbecco's) in 1 L deionized water. Gold disc electrodes (25 mm diameter) with 200nm thick gold layers on glass were supplied from Ssens, Netherland (No. 1-04-02-000). A fluorescence labeled monoclonal anti-progesterone antibody was provided by the Fraunhofer Institute for Cell Therapy and Immunology. The conjugation was accomplished using a published protocol [12].

In addition, a progesterone derivative with the diamino-linker 4,7,10-trioxa-1,13 tridecanediamine ("4,7,10-T") was synthesized . Briefly, 1mg progesterone-3-(O-carboxymethyl)oxime (2.58 μmol) was dissolved in 1 mL DMSO/EtOH solvent (0.5 mL DMSO + 0.5 mL EtOH). 8 mg dicyclohexyl carbodiimide (DCC, 38.8 μmol) and 2 mg N-hydroxysuccinimide (NHS, 17.4 μmol) were added. The carboxyl group was activated during 3 hours at room temperature. 1.7 μL of 4,7,10-trioxa-1,13 tridecanediamine (8 μmol) was added to the mixed solution and incubated for 7 days at 4˚C to accomplish amide formation. 0.5 mL water was added, and the resulting precipitate (the isourea side-product from DCC) was sedimented by centrifugation at 10000 g for 15 min. The supernatant was transferred into a fresh glass vial and 1 ml water was added dropwise to it (final water concent was 60% v/v). This solution was kept at 4˚C overnight, resulting in the formation of aprecipitate of the progesterone-4, 7, 10-T product. After centrifugation at 10000 g for 15 min this precipitate was separated from the supernatant and collected. The product of progesterone-4,7,10-T conjugate was re-dissolved in absolute ethanol and stored at 40C before being used. The identity and concentration was estimated by UV-vis spectrophotometry using a nanodrop ND-1000, Thermo Scientific) (data not displayed).

II.2. Instruments

All electrochemical experiments were carried out using a Gamry potentiostat Reference 600 (Gamry, USA) with gold disc working electrode (Ssens, No. 1-04-02-000), Ag/AgCl (1 M KCl) reference electrode and Pt counter electrode. The contact angle measurement was performed using sitting drop shape analysis method (Kruss, Germany). Besides, the antigen-antibody reaction was detected using a fluorescence microarray scanner (Tecan LS, USA)

II.3. Method for preparation of self-assembled monolayer lipoic acid

Prior to use, gold disc electrodes were pretreated with cyclic voltammetry, in 1 M H_2SO_4 solution, ranging from 0 to 1.5 V versus Pt reference electrode with a scan rate of 200 mV/s for 30 cycles. $(\pm) - \alpha$-lipoic acid , a naturally occurring small molecule with an intramolecular disulfide group and a carboxyl group, was dissolved in absolute ethanol and was coupled on the gold disc electrode by immediately immersing pre-treated electrodes in this solution overnight at room temperature. To optimize the monolayer, these electrodes were immersed in lipoic acid at different concentrations 50, 100, 150 and 200 mM. Then, they were washed in absolute ethanol and in distilled water and dried with pure nitrogen gas.

II.4. Method for characterization of the lipoic acid monolayer

The lipoic acid monolayer was characterized with cyclic voltammetry using a 1xPBS solution comprising 5 mM $Fe(CN)_6^{3-}$ and 5 mM $Fe(CN)_6^{4-}$ as a redox probe at a scan rate of 100 mV/s with the above mentioned three-electrode system in three cycles. The voltage range was fixed at -0.2 V at the lower end, while at the higher end it was progressively increased from 0.5 V to 1 V with 50 mV increments. Contact angle measurements were carried out in triplicate with activated

and pre-activated lipoic acid monolayers using deionized water, previously filtered with a 0.2 μm filter, as the probe.

II.5. Method for immobilization of antigen on gold surfaces

Before immobilizing antigens, the carboxyl group of lipoic acid coated gold surface must be activated by dicyclohexyl carbodiimide (DCC, 8 mg/mL) and N-hydroxysuccinimide (NHS, 2 mg/mL). The activation was carried out in aqueous/organic solvent comprising 25% (v/v) DMSO, 25% (v/v) ethanol and 50% (v/v) water at room temperature for 3 hours. Then, the electrodes were rinsed with absolute ethanol and distilled water and dried with nitrogen gas. After this time, the electrodes were incubated with progesterone-4,7,10-T conjugate in the same solvent overnight at room temperature. The modified electrodes were rinsed with buffer containing 1 X PBS and 0.2% Tween 20 (w/v) to remove all non-immobilized material.

III. RESULTS AND DISCUSSION

III.1. Analysis of contact angle results

Before any pretreatment, contact angles on bare gold disc electrodes were close to 90° (Fig. 1a). In the case of electrochemical treatment, the angles were found to be 80° ± 3° for cleaned gold. The decrease values after this step were given by removal of the surface protection layer.

However, when using old gold disc electrodes contact angles of 60° ± 30 were measured (Fig. 1b). These lower contact angles show the contamination of the surface.

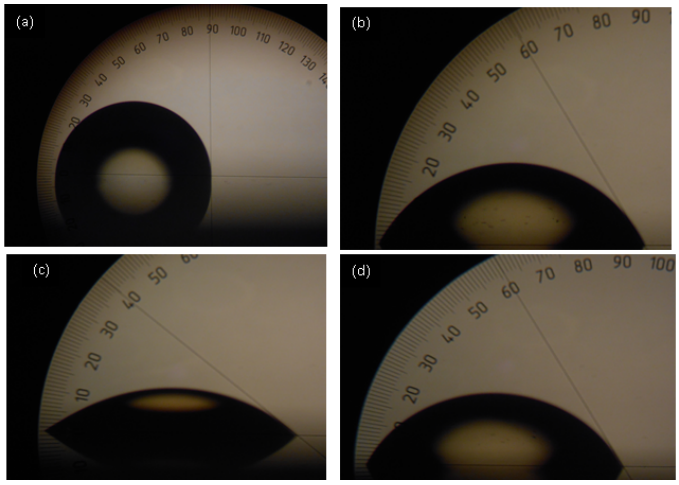

Fig. 1. Contact angle measurement on a gold electrode before (a) and after pre-treatment by cyclic voltammetry (b), gold electrode with lipoic acid monolayer (c) and gold electrode with lipoic acid monolayer after activation of the carboxyl group with DCC/NHS.

The formation of the lipoic acid monolayer and carboxyl group activation were demonstrated by contact angle measurements. The angles were determined at 38° ± 3° (Fig. 1c) for a lipoic acid monolayer (formed from a 50 mM lipoic acid solution in absolute ethanol) and 58° ± 3° after activating the carboxyl group with DCC/NHS (Fig. 1d). The decreased contact angle indicates the significant absorption of lipoic acid onto the gold surface resulting in an abundance of polar carboxyl groups on the surface. The formation of reactive NHS-esters after activation with DCC / NHS was accompanied by a significant increase in contact angle, reflecting the reduced polarity of the surface.

The modified gold electrodes obtained after incubation with higher lipoic acid concentrations (100 mM, 150 mM, 200 mM) returned contact angles of 45° ± 10°. The surprising result that

there were no significant changes in contact angles can be explained by the formation of a dense hydrophilic lipoic acid monolayer even at the lowest of tested concentrations.

So, the contact angle is the first step to characterize the formation of the lipoic acid monolayer onto a gold surface electrode. On the other hand, this method cannot discriminate between monolayers prepared from different lipoic acid concentrations.

III.2. Analysis of cyclic voltammetry measurement

III.2.1. *Formation of lipoic acid monolayer*

To further explore differences between the density of lipoic acid monolayers formed from different solutions, we used cyclic voltammetry in 1xPBS solution comprising 5 mM $Fe(CN)_6^{3-}$ and 5 mM $Fe(CN)_6^{4-}$ as a redox probe at a scan rate of 100 mV/s from -0.2 V to 0.5 V in three cycles. The monolayer film prepared with 50 mM lipoic acid showed a clear quasi-reversible redox wave with an oxidation peak at 0.4 V and a reduction peak at 0.275 V vs. Ag/AgCl (Fig. 2, 50 mM curve). This indicates that the lipoic acid layer formed from this low concentration is not dense enough to inhibit the approach of ferri/ferrocyanide to the gold electrode and therefore it still allows for electron transfer. This redox peaks disappeared when increasing lipoic acid concentration. At 100 mM the formed lipoic acid layer seems to reach its highest density and is effectively blocking electron transfer between ferri/ferrocyanide and gold. Upon further increase of the concentration beyond 100 mM, however, the formed layers seem to be less ordered and dense, as can be judged from the re-appearance of a redox signal at 200 mM lipoic acid (Fig. 2: 100, 150 and 200 mM curves). Hence, we anticipated the effect of the lipoic acid concentration on the formation of the lipoic acid monolayer. 100 mM of lipoic acid was chosen for the coupling of gold disc electrode in further experiments.

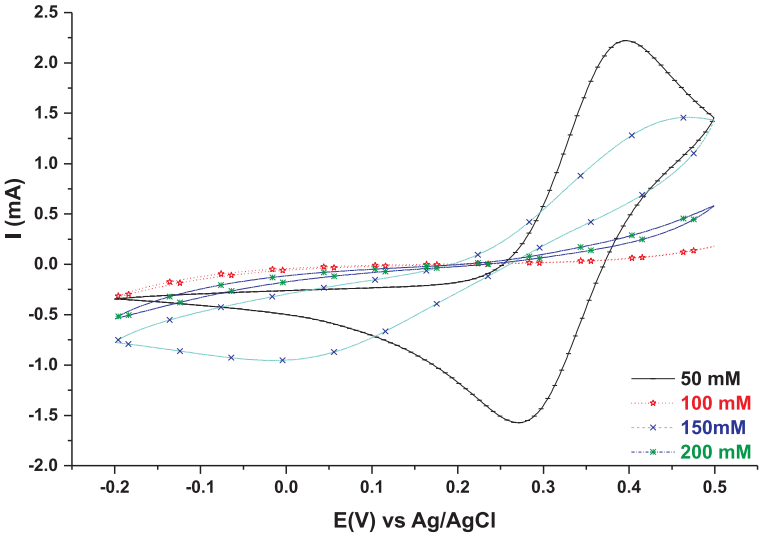

Fig. 2. Cyclic voltammetry of 5 mM $Fe(CN)_6^{3-}$ and 5 mM $Fe(CN)_6^{4-}$ in 1xPBS recorded on gold electrodes modified by lipoic acid layers prepared at different concentrations of lipoic acid.

III.2.2. *Characterization of lipoic acid monolayer*

In order to evaluate the stability of dense lipoic acid monolayers (formed at the optimum concentration of 100 mM), towards electrochemical oxidative desorption, another voltammetric

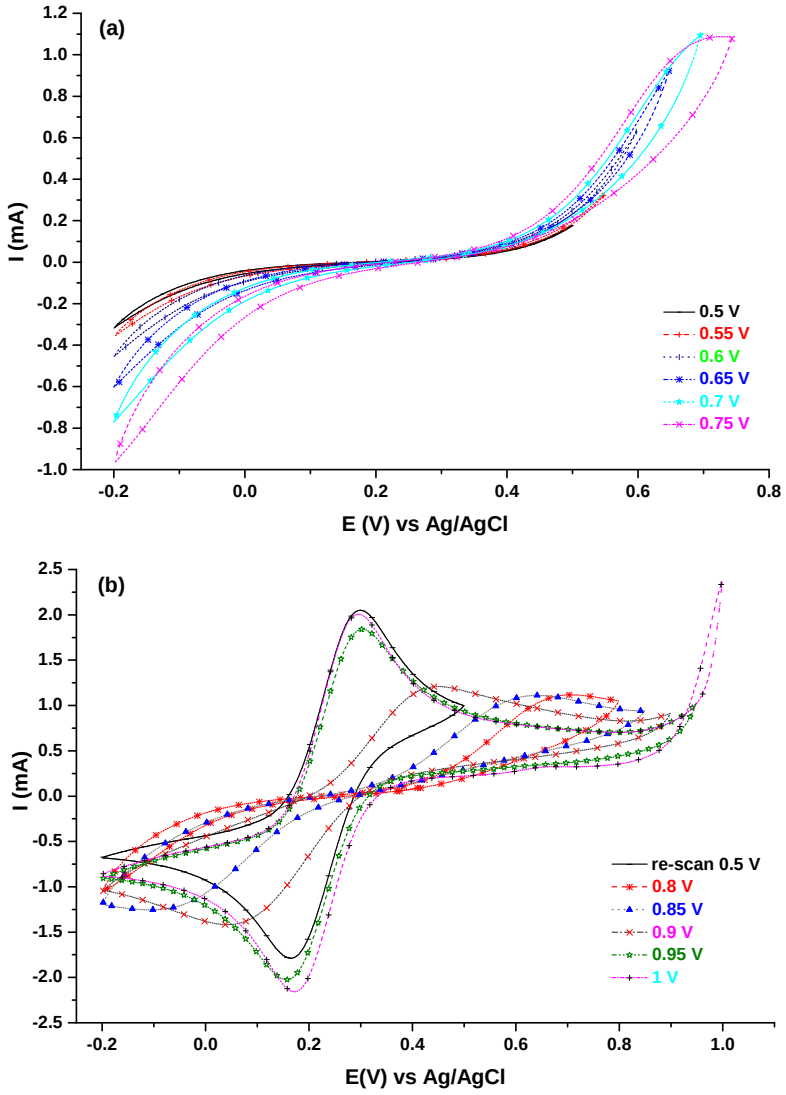

Fig. 3. Cyclic voltammetry of 5 mM $Fe(CN)_6^{3-}$ and 5 mM $Fe(CN)_6^{4-}$ in 1xPBS on a gold electrode modified by a lipoic acid monolayer (at 100 mM).
Increasing the upper switching potential in steps of 0.05 V visualizes the onset of monolayer destabilization (at 0.65 V) and complete monolayer desorption (at 1 V)

experiment was conducted under the same conditions as the previous one, but the upper switching potential was incrementally increased by 50 mV after each full scan cycle. It was shown that beyond 0.65 V the monolayer was destabilized. (Fig. 3a, 0.756 V curve). When applying higher potentials, the observed redox peaks shifted to the standard peak of the potassium ferrocyanide, and when increasing the switching potential to 1 V the redox peaks of ferri/ferrocyanide were fully visible (Fig. 3b, , 1 V curve). It can be assumed that the lipoic acid monolayer was completely

desorbed within one potential half-scan, and that electron transfer between ferri/ferrocyanide and gold was re-established. In other words, the S-Au bond was broken by applying a high potential. To verify this hypothesis, the destroyed monolayer on the electrode was scanned again applying a switching potential of 0.5 V. Fig. 4 shows that after applying 1 V potential the monolayer was more polarized than at the beginning of the formation when scanned at the same potential 0.5 V and compared with the standard cyclic voltammogram.

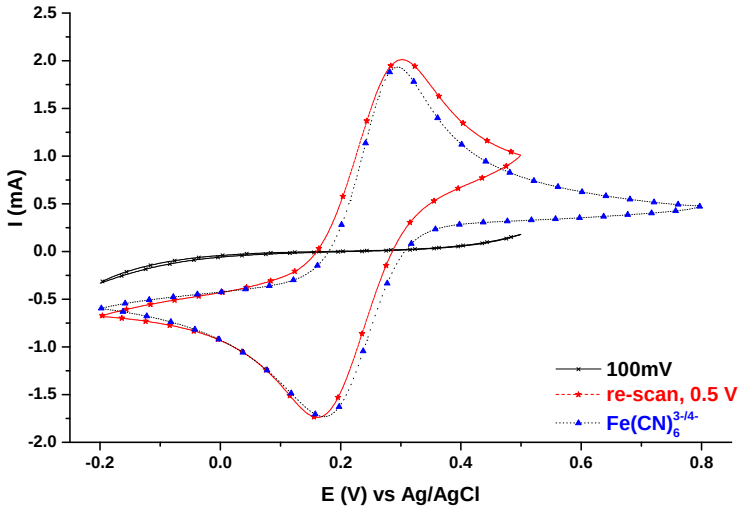

Fig. 4. Cyclic voltammetry of 5 mM $Fe(CN)_6^{3-}$ and 5 mM $Fe(CN)_6^{4-}$ in 1xPBS on a gold electrode modified by a lipoic acid monolayer at 100 mM (100 mV curve), on the same electrode after the monolayer was electrochemically desorbed (by polarization to 1 V, re-scan, 0.5V curve) and, for comparison, on a clean gold electrode ($Fe(CN)_6^{3-/4-}$ curve).

III.3. Analysis of fluorescence microscopic imaging

In order to test the composition and functionality of the lipoic acid monolayers, two experiments using fluorescence microscopy were set up simultaneously. In the first one, a gold disk electrode modified with a lipoic acid monolayer was assessed: one half was the control and the other half was incubated with anti-progesterone antibody-S2314 conjugate for 1 h. In the second one, a gold disk electrode was first modified with lipoic acid and then progesterone-4,7,10-T was covalently coupled to the monolayer. Then, these electrodes were treated in the same manner like in the first experiment. Fig. 5 shows a comparison of the average fluorescence intensity on these four different surfaces. Each scan includes the average fluorescence intensities of the

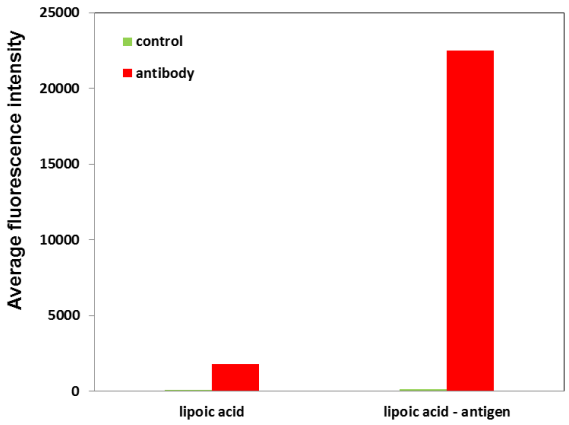

Fig. 5. Average fluorescence intensity (arbitrary units) lipoic acid and lipoic acid-progesterone modified electrodes before (small bars) and after (large bars) incubation with S2314-labeled anti-progesterone antibody.

control samples, which were
used for assessing the background fluorescence from lipoic acid and lipoic acid-progesterone monolayers and the ones incubated with fluorescence labeled antibody. Based on the average intensities the specific signal (due to antibody-antigen binding) was 13-times higher than the non-specific signal (due to unspecific antibody adsorption).

Fig. 6 shows typical fluorescence images of S2314-labeled antibody bound to lipoic acid and lipoic acid-progesterone modified gold electrodes.. The fluorescence measured on the lipoic acid-progesterone monolayer (22508 arbitrary units) can be attributed to the total amount of antibodies bound to the surface by specific and non-specific interaction (Fig. 6c). The fluorescence observed from lipoic acid monolayer (1718 arbitrary units) can be attributed to a small amount of unspecific antibody adsorption (Fig. 6a). The specific binding signal was 13-times higher than the unspecific binding signal, clearly proving the successful surface modification.

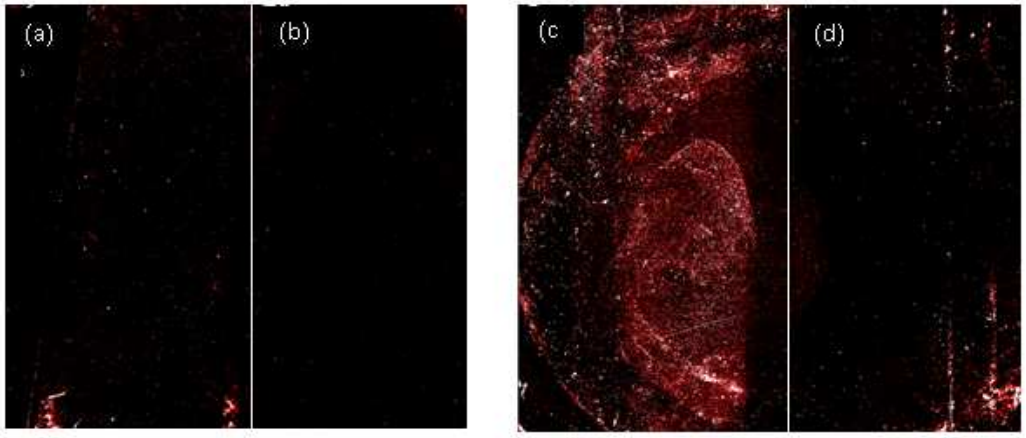

Fig. 6. Fluorescence images of lipoic acid monolayer after (a), before S2314-labeled antibody treatment (b), lipoic acid-progesterone monolayer after (c) andbefore S2314-labeled antibody treatment (d).

IV. CONCLUSIONS

Monolayer formation from the coupling of lipoic acid on a gold electrode is a convenient, one-step method to prepare modified surfaces with antigen functionalities which are suitable for antibody-antigen interaction studies. The presence and stability of these monolayers was confirmed by scanning voltammetry using ferri/ferrocyanide as the redox probe and varying the upper reverse potential between 0.5 V and 1.0 V. The ideal condition for the formation of a monolayer was found by using 100 mM lipoic acid concentration. Such monolayers stayed intact at electrode potentials below 0.65 V (vs. Ag/AgCl). Rapid destruction of the monolayer occured at potentials between 0.7 V and 1.0 V (vs. Ag/AgCl) and the underlying gold electrode showed the electrochemical characteristic of a freshly cleaned one. As previously described for alkanethiol monolayers [13], the lipoic acid monolayer can thus be easily removed by oxidative desorption and a clean gold surface can be obtained. Such a process was described to be especially suited

to the effective cleaning and recycling of micromachined electrodes and structures, e.g. interdigitated electrodes [13]. Compared to alkanethiols with an activatable coupling groups, however, lipoic acid is cheaper, resistant to chemical oxidation, less toxic and environmentally friendly. In this work we proposed an effective immobilization strategy for a small molecular weight antigen, progesterone, on gold and demonstrated that it allows for the sensitive detection of a fluorescence-labeled anti-progesterone antibody by fluorescence imaging. This straightforward approach is not limited to progesterone but may be applied to form monolayers of a variety of small molecules on gold surfaces. Such modified, reusable surfaces may find wide application in biosensor technology, especially for surface plasmon resonance, quartz crystal microbalance and electrochemical biosensors.

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