New probe immobilizations by Lipoate-Diethalonamines or Ethylene-Glycol molecules for Capacitance DNA Chip

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Abstract — **Label–free DNA detection is of crucial role to when developing point-of-care biochips to be used in personalized therapy. Capacitance detection is a promising technology for label-free DNA detection. However, data published in literature often show evident time drift, large standard deviation, scattered data points, and poor reproducibility. To solve these problems, alkanethiol molecules such as mercapto-hexanol are usually considered as blocking agents. The aim of the present paper is to investigate new blocking agents to further improve DNA probe surfaces. Data from AFM, SPR, florescence microscopy, and capacitance measurements are used to demonstrate the new lipoates molecules. Moreover precursor layers obtained by using Ethylene-glycol alkanethiols offer further improvements in terms of diminished detection errors. Film structure is investigated at the nano-scale to justify the detection improvements in terms of probe surface quality. This study demonstrates the superiority of lipoate and Ethylene-glycol molecules as blocking candidates when immobilizing molecular probes onto spot surfaces in label-free DNA biochip.**

I. Introduction

Medical diagnosis requires point-of-care biosensor arrays at the patient's bed. This requirement is due to the new emerging demand for personalized therapies because therapeutic agents in tissue and blood serum are different on a patient-by-patient basis [1]. Therefore, the development of low-cost, point-of-care technologies for array biochips is a necessary step to introduce personalized therapies in clinical practice. The usually considered micro-array technology based on optical detection and molecular labeling is costly and time consuming. Thus, it is not adapted for applications in hospital or home based personalized therapy. Label-free capacitance DNA biochips are a valid solution as they present many advantages. After the initial works of V. M. Mirsky [2,3], the application of capacitance measurements for DNA [4], interleukin [5] and heavy metals [6] detection was extensively investigated in late 90s. After 2003, the capacitance based detection was pursued by increased vigor as demonstrated by more recent works published by different research labs. It was demonstrated that this detection principle can be applied to DNA hybridization by immobilizing single strand probe DNA molecules on gold [7] and on silicon [8], as well as PNA probe molecule [9]. Capacitive detection has been used to reach the femtomolar concentration range to detect metal ions [10], to check the antibody affinity in macroporus silicon [11] and in gold [12]. Finally, the possibility to develop fully integrated DNA biochip was demonstrated [13,14]. Good reviews on this large effort were published summarizing applications for pathogenic detection [15], and mechanisms of impedance changes [16]. However, the usually considered probe immobilization presents not enough stable capacitance properties. Evident time drift [2,13], large standard deviation [14], scattered data points [4], and poor reproducibility [5] usually affect the detection signals. All these phenomena are related to electrode/solution interface not behaving as a perfect insulator [17]. Thus, the sensing capability is reduced. In biosensors, special effort is dedicated word wide to probes surface improvement. For example, new materials based on the lipoamide, lipa-DEA [18,19] and on ethylene-glycol [20, 21] were applied to improve biosensors based on Surface Plasmon Resonance, SPR. A large decrease of non-specific adsorption on probe surfaces was demonstrated for both lipoates [18] and ethylene-glycol alkanethiols [21]. The aim of this paper is to demonstrate that these new probe functionalizations assure an improved stability in DNA detection by using a capacitive biochip. The paper presents original investigations on lipa-DEA and ethylene-glycol monolayers in comparison with those obtained with the usually considered mercapto-hexanol. Results from AFM, fluorescence, SPR and capacitance measurements on chip are used to demonstrate that these new fictionalizations are innovative and suitable to improve label-free DNA biochip detectors.

II. Experimental

A. Chemicals

Ethylene-glycol functionalized alkanethiols differently terminated ($SH-(CH_2)_{11}-(OCH_2CH_2)_3OCH_2COOH$, and $SH(CH_2)_{11}-(OCH2CH2)_3OH$) were purchased from Prochimia, Poland. 11-Mercaptoundecanoic acid ($HS(CH_2)_{10}COOH$), 6-mercapto-1-hexanol, NaCl, Na_2HPO_4, KH_2PO_4, KCl, H_2O_2 (50%) and absolute ethanol were purchased by Sigma, Switzerland.
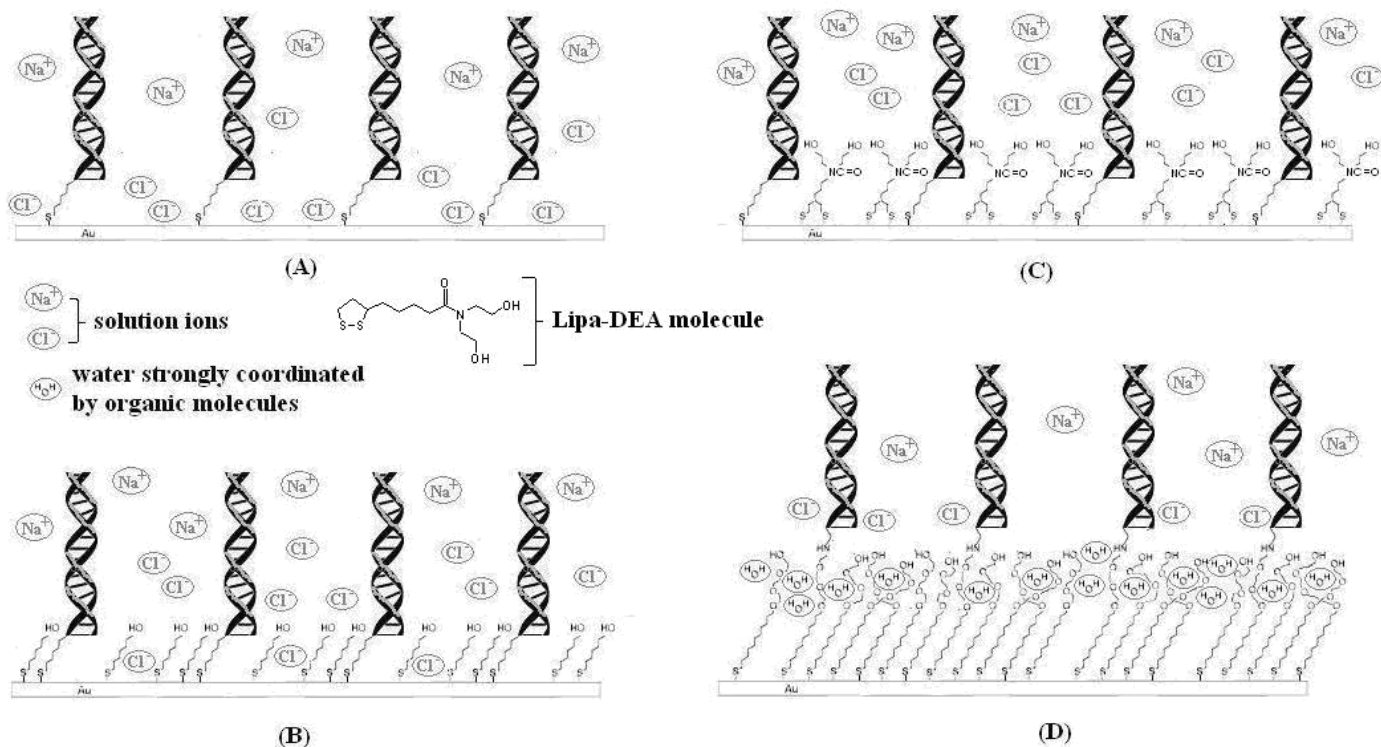
Fig 1: Schematic drawings of DNA hybridizations onto the investigated surfaces: (A) HS-terminated probes onto gold electrodes (A) without blocking agent; (B) with the usually considered 6-mercapto-1-hexanol or (C) with Lipa-DEA; (D) NH-terminated probes onto ethylene-glycol alkanethiols.

Different single-stranded DNA (ssDNA) probe molecules of the same length (25-mer), thiol or amino modified, with a chain of 6 carbon atoms as a spacer were supplied by MWG Biotech, Germany. Single-stranded DNA target were acquired by MWG as well. *N,N*-bis(2-hydroxyethyl)-α-lipoamide (Lipa-DEA) was prepared according to a published method [22]. All the chemicals were used without further purification.

B. Biochip Fabrication

A standard lift-off process is used to pattern the gold electrodes on the glass substrate. To improve adhesion between the substrate and the electrodes first a 20 nm layer of Chromium is deposited, followed by a 200 nm layer of Gold using thermal evaporation. The entire surface is covered by a thick (10 μm) layer of a AZ1512 photoresist that acts as a passivation layer. Individual sensor spots are exposed by developing AZ1512. The chip contains a total of 32 square electrode arranged in groups of four electrode pairs. Figure 2 shows one chip in detail. The electrode square side is 200 μm and the electrode's separation is 20 μm.

C. DNA Probes layer Formation

Different kinds of probe immobilizations were prepared in order to check different sensing monolayers. Mainly, three different immobilization techniques were tested, as shown in Figure 1: SH-terminated ssDNA are immobilized directly onto chip gold electrodes and, then, 6-mercapto-1-hexanol is added as blocking agent (Figure 1A) following a well established procedure [4]. SH-terminated ssDNA are mixed to Lipa-DEA molecules and co-adsorbed onto the chip gold electrodes for immobilization of the probes and the blocking molecule, Lipa-

DEA (Figure 1B). This procedure was found to be more efficient than that when a surface with the DNA probes was post-treated with blocking agent [19]. Ethylene-glycol monolayers are formed as probe precursors onto the electrode surface (Figure 1C). The layers are obtained from a mixture of two differently modified alkanethiol molecules ($HS(CH_2)_{11}(OCH_2CH_2)_3OH$ and $HS(CH_2)_{11}(O-CH_2CH_2)_3OCH_2COOH$). The mixture is prepared into a 2 mM final concentration solution of ethanol with proportion 1.96 mM of -OH terminated thiols and 0.04 mM of –COOH terminated thiols. The samples were incubated overnight, under dark conditions, in such a mixture. The samples were then rinsed and sonicated in ethanol for 10 min. Finally, NH-terminated ssDNA probes are anchored to the precursor monolayers by using well know procedures based on N-hydroxysuccinimide (NHS) and of N-Ethyl-N´-(3-dimethylaminopropyl)carbodiimide (EDC) [21]. By following the same procedures, DNA probe films were also immobilized onto Template Stripped Gold (TSG) and onto Biacore gold chips for AFM and SPR investigations, respectively. The different functionalizations were verified by using fluorescence microscopy, as shown in Figure 3, and SPR as shown in Figure 7.

D. Capacitance Measurements

The capacitance measurements were performed by using an array biochip measurement station developed in our lab to test biosensors for DNA and protein detection. Charge-based measurement technique (CBCM) is employed to improve capacitance estimation.

Fig. 2: Micro-fabricated chip used to investigate the different DNA probe immobilizations showed in Figure 1.

Capacitance values were estimated by measuring the current transient due to the RC behavior of the electrodes/solution interface. The input voltage was a square signal used to drive the working and the reference electrodes of each sensing area of the biochip. A detailed description of the implemented measurement technique has already been published [14]. After the SAM formation and before the capacitance measurements, the electrode chips were left for conditioning in PBS buffer in dark for 24 hours. The conditioning was necessary to further stabilize the capacitance measurements on the so prepared electrode chips.

E. AFM imaging

Atomic force microscopy, AFM imaging was performed in tapping mode with PointProbe nanocontact silicon probes on a Nanoscope IIIa SFM system equipped with a multimode head and a type A piezoelectric scanner (Veeco, Santa Barbara, CA, USA). The images were acquired in ethanol by using a 'liquid cell'. Raw images have been processed only for background removal (flattening) using the microscope manufacturer's image processing software package.

III.   RESULTS

In this section, results of DNA hybridization detection by using the different proposed DNA probe immobilizations are summarized. In particular, data are presented in terms of average detection capacitance signals and standard deviation in series of acquisitions on the same chip spot during 10 minutes. A very good detection has to present very small standard deviations. AFM images are also showed in order to investigate the relationship between different standard deviations and structures at the nano-scale of the formed DNA probe monolayers.

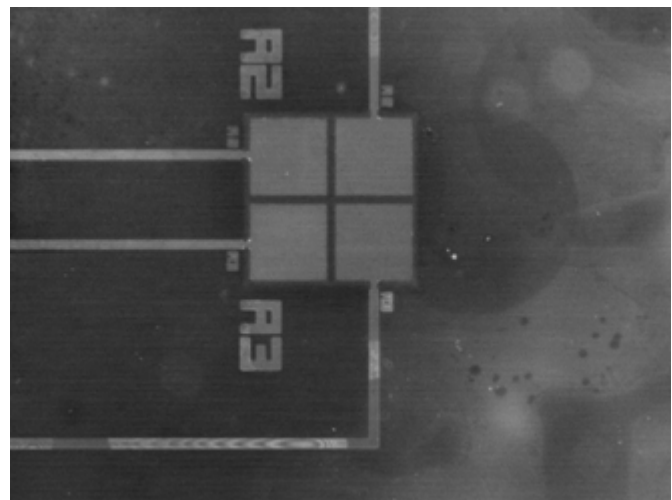### A.   DNA detection with 6- Mercapto-1-Hexanol layer

DNA hybridization detection by using the SH-terminated probes directly immobilized onto gold of the chip electrodes and post-treated with 6-mercapto-1-hexanol is summarized in Figure 4. Acquisitions on different chip spots are similar to that showed by the Figure. In that case, the standard deviations for both the signal on the probe and on the target DNA are not small, as indicated in the Figure. In case of DNA target detection, the data acquired in 10 minutes on the same spot presents a signal variation of 19,7 % from the higher to the lower registered signal. The signal on the target is in the range from 1,10 nF up to 1,37 nF.

B.   DNA detection with Lipa-DEA layer

DNA hybridization detection by using the SH-terminated oligos directly immobilized onto gold of the chip electrodes but pre-mixed with Lipa-DEA is summarized in Figure 6. Acquisitions on different chip spots are similar to that showed by the Figure. In that case, the standard deviations for both the signal on the probe and on the target DNA are much smaller than in the previous case, as indicated in the Figure. In case of DNA target detection, the data acquired in 10 minutes on the same spot presents a signal variation of 1,1 % from the higher to the lower registered signal. The signal on the target is in the range from 1,83 nF up to 1,85 nF. Figure 7 shows the hybridization phase as registered by SPR.
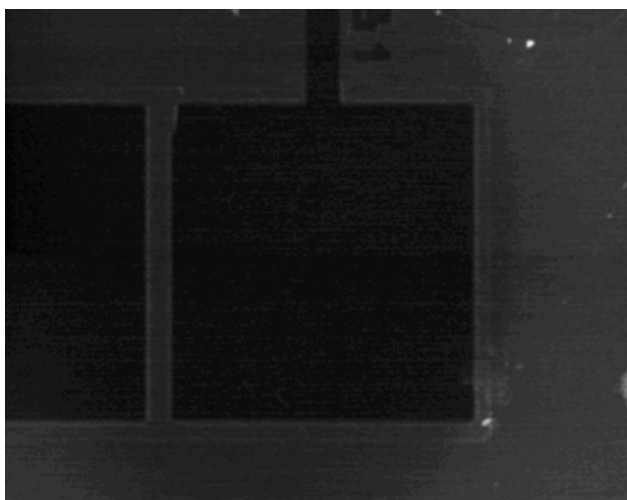
(A)

(B)

Fig 3: Optical microscopy images of chip spots without (A) and with (B) ssDNA probes marked with fluorescence and immobilization onto gold electrodes.
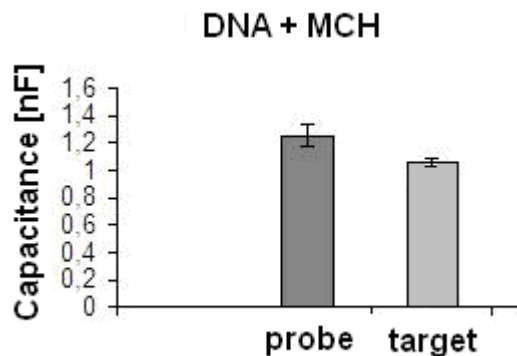
Fig. 4. DNA detection by using 6-mercapto-1-hexanol as blocking agent.

Fig. 5. DNA detection by using DNA probes onto ethylene-glycol monolayers.

C. DNA detection with Ethylene-Glycol layer

DNA hybridization detection by using the NH-terminated probes immobilized onto ethylene-glycol monolayers previously formed on chip gold are summarized in Figure 5. Acquisitions on different chip spots are similar to that showed by Figure 5. In that case, the data are related to capacitance acquired on ethylene-glycol SAM as well as on DNA probes and targets. Standard deviations are now smaller than in the previous case. In case of DNA target detection, the data acquired in 10 minutes on the same spot presents a signal variation of 0,6 % from the higher to the lower registered signal. The signal on the target is in the range from 4,78 nF up to 4,75 nF.

D. AFM imaging on the probe surfaces

AFM imaging is performed in order to verify the probes film structures at the nano-scale. Figure 8 shows AFM image on a probe surface realized onto a Template Striped Gold (TSG) by using SH-terminated ssDNA and 6-mercapto-1-hexanol. The Figure clearly shows evident grooves and distortions on the surface. These large grooves are, instead, absent from the image showed in Figure 9. This image is acquired on a surface prepared onto TSG by using SH-terminated ssDNA and Lipa-DEA.
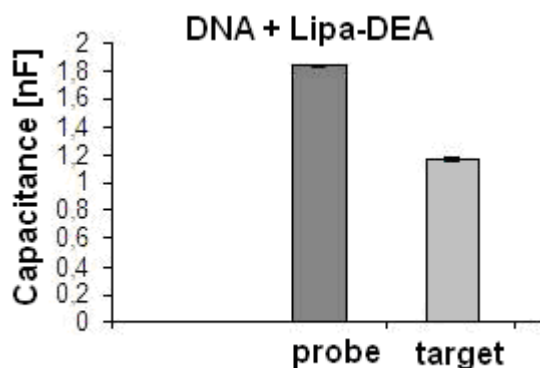
This image presents small clots but grooves are totally absent. The features of Figure 9 are much closely packed with respect features showed in Figure 8. Smoother features are registered on surface prepared by using ethylene-glycol alkanethiols. In fact, Figure 10 shows a surface where neither grooves nor clots are present. In this case the surface average corrugation is smaller than in the two previous cases. Maximum corrugations of 4.2, 1.9 and 0.4 nm were registered on DNA surface immobilized with 6-mercapto-1-hexanol, with Lipa-DEA, and with ehtylene-glycol monolayers, respectively.

IV. DISCUSSION

Figures 4, 5 and 6 compares the detection behaviors of DNA probe surfaces obtained with the different immobilization techniques showed in Figure 1. A decreasing capacitance is observed in all three DNA target detection presented in these graphs. Similar capacitance decrease has also been observed after antibodies immobilization onto alkanethiols without ethylene-glycol function [24]. This decreasing capacitance upon target hybridization has been related to ions removal due to non-hydrophilic target molecules onto the probe surface [2,4]. The capacitance decreases because a conducting aqueous solution is being replaced by a hydrophobic target. Capacitance changes because additional molecules fill up unoccupied sites displacing some of the diffuse layers further out into solution [5].

Fig. 6. Hybridisation of DNA target onto SH-oligo/Lipa-DEA monolayer investigated by chip capacitance measurements.

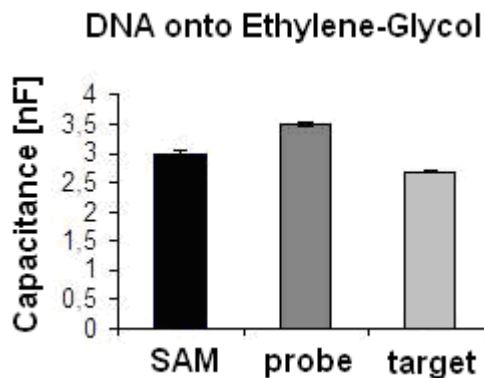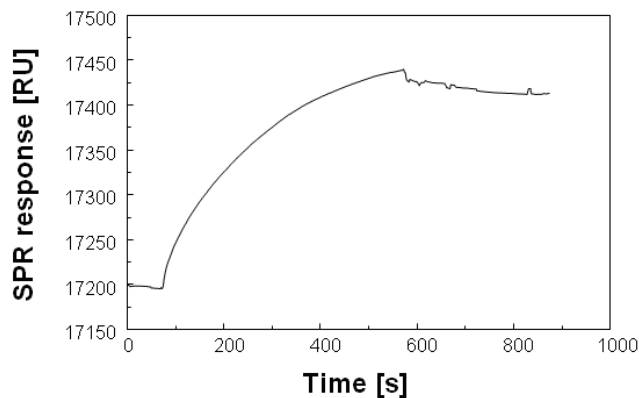Fig. 7. Hybridisation of a 60µg/ml SH-oligo/Lipa-DEA monolayer with 0.5µg/ml complementary DNA investigated by SPR.

Fig. 8. AFM image of a surface of SH-terminated ssDNA probes immobilized with 6-mercapto-1-hexanol.
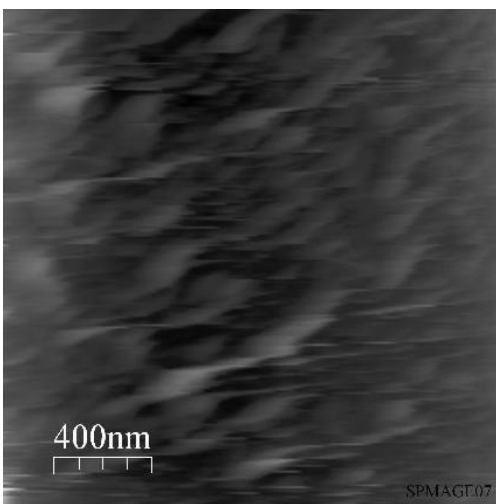

Fig. 9. AFM image of a surface of SH-terminated ssDNA probes immobilized with Lipa-DEA

A capacitance increase is instead showed by Figure 5 after the DNA probe immobilization onto the ethylene-glycol monolayer. Similar increases were also observed after antibody immobilization onto etyylene-glycol monolayers. Such capacitance increases were associated to a net charge contribution of the probes molecules onto a hydrophilic surface [23]. By comparing the three Figures, it is clearly evident that signal errors in Figure 4 are larger than those in Figure 5 and 6. Not only the signal errors are larger on the target DNA but also those on the initial capacitance on the DNA probes are larger. This is due to large time instability registered with 6-mercapto-1-hexanol. In our measurements done with 6-mercapto-1-hexanol as blocking agent, we find capacitance instability in time: data reported in Figure 4 range from 1,10 nF up to 1,37 nF. Similar time instability was registered in case of probes immobilized without blocking agents [13]. Both instabilities are related to ion pathways that are present into the probes layer [17], as schematically shown by Figures 1(A) and 1(B). Such pathways were identified in large grooves showed by AFM images on not well-packed probes layers [23].
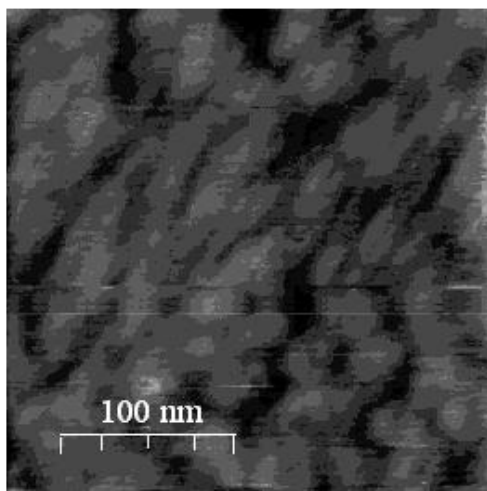

Fig. 10. AFM image of an ethylene-glycol monolayer for NH-terminated ssDNA probes immobilization
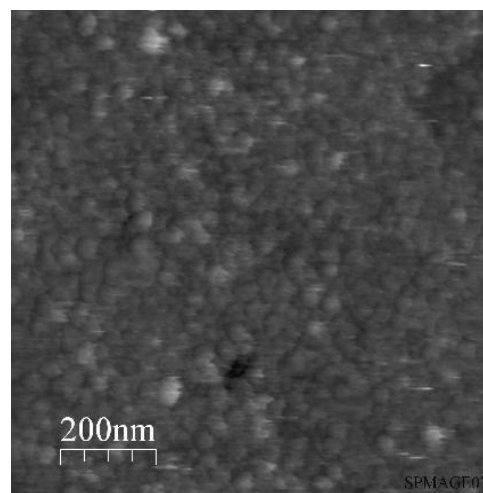
Similarly, Figure 8 shows evident grooves in the AFM image acquired on probe surfaces with 6-mercapto-1-hexanol. On the other hand, DNA detection by using Lipa-DEA as blocking agent or ethylene-glycol anchoring precursors is quite precise resulting in quite stable capacitance signals after DNA targets hybridization. Data related to Figure 5 present only a small 0.6% of capacitance increase within the 10 minutes of the acquisition. While data of Figure 6 present only a small 1,1% of capacitance change. Improved surface stability in case of Lipa-DEA used as blocking agent is also confirmed by SPR investigation, as reported in Figure 7. In both the cases of Lipa-DEA and ethylene-glycol precursors, the AFM images do not present groves, as confirmed by Figures 9 and 10. Figure 9 shows small and high clots which are features related to ssDNA probes on top of short Lipa-DEA molecules. In case of probes precursors, the presence of a stable water layer highly coordinated by the ethylene-glycol chains results in a bit blurred AFM image, as shown in Figure 10. The presence of water strongly coordinated by ethylene-glycol chains was envisaged by *ab initio* calculations and it has been confirmed by infrared spectroscopy and QCM [23]. The strong role played by water in film behavior is also confirmed by the improvement of capacitance stability after water conditioning and by a probe surface behavior close to that of an ideal insulator [23]. More packed structures obtained by using ethylene-glycol precursors or the Lipa-DEA blocking agent are also evident by comparing the average corrugations estimated in AFM images. These are close to 1.9 and 0.4 nm for probe films with Lipa-DEA and ethylene-glycol, while the average corrugation registered in AFM images with 6-mercapto-1-hexanol are close to 4.2 nm. Presence of grooves into the AFM images is so relevant because, in this manner, electron transfer between solution ions and gold electrodes may happen. The presence of groves reported by the AFM image in Figure 8 is a direct proof that the gold electrode surface is accessible to solution ions in the films prepared by using 6-mercapto-1-hexanol. The reason for the Lipa-DEA better performance in DNA detection compared to 6-mercapto-1-hexanol is related to the presence of both two sulfur and hydroxyl groups. In fact,

the two sulfur groups assure a more stable anchoring of the blocking agents onto gold surface while the two hydroxyl groups assure an improved hydrophilic surface to prevent ions penetration into the probes film. Moreover, Lipa-DEA immobilization results in a more wide detection-relative signal with respect to ethylene-glycol immobilization, as clearly showed by a direct comparison of Figure 5 with Figure 6.

V. CONCLUSIONS

The originality of this paper is the identification of surface functionalizations that drastically reduce time drift of interface impedance and, therefore, enhances DNA detection via capacitance measurements. We described new protocols to immobilize single strand DNA probe molecules onto capacitive biochip by means of COOH-terminated ethylene-glycol alkanethiols used as anchoring precursors or by means of Lipa-DEA used as blocking agents. Capacitance measurements were analyzed in terms of signal errors and data ranges. It has been proved that the sensing surfaces prepared with both ethylene-glycol alkanethiols and Lipa-DEA di-thiols present the best performances. Detection with ethylene-glycol precursors presents the smaller standard deviations while that with Lipa-DEA presents higher detection signals. In both the cases, the detection is highly stable in time and it is much better than that with mercapto-hexanol, usually considered as blocking agent The improved stability of these surfaces was explained in terms of ions pathways absence into the probes sensing surface. This explanation was demonstrated by showing the presence of such pathways in AFM images of DNA probes surface obtained by using 6-mercapto-1-hexanol as blocking agent. In opposite, AFM images on ethylene-glycol films and on DNA probes co-immobilized with Lipa-DEA do not present such grooves and show highly packed structures with smaller surface corrugations. In conclusion, we have demonstrated these new fuctionalizations are highly suitable and reliable for applications in label-free capacitance detection in DNA biochip.

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