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Development of a disposable gold electrodes-based sensor for electrochemical measurements of cDNA hybridization

A. Ferrario[a,*], M. Scaramuzza[b], E. Pasqualotto[b], A. De Toni[b], A. Paccagnella[b]

[a]*Department of Biomedical Sciences, University of Padova, 35131 Padova, Italy*
[b]*Department of Information Engineering, University of Padova, 35131, Padova, Italy*

Abstract

This work deals with the development of a disposable electrochemical biosensor for the specific detection of short DNA sequences. The sensor is an amperometric transducer with three planar electrodes, comprising a working, a counter and a pseudo-reference electrode, all made of a gold layer over a polycarbonate substrate. For the development of the genosensor, the working electrode was modified using thiol-tethered 33-mer DNA probe by chemisorptions, in a concentration range from 0.1 μM to 5 μM. Immobilization of ssDNA on gold surface was monitored with electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) in $Fe(CN)_6^{4-/3-}$ and Ruthenium(II)/(III) solutions. The time dependence of ssDNA probe immobilization was also studied. The hybridization detection is then compared with EIS and DPV measurements.

Keywords: DNA sensor, electrochemical impedance spectroscopy, voltammetry, gold electrodes, electron transfer resistance.

1. Introduction

Over the past 20 years, micro fabricated sensors for electrochemical analysis and detection of biological processes, using proteins (enzymes or antibodies), nucleic acids (DNA, RNA, aptamers) or living cells, have been constantly developed and enhanced. A great attention has been focused on new technologies and materials for the sensor interface, in order to improve sensitivity and integration, to minimize dimension and analysis time, and in particular to reduce manufacturing cost.

Silicon-based technology achieved an high miniaturization, due to the well known silicon technology, in very different research fields, from metals analysis [1, 2] to nucleic acids detection [3, 4] or cell manipulation [5]. However, the insufficient long-term stability and, above all, the high cost restrain their practical application. Besides, nanotechnologies offer new interesting possibilities: in recent years, many studies on sensors using nanostructured materials or nanoparticles, which highlight the efficiency and versatility of these kinds of approaches, have been reported. Singh et al. [6] have shown a method to obtained a

*Corresponding author. Tel.: +039-0498277625;
Email address: ferrario@dei.unipd.it (A. Ferrario)

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stable biofunctionalization of a porous silicon surface, for the detection of protein signal. Zhao et al. [7] reported a computer controlled technique to design different nanostructured schemes, suggesting the importance and the sensitivity potential of regular nanostructured surface. Mamuru and Ozoemena [8] and Yet et al. [9] proposed nanoparticles modified surfaces as biosensors, underlying their enhanced electrochemical response. Pan [10] used this technique to improve the sensitivity of a DNA sensor, with the immobilization of gold nanoparticles onto the electrode surface. This work showed that the voltammetric detection of complementary and non-complementary DNA sequences is amplified if DNA probes are combined with gold nanoparticles. While nanostructured sensors show a surprising response, for example in term of electron transfer properties, they are yet far from a low cost mass production [7].

Recently, a variety of papers deals with low cost and disposable devices. In particular, screen printed technology have begun to attract researchers interest for the possibilities of easy large scale production. Erlenkötter et al. [11] proposed a screen printed flexible transducers made of commercial inks and designed for cyclic voltammetric measures. Laschi et al. [12] and Wang [13] developed screen printed biosensors for the detection of DNA hybridisation. These works showed, through electrochemical characterization, that these devices, besides being disposable and easy to prepare, have good electrochemical response, promising a considerable sensitivity in hybridization detection.

A key element for the efficiency of a biosensor is the optimization of the adsorption of the biological substrate [14, 15, 16]. In fact, the organization of the probe self assembly monolayer, which, in turn, depends on the electrode surface characteristics, strongly affects the hybridization efficiency [15, 17, 18]. hence, it is important to study the adsorption process as well as the characteristics of the electrode surface, like the real surface area or the surface roughness factor [19, 20].

Up to now, disposable biosensors, which ensure both low cost, easy reliability and high efficiency, are still a challenge. In this paper we report the characterization of a disposable and low cost gold electrodes based sensor for biomolecular detection. The sensor is an amperometric transducer with gold coplanar integrated electrodes on a polymeric substrate. The process used to create this device is based on the plasma deposition of gold layers on a thin polycarbonate support. The structure of this sensor, i.e. the presence of a counter, reference and working electrodes, allows both Electrochemical Impedance Spectroscopy (EIS) and voltammetric measurements. Moreover, gold working electrodes allow an easy adsorption of thiol-modified DNA sequences.

2. Materials and methods

2.1. Materials

Potassium chloride, sulphuric acid, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, Na_2HPO_4 and Penta Amminechloro Ruthenium(III) chloride (Ruthenium(II)/(III)) were all purchased from Sigma-Aldrich (Milan, Italy). Ethanol 95 % and isopropyl alcohol were from Zetalab (Padova, Italy). Distilled water (Titolchimica, Rovigo, Italy) was used to prepare all the solutions.

Synthetic oligonucleotides were obtained by Diatech (Ancona, Italy). The thiolated probe DNA, abbreviated HS-ssDNA, had the 33-base sequence:

5'-GTA ACA TCA CAG GCT ATT AGT TGC CAA CGT CCT C-3'

and was modified on the 5' to obtained $HS-(CH_2)_6-$ssDNA. The target sequence had the complementary base sequence:

5'-GAG GAC GTT GGC AAC TAA TAG CCT GTG ATG TTA C-3'

without the thiol modification.

2.2. Device fabrication

The sensor layout was designed to be used both with EIS [21] and voltammetric measurements (see Fig. 1). The device consists of four gold coplanar electrodes: a reference (PRE, pseudo reference electrode), a counter and two worker electrode (in this work are called worker and control) of 1 mm^2 area. The gold

layer was prepared by plasma sputter deposition of ~ 80 nm of gold on polycarbonate substrate (~ 1 mm thick). Electrodes were cleaned by exposure to 10 % sulphuric acid 5 min, avoiding the deterioration of polycarbonate [reference policarbonato acido]. An insulator layer is then printed (Dimatix DMP-2800, US) over the device in order to define the electrochemical cell and to insulate the conductive tracks. The free gold pads are suitable for an USB port.

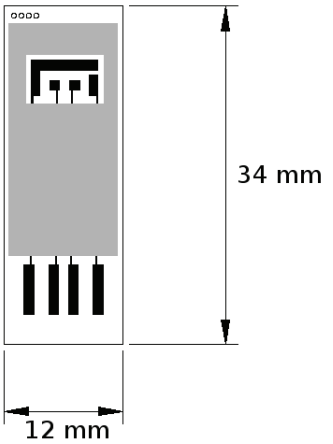


Fig. 1. Layout, not to scale, of the gold electrodes sensor. Polycarbonate substrate is represented in white, gold electrodes in black and the printed insulator layer in grey.

2.3. Electrochemical measurements

Voltammetric measurements were carried out using CH440A potentiostat and CH 8.3 software (CH Instruments Inc., Austin, USA) with the counter and reference electrodes integrated in the device. A commercial Ag|AgCl|KCl 1 M reference electrode (CHI111, CH Instruments Inc., Austin, USA) is used to compared results from the internal reference. Two solutions of 1 mM $Fe(CN)_6^{4-/3-}$ in KCl 100 mM and $[Ru(NH_3)_5Cl]Cl_2$ in KCl 100 mM were prepared. For ciclovoltammetry (CV) the potential was scan from +0.4 to −0.4 V, for hexacyanoferrate(II)/(III) solution, and from +0.2 to −0.6 V for Ruthenium(II)/(III) solution, with a scan rate of 100 mVs^{-1} (unless otherwise specified). Differential pulse voltammetry (DPV) was performed in the same potential range with a pulse amplitude of 5 mV and a pulse width of 0.2 s. Chronocoulometry were performed width a pulse period of 500 ms and a pulse width of 500 mV.

Electrochemical impedance spectroscopy was carried out with Solartron 1260 impedance analyzer (Solartron, US). Measurements were performed in frequency range between 100 mHz and 1 MHz, using alternating voltage of 5 mV over a 0 V bias (vs PRE).

Just prior to electrochemical experiments, the gold electrode was washed with and isopropyl alcohol in a sonicator (Ultrasonic 06, Falc Instruments s.r.l., Treviglio, Italy), and then rinsed with distilled water. Then a drop of 60 µl solution was cast on the active area, waiting 1 min before the measurement. All operations were carried out at room temperature (25 °C). The charge transfer resistance R_{ct} was determined by fitting data to a Randles equivalent circuit, where the R_{ct} resistance is in series to the Warburg element, that accounts for the diffusion of the redox couple. The double-layer capacitance C_{dl} is replaced by a constant phase element CPE, in parallel with R_{ct} and Warburg element [22, 23, 24, 25].

Data were analyzed and fitted with MATLAB (The MathWorks, Inc., US).

2.4. Electrode modification

The gold worker electrode is modified by chemical adsorption of probe solution by casting 0.7 µl HS-ssDNA solution, in a concentration range between 2 µM and 20 µM, for a specific amount of time (2 h,

unless otherwise specified). The DNA buffer solution was Na_2HPO_4 100 mM, pH 8.5. The adsorption process was carried out inside a wet sealed Petri, in order to avoid sample evaporation, at room temperature. Before measurements and hybridization, the electrode surface was washed three times with 20 µl of KCl 100 mM and then was rinsed thoroughly with deionized water.

Before hybridization, the target solution was heated at 90 °C for 1 min. Hybridization was obtained by placing 1 µl of target solution directly onto the probe-modified electrodes for 3 hours, in a wet sealed Petri at 65 °C. The hybridization buffer solution was Na_2HPO_4 100 mM, pH 8.5. After hybridization, the electrode surface was washed three times with 20 µl of KCl 100 mM, to remove non specific adsorbed sequences, and then was rinsed thoroughly with deionized water.

3. Results and discussion

3.1. Electrodes characterization

The surface properties of the Au working electrode and Au reference electrode were investigated by cyclic voltammetry experiments, with 1 mM $Fe(CN)_6^{4-/3-}$ in KCl 100 mM solution, as the surface characteristics govern the electrochemical behaviour.

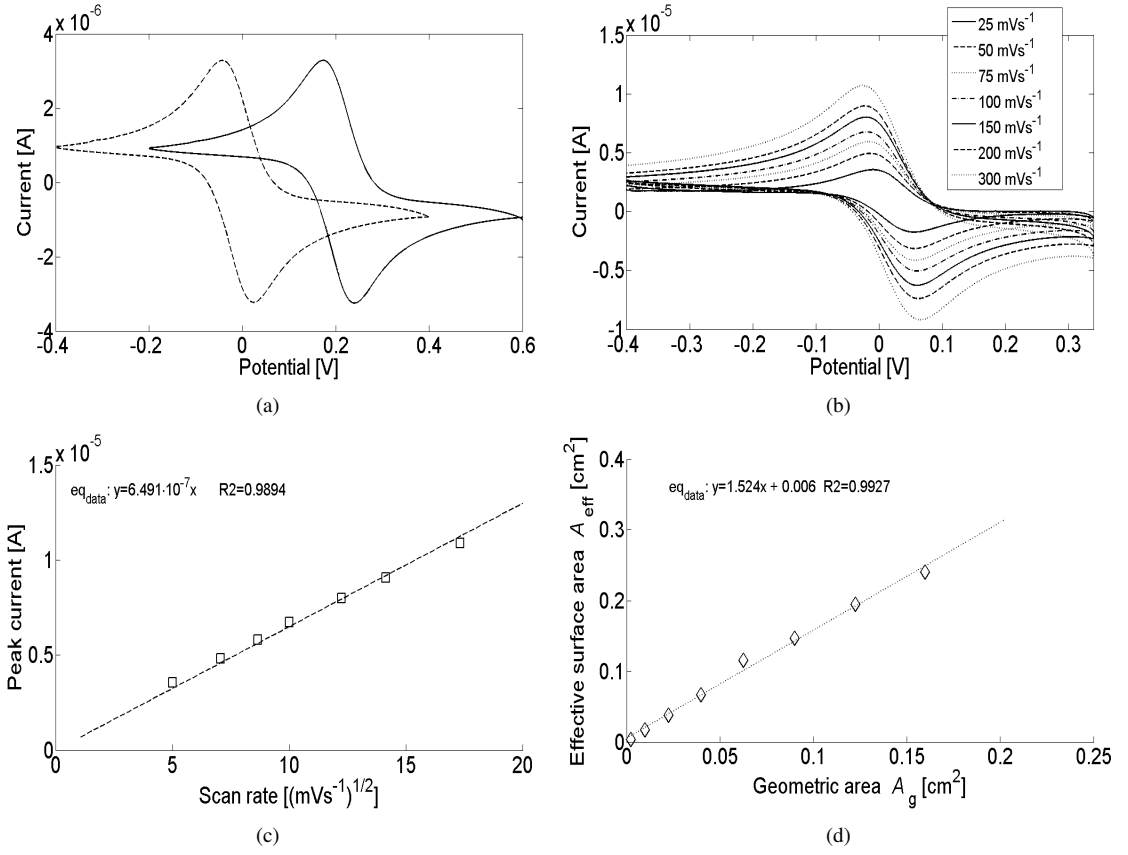


Fig. 2. CV scans of hexacyanoferrate(II)/(III) 1 mM in KCl 100 mM on bare gold electrode: (a) comparison between the sensor pseudo-reference and a standard Ag|AgCl|KCl 1 M reference electrode; (b) CV scans at different scan rates, from 25 mVs^{-1} to 300 mVs^{-1}; c) peak current vs $\nu^{1/2}$ and fitting results; d) relation between the electrode geometric area A_g and effective surface area A_{eff} calculated from CV measurements, where the slope of the fitting curve shows the surface roughness factor f_r.

Fig. 2(a) shows the comparison between cyclic voltammetry measurements obtained with the Au reference and the standard Ag|AgCl|KCl 1 M reference electrode. The electrochemical behaviour with the

two difference electrodes are similar, while a potential difference of 236 ± 5 mV was observed. The difference between E_{pc} and E_{pa} results $\Delta E_p = 0.064 \pm 0.002$ V while the ratio between the current peaks is $I_{pa}/I_{pc} = 1.043 \pm 0.013$. The ΔE_p and I_{pa}/I_{pc} values indicate a nearly reversible electrode reaction: in fact, for a nernstian wave ΔE_p is close to $59/n$ mV, where n is the number of electrons involved in the reaction, and $I_{pa}/I_{pc} \approx 1$. Moreover, no significant potential variation of the gold electrode was observed, thus the gold electrode can be used in replacement for our sensor.

CV measurements of hexacyanoferrate(II)/(III) couple at different scan rates ν (see Fig. 2(b)), from 25 $\mathrm{mVs^{-1}}$ to 300 $\mathrm{mVs^{-1}}$, show that no adsorption effects act on the Au working electrode surface [11]. As shown in Fig. 2(c), a $\nu^{1/2}$ dependence for cathodic peak current I_p was found, as required for these kinds of processes. The linear relationship among I_p vs $\nu^{1/2}$ was $y = 6.49 \cdot 10^{-7} + 2.66 \cdot 10^{-7}$ ($r^2 = 0.9894$).

The electrochemical behaviour of a gold plan electrode depends on the real o microscopic surface area. The latter, also called electrochemical surface area (ESA) or effective surface area, A_{eff}, is usually different from the geometric one, A_g. The ratio between the two areas

$$R_f = \frac{A_{eff}}{A_g}, \tag{1}$$

is called the surface roughness. The surface roughness was investigated with two methods proposed by Trasatti et al. [26]. In the first case the electrochemical effective surface area A_{eff} was calculated from the quasi-reversible reaction [22]

$$I_p = \left(2.69 \cdot 10^5\right) n^{3/2} A D_0^{1/2} c_0 \nu^{1/2}, \tag{2}$$

where the diffusion coefficient D_0 is $7.6 \cdot 10^{-6}$ $\mathrm{cm^{-2}s^{-1}}$ ferricyanide ion [22] and $\nu = 100$ $\mathrm{mVs^{-1}}$. To obtain an accurate estimation, electrodes with different geometrical area were measured with cyclic voltammetry. Fig. 2(d) shows the value of the effective surface area A_{eff}, calculated from Eq. 1 as a function of the geometrical area. Then, the slope of the fitting regression line $y = 1.52 \cdot 10^{-6}$ ($r^2 = 0.9927$) represents the roughness factor, which is $R_f = 1.52$. Moreover, the gold surface area was investigated with the oxygen chemisorption measurements [26]. The standard reference charge of chemisorbed oxygen layer is assumed to be 390 ± 10 $\mu\mathrm{Ccm^{-2}}$ for polycrystalline Au. The determination of the roughness factor was done by extrapolating the charge from chronocoulometry (CC) measurements. The roughness factor is the ratio between measured charge and the reference charge. In this case, a value of 1.47 ± 0.11 was found for our gold electrodes. Carvalhal et al. [19] found a value of 1.42 for a chemically pretreated gold electrode.

3.2. ssDNA probe adsorption

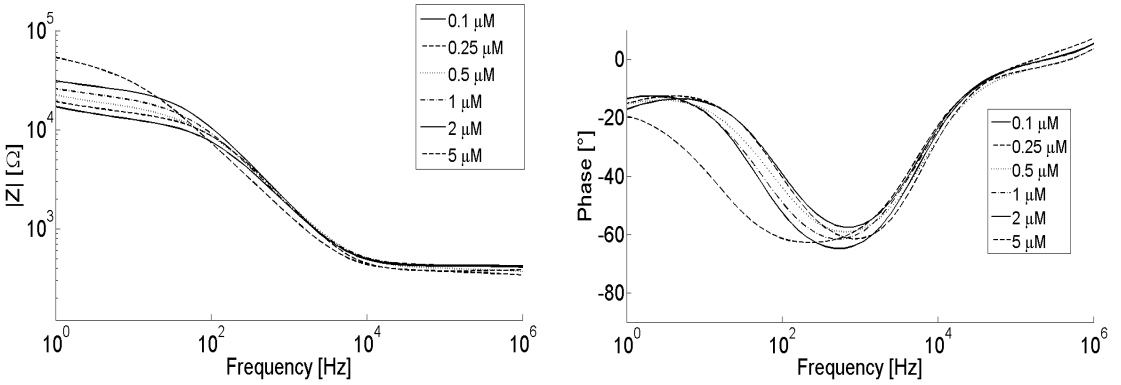
To develop the DNA sensor, the ssDNA probe adsorption was studied as a function of the concentration and the adsorption kinetic. The ssDNA concentration range was between 0.1 μM and 5 μM. Opmtization of probe adsorbtion over the electrode is fundamental for the hybridation detection efficiency.

Fig. 3 depicts Bode diagram of electrical impedance Z obtained from EIS measurements of the worker functionalized with six different concentrations of ssDNA probe concentrations. From these figures can be seen that the impedance magnitude increases as probe concentration increase and that this effect is particularly evident for frequencies below 10 Hz.

Fig. 4(a) shows Nyquist diagrams of the same EIS measurements sets in the range between 1 Hz and 1 MHz: as can be seen, the diameter of the low-frequency impedance semicircle increases with ssDNA probe concentrations.

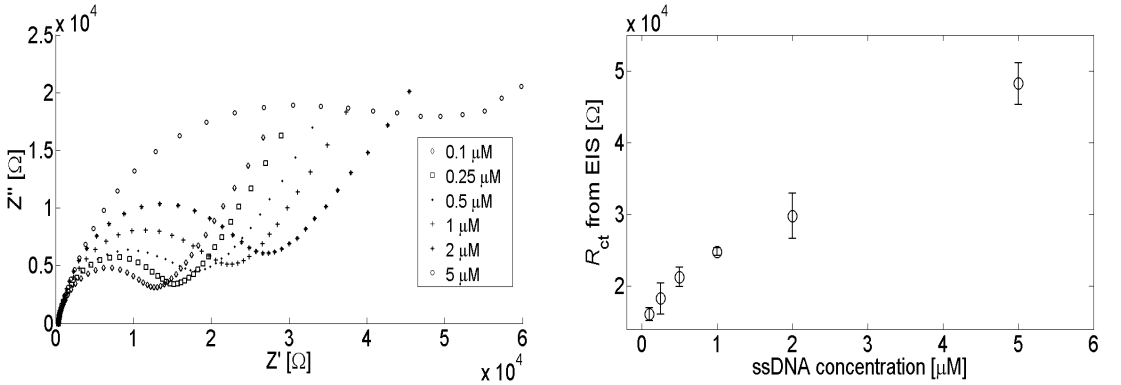
By fitting these values it is possible to evaluate the charge transfer resistance [25] as a function of probe concentration (see Fig. 4(b)): R_{ct} is the parameter that models interfacial charge exchange, therefore its growing values with ssDNA probe concentrations indicate an increase of adsorption process at the working electrode.

DPV measurements have been carried out on electrodes functionalized with different concentrations of ssDNA probe in order to assess the influence of adsorbed molecules on the electrode surface. The results of these measurements are depicted in Fig. 5 , where can be seen the lowering of measured current as probe concentration increase, i.e. as R_{ct} rises.



(a) (b)

Fig. 3. Impedance modulus (a) and phase (b) of EIS measurements of hexacyanoferrate(II)/(III) 1 mM in KCl 100 mM on ssDNA modified electrode as function of ssDNA probe concentration.



(a) (b)

Fig. 4. Nyquist plot (a) of EIS measurements of hexacyanoferrate(II)/(III) 1 mM in KCl 100 mM on ssDNA modified electrode and extrapolated charge transfer resistance R_{ct} (b) as function of ssDNA probe concentration

Fig. 6(a) describes ssDNA probe adsorption behavior as a function of functionalization time. DPV measurements have been carried out on workers functionalized with a solution of ssDNA probe at the concentration of 2 μM. The functionalization solution has been left in contact with the electrodes for a time variable between few minutes and two hours. During the first 20 minutes a sharp decrease of DPV current peaks can be observed, while after this period the decrease is less sharp but nevertheless present.

This situation is better described in Fig. 6(b), where the DPV current peaks are normalized to the non-modified gold electrode DPV peak current: from this figure can be seen that about after one hour the normalized current trend reaches the 90 % of its maximum value that indicates a situation in which the electrode surface is almost completely covered by adsorbed ssDNA probe.

Fig. 7 shows the results of DPV measurements on electrodes functionalized with different concentrations of ssDNA probe in Ruthenium(II)/(III) 1 mM. Positive Ruthenium(II)/(III) ions are attracted by ssDNA backbone [27], therefore a larger concentrations of adsorbed probes lead to an higher DPV current peak value.

The potential peak trend saturates to about −400 mV above 5 μM ssDNA probe concentration, while the peak current one keeps growing linearly from 1.55 μA to about 1.75 μA, proving the positive ions attraction.

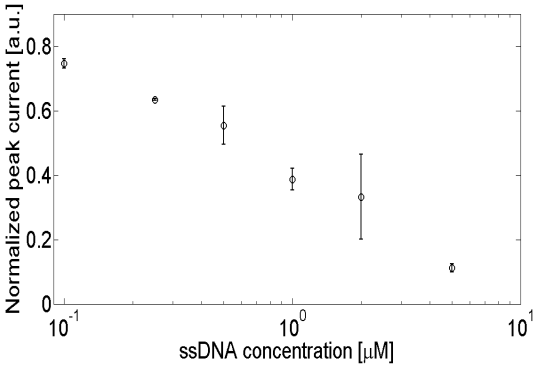


Fig. 5. DPV normalized peak current of hexacyanoferrate(II)/(III) 1 mM in KCl 100 mM on ssDNA modified electrode as function of ssDNA probe concentration.

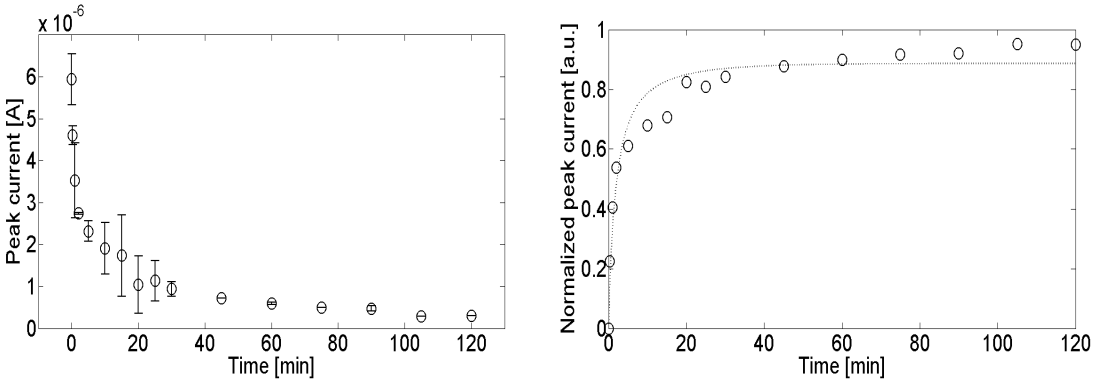


Fig. 6. (DPV peak current (a) and normalized peak (b) of hexacyanoferrate(II)/(III) 1 mM in KCl 100 mM on ssDNA modified electrode as function of time adsorption.

3.3. Hybridization detection

The developes for gold electrodes based sensor was then used to detect the hybridization of a target sequence. The working electroded was modified with 2 μM ssDNA probe, while the control electrode was not modified in order to verify the stability of the pseudo reference electrode and to have a direct mesurements of the bare gold working electrode. The target sequence, described in section 2.1, was at a concentration of 20 μM. Both EIS measurements and DPV measurements were used to perform the detection.

Fig. 8 shows impedance modulus (a) and the nyquist plot (b) of the control electrode, the working electrode modified with ssDNA probe and after hybridization with target sequence. A big impedance variation is clear for frequencies below 100 Hz. Thus, the interaction between the negative hexacyanoferrate(II)/(III) ions and the cDNA backbone induces an increase in the charge tranfers resistance R_{ct}.

The signal variations, with respect to the non modified gold electrode, obtained with EIS measurements and DPV measurements are depicted in Fig. 9. Both electrochemical techniques can be applied to the four electrode sensor to detect the hybridization. DPV measurements gave a signal variation of 57 % for adsorption of probe sequences and a variation of about 76 % for DNA hybridization. On the other hand, EIS measurements seems to be more sensitive in the hybridization detection, as, for the same target concentration, the signal variation is nearly 40 times higher than the ssDNA signal.

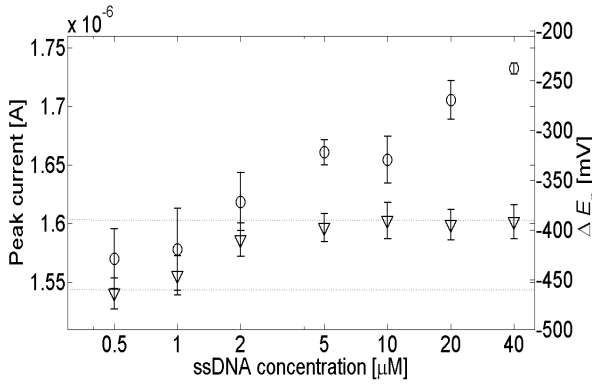
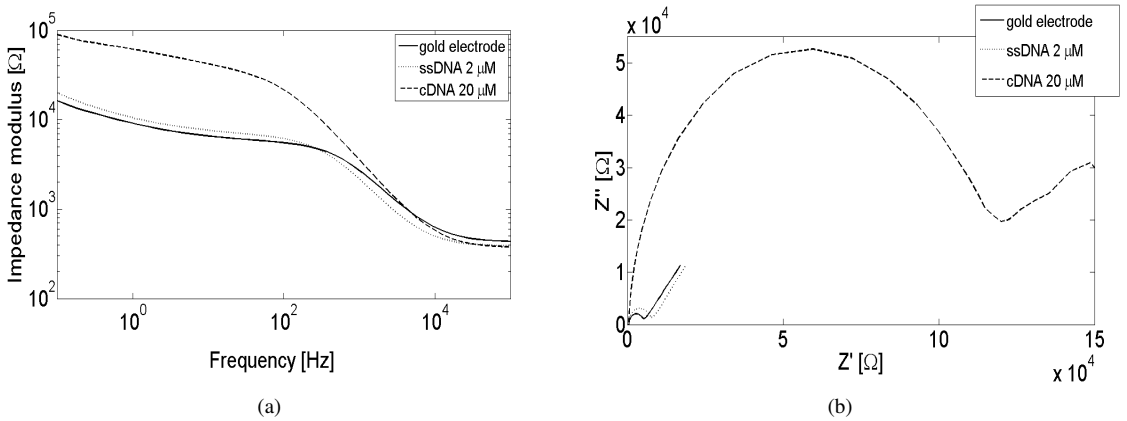


Fig. 7. DPV peak current (a) and peak potential (b) of Ruthenium(II)/(III) 1 mM in KCl 100 mM on ssDNA modified electrode as function of ssDNA probe concentration.



(a) (b)

Fig. 8. Impedance modulus (a) and Nyquist plot (b) of EIS measurements with hexacyanoferrate(II)/(III) 1 mM in KCl 100 mM on bare gold electrode, ssDNA modified electrode and after hybridization with 20 μM target sequences.

4. Conclusions

In this work a disposable and gold electrodes based sensor for biomolecular detection was developed and characterized. The sensor is made of four gold coplanar electrodes on a polymeric substrate. The layout of this sensor allows both Electrochemical Impedance Spectroscopy (EIS) and voltammetric measurements.

The gold pseudo reference alectrode and the working electrode were characterized. Results show a good responce to electrochemical processes. Morever, the active surface area was evaluated in order to improve the pretreatment process.

The adsorption of thiol-tethered 33-mer DNA probe was investigate in a concentration range from 0.1 μM to 5 μM. Moreover, the kinetic was studied from few minute to two hours. Results gave us the ability to crontrol and optimize the immobilization of ssDNA on gold surface, and thus to improve the cDNA detection.

Hybridization was then verified with EIS and DPV measurements. This sensor shows a greater sensitivity to the hybridazation event with electrochemical impedance spectroscopy measurements.

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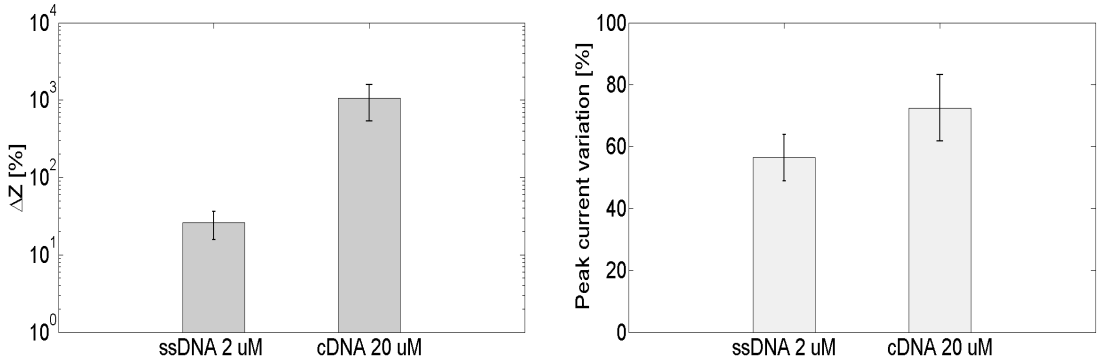

(a) (b)

Fig. 9. Comparison between EIS signal variation (a) and DPV signal variation (b) for 2 μMssDNA probe adsorbtion and 20 μM target hybridization. Variation are referred to the non modified gold electrode.

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