Detection of DNA hybridyzation with impedance amplifying labels

Kuo-Sheng Ma*, Hong Zhou*, Alessandra Fischetti**, Jim Zoval*, Marc Madou*

*Department of Mechanical and Aerospace Engineering
Henry Samueli School of Engineering
University of California, Irvine
**ST Microelectronics, Milan, Italy

ABSTRACT

Traditionally used nucleic acid analysis detection platforms rely on fluorescent or radioactive labels. Optical techniques require large and expensive equipment such as automated colorimetric and fluorimetric instruments that greatly increase the cost of the whole set-up. Working with radioactive labels is an even less attractive option. Electrical measurements of DNA hybridization are expected to involve less complicated and less expensive instrumentation and feature detection limits similar to the traditional optical methods. The diagnostic technique we introduce here will enable low-cost molecular diagnostics methods to become a reality and broaden the extent of the nucleic acid analysis applications.

Keywords: DNA, streptavidin, alkaline phosphatase, electrochemical impedance spectroscopy, impedance

1. INTRODUCTION

Today, the most commonly used method for detection and quantification in nucleic acid analysis involves luminescent measurements using a variety of labels (i.e., fluorescent and/or chemiluminescent probes, photoproteins, etc.). Traditionally used nucleic acid detection techniques rely on fluorescent or radioactive labels [1]. Spectroscopic methods afford the necessary sensitivity but require large and expensive equipment such as automated colorimetric and fluorimetric instruments greatly increasing the cost of the whole detection system. Working with radioactive labels is an even less attractive option since the protocols require well-trained technicians. Electrical detection of oligonucleotide binding is expected to involve less complicated and less expensive instrumentation and have similar detection limits compared to the traditional optical methods.

The success of biosensors based on label-free or label-less impedance sensing has been the topic of debate for years [2][3][4]. The debate is on-going since the promise of a simple impedance detection scheme of this nature remains very attractive. Label-less impedance sensing of affinity binding on functionalized insulators, semiconductors, heterostructures (semiconductor/ dielectric/ electrolyte) and metals have been attempted [5]. While the feasibility of such electrochemical impedance measurements on insulators, functionalized heterostructures and semiconductors was demonstrated [6][7][8], more convincing data were obtained using a metal as support for the DNA hybridization [9]. When metal electrodes such as Pt and Au are used as the substrate for DNA hybridization they exhibited stronger signals (e.g., detection limit of about 10 ng/ml for Si/SiO_2 hetero structures vs. around 10 pg/ml for a gold electrode). A typical example of work in this area by Bergren [9], demonstrates the feasibility of a biosensor for direct detection of DNA hybridization on gold. It must be noted though that even here the specificity was not very high and the reproducibility of the sensors was poor. Martelet et al. [10] presented so far the most convincing antibody/antigen binding detection on gold functionalized electrodes using a differential impedance measurement. This group demonstrated the ability to detect an antibody/antigen bio-recognition event with a functionalized Pt electrode (0.3 mm in diameter) and a functionalized Au electrode (1.0 mm in diameter).

The aim of this work is to provide a more sensitive and more reproducible method for the detection of DNA hybridization by measuring electrical impedance changes upon DNA hybridization.

2. MATERIALS AND METHODS

2.1 Materials

All reagents were purchased from Aldrich Chemical and used as received, unless otherwise noted. All solutions were made up using deionized water (18 MΩcm resistivity). DNA oligonucleotides were purchased from BioSource Inc. (Camarillo, CA). The two different probes used in this work were T1 and T2, DNA sequences (25mers) with the 5' end thio-modified and with a 6-mercaptohexyl spacer. The single-stranded target (CT1) is a 25mer complementary to probe T1 and CT1' has the same sequence as CT1 but with a biotin label on the 3' end. The sequence and modification of each oligo-nucleotide is listed in Table 1.

Name	Labeled	Sequence
T1	5'-SH-$(CH_2)_6$-	5'-CACGACGTTGTAAAACGACGACCAG-3'
T2	5'-SH-$(CH_2)_6$-	5'-GATGCCCGGGTCCGGCTAGATGATC-3'
CT1	none	5'-CTGGTCGTCGTTTTACAACGTCGTG-3'
CT1'	3'-biotin	5'-CTGGTCGTCGTTTTACAACGTCGTG-3'

Table 1 Oligonucleotides Sequences and Modification

2.2 Electrode preparation

Single-crystal Si (001) covered with a 400 nm thick thermal silicon dioxide was used as the substrate material. Before metal deposition the substrates were cleaned using an RCA clean (5 parts deionized water, 1 part NH_4OH, 1 part H_2O_2). In order to promote adhesion of the gold to the silicon dioxide, a thin Cr adhesion layer (50 nm) was deposited by e-beam evaporation, followed by 200 nm of gold.

The measuring electrodes were patterned using photolithography. The lithography process is schematically illustrated in Figure 1. A thin layer of positive photoresist (Shipley 1827) was spun onto the gold coated substrate at 4000 rpm for 40 seconds, followed by a soft bake at 90 °C on a hot plate for 2 minutes. The photoresist was then UV exposed through a patterned iron oxide mask in a Karl-Suss aligner and subsequently developed in MF 319 developer. Au etchant and then Cr etchant were used to remove the unwanted metal areas. Finally, the remaining photoresist was removed by acetone. The dimensions of the working electrode and counter electrode are 200μm x 200μm.

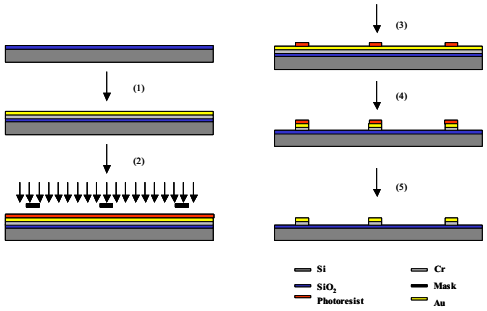


Figure 1. Fabrication process for the detection device

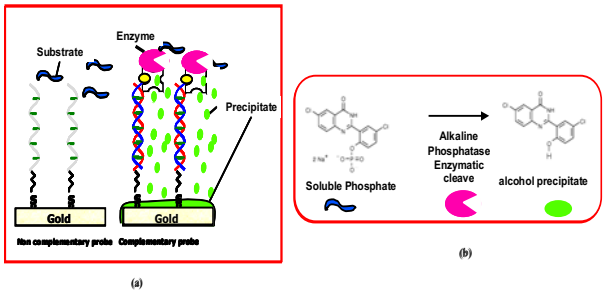
2.3 Self-assembled monolayer films preparation

Prior to the formation of thiolated-ssDNA self-assembled monolayers on the Au electrodes, the Au electrodes were cleaned by dipping into a 3% aqueous HF solution for 10 second followed by extensive rinsing with deionized water (18 MΩcm). Next the electrodes were blown dry using pure nitrogen gas and immediately transferred into a solution of 1μM of oligonucleotide probes in a potassium phosphate buffer (0.5M, pH=7). For the thiolated-ssDNA self-assembled monolayers to form we allowed for 15 hours or more. Finally, the surfaces were rinsed with deionized water for 5 s and dried under a stream of pure nitrogen.

2.4 Procedures

All electrochemical characterizations were performed with an Electrochemical Impedance Spectroscopy Potentiostat from Gamry Inc. (Warminster PA). A probe station (Micromanipulator Inc. Carson City, NV) was used to contact the contact pads of the counter and working electrodes. The solution we used for all the reported impedance measurements is a 100mM NaCl in 1X TE buffer (10mM Tris, pH 7.4 and 1mM EDTA). The bare gold impedance values were measured in this solution after patterning and cleaning. The ssDNA self-assembled monolayer impedance values were measured in the same solution after immobilization of ssDNA onto the electrode surface and after cleaning. Hybridization was performed by spotting the CT1 or CT1' solutions (1μM DNA in 1X TE buffer plus 1M NaCl) on the electrode surfaces. After 4 hours, the electrodes were rinsed with impedance measurement buffer for 10 seconds and dried under a stream of pure nitrogen prior to characterization.

As a method for impedance change amplification upon DNA hybridiziation, we used the commercially available Enzyme-Labeled Fluorescence (ELF 97) signal detection technique (Molecular Probes, Eugene OR). This well-known technology involves the alkaline phosphatase enzyme converting the ELF substrate (ELF-97), a water soluble weak blue fluorescent molecule, to an insoluble, intense green fluorescent molecule. The insoluble product precipitates onto the surface of the solid phase used in the assay. We speculated that this type of a precipitation on an underlying conductor electrode would enhance/amplify the impedance change upon hybridization significantly. The



experimental procedure is schematically illustrated in Figure 2(a) and the chemical reaction involved is shown in Figure 2(b).

Figure 2 (a) Schematic of signal amplification using alkaline phosphatase as an enzyme label. (b) Chemical reaction involved in the amplification process.

3. RESULTS AND DISCUSSIONS

3.1 Theory of DNA hybridization detection with electrochemical impedance spectroscopy (EIS) measurements

When a conductor is placed in an electrolyte solution a potential is generated due to an unequal distribution of charges across the interface (Figure 3(a)). Two oppositely charged layers, one on the electrode surface and one inside the electrolyte form a "double layer," which can be modeled as a parallel plate capacitor. When an additional layer is present at the electrode, such as an oxide or a self-assembled monolayer (SAM), an additional capacitance and/or resistance associated with that layer is added to the

circuit. In principle, the introduction of molecules, such as DNA, to that interfacial region, will affect the measured complex impedance through changes of the local geometry, the dielectric constant, and the amount of charge at the electrode/electrolyte interface.

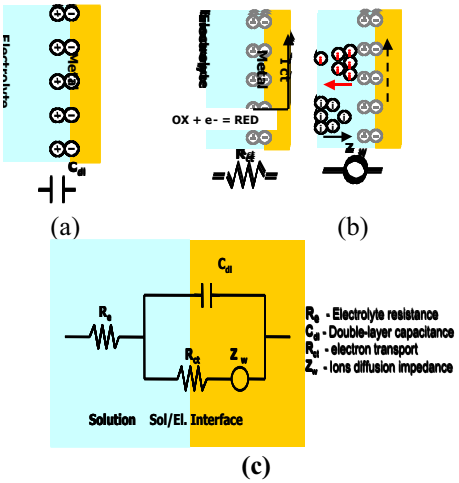


Figure 3. Interface phenomena and Randles equivalent circuit. (a) Electrical double layer capacitance C_{dl}. (b) Charge leaking across the double layer represented by "charge transfer" resistance R_{ct} and diffusion of ions to the interface represented by Warburg impedance Z_w. (c) Randles' equivalent circuit.

A perfect biomolecule recognition layer, for example a self-assembled monolayer of single stranded DNA (a SAM of ssDNA), would cover the electrode completely and the selective binding of complementary biomolecules to that layer (i.e., hybridization in the current example) would be the only contributing impedance element. Figure 3(b) reveals possible competing processes, these include (1) a 'charge transfer' resistance R_{ct}, corresponding to charge leakage across the double layer (this could involve electrons and/or ions), given by

$$R_{ct} = RT / nFi_0 , \qquad [1]$$

Where R is the universal gas constant, T is the absolute temperature, n is the number of charges involved in the electrode reaction, F is Faraday's constant and i_0 is the exchange current density (electrons and/or ions); and (2) the Warburg impedance Z_w, corresponding to the diffusion of ions to the interface from the bulk of the electrolyte. The latter is given by

$$Z_\omega = (1-j)\sigma\omega^{-1/2} , \qquad [2]$$

Where ω is the angular frequency (s^{-1}) and σ is the Warburg coefficient ($\Omega\, s^{-1/2}$). The equivalent circuit of such an interface is represented in Figure 3(c), and is known as the Randles equivalent circuit model, featuring all the above elements as well as a series resistance corresponding to the solution resistance (R_e). To maximize the device's selectivity to a bio-recognition event at the electrode surface, the impedance variation must be dominated by the capacitance change and not by the electrolyte resistance, charge transfer or diffusion effects. To optimize the sensors' sensitivity one consequently works in a frequency regime where the capacitive contributions associated with the binding of biomolecules dominates. For example, in the case of an Au electrode covered with a SAM of ssDNA, at high frequency, the impedance is dominated by the electrolyte resistance R_e. At low frequency the impedance of the sensor exhibits phase and quadrature values that are proportional to $\omega^{-1/2}$, indicative of Warburg impedance control. Only measurements conducted at medium frequencies, with our electrode arrangement we found that to be around 100 Hz, are dominated by the double layer capacitance changes occurring as binding phenomena take place.

3.2 Optimized impedance measurements for DNA hybridization sensing

Based on the theory described in 3.1, the double layer capacitance will change upon surface modifications induced by binding of molecules into that double layer. We will use this property first to measure the double layer capacitance changes upon label-less DNA hybridization onto surface immobilized capture probes. Figure 4 shows the results of impedance measurements made on bare gold electrodes, on gold electrodes with specific and non-specific ssDNA capture probes, and on the same electrodes after hybridization. In the case of specific DNA hybridization, we see an impedance change of 10% at an optimum measuring frequency of 100 Hz . In the case of non-specific DNA hybridization no impedance change is registered. Thus, in principle, label-less DNA hybridization can be monitored using a single frequency impedance measurement.

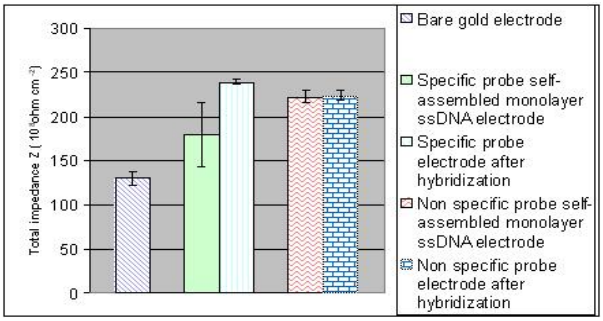


Fig 4. Total impedance measurements at 100Hz made on bare gold electrodes, specific and non-specific ssDNA capture probes on gold electrodes, and the same capture probe electrodes after complementary target strands were hybridized. The dimension of the electrode are 50X 50 um^2. (n=3, error bars = 1 standard deviation)

As pointed out in Section 3.1, in order to selectively monitor a bio-recognition process through its accompanying impedance change, the electrode must be

perfectly polarizable. The impedance changes are expected to be larger and more reproducible if the bio-recognition layer is attached to the electrode surface in a site-specific manner and allows for an optimized interaction with the target DNA. The use of alkanethiol self-assembly methods to fabricate DNA probe-modified gold surfaces is known to form a good surface coverage that exhibits high hybridization activity. But even with this type of surface preparation, thio-labeled ssDNA adsorbs onto the gold surface, both via thiol-gold linkage and non-specific interactions, thus introducing undesirable random impedance variations. The impedance amplifying techniques introduced here, should, in principle, decrease the need for an ideally polarizable interface as the signal to noise ratio (S/N) is improved dramatically by the amplification of the impedance signal.

Impedance measurement results, comparing DNA hybridization with and without enzyme amplification are shown in Figure 5 (we worked again at a fixed frequency of 100 Hz). The impedance data for specific DNA hybridization without the enzyme label is used as a reference point here (normalized to 1). With amplification label, specific hybridization leads to an impedance change of a factor 1.6 (and factor of 4 when compared with bare gold !) . Clearly the insoluble precipitate from the enzyme reaction does amplify the impedance change after hybridization and enzymatic reaction. Similar experimental results have been presented by Willner [11], who also reported a 4 times increase in the double layer capacitance with enzyme amplification technique for the sensing of DNA hybridization.

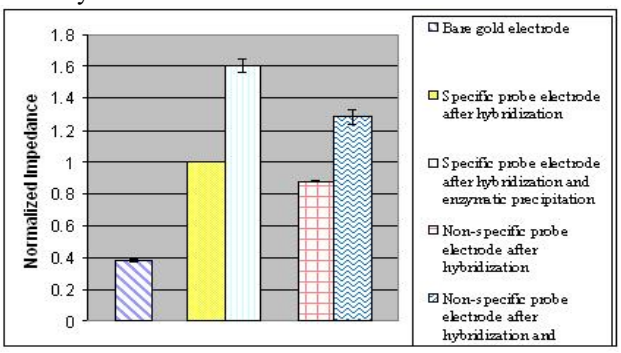


Fig 5. A comparison of the impedance change after DNA hybridization with and without an enzyme labeled. (n=3 error bars = 1 standard deviation)

However, for the non-specific DNA hybridization, the impedance also increased. The reason for that maybe because the streptavidin-alkaline phosphatase conjugate physically adsorbs on the capture probe which also generates some insoluble precipitate onto the electrode.

4. Conclusions

In this work, we have shown that label-less DNA hybridization can be detected using a single frequency impedance measurement. However, the difficulty reproducing such results makes this technique undesirable. The irreproducibility is due to the difficulty in reproducing high quality DNA capture probe layers.

In the case of enzymatic impedance labels, the signal to noise ratio is improved and this could potentially overcome irreproducibility in SAM manufacture but results are still inconsistent. This may be a consequence of the precipitating film being deposited unevenly over the Au/thio-dsDNA electrode. The solution we have come up with and are currently exploring is to use an "Adjacent Impedance Probing" (AIP) technique. In this case, the DNA hybridization site is optimized for the biorecognition event (this site does not need an underlying conductor) and a bare adjacent electrode is optimized for generating the largest possible impedance change through precipitation or passivation. We believe the AIP technique will dramatically increase the sensitivity and reproducibility of the assay. The technique could lead to a more consistent and inexpensive method for impedance measurement of nucleic acid hybridization.

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