A Simple and Highly Sensitive Electrochemical Platform for Detection of MicroRNAs

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***Abstract* — MicroRNAs (miRNAs) in blood can act as fingerprints to many diseases including cancer. However, detection of low levels of these miRNAs in blood demands for very sensitive techniques. Despite many reports available for miRNA detection, the available methodologies have drawbacks such as complexity, use of expensive enzymes, etc. We here report a new and simple electrochemical method to detect miRNAs by exploiting the intrinsic charges of the oligonucleotides and a simple amplification method with positively charged gold nanoparticles (AuNPs). Electrochemical impedance spectroscopy (EIS) was used to monitor the changes in capacitance upon miRNA binding without the need for redox markers. miRNA detection was also obtained through measurements of open circuit potential (OCP) variations. We demonstrate a promising cost effective biosensor using both EIS and OCP with a detection limit of 1 fM, which can easily be expanded into miRNA/DNA microarray platforms.**

Keywords — Biosensor; miRNA; Electrochemical; Gold nanoparticles; Prostate cancer

I. INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA around 22 nucleotides long that are involved in the gene regulation of the human body [1]. They have been reported to be linked with numerous diseases including cancer, making them potential biomarkers [2]. However, in order to get information from these miRNAs, it is important to detect the expression levels. Altered expression levels of these miRNAs can act as fingerprints and provide information about the state of cancer along with its progression [1,2]. Northern blotting [3] and *in situ* hybridization [4] remain the standard detection techniques, but these are laborious and lack sensitivity which has led to the development of more powerful techniques such as microarray technology [5] and quantitative polymerase chain reaction (qPCR) [6]. These techniques, although powerful, are still confined to central laboratories because of their complexity. Hence, there is a pressing need for the development of simpler techniques to quantify the levels of miRNA in a portable or benchtop system.

On similar grounds, electrochemical techniques are gaining significant attention because of easy instrumentation and robustness along with being cost effective and sensitive [7]. There are various electrochemical detection techniques that have been reported in the literature involving e.g. nanoparticles [8] or enzymes [9,10] for the detection of miRNAs. These techniques have significantly improved the state of art in electrochemical miRNA detection. However, they still require further optimization.

In this study, the development of a new simple and sensitive electrochemical detection of miRNA and DNA is presented. We have exploited the inherent charges of oligonucleotides along with a simple amplification step with positively charged gold nanoparticles (AuNPs). Peptide nucleic acids (PNA) probes have been employed as they have advantages such as neutral charge and higher stability than DNA probes. More importantly a PNA/miRNA or PNA/DNA duplex with mismatches are less stable than a DNA/miRNA or DNA/DNA duplex [11,12,13]. Electrochemical Impedance Spectroscopy (EIS) has been used as the detection technique without the use of any redox markers, thus eliminating issues related to the stability of redox markers.

The key aspect of the system is its simplicity, where we monitor the changes in the dielectric properties of the bilayer close to the electrode surface. We also introduce positively charged AuNPs as a simple amplification strategy to disrupt the biolayer once it recognizes the PNA/miRNA or PNA/DNA duplex. With the current setup, changes in the capacitance of the system are observed. Furthermore, for the proof of concept, a DNA sequence was used for easy handling and a detection limit of 1 fM was achieved. We also report another simple detection technique based on the measurement of variations of the open circuit potential (OCP) by employing an ultra-low input bias current instrumentation amplifier circuit, which enables to monitor in real time variations in the interfacial potential.

II. MATERIALS AND METHODS

A. Apparatus

The electrochemical measurements were performed using a CompactStat potentiostat (Ivium Technologies, The Netherlands) with a three-electrode cell setup with a Hg/Hg_2SO_4 (K_2SO_4) reference electrode (BASi, USA) connected via a salt bridge filled with 10 mM phosphate buffer (PB, pH 7.4) and a Pt counter electrode (ALS, Japan). The impedance spectrum was measured in 10 mM PB (pH 7.4) measurement buffer in a frequency range from 100 kHz to 100 mHz, with a 10 mV a.c. voltage superimposed on a bias d.c. voltage of 0 V with respect to the open circuit potential.

B. Biosensor Fabrication

Gold working electrodes with a diameter of 2.0 mm (CH Instruments, USA) were cleaned using a protocol adapted from Keighley et al [14]: electrodes were first mechanically polished for 2 minutes with 1 µm diamond solution (Buehler, IL, USA) and thereafter for 5 minutes with 50 nm alumina slurry (Buehler) on respective polishing pads (Buehler). In between each step, 10 minutes sonication and rinsing in Milli-Q water was performed to remove any remaining particle residues. Additionally, electrodes were then exposed to chemical cleaning using piranha solution (3 parts of concentrated H_2SO_4 with 1 part of H_2O_2) for 10 minutes. Electrodes were then rinsed with ultra-pure water and thereafter electrochemically cleaned in 0.5 M H_2SO_4 (Sigma Aldrich, UK) by scanning the potential between the oxidation and reduction of gold, −0.05 V and +1.1 V versus an Hg/Hg_2SO_4 reference electrode, for 50 cycles until no further changes in the voltammogram were noticed. Finally, electrodes were washed with Milli-Q water.

Clean gold electrodes were co-immobilized with a thiolated ssPNA sequence HS–$(CH_2)_6$–AEEA–AGA AGA AGA AGA AGA G, (Cambridge Research Biochemical, UK) and 6-mercapto-1-hexanol (MCH, Sigma Aldrich, UK) in 50% dimethyl sulfoxide (DMSO, Sigma Aldrich, UK), 50% ultra-pure water (v/v) immobilization solution for 16 h in a humidity chamber. An optimized ratio of 1:5 was adopted from the literature [15]. A high concentration of MCH was prepared in ethanol, which was diluted to working concentration in immobilization solution. After immobilization, electrodes were rinsed with DI water to remove any unattached thiols. In order to ensure complete thiol coverage of the gold surface, the electrodes were thereafter backfilled with 1 mM MCH for 1 hour. Electrodes were then rinsed with ultra-pure water and placed in the measurement buffer for 1 h to stabilize the self-assembled monolayer (SAM).

The functionalized surface was then used to capture target miRNA or DNA sequences, which makes the surface negatively charged; finally, positively charged gold nanoparticles were used to electrostatically bind to the PNA-miRNA or PNA-DNA double-strand (see Fig. 1).

Preparation of positively charged AuNPs was adopted as reported by Kim et al [16]. Shortly, an optimized ratio of hydrogen tetrachloroaurate ($HAuCl_4$, Sigma Aldrich, UK) and branched poly-(ethylenimine) (PEI, MW~25 kDa, Sigma Aldrich, UK) was mixed overnight using vigorous stirring to produce AuNPs. Before experiments the AuNPs were washed three times using centrifugation and re-dispersion in Milli-Q water.

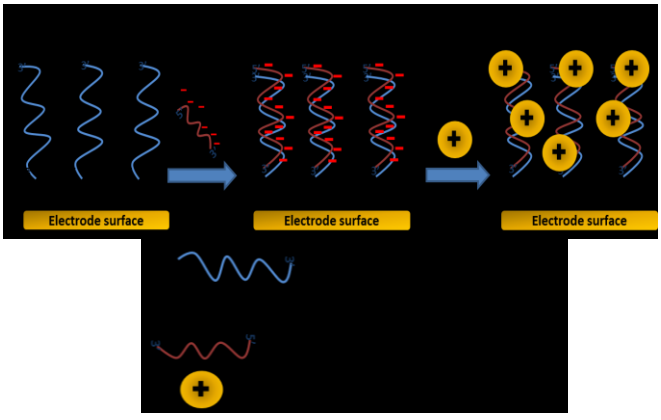

Fig. 1. Schematic of biosensor. (a) Immobilized PNA probes. (b) Capture of target oligo (DNA/miRNA) and finally (c) electrostatic interaction of positively charged AuNPs with PNA/DNA or PNA/miRNA hybrid.

For the binding studies, different concentrations of target DNA, non-complementary DNA and 4% bovine serum album (BSA) were used in 10 mM PB, pH 7.4.

C. Open Circuit Potential

Open Circuit Potential measurements were carried out using a custom made electronic measurement system based on an INA116 ultra low input bias current instrumentation amplifier with a cell [17]. The setup used consisted of 1.6 mm diameter gold disc working electrodes (ALS Ltd, Japan), a Hg/Hg_2SO_4 (K_2SO_4) reference electrode placed into a salt bridge, and a platinum wire for grounding the solution in the electrochemical cell. The OCP was measured in a solution of 10 mM PB (pH 7.4). OCP measurement was recorded in real time and continuously until the experiment was finished. A maximum of three working electrodes were measured at the same time through three different channels simultaneously for the potential changes, by using a common reference electrode.

III. Results and Discussion

A. Electrochemical Impedance Spectroscopy Measurement

Electrochemical impedance spectroscopy (EIS) was used in this study to measure the changes in capacitance of the system at the electrode/electrolyte interface as a result of the binding event and attachment of positively charged AuNPs. The electrodes were initially stabilized in 10 mM PB (pH 7.4) before conducting binding experiments. After stabilization, the electrodes were exposed to different concentrations of complementary oligonucleotide prepared in 10 mM PB buffer (pH 7.4). In order to evaluate the capacitance of the system, a complex capacitance was defined as:

[redacted] (1)

Where C' corresponds to the real part of the capacitance and C'' corresponds to the imaginary part of the capacitance. Z' and Z'' are the real and imaginary components of the measured impedance, respectively, and $\omega=2\pi f$ is the angular frequency of the measurement. From the capacitance data, Cole-Cole capacitance plots can be obtained. With these plots, the diameter of the obtained semicircle corresponds the capacitance of the system (see Fig. 2).

Figure 2 represents a typical Cole-Cole plot obtained with the fabricated biosensor. It can be seen that on hybridization with complementary strand, there is slight decrease in the capacitance denoted by the red curve. The probable reason for this small signal is that the PNA has a PEG linker (AEEA, $C_{21}H_{23}NO_6$), hence the binding event happening far away from the surface and well above the Debye length of the system, leading to screening of the DNA charges. However, when incubated with positively charged AuNPs, (shown by blue

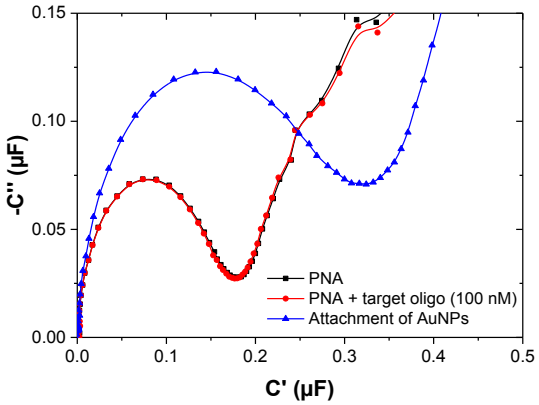

Fig. 2. Cole-Cole plot representing capacitance of the system. Black curve shows the capacitance of PNA probe on the surface. Slight decrease in the capacitance is observed with hybridization of target oligo (red curve) which is significantly increased on binding with AuNPs.

curve), a significant increase in capacitance was observed due to disruption of the dielectric biolayer. The AEEA linker was used in order to decrease non-specific binding.

Selectivity studies were performed using (i) just positively charged AuNPs on the immobilized PNA probes, (ii) 100 nM of non-complementary oligonucleotides and (iv) 4% Bovine Serum Albumin (BSA, Sigma Aldrich, UK). The results demonstrated less than 1% changes in capacitance of the system making it a potential platform for real clinical samples.

Finally, a dose response was obtained with the current detection strategy where a very low detection limit of 1 fM was observed with the AuNP amplification system (see Fig. 3).

B. Open Circuit potential measurements

Open Circuit Potential measurement was used to measure the potential changes due to charge changes at the electrochemical interface upon DNA hybridization and attachment of positively charged AuNPs. All the working electrodes with a common reference electrode were immersed in a cell filled with 10 mM PB buffer (pH 7.4) and were measured simultaneously in real time (see fig 4). After the system reaching a stable value, the first injection of 100 nM complementary DNA was injected into the cell. As DNA hybridization occurs, the potential drops due to the attachment of negatively charged complementary strand DNA. A stable value is achieved after half an hour and a total change of -3.1 mV is observed. Then, positively charged AuNPs were injected and an increase of +14.7 mV was achieved. This is attributed to the attraction of the positive charges on the AuNPs to the negative charges on the PNA/DNA layer.

The experiment was done without a flow cell and the OCP results in different concentrations are shown in figure 5. The potential changes upon DNA hybridization were lower than expected: < 5 mV for 1 μM of DNA target (the highest concentration tested). This is likely due to the presence of the long linker used for the PNA probe along with the excess of complementary strand DNA that is flowing in the solution, which hinders the potential change due to charge floating in the bulk solution. This possible reason also causes a higher potential change upon injecting AuNPs to the blank test. Although the current results do show that the changes due to attachment of AuNPs are distinguishable between blank and PNA/DNA, a further improvement can be made to enhance the measurement result. Adding an extra rinsing step after each injection, which can rinse away the excessive molecules, can help decrease the background signal. Nevertheless, there is still a clear difference between potential changes on blank and the tested concentrations of target DNA. Additionally, the decrease in the measurement cell volume can significantly decrease the time needed to get a stable signal on a binding event, which would be investigated in our future experiments.

IV. CONCLUSIONS

In the current work, a simple electrochemical biosensor with gold nanoparticle amplification for the detection of miRNA or DNA is presented. It has been presented how gold nanoparticles can disrupt the dielectric biolayer of the sensor, leading to significant changes in the capacitance of the system. With the current system, a limit of 1 fM concentration detection has been achieved. Furthermore, open circuit potential measurements, as another simple cost effective

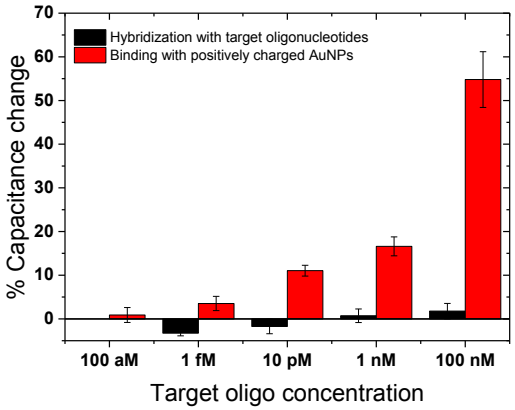

Fig. 3. Dose response. Black bars represent changes in capacitance on binding with target oligo while red bars represent amplification in capacitance change on binding with AuNPs.

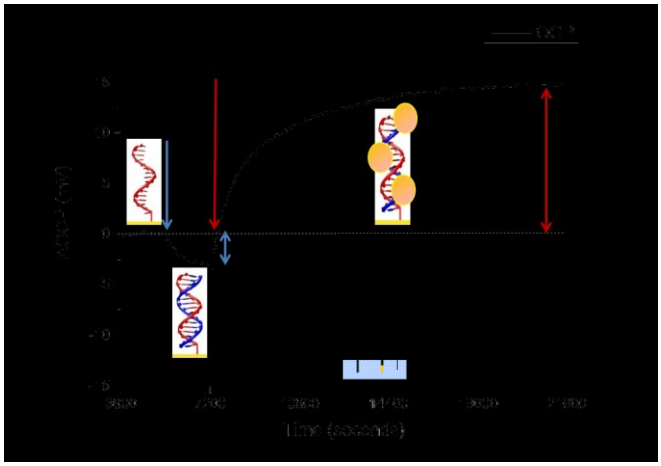

Fig. 4. Real time OCP signal. Insert shows schematic design of instrumentation amplifier set-up for OCP measurement.

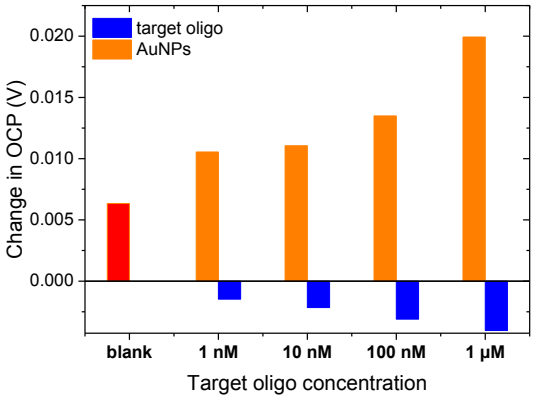

Fig. 5. Dose response with OCP setup. Blue bars represent binding with target oligo while yellow bars represent AuNPs amplification.

detection tool, has been demonstrated to detect low levels of target oligonucleotides. The presented application can be extended to the detection of oligonucleotides specific for various other diseases. Of significant importance is that the detection techniques can easily be expanded into arrays for the parallel screening of panels of DNAs or miRNAs.

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