

Electronic Detection of Selective Proteins using Non Antibody-Based CMOS Chip

Priyanka P. Ramachandran

Department of Biomedical Engineering
Nanotechnology Research and
Teaching Facility
University of Texas at Arlington
Arlington, TX
ppr6333@exchange.uta.edu

Shawn M. Christensen

Department of Biology
University of Texas at
Arlington
Arlington, TX
shawnc@uta.edu

Samir M. Iqbal

Department of Electrical Engineering
Nanotechnology Research and
Teaching Facility
University of Texas at Arlington
Arlington, TX
smiqbal@uta.edu

Abstract—Complementary Metal Oxide Semiconductor (CMOS) technology has enabled fabrication at nanoscale. We have fabricated 500 nm spaced metal electrodes on silicon chips to selectively capture and detect R2Bm protein molecules. The protein is captured using a dsDNA chemically attached on the SiO₂ surface between the electrodes. Surface functionalization and protein-DNA interaction is characterized for specific binding and direct current (DC) measurements are performed as proof of principle of the new modality. Electronic recognition of proteins using nucleic acids can enable more robust detection of a number of important biomarkers. We term such electronics platform for specific protein detection with non-antibody means as Proteonic Biochip. Current-voltage measurements show more than two order decrease of resistance between electrodes due to the capture of the protein.

I. INTRODUCTION

Complementary Metal-Oxide-Semiconductor (CMOS) technology has provided new tools and techniques to fabricate nano-scale devices. CMOS chips can be made with many materials and smart-surface approaches provide capabilities to covalently attach a number of biomolecules on these materials. CMOS can play another crucial role of providing same-chip data-processing and read-out interface. Antibodies are commonly used to functionalize nano-devices and nano-objects for detection of specific biomarkers. Such antibody-based molecular recognition has limited capability for field-deployable or point-of-care modalities, as antibodies need certain range of temperatures, humidity and solution conditions to retain their structure. In terms of the solution conditions, an important parameter is the need for low ionic strength. Low ionic strength of the buffer solutions is needed to overcome surface Debye screening, but it also results in weak interactions between the surface probe and solution target. A possible solution is the development of non antibody-based interactions that are at least as selective as antibody-based assays. A huge research effort has focused lately on developing aptamers against a number of important biomarkers and bio-warfare agents [1-4].

We report a CMOS chip integrating nano-electrodes and aptamers for the detection of a specific protein using electronic signatures. We call it a “Proteonic” biochip as we use direct current electronic signatures for the detection of a protein in low concentrations exploiting the binding interactions that exist between proteins and DNA.

There have been reports of protein detection from alternative methods as well. These include optical and amperometric detection modalities. In the case of optical detection, a fluorescent tag is usually attached to the DNA or the protein and the change in fluorescent intensity is measured after binding. Electrical detection methods include capacitive, impedometric or voltametric detection [5]. We believe a proteonic biochip can leverage the benefits of CMOS to integrate sensing, characterization, comparative analysis and decision making all on-board a single chip, while sustaining or doing even better on sensitivity and specificity.

As a proof of concept, this paper reports direct current detection of a protein binding to a ‘proteonic chip’ affixed DNA aptamer. The aptamers are essentially DNA or RNA that are found to “bind nucleic acids, proteins, small organic compounds, and even entire organisms” [6]. Importantly, aptamers can be easily synthesized and attached to various reporter tags and payloads. In this work, we show the effective attachment of the double-stranded DNA (dsDNA) fragment on the chemically modified silicon surface using previously published attachment chemistry [7]. Protein binding to the dsDNA is detected electrically. We also present optical measurements that are carried out to verify the interactions of DNA-protein complex.

II. MATERIALS AND METHOD

A. Materials:

The chemicals used were 3'-aminopropyltrimethoxysilane (APTMS); 1,4-phenylene diisothiocyanate (PDITC); N,N-dimethylformamide (DMF); 1,2-dichloroethane; N,N-diisopropylethylamine (DPEA); 6-amino-1-hexanol; and methanol. Autoclaved de-ionized water (DIW) was used to make the buffer solutions. The chemicals were purchased

from Sigma-Aldrich (Saint Louis, MO). The 3'-amino modified DNA strands were purchased from Alpha DNA (Montreal, Quebec). The DNA binding domain from the *Bombyx mori* retrotransposon protein R2Bm was made in house (See [8] for details). The zinc finger and myb motif of the R2Bm protein binds to a specific dsDNA sequence (5'-CTTAAGGTAGCAAATGCCTCGTC-3') within the gene coding for the large ribosomal subunit [8, 9].

B. Method

The reported work consists of three major parts that were carried out in parallel: a) Fabrication of the CMOS chip; b) SiO₂ surface preparation, modification and attachment of dsDNA on the Chip; c) Binding between protein and DNA and optical/electrical detection.

a) Fabrication of the CMOS chip:

Chips were fabricated in two steps of lithography. On the first layers Ti/Au (Thickness 50 Å/150 Å) metal pads 500 nm apart were made using e-beam lithography. Metal lift-off resulted in well-defined structures. In the second step, optical lithography was used to fabricate probing pads to contact the thin film electrodes. The chips were partially fabricated at Birck Nanotechnology Center (Purdue University), Nanotechnology Core Facility (University of Illinois at Chicago) and Nanotechnology Research and Teaching Facility (University of Texas at Arlington). The e-beam lithography was done by xlith (Ulm, Germany).

b) Surface modification and attachment of dsDNA:

The silicon chip was cleaned using UV Ozone plasma system. This also resulted in hydrophilic SiO₂ surface. The attachment chemistry was performed in a nitrogen glovebox in a controlled environment, as reported previously [7]. Briefly, the chips were silanized in a 3% APTMS solution (made with 19:1 methanol-DIW solution) for over 12 hours. The chips were then cured at 110 °C for 15 minutes. These were then washed with methanol, DIW and dried with nitrogen gas. The chips were then immediately immersed in a DMF solution containing 10% pyridine and 1 mM PDITC overnight. After this, the chips were sequentially washed with DMF and 1,2-dichloroethane and dried under nitrogen gas. The dsDNA sequence solution was prepared at a concentration of 1 pmole/μl and chips were immersed in it immediately. The chips were incubated at 37 °C overnight in order to facilitate the covalent attachment of the 3'-amino modified dsDNA with the PDITC cross linker molecules. The chips were again washed with DIW, methanol and dried under nitrogen. The unbound reactive groups from PDITC were deactivated by immersing the chips in a solution of 50 mM 6-amino-1-hexanol and 150 mM DPEA in DMF for 24 hours. The chips were then washed with DMF, acetone, DIW and dried with nitrogen gas.

In order to confirm the surface modification, Energy-dispersive X-ray spectroscopy (EDAX), contact angle and ellipsometry measurements were carried out at every step.

The presence of dsDNA immobilized on the silicon surface was confirmed by fluorescence measurements of Acridine Orange stain at 525 nm wavelength using, using Zeiss Confocal Microscope.

c) Binding between protein and DNA:

The dsDNA used in these experiments was a 23 base-pair (bp) fragment of the ribosome gene that corresponds to the binding site of the R2Bm derived polypeptide [9]. In order to confirm that the purified R2Bm polypeptide is capable of binding to our short dsDNA, we ran an electrophoretic mobility shift assay (EMSA).

Chips with covalently attached dsDNA were then incubated with 2.8 fmole/μl of R2Bm polypeptide for 30 minutes in binding buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl₂). The presence of the protein on the chip was initially confirmed by optical detection of fluorescent *Sypro Ruby Protein Blot* stain at 488 nm wavelength. The fluorescence intensity analysis was done with *ImageJ* software [10]. The presence of protein bound to the dsDNA was also detected electrically by direct current electrical measurements.

III. RESULTS

The EDAX analysis was used to identify the elemental composition of the silicon surface as the different modifications were added. The data in Table I show the elemental increase in Carbon and Nitrogen after dsDNA immobilization. Control chips without dsDNA showed no change in carbon and nitrogen.

TABLE I
EDAX ANALYSIS – WEIGHT % OF SIGNIFICANT ELEMENTS ON CHIPS WITH AND WITHOUT MODIFICATIONS

	C	N	O
Clean Chip	0.2	8.2	320.4
PDITC	7.3	9.1	329.2
DNA	10.7	25.8	391.4

The Contact angle measurements showed the silicon surface becoming hydrophilic after plasma treatment and later less hydrophilic when functionalized with APTMS and PDITC. This showed that the surface of silicon chip was hydroxyl (-OH) rich after plasma etching. Functionalization with APTMS/PDITC reduced available -OH groups on the surface and thus showed reduced hydrophilicity. This also proved that OH bonds were used up in effective covalent attachment of silane.

The Ellipsometry measurements gave the thickness of the self-assembled monolayers (SAM) of silane modification as shown in Table II. The difference in the two thicknesses is around ~10 nm. Reaction conditions like temperature, silane concentration, nature of the aminosilane, solvent type,

incubation time and more importantly, the amount of adsorbed water, all contribute to the reproducibility of the final structure of the adsorbed aminosilane layer. Our functionalization setup carefully maintained these conditions resulting in reproducible results.

TABLE II
ELLIPSOMETRY MEASUREMENTS (in nm)

	Thickness	SD
Silicon dioxide	1203.66	2.18
APTMS	1213.35	7.32

The presence of dsDNA immobilized on the silicon surface was confirmed by Acridine Orange (Fig 1). Acridine Orange gives a green fluorescence when it interacts with dsDNA [11]. The Acridine Orange stain bears a positive charge and binds electrostatically with the dsDNA. Electrostatic interactions with non-specific polyanions is avoided by using a very low concentration of the stain (0.2% v/v) and by including other cations like Mg^{2+} , Na^+ in the buffer solution that would compete for the binding with the dsDNA [12]. So the Acridine Orange stain fluorescence obtained could be taken as a conclusive result of the covalent attachment of dsDNA on CMOS chip surface.

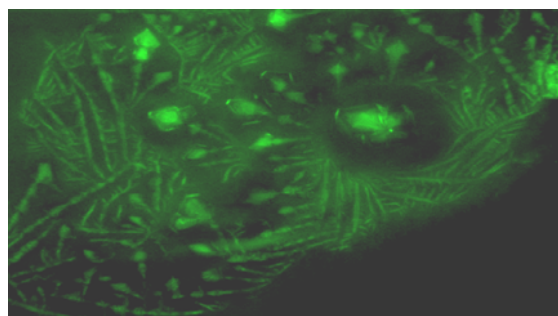


Fig. 1. Fluorescence image of dsDNA stained with Acridine Orange on the surface of CMOS chip. Acridine Orange fluoresces green when it interacts with dsDNA.

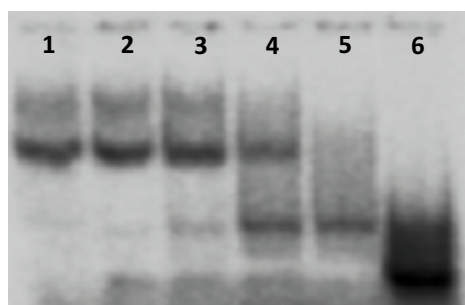


Fig. 2. EMSA PAGE gel. The first 4 lanes (from L to R) 8.4 pmole, 2.8 pmole, 0.84 pmole, and 0.28 pmole protein, respectively, of R2Bm protein bound to 1 pM of dsDNA. The last two lanes are dsDNA and ssDNA, respectively, in the absence of protein.

Prior to functionalizing the chips, the polypeptide binding to the 23 bp dsDNA fragment was confirmed using EMSA—a polyacrylamide gel electrophoresis based method to detect protein-DNA interactions (Fig. 2). Importantly, we can also see the peptide binding to the DNA on the functionalized chip; Fig. 3 shows the data for the protein stain *Sypro Ruby* confirming polypeptide binding to dsDNA on chip. The *Sypro Ruby* stain is a ruthenium based stain that detects the amino acids lysine, arginine & histidine [13].

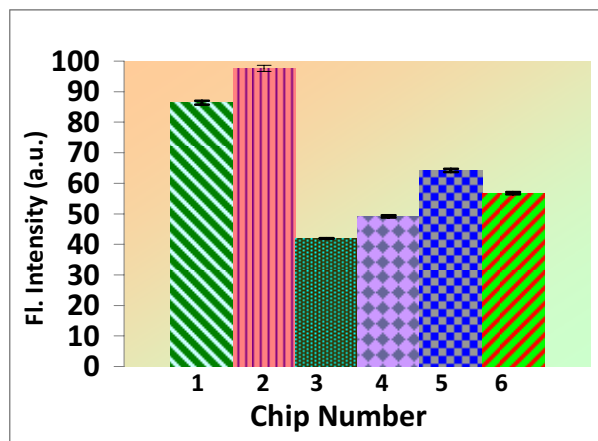


Fig. 3. Sypro stain intensity measurements on chips with the following surface modifications: Chip 1 and 2: DNA & protein attached on chip; Chip 3: Only DNA immobilized on chip; Chip 4: Only protein on chip (No DNA); Chip 5: Only APTMS modification on chip surface; Chip 6: Piranha cleaned chip surface (no biomolecule). These results are averages of 10 chips (n=10).

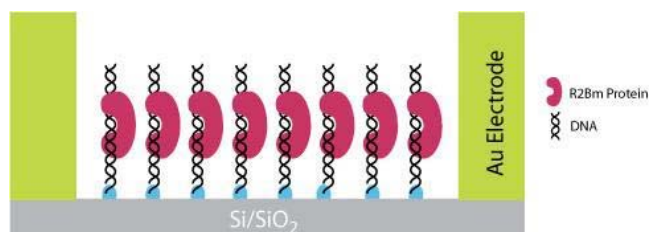


Fig. 4. Schematic representation of the attachment of dsDNA and protein on the proteonic biochip

Once the dsDNA immobilization on silicon chips and selective DNA-protein binding was verified with stains, the dsDNA and protein detection was done on nano-electrode CMOS chip, without any staining. The Fig. 4 shows a schematic of the surface bound dsDNA and protein bound to dsDNA between nano-electrodes and inset to Fig. 5 shows an SEM micrograph of the nano-electrodes. The current-voltage ($I-V$) measurements were performed using Agilent Semiconductor Parameter Analyzer (4155C) on a probe station. A chip without any biomolecules was used as a control. The $I-V$ data was recorded from -1 V to +1 V across the metal electrodes 500 nm apart. The $I-V$ data showed linear trend after the capture of proteins on surface immobilized dsDNA. The yield of devices was 20%, which can be substantially increased by using electrodes with lesser separation or by tagging the protein molecule with

conducting nanoparticles, e.g. of gold. The I - V data showed a linear trend indicating conducting behavior of the protein. The control chip showed open circuit behavior before and after the functionalization (red triangles in Fig. 5). The resistances of the devices after protein capture ranged from few ohms to $G\Omega$, indicating a varying number of proteins bridging the gaps between the electrodes.

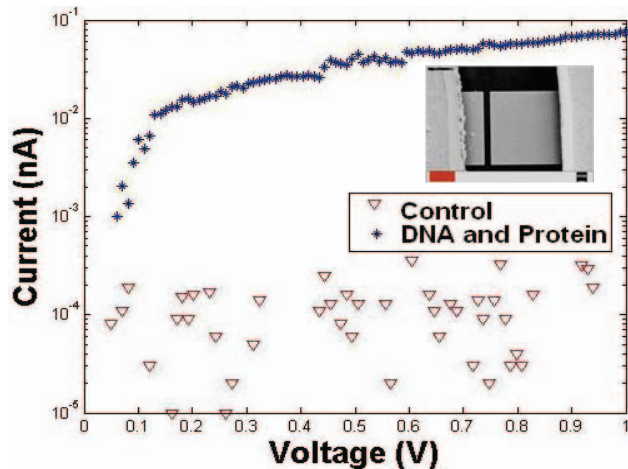


Fig. 5. I - V measurements comparing current measured between metal nano-electrodes. Control data is red triangles with no surface bound dsDNA and protein molecules. The blue stars show I - V data for the chip with the DNA and the protein immobilized on its surface. The inset is an SEM micrograph of the pads. Scale bar: 2 μ m.

A CMOS chip is presented, with electronic recognition of selective protein. The selectivity is achieved by using a dsDNA fragment. The I - V measurements are carried out to detect the capture of the protein. Works describing the capture of folate binding protein using antibodies as the capturing agent report sensitivities such as 130 ng/ml by Surface Plasmon Resonance; 1.5 ng/ml by Quartz Crystal Microbalance; 5–100 ng/ml by Enzyme-Linked Immunosorbent Assay (ELISA) and 50 pg/ml by optical diffraction [14–16]. Using DNA as the capturing agent we report a detection capability down to 0.28 pmol (less than a pg/ml) of a protein [15]. Such framework can be easily extended to carry out data acquisition, analysis and decision making on-board the same chip. We call this new approach Proteonic Biochip. With the advent of so called “Omics” revolution, diseases can be defined at both the molecular and the genomic/protein network levels, and proteonic chips can be used to detect disease linked protein biomarkers to speed up diagnosis and therapy. The application of the chips could also be extended to environmental sample analysis as well, such as in bioterrorism to identify dangerous virus or bacteria or to identify contaminants in food and water, etc. Samples from suspect fluids or tissues can be electrically tested for the presence of important biomolecules.

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