Nanoelectrodes with Selective Molecular Receptors for Label-Free Electronic Detection of Thrombin

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ABSTRACT

In this paper, we present the construction of an array of current blocking devices capable of translating thrombin and DNA oligonucleotide aptamer interaction into a change in electrical resistance. Each sensor in the array consists of a counter electrode and two 75 nm tall measurement electrodes: control and working electrodes. Using self-assembly and electrochemical desorption, the end surface of the working electrode is functionalized with a self-assembled monolayer (SAM) of thrombin aptamer oligonucleotides, while that of the counter electrode with a protein nonfouling SAM. We used various surface characterization methods to confirm the formation of SAM on gold electrode surface, electrochemical surface programming, and binding of thrombin to the aptamer SAM. By monitoring the differential conductance signal between the two electrodes, we demonstrated successful detection of aptamer-thrombin binding.

Keywords: biosensors, self-assembled monolayer, electrochemical sensors and oligonucleotides

1. INTRODUCTION

The enzyme thrombin plays a crucial role in the regulation of blood coagulation. Active thrombin is generated through the processing of prothrombin by factor Xa and has a molecular weight of 36,000. Its activity analysis is important both in clinical trials and life science research[1]. Traditional methods to quantify the thrombin include biochemical approaches such as enzyme activity assay or immunoassay. However, such assays take hours to acquire the results and typically require large amount of samples. In early 1990's it was determined that some synthetic DNA oligonucleotides, or so called aptamers tend to bind to thrombin with high specific affinity[2]. In recent years, these aptamers have been used as specific ligands for constructing electrochemical sensors to quantify thrombin concentration. Nevertheless, these sensors still require lengthy chemical labeling of aptamer using redox moiety such as ferrocene[3].

In this paper, we present a sensor that can directly detect thrombin and generate an electronic signal. The sensor structure (Fig. 1) consists of three electrodes that are partially exposed to the environment under test. A bias voltage is applied between the larger counter electrode and two smaller working and control electrodes and the current passing through the electrodes and the test medium is monitored. The surface of the working electrode is functionalized with a custom-designed aptamer receptor molecule that can specifically bind to the thrombin. The preferential binding of the enzyme to the working electrode changes the ratio of the currents passing through the electrodes. This differential signal is used for detection of the enzyme. Besides its differential signal output, the uniqueness of this sensor also lies on its label-free detection, expandable specificity spectrum by using aptamers specifically binding to other proteins and capability of being integrated with CMOS circuitry and scaled down to improve sensitivity.

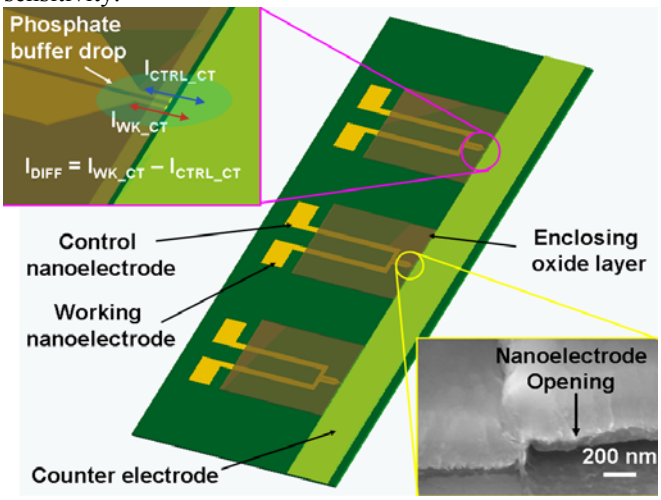

Figure 1. Schematic of a chip containing three current blocking sensors. Insets show the magnified view on the nanoelectrode tip, phosphate buffer drop, current flow between electrodes and SEM picture of the oxide opening leading to the end surface of one nanoelectrode.

2. FABRICATION OF THE MICROELECTRODES

The devices were fabricated on a single-side polished 3" Si wafer (Wafer World Inc., Phosphorus doped, 0.004~0.01 Ω·cm resistivity, 1-0-0). We grew a 200 nm thick thermal oxide on the silicon wafer as the bottom insulating layer for the electrodes by dry oxidation at 1100°C. We then used photolithography to pattern an AZ1512 photoresist layer

followed by metal evaporation and a liftoff in acetone to define the working, control, and counter electrodes. The metal layers were comprised of a 2 nm Cr adhesion layer and a 75 nm Au layer. We then performed another photolithography step followed by silicon dioxide sputtering and acetone liftoff to define the 400 nm thick top and sidewall-covering oxide layer. At this stage, tips of both the working and control electrodes protruded out of the oxide. Then we used isotropic wet Au etching and Cr etching to remove the protruding gold wires so only the end surface of these electrodes were exposed to the outside environment. Exposed metal on the counter electrode was also etched back to the oxide layer. The fabrication process is shown in Fig. 2. This procedure readily generates electrodes that have nanometer-scale sizes in one dimension by taking advantage of thin film deposition, protection, and etching.

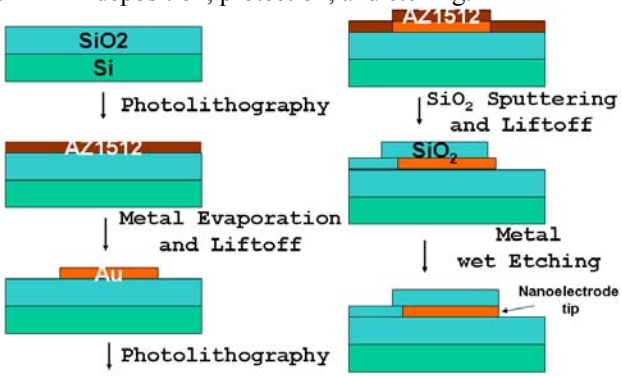

Figure 2. Schematic showing the fabrication process of a current blocking sensor.

3. SURFACE MODIFICAIOTN OF NANOELECTRODES

After completing the fabrication of the electrodes we diced the wafer into 2 cm × 1.5 cm chips. Each chip has three devices as shown in Fig. 1. The aptamer thiol conjugate molecules were ordered from Integrated DNA Technology (Coralville, IA) with structure shown in Fig. 3. The 3' end of the oligonucleotides is linked to a 3-mercapto-1-propanol (MPA) molecule via a hexa(ethylene-glycol) linker. This molecule from the commercial supplier cannot be directly used to assemble the DNA SAM since its thiol terminal is blocked by another MPA molecule through a disulfide bond. The thiol-blocking MPA molecule is released by reduction with either dithiothreitol or tris(carboxylethyl)phosphine (TCEP) [4].

We first rinsed a chip using acetone, isopropanol and water. Then we cleaned it by an oxygen plasma treatment for 5 minutes. To reduce the nonspecific protein adhesion on SiO_2 surfaces, we made the chip nonfouling by incubating it in 0.1 mM 2-(Methoxy-(polyethyleneoxy)-propyl)-trimethoxysilane toluene solution. We then used isotropic wet Au etching and wet Cr etching to remove the portions of the gold lines not protected by oxide so that only the end surface of these electrodes were exposed to the test environment. Immediately after wet etching, we incubated the chip in ethanol containing 10 μM DNA aptamer conjugate, 1 mM hexa(ethylene glycol)undecanethiol (EG6SH) and 2 mM TCEP for 12 hrs to form the binary self-assembled molecular monolayer (SAM). Since this SAM covered all the gold surfaces on the chip, we used electrochemical desorption to remove the SAM from the control and the counter electrodes [5]. To reduce the nonspecific binding of thrombin to the re-exposed gold surface on control and counter electrodes, we incubated the chip in 1 mM EG6SH ethanol solution for 12 hr to form a hexa(ethylene glycol) terminated SAM. We heated the chip to 95°C in a 200 mM pH 7.4 sodium phosphate buffer solution containing 1 mM $MgCl_2$ followed by slowly cooling to anneal the immobilized DNA aptamer to its correct conformation prior to the incubation of chip with thrombin solution.

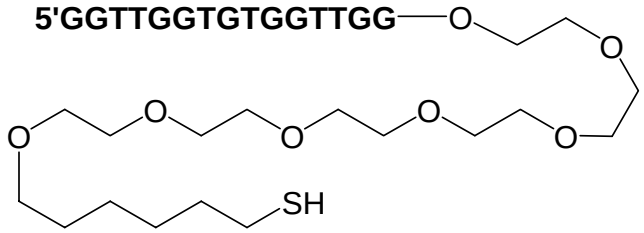

Figure 3. Sequence and chemical formula of the DNA aptamer thiol conjugate custom-designed for specific binding to thrombin.

In parallel to the surface modification to the exposed device electrode surfaces, we formed aptamer/EG6SH binary SAM on gold coated silicon chips to confirm the existence of SAMs, electrochemical desorption of SAM and thrombin binding to SAM by Fourier Transform InfraRed spectroscopy (FTIR). We also formed this SAM on atomically flat gold substrate for atomic force microscopy (AFM) analysis.

4. CONDUCTANCE MEASUREMENTS

Electrical conductance measurements were carried out using a Keithley 6430 remote sourcemeter. We immersed the electrodes in 10 mM pH 7.4 sodium phosphate buffer solution and measured the current by sweeping the voltage from -0.2 V to 0.2 V at 10 mV intervals. We then incubated the chip in 200 mM phosphate buffer solution containing 1 mM $MgCl_2$ and 27 nM thrombin for 30 minutes followed by rinsing the chips with phosophate buffer solution and deionized water. We then blew the chip dry with air and repeated the electrical current measurements in phosphate buffer.

5. EXPERIMENTAL RESULTS

After the immobilization of oligonucleotide ligand molecules on gold surface, we have used the FTIR and AFM to characterize the formation of SAM, electrochemical desorption of SAM and binding of target molecules to the SAM modified gold surface. Fig. 4 shows the FTIR spectra

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of gold surface with EG6SH SAM and thrombin aptamer/EG6SH composite SAM and after the incubation of aptamer/EG6SH with 28 nM thrombin solution. The absorbance in these curves have the background absorbance of the bare gold subtracted. In the spectrum of gold coated with EG6SH SAM, we can clearly see the strong peaks at 2850 and 2950 cm^{-1} which correspond to the -CH_2- stretching[6]. Moreover, in the curves of thrombin aptamer SAM, we observed the peak at 1100 cm^{-1} which is caused by the PO_2^- vibration[7]. These peaks clearly indicated the successful immobilization of the EG6SH SAM, DNA oligonucleotides aptamer SAM. After comparison of the curves of aptamer/EG6SH before and after its incubation with thrombin, we can clearly see the additional absorbance peaks at 1545 cm^{-1} which is caused by the stretching of the peptide bond[8]. This peak provides clear evidence that thrombin binds to the EG6SH/aptamer composite SAM. Fig. 5 shows the FTIR curves of EG6SH/aptamer SAM before and after the electrochemical desorption. Before the desorption, the CH_2 stretch peaks at 2850 cm^{-1} are visible but after the desorption, these peaks disappeared, suggesting the removal of the EG6SH/aptamer SAM. Independently from the FTIR experiments, the current/voltage curves during the electrochemical desorption of EG6SH/aptamer showing the successively reduced peak height at 0.55 V in reference to Ag/AgCl electrode in Fig. 6 further suggested that SAMs were removed from the gold surface.

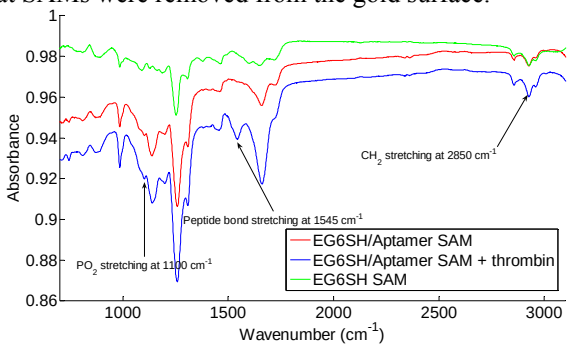

Figure 4. FTIR spectra of EG6SH SAM coated gold, EG6SH/aptamer composite SAM coated gold before and after incubation with thrombin.

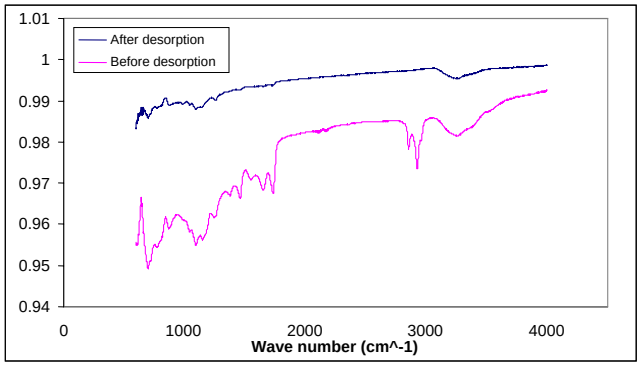

Figure 5. FTIR spectra of EG6SH/aptamer composite SAM before and after electrochemical desorption.

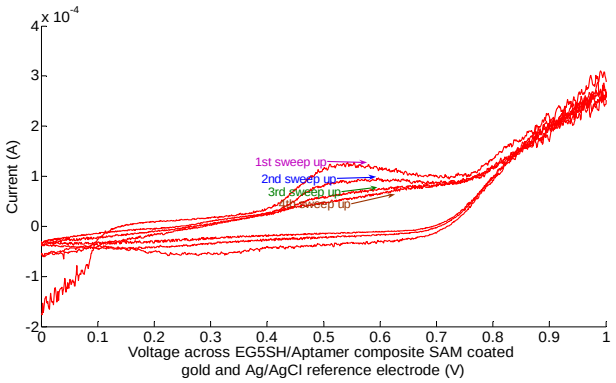

Figure 6. Current versus swept voltage of electrochemical desorption of EG6SH/aptamer composite SAM.

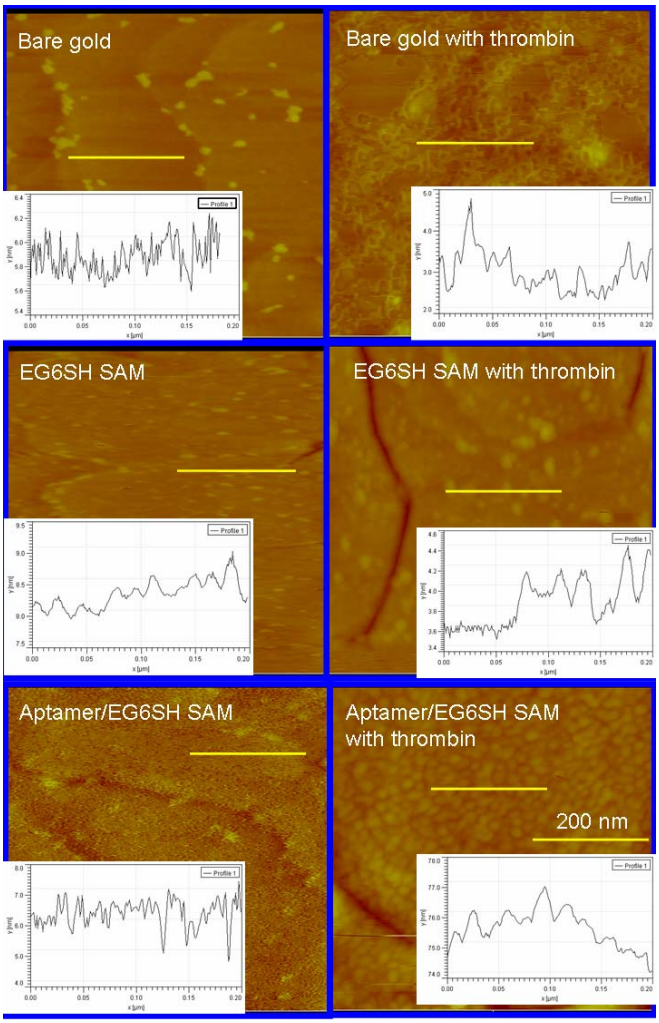

Figure 7. AFM picture and sectional analysis of SAMs formed in the experiments before and after the binding of thrombin. The yellow lines show the traces to produce the sectional views.

Fig. 8 shows that the effective conductance between the working and counter electrodes was significantly decreased after the incubation of aptamer SAM coated chip in

phosphate buffer containing thrombin. The lower conductance is likely due to the increased tunneling length of the thrombin-aptamer SAM compared to the tunneling length before the thrombin binding.

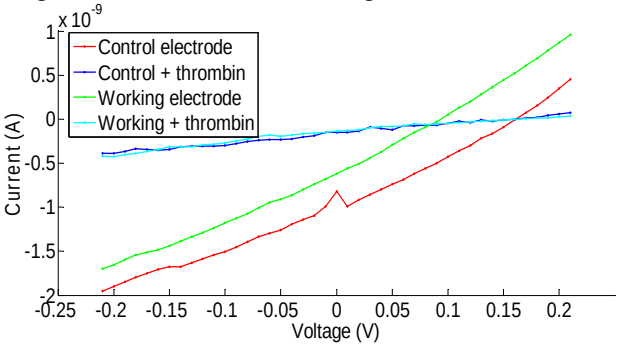

Figure 8. Electrical current change before and after the thrombin binding.

6. DISCUSSIONS

In this paper, we have successfully formed thrombin aptamer SAM on the gold surface of nano-scale electrodes and used electrochemical desorption to program the affinity to thrombin on different electrode surfaces. We have demonstrated the concept of a current blocking sensor and implemented such a sensor array capable of detecting thrombin binding. When compared to other current biosensors, the current blocking sensor has four advantages. First, its fabrication process only uses standard microfabrication techniques which are fully compatible with the complementary metal oxide semiconductor (CMOS) integrated circuit fabrication process. Therefore, integration of this device with CMOS readout circuitry is very straightforward and requires only four post-process steps. Second, this device is readily scalable hence large arrays of these sensors can be built with extremely high density. Even though in this research, the exposed tip area of the working and control electrodes are 10 μm × 75 nm, the width can easily be reduced to 100 nm without adversely affecting its functionality. Third, the specificity of this device can easily be altered by changing the functionality of the chemical group immobilized on the gold electrode surface. More specifically, it is feasible to use the instrumentation spotting oligonucleotide probe to make DNA or RNA microarray chip to immobilize oligonucleotides with various sequences on the current blocking sensor array. The ability to effectively and electronically program the molecular selectivity of the sensor, as demonstrated here, is a powerful tool for constructing multiplexed bio-marker identifiers. Fourth, current readings from our current blocking device can be made differential hence the signal to noise ratio could be very good with properly designed layouts.

ACKNOWLEDGEMENTS

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