Nanomonitors: Electrical Immunoassays for Protein Biomarker Profiling

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ABSTRACT

The objective of this research is to develop a "point-of-care" device for early disease diagnosis through protein biomarker characterization. Here we present label-free, high sensitivity detection of proteins with the use of electrical immunoassays that we call Nanomonitors. The basis of the detection principle lies in the formation of an electrical double layer and its perturbations caused by proteins trapped in a nanoporous alumina membrane over a microelectrode array platform. High sensitivity and rapid detection of two inflammatory biomarkers, C-reactive protein (CRP) and Myeloperoxidase (MPO) in pure and clinical samples through label-free electrical detection were achieved. The performance metrics achieved by this device makes it suitable as a "lab-on-a-chip" device for protein biomarker profiling and hence early disease diagnosis.

Keywords: point-of-care, protein detection, electrical immunoassays, nanopores, ELISA

1 INTRODUCTION

Recent research in the field of proteomics has revealed that proteins can be utilized as biomarkers that facilitate disease diagnostics [1-4]. New trends have shown that a combination of biomarkers can significantly improve the reliability of disease detection [5-7]. Increased number of biomarkers for a disease can also give access to early detection capability. Fast and multiplexed protein detection techniques with high sensitivity and selectivity are imperative to realize the true potential of biomarkers in healthcare. Conventional immunoassay techniques, including enzyme linked immunosorbent assay (ELISA) have not been able to achieve this goal [6-10]. These techniques have several limitations such as the need for use of labels, time of detection in several hours, large volume of reagents, issues with concurrent multiple protein detection due to the associated expense and cross-reactivity [6-10].

Within the last two decades, label free techniques such as surface plasmon resonance, piezoelectric oscillators and electrochemical devices have been developed with higher speed of detection, lower cost and medium throughput [11-13]. The trend over the past few years is towards high throughput systems with device scaling approaching the molecular level to minimize the current limitations of low signal strength, signal variability due to cross reactivity and non-specific binding, which are not completely addressed by the existing label free techniques. Thus, in the last decade, nanotechnology has been applied to biomolecule detection as it provides the advantages of size matching with proteins thereby resulting in higher sensitivity due to increased signal to noise ratio and improved surface area to volume which in turn decreases signal variability. Nanotubes, nanowires and nanospheres are some of the nanomaterial architectures that have been utilized to improve the performance of label free biosensors by targeting the current limitations [13].

This paper describes a device that uses a nanoporous alumina (Al_2O_3) membrane in conjunction with microfabricated gold, Au, electrode platform to form an array of nanoscale well structures to selectively localize proteins onto metallized measurement surfaces in confined volumes. The performance parameters of the device are compared with the traditional assay methods showing that apart from being a label-free technique, it can also provide several improvements such as highly increased speed of detection on the order of minutes as compared to several hours for ELISA, significant reduction in volume of reagents to a few µl, large reduction in cost per assay and the reduction in the size of assay thus making it a candidate for a clinical diagnostic "lab-on-a-chip" device that we call nanomonitor (NM).

2 MATERIALS AND METHODS

2.1 Device Fabrication

The NM comprises of two parts: the microelectrode array base platform that is fabricated using standard photolithography principles and a nanoporous alumina membrane over layer.

The base platform comprises of an array of metallized circular measurement/sensing sites where the binding of the protein molecules occurs in a controlled manner (Figure 1a). Each sensing site is constituted of a working electrode (WE) – and a counter electrode (CE), wherein the surface area ratio of the CE to WE is 225:1 (Figure 1b). Active protein sensing happens at these electrodes (sensing sites), which are in turn connected, to input/output pads for electrical read-out (Figure 1c). Both the electrodes were

designed to be circular in shape to attain maximum surface area of interaction and to avoid any possible edge effects. The outer diameter of CE is 150 µm and that of WE is 10 µm, separated by a gap of 5 µm, as shown in Figure 1b.

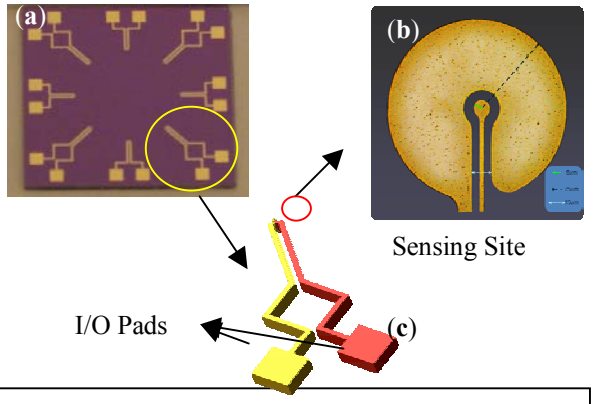

Figure 1: (**a**) Nanomonitor device (**b**) Sensing Site - Working and Counter electrodes layout (**c**) I/O pads connected to the sensing sites (electrodes) through interconnects for electrical read-out

The second part of the NM is the nanoporous Al_2O_3 membrane. A 250 nm thick layer of aluminum is thermally evaporated on the microfabricated platform. This is used as the anode for the electrophoresis reaction that results in a nanoporous Al_2O_3 membrane (Figure 2).

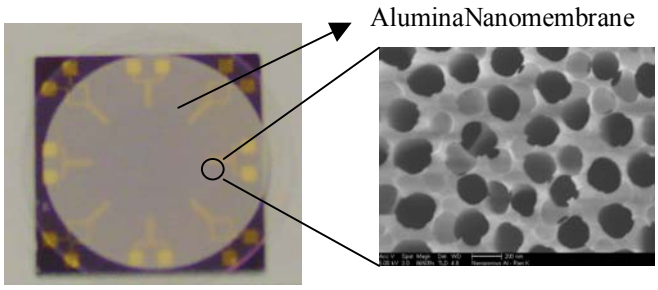

Figure 2: Nanoporous alumina membrane over the microfabricated platform. SEM micrograph showing the nanopores in the membrane.

The pore diameter can be tailored to match the protein size so that each pore forms a well corresponding to a single antibody-antigen conjugate. At 200 nm diameter there are approximately quarter million nanowells on a single sensing site. The platform is fabricated with eight sensing sites and hence can be readily used for multiplexed detection [14].

2.2 Device Operation

Nanomonitor device works on the principle of formation of the electrical double layer. An ionic buffer solution is always present between the electrodes and is used as the basic platform and the medium for protein detection. An electrical double layer occurs whenever an array of charged particles and oriented dipoles are present near the liquid/metal interface. When an electrode is charged, it attracts oppositely charged species and forms a neutral region around the electrode (Figure 3). This neutral layer creates other solvent ions in solution. The inner layer, which is closest to the electrode is called inner Helmholtz plane (iHp) and it contains solvent molecules, specifically adsorbed ions. The next layer is called outer Helmholtz plane (oHp) and the layer after this is called the diffuse layer [15]. When a protein binds at the metal/liquid interface, it perturbs the surface charge distribution at the inner Helmholtz layer as the protein is electrically charged. With more proteins binding at the interface, the associated surface charges also changes significantly. Hence, in the NM device the measurement of the protein biomolecule binding occurs by measuring the surface charge perturbations at the electrical double layer resulting in a measurable electrochemical capacitance change.

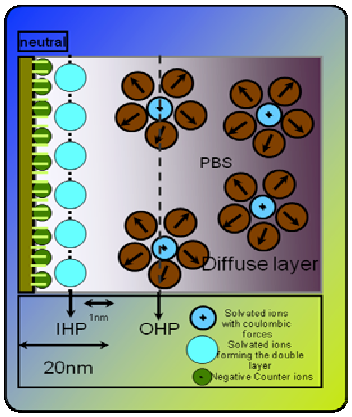

Figure 3: Charge distribution across the liquid/electrode interface forming the double layer

2.3 Methods

Two inflammatory biomarkers, C-reactive protein (CRP) and Myeloperoxidase (MPO), were identified as proteins of study for the development of the device and to demonstrate the detection. It has been suggested that both these biomarkers have pro-inflammatory and pro-coagulant effects, hence making them ideal as study proteins for early disease diagnosis of cardiovascular diseases [16, 17]. Proteins CRP and MPO (antigens) specifically bind with their antibodies or protein receptors, anti-CRP and anti-MPO, respectively. This property of protein binding was incorporated to bring in high selectivity of detection in this device.

The NM was first cleaned in 75% ethanol and de-ionized (DI) water, followed by a DI water rinse and blow drying under dry nitrogen to create a bacteria free device. The nanomembranes/metallic measurement sites were then functionalized by coating a layer of the chemical cross-linker dithiobis (succinimidyl propionate) (DSP), a homo-bifunctional, amine-reactive cross-linker. At this point, the capacitance was measured between the two electrodes to mark the baseline measurements for instrument calibration. Immediately the electrodes were saturated with 3 ul solution of antibodies, as the NHS groups tend to hydrolyze very quickly. There was an increase in impedance due to

the increasing capacitive reactance formed between the antibodies and the electrodes. However, impedance saturated at a particular concentration of proteins, which corresponded to what was called the saturation concentration when almost all the nanopores are occupied with antibodies for protein binding (Figure 4).

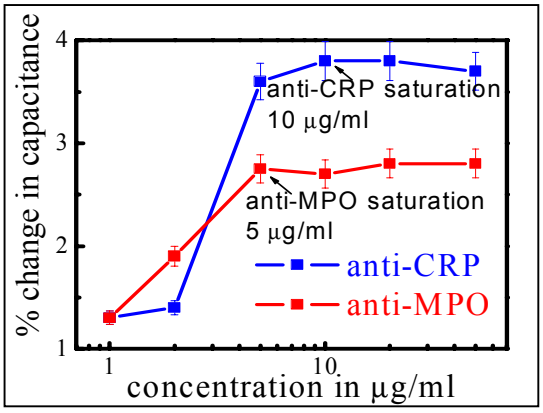

Figure 4: Antibody saturation measurements for proteins CRP and MPO.

After saturating the sensing site with antibodies, a block step with Bovine Serum Albumin (BSA) was performed to block all unoccupied sites and avoid non-specific binding of proteins. A 5% BSA/PBS solution was used to wash the nanowells for few minutes and then left to incubate at room temperature for 15 minutes. After this block step, the protein to be detected was inoculated onto the sensing site (Figure 5).

The protein solutions were prepared by diluting in the pure protein in 1X PBS as well as in human serum (U.S. Biological, MA) to demonstrate the detection from physiological fluids. The capacitance measured after the block step marked the final reference value for quantification of protein characterization. The change in capacitance measured after the exposure to the proteins corresponds to the action of protein binding. Hence the magnitude of change in capacitance can be directly attributed to the protein concentration present. This correlation between the change in capacitance and protein concentration is plotted and used for device calibration for detection. In the case of multiplexed detection, the alternate sites were inoculated with different antibodies and the corresponding change in capacitance induced by the respective protein on each of the sites was recorded for their respective protein characterizations.

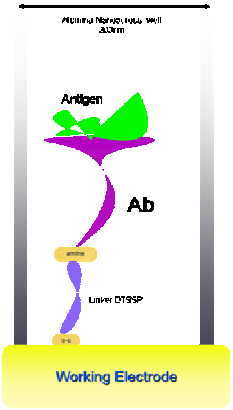

Figure 5: View inside a single nanowell showing the series binding of species.

For protein detection in clinical samples, known concentrations of proteins were spiked in the serum, Human Serum Albumin (HSA) and detected using the same protocol as mentioned above. HSA is constituted of many competing proteins which also includes CRP, the concentrations of which are known. After recording the concentration of CRP already present in HSA, appropriate spiking with known concentrations of CRP and/or dilution with 1X PBS control buffer is done to achieve a 50% CRP spiked serum by volume. The change in capacitance measured while detecting the CRP in the serum was compared with the reference created by the pure sample detection.

3 RESULTS

This section shows the various responses observed while detecting the proteins CRP and MPO.

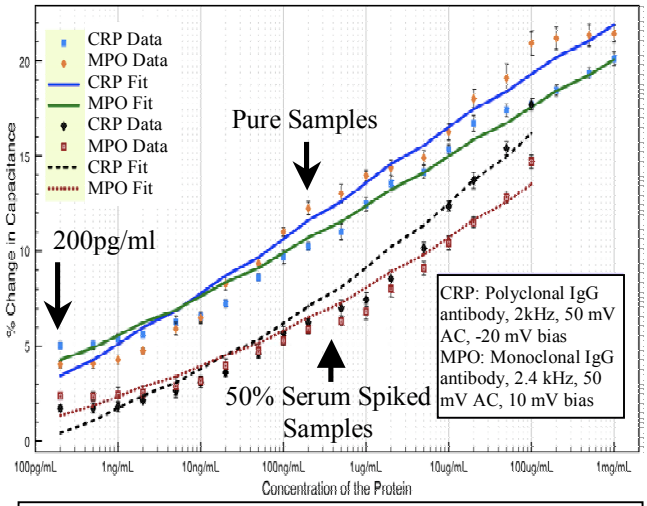

Figure 6: Dose response curves for proteins CRP and MPO when detected in pure and serum samples.

Figure 6 shows the dose response curves for the proteins CRP and MPO detected in pure form and from samples containing 50% serum by volume. Figure 8 shows the response for various concentrations of multiplexed samples. The values in these plots were obtained at 4 kHz, where the change in capacitance was found to be the highest. The detection limit was found to be ~200 pg/ml for CRP and ~500 pg/ml for MPO. The time taken for the detection of proteins was about 120 – 180 seconds. Figure 7 also shows the linear dynamic range of detection extends from ~200 pg/ml to ~100 µg/ml, which is about 6 orders of magnitude.

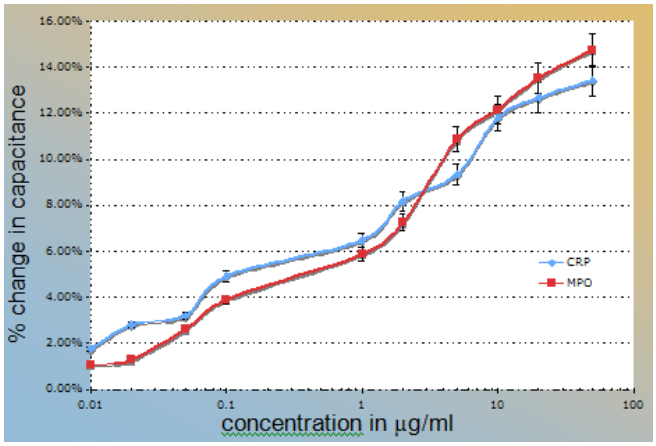

Figure 7: Multiplexed detection dose response curves for proteins CRP and MPO

4 DISCUSSION

A highly sensitive device for protein detection was designed and fabricated using label-free electrical immunoassay techniques. In addition to being highly portable, it is quite inexpensive and very quick in detection when compared to current predominant labeled immunoassay techniques like ELISA. The lower limit of detection achieved by ELISA is better than what is achieved by the NM device. This is attributed to the presence of some non-specific binding or cross-reactivity occurring when competing antigens are present. Competing antigens are those whose structures and chemical properties are very similar in nature and hence they create a noise margin which currently inhibits the limit of detection to 200 pg/ml. The second reason would be because of the background noise present from the substrate, the external circuitry and the environment.

5 CONCLUSIONS AND FUTURE WORK

Multiplexed detection of protein biomarkers from small volumes in a rapid manner is important for cost effective disease diagnosis. The NM device technology has been demonstrated here to detect multiple proteins in pure and serum samples. To address the issues due to non-specific binding, many more nanopores could be introduced over the sensing site for more protein trapping and reducing signal variability. This increases the amount of antibodies present, hence increasing the average signal read out from protein binding. This increases the overall signal-to-noise ratio and hence would help achieve a lower limit of detection.

One of the primary objectives of this research when it started was to make a handheld device. So to move towards that destination, an external electrical circuitry will be built to process the signal read from the device and give an electrical read-out in the form of an electrical display or an LED based output display.

It has the potential for "point-of-care" disease diagnostics, as it is highly portable and inexpensive. There is a great promise for this technology in the areas of laboratory clinical diagnostics, healthcare and in pharmaceutical sectors.

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