

# Nanoporous Alumina Membranes Based Microdevices for Ultrasensitive Protein Detection

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## ABSTRACT

Electrical and chemical properties of nanostructured alumina membranes have been used to improve sensitivity and performance of Si-based microdevices for protein sensing. Trapping of proteins within the nanoscale well-like structures is an experimental demonstration of “molecular crowding” phenomenon retaining the functionality of proteins due to confinement in small spaces i.e. nanowells. Protein conjugation in nanowells causes charge perturbation in the electrical double layer at the interface between biomolecule and gold electrode, measured as capacitance change. We have demonstrated the detection of cardiovascular biomarkers, C-reactive protein (CRP) and Myeloperoxidase (MPO), from purified samples and human serum. The device performance metrics - sensitivity, selectivity, speed and dynamic range of detection - have been measured to quantify the efficacy of these nanomonitors and compared to standard immunoassays.

**Keywords:** alumina, nanopore, biomolecule detection, macromolecular crowding, cardiovascular

## 1 INTRODUCTION

Every year about 10% of the population of United States undergoes cardiovascular surgery. One million of these patients have adverse effects after the surgery. It has been projected that within the next two decades surgical patients would increase by 25%, costs by 50%, and complications by 100% as the population ages [1-4]. The current surgical burden is burgeoning as a surgical crisis. Our best ally is to improve the outcomes after surgery to reduce the after effects. Currently, this is being addressed by using medicines, which were developed to address the perioperative ischemia. Perioperative ischemia is the best known predictor for postoperative cardiovascular morbidity and mortality [5-8]. Perioperative ischemia and infarction are thought to occur from plaque rupture of an unstable or vulnerable plaque in most cases.

Due to the complex nature of the problem at hand, one single biomarker is not sufficient to predict the risk factor of a patient (Fig. 1). The heterogeneity of the condition stems from the fact that many markers have to be considered as potential factors for this condition.

Monitoring multiple biomarkers at the same time would provide a holistic image of the patient's condition. To perform such a test there are currently no tests that could do these proteins at the same time, which would require a patient to make multiple visits and also trained technicians to run the tests. We wanted to address these problems by creating a multiplexed test that is easy to perform in a clinical environment (doctor's office), which would require very low quantities of patient's samples. The various proteins identified as potential markers are as follows:

1. CRP
2. Myeloperoxidase (MPO)
3. Ox-LDL
4. Tissue factor
5. CD40 ligand.

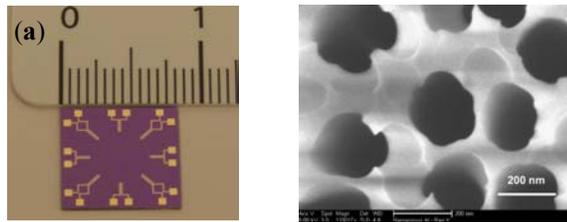
In this current work, we use the first two proteins CRP and MPO as a proof of concept demonstration of the technology. This paper describes the development of a biomimetic electrochemical device for protein sensing that works on the principle of excluded volume associated with the phenomenon of macromolecular crowding [9]. The macromolecular crowding phenomenon refers to the influence of mutual volume exclusion upon the energetics and transport properties of protein molecules within a crowded, or highly volume-occupied, medium. Because of steric repulsion, no part of any two macromolecules can be in the same place at the same time. We have developed a nanoarray membrane based system that has utilized this phenomenon towards building an electrical immunoassay with selective protein biomarker localization in confined spaces.

## 2 MATERIALS AND METHODS

### 2.1 Materials

We have developed an electrical immunoassay technology that works on the principle of creation of the electrical double layer at the liquid/metal interface and modulation of the double layer due to the addition of charged species such as proteins. Integration of both detection and the measurement makes the device label free and easier to use. The Nanomonitor comprises of two parts. They are the micro-fabricated platform fabricated by overlaying the Au electrodes on the Si substrate using

standard lithographic techniques; and the nanoporous membrane implanted on the platform as represented in Fig. 1(A).



**Figure 1:** (a) Microfabricated platform with 8 sensing sites. (b) SEM micrograph showing the nanopores in the membrane.

Alumina was chosen as the membrane materials because the chemistry to make them is well known and the pore diameter could be well controlled. Alumina membranes are helpful:

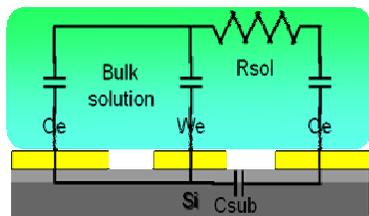
1. Adsorption of protein onto their surfaces due to simple van der Waals bonds. When sample liquid flows

on top of the membrane due to capillary effect the molecules in the solution flow to the transducer element

(microfabricated platform) at the bottom of the well. Due to the flow of charge on the electrodes, this causes the accumulation of the ions in solution to adsorb on the surface creating the double layer. The electrical double layer consists of an array of charged species and/or oriented dipoles existing at electrode interface. A simple equivalent circuit is given in Fig. 2.

2. Micro encapsulation of the biomarkers in each well facilitates ease of the transfer of electrons and ions exclusive to that well, providing very little cross talk electrically across wells.

3. Entrapment of the biomarker very close to the transducer element (microfabricated platform of gold) improves the signal transduction and sensitivity of the device.



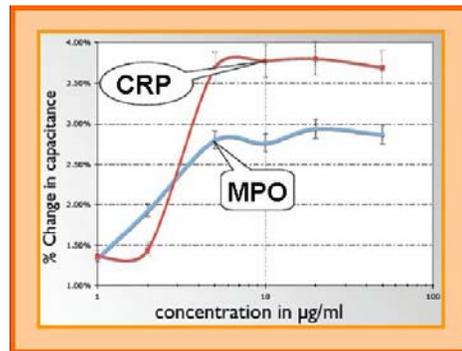
**Figure 2:** Charge distribution across liquid-electrode interface forming the equivalent circuit.

A 250nm thick layer of aluminum film is thermally evaporated onto the microelectrode platform. This acts as the anode for the two-step anodization process required for fabricating the nanopores. The cathode is a platinum wire. Both the anode and cathode were immersed in an electrochemical bath. A constant voltage of 45 V and a constant current density of 20 mA/cm<sup>2</sup> are applied across the electrodes. A mixture of 0.1 M sulfuric acid and 0.4 M oxalic acid is used as the electrolyte to obtain a uniform structure of the pores. The conditions for the electrochemical bath were optimized to get a uniform pore size of 200 nm. The pore size is modified to match the protein dimensions suitable for protein entrapment. This

results in the formation of nanowells. Hence on an individual sensing site there area approximately quarter million nanowells and the electrical conductance signal is cumulated and averaged out over all the nanowells on the sensing site. The pore dimensions were tailored to 200 nm for the two cardiac markers that were evaluated: C-reactive protein and Myeloperoxidase.

## 2.2 Methods

The first step in detection of the proteins was to saturate the nanoporous membrane with the antibody and then inoculate the region with sample for detection. 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. The purified samples were obtained from EMD Biosciences, San Diego, CA. The baseline measurements were recorded with 0.1X PBS because the dilutions for both antibody and the samples were made in 0.1X PBS. The interfacial voltage and the frequency of voltage signals are recorded. Experimental protocol included the cleaning of the chip before the experiment and then embedding the nanoporous membrane on the chip. Baseline measurements with 0.1X PBS were done, and then the NM was functionalized with the chemical cross-linker dithiobis (succinimidyl propionate) (DSP), a homobifunctional, amine-reactive cross-linker. The antibodies that were then inoculated on the surface are immobilized by the covalent linking with DSP. The chip was incubated for a period of 15 min at room temperature after measurements were made. The antibody saturation for both CRP and MPO are shown in Figure 3.

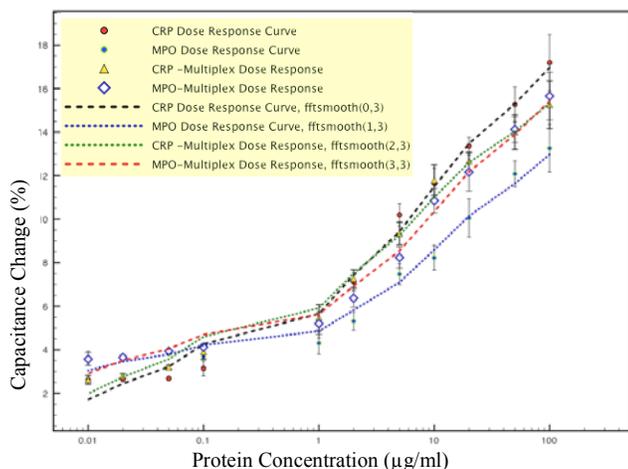


**Figure 3:** Antibody saturation measurements for proteins CRP and MPO.

Next, the proteins were inoculated followed by 15 min incubation and measurements. The multiplexed data with both the antibodies of CRP and MPO immobilized on the surface are tested with a solution of both the proteins. Further experiments were conducted with the human serum (Fischer Scientific) to check for the nonspecific binding issues associated with most immunoassay setups.

### 3 RESULTS

The results for both CRP and MPO detection in both pure and serum samples are presented here (Fig. 4). There were



**Figure 4:** Dose response curves for proteins CRP and MPO when detected in pure and serum

several data points in the dose response curve from 100pg/ml to 100µg/mL. Each data point was evaluated in triplicate. There was linear correlation between the % change in capacitance and the concentration.

The values in this plot 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.

### 4 DISCUSSION

In the studies conducted, the device has been tested with standard synthesized samples obtained from the vendor. The samples have also been validated with ELISA Vendor validation datasheets.

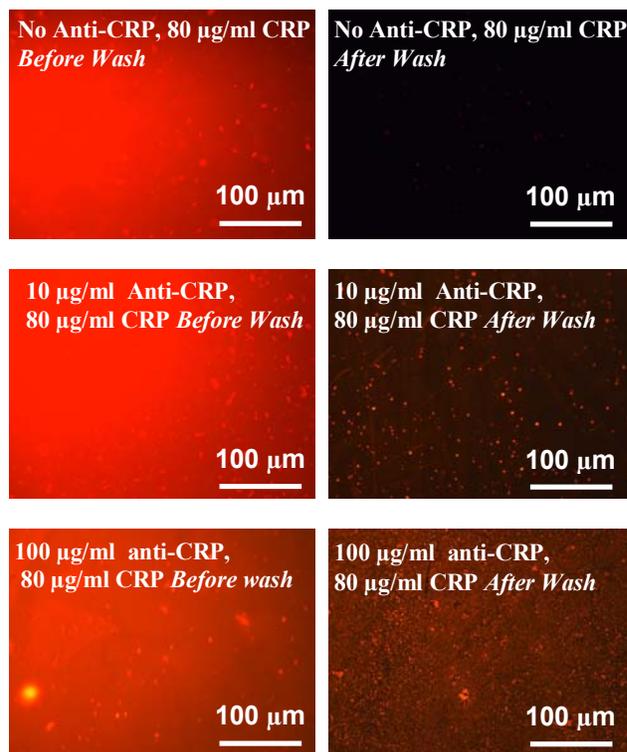
#### 4.1 Validation Experiments

Optical detection through fluorescence markers and ELISA are two commonly used protein detection techniques. Thus in order to validate the NM results we designed two experiments to qualitatively and quantitatively demonstrate antigen-antibody conjugation on the NM.

##### Fluorescence Validation

Three NM chips were prepared such that the first chip (negative control) was coated with only the DSP linkers, while the remaining two NMs were functionalized with DSP and the anti-CRP with the concentrations of anti-CRP being 10 µg/ml and 100 µg/ml according to the procedure

described before. Fluorescence marker kit Alexa 594 (Invitrogen Corporation, OR) was used to tag the CRP-protein according to the specified protocol. This tagged protein was reconstituted to a concentration of 80 µg/ml in 1X PBS and introduced on the control and the two experimental NMs and incubated for 30 minutes. All the NMs were washed under 1 ml of flowing 1X PBS. The NMs were observed under fluorescence microscope activated by red light (~594 nm wavelength) before and after the washing step. From the images shown in Figure 5 it is evident that with the increase in anti-CRP concentration, the amount of CRP localized on the NM increased. Also there was no binding of the CRP on the NM without the anti-CRP in the control experiment.



**Figure 5:** Sequence of images taken before and after wash with varying concentrations of anti-CRP showing increasing fluorescence, thereby indicating higher amount of anti-CRP and CRP conjugation on

##### Gold ELISA Validation

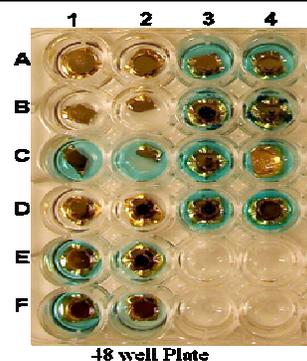
The second validation experiment was conducted by placing small pieces of Au coated Si wafer in an ELISA plate and treating the pieces with the DSP linker and antibody solutions in the ELISA plate. Then a HRP (horseradish peroxidase) conjugated detection antibody was added followed by the TMB (3,3',5,5'-tetramethylbenzidine) substrate to give the change in color as seen in Figure 8. The plate was washed three times after each step with 3% BSA/PBS solution. A total of twelve

samples consisting of four controls, six test samples and two blank samples were tested in duplicates. Table 1 gives the details of the testing conditions. Rows A-D in columns 1 and 2 were set as four negative controls. The next three samples (cells E1, E2, F1, F2, A3 and A4) were test samples with increasing concentrations of anti-CRP. Similarly, three different concentrations of a second antibody, anti-HAPT (anti- haptoglobin) were tested in columns 3 and 4 of rows B-D. The last four cells (E3, E4, F3 and F4) served as blanks without any Au coated Si-pieces.

	1&2	3&4
A	Au, HRP-Antibody, TMB (No DSP, No Anti-CRP)	Au, DSP, Anti-CRP (100 µg/ml), HRP-Antibody, TMB
B	Au, DSP, HRP-Antibody, TMB (No Anti-CRP)	Au, DSP, Anti-HAPT (0.4 µg/ml), HRP-Antibody, TMB
C	Au, Anti-CRP, HRP-Antibody, TMB (No DSP)	Au, DSP, Anti-HAPT (2 µg/ml), HRP-Antibody, TMB
D	Au, DSP, Anti-CRP (50 µg/ml), TMB (No HRP-Antibody)	Au, DSP, Anti-HAPT (10 µg/ml), HAPT, HRP-Antibody, TMB
E	Au, DSP, Anti-CRP (10 µg/ml), HRP-Antibody, TMB	Blank
F	Au, DSP, Anti-CRP (50 µg/ml), HRP-Antibody, TMB	Blank

**Table 1:** Details of the solutions added for the twelve samples in Gold ELISA experiment

Figure 6 shows a digital photograph of the Gold ELISA plate. The cells in which the TMB substrate solution turned blue indicated that HRP-conjugated antibody had been captured by the antibody attached to the Au in that cell. This in turn validated the binding of the antibody on Au coated Si piece which forms the metallized platform for NM electrical detection.



**Figure 6:** Digital image of the Gold ELISA plate

## 5 CONCLUSIONS AND FUTURE WORK

In this article, we have demonstrated the design, fabrication, and operation of electrical biosensor for protein biomarker detection. We have selected two inflammatory proteins, CRP and MPO, as the study proteins to demonstrate the operation of the device prototype. These protein biomarkers were chosen as they are thought to be biomarkers of the vulnerable coronary plaque rupture state. The basis of the electrical biosensor functioning is the perturbation to the Helmholtz layer due to the binding of the relevant proteins from a test sample. This perturbation results in a modulation to the electro-ionic distribution of the interfacial electrical double layer formed in each of the nanowells/pores. These electrical signals can be measured as change in capacitance of the electrical double layer and be correlated to the concentration of the protein. It is clear from the current work that it is possible to selectively identify surface-charged proteins by measuring the variations to the Helmholtz layer in nanoscale confined spaces. The current work is a feasibility study indicating that changes at the nanoscale can be captured and correlated to protein-binding events thus indicating a promise in the development of a technique that is rapid and label free for the detection of protein molecules.

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