Acoustic Manipulation of Biological Samples for Improved Sensors

Stefan Cular[1], Darren W. Branch[2], Grant D. Meyer[3], Venkat R. Bhethanabotla[1]
[1]Sensors Research Laboratory, Department of Chemical Engineering, University of South Florida, Tampa, FL, USA 33620
[2]Microsensor R&D Department, Sandia National Laboratories, Albuquerque, NM, USA 87185
[3]School of Applied Physics, Cornell University, Ithaca, NY, USA 14853

Abstract

Non-specific binding is an ongoing problem that dramatically reduces the sensitivity and selectivity of biosensors. We demonstrate that ultrasonic waves generated by surface acoustic wave (SAW) devices remove nonspecifically bound proteins from the sensing and non-sensing regions of the micro-patterns. Our approach is proven for controllably and non-destructively cleaning the sensor interface to recover signals from a fouled background. In this work, 128^{o} YX lithium niobate was chosen for its high coupling coefficient and efficient power transfer to mechanical motion. Ultrasonic waves propagating along the surface were coupled into specifically bound and non-specifically bound proteins on 40 μm pattern feature size. Fluorescence intensity was used to assess cleaning efficacy of the micro-patterns. Our results have shown that excess protein layers and aggregates are removed leaving highly uniform films as evidenced by fluorescent intensity profiles. Selected antigen-receptor interactions remained bound during the acoustic cleaning process while being subjected to 11.25 mW of power and retained their efficacy for subsequent antigen capture. Results demonstrate proper fluorescent signal recovery for both the sensing and non-sensing regions of the micro-patterns. Of significance is that our approach can be integrated into existing array technologies where sensing and non-sensing regions are extensively fouled. We believe that this technology will be pivotal in the development and advancement of micro-fluidic devices and applications.

1.0 Introduction

Many chemical sensors are relatively easy to handle and are reusable many times because of known reversible interactions between the analyte and sensing film. Although biological sensors have known interactions that are relied on to make determinations; they also have non-specific interactions with biological species. These non-specific interactions occur from a combination of intermolecular forces such as ionic, hydrophobic and van der Waals and result in large unwanted effects on critical determinations [1, 2]. Non-specific binding can take the form of general fouling which is a more often used in industrial processes to describe the build up of matter on a device such as a heat exchanger [3].

Biological sensors are prone to many challenges, including the binding of un-wanted proteins, toxicity, and binding of desired proteins to appropriate locations [4]. Non-specific binding is a significant problem when tiny portions of

rare and expensive samples are in use [5]. It has been described by Blawas and Reichert [1] as “the most difficult issue to address for protein patterning.” They continue on to define non-specific binding as “indiscriminant adhesion of proteins to a surface due to weak attractive interactions or entropic forces.” [1]. More general than micro-patterning, non-specific binding is a complication in all types of measurements including: optical, electrical, and gravimetric techniques. To address this problem, chemical techniques and processes have been developed to reduce non-specific binding [2, 6]. One of the more common methods is to use patterning followed by applying a blocking agent to the un-reacted sites. Not only will this terminate the active surface groups, but also block other proteins from being able to bind [6-10]. This causes problems such as false positive/negative identifications and decreased sensitivity limiting the usefulness of automation when individual determinations need to be made [11].

Surface Acoustic Waves (SAWs) have been used for many years as chemical sensors directly [2]. Within the past few years, these sensors and more generally acoustic wave sensors have been tested for application in biological sensor applications for the same reasons they were chosen for general chemical applications, such as: high sensitivity, ease of integration to circuits, and all around hardiness [12]. For instance, SAW sensors have been used for rapid assays of antigens present in foods and liquids [2, 4, 13]. The SAW sensor primarily is a mass sensor, but also can be influenced by temperature, pressure, and surface film electrical properties [2]. As we present the SAW device, it is not used as a sensor, but as a tool to clean a protein array, minimizing non-specific binding and reducing other fouling.

2.0 SAW device and RF design

A Rayleigh SAW device utilizing 128^{o} $LiNbO_3$ was used as the substrate with an electrode pattern having 40 double split finger pairs per Interdigital Tranducer (IDT). The aperature was 38 λ and the delay path of the devices was 120 λ wavelengths. The SAW devices were powered with a Hewlett Packard 8656B Frequency Generator and an EVI 420A 20 W RF amplifier.

2.1 Silanization of lithium niobate surface

A 1 volume percent solution of (3-glycidoxypropyl)dimethylethoxysilane (Sigma Aldrich), in toluene was used to form the silane film on lithium niobate surface, Figure 1. Prior to this treatment, the SAW devices were washed with a typical acetone, methanol, de-ionized water cleaning to remove any photo-resist used to protect the IDT structures as

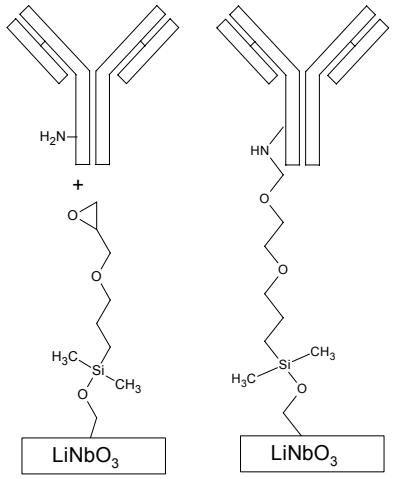

Figure 1. Structure and chemical reaction of organo-silane used to bond antibodies to lithium niobate surfaces

well as other surface contaminants. The general cleaning was followed by a brief, 2 minute, air plasma cleaning in a Harrick plasma cleaning system set on the lowest setting of 6.8 watts. The SAW devices were placed in the silane-toluene solution for one hour to allow a monolayer of silane to be formed. The devices were subsequently rinsed with toluene to remove the excess silane on the surface, and dried in a nitrogen stream. The final step in forming the silane layer was a cure for one hour at 125°C.

2.2 Patterning of SAW Devices

To form a representative micro-pattern array of 40 μm squares on the delay path of the SAW devices a photo-lithographic process was used [1]. This entailed spin coating the individual devices with AZ5214 photo-resist for 30 seconds at 3000 rpm, providing a uniform coating of 1.6 μm that was subsequently soft-baked for 20 minutes at 95°C. The patterning in the photo-resist was accomplished on the bench top with a long wave UV lamp and a dark field emulsion mask. Devices were aligned using a custom fabricated mask aligner, followed by exposure to the UV light for an optimized time of 30 seconds. Devices were removed from the aligner and the photo-resist was developed for 1 minute in a 1:5 400K developer water solution. Immediately following the development, the devices were rinsed for 2 minutes in de-ionized water to terminate the development reaction. This process was used for both non-treated and organo-silane treated devices.

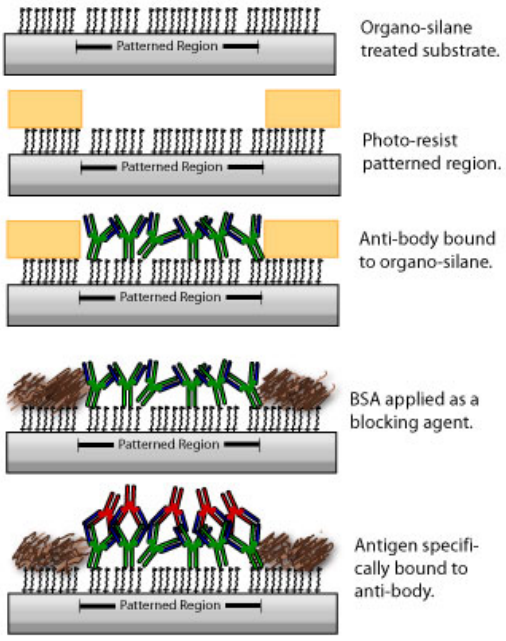

Figure 2. Pictorial representation of an ideal photolithographic protein patterning process.

2.3 Deposition of Protein Films

The deposition of protein films was implemented to mimic typical current microbiology techniques. The photo-resist patterned SAW devices were placed in a humidity chamber and had 3-6 μL 0.2 mg/mL of the desired protein in PBS pipetted onto the pattern. Special care was taken to prevent damage to the existing deposited films from physical contact. The devices were then sealed in the humidity chamber to prevent evaporation for 30 minutes. Following the incubation period, the devices were rinsed with PBS to remove any excess proteins and placed in PBS while awaiting the next processing step. This procedure is pictorially represented in the first three steps of Figure 2.

For the first protein layer, directly patterned with the photo-resist, acetone was used to rinse away the photo-resist. The acetone rinse was followed by a PBS rinse and a PBS immersion while waiting for the next processing step.

2.4 Fluorescent Imaging

Images of the fluorescently labeled devices were taken out of the micro-fluidic fixture to increase the clarity of images. Although the additional handling of the device introduced more sources for error, the image quality more than made up for it. The process was to apply a large drop of PBS to a cleaned glass slide and invert the coated device onto the slide. The drop of PBS protected the films on the surface of the SAW device. A 20x objective lens was used for all image capturing in conjunction with a Roper Scientific Cool Snap ES® CCD camera. The chassis for the fluorescence measurements was an Olympus® IX-70 Microscope equipped with Chroma Technology Corp. filter sets specific for the Alexa-488 and Alexa-594 flurophores used. For the Alexa-488 labeled proteins, 41001 FITC/ RSGFP/ Bodipy/ Fluo 3/ DiO filter set was used, and a Chroma 41002b, TRITC (Rhodamine with narrow-band excitation filter) was used for the Alexa-594 labeled proteins.

2.5 Experiments

Many small experiments were used to build up to more encompassing experimental procedures. Collectively, the experiments show that surface acoustic waves can be used to enhance sensor response by removing loosely bound proteins from the surface. Such a removal provides the benefit of reducing background noise, and reducing excessively bound proteins from the foreground.

2.5.1 Proteins are Still Active

One consideration for the application of surface acoustic waves to cleaning of protein arrays is that the waves do not stop the proteins from functioning as they would normally. For this experiment, the SAW devices were prepared according to procedures given previously in this document. The first protein deposited onto the organo-silane treated lithium niobate was Goat anti-mouse IgG labeled with the fluorophore Alexa-488. Following the deposition of this protein for 30 minutes, acetone wash, and a PBS wash, unlabeled BSA was applied for 30 minutes. It

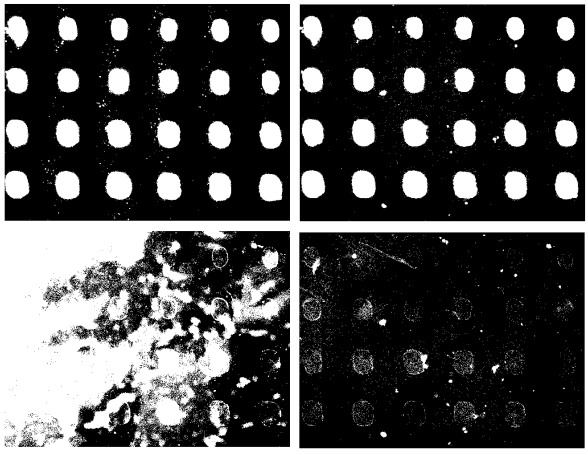

Figure 3. Green (top) and Red (bottom) patterns as shown in a before and after acoustic cleaning.

was found that longer periods of times were not necessary to have uniform high coverage films, also the 30 minute time frame allowed enough time to prepare future processing steps. The BSA was used as a blocking agent to prevent any further binding of proteins to the non-patterned regions of the SAW devices delay path [1]. Once the excess BSA was washed away with PBS, the SAW device was placed into the probing fixture with a film of PBS remaining between the IDT's. The SAW device was then driven at various RF levels and the results were recorded via fluorescent microscopy.

After the device was powered and the results were recorded, Mouse anti-rabbit IgG labeled with the fluorophore Alexa-594 following section 2.3 Deposition of Protein Films was deposited. After the 30 minutes of incubation/reaction, the excess protein was washed away with PBS. The resulting fluorescent images showed the antigen not only bound to the antibody, but also bound heavily to the BSA blocking agent to such a level that the specifically bound patterns were very difficult to distinguish as depicted in the Figure 3 (bottom). To further show that the antibody did not loose its ability to specifically bind to its antigen, the SAW devices were again driven with RF power. The results were recorded and analyzed; they show that the antigen that had been bound to the BSA blocking agent was nearly completely removed whereas the antigen bound to the antibody remained.

2.5.2 Non-specifically Bound Protein Removal

In this experiment, the same procedure was followed as in section 2.5.1 Proteins are Still Active with the exception of the applied Goat anti-mouse IgG was unlabeled. Only two different fluorophores were used throughout all of the experiments to simplify equipment needs. In this experiment the Mouse anti-rabbit IgG was still labeled with Alexa-594, and BSA, not used as the blocking agent, was labeled with Alexa-488. With this scheme, the antibody-antigen bond was made, and the labeled BSA was applied over the two proteins. Here, the labeled BSA non-specifically bound across the entire surface. As the acoustic waves hit the protein film, the BSA was removed and the specifically bound proteins remained relatively unchanged in the fluorescent measurements.

2.5.3 Control Sample

As a control for the previous experiments where surface acoustic waves were used a number of times to clean the surface/films of non-specifically bound proteins and general fouling, 2.5.2 Non-specifically Bound Protein Removal was used. This experimental procedure was to perform all of the same steps as the previous with the difference of vigorously washing the substrate in PBS as opposed to using acoustic waves.

The results of this experiment were as expected as the patterns on the substratum were difficult to distinguish from the surrounding regions. Only very minor improvements were a result of the vigorous washings. This implies that

more complicated procedures are necessary for non-acoustically cleaned patterns to have any significance in comparison.

3.0 Results

All results have shown that it is possible to use acoustic waves to clean samples by removing non-specifically bound proteins and reducing fouling. From the first experiments, general concepts were proven that first the acoustic waves generated from the available RF power was able to manipulate the proteins bound to the surfaces. This was shown by section *2.5 Experiments* with a configuration that took a lithium niobate SAW device held in a fixture and subjected it to high concentration of labeled proteins.

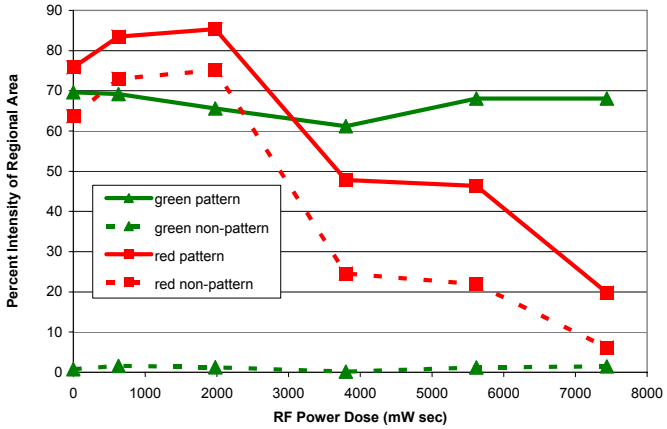

Figure 4. Red and green pattern and non-patterned intensities versus the RF power dose.

Having shown that the SAW has the ability to do some removal on bound proteins, progression to specifically bound proteins was made. This was done following the procedure as outlined in section *2.5 Experiments*, and the resulting data is shown in Figure 4. As illustrated in the data, the general trend of the intensity was to decrease as the RF power was applied; however, the covalently linked proteins labeled with the green fluorophore only decreased slightly, whereas the intensity of the protein labeled with the red fluorophore decreased from ~60% down to ~15%, nearly equivalent to the green labeled protein to which it was specifically bound. For a clearer picture of the results, see Figure 3 which shows the data as record in an image format. Note that it is difficult to determine the red's pattern without the use of some image processing to help enhance the image for our eyes. To assist with

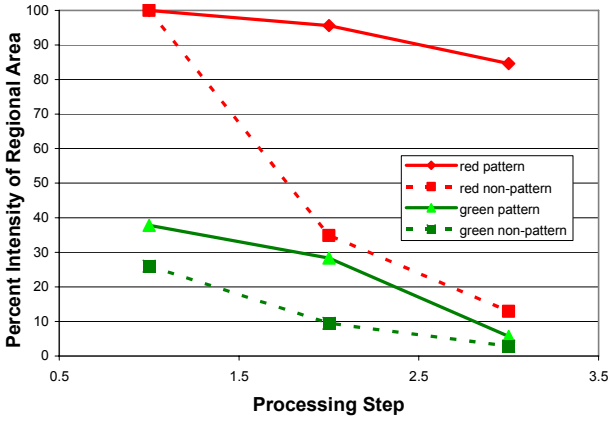

Figure 5. Red and green pattern and non-patterned percent of defined region intensity versus the processing step of repeated RF power.

the analysis, defined regions were used in the MetaMorph software to correspond to the lithographic patterns. Detailed information for each region was collected and processed to give the results shown in Figure 4.

The last important data set is for the proof that the acoustic waves do not input enough energy to damage the proteins where they become unable to function as they would normally. For this proof the SAW device was turned on after each step of the process to remove non-specifically bound proteins, and to stress the specifically bound proteins. After each step of reacting the protein and then running the acoustic cleaning, measurements were made to show the proteins were still on the device. Since the cleanings did not harm the proteins, specific binding was still possible as shown in Figure 5; however, the non-patterned intensity decreased significantly.

4.0 Conclusion

The experiments described here lead to the conclusion that SAWs are capable of cleaning surfaces; in particular SAWs can be used to remove non-specifically bound materials. Experimental evidence shows that the acoustic waves do not damage the proteins bound to the surface. This can become a valuable tool for increasing the sensitivity of biological sensors. The concept of applying SAW cleaning technology to biological sensors can be applied to a number of different applications. Additionally, the potential was demonstrated to remove specifically bound proteins at a higher input power for a longer exposure times. Directly from these experiments, it was shown that there is a large increase in the pattern to non-patterned ratio of intensity and area.

Acknowledgements

We would like to thank Mrs. Erika Cooley and Ms. Lauren Meyer for assistance with ellipsometry measurement and general laboratory help; also the support of Sandia National Laboratories MESA Institute, University of South Florida's IGERT-SKINS program (NSF grant DGE-0221681).

Sandia is a multi-program laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000.

References

[1] A. S. Blawas and W. M. Reichert, "Protein Patterning," *Biomaterials*, vol. 19, pp. 595-609, 1998.

[2] D. S. Ballantine, *Acoustic wave sensors: theory, design, and physico-chemical applications*. San Diego: Academic Press, 1997.

[3] S. M. Walas, *Chemical Process and Equipment*. Boston: Butterworth-Heinemann, 1990.

[4] J. E. Roederer and G. J. Bastiaans, "Microgravimetric Immunoassay with Piezoelectric Crystals," *Analytical Chemistry*, vol. 55, pp. 2333-2336, 1983.

[5] P. Mitchell, "A perspective on protein microarrays," *Nature Biotechnology*, vol. 20, pp. 225-229, 2002.

[6] S.-H. Lee, C.-S. Lee, D.-S. Shin, B.-G. Kim, Y.-S. Lee, and Y.-K. Kim, "Micro protein patterning using lift-off process with fluorocarbon thin film," *Sensors and Actuators B Chemical*, vol. 99, pp. 623-632, 2004.

[7] G. MacBeath and S. L. Schreiber, "Printing Proteins as Microarrays for High-Throughput Function Determination," *Science*, vol. 289, pp. 1760-1763, 2000.

[8] J. G. Kenna, G. N. Major, and R. S. Williams, "Methods for Reducing Non-specific Antibody Binding in Enzyme-Linked Immunosorbent Assays," *Journal of Immunological Methods*, vol. 85, pp. 409-419, 1985.

[9] J. Robert F. Vogt, D. L. Phillips, L. O. Henderson, W. Whitfield, and F. W. Spierto, "Quantitative differences amoung various proteins as blocking agents for ELISA microtiter plates," *Journal of Immunological Methods*, vol. 101, pp. 43-50, 1987.

[10] H. Schonheyder and P. Anderson, "Effects of Bovine Serum Albumin on Antibody Determination by Enzyme -Linked Immunosorbent Assay," *Journal of Immunological Methods*, vol. 72, pp. 251-259, 1984.

[11] G. D. Meyer, J. M. Moran-Mirabal, D. W. Branch, and H. G. Craighead, "Nonspecific Binding Removal from Protein Microassays using Thickness Shear Mode Resonators," *Accepted IEEE Sensors*, 2004.

[12] R. Dahint, R. R. Seigel, P. Harder, M. Grunze, and F. Josse, "Detection of non-specific protein adsorption at artificial surfaces by the use of acoustic plate mode sensors," *Sensors and Actuators B Chemical*, vol. 35-36, pp. 497-505, 1996.

[13] C. D. Morgan, D. J. Newman, and C. P. Price, "Immunosensors: technology and opputunities in laboratory medicine," *Clinical Chemistry*, pp. 193-209, 1996.

[14] D. W. Branch, G. D. Meyer, C. J. Bourdon, and H. G. Craighead, "Active Mixing in Microchannels using Surface Acoustic Wave Streaming on 128^{o} YX $LiNbO_3$," *Submitted JMEMS*, 2005.