Quantification of CEA from Human Plasma using Plasmonic Enhancement of Fluorescence and Acoustic Streaming

Yuqi Huang, and Venkat R. Bhethanabotla, *Senior Member, IEEE*

Department of Chemical & Biomedical Engineering, University of South Florida, Tampa, FL 33620 USA
yuqi@usf.edu
bhethana@usf.edu

***Abstract*—Carcinoembryonic antigen (CEA) is a prognostic biomarker for colon cancer and is elevated in many other cancers such as ovarian cancer and pancreatic cancers. We introduce an optimized technique based on plasmonic enhancement of fluorescence to lower the detection limit of CEA to sub ng/ml levels. Nanostructures generated using rapid thermal annealing of thin silver films, covered by a silica layer, enhanced fluorescence intensity to lower the detection limit to about 100 pg/ml and increase sensitivity by a factor of 18. Additionally, acoustic streaming generated by Rayleigh surface acoustic waves (SAWs) was utilized to address interference from non-specifically bound (NSB) proteins in the quantification of CEA from human plasma. It is shown that NSB proteins in human plasma can be successfully reduced using acoustic streaming to achieve a CEA detection limit of 500pg/mL in plasma.**

Keywords—Immunofluorescence; Plasmonic enhancement of fluorescence; Rayleigh surface acoustic wave; antibody-antigen; nonspecifically bound proteins

I. Introduction

Immunofluorescence (IF) is a primary technique to identify and quantify cellular antigens [1]. By detecting the intensity from the excitation light of the fluorophore dye, IF is quite easy to operate, and the results are direct. The quality of immunofluorescence assay is largely dependent on the specific attachment of antigen to antibody [2], and the sensitivity of apparatus to detect and analyze excitation light. However, detecting specific antigens in complex media such as urine, blood, sera and plasma involves dealing with the problem of non-specific adsorption proteins physically absorbed to the sensor surface and further attached to the open sites from specific antibodies, hindering the binding between antibodies and antigens [3]. Non-specifically bound (NSB) proteins, raise the background noise and decrease the amount of specific attachment from antibody/antigen pairs to the sensor surface, which weaken the signal and lead to poor sensor performance [4].

Our previous studies have shown that by utilizing acoustic streaming from Rayleigh surface acoustic waves (SAW) to the pretreated surface, NSB proteins can be effectively removed [5,6]. We have recently described a small, portable electronic system to provide power to a delay line SAW device for generation of acoustic streaming [6]. Also, recently, we have demonstrated plasmonic enhancement of fluorescence utilizing silver nanocubes incubated on a glass substrate prior to the CEA capture antibodies/antigen assembly [7]. An eight-fold increase in sensitivity was achieved, and a limit of detection below 1 ng/ml CEA was demonstrated in that study.

In this contribution, we apply plasmonic enhancement of the fluorescence signal for detection, and acoustic streaming for NSB removal together to quantify CEA directly from human plasma, to demonstrate sensing of a cancer marker in the presence of interfering proteins. The portable electronic device from reference [7] is utilized here to move towards point of need (PON) detection, while the plasmonic structures were generated using a simpler technique than that of dispersing colloidally synthesized nanocubes. These silver nanostructures were generated using rapid thermal annealing (RTA) of a thin silver film deposited by electron beam evaporation. These structures were further protected against oxidation by a thin silica film generated using plasma-enhanced chemical vapor deposition or sputtering, with either technique working equally well. The silica film served the dual purpose of protecting the silver structures against oxidation and of allowing for maximization of the plasmonic enhancement through optimization of its thickness. Combined, this work led to 18x increase in sensitivity compared to not using the plasmonic structures and lowered the detection limit of CEA to 500 pg/ml directly from human plasma in the presence of all the interfering proteins present. These developments take us one step closer to PON detection of cancer markers at the clinically relevant pg/ml levels directly from a small blood sample.

II. Experimental

A. Reagents and Apparatus

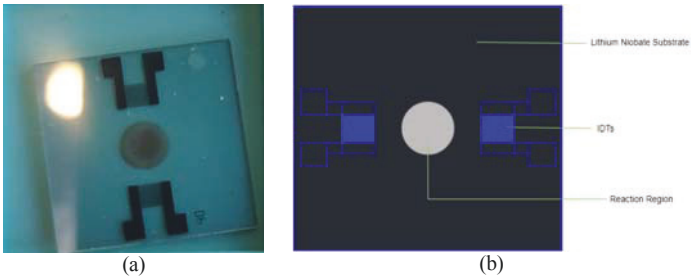
All materials and reagent utilized in this experiment are of analytical grade, which include: CEA capture antibody (Fitzgerald, 10-C10D), CEA detection antibody labeled with Alexa488 (Fitzgerald, 10-C10E) (tagged with Alexa Fluor 488 Protein Labeling Kit, Thermo Fisher), CEA antigen (Abcam, ab742), PBS (Life Technologies, pH 7.4), bovine serum albumin (BSA, Fisher Scientific), Protein A (abcam), and

Single Donor Human Plasma (whole blood derived, Innovative Research, Inc).

Instrument used in this experiment include a network analyzer (Agilent 8753ES); and a Nikon FN1 fluorescence microscope.

B. Surface Acoustic Wave Device with Plasmonic Region

The delay-line SAW devices were made on a 4-inch 0.5mm 128° YX lithium niobate wafer, deposited with two pairs of aluminum interdigital electrodes using E-beam evaporation. Each of the electrodes has 60 fingers of 10 by 4μm dimensions and can generate Rayleigh waves with a fundamental frequency of about 98.1MHz. The plasmonic region was then fabricated by e-beam evaporating a 15nm silver film at the delay path region between the two electrodes, then followed by a rapid thermal annealing procedure. The SAW chips were heated up to 500 °C in 25 seconds and remained at 500 °C for 60 seconds, then the chips were cooled down to room temperature. Silver nanoparticles of about 200nm diameter were formed by this process. Then, a 10nm silica film was metal sputtered on top of the silver nanoparticles to protect the silver structures and provide spacing between metallic structures and fluorophores, which can further enhance the resulting fluorescence signal. The fabricated SAW chip is shown in Fig.1.



(a) (b)

Fig. 1. (a) Image of Surface Acoustic wave device. (b) Schematic diagram of SAW device.

C. Immunofluorescence Assays

Bio-assembly of CEA capture antibody, CEA antigens, and CEA detection antibody with prelabeled fluorophore was conducted on the fabricated SAW devices. SAW substrates were first pretreated with 3-APTES to silanize the surface and after 1-hour, the remaining solution was rinsed with ethyl alcohol and dried with nitrogen gas. Protein A was then added to immobilize CEA capture antibody. Then, the chip was PBS rinsed and dried in nitrogen gas, PBS rinsing and drying was done for each of the subsequent steps. First, the CEA capture antibody is added to the surface. Then, 1% BSA in PBS was added to rough block the surface from absorbing NSB proteins. CEA antigen diluted with either PBS or human plasma in the concentration range of 100pg/mL to 10μg/mL is added to the surface. The final step involved adding CEA detection antibodies labeled with Alex-488, which has an excitation peak of 494 nm and an emission peak of 519 nm.

Several experiments were done to measure the plasmonic enhancement and SAW removal effects, all with similar bio-assembly steps. The first experiment compares the enhancement by adding biostructures on both bare lithium niobate substrates and SAW substrate with the plasmonic region, to quantify CEA antigen diluted in PBS solution with a concentration range of 0.1ng/mL to 10μg/mL. The second experiment compares the plasmonic enhancement for CEA in PBS with a concentration range of 0.1ng/mL to 10μg/mL and a further diluted CEA sample in human plasma with a concentration range of 0.5ng/mL to 5μg/mL, to study the effect of NSB interference to fluorescence signals. The third experiment utilizes SAW devices with a plasmonic region and adds a SAW streaming step after 1-hour CEA antigen incubation, by adding 10μL PBS to the incubation surface and streaming at a power of 5mW for 5 minutes.

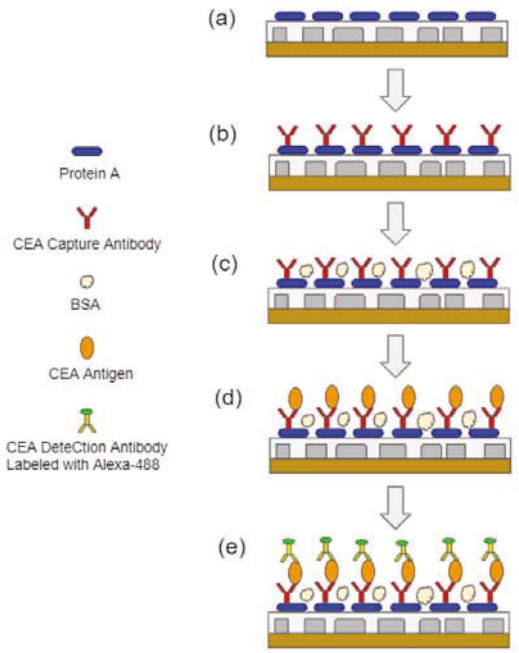


Fig. 2. Procedures of antibodies assembly on annealed silver nanostructures.

III. Results and Discussion

Fluorescence images were taken using a Nikon FN1 fluorescence microscope with a 10s exposure time and 1X gain. The measured green channel values of the RGB system (255,255,255) were quantified using ImageJ software. Fluorescence enhancement factors from the plasmonic substrates were calculated from the measured intensities. Removal of NSB protein removal from acoustic streaming was also demonstrated using microscopy.

A. CEA diluted in PBS

A comparison of the effectiveness on enhancing the fluorescence signal clearly shows that for the non-enhanced group, the signals from CEA concentration of 100pg/mL to 1μg/mL are indistinguishable, but somewhere between 1μg/mL and 10μg/mL, the intensity starts to increase. The results are presented in Table 1. However, with the same experiment done with metal nanostructures, the trend is clear for all

concentrations, from 100pg/mL to 10µg/mL. The detection limit is clearly lowered to about 100 pg/ml.

TABLE I. INTENSITIES FOR CEA DILUTED IN PBS

CEA Concentrations in PBS	Fluorescence Intensity with plasmonic enhancement	Fluorescence Intensity without plasmonic enhancement
100 pg/mL	56.83	25.27
1 ng/mL	66.83	24.8
10 ng/mL	77.45	24.8
100 ng/mL	94.08	25.33
1 µg/mL	113.42	25.319
10 µg/mL	165.04	33.66

B. *CEA from Human Plasma*

Utilizing the same experimental procedures, but with CEA diluted in human plasma, intensity for each concentration group decreased for plasma samples. More significantly, even with the plasmonic substrates, the limit of detection increased from 100 pg/mL to 50 ng/mL, as can be seen in Table 2. A reasonable explanation of the increase of LOD is the existence of unrelated antibodies, proteins, cells, etc. blocking the binding sites from CEA capture antibodies and preventing the incubation of CEA antigens, as shown in Fig.3. Even with the same experimental procedures which include PBS wash following every protein incubation, a significant amount of remnant NSB proteins still adhere to the incubation surface. These NSB proteins could attach to the capture antibody and block the open sites, while also absorb the fluorophore labeled CEA detection antibody in the following procedure and causing false signals. The blocking of open sites from CEA capture antibodies prevents conjugations of CEA antigen to the capture antibodies and to the fluorophore labeled detection antibodies. Thus, it decreases signal intensity for all concentrations.

TABLE II. INTENSITIES FOR CEA DILUTED IN HUMAN PLASMA

CEA Concentrations in PBS	Fluorescence Intensity with MEF	Fluorescence Intensity without MEF
0.5 ng/mL	50.79	31.40
5 ng/mL	49.77	32.21
50 ng/mL	49.53	30.52
500 ng/mL	70.28	31.47
5 µg/mL	74.74	30.67

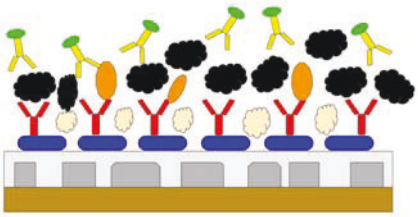


Fig. 3. NSB proteins interfere with the incubation of capture antibodies and detection antigens.

C. *Removal of NSB Proteins with Acoustic Streaming*

To address the issue of NSB proteins blocking the open sites from CEA capture antibodies, SAW streaming with a removal power of 5mW (7dBm) was applied for 5 mins after a 1-hour incubation of CEA antigen from human plasma. Although signals of all concentrations are weakened, there is a larger signal decrease for lower CEA concentration, making differences distinguishable. Applying the SAW wave to the incubated surface decreases the detection limit from 50 ng/mL back to nanogram level. A possible explanation of this reduction in LOD is that, NSB proteins are removed upon streaming, as shown in Fig.4. Lower concentration CEA samples usually have larger NSB proteins to CEA antigen ratio, and NSB proteins are removed by SAW streaming at a much higher rate.

TABLE III. FLUORESCENCE INTENSITIES OF CEA WITH AND WITHOUT SAW REMOVAL

CEA Concentrations in Plasma	Fluorescence Intensity with SAW Removal	Fluorescence Intensity without SAW Removal
0.5 ng/mL	34.07	50.79
5 ng/mL	42.51	49.77
50 ng/mL	45.16	49.53
500 ng/mL	52.29	70.28
5 µg/mL	61.46	74.74

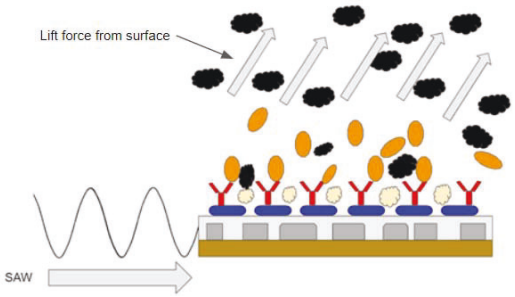


Fig. 4. Schematic illustration of SAW removing NSB proteins.

I. CONCLUSION

A novel CEA antigen quantification approach combing the characteristics of surface acoustic wave's removal on NSB proteins and plasmonic enhancement of fluorescence to reduce the background noise and enhance the fluorescence signal is presented. Using this approach, the detection limit of CEA antigen from human plasma was brought down to picogram per milliliter levels. The small, low-cost biomarker detection system made possible from this work upon the integration of optical components to the already developed electronics will enable quantification of biomarkers directly from blood at clinically relevant concentrations.

ACKNOWLEDGMENT

NSF funding via grant IIP-1640668 and USF funding via a Strategic Investment Pool grant are gratefully acknowledged.

REFERENCES

[1] J. Owen, J. Punt, S. Stranford, "Kuby Immunology," New York : W.H. Freeman, pp. 699-700, 2013.

[2] I. Odell, D. Cook, "Immunofluorescence Techniques," Journal of Investigative Dermatology,133, e4, 2013.

[3] E. Güven, K. Duus, et al. " Non-specific binding in solid phase immunoassays for autoantibodies correlates with inflammation markers ", Journal of Immunological Methods, vol. 403, 1-2, 2014.

[4] J. Lichtenberg, Y Ling, S. Kim, "Non-Specific Adsorption Reduction Methods in Biosensing," Sensors (Basel, Switzerland) vol. 19,11 2488. May 2019.

[5] S. Cular, D. W. Branch, V. R. Bhethanabotla, G. D. Meyer and H. G. Craighead, "Removal of Nonspecifically Bound Proteins on Microarrays Using Surface Acoustic Waves," in IEEE Sensors Journal, vol. 8, no. 3, pp. 314-320, March 2008.

[6] S. Li, V. R. Bhethanabotla, "Design of a Portable Orthogonal Surface Acoustic Wave Sensor System for Simultaneous Sensing and Removal of Nonspecifically Bound Proteins," Sensors (Basel), vol. 19,18 3876, 2019.

[7] J. Liu, S. Li, V. Bhethanabotla, "Integrating Metal-Enhanced Fluorescence and Surface Acoustic Waves for Sensitive and Rapid Quantification of Cancer Biomarkers from Real Matrices," ACS Sensors, vol 3, 222-229, 2018.