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# Biomarker Detection at Risk Forecasting Level Using Metal-Enhanced Fluorescence Combined with Surface Acoustic Wave

Jun Liu
*University of South Florida*, liuj@mail.usf.edu

# Biomarker Detection at Risk Forecasting Level Using Metal-Enhanced Fluorescence Combined with Surface Acoustic Wave

by

Jun Liu

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Major Professor: Venkat Bhethanabotla, Ph.D.
John Kuhn, Ph.D.
Anna Pyayt, Ph.D.

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## DEDICATION

I dedicate this work to my family and friends for always supporting and encouraging me to go further both in college and in life. I dedicate to my parents and wife especially for them being available to help all the time. I would also like to dedicate this manuscript to everyone in the lab, just like my family members for being so kind and warm to me.

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## ABSTRACT

In this paper, metal-enhanced fluorescence (MEF) technique is used to lower the detection limit of carcinoembryonic antigen (CEA) which is able to be utilized in forecasting the risk of having certain kinds of cancers, especially colon and rectal cancer. By incubating silver nanocubes (Ag NCs) on the surface of the chips, the detection limit goes down to below 1ng/mL of CEA. Also, when combining MEF with surface acoustic wave (SAW) devices, the incubation time between antigen and antibody will decrease significantly with the fluorescence signal keeping similar or higher level.

# CHAPTER 1: INTRODUCTION

Immunofluorescence assays are used to detect the quantity level as well as the distribution of the protein or tissues within the cell or on other substrates. The operations and the principles of this technique are fairly intuitive and accessible, however, it still suffers from some severe limitations originally, for example, low detection limit and photobleaching. Hence, the application will confront problems like irresponsive signal when detecting lower sample concentrations or loss of signal in a short period. Besides technical problems, another negative factor existing here is that incubation between a pair of antigen and antibody sometimes can take from dozens of hours to a couple of days, with some incubations being repeated in several different pairs taking much longer time.

In the paper, metal-enhanced fluorescence is applied to increase the sensitivity of the detection and resisting the possible photobleaching. Here, Silver nanocubes (Ag NCs) were found to be able to effectively increase the signal by around 4.6 times and improve the photobleaching. With the enhancement provided by the MEF, it is practical and reliable that the detection limit can be lowered down to nano level.

Gold and Freedman first introduced carcinoembryonic as the tumor associated antigen in 1965.[1] Since that, considerable research has been made to investigate the structure, potential functions and expression of CEA.[2] CEA antigen is categorized to a type of glycoprotein which is related to cell adhesion. Though lots of work has been done to clarify the relationship between the content of CEA and the risk level having cancer diseases, it is still not clear of its clinical value.

However, CEA has been used as one of most widely accepted tumor marker for over last forty years, especially in gastrointestinal cancers. Much of that evidence has proved that CEA has a direct and strong relevance to colon cancer in clinical experiment and research.[3] It was found that the overwhelming majority of patients with colon cancer had a higher level of CEA than normal individuals.[4] With many measurements carefully done by many researchers, it has been acknowledged world widely that 2.5ng/mL of CEA is the critical value to estimate the possibility of having gastrointestinal cancers or malignancy.[3-5]

Metal-Enhanced Fluorescence has been drawing great attention from many researchers over the last decades due to its incredible and unique properties performed in fluoroscopy. MEF is also termed as radiative decay engineering or surface-enhanced fluorescence. It has been applied in biosensing and detection technology to lower the detection limit and conquer the photobleaching issue encountered in practice. As a very sensitive technique, MEF also has surprising advantages in keeping photostability of fluorophores and decreasing the self-luminous phenomenon.[6] The most studied and used in MEF is metallic nanostructures, especially silver and gold nanoparticles.[7-10] In addition to these two metals, aluminum, copper, some metallic oxides, like zinc oxide, even alloys of different composition have been investigated and modified to measure their performance in MEF.[11, 12] Among them, silver has relative narrow surface plasmon resonance peak which is decided by its nature property. In this paper, silver nanocubes were chosen in the experiment due to their particular structure and performance.

Lord Rayleigh first described surface acoustic wave as a pattern of propagation along the surface of elastic solid in 1885.[13] SAW is also called as Rayleigh wave to honor its discoverer. The motion mode of particles in Rayleigh wave is similar to sea wave that vibrates elliptically and counterclockwise in a vertical plane while vibration amplitude will decrease along the depth into

the substrate. Before scientists and engineers putting SAW devices into the use of biosensing, the most widely used and commercialized application of them was in the field of telecommunication. Still, they have been playing a critical role in mobile data processing technology since then.[14] SAW devices utilize interdigital transducers (IDT) on the surface of piezoelectric materials to realize conversion between electric and acoustical signals as well as detection signals.[15]

### 1.1. Primary Detection Techniques

Immunoassays are mostly developed and used in the field of detecting proteins at present. They are categorized into several assays including Enzyme-Linked Immunosorbent Assay (ELISA), Precipitation Reactions (PR), Agglutination Reactions (AR), Radioimmunoassay, Western Blotting (WB), Immunofluorescence, etc. Each of these methods has its own benefits and drawbacks. In this section, a couple of important techniques will be discussed. The primary information was taken from the fifth edition of *Immunology*.[16]

#### 1.1.1. Precipitation Reactions

Precipitation reactions are for the detection of immunoglobulin concentration by antibody and antigen interacting and finally forming a precipitate. There are some severe constraints existing in this method. One of the problems is that it takes as long as one or two days to finish the precipitation completely between antigen and antibody. This can be an issue when it comes to detecting the immunoglobulin level of affected patients in urgent or in other circumstances. Another defect is the precipitation reactions are only useful to some certain antigens and antibodies. They require that the antibody has to be bivalent meanwhile the antigen must be at least bivalent and soluble in aqueous to allow the precipitation go on. Therefore, a large portion of antigen/antibody is unqualified for this method. Besides those two aspects, what makes precipitation reactions a worse means is their low sensitivities which typically is restricted within

10-200 μg antibody/mL, though 2 μg/mL can be reached in some cases. Table 1-1 below provides the sensitivities of various PR branches adapted from *Immunology*.[16]

Table 1-1. Sensitivities of precipitation reactions. Data adapted from *Immunology*[16]

| Assay | Sensitivity (μg antibody/mL) |
|---|---|
| Precipitation reaction in fluids | 20-200 |
| Precipitation reactions in gels | |
| Mancini radial immunodiffusion | 10-50 |
| Ouchterlony double immunodiffusion | 20-200 |
| Immunoelectrophoresis | 20-200 |
| Rocket electrophoresis | 2 |

Considering the bad sensitivity of precipitation reactions, they are not used in detecting biomarkers that often requires the minimum detection limit of 1 μg/mL. However, they still prove useful for detecting antigens and antibodies in relatively high concentrated solutions, although long latency time is reported for most cases.

### 1.1.2. Agglutination Reactions

Close to precipitation reactions, agglutination reactions refer to the reactions where a specific antigen interacts with an antibody and forms clumping as agglutination. Agglutination reactions also rely on the polyvalent antigens via crosslinking effect. One important phenomenon here is the prozone effect which is caused by an excess of antibody inhibiting agglutination reactions. Inhibition reactions were developed based on the prozone effect. More information about the mechanism can be found in the book of *Immunology*. Besides, agglutination reactions have also extended to direct and passive reactions.

In general, the sensitivity of agglutination reactions is much higher than precipitation reactions as shown in Table 1-2.[16] The introduction of artificial beads as the reaction matrix other than blood cell cuts down the reaction time to several minutes compared to one or two days in precipitation reactions. Moreover, the sensitivity of agglutination reactions has a strong

advantage over precipitation reactions. Table 1-2 below provides the information which is adapted from *Immunology*.[16]

Table 1-2. Sensitivities of agglutination reactions. Data adapted from *Immunology*[16]

| Assay | Sensitivity (μg antibody/mL) |
|---|---|
| Direct | 0.3 |
| Passive agglutination | 0.006-0.06 |
| Agglutination inhibition | 0.006-0.06 |

Due to their fast reactions and high sensitivity, agglutination reactions are utilized primarily for diagnostic infection and blood typing, as well as in detecting illegal drugs by an individual.[16] However, agglutination reactions will no longer have effect when the concentration of antigen goes down to lower than 6 ng/mL since it reaches the limits of detection.

### 1.1.3. Radioimmunoassay

Radioimmunoassay (RIA) is one of the most sensitive techniques in immunology where it can detect antigens lower than the concentration of 1 ng/mL for various substances.[16] The basic principle involved in RIA is the competitive mechanism. First, a known quantity of radiolabeled antigen is mixed with a known quantity of its corresponding antibody and they will specifically bind to each other. Then, unlabeled antigen at an unknown concentration in overwhelmed amount is added to the mixture. At this spot, the unlabeled antigen will compete with labeled ones to bind to the antibody. Afterward, the decrease of the amount of labeled antigen bound to the antibody is determined by a gamma counter. The concentration of unknown antigen can then be measured according to the comparison to a reference amount.

RIA is widely used for detecting hormone levels in serum or some other forbidden drugs in society due to its high sensitivity and specificity. Though RIA has a quite good capability for detecting many antigens and not constrained by serum, it will require expensive equipment for

counting and dangerous radiative substances involved in the experiment whereby well trained and fully prepared operators are required. These drawbacks limit the promotion of RIA as a popular tool to some degree.

### 1.1.4. Enzyme-Linked Immunosorbent Assay

Enzyme-linked Immunosorbent assay, generally called as ELISA, is able to detect macromolecule antigens and specific antibodies. The mechanism is close to RIA that it conjugates with an enzyme instead of a radioactive label. An antibody labeled with enzyme will produce colored products when reacting with colorless substrate. Different enzymes are chosen for antibody in ELISA based on their properties and compatibilities, such as alkaline phosphatase and horseradish peroxidase.[16] According to the format structure, ELISA can be sorted into four different types, including direct, indirect, sandwich and competitive these. Figure 1-1 shown below gives the schematic diagram of four types in principle gathered from the web.[17]

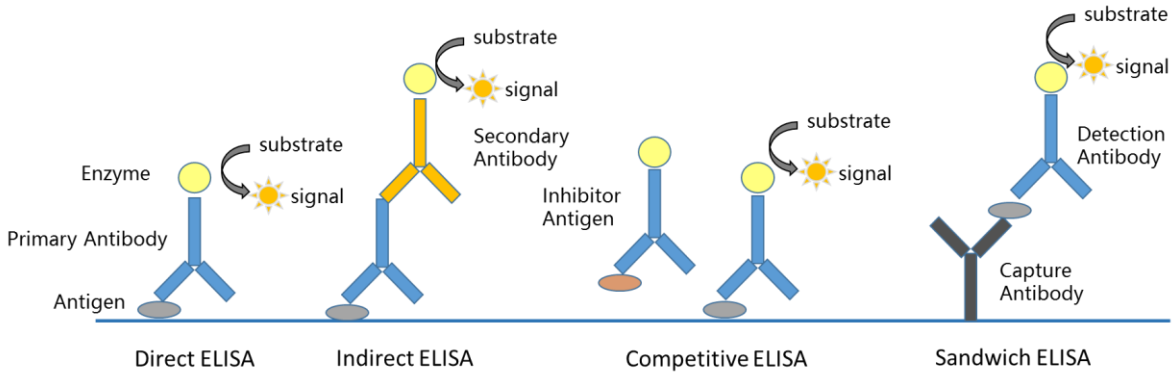


Figure 1-1. Schematic diagram of several types of ELISA.

As shown in the figure, these methods are similar to each other except the order and the species in which the antigens or antibodies are introduced. Direct ELISA, the easiest one among them, only requires one primary antibody conjugated with an enzyme to be bound to the immobilized antigen and then another substrate will be added in for the chromogenic reaction.

Similar to direct, the indirect method also needs another secondary antibody after adding the primary antibody. The only difference is that the secondary antibody is conjugated with enzyme other than the primary. Sandwich ELISA is the most sensitive and robust tool compared to the others. The antigen is added after the immobilization of capture antibody. Afterward, the detection antibody is added to move on the reaction. The detection antibody is either carried with an enzyme or it will bind to another second antibody which conjugated with an enzyme in order to realize the color change. As for competitive ELISA, the mechanism is quite different from previous ones. First, the antibody is mixed with its sample antigen. The complexes are then added to the well where antigen coated already. Then the unbound antibodies are washed off where if more sample antigen exists, the less unbound antibodies available for antigen in the well. After adding a second antibody labeled with the enzyme, the concentration of sample antigen can be calculated according to the color signal.

The ELISA approaches the same sensitivity as RIA when it is performed in a safer environment and costs much less. For this reason, ELISA has been well developed over the years and many companies have built mature systems and have provided commercial services for determining a lot of serum antibody concentrations and the presence of the certain antigen.

### 1.1.5. Western Blotting

Western blotting is a good tool for identifying a specific protein within a mixture of many proteins. Basically, the proteins are separated by gel electrophoresis and then transferred to a membrane. Then specific antibody labeled with an enzyme is added to generate the color reaction.[16]

### 1.1.6. Immunofluorescence

Since Albert Coons found that antibodies labeled with some specific molecules were able to generate fluorescence in 1944, immunofluorescence (IF) assays have been developed tremendously over the last decades.[16] The technique is now widely utilized in detection of antigen or antibody, some even used for DNA and protein detection.[6, 11, 12, 18-22] In general, immunofluorescence is a microscopic technique that visualizes specific antigens or cell tissues by binding corresponding antibodies labeled with fluorescent compounds. There are several fluorescent compounds that can be used in immunofluorescences, such as rhodamine, fluorescein, and some other dyes. At first, these compounds are conjugated to the antibodies by chemical bonds without affecting their biological activities and binding specificities to the antigens. Afterward, fluorescent groups will absorb at a certain wavelength when exposed to a UV light source and then emit at a slightly longer wavelength. The emission light will thereby be detected by fluorescence microscope or the signal will be transferred to a monitor in real time.

However, there are some limitations in this technique. The weak signal should be an annoying issue here. The fluorophores often lose their activities and produce a low signal which hinders the application for concentrations lower than 1 μg/mL.[16] Also, the background noise or self-luminescence makes it harder to take a distinct picture. Time factor needs to be considered as well since it can take up to days for incubation between antigen and antibody. This paper is dedicated to solving or improving these problems by introducing MEF and combining with SAW device that can approach lower limits of detection for real biomarker detection. Johnson et al. pointed out the photobleaching problem with immunofluorescence.[23] This is another reason why MEF was chosen. MEF is able to increase the sensitivity while eliminating or reducing the photobleaching effect. The photostability can be increased as a shorter lifetime allows less time

for adverse reactions to occur.[24] Moreover, the employment of SAW will significantly short the incubation time by increasing the mixing between antigen and antibody.

### 1.2. History and Diagnostic Significance of Carcinoembryonic (CEA)

Since Gold and Freeman first introduced CEA as a tumor marker in 1965,[1] much work has been done trying to identify the relationship between CEA and some certain types of cancer. Unfortunately, the intrinsic clinical value of CEA is still vague and more research is needed. However, it does not prevent CEA from becoming one of most used tumor markers over years.

The most common application of CEA is in colorectal cancers as well as in some other non-neoplastic diseases including ulcerative colitis, pancreatitis, cirrhosis, etc.[3, 25] Thomson et al. demonstrated that CEA in the serum of colorectal cancer patients increased in overwhelming majority (for 35 among 36 patients). However, the growth of CEA content did not happen with those patients with non-gastrointestinal cancers, either normal subjects or with miscellaneous benign gastrointestinal diseases.[4] Similar results were also obtained by many other independent researchers and CEA is monitored in preoperative and postoperative surgical treatment to help surgeons determine the condition and recovery of patients with colorectal cancers.[25-28]

Typically, 2.5 ng/mL level of CEA is widely accepted to be the critical value when evaluating the risk of having colorectal cancers.[5] However, according to *Immunology*[16], the limit of detection of traditional immunofluorescence is 1 μg/mL which is much larger than the risk-level of CEA concentration. It is thus revealed that the effective MEF dose for successful detection of CEA concentrations by IF is at least 2.5 ng/mL or lower.

### 1.3. Metal-Enhanced Fluorescence

The studies of fluorophores-metal nanoparticles in close proximity interactions have been well documented and reported over the past decades.[29] The ability of some metal nanoparticles

with various characteristics to enhance the intensity in IF has drawn great attention worldwide. Silver and gold have shown satisfactory effects on improving fluorescence among many metals while other materials including copper, aluminum or zinc oxide also present the ability of MEF.[7-12] Many examples of metal nanoparticles giving enhancement in IF can be easily found throughout literature. MEF has been a very useful tool in biotechnology in detecting proteins or DNA in a cell or in vitro according to the reports. Malicka et al. first discovered that MEF worked for DNA hybridization.[30] However, the work in combination with SAW devices is still a huge vacancy. Morrill introduced a SAW device into MEF on the quartz surface and demonstrated that MEF-SAW combination was feasible.[31] Non-specific binding removal and accelerated mixing were realized in his device with the help of SAW which could be the two main issues in current IF.[31]

### 1.3.1. Origin of MEF

As mentioned earlier, the MEF phenomenon is caused by the interactions between excited states of the fluorophores and the induced surface plasmons of metal nanoparticles.[32] Due to the enlarged electric fields around the metal nanoparticles, fluorophores are able to absorb light with great enhancement.[33] The sample geometries in fluorescence experiments are generally larger than the size of fluorophores as well larger than the absorption and emission wavelengths which cause the fluorophores to radiate into free space. This leads to a low quantum efficiency in fluorescence experiments. The presence of metal nanoparticles typically within 20 nanometers changes the light radiation condition from the free-space condition and causes a significant spectral difference.[6] Thus, radiative decay rates (Γ) of fluorophores are altered by the interaction with the metal nanoparticles which results in the enhancement.[34]

For those theoretical facts to be more clearly illustrated, quantum yield ($Q_0$) and lifetime of the fluorophores ($\tau_0$) here are introduced. Quantum yield is defined as the ratio of the number of photons emitted to the number of photons absorbed whereas lifetime of the fluorophores refers to the period that a photon stays in the excited state before returning to the ground state which can vary over a huge range. When there are no metal nanoparticles, these equations are given as:[6]

$$Q_0 = \frac{\Gamma}{\Gamma + k_{nr}} \tag{1}$$

$$\tau_0 = \frac{1}{\Gamma + k_{nr}} \tag{2}$$

When applying metal nanoparticles, these equations are modified to:

$$Q_M = \frac{\Gamma + \Gamma_M}{\Gamma + \Gamma_M + k_{nr}} \tag{3}$$

$$\tau_M = \frac{1}{\Gamma + \Gamma_M + k_{nr}} \tag{4}$$

where $k_{nr}$ is referring to the non-radiative rate and $\Gamma_m$ is generated by the presence of the metal nanoparticles.[6] These four equations clearly explain the reason why the quantum yield will increase while the lifetime of fluorophores will decrease when applying the metals.

### 1.3.2. Plasmonics and Localized Surface Plasmon Resonance

Plasmonics is dedicated to the study of the ability and performance of metal at the nanoscale to manipulate light.[35] As categorized into this field, MEF has already gained much development along with other subtopics, i.e. Surface-Enhanced Raman Scattering (SERS). The breakthrough in synthesis, characterization, and equipment of metal nanoparticles has accelerated MEF to achieve many exciting results and progress.[35]

Localized Surface Plasmon Resonance (LSPR) is the theoretical basis of MEF. It expresses the resonant oscillation of free electrons confined at the surface of metal particles generated by

incident light. LSPR happens due to the overlap between the wavelengths of incident light and the oscillation of free electrons on the surface. The resonance is then able to cause spectral absorption, scattering peaks and strong electromagnetic enhancements.[36]

### 1.3.3. Influencing Factors in MEF

There are many characteristics of metal particles that can vary the effects on the enhancement of fluorescence, for example, particle shape, size, metal material, even alloy, etc.[11, 12, 34, 36] Massive information can be collected and reached easily from the literature on how different factors work with MEF. On the other hand, the enhancement is greatly controlled by the matching up between the excitation peak of the fluorophores and the plasmonic peak of the metal structure. After careful studying all kinds of aspects as well as based on the work which Morrill[31] has done before, silver nanocubes were chosen for MEF experiment due to their superior performance in biotechnology especially for sensing.[37]

## 1.4. Surface Acoustic Wave

The SAW device is designed to propagate the waves that can be converted from electric to mechanical signals by IDTs and vice versa. which are well confined along the surface of piezoelectric substances like quartz and lithium niobate ($LiNbO_3$). The device sends Rayleigh waves through the piezoelectric material[38] and as mentioned, Raleigh waves vibrate elliptically and counterclockwise in the vertical plane. This vibration can be used to realize non-specific binding removal and speeding mixing effects.[31] This will be discussed more in Chapter 3.

In general, a SAW device includes a piezoelectric substrate, the input and output IDTs and conductive areas for connecting with the signal generator (Rohde & Schwarz, SMA100A). The substrate of SAW device used here was $LiNbO_3$ 128 $^{o}$ rotated Y-cut X-propagation in the size of 20 $\times$ 20 mm. A schematic diagram of SAW device is shown below in Figure 1-2. There are two

IDTs on the top of the lithium niobate substrate and a polydimethylsiloxane (PDMS) structure with a reaction cell is bound on the path between the IDTs. The incubation of antigen and antibody happens in the small cell which diameter is set at 5mm. The use of PDMS as a reaction hole prevents the leaking of liquid as well as makes it more explicit area when rinsing with Phosphate-buffered saline (PBS) or adding following antigen/antibody.

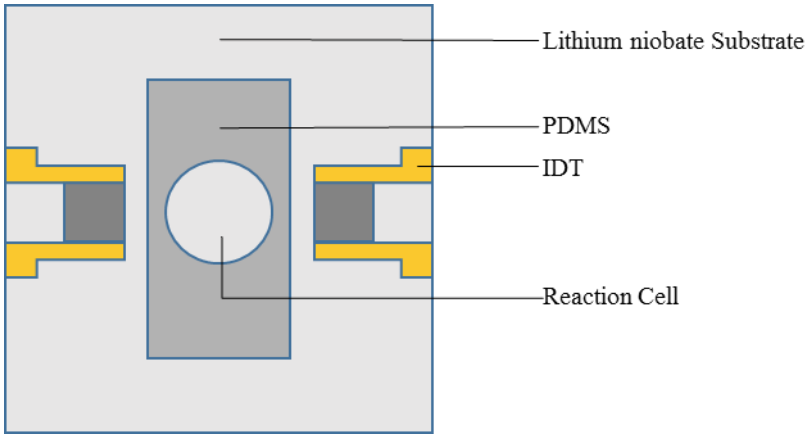


Figure 1-2. Schematic diagram of SAW chip on lithium niobate substrate.

One main issue in current IF is the “noise” generated by the background which causes a false signal or can overwhelm the desired signal by target analytes. These noises are produced by other fluorophores that have overlap spectral with the one being detected or by biofouling which is caused by particles like dust or aggregations reflecting lights interfering with the signal. For these reasons, when detecting the signal of a biomarker like CEA could be either lower or higher when background noises exist. Therefore, it is very likely to result in a misjudgment or false data in many cases especially when marker levels are at low concentrations. Noises have been a threat to spread IF to a wider use. There are some ways to reduce the impact of noises. However, more workload and time will also need to be done while the effect is not very good. One method applied in spectrofluorometer is to test twice before and after adding fluorophores and then do subtracting

to get the result. This way not only requires more work and time but also assumes that all added fluorophores are bound to the target which is impossible. Also in some other modified methods, the operator has to determine how many fluorophores are bound to the antigen or antibody which also increases the work.

It is clear that the best way to avoid the noises from the background is that they do not exist at first. Fortunately, SAW devices seem to give a good option to realize the removal from the background. Cular et al. demonstrated the effective removal on a lithium niobate substrate with a SAW device. The work shows a good result towards removal of biofouling and nonspecifically bound proteins.[38]

On the other side, SAW devices are also able to reduce the time required for specific binding between antigen and antibody by speeding up the mixing process. This will appear more meaningful especially when some experiments may take up to days for incubation. The basic principle is that increasing the possibility that antibody finds a binding site by acoustic wave will make specific attachment achieved in a much shorter period. Paxton et al. first demonstrated the mixing effect of the acoustic wave by ion exchange which accelerated by a 5.8-times factor[39] and Samuel showed the feasibility of applying mixing effect on specific attachment between antigen and antibody.[31] These work indicate that SAW devices have much potential both in non-specific binding removal and accelerating mixing.

# CHAPTER 2: METAL ENHANCEMENT FLUORESCENCE EXPERIMENT

This chapter talks about the detailed records of experimental procedures and operations about MEF experiments in this work. The first step was to determine the material and the geometry of noble metallic nanoparticles that would be used for MEF. As mentioned before, silver nanocubes (Ag NCs) was finally decided to be deployed. Silver nanoparticles have shown remarkable properties in many fields including sensing, electronics, catalysis, etc.[7, 40, 41] Among all types of particles, nanocubes give a unique performance as a result of their sharp edges/corners and more uniformed size distribution especially in LSPR.[42, 43] A lot of effort has been made to synthesize Ag NCs at desired various sizes for different purposes. Zhang et al. utilized a novel method with the use of polyol as the solvent and silver trifluoroacetate ($CF_3COOAg$) as the precursor to synthesize Ag NCs. This method produces nanocubes having uniform shapes, narrow size distribution, and exhibits good repeatability.[37]

## 2.1. Synthesis of Silver Nanocubes

The silver nanocubes were synthesized according to the protocol developed by Zhang et al.[37] Before synthesizing silver nanocubes, all the glassware and containers used in the experiment was dealt with acid wash comprised of 10% in mass of hydrochloric acid (HCl) in water overnight (by adding deionized water a factor of 2.7 to 37% HCl in weight). First, 5 mL of ethylene glycol (Sigma-Aldrich) was added into a 50-mL round bottom flask and heated on the plate in an oil bath with magnetic stirring where the temperature was preset to 150 $^{o}$C. After the temperature reached the set point, 0.06 mL of 3 mM NaSH (Sigma-Aldrich) in ethylene glycol

was quickly injected into the flask. Waiting for 2 minutes, 0.5 mL of 3 mM HCl (Sigma-Aldrich) (by adding 2.5 μL 37% in weight HCl into 10.30 mL of ethylene glycol) was added to the heated reaction solution, followed by the injection of 1.25 mL of 20 mg/mL poly(vinyl pyrrolidone) (PVP) (Alfa Aesar) in ethylene glycol without delay. Then another 2 minutes later, 0.4 mL of 282 mM $CF_3COOAg$ (Sigma-Aldrich) in ethylene glycol was added to the solution. The flask was capped with the plug during the entire process expect during the addition of reagents. The transparent solution turned quickly to whitish gray and then slightly yellow within 1 minute after the addition of the $CF_3COOAg$. This indicated that the Ag seed started to form and grow. The total reaction lasted for 30 minutes to approach the desired size of nanocubes during which the color shifted from red to reddish gray and brown finally as the edge sizes increased gradually.

Afterward, the solution was quenched in the ice water bath for 10 minutes to prevent Ag seed from growing to bigger sizes. The Ag nanocubes were divided equally into several centrifuge tubes washed with acetone once to remove the residual $CF_3COOAg$ and ethylene glycol followed by rinsing with deionized water (DI water) three times to remove excess PVP all via centrifugation. The Ag nanocubes were then dissolved in DI water for storage and future use. The LSPR spectrum was obtained by UV-Vis spectroscopy (PerkinElmer Lambda 35) and characteristic peaks were recorded for analyzing the size distribution. SEM (Hitachi, S800 SEM) images were gained to ascertain the shape and size of Ag nanocubes.

The UV-Vis result was given below in Figure 2-1. The extinction spectrum was measured by the machine and the characteristic peaks were shown clearly at 429 nm wavelength according to the data. It indicated that the size of the Ag nanocubes located around 50 nm. In Figure 2-2, SEM image of the Ag nanocubes was given here. Many SEM images were taken after the synthesis

of nanocubes and this image was chosen as the representative of the samples to show the size and distribution.

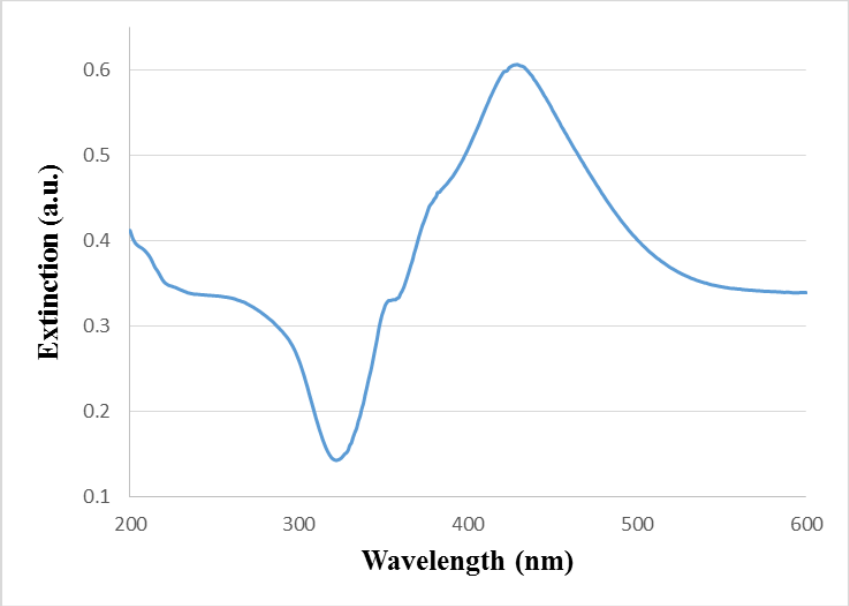


Figure 2-1. Extinction of silver nanocubes in DI water.

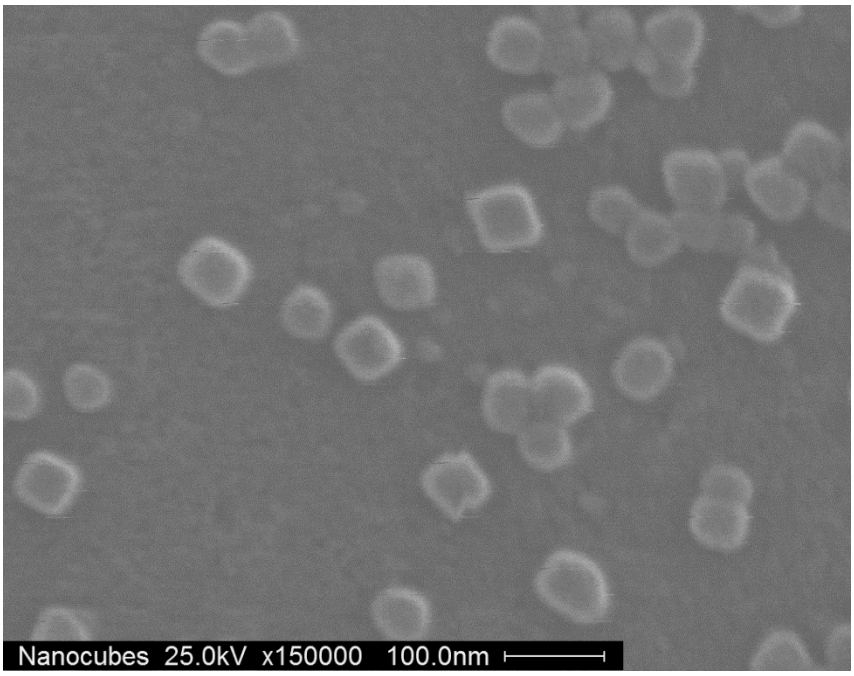


Figure 2-2. SEM image of the Ag nanocubes synthesized. The image was taken at 25 KeV accelerated voltage and enlarged up to 150,000 times.

As shown in Figure 2-2, most particles were cubes with some spheres, irregular shapes present. Although the extinction spectrum did not match up the excitation spectrum of Alexa-488 exactly at the characteristic peak 488 nm, there was a huge overlap between these two which enabled the MEF to be utilized in this experiment.

## 2.2. MEF with Silver Nanocubes

A number of experiments were done to show MEF in this work. The materials involved throughout all the experiments were CEA capture antibody (Fitzgerald, 10-C10D), CEA detection antibody labeled with Alexa-488 (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). The Figure 2-3 shown below gives the schematic diagram of MEF experiment. The silver nanocubes synthesized before were stored in DI water and applied throughout the experiments both in MEF and SAW parts.

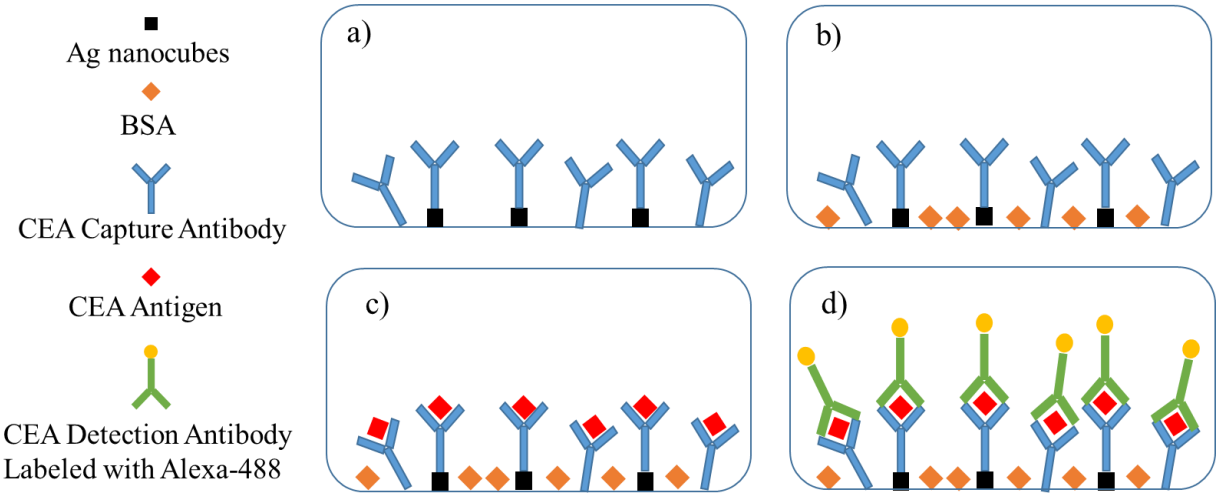


Figure 2-3. Schematic diagram of MEF experiment. a) Ag nanocubes were immobilized on the surface of the glass slides via APTES and CEA capture antibody was incubated above them; b) BSA was added to block the excess binding sites; c) CEA antigen was then added and bound to the capture antibody; d) CEA detection antibody labeled with Alexa-488 was added and bound to the CEA antigen.

The Ag nanocubes were first immobilized on the surface of the glass slides via 3-aminopropyltriethoxysilane (3-APTES) due to the charge effect between the positive-charged amino from APTES and the negative charged Ag nanocubes. The interaction between Ag nanocubes and APTES was shown in Figure 2-4. After the immobilization of metal particles, CEA capture antibody was added and bound either on the top of silver nanocubes or on the APTES followed by PBS rinse to remove excess unbound antibody. PBS rinsing followed by every incubation procedure if not mentioned intentionally. BSA then was added to block extra binding sites due to adsorption effect in the case that the following CEA antigen or detection antibody would be bound directly on the surface. Afterward, CEA antigen and detection antibody labeled with dye Alexa-488 were also added and incubated respectively in the next two steps. After rinsing with PBS, the glass slides were taken pictures with a Leica DMI4000 B fluorescence microscope.

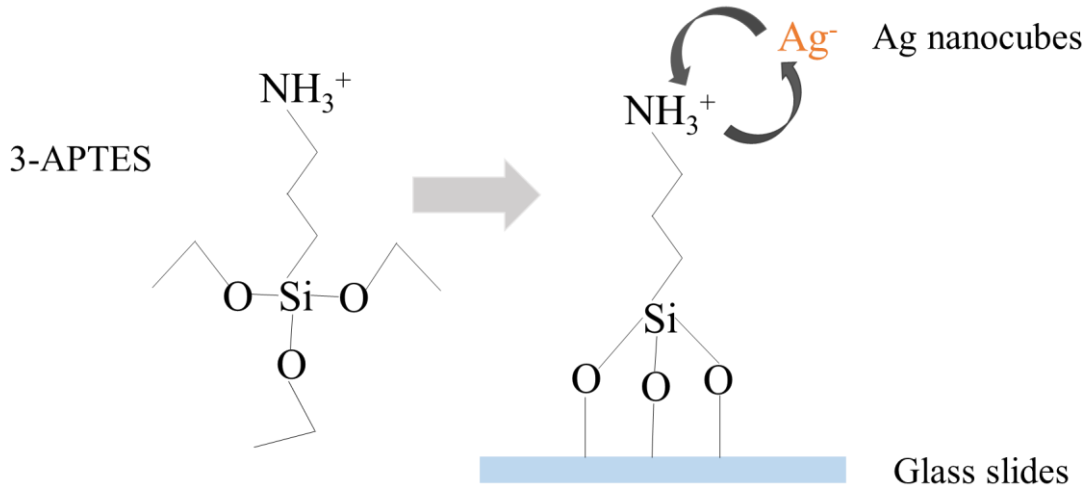


Figure 2-4. Diagram of interaction between APTES and Ag nanocubes.

To demonstrate that MEF with silver nanocubes was effective and determine the enhancement, a control group was set up where Ag nanocubes were not coated on the top of the surface. Two different sets of glass slides were prepared by cutting slides (Fisherbrand microscope

slides precleaned, 25 × 75 mm) into around 1″ × 1″ of squares. Each set contained 5 pieces of slides. Both two sets of slides were silanized with 3-APTES using the protocol from Chowdhury et al. and Aslan et al.[11, 44] while some modifications were made in the experiment. The slides were first immersed in piranha solution (consisting of 3 parts of concentrated sulfuric acid and 1 part of 30% $H_2O_2$ in volume) for 20 minutes to remove any organic or inorganic contaminants on the surface as well as generate a positive charge. The slides were rinsed with DI water thoroughly and blown to dry under nitrogen ($N_2$) flow.

To prevent the liquid leaking from the surface and make the reaction area more explicit, a PDMS reaction cell was applied on the surface of the slides by charging in plasma cleaner. The cells were fabricated via Sylgard 184 Silicone Elastomer Kit (Dow Corning). First, 10 parts of base agent with 1 part of curing agent (in weight) were mixed under stirring followed by putting the mixture in a vacuum container with the pump turned on to remove the air bubbles for 1 hour. Then poured the liquid into a container with an aluminum foil as an underlay and dried the mixture at 100 ºC for 30 minutes or kept at room temperature overnight. Afterward, the dried polymer with a smooth upper surface was cut into small pieces around 8 × 8 mm and 5 mm in thickness. Finally, pierced the pieces by a punch (Miltex, Biopsy punch) with 5-mm diameter. The PDMS pieces were then cleaned by isopropyl (Sigma-Aldrich), methanol (Sigma-Aldrich), and DI water followed by drying off under $N_2$ flow.

The cleaned slides and PDMS pieces were treated with a plasma cleaner (Harrick Plasma, PDC-32G) for 1 minute and then put PDMS on the surface of the slides to form binding followed by heating in the oven at 120 ºC for 10 minutes to complete. 30 μL of one percent APTES in pure ethanol was added in the hole for 30 minutes. The slides were washed with ethanol three times,

dried with $N_2$ flow and heated in the oven set at 120 $^oC$ for 15 minutes to accomplish the silanization procedure.

One set of the slides then were coated with the 30 μL of silver nanocubes solutions in DI water overnight then washed three times with DI water, meanwhile, the other set of slides had nothing added to them as a control group.

Before starting bio-test, five different concentrations of CEA antigen were prepared by diluting from concentration at 1 mg/mL to obtain 1 ng/mL, 10 ng/mL, 100 ng/mL, 1 μg/mL, and 10 μg/mL CEA antigen respectively. PBS was used as a solvent in diluting all antigen/antibody and BSA in the experiment.

First, 30 μL of 10 μg/mL CEA capture antibody was added to the surface and incubated for 1 hour at room temperature which followed by a PBS rinse three times thoroughly. 1% of BSA was used to block the extra binding sites of APTES and on the surface. 30 μL of BSA was added and also incubated for 1 hour. After rinsing, 20 μL of different concentrations of CEA antigen from 1 ng/mL to 10 μg/mL were added to incubate for 45 minutes. Following this, 10 μL of approximate 10 μg/mL (the concentration varied from original due to labeling work) of CEA detection antibody labeled with Alexa-488 was added and incubated for another 45 minutes. Finally, the slides were taken fluorescence pictures under Leica DMI4000 B microscope. The concentration of CEA capture antibody and CEA detection antibody labeled with dye were kept constantly in the experiment. Three different sets of experiments were conducted for each different concentration of CEA antigen.

### 2.3. Discussion on MEF Results

The fluorescence pictures were obtained by the microscope and the pixel intensity was determined and color coded with ImageJ. The images can be seen in Figure 2-5 below. Five

concentrations were detected to measure MEF with and without silver nanocubes and the results showed a relatively stable enhancement when metal particles were applied. Specific values of each intensity can be seen in Table 2-1.

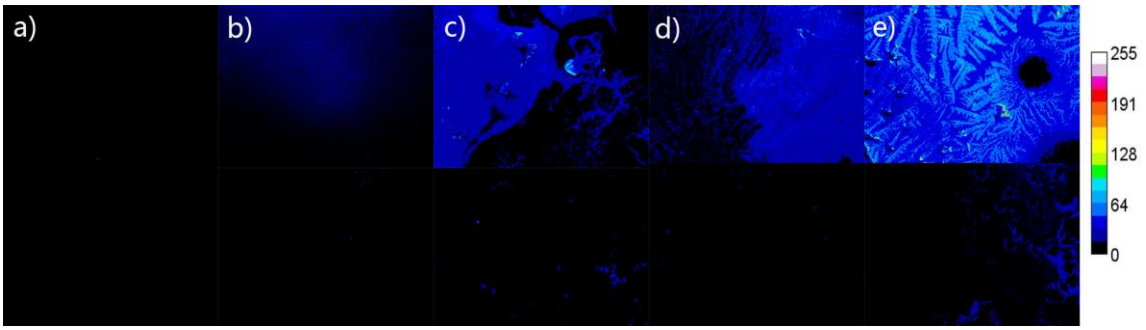


Figure 2-5. Results of MEF experiment. The top row gives the result of the group with silver nanocubes approximate 50 nm in size. The bottom row gives the result of the control group which had no metal particles. The concentration of CEA antigen used was a) 1 ng/mL, b) 10 ng/mL, c) 100 ng/mL, d) 1 μg/mL, and e) 10 μg/mL.

Table 2-1. Intensity values with/without silver nanocubes and the enhancement.

| Concentrations of CEA antigen | Intensity with silver | Intensity without silver | Enhancement |
|---|---|---|---|
| 1 ng/mL | 6.147 | 1.132 | 5.430 |
| 10 ng/mL | 14.573 | 2.787 | 5.229 |
| 100 ng/mL | 18.875 | 4.820 | 3.916 |
| 1 μg/mL | 22.582 | 5.254 | 4.298 |
| 10 μg/mL | 42.714 | 10.565 | 4.043 |

As shown clearly in Table 2-1, the factors of enhancement for different concentration varies in a small range from 3.916 to 5.430. It was testified that silver nanocubes performed much better than the control group in fluorescence experiment in a relatively stable pattern. The experiment also proved that the traditional IF has a high limit of detection when it comes down to ng/mL level as shown in Table 2-1.For example, the signal of 1 ng/mL CEA antigen without silver was measured to be 1.132 which was at very low level and hard to distinguish from the noise. One important reason to test MEF in this experiment was to lower the limit of detection to at least 2.5

ng/mL since this number is regard as the critical value when diagnosing the risk having a colon cancer. The traditional IF is helpless at this low-level concentration as mentioned. However, with the existence of silver nanocubes, it is possible to down the limit of detection from 1 μg/mL to 1 ng/mL. As shown in the table, the intensity values of 1 ng/mL and 10 ng/mL in control group were 1.132 and 2.787 respectively. This small difference was very hard to determine and this would lead to an entirely different result between a positive and a negative diagnosis, not to mention that noises could be as high as a signal in many cases. Whereas this situation improved much better with the help of MEF in the experiment. The signal level of 1 ng/mL was 6.147 which was much higher than the background and also easy to distinguish from the intensity of 14.573 at 10 ng/mL. It would significantly decrease the possibilities of misdiagnosis to help patients more effectively and correctly.

Although MEF shows a good improvement compared to control group, there are still some problems existing but not in urgent at this point since MEF still works. One of these issues here is the enhancement is not quite large. It would be much useful and convinced if the enhancement could expand to around 10 times or larger. The reasons for this are numerous. One most likely but also complex one is the nanocubes themselves. Many factors of metal nanoparticles affect the performance of MEF drastically, including size, shape, aggregation, uniformity, distribution on the surface, etc. Every single factor is able to vary the interaction between the fluorophores and needs to be optimized which means there is still huge room to increase the enhancement from metal particles. Another potential aspect is the purity of nanocubes. Not all the particles are in the shape of a cube, rods and spheres being the other major prevalent shapes. What should be emphasized is that not only is metal nanoparticles able to enhance the fluorescence, but also depress the fluorescence by decreasing the quantum yield as the $\Gamma_m$ to be negative coefficient as shown in

equation (1). As a result, more work is required in the future and still MEF in this experiment has been proven to be effective and valuable compared to traditional IF.

# CHAPTER 3: NON-SPECIFIC BINDING REMOVAL AND MIXING

This chapter discusses the experimental procedure and results of non-specific binding removal and increased mixing using a SAW device. As mentioned in Chapter 1, background signals also known as noise can be an annoying issue in MEF since they can result in fake signals that misguide the judgment of a case seriously. Therefore, a complete rinse process is essential after every step of incubation to wash excess antigen/antibody and non-specific binding to minimize the effect from the background. However, due to the small volume of the solution and careful mild operation involved, it is very difficult to remove all the background signals during most rinsing steps. In this chapter, a SAW device was applied to show the ability to be a supplementary even a primary method to reduce the background signals by removing non-specific binding.

Another aspect that has the potential to improve with MEF is the long time it takes for attachment. This can vary from hours to days for different antigen and antibody and maybe longer time for those complicated sandwich structures. It will save a lot of time and resources if it takes one hour or less and more work can be done compared to a three-hour workload. SAW device was applied to increase the mixing effect by expanding the possibilities of attachment between antigen and antibody. The acoustic wave would speed up the movement of proteins which increased the chance of proteins contacting with the binding sites. Therefore, the attachment could be done in a much shorter period. Moreover, Toegl et al. demonstrated that SAW could also overcome the diffusion limit to large the signal-to-noise ratio by a factor of 6 in the hybridization experiment of

DNA besides reducing the time and improving homogeneity.[45] This was confirmed in this work, however, the factor was not as large as in the literature.

### 3.1. Non-Specific Binding Removal by SAW

The non-specific binding in this work mainly existed in the process when incubating CEA antigen and CEA detection antibody. All CEA antigen should have bound to capture antibody specifically, but still, some of them could bind to BSA or themselves. Similarly, some detection antibody could also bind to BSA and capture antibody instead of binding to CEA antigen specifically. Detection antibody was used to demonstrate the non-specific binding removal since it was labeled with a dye which would be measured with the fluorescence microscope to show the results. Lithium niobate substrate was chosen other than the glass slides used in the MEF experiment part because it performs good piezoelectric effect that allows the transfer between the acoustic signal and electric signal along the surface as well as it is transparent for a large range of wavelengths including the UV light used in the experiment.

#### 3.1.1. Experimental Procedure of Removal

The experiment was designed to show the removal by adding CEA detection antibody on the top of incubated BSA followed by a SAW device.

A chip shown in Figure 1-2 was set up. The chip was first cleaned by acetone, methanol and DI water rinse then dried with nitrogen flow. The PDMS cell was again bound on the surface the same as that processed in section 2-2. Silanization and incubating silver nanocubes were again done for the chip. At this point, 30 μL of 1% BSA was added to the surface and incubated for 1 hour. After rinsing with PBS, 20 μL of around 10 μg/mL of detection antibody labeled with dye was added and incubated for another 1 hour. When PBS washing was done, the slide was first taken pictures under Leica DMI4000 B microscope. Next, the chip was set up with the signal

generator SMA100A to apply SAW for removal. 10 μL of PBS was added before turning on the power. The initial power was set at 0 dBm (equal to 1 mW) and applied for 10 minutes. Pictures were again taken after each step of removal. Another 10 minutes of applying SAW was done at still 0 dBm after that. Finally, power was set to 3 dBm (around 2 mW) and applied for a third 10 minutes with the chip. All images were collected and analyzed by ImageJ. Figure 3-1 below gives the schematic diagram of removal. The experiment was repeated two times because of the lack of lithium niobate chips as well as their vulnerability.

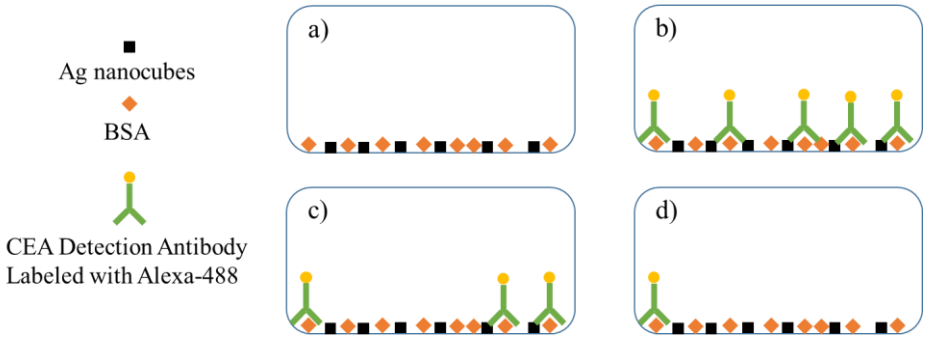


Figure 3-1. Schematic diagram of removal with SAW. a) Ag nanocubes were immobilized on the surface of the chip via APTES and BSA was used to block the surface; b) CEA detection antibody labeled with Alexa-488 was added and incubated; c) Rinsed with PBS three times; d) Removal was done with SAW.

### 3.1.2. Discussion on Removal Results

The fluorescence pictures were taken with a microscope and measured using ImageJ. The results can be seen in Figure 3-2 below. Four fluorescence pictures were taken at different stages. They were taken after PBS rinse, after 10 minutes removing with SAW at 0 dBm, another 10 minutes removing at 0 dBm and a third 10 minutes dealing at 3 dBm respectively. Specific values of each intensity can be seen in Table 3-1. As shown in the figure, the ideal signal after rinse with PBS should have been close to “zero” due to it was non-specific binding between CEA detection antibody and BSA. However, the fact is that the signal reached a relatively high level in the

experiment approximately 25 which could cause a false data seriously. By applying SAW to remove non-specific binding, the signal could get down to 1/6 of its original level which decreased significantly.

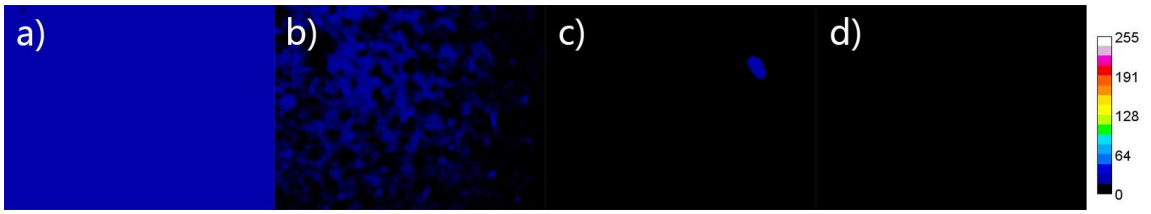


Figure 3-2. Fluorescence results for removal. a) After PBS rinse but before removal; b) After 10 minutes at 0 dBm; c) After another 10 minutes at 0 dBm; d) After a third 10 minutes at 3 dBm.

Table 3-1. Fluorescence intensity results of removal.

| Stage | Intensity |
|---|---|
| After rinsing before removal | 24.689 |
| After 10 minutes removal at 0 dBm | 14.538 |
| After second 10 minutes removal at 0 dBm | 6.530 |
| After third 10 minutes removal at 3 dBm | 4.078 |

The fluorescence pictures in Figure 3-1 show clearly that removal was done by SAW in a fast and good way. As listed in Table 3-1, the signal of non-specific binding went down to its 1/6 of original level in 30 minutes when applied with SAW. The results indicate that SAW has a great potential to minimize the effect of background signal since it is fast and powerful. Even within 10 minutes dealing with SAW device, the background signal could reduce by 42%. Also, the first 20 minutes dealt was set at 0 dBm which was the only half power to 3 dBm. In general, SAW reduced background signal by a factor of 6 in this work and it forecasts that SAW has a strong ability to remove loose bound structures quickly and effectively.

### 3.2. Increased Mixing from SAW

Other than the removal of non-specific binding, SAW was also reported in the use of accelerating mixing. Traditional IF can be a time-consuming technique which takes from hours up to days. This issue may get worse when having complex sandwich structures. IF will be much more useful if work can be done in one hour or shorter time as lots of cases require fast diagnostics.

The increased mixing can be applied during many periods in this work as an improvement, including adding CEA antigen, detection antibody, etc. $LiNbO_3$ chip was still selected in this part due to high electromechanical coupling coefficient. The mixing can be regarded as a reverse process of removal loose bound structures. Acoustic waves accelerate the motion of protein in a pattern and allow them to have a greater chance to find the binding sites. That is, removal of non-specific binding and accelerating specific binding happen at the same time with SAW. They are two different results led by the same mechanism.

#### 3.2.1. Experimental Procedure of Mixing

The experiment was designed to show the mixing by comparing several chips treated differently. Two chips, loaded with detection antibodies, were treated with SAW for different times while the other two was just left for incubation without the use of SAW.

The chips shown in Figure 1-2 were used. The chips were first cleaned and dried according to the same procedure used before. The PDMS cell was again bound. Silanization and silver nanocubes were again done for the chips. The first steps were similar to the procedure in section 2-2. To start with, 30 μL of 100 μg/mL of CEA capture antibody was added to the surface and incubated for 1 hour. Then BSA blocked the excess binding sites. At this point, 20 μL of 10 μg/mL of CEA antigen was added to all of the chips and incubated for another 1 hour. Then 20 μL of around 10 μg/mL of detection antibody with dye was added but incubated in different conditions.

Two chips were incubated without SAW for 10 and 60 minutes respectively while the other two were incubated with SAW at 0 dBm for 5 and 10 minutes respectively. Slides were taken pictures with a microscope after rinsing with PBS. The experiment was repeated two times because of the lack of lithium niobate chips as well their vulnerability.

### 3.2.2. Discussion on Increased Mixing Results

The fluorescence pictures were taken and measured via ImageJ. Figure 3-3 and 3-4 below give the fluorescence results of increased mixing with SAW device.

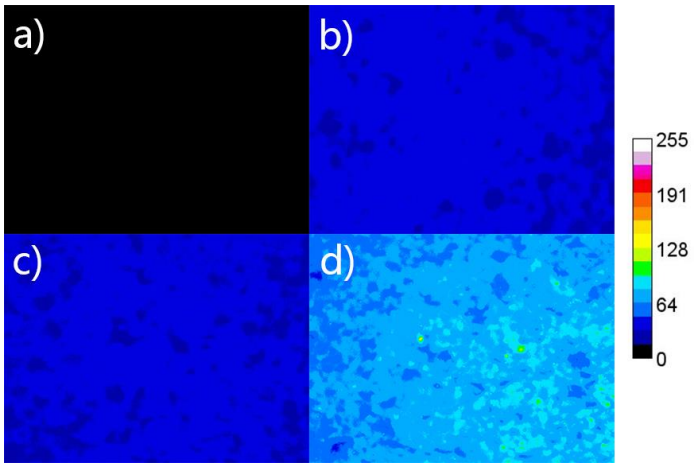


Figure 3-3. Results of increased mixing experiment. a) Without SAW for 10 minutes; b) Without SAW for 60 minutes; c) With SAW for 5 minutes at 0 dBm; d) With SAW 10 minutes at 0 dBm.

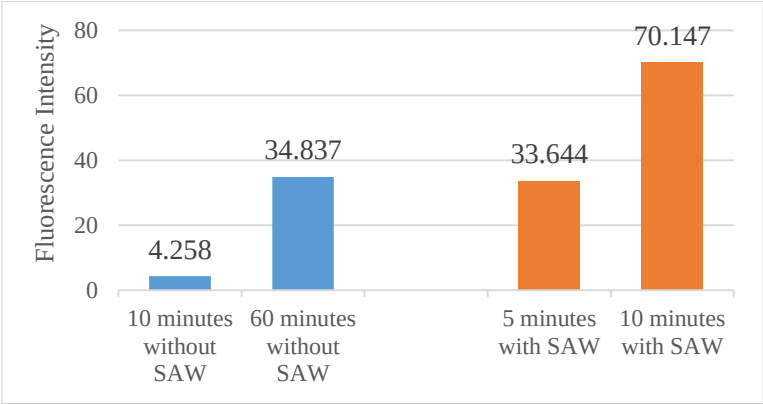


Figure 3-4. Comparing the use of SAW to not using SAW for mixing. The intensity of 5 minutes with SAW was much better than the result of 10 minutes without SAW and close to the level of 60 minutes without SAW while the intensity of 10 minutes with SAW increased the signal over the limitation of 60 minutes without SAW very much.

As shown above, the results of 5 minutes with SAW was much stronger than the signal of 10 minutes without SAW and close to the signal of 60 minutes without SAW. Moreover, the signal of 10 minutes with SAW could break the limit of self-mixing by a factor of 2 as incubation often stayed stable after one hour. Toegl et al. had a larger signal-to-noise ratio by a factor of 6 in their work using SAW to overcome the diffusion limit.[45] However, only 2 times of enhancement was obtained here. Many reasons could result in it such as applying time of SAW, signal generator power, different concentrations of substrate, etc. Toegl received 6 times of enhancement in totally 16 hours which was much longer than 10 minutes set in this work. This also implies the large room to improve in this work that can be done in the future to verify the optimized condition to have a better mixing effect. Unfortunately, the ability of SAW can overcome the diffusion limit and increase the signal significantly has not drawn much attention and more research needs to be done to clarify the mechanism of increased mixing.

## CHAPTER 4: SUMMARY AND FUTURE PROSPECT

In this work, both MEF and SAW were proved to work and results were given in Chapter 2 and 3.

MEF is able to increase the fluorescence signal by a factor around 4.6 in average and it is possible to distinguish the concentration of CEA antigen at ng/mL level compared to the control group. It will enable immunofluorescence to play an important part in diagnosing and forecasting the risk of having diseases, in this case, colon cancer can be predicted or monitored by detecting CEA antigen concentration level above 2.5 ng/mL as this number is thought to be the critical value. MEF is shown to overcome the shortage of high limit of detection by increasing the quantum yield of fluorophores. Silver nanocubes were selected to perform MEF in the work due to their unique properties and sharp corner/edges. The size control of cubes is of huge importance because it not only affects the distribution on the surface of slides but also changes the spectrum overlap with the fluorophores absorption spectrum which will directly influence the quantum yield drastically. A silane method was used to bind the silver nanocubes and antibody on the surface. The effect of silanization of the surface is another crucial aspect of MEF since nanocubes and antibody would be bound on the top of that. Thus, thoroughly clean and positive charge need to be done very carefully. Last but not least one in MEF experiment is the rinse step. It has to be completely and consistently after each incubation though it is somewhat impossible for manual operations. Otherwise, it is able to vary the results in a bad way.

SAW has been demonstrated that it is able to remove the non-specific binding and increase the mixing effect significantly. It reduces the background signal of non-specific binding by a factor

of 6 and increases the mixing effect by overcoming the diffusion limit. There are lots of aspects that can be adjusted in SAW, for example, signal generator power, processing period, frequency, etc. With the help of SAW, a much shorter time is required for incubation and noises can be removed to a large extent.

However, still, some problems need to be considered in the work. One issue is that the enhancement is not quite large as it being at around 4.6. It would be much better if the enhancement reached to 10 fold times or higher that lower concentration than 1 ng/mL could be detected and the accuracy could be increased as well. Many factors need to be optimized to realize higher enhancement, such as better fluorophores, a more uniform size distribution of nanocubes, high-quality silanization method and so on.

As a result, the next step in this work will focus on improving the enhancement of MEF and real biotest. More work will be involved as there are still many problems existing and the combination of MEF and SAW requires more attention and input to have a more bright future.

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