Impedimetric immunosensor based on humidity sensing properties of BST composite film

Mahdi Rasouli

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**AN IMPEDIMETRIC IMMUNOSENSOR
BASED ON HUMIDITY SENSING PROPERTIES OF
BST COMPOSITE FILM**

**MAHDI RASOULI
SCHOOL OF ELECTRICAL & ELECTRONIC ENGINEERING
2008**

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BASED ON HUMIDITY SENSING PROPERTIES OF
BST COMPOSITE FILM**

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*To my sister,
Zahra*

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Abstract

In this project, a label-free impedimetric immunosensor with a signal amplification mechanism based on humidity sensing properties of Barium Strontium Titanate (BST) composite film has been developed.

The immunosensor was constructed as a capacitor structure. A layer of BST composite film was deposited onto a platinum coated silicon substrate, and then were modified by Poly(TMS-r-NHSMA) [TMS: 3-(trimethoxysilyl)propyl methacrylate; NHSMA: N-hydroxysuccinimide methacrylate] as a functionalized polymer to be able to bind to bio-molecules covalently. To demonstrate the sensing capability of this structure, Bovine Serum Albumin (BSA) protein was immobilized on the surface to detect its conjugate protein, anti-BSA, as a target analyte. The sample was then exposed to Phosphate Buffer Solution (PBS) buffer solution containing anti-BSA protein so that anti-BSA could bind with the immobilized BSA to form the antigen-antibody complex. The electrochemical detection was finally achieved by impedance spectroscopy after applying a drop of DI water between a top gold electrode and the sample as the bottom electrode.

It was observed that the formation of biorecognition complex could change the impedance of the system. Higher concentrations of anti-BSA could result in higher impedance values. These impedance increments (The sensing capability) were attributed to three parallel mechanisms upon the biorecognition reaction. First, the interfacial impedance of the system increases. Formation of the antigen-antibody complex reduces the charges transfer at the solid/liquid interface, and hence, results in an increase in the electron transfer resistance of the system. Moreover, the thickness of the interfacial layer also increases which causes a decrease in capacitance of the double layer and hence an increase in the interfacial impedance of the system. Second,

impedance of the DI water increases. BST is a highly polar material which can dissociate water molecules into H^+ and OH^- ions. Immobilized biomolecules could prevent the water molecules from contacting BST grains and induce less water molecules to dissociate into mobile ions. This will result in a decrease in amount of charge carriers, and hence, an increase in the impedance of the DI water. Third, impedance of the BST composite film also increases when DI water is prevented from penetrating into the BST structure. This is due to the fact that the BST film is a humidity sensor and shows higher impedance for lower water contents.

The proposed immunosensor seems to be a good alternative to the conventional electrochemical immunosensors by utilizing two signal amplification mechanisms without using the harmful redox labels. Moreover, the proposed structure might be useful for detection of different analytes since the applied functionalized polymer is capable of immobilizing different proteins.

1 Introduction

1.1 Background and Motivations

An immunosensor is an analytical device comprising antigen or antibody molecules coupled to a signal transducer to detect the binding of complementary species [1-4]. The aim is to produce a signal which is proportional to concentration of a desired analyte. Immunosensors have been the subject of an increasing interest during the past few years. That is mainly because of their high selectivity and specificity, short response time, relatively low cost, potential for miniaturization, portability, and ease of use [5-6]. The selectivity and specificity of the antibody-antigen reaction has made immunosensors suitable for a wide range of applications including clinical diagnosis [7], food quality control [8], environmental analysis [9, 10], detection of pathogens or toxins [11, 12], and even detection of explosives or drugs [13-14].

Several techniques have been successfully employed for transduction of biological reactions occurring at the electronic supports [15-18]. Impedance spectroscopy represents a powerful technique for characterization of chemically modified electrodes, and hence, transduction of biorecognition reactions [19-22]. Most of the biosensing devices involve formation of a biorecognition complex. Formation of the biorecognition complex alters the capacitance and the resistance at the electrode/electrolyte interface which could be translated to a meaningful electrical signal using impedance spectroscopy [23]. This forms the basis of impedimetric immunosensors. Impedimetric immunosensors are attracting increasing interest, since they are direct and label free and have many advantages with respect to speed, low driving voltage, and the potential for miniaturization and large scale production [24-26]. Although these immunosensors are very simple in operation and have many advantages, but they still suffer from several drawbacks. One of the major problems of

these immunosensors is the relatively small change of the impedance spectra upon the direct binding of the analyte to the antigen/antibody functionalized electrodes, especially when the analyte concentration is low and the surface coverage of the antigen-antibody complex is far from saturation [23]. This could result in low detection limit and low signal to noise ratio. To overcome this problem, the concept of amplified impedimetric immunosensors was introduced. In general, this approach involves the application of enzyme-labels that perform biocatalytic reactions and precipitate an insoluble product on the sensing interface upon the antigen-antibody recognition [23-25]. Although these signal amplification mechanisms improve the sensitivity of the device, however, they involve labeling of biomolecules which is not desired.

In this study, a novel label-free amplification mechanism based on humidity sensing properties of perovskite metal oxides was proposed, and a prototype immunosensor was fabricated to demonstrate the capability of this sensing mechanism. The suggested sensing mechanism also eliminates the need for using harmful redox probes, such as $[\text{Fe}(\text{CN})_6]^{4-/3-}$ [26], for Faradaic impedance measurements.

The prototype immunosensor was constructed using Barium Strontium Titanate (BST) composite film as the main sensing element because of its unique characteristics [28-30], including high polarity [31, 32] and humidity sensing [33-36], and our previous background on this material. BST is a Ferroelectric material which has been widely investigated as a potential material for application in microelectronic devices, such as DRAM [37-40], infrared detectors [41], microwave phase shifters [42, 43], voltage tunable devices [44, 45], humidity sensors [34-36], and gas sensors [46-49]. It is of great interest to explore the possibility of BST to be applied as an immunosensor which could open up new applications for this material.

1.2 Objectives

The general aim of this study is to develop a novel immunosensor based on humidity sensing properties of BST composite film. BSA and anti-BSA are used as test proteins to demonstrate the sensing capability of the immunosensor. It is hoped that with the successful detection of anti-BSA, the device could be applied for detection of other biomolecules. Several sub-objectives are listed as follows, which will enable the successful fulfillment of the project:

- 1- To fabricate uniform and crack-free BST composite films; the BST composite film plays an important role in sensing performance characteristics of the immunosensor. It is directly involved in the sensing mechanism, and also affects other surface modification steps. Thus, it is very important to establish a robust and repeatable fabrication process for synthesis of uniform and crack-free BST composite films to eliminate the cross-sample and sample-to-sample variations.
- 2- To modify the BST surface for covalent immobilization of bio-receptors; in order to make a viable biosensor, the biological component has to be properly attached to the transducer. This process is known as immobilization. Among several possible methods for immobilization of biomolecules, covalent immobilization is an appealing technique. It involves modifying the surface with functionalized sites which are able to catch and bind to biomolecular species covalently.
- 3- To characterize the immunosensor using impedance spectroscopy; impedance spectroscopy is to be utilized as the main characterization and transduction technique in this study. Formation of the biorecognition complex on the electrode surface alters the impedance features of the system which could be translated into a detectable electrical signal using impedance spectroscopy.

4- To set up an equivalent circuit model to analyze the impedance data; the immunosensor incorporates several electrical conduction mechanism, including electrical conduction across the BST composite film, ion transfer in the solvent solution, capacitive electrical double layer, and charge transfer at the electrode/electrolyte interface. To have a better understanding of these conduction mechanisms, an equivalent circuit model must be developed. It helps to analyze the experimental data and explain the sensing mechanism. Moreover, the effects of different modification steps on device performance can be investigated.

5- To optimize the fabrication process to improve the sensitivity and reduce the fabrication cost. To achieve this goal, fabrication process of the BST composite film, polymer modification procedure, and biomolecule immobilization protocol have to be optimized.

6- To establish a simple and robust measurement protocol; the aim is to develop a sample treatment and measurement protocol so that signal variations due to undesired elements become minimized. It includes development of fabrication processes which could eliminate cross-sample and sample-to-sample variations, and measurement protocols which are non-sensitive to environmental conditions.

1.3 Major Contributions of the thesis

1- A modified sol-gel process has been developed for fabrication of BST composite films using commercial nano-size BST powder. Using this procedure, uniform and crack-free BST composite films with thickness of around 3 microns were synthesized. The effects of different parameters involved in the fabrication process (including pH value of the BST sol-gel precursor solution, powder-to-solution mass ratio, and annealing temperature) were investigated. It was shown that the fabrication procedure

was robust with respect to small changes in the fabrication process or environmental conditions.

2- An effective and simple approach to modify and functionalize the BST composite film surface was developed based on the “grafting to” technique. Poly(TMS-r-NHSMA) [TMS: 3-(trimethoxysilyl)propyl methacrylate; NHSMA: N-hydroxysuccinimide methacrylate] was utilized to functionalize the surface for covalent binding to bio-molecules. It was shown that the Poly(TMS-r-NHSMA) can be successfully applied for immobilization of biomolecular species onto the BST composite film surface. Moreover, the effects of different parameters involved in the polymer modification (including concentration of the polymer solution, length of the soaking period, and number of the soaking steps) on water affinity and biomolecule-binding capability of the modified surface were investigated.

3- The sensing capability of the proposed immunosensor was demonstrated for the detection of anti-BSA protein. Impedance spectroscopy was used as the transduction technique. A proportional relationship between the impedance changes and anti-BSA concentration in the range of 10 ng/ml to 1 mg/ml was obtained. This indicates that the immunosensor could be used for the detection and quantification of anti-BSA.

4- An equivalent circuit model was developed to fit the measurement data and explain the sensing mechanism. The effects of different modification steps (including BST composite film deposition, phosphoric acid modification, polymer modification, and immobilization of biomolecules) on impedance of the system were investigated. The model confirmed the proposed sensing mechanism and conduction processes.

5- Different fabrication steps were optimized to reduce the fabrication cost and improve the sensitivity of the immunosensor. This involved development of a robust fabrication process for synthesis of crack-free BST composite films, determining the

polymer concentration which could result in maximum sensitivity, and determining the minimum BSA concentration which could cover and saturate the sample's surface.

6- A sample treatment and measurement protocol was developed to minimize the signal variations due to undesired elements such as environmental conditions. The developed protocol involves a precise control over time and washing procedures.

1.4 Organization of the thesis

The thesis emphasizes on the fabrication, structural and electrical characterization of a capacitive-structured immunosensor. A detailed discussion on fabrication procedures, as well as the experimental results to confirm the successfulness of each of the steps, is provided. The general organization of the report is as follow:

Chapter 1 provides an introduction to the thesis, including background and motivations, objectives, major contributions of the thesis, and organization of the thesis.

In chapter 2, the concepts of immunosensors, their applications and transduction mechanisms; Barium Strontium Titanate (BST), its applications and humidity sensing properties; and Impedance Spectroscopy technique are reviewed.

In chapter 3, detailed procedures for the fabrication of the BST composite film, surface modification of the BST composite film, and immobilization of the bioreceptors are provided. It is followed by an introduction to the experimental setup and procedures for the structural and electrical characterization of the immunosensor.

Chapter 4 summarizes the results which are related to structural characterization of the immunosensor. This chapter consists of two main sections. One is about the characterization of the BST composite film, and the other discusses on the surface modification procedures and effectiveness of each procedure.

Chapter 5 focuses mainly on the impedance spectroscopy results. The characteristics curve of the immunosensor, a detailed discussion on sensing mechanism, and the equivalent circuit analysis results are presented in this chapter. Furthermore, a detailed route to improve the biomolecular sensing performance of the immunosensor is provided. It includes establishment of a sample treatment and measurement protocol, determining the polymer concentration which results in maximum sensitivity, and determining the minimum BSA concentration which could cover and saturate the sample's surface. The observed phenomena and experimental results which lead to the establishment of the proposed sample treatment and measurement protocol are also described.

Chapter 6 concludes this study and provides some possible directions and recommendations for future research in this field.

2 Literature Review

2.1 Immunosensors

2.1.1 Introduction

A biosensor is an analytical device which integrates a biological sensing element with a transducer to produce a signal which is proportional to concentration of a specific analyte or a group of analytes [1-4]. If antibodies or antibody fragments are applied as biological element the device is termed immunosensor [1-4]. A schematic view of an immunosensor is illustrated in Figure 2.1.

Figure 2.1 General configuration of an immunosensor

The selectivity and specificity of the antibody-antigen reaction has made immunosensors suitable for a wide range of applications including clinical diagnosis [7, 3], food quality control [8], environmental analysis [9, 10], detection of pathogens or toxins [11-12], and even detection of explosives or drugs [13, 14]. Immunosensors have many advantages over the traditional immunoassay techniques with respect to short response time, relatively low cost, potential for miniaturization, portability, and ease of use [5-6].

Immunosensors can be classified into two main categories based on their sensing mechanism: direct and indirect [1, 15]. In direct immunosensors, the sensing signal is

produced directly by the antigen–antibody binding reaction and is detected in real time. In these systems, the biological reaction may result in a change in potential difference, current, resistance, mass, heat, or optical properties. Indirect immunosensors are based on labeling of one of the biomolecules involved which makes them more expensive and time consuming [2]. Moreover, it decreases selectivity by introducing secondary species and antibodies. However, the indirect immunosensors are more sensitive because of the enzymatic amplification effect. Although direct label-free immunosensors are less sensitive compared to the indirect immunosensors, they are more attractive because of their simplicity and fast speed [2].

2.1.2 Transduction mechanisms

The formation of the biorecognition complex upon the antigen-antibody reaction induces changes in physical properties (such as impedance, refractive index, reflectivity, mass, or surface intensity) of the surrounding environment. Various physical features can be employed for the electronic transduction of these biorecognition reactions. The most commonly studied techniques are electrochemical, optical, microgravimetric, and thermal [15-17].

2.1.2.1 Electrochemical immunosensors

Electrochemical immunosensors have been the subject of an increasing interest in the past few years. They offer benefits in terms of technical simplicity and fast response due to the direct transduction of the biomolecular recognition event into electronic signals [16-17]. However, they have lower sensitivity comparing to optical biosensors [17]. Electrochemical immunosensors are divided into three main categories: potentiometric, amperometric, and conductometric/impedimetric.

Potentiometric immunosensors are based on the potential difference between a biochemical sensitive electrode and a reference electrode at near-zero current [3]. Potentiometric immunosensors are advantageous with respect to simplicity of operation and possibility of applying in solid-state FET sensors [16]. However, they suffer from lack of sensitivity and low signal-to-noise ratio [3].

Amperometric transduction is the most studied technique among electrochemical methods [15]. Amperometric immunosensors measure the current produced during the oxidation or reduction of a product or reactant. They have a better sensitivity comparing to potentiometric immunosensors. The main disadvantage of amperometric immunosensors is their indirect sensing mechanism. [16]

Conductometric/impedimetric immunosensors are based on measuring changes in the impedance of a biosensing system upon the biorecognition reactions. These sensors are attracting increasing interest because they are direct and label free and have many advantages with respect to speed, low driving voltage, simple instrumentation, and the potential for miniaturization and large scale production [24-26]. However, they suffer from low sensitivity. To overcome this problem, the concept of amplified impedimetric immunosensors was introduced to enhance the detecting signal.

2.1.2.1 Optical immunosensors

Optical immunosensors are probably today's largest group of transducers because of their high selectivity and specificity [3, 16]. Moreover, they are advantageous in terms of the nondestructive operation mode, the rapid signal generation and reading, and applying visible radiation compared to other transduction techniques [16]. Optical transduction is based on measurement of changes in adsorption, fluorescence, luminescence, scatter, or refractive index of the biosensing surface. This may be

achieved with or without the use of a label (commonly enzyme or fluorophore). Direct optical sensors make up a substantial proportion of the immunosensors currently available. These include attenuated total internal reflection, ellipsometry, and Surface plasmon resonance (SPR). SPR is the most popular optical technique because of its high sensitivity and low detection limit [3].

2.1.2.3 Microgravimetric immunosensors

An alternative property used in immunosensor design is mass change, which is measured with the use of piezoelectric crystals and acoustic wave techniques [15-17]. These devices are based on generation of an electric signal from a vibrating crystal. The frequency of vibration is affected by the mass of material adsorbed on its surface, which could be applied for transduction of biorecognition reactions. The main drawback of microgravimetric immunosensors is the lack of selectivity due to non-specific binding problems. Surface acoustic wave resonator (SAW), Quartz Crystal Microbalance (QCM), and Shear-Horizontal Acoustic-Plate-Mode resonator (SH-APM) are some popular sub-categories of microgravimetric immunosensors.

2.1.2.4 Thermal immunosensors

All chemical and biochemical processes involve the production or absorption of heat. This produced or absorbed heat can be measured by sensitive thermistors, and hence, be related to the amount of substances to be analyzed. This forms the basis of thermal immunosensors. These immunosensors have not gained considerable attention because of some major drawbacks. The main disadvantage of these immunosensors is the lack of selectivity [3].

2.1.3 Immobilization techniques

A critical step in developing an immunosensor is the proper attachment of the protein to the transducer. This process is called immobilization, and can significantly affect the sensing performance of the immunosensor. The activity of biomolecules and life time of the device may be greatly enhanced with proper immobilization techniques [50]. Immobilization can be achieved using different approaches. Some of the popular immobilization techniques are as follow:

- (i) *Adsorption*: adsorption is an experimentally simple immobilization method in which analytes from solution can be immobilized non-covalently on the solid surface through physical or chemical adsorption. Hydrogen bonding, Van der Waals forces, and hydrophobic interactions are different mechanisms which might be involved in this type of immobilization. Adsorption is considered as a mild coupling method that can preserve protein activity. However, the bonding is weak, and it is relatively simple to reverse the immobilization process. This results in instability and sensitivity degradation over time [51].
- (ii) *Entrapment*: in this method, the protein is introduced into and trapped in matrix of a gel, paste or polymer. Analytes must diffuse through the trapping material to interact with entrapped proteins. This method is very mild and does not affect the protein activity. However, it has several disadvantages. It has a fragile structure and also limited applications as the trapping material limits the size of substrates or proteins [52].
- (iii) *Cross-Linking*: this technique is based on inter-molecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix such as a gel. It can be useful for stabilizing adsorbed biomaterials. However, the mechanical strength of the system is poor and there is some

diffusion limitation. Moreover, this method may result in loss of activity of biomaterials as it involves severe conditions [50, 53].

(iv) *Covalent Binding*: in this method, covalent chemical bonds are formed between the protein molecules and the transducer. This is the most desirable immobilization technique because of its high stability and high surface loading. It is considered as the most stable and highest irreversible immobilization. However, it may damage the protein activity as it involves harsh chemical treatment [53, 54].

Table 2.1 lists the common immobilization techniques and their advantages and disadvantages. It should be emphasized that the best method of immobilization depends on a variety of factors, including the application of the immunosensor, the transducer type, the nature of the biological components, and the surface chemistry of the device [50].

Method	Advantages	Disadvantages
Physical adsorption	Simple, mild conditions, less disruptive to enzyme protein, low cost	Enzyme linkages are highly dependent on pH, solvent and temperature; insensitive
Entrapment	Universal for any enzyme or protein, mild procedure	Large diffusional barriers, loss of enzyme activity by leakage, possible denaturation of the enzyme molecules as a result of free radicals
Cross-linking	Simple procedure, strong chemical binding of the biomolecules, widely used in stabilizing physically adsorbed enzymes or proteins that are covalently bound onto a support	Difficult to control the reaction, requires a large amount of enzyme, the protein layer has a gelation nature (lack of rigidity), relatively low enzyme activity
Covalent binding	Stable enzyme-support complex, leakage of the biomolecule is very unlikely, ideal for mass production and commercialization	Complicated and time-consuming, possibility of activity losses due to the reaction involving groups essential for the biological activity (can be minimized by immobilization in the presence of the substrate or inhibitor of the enzyme)

Table 2.1 Conventional immobilization techniques (modified from [55])

2.2 Barium Strontium Titanate (BST)

2.2.1 Introduction

Barium Strontium Titanate (BST) is a ferroelectric material with perovskite structure. As it is shown in figure 2.2, it has a cubic structure above Curie temperature which transfers to an asymmetric structure below Curie temperature.

BST has been intensely studied and employed in different applications because of its unique combination of characteristics, including large dielectric constant, low dc leakage, low loss tangent, and mechanical and thermal stability [28-30]. It has been successfully applied in microelectronic devices, such as DRAM [37-40], infrared detectors [41], microwave phase shifters [42, 43], voltage tunable devices [44, 45], humidity sensors [34-36], and gas sensors [46-49].

Figure 2.2 Crystallographic structure of BST

2.2.2 Sol-Gel Derived BST film

Several methods have been developed for the fabrication of BST films, such as RF sputtering [56, 57], metal organic chemical vapor deposition (MOCVD) [58], pulse laser deposition [59] and sol-gel deposition [60, 61]. Among these methods, sol-gel

process has been widely used due to its low cost, simplicity, low temperature, good homogeneity, ease of doping, and compatibility with IC processes [62].

Sol-gel is a simple and low temperature technique for synthesis of powders and films. It involves preparation of a solution of the elements of the desired compound in an organic solvent using inorganic or organometallic compounds, such as alkoxides of appropriate solubility. This is followed by polymerizing the solution to form a gel, and drying the gel at an appropriate temperature to decompose the residual organic components to form a final inorganic oxide [62].

BST thin films of different composition have been prepared by using sol-gel method with different precursors and solvents [63-72]. The most commonly used precursors as Ba and Sr sources are Ba- and Sr- acetates [$\text{Ba}(\text{CH}_3\text{COO})_2$ and $\text{Sr}(\text{CH}_3\text{COO})_2$] and alkoxides. The commonly used titanium source is a titanium alkoxide, such as titanium isopropoxide [$\text{Ti}(\text{OC}_3\text{H}_7)_4$], and the solvent is acetic acid or 2-methoxy ethanol, depending on the precursor. In addition, to prevent Ti-alkoxide from rapid hydrolysis, acetylacetonate might be added to the solution as a chelating agent.

2.2.3 Humidity sensing properties of BST

When water molecules interact with the perovskite BST grains, the electrical properties of the BST film change. As humidity increases, the capacitance of the BST film increases and the resistance decreases in Metal-Insulator-Metal (MIM) structured BST [32-34]. This property has been used for humidity sensing applications. Figure 2.3 plots a sample curve for BST impedance at various relative humidity values.

The changes in the capacitance and resistance of the BST film are explained by the adsorbed water on the grain surface and capillary condensed water within the pores. The porous structure of the BST film favors both of these processes.

Figure 2.3 Capacitance and resistance of BST vs. humidity [34]

As it is illustrates in figures 2.4, and 2.5, two types of adsorption of water molecules occur on the BST grains: chemisorption at low humidity levels, followed by physisorption at higher humidity levels [35, 36]. When adsorption commences at the BST grain surface, the hydroxyl group (OH^-) of water molecule is attracted to metal cations (Ba/Sr) present on the surface layer of the grains, and the proton (H^+) reacts with an adjacent surface O^{2-} group to form a second OH^- group. Thus, a layer of hydroxyl groups is formed through the chemisorption process. As humidity increases, additional layers of water molecules start to form by physical adsorption through hydrogen bonding. With more than one layer of physisorbed water, water starts to condense within the capillary pores. The neck of the pore is filled according to its radius and thickness of the physisorbed layer [35, 36].

Higher water content in the film results in higher capacitance of the film because the dielectric constant of the water is very high (≈ 80). Capacitance of the BST increases due to polarization of the adsorbed and capillary condensed water molecules.

Figure 2.4 Four stages of water adsorption on metal oxide surfaces: at the first stage of adsorption, a water molecule is chemically adsorbed on an activated site (a) to form an adsorption complex (b), which subsequently transfers to surface hydroxyl groups (c). Then another water molecule comes to be adsorbed through hydrogen bonding on the two neighboring hydroxyl groups as shown in (d) [36].

Figure 2.5 Multilayer structure of condensed water: as the water continues to condense on the surface of the ceramic, another layer on top of the first physisorbed layer forms through only one hydrogen bond [36].

Electrical conduction can be separated into four regimes depending on the water content in the film [35-36]: (a) when only hydroxyl groups are present on the grain surface, the charge carriers are protons, which hop between adjacent hydroxyl groups.

The protons are originated from the dissociated hydroxyl groups ($\text{OH}^- \rightarrow \text{O}^{2-} + \text{H}^+$). This requires high activation energy; (b) when water is present, but one monolayer of surface coverage is not complete, H_3O^+ diffusion on hydroxyl groups dominates ($\text{H}_3\text{O}^+ + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{O} + \text{H}_3\text{O}^+$). Simultaneously, proton transfer between adjacent water molecules in cluster also takes place. The physisorbed water dissociates to form hydronium (H_3O^+), $2\text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{OH}^-$. Hydronium is the hydrated hydrogen ion, as there is no free existence of naked protons; (c) when the first physisorbed water layer is continuous, the charge transport occurs when H_3O^+ ions release a proton to neighboring water molecules, which accepts it while releasing another proton and so on. This is known as the Grotthuss's chain reaction. The brief illustration of the Grotthuss's chain reaction is shown in figure 2.6. The Grotthuss's chain reaction provides easy paths for electrical conduction through the interface between electrode and film as well as BST grain boundaries. Thus, it leads to a great increase in conductivity of the film; (d) with further increase in relative humidity (RH), the thickness of the adsorbed layer of water increases, leading to the filling of successively higher diameter necks. Liquid water condenses within capillary pores, and electrolytic conduction occurs in the liquid layer of water condensed which enhances the conductivity of the film.

Figure 2.6 An illustration of the Grotthuss mechanism [36]

2.3 Impedance Spectroscopy

2.3.1 Introduction

Impedance Spectroscopy is a powerful and effective technique for the characterization of chemically modified electrodes and biomaterial-functionalized electrodes [23]. It can be utilized as a transduction mechanism in electrochemical sensors, especially biosensors, as it can provide detailed information on interfacial changes occurring at the electrode surface.

Impedance spectroscopy involves applying a small-amplitude perturbing sinusoidal voltage signal to an electrochemical system, and measuring the resulting current response to calculate the electrical impedance. The concept of electrical impedance was first introduced by Oliver Heaviside in the 1880s and was soon after developed in terms of vector diagrams and complex presentation by A.E. Kennelly and C.P. Steinmetz [19]. Impedance is a more general concept than resistance. It is a measure of the ability of an element to resist the flow of an ac electrical current. Impedance can be calculated as the ratio between the system voltage phasor, $V(j\omega)$, and the responding current phasor, $I(j\omega)$, where $j=\sqrt{-1}$ and $\omega=2\pi f$ in which f is the excitation frequency [19]:

$$Z(j\omega) = \frac{V(j\omega)}{I(j\omega)}; \text{ where } \omega=2\pi f \quad (2-1)$$

2.3.2 Electrical Circuit Elements

Impedance spectroscopy data is commonly analyzed by fitting it to an equivalent electrical circuit model [18]. Most of the circuit elements in the model are common electrical elements such as resistors, capacitors, and inductors. Thus, it is very useful to have some knowledge of standard circuit components. A list of common circuit elements and their impedances is provided in Table 2.2.

Component	Current vs. Voltage	Impedance
Resistor	$I = V/R$	$Z = R$
Capacitor	$I = C \cdot dv/dt$	$Z = 1/j\omega C$
Inductor	$V = L \cdot di/dt$	$Z = j\omega L$

Table 2.2 Impedances of common electrical elements

As shown in Table 2.2, the impedance of a resistor is independent of frequency and has only a real component. The current through a resistor is always in phase with the voltage. The current through a capacitor is phase shifted -90 degrees with respect to the voltage. The impedance of a capacitor decreases as frequency increases. The impedance versus frequency behavior of an inductor is opposite to that of a capacitor. The impedance of an inductor increases as frequency increases. Inductors have only an imaginary impedance component.

2.3.3 The electrical analogs of physical and chemical processes

As mentioned earlier, the impedance data is commonly analyzed by fitting the data to an appropriate equivalent electrical circuit. The equivalent circuit model is used to simulate the electrical behavior of the respective electrochemical system to obtain a better understanding of the electrochemical behavior of the system. To be useful, the elements in the equivalent circuit should have a basis in physical electrochemistry of the system. For example, in most cases, the solution resistance can be modeled by a resistor, and the double-layer capacitance can be represented by a capacitor since it closely resembles a pure capacitance. Some of the common physical and chemical processes and their electrical analogs are provided in the following sub-sections. A typical electrochemical cell is illustrated in Figure 2.7 for a better understanding of electrochemical systems.

Figure 2.7 An electrolytic electrochemical cell [22]

2.3.3.1 Electrolyte Resistance

The electrolyte impedance of electrochemical cells is commonly modeled by a resistor. The electrolyte is an ionic conductor, and its resistance depends on the ionic concentration, type of ions, temperature and the geometry of the area in which current is carried [18]. The resistance of an electrolyte in a cell with area, A , and length, l , carrying a uniform current is given as:

$$R = \frac{l}{kA}, \quad (2-2)$$

where k is the solution conductivity. In most cases it is difficult to determine the ionic concentration, the current flow path, or the geometry of the electrolyte that carries the current. Thus, the solution resistance is not usually calculated from the ionic conductance. Instead, it is calculated based on fitted impedance data.

2.3.3.2 Double Layer Capacitance

When an electrical voltage is applied to an electrode, ions of the opposite electrical charge will be attracted toward the electrode. As illustrated in Figure 2.8, these ionic

charges are separated from the electrode charges by a short distance, often in the order of angstroms. This will result in a small capacitance at the electrode/electrolyte. The value of the double layer capacitance depends on many variables such as the electrode potential, temperature, ionic concentrations, types of ions, oxide layers, and the electrode roughness [18].

Figure 2.8 The electrical double layer [22]

2.3.3.3 Polarization Resistance

Polarization is termed to a situation in which the potential of an electrode is forced away from its value at open circuit [18, 19]. When an electrode is polarized, it can cause current to flow through electrochemical reactions that occur at the electrode surface. The amount of current is controlled by the kinetics of the reactions and the diffusion of reactants both towards and away from the electrode.

2.3.3.4 Charge Transfer Resistance

The charge transfer resistance is a measure of difficulty in transferring charged species across the electrode/electrolyte interface [18]. This process controls the electron

transfer kinetics of the redox probe. In the case of a metal electrode, the metal molecules can electrolytically dissolve into the electrolyte, according to:

In the forward reaction, electrons enter the metal and metal ions diffuse into the electrolyte. Charge is being transferred. This charge transfer reaction has a certain speed. The speed depends on the kind of reaction, the temperature, the concentration of the reaction products and the potential [18].

2.3.3.5 Warburg Impedance

Diffusion is the transport of species from a location of high concentration to a location of lower concentration. It occurs at a rate proportional to the concentration gradient. Concentration of reactants and redox labels may vary at the electrode side or in the electrolyte due to diffusion processes. This results in frequency-dependent impedance which is recognized as the Warburg impedance. The Warburg impedance is small at high frequencies because diffusing reactants do not need to move very far. At low frequencies the Warburg impedance increases as the reactants have to diffuse farther [18, 19].

2.3.3.6 Constant Phase Element

Capacitors in Electrochemical systems often do not behave ideally, e.g., because of the roughness of the surface and the complicated structure of an immobilized antibody layer [19, 20]. A constant-phase element (Q) is used to describe these systems more appropriately:

$$Z = A(j\omega)^{-\alpha}, \quad (2-4)$$

where $A = 1/C$ is the inverse of the capacitance.

For a constant phase element, α is less than one, but it equals one for a capacitor. The "double layer capacitor" in real cells often behaves like a CPE, not a capacitor.

2.3.3.7 Coating Capacitance

Coating capacitances are related to non-conducting layers in the structure of the electrochemical cell. Like all capacitors, the value of the coating capacitance also depends on the size of its plates, the distance between the plates, and the dielectric properties of the coating material [18].

2.3.4 Randles and Ershler Equivalent Circuit Model

A simple and general equivalent circuit to model the interfacial charge transfers (Randles and Ershler model) is illustrated in figure 2.9. R_s , C_{dl} , R_{et} , and Z_w represent the ohmic resistance of the electrolyte solution, the double layer capacitance, the electron transfer resistance, and the Warburg impedance of the system [23]. The Warburg impedance is the result of diffusion of ions from the bulk electrolyte to the electrode interface, and the electron transfer resistance exists if a redox probe is present in the electrolyte solution. The parallel elements (C_{dl} and $R_{et} + Z_w$) are introduced because the total current through the working electrode is the sum of distinct contributions from the double layer charging and Faradaic process. Since all the current must pass through the electrolyte, R_s is inserted as a series element in the equivalent circuit.

A typical Nyquist diagram of a Faradaic impedance spectrum is illustrated in figure 2.10 [19, 20, and 23]. It consists of a semi-circle region followed by a straight line. The semi-circle portion corresponds to the electron transfer limited process, whereas the linear part is characteristic of the lower frequency range and represents the

diffusionally limited electrochemical process. This diagram can be used for extraction of the electron transfer kinetics and diffusional characteristics of the system. The semi-circle diameter equals the electron transfer resistance, and the intercept of the semi-circle with real-axis at high frequencies is equal to the solution resistance, R_s . Having the characteristic frequency (ω_0), C_{dl} can be calculated using the formula

$$\omega_0 = 1/(C_{dl} \cdot R_{et}) \quad (2-5)$$

In the absence of any redox label in the electrolyte solution, only non-Faradaic impedance exists. In this case, the electron transfer parameters, R_{et} and Z_W become infinite, and the equivalent circuit can be simplified.

Figure 2.9 Randles and Erschler Equivalent Circuit Model [23]

Figure 2.10 A typical Nyquist diagram of a Faradaic impedance spectrum [20]

2.3.5 Impedimetric immunosensors

Formation of the biorecognition complex on a conductive or semiconductive surface alters the capacitance and the resistance at the surface/electrolyte interface which could be translated to a meaningful electrical signal using impedance spectroscopy [23-25]. This forms the basis of impedimetric immunosensors. Impedimetric immunosensors are attracting increasing interest, since they are direct and label free and have many advantages with respect to speed, low driving voltage, and the potential for miniaturization and large scale production [25, 26]. Although these immunosensors are very simple in operation and have many advantages, but they still suffer from several drawbacks. One of the major problems of this type of immunosensors is the relatively small change of the impedance spectra upon the direct binding of analyte to the antigen/antibody functionalized electrodes, especially when the analyte concentration is low and the surface coverage of the antigen-antibody complex is far from saturation [23]. This could result in low detection limit and low signal to noise ratio. To overcome this problem, the concept of amplified impedimetric immunosensors was introduced. In general, this approach includes the application of enzyme-labels that perform biocatalytic reactions and precipitate an insoluble product on the sensing interface upon the antigen-antibody recognition [23]. However, most of these amplification methods involve labeling of biomolecules which is not desired.

3 Experimental procedures

Figure 3.1 illustrates the cross-sectional schematic view of our prototype device. As it is shown, it consists of a layer of BST composite film deposited on top of a platinum coated silicon wafer, modified by a layer of functionalized polymer, and immobilized with biomolecules. The fabrication process of the BST composite film is described in section 3.1, and the surface modification procedures are presented in sections 3.2 and 3.3. Section 3.4 provides an overview of the characterization techniques applied.

Figure 3.1 Cross-sectional view of the prototype immunosensor

3.1 Fabrication of the BST composite film

Several methods have been developed for the fabrication of BST films, such as RF sputtering [7, 8], metal organic chemical vapor deposition (MOCVD) [9], pulse laser deposition [10, 11] and sol-gel deposition [12, 13]. Among these methods, sol-gel process has been widely used due to its low cost, simplicity, low temperature, and compatibility with IC processes. However, a major limit of the sol-gel process is the maximum film thickness that can be achieved. It is difficult to deposit films with thickness of more than few hundred nanometers because of crack formation. In this study, a modified sol-gel method has been developed to fabricate crack-free

$\text{Ba}_{0.6}\text{Sr}_{0.4}\text{TiO}_3$ films with thickness of up to several microns which is not achievable by using conventional sol-gel technique [71, 72]. This method involves dispersing BST nano-size powders in BST sol-gel precursor using ball mill technique to form a uniform slurry. The slurry is then deposited onto the platinum coated silicon wafer using the spin-coating technique. By applying this method, uniform and crack-free BST composite films with thickness of around 3 μm were fabricated. The general scheme of the process is illustrated in Figure 3.2.

Figure 3.2 Schematic illustration of the modified sol-gel process

3.1.1 Materials

The starting materials used to prepare the BST slurry were:

Barium Strontium Titanate ($\text{Ba}_{0.6}\text{Sr}_{0.4}\text{TiO}_3$) powder (average grain size: 50 nm, PI-KEM Ltd), Titanium (IV) butoxide (99%, Aldrich), acetylacetone (99 %, Merck), barium acetate (99%, Merck), strontium acetate (Nacalai Tesque), 2-methoxyethanol (99%, Panerac Sintesis), Acetic acid (Glocial, Best chemical), and Amonium Hydroxide (25%, Ashland).

3.1.2 Preparation of the sol-gel precursor solution

Titanium (IV) butoxide was mixed with acetylacetone in a molar ratio of 1:4 to form a stable chelate complex. Barium acetate and strontium acetate powders were mixed in proper molar ratios ($M_{Ti}:M_{Ba}:M_{Sr} = 1:0.6:0.4$) and were dissolved in acetic acid. De-ionized water, in a molar ratio of 1:4 ($M_{Ti}:M_{DI}$), was added to the solution to promote the dissolution process. The dissolved (Ba,Sr) precursor solution was added to the titanium butoxide precursor solution. Then, 2-methoxyethanol was added to achieve a final concentration of 0.2 M. The solution was filtered through a 0.1 μm Teflon micro-pore filter to eliminate dust and other contaminating particles. The detailed procedure is illustrated in Figure 3.3.

Figure 3.3 Preparation of the BST pure so-gel precursor solution

3.1.3 Preparation of the sol-gel slurry

The BST powder and sol-gel solution were mixed in proper mass ratios (4:1), and were ball milled to form a uniform slurry. A Fritsch “Pulverisette 5” planetary ball-milling machine was used together with tungsten carbide vials (with volume of 250 ml, and an inner diameter of 75 mm) and balls (with diameters of 10 mm and 20 mm). A powder-to-ball mass ratio of 1:20 was used, and the milling speed was set to 200 rpm. The milling was carried out in 30 minutes cycles with 20 minutes of milling followed by 10 minutes of pause in order to prevent overheating of the machine. The slurry was collected after 12 hours of ball mill.

3.1.4 Deposition of the BST composite film

The BST composite films were deposited on Platinum coated Silicon wafers using the spin coating technique. The deposition procedure is illustrated in 3.4. First, a pure sol-gel precursor layer was deposited on the wafer to enhance the adhesion between the substrate and BST composite layers. Then, three BST composite layers (using the BST slurry) were deposited to achieve a film thickness of around 3 microns. A final capping layer of pure sol-gel precursor solution was applied to make the BST surface smoother. Each BST layer was spun on at 3000 rpm for 30 seconds then dried at 150 °C and pyrolyzed at 475 °C. The final spin coated film was annealed for 1 hour at 600 °C in air.

3.2 Surface Modification of the BST composite film

An effective and simple approach to modify and functionalize the BST composite film surface was developed with the help of Dr. Mao Baowei, a surface chemist from the National Institute of Education (NIE). Organic block copolymer of 3-

(trimethoxysilyl)propyl methacrylate (TMS) and N-hydroxysuccinimide methacrylate (NHSMA) was synthesized using the Atom Transfer Radical Polymerization (ATRP) technique [74-77]. These copolymers consist of two segments: the segments of TMS are end-capped with trimethoxysilane moieties and can facilitate the cross-linking between the functionalized polymer and inorganic oxide network [78-81], while, NHSMA segments are capable of tethering of biomolecular species through aminolysis reaction [82-86].

Figure 3.4 Deposition of the sol-gel slurry

3.2.1 Chemicals used

The chemicals used in this modification process were:

3-(trimethoxysilyl)propyl methacrylate (TMS) (99%, Fluka) which was stirred over calcium hydride for 2 hours, distilled under vacuum, and stored at -5 °C under an inert gas (Ar) atmosphere; Methacrylic acid (99%, Aldrich) stored at -5 °C; N-hydroxysuccinimide (99%, Sigma), ethyl 2-bromoisobutyrate (EBriB) (98%, Aldrich), CuCl (99.99% Aldrich), 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA) (97%, Aldrich), n-heptane (99%, Merck), n-hexane (99%, Merck), methanol (99.9%, Fisher), acetone (99.5%, Merck), phosphoric acid (85%, Fluka) and 1,4-dioxane (anhydrous 99%, Aldrich) were used as received.

3.2.2 Synthesis of N-hydroxysuccinimide methacrylate (NHSMA)

NHSMA was synthesized and used to prepare a random block copolymer with TMS via Atom Transfer Radical Polymerization (ATRP). NHSMA monomers were synthesized by coupling methacrylic acid with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide [73]. The procedure of synthesis of NHSMA monomers is illustrated in Figure 3.5. To a stirred solution of g N-hydroxysuccinimide in 40 ml of dry dioxane, cooled at 5 °C with an ice-bath, 3.5 ml methacrylic acid and 8.66 g N,N'-dicyclohexylcarbodiimide were added. The mixture was stirred at 0 °C for 2 hours. Then, the cooling bath was removed and the mixture was stirred for 4 hours to reach the room temperature. The precipitated N,N'-dicyclohexylurea was then removed by filtration and the solvent was evaporated in vacuum. The residue was extracted exhaustively with hot n-heptane. The combined n-heptane extracts were cooled to 0 °C for crystallization of the product. The product was then recrystallized from n-heptane, m.p. 106 °C. The Proton Nuclear Magnetic Resonance (^1H NMR) spectrum of

NHSMA is presented in Figure 3.6. The ^1H NMR spectra were recorded using a Bruker DRX400 spectrometer.

Figure 3.5 The procedure for synthesis of NHSMA

Figure 3.6 ^1H NMR spectrum of NHSMA in CDCl_3

3.2.3 Synthesis of poly(TMS-r-NHSMA)

ATRP technique was used to prepare the random block copolymer of TMS and NHSMA [73]. The synthetic route is illustrated in Figure 3.7. To a dry 25 ml Schlenk flask with a magnetic stirring bar, CuCl (0.027 g, 0.268 mmol) and NHSMA (0.2 g, 1.08 mmol) were added. The flask was evacuated and flushed with argon. Degassed 1,4-dioxane (3 ml), HMTETA (0.073 ml, 0.268 mmol) and TMS (2 ml, 4.02 mmol) were introduced to the Schlenk flask using degassed syringes. The mixture was then stirred for 10 minutes and degassed by three freeze-pump-thaw cycles. Finally, EBrIB (0.020 ml, 0.134 mmol) was added using a degassed syringe. The flask was then placed in a preheated oil bath at $50\text{ }^\circ\text{C}$. The reaction was allowed to proceed with

constant stirring for 4.2 hours. The polymer was precipitated in n-hexane and resolved in dry Tetrahydrofuran (THF). The ^1H NMR spectrum of poly(TMS-*r*-NHS) is presented in Figure 3.8. The Number Average Molecular Weight (M_n) and polydispersity index (M_w/M_n) of the polymers were determined by gel permeation chromatography (GPC). The Number Average Molecular Weight (M_n , GPC) and polydispersity index (PDI) of the synthesized polymer were 11,100 and 1.26, respectively. Moreover, molar ratio of TMS and NHSMA was calculated based on the integrated signals of ($\text{OCH}_2\text{-CH}_2\text{O}$) of NHSMA at 2.81 ppm and (OCH_3) of TMS at 3.60 ppm. It was found to be around 6.

Figure 3.7 Synthetic route for the poly(TMS-*r*-NHSMA)

Figure 3.8 ^1H NMR spectrum of poly(TMS-*r*-NHSMA)

3.2.4 General protocol for modification of the BST composite film surface

BST composite films were soaked in Nitrogen bubbling DI water for 10 minutes to remove the soluble ions or contaminations, and were dried at room temperature. Clean BST films were treated with phosphoric acid to have more OH function groups for a better polymer modification. Films were immersed into 9.5 % phosphoric acid aqueous solution for 10 minutes and dried at 60 °C for 8 hours, then washed in constant Argon bubbling water for 30 minutes and dried at 60 °C under vacuum condition for 6 hours. Then, the acid modified BST films were immersed in poly(TMS-*r*-NHS) methanol solution (8.8 mg/ml) for 10 minutes and dried at 200 °C for 16 hours.

Figure 3.9 BST surface modification using phosphoric acid and poly(TMS-*r*-NHSMA)

3.3 Immobilization of bioreceptors

The polymer modified BST composite film can catch and bind to bimolecular species covalently through the carbonyl group of NHSMA. The carbonyl group in NHSMA could bind to biomolecules via nucleophilic displacement [82-86]. To immobilize the Bovine Serum Albumin (BSA) molecules on the surface, polymer modified samples were soaked in BSA solution for 16 hours at 4 °C. The BSA solution was prepared by dissolving BSA in Phosphate Buffer Solution (PBS). Then, the excess protein

molecules were removed by rinsing the samples with PBS. The BSA soaking time was chosen based on the advice of the biologist of our group to prevent denaturation of protein molecules. However, even shorter soaking periods, commonly at room temperature, were reported by some researchers [87-91].

3.4 Characterization techniques

3.4.1 Characterization of the BST powders and composite films

3.4.1.1 Zeta Potential

Colloidal particles dispersed in a solution are usually electrically charged due to their ionic characteristics and dipolar attributes. The zeta potential is defined as the overall charge of a particle in a specific medium. Therefore, it is a good measure of the magnitude of repulsion or attraction between colloidal particles, and hence, the potential stability of a system. If all the particles have a large negative or positive zeta potential they will repel each other, which results in good dispersion stability. However, if the particles have low Zeta potential then the coagulation (formation of larger assemblies of particles) may occur because there is no force to prevent the particles from coming together. This will result in a fast sedimentation.

A Malvern Zetasizer Nano ZS was used to measure the zeta potential of BST powders dispersed into BST sol-gel solutions of different pH values to study the effect of pH value on the structural characteristics of the BST composite film.

3.4.1.2 Thermogravimetric Analysis (TGA)

Thermogravimetric Analysis (TGA) involves subjecting a sample to a controlled heat treatment and measuring its weight loss with increasing temperature. It can be used to study the decomposition behavior of the sample and to determine its moisture content

and organic residues with respect to temperature. The experiment was carried out to characterize the dried BST sol-gel slurry by using a Perkin Elmer Thermogravimetric Analyzer (TGA-7).

3.4.1.3 Differential Thermal Analysis (DTA)

Differential Thermal Analysis (DTA) involves heating a sample and comparing its temperature with a thermally inert reference. When the sample experiences an exothermic or endothermic reaction, its temperature will be higher or lower than that of the reference, respectively. Using this technique, thermal reactions such as phase transition, reduction, oxidation, decomposition, and crystallization can be determined as they are correlated to a temperature range. A Perkin Elmer Differential Thermal Analyzer (DTA-7) was used to study the thermal properties of the dried BST sol-gel slurry

3.4.1.4 X-Ray Diffraction (XRD)

X-Ray diffraction Spectroscopy (XRD) is a non-destructive analytical technique to identify and characterize the chemical composition and crystallographic structure of solid materials and powders. In this technique, the sample is exposed to an x-ray beam. The incident beam is collected after being diffracted off planes of atoms which satisfy the Bragg's equation. This results in a diffractogram which can be used to identify the presence of different materials and phases (peak positions), their concentrations (peak heights), amorphous content (background hump) and grain size (peak width).

A Shimadzu XRD-6000 X-ray Diffractometer was used to study the crystallographic structure of the BST composite films with a scan range from 20° to 60° and a sweep rate of 2° per minute. Cu $K\alpha$, at 40 KV and 30 mA was chosen as the X-ray source.

3.4.1.5 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is a powerful technique to study the surface morphology and structural characteristics of a specimen. It can produce high resolution images of a sample surface by rastering a focused electron beam over the sample surface. In a typical SEM, the electron beam is formed via thermionical emissions from a tungsten or lanthanum hexaboride or via field emission (FE), and then is focused through a series of electromagnetic lenses. The image of the surface is then produced through a cathode ray screen which maps the intensity of the detected signal as a function of the position.

SEM was used to study the effect of different fabrication parameters on surface morphology of BST composite films. Moreover, cross sections of the films were observed and thicknesses of films were estimated. The experiment was carried out using a JEOL JSM-6700F Scanning electron microscope.

3.4.1.6 Energy Dispersive X-ray analysis (EDX)

EDX is an analytical technique to identify the elemental composition of a specimen or an area of interest down to a spot size of 1 micron. The EDX analysis system is integrated with a scanning electron microscope (SEM). It utilizes the x-rays that are emitted from the sample during bombardment with the electron beam of the SEM. When the sample is bombarded by the electron beam, some electrons are ejected from the atoms on the surface. This results in electron vacancies. These electron vacancies are being filled by electrons from higher shells. To balance the energy difference between the two electrons, a characteristic x-ray is emitted. Each element has a unique set of energy levels, so it produces x-rays at a unique set of energies. The EDS x-ray

detector measures the number of emitted x-rays versus their energy which can be used to determine the elemental composition of the material.

3.4.2 Surface Characterization of the modified BST composite film

3.4.2.1 Contact Angle Measurement

The contact angle measurement is a simple and fast way to measure the water affinity of a surface. It could provide some insight into the surface content and morphology after different modification steps. The measurement was carried out using a FTA200 Dynamic Contact Angle Analyzer. FTA32 Video 20 software was used to fit the contour and calculate the contact angle.

3.4.2.2 Fourier Transform Infrared Reflection (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a powerful technique for identifying the chemical bonds in a specimen by producing an infrared absorption spectrum. The infrared spectrum of the sample is obtained by passing a beam of infrared light through the sample and collecting the transmitted or reflected spectra. Then the amount of energy absorbed at each wavelength is calculated. This spectrum is a characteristic of the chemical bond. Different molecular bonds absorb different wavelengths since they vibrate at various frequencies depending on the elements and type of bonds. Hence, molecular structure of the sample can be recognized by analyzing the absorption spectrum. A Perkin-Elmer 2000 FTIR spectrometer (model 1725 X) was used to analyze the surface content after different surface modifications. The experiment was carried out in the Reflection mode because the BST composite films were not transparent.

3.4.2.3 X-ray Photoelectron Spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS) is a quantitative technique that can be used to analyze the surface chemistry of a specimen. Using this technique, the elemental composition, empirical formula, chemical state and electronic state of the elements of materials can be determined [74]. XPS involves irradiating a material with an x-ray beam in ultra-high vacuum (UHV) conditions, and measuring the kinetic energy (KE) and number of electrons that are ejected from the top 1 to 10 nm of the surface. XPS was used to analyze the BST composite film surface after different surface modifications to investigate the effectiveness of each modification step.

3.4.2.4 Fluorescence Microscopy

Fluorescent Microscopy was carried out to evaluate the protein binding properties of polymer-modified surface and to gain further insight into the spatial distribution of bimolecular species on the surface.

Fluorescence microscopy is based on the fact that certain materials can emit energy in the visible light range when irradiated with the light of a specific wavelength. The specimen can either be fluorescing in its natural form or be labeled with a fluorescent molecule called fluorophore (such as Green fluorescent protein, fluorescein or DyLight 488). When the specimen is illuminated with light of the specific wavelength, it emits longer wavelengths of light. The emitted light is then separated from the much stronger illumination light through an emission filter [76]. A Leica DFC 300FX microscope was used to study the surface coverage of fluorescein isothiocyanate (FITC) labeled BSA molecules.

3.4.3 Electrical Characterization

Impedance Spectroscopy is a powerful technique for characterization of the chemically-modified surfaces. It can provide an understanding of interfacial electrochemical reactions occurring at conductive or semiconductive contacts. In this technique, a small-amplitude perturbing sinusoidal voltage is applied to the electrochemical system and the resulting current is then measured to calculate the impedance. A detailed introduction to this technique has been provided earlier in section 2.3.

In this study, impedance spectroscopy was utilized to study the transduction of the biorecognition events by monitoring the impedance changes upon different reactions. The experiment was carried out using a Solartron SI1260 impedance/gain-phase analyzer in the frequency range from 1 Hz to 10^6 Hz with an ac level of 50 mV. The impedance data was analyzed by fitting to an equivalent circuit using the Zview software.

3.4.3.1 *A prototype measurement setup*

To measure the impedance value, a measurement setup was developed to hold the sample and facilitate the connection between sample's electrodes and electrical probes of the impedance analyzer. The impedance measurement setup consists of two separate parts; a gold top electrode which is fabricated using e-beam evaporation on a glass substrate, and a platform made of poly-dimethylsiloxane (PDMS) for holding the sample and the top electrode. Figure 3.10 depicts the experimental setup used. After fixing the sample on the platform, 10 μ l of DI water was dropped onto the sample surface using micropipette. Then, the gold top electrode was covered over the drop of

water to form a capacitor structure for impedance measurement. A detailed sample preparation and measurement protocol is provided in the following section.

Figure 3.10 Experimental setup for impedance measurement

3.4.3.2 A typical sample treatment and impedance measurement protocol

A sample treatment procedure was set up to minimize the signal variations caused by other effects other than the real signal which comes from the biorecognition reaction. The procedure is as follows:

1- Preparation of the samples:

- Size: 0.9cm×1.8 cm
- Expose the bottom electrode by etching the corner of the BST film using diluted HF solution
- Soak the sample in N₂ bubbling DI water for 10 minutes after etching
- Modify and functionalize the surface with polymer

2- Measurement of Impedance on the polymer surface:

- Drop 10 μL of fresh DI water on the polymer surface and start the timer
- Start the measurement after 2 minutes

(Sweep frequency range: 0.1Hz-1MHz, ac signal, level: 50 mV)

- After measurement, dry the sample using N_2 gun blow

3- Immobilization of the first protein layer (bioreceptor: BSA):

- Cover the sample with 200 μL of PBS buffered BSA solution (with concentration of 1 mg/ml). Keep it in fridge for 16 hours at 4 °C
- Take the sample out of the fridge and rinse it with PBS for 90 seconds
- Dry the sample with N_2 gas for 10 seconds (N_2 gun pressure is set to 0.5 Kg/cm²)

4- Immobilization of the second protein layer (analyte: anti-BSA):

- Cover the sample with 200 μL of PBS buffered anti-BSA solution. Keep it in fridge for 16 hours at 4 °C
- Take the sample out of the fridge and rinse it with DI water for 90 seconds
- Dry the sample with N_2 gas flow for 10 seconds (N_2 gun pressure: 0.5kg/cm²)
- Let the sample dry at fridge for a determined period of time

5- Measurement of Impedance:

- Wait for one minute before dropping 10 μL of DI water on the surface
- Start measuring the impedance after 2 minutes of dropping the DI water on the surface

4 Structural Characterization of the Immunosensor

4.1 Characterization of the BST powders and composite films

4.1.1 Potential stability of the BST slurry

Stability of the sol-gel slurry has a significant effect on the quality of films being fabricated by the sol-gel technique. If the slurry is not stable, particles will agglomerate during deposition or even in the slurry phase. As a result, films may have cracks or non-uniformity on the surface.

Zeta potential is a good measure of interactions between colloidal particles. Hence the magnitude of Zeta potential gives an indication of the potential stability of a colloidal system such as the BST slurry. When the absolute value of zeta potential is above 30 mV the slurry is very stable due to mutual electrostatic repulsion; and when the zeta potential is close to zero, the coagulation (formation of larger assemblies of particles) may occurs which results in a fast sedimentation.

One of the most important factors that affects the zeta potential is the pH value of the solution. Variation in the pH value of the solution alters the zeta potential which can be used to adjust the electrostatic forces between particles.

The pH value of BST precursor solution was varied between 4.0 and 6.0 by adding acetic acid or ammonium hydroxide. The pH modified BST sol-gel solution was then added and mixed with the powder and was ball milled for 12 hours to obtain a uniform slurry. The slurry was centrifuged to obtain a transparent solution which could be used for zeta potential measurement. A Brookhaven ZetaPALS zeta potential analyzer was used to perform zeta potential measurement. Results are provided in Figure 4.1. It was observed that the slurry is stable in a wide range of pH values between 4.0 and 6.0. The pH value of the original sol-gel precursor solution was 4.5 ± 0.1 .

Figure 4.1 Effect of sol-gel solution pH value on Zeta potential of the BST powder

4.1.2 Thermal characterization

In order to determine a suitable heat-treatment for eliminating organic components from the film during deposition, dried sol-gel slurry was characterized by differential thermal analysis (DTA) and thermal gravimetric analysis (TGA) techniques at a heating rate of 2°C/min at air. Figure 4.2 illustrates the thermal decomposition behavior of the dried BST slurry.

Figure 4.2 DTA and TGA curves of the dried BST slurry

As it is shown in Figure 4.2, there is an endothermic peak at 80 °C accompanied by a weight loss in TGA curve which is related to evaporation of residual solvent in the xerogel. The pyrolysis of bound organics is recognized by the significant weight loss and the two-step exothermic process which occurs between 260 °C and 420 °C [66-71]. Accordingly, preheating treatments at 150 °C and 475 °C were chosen to eliminate the residual solvent and organic components from the film, respectively. With further increase in the temperature, there is an exothermic peak near 600 °C which is attributed to the crystallization of BST [66-71]. Analysis of XRD measurements also suggests that the exothermic peak at 600 °C is associated with the formation of perovskite phase. Hence, an annealing temperature of 600°C is chosen to fabricate perovskite BST composite film. Annealing temperature should be below the limiting temperature of the commercial Pt-coated Si wafer.

4.1.3 Surface Morphology

The BST composite film plays an important role in performance characteristics of the immunosensor. It affects the subsequent surface modification steps. Moreover, BST layer is directly involved in the sensing mechanism as a signal amplifying element. Hence, it is very important to establish a stable and repeatable fabrication process for synthesis of uniform and crack-free BST composite films.

In this section, the effect of different parameters involved in film deposition on structure and surface morphology of BST composite film was investigated. This can help to recognize the effective and critical parameters involved in the fabrication process to control the characteristics of BST composite films and ensure it is insensitive to probable handling errors and changes in the procedure. SEM experiment was carried out to observe surface morphology and cross section of the films. Figure

4.3 illustrates the cross section of a typical fabricated BST composite film. As it is shown, the film has a uniform thickness of about 3 μm .

Figure 4.3 Cross section of the BST composite film, Pt substrate, pH: 4.5,

Powder to solution mass ratio=1:4, annealed at 600 °C

Four different batches of BST composite films using different powder to solution mass ratios of 1:2, 1:3, 1:4, and 1:5 were fabricated. SEM pictures with magnification of 4000 and 30000 times are provided in Figure 4.4. It was observed that higher powder content in the solution could result in cracks on the surface of the films. The reason for this phenomenon might be that the pure sol-gel solution phase was not enough to encompass and bind the powders together. Crack-free composite films were obtained by using powder to solution mass ratios of less than or equal to 1:3. Powder to solution mass ratio of 1:4 was chosen for fabrication of BST composite films as it was not marginal and could result in crack-free films.

To investigate the effect of pH value on film's structure and to ensure that the process is stable with respect to small changes in pH value of the pure sol-gel solution, different batches of BST composite films were fabricated by adjusting the pH value of the sol-gel solution to 4.5, 5, 5.5, and 6. SEM pictures with magnification of 4000 and 50000 times are provided in Figure 4.5. As it is shown, the films have similar surface morphologies. Hence, it could be concluded that the fabrication process is insensitive

Figure 4.4 Effect of the powder-to-solution mass ratio on surface morphology of the BST composite film; Pt Substrate, annealed at 600 °C, pH 4.5

(a) pH: 4.5

(b) pH: 5.0

(c) pH: 5.5

(d) pH: 6.0

Figure 4.5 Effect of the pH value of the sol-gel precursor solution on surface morphology of the BST composite film; Pt substrate, annealed at 600 °C, powder to solution mass ratio 1:4

to small changes in pH value of the BST sol-gel solution and there is no need to adjust the pH value of the BST sol-gel solution. This is consistent with the zeta potential measurement results which show that the BST slurry is potentially stable in the pH range of 4.0-6.0.

Looking at SEM pictures in Figures 4.4 and 4.5, two different regions with different colors and patterns could be recognized on the surface of the BST composite films. It was suspected that the dark color patterns might be due to the pure sol-gel solution phase itself and not any other contaminations. To confirm this, EDX experiment was performed. EDX results, illustrated in Figure 4.6, prove that those strange patterns are also composed of BST. It is believed that one region is originated from the BST powder introduced into the sol-gel slurry, and the other is grown and crystallized from the dried sol-gel solution.

Figure 4.6 EDX observation of the BST composite film surface
at two different regions of film surface

Figure 4.7 Effect of the annealing temperature on surface morphology of the BST composite film; Si Substrate, pH 4.5, powder to solution mass ratio 1:4

To further understand the grain growth and crystallization behavior during high temperature sintering, films were fabricated on SiO₂/Si substrate and annealed at different temperatures. Figure 4.7 illustrates the SEM pictures of the films annealed at different temperatures. As it is shown in Figure 4.7, the grain size grew with increase in temperature. The final submicron-size grains were fully developed at 1100 °C. At this stage, the powder and sol-gel grains joined together, and a totally new homogenous polycrystalline matrix were formed, although the film originally consists of both powder and sol-gel dried phases.

4.1.4 Crystallographic structure

Figure 4.8 illustrates the XRD patterns of the commercial nano-size BST powder and BST composite films annealed at 600 °C and 700 °C. Films were fabricated on Platinum substrates by using the powder to solution mass ratio of 1:4. As it is shown, the XRD patterns of the BST composite films annealed at 600 °C and 700 °C matched closely with the standard crystallographic pattern of the perovskite BST.

Figure 4.8 XRD patterns of the commercial nano-size BST powder and BST composite films annealed at 600 °C and 700 °C

4.2 Surface characterization after different surface modifications

4.2.1 Fourier Transform Infrared Reflection (FTIR) Spectroscopy

Figure 4.9 shows the FTIR spectrum of the BST composite film after the phosphoric acid modification, using a BST composite film as the background. Intense and broad phosphorous-oxygen bonding were observed at 968 (P-OH) and 1187 (P=O) cm^{-1} [92]. The FTIR spectra of the BST composite film after polymer modification and protein immobilization steps, using a BST composite film as background, are also illustrated in Figure 4.10. The spectrum of the polymer-modified film shows the characteristic bonding peaks for poly(TMS-r-NHS). Starting from the left, the spectrum shows stretching vibrations of CH_2 and CH_3 around 2919 cm^{-1} , C=O at 1724 cm^{-1} , Si- CH_2 at 1520 cm^{-1} , Si-C at 1265 cm^{-1} , and Si-O-Si around 1027 cm^{-1} , respectively [93, 94]. Regarding the spectrum of the protein-immobilized surface in Figure 4.10, the signal of N-H is present at 3254 cm^{-1} . Due to the effect of N-C, the signal of C=O shifted to 1635 cm^{-1} . The signals became broad from 3100 to 2000 cm^{-1} . These spectra show the successful surface modification. FTIR spectra of protein immobilized and TMS-r-NHS modified surface without prior phosphoric acid modification are presented in Figures 4.11 and 4.12, respectively. The signals related to polymer modification step are available, but they are very weak in comparison to the phosphoric acid modified samples. Although the FTIR results show that there exist some signals related to polymer modification and protein immobilization for the films modified without a prior phosphoric acid modification, but it is believed those signals are due to physical adsorption of molecules and not covalently coupled ones. Therefore phosphoric acid modification before polymer modification is necessary for a better polymer modification and immobilization of biological samples by providing more functional OH groups on the film surface.

Figure 4.9 FTIR spectrum of the BST composite film after phosphoric acid modification, using a BST composite film as the background

Figure 4.10 FTIR spectra of the BST composite film after (a) TMS-NHS modification, and (b) Protein immobilization, using a BST composite film as the background

Figure 4.11 FTIR spectrum of the BST composite film after TMS-NHS modification without a prior Phosphoric acid modification, using a BST composite film as the background

Figure 4.12 FTIR spectrum of the BST composite film after BSA and TMS-NHS modification without a prior Phosphoric acid modification using a TMS-NHS modified BST composite film as the background

4.2.2 X-ray Photoelectron Spectroscopy (XPS)

To confirm the FTIR results and to investigate the effectiveness of each modification step, X-Ray Photoelectron Spectroscopy (XPS) was carried out. Figure 4.13 illustrates the XPS spectra of the BST composite film, phosphoric acid modified BST composite film, polymer modified BST film, and BSA-immobilized BST film. Atomic concentrations of Ba, Sr, Ti, O, C, N and P after different modifications were analyzed and calculated. Table 4.1 summarizes the results for this experiment.

Figure 4.13 XPS spectra of (i) BST composite film; (ii) phosphoric acid modified BST; (iii) polymer modified BST film, and (iv) Protein-immobilized BST film

XPS results obtained by analyzing the atomic concentrations of the related elements are in good agreement with the results of FTIR experiment, and prove that each modification step was successful. According to Table 4.1, the atomic concentration of Ba, Sr, and Ti decreases as surface of BST composite film goes through each of the modification steps. Effectiveness of phosphoric acid treatment is demonstrated by a

clear phosphor peak in XPS spectrum of acid-modified BST film which is also confirmed by calculation of elemental atomic concentrations. It indicates that the BST surface was covered by a layer of phosphoric acid through covalent bonding. The photoelectron peak at 407 eV is related to the presence of Nitrogen atom after the immobilization of biomolecules on the surface which proves that the polymer-modified BST composite film has the ability to bind to bimolecular species. Furthermore, the atomic concentration of carbon increases after the polymer modification and BSA immobilization steps. This is consistent with FTIR results. Although elemental Si comes from the polymer layer, but its photoelectron peak could be observed in XPS spectra of bare BST composite film and all other subsequent modification steps. It is suspected that the Si element is likely coming from the contaminated chambers of the XPS instrument since its atomic concentration has little changes after different modification steps.

	Ba 3d	Ti 2p	Sr 3p	C 1s	O 1s	N 1s	P 2s	Si 2p
BST	7.90	12.07	4.21	19.93	41.63	0	0	2.54
BST-P	6.09	12.54	3.88	16.10	46.73	0	4.64	3.73
BST-P-Polymer	4.41	9.14	3.13	25.00	45.74	0	1.10	3.63
BST-P-Polymer-BSA	1.56	4.31	1.59	46.12	30.79	9.12	0.22	2.41

Table 4.1 Elemental atomic concentration for respective samples

4.2.3 Fluorescent Microscopy

Fluorescent Microscopy was carried out to evaluate the protein binding capability of the polymer-modified surface and to gain further insight into the spatial distribution of the bimolecular species on the surface. Samples were treated with Fluorescent-labeled BSA. They were soaked in BSA-FITC solution of a desired concentration for 20 hours

at 4 °C, rinsed with PBS solution, and then characterized by fluorescent microscopy. It should be pointed out that all the procedures for this experiment were carried out in darkness to avoid the BSA-FITC from being exposed to light. Figure 4.14 illustrates the fluorescence images of (a) the bare BST film, (b) the bare BST film treated with BSA-FITC, and (c) the polymer- modified BST film treated with BSA-FITC.

Figure 4.14 Fluorescence images of (a) bare BST film, (b) bare BST film treated with BSA-FITC, and (c) polymer- modified BST film treated with BSA-FITC

As it was expected, the surface of the polymer-modified BST film was much brighter than that of the bare BST film. It indicates qualitatively that the polymer-modified BST film has the ability to catch and covalently bind to biomolecules. It should be pointed out that there was some physical adsorption of BSA-FITC onto the rough surface of the BST composite film.

4.3 Effective parameters in polymer modification

Although it was shown in the previous section that all the modification steps were successful, however, the distribution of functional sites on the surface, which we will refer to it as the surface coverage, was not discussed. The coverage and special distribution of immobilized biomolecules on the surface are significantly affected by the distribution of polymer functional sites. Hence, it is very important to investigate

the effect of different parameters involved in polymer modification to be able to control the process and its surface coverage. In this section, the effects of different parameters involved in polymer modification are investigated. There are three main parameters involved in polymer modification: 1) concentration of polymer solution, 2) soaking time, and 3) number of soaking steps. Contact angle measurement and Fluorescent Microscopy were carried out to measure the surface water affinity (which gives a rough indication of surface content and morphology) and to evaluate the protein binding properties of the polymer-modified surface, respectively.

4.3.1 Soaking time (t_s)

Samples were soaked in a dilute polymer solution (4.4 mg/ml) for various periods, and then were dried at 100 °C for 16 hours. Table 4.2 and Figures 4.15-4.18 summarize the results. As it is shown, there was no need to soak the samples in polymer solution for a long time and even a short soaking period was sufficient for a successful polymer modification. However, the soaking time was set to 10 minutes to be on a safe margin and to let the physical absorption occur.

Figure 4.15 Fluorescent microscopy images for samples fabricated using various polymer-soaking periods

Figure 4.16 Contact angle measurements for samples fabricated using various polymer-soaking periods

Soaking time	Contact angle (degree)	Fluorescent intensity
1 s	49	130
10 s	45	130
30 s	-	110
1 min	67	172
5 min	43	147
10 min	55	139
30 min	52	177
60 min	56	185

Table 4.2 Contact angle and fluorescent intensity for various polymer-soaking periods

Figure 4.17 Effect of polymer-soaking time on contact angle

Figure 4.18 Effect of polymer-soaking time on fluorescent intensity

4.3.2 Number of soaking steps

A dilute polymer solution (4.4 mg/ml) was utilized and the polymer soaking time was set to 10 minutes. Each time, samples were soaked in the polymer solution for 10 minutes, and then were dried at 100 °C for 5 minutes. Different samples with different soaking steps were prepared and were dried at 100 °C for 16 hours. Contact angle measurement and Fluorescent microscopy results are provided in Table 4.3, and Figures 4.19-4.22.

Figure 4.19 Fluorescent microscopy images for samples fabricated using various numbers of polymer-soaking steps

Figure 4.20 Contact angle measurements for samples fabricated using various number of polymer-soaking steps

Repeating time(s)	Contact angle (degree)	Fluorescent intensity
1	39	116
2	70	184
3	88	176
4	96	246
5	98.5	190
6	99	192

Table 4.3 Contact angle and fluorescent intensity for various number of polymer soaking steps

Figure 4.21 Effect of number of polymer-soaking steps on contact angle

Figure 4.22 Effect of number of polymer-soaking steps on fluorescent intensity

Although it seems that the surface is going to saturate after several soaking steps, however, it is believed that the surface reaches its final state for that concentration and it may not have been fully covered by the polymer as the fluorescence intensity is not significant compared to higher polymer concentrations (will be discussed in the following section). It should be mentioned that the curve's peak (at Number of polymer soaking steps=4) in Figure 4.22 might have been due to experimental errors. However, we had not the opportunity to repeat the experiment due to some constraints, including limited number of samples with similar backgrounds.

4.3.3 Polymer concentration

Samples were soaked in polymer solutions of different concentrations for 10 minutes, and then were dried at 100 °C for 16 hours. The surface coverage was investigated using contact angle measurement and Fluorescent microscopy. Results are provided in Table 4.4 and Figures 4.23-4-26.

As it is shown in Figure 4.26, the contact angle increased with the increase in the polymer concentration up to a polymer concentration of around 25 mg/ml, and then

tended to saturate. The saturation point in the contact angle curve might have been due to the full coverage of the surface with polymer chains. Although the saturation point in the contact angle curve was around 25 mg/ml, however, fluorescent intensity saturated in a slightly lower polymer concentration of 13.2 mg/ml. This might have been due to the full coverage of the surface with biomolecules or even cross-linking of polymer chains which could result in non-functional polymer sites on the surface. In both cases, the fluorescent intensity saturates because no more polymer binding sites are available to interact with the biomolecules. These results only provide a rough indication of the surface content, and further experiments and investigations are needed to confirm the possible reasons.

Figure 4.23 Fluorescent microscopy images for various polymer concentrations

Figure 4.24 Contact angle measurements for various polymer concentrations

Polymer Concentration	Contact angle (degree)	Fluorescent intensity
2.2	30	87
4.4	32	117
8.8	41	138
13.2	50	236
17.6	62	245
26.4	77	251
35.2	75	251

Table 4.4 Contact angle and fluorescent intensity for various polymer concentrations

Figure 4.25 Effect of polymer concentration on contact angle

Figure 4.26 Effect of polymer concentration on fluorescent intensity

4.4 Concluding Remarks

A modified sol-gel process was developed for fabrication of BST composite films based on commercial nano-size BST powder. Using this procedure, uniform and crack-free BST composite films with thickness of around 3 microns were synthesized. Surface morphology and structural characteristics of the fabricated BST composite films were investigated by using XRD, EDX, SEM, and FTIR experiments. Moreover, effects of different parameters involved in the fabrication process were investigated. It was shown that the fabrication procedure is robust with respect to small changes in fabrication parameters and environmental conditions.

Poly(TMS-r-NHSMA) was successfully applied to functionalize the surface for covalent binding to bio-molecules. The effectiveness of each of the modification steps was confirmed by FTIR, XPS, and Fluorescent microscopy observations. It was shown that the modified surface is capable of binding to biomolecular species.

Effects of different parameters involved in polymer modification on surface properties, such as water affinity and bioactivity, were investigated. It was observed that the polymer soaking time is not a critical parameter in the modification step. Moreover, polymer concentration was found to be an effective parameter to control the amount and distribution of polymer functional sites, and hence, the bioactive sites.

5 Biomolecule-sensing performance characterization of the immunosensor

5.1 Characteristics curve of the immunosensor

Several samples with similar treatment procedure and background impedances were chosen. The samples were first immobilized with BSA solution with concentration of 1 mg/ml (the choice of this value will be discussed in section 5.4.3), and then were reacted with different concentrations of anti-BSA. Impedance spectroscopy was carried out and differences between impedances of the anti-BSA immobilized samples and impedance of a reference sample were calculated. An interpolated curve of the impedance changes for various anti-BSA concentrations is plotted in Figure 5.1. This curve represents the characteristics curve of the immunosensor and could be applied for the detection and quantification of anti-BSA concentration in a solution. The impedance value is proportional to the anti-BSA concentration; higher anti-BSA concentrations result in higher impedance values. This relationship is almost linear for anti-BSA concentrations in the range of 1 - 100 $\mu\text{g/ml}$.

Figure 5.1 Impedance (at 1 kHz) vs. anti-BSA concentration

As it is shown in Figure 5.1, the characteristics curve tends to saturate at high anti-BSA concentrations. This might be due to the full coverage of all bioreceptors with complimentary analytes (which impedes the binding of further spices). A low detection limit of 10 ng/ml could also be identified, which might be due to the protein association/dissociation constraints at low concentrations. This detection limit is close to the lowest reported one, to our knowledge, which is about 2 ng/ml [95].

5.2 Discussion on sensing mechanism

To have a better understanding of the sensing mechanism, impedance spectroscopy was carried out using a gold top electrode and various substrates as the bottom electrode. Impedance spectra for the Pt /DI water /Au and the Pt /BST /DI water /Au structures are illustrated in figure 5.2. As it is shown, the measured impedance of the Pt /DI water /Au structure was much higher than that of the Pt /BST /DI water /Au structure. This indicates that there exist more charge carriers in the water which has been exposed to the BST composite film.

The perovskite BST is a highly polar material. When water molecules contact BST grain surface, the negatively charged oxygen atoms of water molecules will be electrically attracted to the positively charged Ba^{2+}/Sr^{2+} of BST. High electrostatic field between Ba^{2+}/Sr^{2+} and oxygen atoms of the chemisorbed water layer will result in dissociation of water molecules into H^+ and OH^- ions, given as:

Once the first layer is formed, subsequent layers of water molecules are physically adsorbed. These physisorbed water molecules will be dissociated into H^+ and OH^- ions because of the high electrical field in the chemisorbed water layer. Existence of these

ions results in a significant decrease in electrical impedance of the system by increasing the conductivity of DI water and amount of charge carriers at the electrode/electrolyte interface.

Figure 5.2 Impedance spectra of the Pt/DI water/Au and the Pt /BST/DI water /Au structures, (a) Bode plot; (b) Nyquist plot

Figure 5.3 plots the impedances of the bare BST composite film, the poly(TMS-*r*-NHSMA) modified BST composite film, the BSA immobilized film, and the anti-BSA immobilized film (after being immobilized with BSA and reacting with 1 $\mu\text{g/ml}$ anti-BSA solution). After being modified with poly(TMS-*r*-NHSMA), the BST composite film showed higher interfacial resistance. This indicates that the poly(TMS-*r*-NHSMA) could obstruct the electron transfer at the solid-liquid interface. After immobilization of BSA molecules, even a further increase in the impedance value was observed which indicates that BSA molecules could further block the surface. A similar behavior was also observed after immobilization of anti-BSA molecules. These results prove that the hindrance of the surface after various immobilization steps increases the impedance of the system.

Figure 5.3 Impedance spectra of bare BST film, poly(TMS-r-NHS) modified BST film, BSA immobilized film, and anti-BSA immobilized film

Figure 5.4 Impedance spectra for various concentrations of anti-BSA

As it is shown in Figure 5.4, higher concentrations of anti-BSA result in higher impedance values. This can be applied for quantification of anti-BSA concentration in a solution by calculating the impedance changes upon the anti-BSA immobilization and using the characteristics curve which was presented in section 5.1.

Considering the impedance spectroscopy results and the equivalent circuit analysis, it is concluded that the sensing capability is based on four parallel mechanisms upon the biorecognition reaction: 1) obstruction of charge transfer at the interface, 2) increase in the thickness of the interfacial double layer, 3) prevention of DI water from being dissociated into H^+ and OH^- ions, and 4) prevention of DI water from penetrating into the BST film. When protein molecules bind to the surface, they will block the surface and reduce the charge transfer at the interface. This results in an increase in the interfacial impedance of the system. The thickness of the interfacial layer also increases which causes a decrease in the capacitance of the double layer and hence an increase in the interfacial impedance of the system. Moreover, the impedance of the BST composite film increases upon prevention of the DI water from penetrating into the BST structure. This is due to the fact that the BST film is a humidity sensor and shows higher impedances for lower water contents. Beside this, impedance of the DI water also increases since the dissociation of DI water molecules, and hence the amount of charge carriers, decreases. Compared to the conventional electrochemical biosensors, our immunosensor suggests a new sensing mechanism by utilizing two signal amplification methods.

5.3 Equivalent Circuit Modeling

An equivalent circuit model was set up to fit the measurement data and explain the sensing mechanism. The suggested circuit model and its simulated impedance spectrum are illustrated in Figures 5.5 and 5.6, respectively. As it is shown, the simulated impedance spectrum well matches the experimental data. The proposed model includes the ohmic resistance of the water, R_s ; the Warburg impedance, Z_w , which is due to diffusion of ions from bulk water to the electrode/water interface; the

double layer capacitance at the electrode/electrolyte interface, C_{dl} ; charge transfer resistance at the electrode/electrolyte interface, R_{ct} ; resistance of the BST layer, R_m , and capacitance of the BST layer, C_m . R_s and Z_w depend on bulk properties of the water and diffusion of dissociated ions, respectively. C_{dl} and R_{ct} represent the impedance features of the electrode/water interface, while R_m and C_m represent the dielectric and electric properties of the BST layer.

Figure 5.5 An equivalent circuit model for analyzing the impedance data, including: R_s , ohmic resistance of the DI water; Z_w , Warburg impedance; C_{dl} , double layer capacitance; R_{ct} , charge transfer resistance; R_m , the BST composite film resistance; and C_m , the BST composite film capacitance

Figure 5.6 The fitted impedance curve for the impedance data measured after recognition of 1 $\mu\text{g/ml}$ anti-BSA. Symbol: the experimental value, solid line: numerical fits of the experiment data by equivalent circuit

Impedance data measured after different surface modifications was fitted to the proposed circuit model and corresponding parameters were extracted. The results are summarized in Table 5.1. It could be concluded that the blockage of the surface after the biorecognition reaction increases the impedance values of the DI water (R_s) and the BST composite film (R_m , and C_m), as well as interfacial impedance of the system (R_{ct} , C_{dl}). R_s , R_{ct} and R_m increase, and C_{dl} and C_m decrease as the anti-BSA concentration increases from 100 ng/ml to 1 mg/ml.

It is believed that the increase in R_s and R_{ct} values is because of the reduced amount of dissociated H^+ and OH^- ions and charge transfer rate after further blockage of the surface, respectively. Moreover, C_{dl} decreases since the formation of antigen-antibody complex increases the thickness of the charge double layer at the electrode/DI water interface. Upon the surface blockage, less water molecules can penetrate into the BST composite film which results in an increase in impedance value of the BST composite film layer. R_m increases because the charge transfer at BST grain boundaries reduces, and C_m decreases because dielectric constant of the BST film decreases due to less polarized water molecules.

Anti-BSA concentration	R_s (k Ω)	R_{ct} (k Ω)	C_{dl} (pF)	Z_w			R_m (k Ω)	C_m (pF)
				R (k Ω)	T	P		
1 mg/ml	2.7	106	40	72	0.0074	0.42	561	6.2
100 μ g/ml	2.5	61	68	89	0.0086	0.41	455	6.8
10 μ g/ml	2.2	29	110	85	0.0073	0.41	372	7.4
1 μ g/ml	1.9	14	230	70	0.0086	0.42	317	8.5
100 ng/ml	1.6	8	420	76	0.0073	0.40	264	9.9

Table 5.1 Corresponding parameters of the equivalent circuit model after different surface modifications and biorecognition reactions at various analyte concentrations

The proposed immunosensor seems to be a good alternative to the conventional electrochemical immunosensors by utilizing two signal amplification mechanisms based on high polarity and humidity sensing properties of the BST composite film.

5.4 Optimization of the biomolecule sensing performance

The aim was to optimize the different fabrication procedures to improve the reproducibility, stability, and sensitivity of the immunosensor. To achieve this goal, at the first step, a robust fabrication process was developed to minimize the cross-sample and sample-to-sample variations. Then, a measurement protocol was developed to reduce the effects of environmental conditions on the measured data. Having established robust fabrication and characterization procedures, effect of polymer concentration on sensitivity of the immunosensor was investigated and the optimum polymer concentration to maximize the sensitivity was obtained. Moreover, the minimum BSA concentration which could cover and saturate the sample's surface was determined to minimize the biological resources and reduce the fabrication cost.

5.4.1 Establishment of a robust fabrication process

Cross-sample and sample-to-sample variations could lead to erroneous results. Thus, development of a robust fabrication process for synthesis of similar samples with identical behaviors and background signals is of great importance.

BST composite film, as the underlying hosting layer for further surface modifications and the main sensing element of the system, plays an important role in sensing performance of the immunosensor. Hence, it is very important to establish a robust and repeatable fabrication process for synthesis of uniform and crack-free BST composite films to reduce the cross-sample and sample-to-sample signal variations. This goal

was achieved by developing a modified sol-gel process which could result in crack-free BST composite films with thickness of around 3 microns. It was shown that the fabrication procedure was robust with respect to small changes in fabrication and environmental conditions. Surface modification of the BST composite films was also well controlled, and effects of various parameters involved in polymer modification were investigated. A detailed description of the fabrication procedures and characterization results were provided in chapters 3 and 4, respectively.

5.4.2 Establishment of a sample treatment and measurement protocol

During impedance spectroscopy, it was noticed that the environmental conditions and handling procedures could significantly affect the measurement results. Hence, a set of experiments were carried out to recognize the parameters involved. The aim was to set up a sample treatment and measurement protocol so that the signal variations due to undesired elements become minimized. The established sample treatment and measurement protocol was described in section 3.4.3.2. In the following subsections, some of the supporting experiments and observations which lead to the development of the proposed protocol will be discussed.

5.4.2.1 DI water aging effect

Measuring impedance at various time intervals showed that the impedance value on the protein-immobilized surface decreases as the time passes. It was suspected that the drop in the impedance value was due to some reactions between the substrate and the DI water. Hence, the experiment was repeated on the polymer-modified surface, the BST surface, a gold electrode, and a platinum electrode. Similar phenomena were observed for all the cases. The impedance value dropped very fast in the first seconds,

and then tended to saturate after sometime. Thus, it was concluded that the drop was not because of any reaction at the substrate's surface as there should be no reaction for the Pt-DI water-Pt structure.

It is well known that when the pure water is exposed to the atmosphere, carbon dioxide will be taken in and reacts with water to form carbonic acid and hydrogen ion, thereby causes a decrease in its impedance and pH value. It was suspected that the drop in the impedance value might be due to a similar scenario, i.e. some contaminations from the air. Thus, an experiment was carried out in which 1 mL fresh DI water was kept in a small bottle and was exposed to air. The impedance of the DI water was measured after different holding times. Figure 5.7 plots the measured impedance at various time intervals. The pH value of the DI water was also measured and the concentration of H^+ ions was calculated. These values are plotted in Figure 5.8. It was concluded that the drop in the impedance value has been because of some contaminations from the air, most probably carbon dioxide.

Another corroborative experiment was carried out to confirm the observed phenomenon. Impedance of DI water droplets with different volumes were measured on the Pt substrate. Impedance values are depicted in Figure 5.9. As it is shown, the dropping rate depended on the surface area to volume ratio. This is well consistent with the proposed reason. Smaller droplets have a larger surface area to volume ratio, and hence, will more easily absorb the contaminations from the air. This causes their impedance to drop faster.

This phenomenon, which we will refer to it as the water aging effect, makes the sensor very sensitive to the environmental conditions and timing errors. Thus, it is necessary to fix and precisely control the time intervals, especially the time interval between dropping the DI water on the sample's surface and measuring the impedance.

Figure 5.7 Impedance of DI water kept in small containers exposed to air

Figure 5.8 pH value of DI water and calculated H⁺ concentration vs. holding time

Figure 5.9 Impedance (at 1 kHz) vs. time for DI water drops of various volumes

5.4.2.2 Stability of the measured impedance on the BST surface

It was observed that repeating the impedance measurement on the BST surface makes a gradual increase in the impedance value toward a saturation point. This phenomenon was not observed for the samples which were pretreated with N₂ bubbling DI water. It was believed that there exist some dissolvable impurities in the films which could be removed by DI water treatment. Thus, BST composite films were treated with N₂ bubbling DI water, and then were dried before polymer modification.

5.4.2.3 Polymer swelling

A gradual increase in the impedance value was observed while repeating the impedance measurement for the polymer-modified films for several times. Immersing the sample in DI water could also result in an increase in the measured impedance value. The impedance values decreased after leaving the samples in air for a long period of time. Experimental results are provided in Figures 5.10 and 5.11. It is believed that this phenomenon is because of polymer swelling. When wetting, polymer absorbs water and increases in its volume which results in higher impedance, and while drying, swollen polymer gives out the bonded water molecules and shrinks back to its original size. To avoid impedance changes due to the polymer swelling effect, it was decided to perform only one impedance measurement for each of the samples.

5.4.2.4 Removing the extra biomolecules after protein immobilization

The BST composite film has a rough surface and porous structure, so it can trap a lot of biomolecules physically in addition to those chemically reacted with functional groups of the polymer. If the physically trapped biomolecules are not removed from the BST surface, sensitivity and stability of the constructed sensor will be deteriorated.

Thus, a washing procedure had to be established to wash away and remove the physically absorbed and unstable proteins from the surface after each protein immobilization step.

Figure 5.10 Impedance vs. repeating times

Figure 5.11 Impedance vs. time (drying period)

The polymer-modified samples were immobilized with FITC-BSA. Then, PBS was utilized to wash away the extra protein molecules from the surface. Fluorescent microscopy was carried out to find the washing time needed to remove the extra proteins. The experiment was done for various PBS rinsing periods. The results are provided in Figures 5.12. As it is shown, after 90 seconds of rinsing, the fluorescent intensity does not change that much. This indicates that even 90 seconds of rinsing with PBS has been sufficient to remove the extra protein molecules from the surface. However, there exist some points with higher brightness for longer rinsing times. This might have been due to some other factors including photo bleaching or surface non-uniformity. The samples might have been exposed to the light for different periods of time. This causes the samples to have different background fluorescent intensities. Surface non-uniformity might also result in different background signals due to the variation in polymer concentration on different samples or at different points on a sample.

Rinsing with PBS is applied to remove the physically absorbed proteins after the first protein modification step. This is due to the fact that the samples should only be rinsed with PBS to avoid denaturation of the bioreceptors (protein molecules).

Figure 5.12 Fluorescent intensity of BSA-FITC immobilized BST composite films for various PBS washing periods (exposure time: 50 ms)

A similar experiment was carried out for various DI water rinsing periods. This procedure could be applied after immobilization of analytes (second protein species) to remove the extra protein molecules and ions from the surface before Impedance spectroscopy. It should be pointed out that the PBS rinsing could not be applied at this step because the PBS could act as an ion-source itself. Moreover, denaturation of the protein molecules is not a concern at this step as the biorecognition event has already happened and the sample is ready for impedance measurement. Figure 5.13 illustrates the results for this experiment. As it is shown, 90 seconds of rinsing with DI water was sufficient to remove the extra protein molecules from the surface.

Figure 5.13 Fluorescent intensity of BSA-FITC immobilized BST composite films for various DI water washing periods (exposure time: 50 ms)

5.4.3 Determining the BSA saturation concentration for immobilization

To eliminate non-specific bindings, BSA must cover all the binding sites of the underlying polymer layer. Hence, a concentrated BSA solution is usually used. It is useful to find the minimum BSA concentration which is needed to cover the surface. It will help to save on the expensive biological resources. Figure 5.14 illustrates the

fluorescent images of the samples modified with different concentration of FITC-BSA. A plot of the fluorescent intensity vs. concentration of BSA-FITC is shown in Figure 5.15. As it is shown, the fluorescent intensity of the film increased significantly with increase in the concentration of FITC-BSA and reached its saturation point at the BSA concentration of 1mg/ml. BSA concentration of 1mg/ml seemed to be sufficient to cover all the functional groups on the sample's surface. Therefore, BSA concentration of 1 mg/ml was chosen to be used for immobilization experiments.

Figure 5.14 Fluorescent microscopy images for various concentrations of FITC-BSA

Figure 5.15 Fluorescent intensity vs. BSA-FITC concentration

5.4.4 Optimization of the polymer concentration

As it was mentioned in section 5.2, the signal amplification mechanism is based on prevention of DI water from contacting BST grains after the biorecognition reaction.

Hence, it seems that the surface coverage plays an important role on the sensing characteristics of the immunosensor.

Surface coverage of the first bimolecular layer (BSA) depends on the concentration of polymer binding sites. A high concentration of BSA is used to cover all the binding sites of the polymer. This is because no non-specific polymer binding sites should exist due to specificity considerations. Therefore, only the polymer surface coverage could be used as a critical parameter to adjust and control the sensing characteristics of the immunosensor. Thus, sensitivity of immunosensors synthesized using various polymer concentrations were measured to investigate the effect of polymer concentration on sensing characteristics of the immunosensor. Sensitivity for a given device is defined as the ratio of the change in the impedance over the change in the concentration of anti-BSA in the linear range of the characteristics curve. Figure 5.16 illustrates the sensitivity for several polymer concentrations.

Figure 5.16 Sensitivity of the immunosensor vs. polymer concentration

As it is shown, the sensitivity increases as the polymer concentrations increases until it reach its maximum at polymer concentration of 8.8 mg/ml, and then drops for higher polymer concentrations. A possible reason for low sensitivity at low polymer

concentrations might be the fact that at low polymer concentrations there would not be enough bioreceptors to detect the complimentary analytes. This will result in low sensitivity and low device performance. Low sensitivity at high polymer concentrations might be due two main factors: 1) difficulty in controlling the modification process which results in different background signals because of cross-sample and sample-to-sample variations, and 2) full blockage of the surface at high polymer concentrations which nullifies the signal amplification mechanisms. Although these phenomena are consistent with the experimental results, they may not be the actual reason behind this behavior and further investigations are needed to confirm those reasons.

However, it was noticed that high polymer concentrations are not desired due to four main reasons: 1) Difficulty in controlling the polymer modification process, 2) Surface non-uniformity, 3) Polymer swelling effect which deteriorates the performance of the device, and 4) low sensitivity. Thus, it is very important to determine the minimum polymer concentration needed to achieve the best device performance. This value is found to be about 8.8 mg/ml for the fabricated device.

5.5 Concluding Remarks

A novel label-free biosensing mechanism based on high polarity and humidity sensing properties of BST composite film was proposed, and the sensing capability of the proposed sensing mechanism was demonstrated for detection of anti-BSA as a target analyte.

Impedance spectroscopy was applied as a powerful technique for characterization of the chemically modified surfaces, and hence, transduction of biorecognition reactions. It was shown that the formation of biorecognition complex could change the

impedance of the system. A proportional relation between the impedance changes and analyte concentration in the range of 10ng/ml to 1 mg/ml was obtained. This indicates the immunosensor could be used for detection and quantification of target analytes.

An equivalent circuit model was set up to fit the measurement data and explain the sensing mechanism. Effect of different modification steps was investigated to provide a better understanding of the conduction mechanisms and sensing performance.

It was shown that the sensing capability is based on three parallel mechanisms upon the biorecognition reaction: 1) an increase in interfacial impedance of the system due to obstruction of charge transfer at the electrode/DI water interface and increase in the thickness of the interfacial double layer, 2) an increase in impedance of the DI water because of prevention of DI water from being dissociated into H^+ and OH^- ions, and 3) an increase in impedance of the BST composite films layer upon prevention of DI water from penetrating into the BST film.

Surface modification steps were optimized to reduce the fabrication variations and improve the sensitivity of the immunosensor. The minimum BSA concentration needed to fully cover and saturate the surface was determined to be 1 mg/ml. It helps to save on the expensive biological resources and reduce the fabrication cost. Moreover, the polymer concentration was optimized at 8.8 mg/ml in order to have the maximum sensitivity.

Some of the environmental conditions and fabrication parameters which could influence the measured data and result in measurement errors were recognized. A sample treatment and measurement protocol was then established to minimize the undesired signal variations.

6 Conclusions and recommendations for future direction

6.1 Conclusions

In this study, a novel label-free immunosensor with a signal amplification mechanism based on high polarity and humidity sensing properties of BST composite film was developed, and the sensing capability of the immunosensor was demonstrated for detection of anti-BSA as a target analyte.

It was shown that impedance spectroscopy could be applied as a powerful technique for characterization of chemically modified device surfaces, and hence, transduction of biorecognition reactions. Formation of the biorecognition complex could alter the impedance features of the system. Higher concentrations of analyte resulted in higher impedance values. This impedance increment (The sensing capability) was attributed to three parallel mechanisms upon the biorecognition reaction. First, the interfacial impedance of the system increases. Formation of the antigen-antibody complex obstacles the charges transfer at the solid/liquid interface, and hence results in an increase in the electron transfer resistance of the system. Moreover, the thickness of the interfacial layer also increases which causes a decrease in capacitance of the double layer and hence an increase in the interfacial impedance of the system. Second, impedance of the DI water increases. BST is a highly polar material which can dissociate water molecules into H^+ and OH^- ions. Immobilized biomolecules could prevent the water molecules from contacting BST grains and induce less water molecules to dissociate into mobile ions. This will result in a decrease in amount of charge carriers, and hence, an increase in the impedance of the DI water. Third, impedance of the BST composite film also increases upon prevention of DI water from penetrating into the BST structure. This is due to the fact that the BST film is a humidity sensor and shows higher impedance for lower water contents.

Compared to the conventional electrochemical biosensors, the proposed immunosensor suggests higher sensitivity by utilizing two signal amplification mechanisms without using bioactive labels. Moreover, it eliminates the need for using harmful redox reagents for Faradaic impedance measurements.

Although the fabricated immunosensor was only used to detect anti-BSA, however, it has the potential to be applied for detection of a wide range of analytes. The functionalized surface has the ability to bind to various bioactive species covalently. Thus, the BST film provides a suitable platform for immobilization of desired bioreceptors to detect their complimentary species.

6.2 Recommendations for future direction

The aim of this study was to investigate the feasibility of applying the proposed sensing mechanism for biosensing applications. Although the main goal of this research was achieved, however, further investigation is still needed to improve the sensing characteristics of the device. Moreover, it is of great interest to extend the application of the proposed immunosensor structure by developing modified biosensors. Some of possible directions for future studies are as follows:

- 1) The proposed immunosensor has the potential to be applied for detection of a wide range of analytes. It might be useful to develop a modified immunosensor for detection of other types of analytes. This might be achievable by immobilizing a suitable bioreceptor instead of immobilizing BSA.
- 2) It is interesting to study the effect of BST composite film structure, such as surface roughness and porosity, on sensitivity of the immunosensor. It might be useful to adjust the BST composite film fabrication process to improve the sensitivity by optimizing the BST composite film structure.

3) Feasibility of applying high polarity and humidity sensing properties of BST composite film for biosensing applications opened the door for application of other materials with similar characteristics as biosensors. Thus, a possible direction for future studies is to investigate the effectiveness of new materials and even new structures, such as interdigitated electrodes.

4) As it was mentioned earlier, distribution of functional sites on the surface plays an important role on sensing characteristics of the device. Although, a rough insight into the surface morphology and content was obtained through contact angle measurements and fluorescent microscopy, however, a quantitative analysis is needed to confirm those results and to determine the amount and distribution of the binding on the surface. It could help to understand the relationship between the sensitivity and the polymer surface coverage. Moreover, effect of different parameters involved in polymer modification on surface characteristics could be investigated.

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