Detection of biomarkers using field effect transistor (FET)-based biosensors for disease diagnosis

疾患診断に向けた電界効果トランジスタ型バイオセンサを利用したバイオマーカーの検出

July, 2015

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Chapter 1  General Introduction

1.1  Detection of biomarkers for early diagnosis
1.1.1 Disease biomarkers
1.1.2 Current standard methods for biomarker detection

1.2  Label-free biosensors as diagnostic tools

1.3  Field effect transistor (FET) biosensors
1.3.1 Enzyme ion-selective field effect transistor (ISFET)
1.3.2 Immuno ISFET
1.3.3 DNA-based ISFET
1.3.4 Cell-based ISFET

1.4  Nanomaterials application in FET biosensing
1.4.1 Silicon nanowire (SiNW)-based FET biosensors
1.4.2 Carbon nanotube (CNT)-based FET biosensors

1.5  Objective for this dissertation

References

Chapter 2  Development of FET-based biosensor for high-sensitive detection of biomarkers using small receptors
2.1 Detection of immunoglobulin E (IgE) using antigen-immobilized FET biosensor for egg allergy diagnosis ................................................ 37

2.1.1 Introduction ........................................................................................ 37
2.1.2 Experimental ..................................................................................... 40
2.1.3 Results and discussion ..................................................................... 43
2.1.4 Summary ........................................................................................... 50

References .................................................................................................. 51

2.2 Detection of α-fetoprotein (AFP) using antigen binding fragment (Fab)-immobilized FET biosensor for liver cancer diagnosis ... 55

2.2.1 Introduction ..................................................................................... 55
2.2.2 Experimental ..................................................................................... 56
2.2.3 Results and discussion ..................................................................... 59
2.2.4 Summary ........................................................................................... 69

References .................................................................................................. 70

Chapter 3 Development of FET biosensors for multiplexed detection of tumor markers for cancer diagnosis ........................................................................................................ 75

3.1 Multiplexed detection of Cytokeratin fragment 21-1 (CYFRA 21-1) and neuron-specific enolase (NSE) using multi-analyte FET
Chapter 3  

3.1  Biosensor for lung cancer differential diagnosis ................................. 77

3.1.1  Introduction ................................................................................ 77
3.1.2  Experimental .............................................................................. 80
3.1.3  Results and discussion .............................................................. 82
3.1.4  Summary .................................................................................... 93

References ............................................................................................ 94

3.2  Concentration-dependence detection of CYFRA 21-1 and AFP using multi-analyte FET biosensor for cancer diagnosis .............. 97

3.2.1  Introduction ................................................................................ 97
3.2.2  Experimental ............................................................................ 100
3.2.3  Results and discussion .............................................................. 102
3.2.4  Summary .................................................................................. 111

References .......................................................................................... 112

Chapter 4  General Conclusions ............................................................ 117

4.1  Conclusions .................................................................................. 119
4.2  Future work .................................................................................. 122

List of Achievements ............................................................................. 125
Acknowledgement.................................................................................. 129
Chapter 1

General Introduction
1.1 Detection of disease biomarkers for early diagnosis

Combining with the effective treatment, the early diagnosis of the disease is crucial for the survival of the patients [1-11]. The biomarkers are indicators of specific biological state of disease, which are either produced by the body or by the diseased organ. The biomarkers are useful for the process of whole disease. Selection of initial therapy, staging and grading could be determined by the biomarkers during diagnosis [12, 13]. A panel of biomarkers has been studied for disease diagnoses. The detection of an effective biomarker would stimulate therapeutic trials and facilitate improved diagnosis for early disease diagnosis [13]. Because of multi-disciplinary clinical disease diagnosis requires people who are seldom available outside of hospitals. In addition, the test need long analytical time and costs much that until the disease has been progressed beyond stages, the patients could receive effective diagnosis. Therefore, the detection of proper biomarkers is very important to be developed for early disease diagnoses [1-20].

Fig. 1.1 Schematic description of the uses of biomarkers along the diseases diagnosis.
1.1.1 Disease biomarkers

The level of biomarkers could provide us information on the method to treat and diagnose the disease with appropriate measurements at desired time [1, 2, 5]. Biomarkers have been discovered for many diseases. DNA fragments, RNA fragments, proteins, and antibody recognition parts are the base of biomarkers [11, 21]. Increased the concentration of DNA in serum are associated with different cancer types and with other diseases including autoimmune and sepsis disease [22-24]. For examples, mutations in the oncogene KRAS is a prediction of metastatic spread in different types of tumors [25-27]. Most RNA-based biomarkers are used in the field of clinical evaluation, such as multi-gene molecular patterns [28, 29]. Breast cancers were classified into subtypes distinguished by pattern-based RNA-expression analysis [30-36]. Mostly, proteins are used as biomarkers in clinical [37-41].

This dissertation focuses on the protein biomarkers for diagnosis of allergy and cancer. Currently, several markers have been proposed to detect allergic inflammation, such as tryptase in nasal fluid, eosinophil counts or ECP levels in sputum, which are related to inflammatory symptoms [42, 43]. In Chapter 2.1, the specific anti-ovalbumin (OVA) immunoglobulin E (IgE) was detected for egg allergy. Cancer is the biggest cause of death worldwide [3], the cancer biomarkers could be used to identify the molecular composition of a tumor indirectly by analyzing blood samples, instead of examining the tumor cells themselves. There is an enormous of biomarkers, both on the proteomic and on the genomic level, which are used to identify the certain state of cancer [3, 6]. In Chapter 2.2, the liver cancer biomarker alpha-fetoprotein (AFP) was detected. In Chapter 3.1, cytokeratinfragment 21-1 (CYFRA 21-1) and neuron-specific enolase (NSE) were detected for lung cancer differential diagnosis. In Chapter 3.2, CYFRA 21-1 and AFP were detected for the differential diagnosis between lung cancer and liver cancer. Some of the current cancer biomarkers in use are listed below (Table 1.1).
<table>
<thead>
<tr>
<th>Cancer</th>
<th>Markers</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>PSA (Prostate-specific antigen)</td>
<td>High sensitivity in all stages; also elevated from some non-cancer causes</td>
</tr>
<tr>
<td></td>
<td>PSMA (Prostate-specific membrane antigen)</td>
<td>Levels tend to increase with age</td>
</tr>
<tr>
<td>Breast</td>
<td>CA 15-3, 27, 29 (Cancer antigen 15-3, 27, 29)</td>
<td>Elevated in benign breast conditions. Either CA 15-3 or CA 27, 29 could be used as marker</td>
</tr>
<tr>
<td></td>
<td>Estrogen receptors</td>
<td>Overexpressed in hormone-dependent cancer</td>
</tr>
<tr>
<td></td>
<td>Progesterone receptors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Her-2/neu</td>
<td>Only 20–30% of patients are positive to Her-2 oncogene that is present in multiple copies</td>
</tr>
<tr>
<td>Lung</td>
<td>CYFRA 21-1 (Cytokeratin fragment 21-1)</td>
<td>Used in combination with NSA to increase specificity, used also for colon cancer detection</td>
</tr>
<tr>
<td>(non-small cell)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>NSEn (Neuron-specific enolase)</td>
<td>Better sensitivity towards specific types of lung cancer</td>
</tr>
<tr>
<td>(small cell)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>NMP22 (Matriarch’s nuclear matrix protein), BTA</td>
<td>NMP-22 assays tend to have greater sensitivity than BTA assays</td>
</tr>
<tr>
<td></td>
<td>Bladder tumor antigen</td>
<td></td>
</tr>
<tr>
<td>Pancreatic</td>
<td></td>
<td>Composed of basement membrane complexes</td>
</tr>
<tr>
<td></td>
<td>BTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CA 19-9 (Carbohydrate antigen 19-9)</td>
<td>Elevated also in inflammatory bowel disease, sometimes used as colorectal cancer biomarker</td>
</tr>
</tbody>
</table>
Table 1.1 Cont [11].

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Markers</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial ovarian cancer (90% of all ovarian cancer)</td>
<td>CA 125 (Cancer antigen 125)</td>
<td>High sensitivity in advanced stage; also elevated with endometriosis, some other diseases and benign conditions</td>
</tr>
<tr>
<td>Genital cell cancer of ovaries</td>
<td>CA 72-4 (Cancer antigen 72-4)</td>
<td>No evidence that this biomarker is better than CA-125 but may be useful when used in combination</td>
</tr>
<tr>
<td></td>
<td>AFP (Alpha-fetoprotein)</td>
<td>Also elevated during pregnancy and liver cancer</td>
</tr>
<tr>
<td>Multiple myeloma and lymphomas</td>
<td>B2M (Beta-2 microglobulin)</td>
<td>Present in many other conditions, including prostate cancer and renal cell carcinoma</td>
</tr>
<tr>
<td></td>
<td>Monoclonal immunoglobulins</td>
<td>Overproduction of an immunoglobulin or antibody, usually detected by protein electrophoresis</td>
</tr>
<tr>
<td>Metastatic melanoma</td>
<td>S100B</td>
<td>Subunit of the S100 protein family</td>
</tr>
<tr>
<td></td>
<td>TA-90 (Tumor-associated glycoprotein Antigen)</td>
<td>Could be used to monitor patients with high risks of developing the disease</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Thyroglobulin</td>
<td>Principal iodoprotein of the thyroid gland</td>
</tr>
<tr>
<td>Testicular</td>
<td>hCG (Human chorionic gonadotropin)</td>
<td>May regulate vascular neoformation through vascular endothelial growth factor (VEGF)</td>
</tr>
<tr>
<td>Colorectal, lung, breast, pancreatic, and bladder</td>
<td>CRA (Carcinoembryonic antigen)</td>
<td>Subtle posttranslational modifications might create differences between tumor CRA and normal CRA</td>
</tr>
</tbody>
</table>
1.1.2 Current standard methods for biomarker detection

The measurement of biomarkers is definitely important in the assistance of disease diagnosis [1-20]. Enzyme-linked immunosorbent assay (ELISA) is one of the most common methods to detect biomarkers [44-47]. However, it is a method that requires hours or even days to provide results. Besides this issue, this method also relies on expensive instruments and extra care, making early diagnosis more complicate for the patients [48, 49]. A quicker and more sensitive technology is necessary in order to achieve the requirements biomarkers have to detect disease in its early stages. Many researches on the detection of biomarkers have been carried out at present, such as fluorescence immunoassay [50-53], chemiluminescence analysis [54-57], optical methods [58-61], and electrochemical analysis [62-66]. Among these detection methods, the latter is one of the most sensitive for the assay of biomarkers. The application of electrochemical assay can potentially solve some of the problems involving sensitivity, speed, and the cost of measurements, as it can detect biomarkers electrically with good sensitivity and specificity in a wide range [48, 49, 62-66]. Under this consideration, an electrochemical sensor allows high sensitivity and low cost, thus becoming a promising alternative to assist in rapid diagnosis, reducing the analytical-time, which is highly significant in reducing patient stress.
1.2 Label-free biosensors

A biosensor is an analytical device converting a biological information into a quantifiable and processable signal [67]. Since the research by Clark and Lyons in 1962 on the first biosensor [48, 68-70], the demand on such analytical tools in diagnostic applications was increasing. With the growing number of deaths due to late diagnosis and the high number of diseases being diagnosed in the world, biosensors are a critical tool in the early disease diagnosis [6].

In clinical laboratories, as described above, ELISA is the most common biomarkers detection method. It can detect more than hundred types of diseases. In biomarkers detection, most of the techniques currently employed use various labels or reagents like enzymatic, fluorescent or radiochemical labels [6, 71]. The labelling processes may hinder monitoring of the probe/target interaction rapidly. In other words, the labeling may affect the target-receptor interactions, which are caused by steric hindrance, induced by the label. In spite of the low detection limits and high sensitivity, these methods are still expensive, complex and time-consuming. Additionally, such methods often require laboratory personnel with high skills [5, 6, 9, 10]. Thus, the development of sensitive, simple and high-throughput biosensors that are able to detect biomarkers without involving labeling is of great interest. Label-free biosensing would eliminate the process steps for the modification and preparation of the target molecules, which significantly save the costs and time needed for sample preparation. Label-free biosensors are potentially very useful as clinical diagnostic tool, thanks to real-time and multi-analyte tests, quick detection and inexpensiveness.

Label-free biosensing are performed without any labeled process. The signal response varies when target molecules adsorb onto the surface of biosensor. In other words, it is a process that goes on in a single step, without any other reagents, and has advantages of the simplicity and inexpensiveness. However, the fact that detection is simple might become a disadvantage when detecting complex samples (e.g., serum), because other nonspecific components in the sample might also adsorb onto the transducer surface, creating false-positive signals. For direct detection without labelling, one thing has to ensure that not any nonspecific adsorption with the complex samples, only the target molecules which bind to their corresponding receptor molecules immobilized on the biosensor surface [5, 6, 72, 73].

Many transducers for label-free detection have been developed base on optical,
acoustic and electrochemical mechanisms. Optical detection methods work by using reflectometry or refractometry [74]. Quartz crystal microbalances (QCM) are the best-known acoustic biosensors [75, 76], others including cantilever biosensors [77] and surface acoustic wave biosensors [78]. Electrochemical biosensors use conductometric/impedimetric, potentiometric, or amperometric transduction principles [79, 80]. So far, electrochemical transducers are being extensively used in biosensor. Among the variety of electrochemical transducers proposed for detecting biomolecules without labeling, the field effect transistor (FET) is an attractive approach. The FET biosensor can analyze kinds of biomarkers such as DNA [81-83] and proteins [84-88] without labeling and in a rapid, simple and inexpensive way.
1.3 Field effect transistor (FET) biosensors

Many types of biosensor exist in the fields of biomarker detection. As introduced above, some detection methods like fluorescence, surface plasmon resonance (SPR), chemiluminescence, QCM and electrical current can detect biorecognition events. FETs have been also used for the label-free detection of biorecognition events, such as antibody-antigen reactions, glycan-protein interactions, DNA hybridization reactions and cell identification [81-89].

The FET biosensors are sensitive to the intrinsic electric charge of biomolecules. By modification of the FET gate surface with a biomolecular layer as a receptor, the changes in the gate voltage-drain current caused by the adsorption of analyte biomolecules can be detected. Since target molecules are charged in an aqueous solutions, the charge on the gate surface changes with biorecognition, and its density variation is transduced by field effect into an electrical signal. The biorecognition events on the FET gate surface can be found by detecting a shift of the threshold voltage. The immobilization of various biorecognition materials on the FET gate surface for biomarker analysis has been developed through surface modification technology. For examples, the immobilization of probes or receptors on aminosilane self-assembled monolayer (SAM)-modified Si$_3$N$_4$/SiO$_2$ gate insulator [81-83]; on a thiol SAM-modified gold electrode for extended gate [90]; on aminosilane SAM-modified SiO$_2$ gate insulator [85-88, 91].

Among many different types of FET devices, (MOSFET, MESFET, JFET and CHEMFET among others), commonly biosensing applications are focused on ISFET (ion-selective field-effect transistor) devices [92-95]. ISFET was firstly described by Bergveld in 1970, which was used as a transducer in electrochemical sensors for electrophysiological measurement of ion composition around nerve tissues [96]. Several types of ISFET-based biosensors exist. Some forms of ISFET-based biosensors, such as enzyme ISFET, immuno ISFET, DNA-based ISFET and cell-based ISFET are explained in detail in this section.
1.3.1 Enzyme ISFET

The concept of Enzyme ISFET revolves around the principle of pH-sensitive ISFET, stating that the enzymes are immobilized on the gate surface of the ISFET device. So far, enzyme ISFET is applied on the detection of numerous analytes such as penicillin, urea, glucose, pesticides, etc [97-101]. Janata and Moss firstly suggested the ISFET-based enzyme biosensor in 1976 [102]. A report on an application of ISFET for practical detection of penicillin appeared in 1980 [93]. In that report, it was proven that the enzyme ISFET might be suitable for quick and sensitive detection of small amount of analytes, with the advantage of saving analysis time. Thus, this ISFET enzyme biosensor has been found to have potential of detection of complex samples.

Fig. 1.2 Structure and principal function of an ISFET-based enzyme biosensor for penicillin analysis. The enzyme penicillinase is immobilized on the Ta$_2$O$_5$ surface. Reprinted with permission from [48]. Copyright © 2002 Royal Society of Chemistry.
The purpose of several methods have been developed is the stable immobilization of enzymes at the ISFETs gate surface [94, 103]. As shown in Fig. 1.3, magnetic nanoparticles were used for keeping the enzyme at the gate, where lipase was immobilized onto magnetic nanoparticles. As a result, the enzyme-immobilized nanoparticles could be maintained at the gate surface thank to the magnet continuing to run from the bottom of ISFET gate [104].

Fig. 1.3 Schematic diagram of an enzyme ISFET using magnetic nanoparticles. Reprinted with permission from [105]. Open access.
1.3.2 Immuno ISFET

An immuno ISFET sensor is constructed using immunoactive probe molecules recognizing corresponding proteins immobilized on the gate surface and an ISFET. Antibodies are the most commonly used recognition elements that are capable of recognizing individual biomarkers, thanks to the specificity of antigen-antibody reactions [106].

As a method to enhance the sensitivity, the use of small receptors has been considered due to they allow a binding reaction to occur within the Debye length, defined as the distance where moving charge carriers screen out the external electric field. Such distance has to be taken into consideration as one of the main problems for biomarker detection using FET-based biosensor, is to make sure that the biosensing takes place within the Debye length. Many approaches to enhance sensitivity use small molecules such as glycan, antigens, aptamers, polypeptides, and aromatic compounds [84, 86, 87, 107-110].

An immuno ISFET sensor for detecting of lysozyme and thrombin was also developed by Goda et al. [84]. As shown in Fig. 1.4, oligonucleotide aptamers with small size were immobilized on a gold electrode extended to the gate of FET. The use of the aptamers allows the target recognition events to occur within the electrical double layer, therefore minimizing the effect of charge screening.

![Fig. 1.4](image_url)

(a) Circuit diagram and photos of the immuno sensor based on ISFET. (b) Conceptual representation of aptamer induced target protein recognition in an electrical double layer, at the interface between the gate insulator and an aqueous solution. Reprinted with permission from [84]. Copyright © 2013 Elsevier B.V.
1.3.3 DNA-based ISFET

DNA molecules are negatively charged in an aqueous solution. The amount of negative charge at the gate surface increases when DNA strands bind to the ISFET gate surface. The FET could transduce the variation of charge density into an electrical signal, thereby allowing for excellent performance of DNA analysis.

The first report on the genetic FET for the detection of the hybridization event was appeared in 1997 by Souteyrand et al. [111] They immobilized homo-oligomer DNA strands with 3-aminopropyltriethoxysilane on silicon/silicon dioxide electrodes. The variation in the surface charge induced by recognition and hybridization between the complementary homo-oligomer strands was examined as a response (Fig. 1.5).

![Diagram](image)

Fig. 1.5 Detection of the hybridization of synthetic homo-oligomer DNA sequences using ISFET. (a) Electrical effects induced by hybridization between complementary strands. (b) Sensitivity of the FET to the introduction of poly (dA). Reprinted with permission from [111]. Copyright © 1997 American Chemical Society.
Single nucleotide polymorphisms (SNPs) are among of the most frequent genetic alterations in humans [112]. Detection of SNPs has the great potential of disease diagnosis, screening and personalised drug therapy. Purushothaman et al. developed a DNA-based ISFET with its own DNA probes for the detection of SNPs [113].

Fig. 1.6 Illustration of the concept of the DNA-based ISFET for SNPs detection. The concentration of SNPs is determined through measurement of the drain current or of the voltage. Reprinted with permission from [113]. Copyright © 2005 Elsevier B.V.
1.3.4 Cell-based ISFET

Cells' activity during their life can be monitored, and these data have applications in biomedicine and pharmacology. Cell-based ISFET has been considered as a promising tool for providing a variety of biologically active information of living cells [114].

Recent advances in cell-based ISFET applications allow the monitoring of various cellular behaviors, like cellular respiration and acidification at the same locus. Lehmann et al. reported a single cell-based ISFET which measures both pH and oxygen partial pressure simultaneously [115]. Milgrewa et al. reported a large sensor array chip based on pH-sensitive ISFET for direct extracellular imaging [116].

![Fig. 1.7 Cross-section through an ISFET made by an unmodified CMOS process. Reprinted with permission from [116]. Copyright © 2005 Elsevier B.V.](image-url)
1.4 Nanomaterials applications in FET biosensing for disease diagnosis

In FET biosensing, the use of nanomaterials-based FET is a possibility that has been appreciated in the recent years, due to unique physicochemical properties and ultrasmall size of nanomaterials. Nanomaterials offer the high surface/volume ratio, that enhance the performances of the biosensors in terms of sensitivity and detection limits down to even individual molecules detection, which is attractive in monitoring of contaminant such as toxins [71, 117]. Additionally, nanomaterials can attach to antibodies and other receptors, which are molecules that produce multiplex sensor array to detect different biomarkers at the same time [118]. Several kinds of nanomaterials, such as carbon nanotubes and nanowires, have been widely used for disease diagnosis in combination with FET technology [79]. However, the immobilization of nanomaterials on a sublayer for reproducible detection remains a challenge to date [119]. After overcome these serious issues such as reproducibility, this FET technique may be suitable for clinical applications and commercialization. This section described recent remarkable advances in the area of disease diagnosis using FET based on nanomaterials (nanowires or carbon nanotubes).
1.4.1 Silicon nanowire (SiNW)-based FET biosensors

Fig. 1.8 shows a SiNW-FET as presented by Gao et al. [120]. In such a biosensor, the source and drain of the FET device is bridged by a silicon nanowire. Apart from several significant advantages of SiNW-FET, such as the fact that it allows label-free, direct and real-time electrical detection, the potential for multiplexed sensor array with ultrahigh sensitivity and excellent selectivity, has generated an increasing attention in the past years. It is possible to integrate a large number of NWs immobilized with different probe molecules on a significantly small footprint of an array, because of their ultrasmall size.

A SiNW-FET array for the multiplexed detection of several cancer markers was developed by Zheng et al. [121]. As shown in Fig. 1.9, the array consists of three independent SiNW-FET devices immobilized with different antibodies. Target cancer markers were simultaneously detected at femtomolar concentrations with high selectivity.
Fig. 1.8 Illustration of a SiNW array biosensor for DNA. Reprinted with permission from [120]. Copyright © 2007 American Chemical Society.
Fig. 1.9 SiNW-FET array for multiplexed detection of cancer markers. (a) Optical image (top) of a SiNW-FET array. (b) Schematic illustrating multiplexed protein detection by three SiNW devices in an array. (c) Conductance versus time data recorded for the simultaneous detection of three markers. Reprinted with permission from [121]. Copyright © 2005 Nature Publishing Group.
1.4.2 Carbon nanotube (CNT)-based FET biosensors

CNT act as the conducting channel in FET configuration and interact with introduced target analytes. The first biological application of CNT-FET was suggested in 2003 by Chen et al. [122]. The CNT-based FET biosensors are high-sensitive to variations in the environment as the electrical current flows through the outermost layer of the CNT, contacting directly with the target molecules.

In a study on CNT-FET for a real-time detection of prostate cancer biomarker, the sensitivity of the CNT-based FET biosensor was maximized by providing enough space between each antibody on the CNT (Fig. 1.10). The conductance of CNT-FET was largely enhanced by controlling the specific linker/spacer ratio on CNTs. In this way, the detection limit of 1.0 ng/mL, without cutting antibody or labeling was achieved [123].

![Cross-section of the CNT-based FET biosensor for prostate cancer biomarker detection.](image)

Fig. 1.10 Cross-section of the CNT-based FET biosensor for prostate cancer biomarker detection. Reprinted with permission from [123]. Copyright © 2009 Elsevier B.V.
1.5 Objective for this dissertation

The early detection of biomarkers is very important to be developed for disease diagnoses. FETs have emerged as an important new technique since they do not require any labeling and enable high-sensitive detections. However, FET biosensors need to be further developed to meet the requirements of practical diagnostic applications, for examples, (1) the detection at low concentrations where the sensitivity of FET need to be enhanced; (2) multiplexed detection of different biomarkers where arrays of sensors need to be developed on the same chip. This dissertation described an approach to overcome the above two big problems that will bring FET biosensors close to diagnostic tools in practical application.

Chapter 2 describes approaches resolving the first problem, which is the detection of biomarkers at low concentrations using FET biosensors is hampered by charge screening effects. To enhance the sensitivity, the use of small receptors enables the biomolecular interaction to occur within the Debye length, which is related to the detection range of the charged target protein in solution. This resulting in an enhancement of sensitivity and lower detection limits of the sensing system. In this section, two types of small probe molecules, antigen and antigen binding fragment (Fab) were examined.

These works demonstrate an effective method to overcome the restriction of the FET biosensor for biomarker detection, that the sensor responsiveness may be reduced by ionic screening when the large probe molecules adsorbed to the transistor gate surface. The small probe receptors, as examined above, could ideally fix close to the sensor gate surface, resulting in the improvement of the sensitivity to biomarkers. The FET biosensor immobilized with small receptors, as described herein, will help for the high-sensitive detection of biomarkers for disease diagnosis.

Chapter 3 describes the approach for the second problem, detecting two biomarkers at the same time by integrating two receptor types on the same chip. A multi-analyte FET biosensor was developed for multiplexed detection of different biomarkers. The multi-analyte FET biosensor consists of two transistors on the same chip. Each transistor was designed to detect one specific biomarker by functionalizing its gate surface with the corresponding probe molecules. To test the capabilities of a multi-analyte FET biosensor for multiplexed detection of biomarkers, an application toward diagnosis of cancer, which is the second most common cause of mortality and
morbidity worldwide, was discussed.

These works provide a step towards the realization of sensor arrays for multiplexed detection of panels of biomarkers. The multi-analyte FET biosensor, as described herein, will help not only for the multiplexed detection of different biomarkers, but will also provide significant advantages over single-analyte biosensors in terms of convenience, measurement time, sample volume and financial resources, taking simultaneously exclusive bio-molecule data will reduce the probability of error, both in false negative and false positive. In the future, by integrating more sensors on one chip, the sensor system would be useful for more other cancers or severe diseases.

In my dissertation, the detection based on FET was developed in two ways. I introduced small probe molecules (antigen and Fab), instead of the conventional receptor, antibody, with big molecular size, to reduce the ionic screening effect. Therefore, the sensitivity of FET biosensor was improved and biomarker at low concentrations (lower than the cut off value) could be detected. On the other hand, the FET biosensor, which allows multi-analyte detection, was well designed by controlling the modification of the sensing surface. Thus, the FET biosensor could provide more accurate diagnostic information for disease diagnosis that leads FET to a practical application.
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Chapter 2

Development of FET-based biosensor for high-sensitive detection of biomarkers using small receptors
2.1 Detection of immunoglobulin E (IgE) using antigen-immobilized FET biosensor for egg allergy diagnosis

2.1.1 Introduction

Recently, allergic diseases (food allergies, etc.) have been receiving increasing attention to develop effective treatments for the patients [1-3]. Because of the success in numerous researches on allergy, some allergy tests have been used for practical diagnosis more than 10 years [4-6]. The preventive tests become more and more important due to some allergy diseases cause serious symptoms, although a standard procedure during overall health examination does not include allergy diagnosis. Recently, two types of representative allergy tests were allowed; ELISA and immunochromatography. Unfortunately, ELISA, a conventional and commercial method needs many handling processes (e.g. labeling) for the target analyte, resulting in the lack of swiftness and ease. The other method based on immunochromatography is quick and simple, but it is not useful for quantitative diagnosis. To overcome those weaknesses should contribute to the advancement of allergy diagnosis. FET biosensor detects the surface-charge density changes caused by the intrinsic charge of the adsorbed biomolecules. The detection process does not require any labeling; therefore, their successful applications in medical fields have been made due to the simplicity and rapidity [7-14]. The FET biosensing device with chemical durability was developed by Osaka’s group, and its fabrication process has been transferred to a company for 10 years. The FET sensor devices, which are easily manufactured by using semiconductor technology, would be cost-competitive for the full-scale practical applications. The technology of FET biosensor, which will be matured by huge up-front costs for the developments, is now beginning to be accepted by society [14]. Further development of the FET biosensors will be accelerated by the expanding application in medical fields. The FET, which could be applied to any fields only by changing the type of probe molecules for the target analytes, is not required to be manufactured specially for one specific application. Therefore, the creation of many kinds of FET biosensors applications for practical use, would lead a mass production and mass consumption, resulting in cost reduction. Herein, an application of FET biosensors toward allergy diagnosis was shown.

A limited charge-detectable region of the solution Debye length for target analytes is one of the biggest problems, in the detection using FET biosensors [10, 11]. In ionic solution, the Debye length is formed near the sensing surface of the FET. The potential
decays exponentially with distance from the surface. The Debye length at the gate/solution interface is calculated to be approximately 7.5 nm for the diluted buffer solutions (i.e. 0.01 \times \text{phosphate buffered saline}, PBS). The intrinsic charges of the target molecules within the Debye length could be detected by using the FET, thus the charge-detectable region needs to be effectively used. Often-used probes, such as antibody, are relatively large (ca. 4-14 nm), resulting in a large area of the charge-detectable region was occupied by the probe, suggesting that the signals caused by the charged target adsorption will be reduced when a binding reaction occurs. Recently, some reports on the application of small receptors for FET-based detection to improve the sensitivity is appeared [15, 16]. In this section the use of a smaller receptor, antigen (ca. 4-7 nm), to realize an efficient application of the region than the antibodies is proposed. Additionally, the receptors in small size are expected to be packed more closely, suggesting that the number of the recognition sites for sensing could be increased. Ovalbumin (OVA), a useful biomarker for the egg allergy diagnosis, was selected as a receptor [17, 18].

An OVA-immobilized FET sensor was fabricated to detect the specific anti-OVA IgE for egg allergy. The purpose was to develop a method for practical examination of the antigen-immobilized FET to determine the amount of specific IgE. The objectives were as follows: (1) optimization of the surface condition of OVA-immobilization, (2) comparison between the IgE response of OVA-immobilized FET and that of anti-IgE antibody-immobilized FET, (3) quantitative detection of IgE in PBS using the OVA-immobilized FET.

The use of small probe molecules for high-sensitive detection based on FET was demonstrated. Antigen and Fab were introduced in Chapter 2.1 and Chapter 2.2, respectively.
Fig. 2.1.1 Schematic illustration of FET-based biosensor for allergy diagnosis. (A) Immobilizations of antigen (OVA) and antibody (anti-IgE antibody) onto the FET gate surface. (B) Electrical detection of IgE using the OVA-immobilized FET. Reprinted with permission from [19]. Copyright © 2013 Elsevier Ltd.
2.1.2 Experimental

2.1.2.1 Materials

The antigen, OVA from chicken egg white, and human serum albumin (HSA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). IgE was purchased from Abbiotec, LLC (San Diego, CA, USA). The anti-OVA IgE was included in Mouse OVA-IgE ELISA KIT (AKRIE-030, Shibayagi, Gunma, Japan). The anti-IgE antibody (MouseAnti-Human IgE (Fc)-UNLB (Clone HP6029)) was purchased from Beckman Coulter (Brea, CA, USA). 3-aminopropyltriethoxysilane (APTES) was purchased from Sigma-Aldrich Inc. Bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). All other chemicals were purchased from Kanto Chemical Co. Inc. All the proteins were used without further purification. Phosphate buffered saline (1 × PBS) of pH 7.4 was made in the laboratory using 137 mM NaCl, 8.1 mM Na₂HPO₄·12H₂O, 2.7 mM KCl, and 1.5 mM KH₂PO₄. Antigen and antibody were diluted in 1 × PBS. Diluted PBS, 0.01 × PBS (pH 7.4), was prepared by diluting 1 × PBS with ultrapure water.

2.1.2.2 Fabrication of antigen-immobilized FETs

As Fig. 2.1.1A shown, the antigen OVA was immobilized on the FET gate surface. The modification process of the APTES and the cross-linker glutaraldehyde (GA) in this section is the same as the one described in the previous work [12, 13]. The antigen OVA was deposited on the GA-modified FET at room temperature for 1 h. The residue probes were washed by PBS to obtain the antigen-immobilized FET. To evaluate the advantage of the antigen-immobilized FET, an antibody-immobilized FET was prepared as described in the previous work [12, 13].

2.1.2.3 Electric measurements

The gate voltage ($V_g$)-drain current ($I_{ds}$) characteristics of the antigen-immobilized FET was measured and used as the reference (Fig. 2.1.1B). The measurements were made in the dark with a semiconductor parameter analyzer (2612A, Keithley Instruments Inc.; Cleveland, OH, USA) at room temperature in 0.01 × PBS (pH 7.4) by sweeping the $V_g$ from −3 V to 1 V with a 1 V drain voltage. Antigen-immobilized FET was immersed in IgE solutions for 1 h. The $V_g$-$I_{ds}$ relationship of the IgE-reacted FET was measured and compared with the reference. A threshold voltage shift, $\Delta V_g$ was...
2.1.2.4. Evaluation of antigen-immobilized surface

Surface morphologies before and after the addition of IgE onto the FET surface and the density of immobilized probe antigens on the FET were examined using atomic force microscopy (AFM) and fluorescence measurement, respectively. The topographic images of the FETs gate surface were investigated by AFM (Shimadzu Co., SPM-9600) in dynamic mode. Silicon cantilever (Olympus Co., OMCL-AC240TSC2, spring constant 2 N/m, resonance frequency 70 kHz) was used in the measurement. The image size was 1 μm × 1 μm with 512 × 512 pixels. Roughness parameters were obtained from AFM images. The amount of the immobilized OVA on the substrate was examined using a fluorescent imager (GE Healthcare, Typhoon 9410). In the fluorescence measurement, the OVA-immobilized substrates were rinsed with PBS containing 1% Tween 20. OVA was stained by an NH₂-reactive fluorescent staining kit for protein (Dojindo, HiLyte Fluor®647 Labeling Kit-NH₂).
Fig. 2.1.2 AFM images of surfaces of OVA-immobilized FETs at 0.1 mg/mL (A, B, C) and 0.01 mg/mL (D, E, F). The change of the surface morphologies before (A, D) and after the addition of 10 (B, E) or 100 ng/mL (C, F) anti-OVA IgE. Z range = 10 nm. Reprinted with permission from [19]. Copyright © 2013 Elsevier Ltd.
2.1.3  Results and discussion

2.1.3.1 Optimization of the surface condition of antigen-immobilized FET surface

AFM and fluorescent measurements were carried out for the optimization of the OVA-immobilized surface. Fig. 2.1.2 shows AFM images of the surfaces of OVA-immobilized FETs after addition of anti-OVA IgE (0, 10, and 100 ng/mL) at various concentrations ranging from 0 to 1 mg/mL, showing that the asperity of the OVA-immobilized surface depended on the concentration of OVA. From the AFM images of the OVA-immobilized gates, roughness parameters were obtained. As shown in Fig. 2.1.3A, the maximum value of $S_{ku}$ (kurtosis, “peakedness” of height distribution) and $S_{sk}$ (skewness, the asymmetry of height distribution) were obtained when the surface was immobilized with 0.01 mg/mL OVA. Large values of $S_{ku}$ (nearly equal to 100) and large positive $S_{sk}$ (>1) indicate that the surface was honed and ground with the presence of a few spikes on the surface [20], which means a nearly flat surface with a few aggregates was formed after the immobilization of 0.01 mg/mL OVA. This result suggested that an OVA layer starts to form beginning with the OVA concentration of 0.01 mg/mL. The change of $S_a$ (arithmetic mean deviation of roughness) caused by the addition of the anti-OVA IgE was the most significant at 0.1 mg/mL OVA (Fig. 2.1.3B), when the OVA concentration was increased greater than 0.01 mg/mL OVA. This result suggesting that the FET gate surface immobilized with 0.1 mg/mL OVA could be effective for the specific detection of anti-OVA IgE. AFM provides more accurate information for vertical direction than that for lateral direction [21], which is useful for discussing the change in the asperity height as shown in Fig. 2.1.2A. The 4-6 nanometer-high asperity, corresponding to the size of OVA molecule, was found in the images of the surface immobilized with 0.1 mg/mL OVA, indicating that the surface composed of smooth and flat biomolecular layer with some aggregates of OVA molecules. Therefore, the best immobilization condition for IgE detection was found to be with 0.1 mg/mL OVA from AFM observation.
Fig. 2.1.3 Roughness parameters of the OVA-immobilized FET gate surfaces and density of immobilized OVA. (A) $S_{ku}$ (kurtosis, “peakedness” of height distribution) and $S_{sk}$ (skewness, the asymmetry of height distribution), (B) $S_a$ (arithmetic mean deviation of roughness). (C) Density of immobilized OVA. Reprinted with permission from [19].

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Fluorescent labeling measurements were also conducted (Fig. 2.1.3C) to estimate the surface coverage of immobilized OVA at various concentrations (0.01, 0.10, 0.50, and 0.86 mg/mL). The maximum immobilization density (1.02 × 10^{13} \text{ molecules/cm}^2) was obtained by OVA at the concentration of 0.1 mg/mL. Similarly, the area occupied by a single OVA molecule was calculated to be 9.8 nm^2 by using the reciprocal of the immobilization density, when immobilized with 0.1 mg/mL OVA. An OVA molecule is an ellipsoid with the longer axis of 7.3 nm and the shorter axis 3.9 nm, according to the X-ray structural analysis result (Protein data bank ID, 1OVA), as shown in Fig. 2.1.4. By using the filling rate (\pi/\sqrt{12}) of the ellipsoidal close packing model, the area occupied by a single OVA molecule is calculated to be 13.3 nm^2 with its long axis vertical to the substrate surface (Fig. 2.1.4A) and 25.2 nm^2 with its long axis in parallel to the surface (Fig. 2.1.4B). The number of OVA layers was estimated to be 1.35 (Fig. 2.1.4A) and 2.56 (Fig. 2.1.4B) from an assumption. Because the nonspecifically adsorbed proteins should be removed by washing the surface with the surfactant Tween 20, the number of the OVA layer was suggested to be 1.35.

According the results above, both the experimental data and the calculation, a monolayer of OVA is formed at the OVA concentration of 0.1 mg/mL, with its long axis perpendicular to the substrate surface. The OVA layer starts to form on the substrate at 0.01 mg/mL OVA, and a monolayer of OVA is completely formed at 0.1 mg/mL. The coverage of the antibody-immobilized surface was estimated to be 61% in the previous report [13], suggesting that the immobilized OVA were packed twice more closely than the antibody. Thus, the concentration of 0.1 mg/mL OVA was selected as the optimal condition of OVA immobilization for the anti-OVA IgE detection.
Fig. 2.1.4 Schematic illustration representing various orientations of immobilized OVA (A, B) and antibody (IgG) (C, D), and the OVA (E) makes more effective use of the charge-detectable region than the antibody (F) for FET detection. Dash line means the Debye length. Reprinted with permission from [19]. Copyright © 2013 Elsevier Ltd.
2.1.3.2. Effect of probe size on the detection of IgE

It is demonstrated that antigen-immobilized FETs exhibited a higher response to IgE than antibody-immobilized FETs. The $\Delta V_g$ shifted in the positive direction after the addition of IgE, due to the intrinsic charge of IgE is negative in PBS (pH 7.4), as expected from its isoelectric point [pI] that equal to be 5.2-5.8 [22]. The magnitude of the $\Delta V_g$ observed upon incubation of 10 ng/ml IgE was 112 mV for the antigen (OVA)-immobilized FET, while that was 39 mV for anti-IgE antibody-immobilized FET (Fig. 2.1.5, inset), suggesting that the difference in the magnitude of FET response came from the difference in size between antigen and antibody. Biomolecular detection using FET devices always is subject to Debye length limitation, which means that intrinsic charges of proteins within the Debye length may be detected [10-12]. Herein, the size of the probe molecules influenced the difference in the magnitude of $\Delta V_g$ under the same Debye length condition (7.5 nm in 0.01 × PBS). As shown in Fig. 2.1.4, the size of the antigen (OVA) is 7.3 nm × 4.0 nm × 3.9 nm from the PDB data, suggesting that almost all antigen molecules may be immobilized in height of 4-7 nm on the substrate as receptors. As a result, the OVA-IgE interaction might occur within the Debye length (7.5 nm). In comparison, the size of the antibody (anti-IgE IgG) is 14.2 nm × 8.5 nm × 3.8 nm [23]. The height of the immobilized IgG is estimated to be equal to 6 nm (Fig. 2.1.4D) because of the orientation of IgG molecules tend to be flat-on the surface [12]. However, some of the immobilized IgG might be larger than 10 nm when they are immobilized with an end-on orientation (Fig. 2.1.4C). It is difficult to judge the orientation of each individual IgG. Therefore, the IgG-IgE interaction might occur outside the charge-detectable region, resulting in a decrease of the effective FET response. It should be noted that the dissociation constant between IgE and antigen (around $10^{-11}$ to $10^{-9}$ M [24]) is known to be similar to that between antibody and antigen [25], suggesting that the use of small receptor might provide more recognition sites for target analytes, which contributes to the enhancement of the FET response.
Fig. 2.1.5 Quantitative detection of IgE using antigen (OVA)-immobilized FET. (Inset: Effect of the size of receptor in IgE detection using OVA-immobilized FET and BSA-blocked anti-IgE antibody (IgG)-immobilized FET). Reprinted with permission from [19]. Copyright © 2013 Elsevier Ltd.
2.1.3.3. Quantitative detection of IgE using antigen-immobilized FETs

It is demonstrated that the antigen-immobilized FET quantitatively detected an allergy-associated IgE (anti-OVA IgE), which is related with egg allergy, in PBS. The use of the antigen as a receptor contributes to the determination of specific IgE antibody. The antigens were immobilized on the FET gate surface to capture the anti-OVA IgE. Here, the biosensing system is useful for the egg allergy diagnosis. The antigen-immobilized FET quantitatively detected anti-OVA IgE at varying target protein concentrations ranging from 1 ng/mL to 100 ng/mL. The magnitude of $\Delta V_g$ proportionally increased as the target protein concentration was increased in the above range (Fig. 2.1.5). The dissociation constant ($K_d$) between OVA and anti-OVA IgE was calculated equal to be $1.5 \times 10^{-10}$ M, according to the Langmuir isotherm, which is more or less a normal $K_d$ value for the antibody-antigen binding event. Compared with the magnitude of $\Delta V_g$ observed after the addition of target protein-free PBS or the addition of 100 mg/mL non-specific analyte HSA (twice of human blood level), the magnitude of the $\Delta V_g$ observed upon incubation of 1 ng/mL anti-OVA IgE was significant. This number is significantly lower than the IgE level of a normal individual (25-100 ng/mL [26]). Therefore, the detection limit of the OVA-immobilized FET satisfies the need of the detection of IgE in clinical level for allergy diagnosis. Furthermore, as shown in Fig. 2.1.5, inset, the non-specific adsorption of non-related protein (HSA) on this proposed OVA-immobilized FET biosensor was minimal, even without any blocking treatments to the sensing surface. These results suggested that the antigenic protein immobilized FET is a promising tool for detecting specific allergens in allergy diagnostic field.
2.1.4 Summary

IgE detection using FET biosensors for the purpose of allergic diagnosis was demonstrated. The antigen-immobilized FETs exhibited a higher response to IgE compared with the antibody-immobilized FETs, suggesting that the small receptor makes effective use of the charge-detectable region for FET detection in terms of Debye length limit, provides more recognition sites for target molecules and greater ability to block nonspecific adsorption due to the closely-packed immobilized probe molecules. Thus, the antigenic protein immobilized FET, as described herein, was shown to be useful as an allergy sensor.
References


2.2 Detection of α-fetoprotein (AFP) using antigen binding fragment (Fab)-immobilized FET biosensor for liver cancer diagnosis

2.2.1 Introduction

Early detection of tumor markers is critical for the survival of cancer patients [1-4]. A variety of technologies have been developed for biomarker detection, such as the ELISA. However, many of these methods feature complicated and time-consuming labeling processes. Label-free detection for medical diagnosis has attracted much attention, and detection based on FET biosensors may have advantages in terms of their rapidity and sensitivity [5-11]. However, the detection of proteins at low concentrations using FET biosensors is hampered by charge screening effects. Especially in clinical conditions, contaminating proteins such as HSA in blood serum might prevent the target proteins from specifically adsorbing onto the sensing surface, suggesting that the sensitivity needs to be improved. To enhance the sensitivity, the use of small receptors enables a binding reaction to occur within the Debye length, which is related to the detection range of the charged target protein in solution. This results in an enhancement of sensitivity and lower detection limits of the sensing system. Many approaches to enhance sensitivity are based on the use of small molecules, such as glycan, antigens, aptamers, polypeptides, and aromatic compounds [12-16]. An alternative, efficient approach is to use a Fab [17-19]. A Fab is capable of recognizing a target molecule with high binding affinity and specificity. Because the vertical length of a Fab is approximately 6 nm [20], which is smaller than the whole antibody, Fabs can recognize and bind target antigens near the FET surface. Here, the use of a Fab as a receptor in a FET biosensor was demonstrated. The response of Fab-immobilized and antibody-immobilized FET biosensors on protein detection were compared and the adsorption mechanism of proteins onto the Fab-immobilized surface was considered. Furthermore, to determine effective blocking reagents for the Fab-immobilized surface, the surface blocking effects of BSA and ethanolamine (EA) were evaluated. Also explored the possibility of the quantitative detection of a liver cancer tumor marker, AFP, and performed a series of electrical experiments under various concentrations of AFP in human serum using the Fab-immobilized FET.
2.2.2 Experimental

2.2.2.1 Materials

The antigen, human AFP was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). Monoclonal anti-AFP (human) was purchased from Nippon Bio-test Laboratories Inc. (Tokyo, Japan). The Fab Preparation kit was purchased from Thermo Scientific Inc. (Waltham, MA, USA). Foetal calf serum was purchased from A&E Scientific (Hainaut, Belgium). Human serum and a self-assembled monolayer reagent, 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). BSA was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), and all other chemicals were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Fab was prepared by using the Fab Preparation kit according to the manufacturer’s instructions. The proteins were used without further purification. PBS (pH 7.4) was made in the laboratory and was prepared using 137 mM NaCl, 8.1 mM Na₂HPO₄·12H₂O, 2.7 mM KCl, and 1.5 mM KH₂PO₄. Diluted PBS, 0.01 × PBS (pH 7.4), was prepared by diluting 1 × PBS with ultrapure water.

2.2.2.2 Detailed information for the FET device

SiO₂-gate FETs was successfully developed with a high degree of chemical durability through surface modification technology using a self-assembled monolayer (SAM) [5], and was successfully engaged in the technology transfer of the fabrication process to Toppan Printing Co., Ltd. (Tokyo, Japan). A photograph of the FET device is shown in Fig. 2.2.1. The gate size of the FET device is 10 μm (length) × 1000 μm (width). The FET detects potential changes on its gate surface in terms of the intrinsic charge of target proteins that bind specifically to probe molecules immobilized on the gate surface, such as the threshold voltage shift (ΔVg), or the gate voltage (Vg)-drain current (Id) characteristics.
Fig. 2.2.1 (a) Optical microscopic image of the FET device fabricated through the semiconductor processes. The bar indicates 10 mm; (b) Change in the $V_g$-$I_{ds}$ characteristics of FET device before and after the addition of the proteins. Reprinted with permission from [26]. Open access.
2.2.2.3 Fabrication of Fab-immobilized FET

The silicon dioxide surface of our transistor substrate was exposed to O$_2$ plasma (200 W for 1 min) to introduce hydroxyl groups onto the surface, followed by coating with a APTES SAM. The SAM was formed on the silicon dioxide surface by immersing in 1% (v/w) APTES toluene solution at 60 °C for 7 min in an argon atmosphere. After the SAM modification, the cross-linker, GA, was allowed to react with the amino-terminated surface by immersing the gate area of the APTES-modified FET in a solution of 2.5% GA in 1 × PBS for 30 min. The probe Fab molecules, which were obtained by cleaving the whole anti-AFP antibody, were allowed to react with the aldehyde moiety of the GA-modified surface for 60 min. Subsequently, blocking treatment to avoid non-specific adsorption was performed using EA or BSA. The Fab-immobilized surface was allowed to react with EA (10 mM) or BSA (1 wt%) for 60 min.

2.2.2.4 Electrical measurements

The gate voltage-drain current ($V_g$-$I_{ds}$) relation of the Fab-immobilized FET was measured and used as a reference. The measurements were made in the dark with a semiconductor parameter analyzer (2612A, Keithley Instruments Inc., Cleveland, OH, USA) at room temperature in 0.01 × PBS (pH 7.4) by sweeping the $V_g$ from −3 V to 0.5 V with a 0.1 V drain voltage. The reference electrode was Hg/Hg$_2$SO$_4$. The Fab-immobilized FETs were immersed in the antigen AFP solutions for 60 min. After the immersion, the residue was washed with 0.01 × PBS. The characteristics of $V_g$-$I_{ds}$ relation of the antigen-reacted FET was measured in 0.01 × PBS and compared with the reference. The threshold voltage shift ($\Delta V_g$) was then calculated.

2.2.2.5 Surface analysis by using atomic force microscopy

Surface morphologies on the FET surface were analyzed using AFM. The topographic images of the gate surfaces of the FETs were investigated by dynamic mode AFM (SPM-9600, Shimadzu Co., Kyoto, Japan). A silicon cantilever (OMCL-AC240TSC2, Olympus Co., Tokyo, Japan, spring constant 2 N/m, resonance frequency 70 kHz) was used, and the image size was 1 μm × 1 μm with 512 × 512 pixels. Roughness parameters were obtained from the AFM images.
2.2.3 Results and discussion

2.2.3.1 Analyses of the surface of Fab-immobilized FET

First, AFM measurements of the FET sensing surface were performed to examine the height of the immobilized Fab molecules and the change in surface morphology following Fab immobilization. The gate surface of the GA-modified FET (Figure 1a), 0.778 nm, and Fab-immobilized FET (Fig. 2.2.2b), 0.998 nm showed differences in the root mean square roughness ($R_q$). The increase in the $R_q$ value from the introduction of Fab molecules suggests that Fab molecules were immobilized on the GA-modified FET gate surface. The height of the immobilized Fab surface was 2-3 nm, which was in good agreement with the size of Fab [19]. However, as shown in Fig. 2.2.2c, the $R_q$ value for the antibody-immobilized surface was greater than that of the Fab-immobilized surface. In line with the previous reports [9], the height of the immobilized antibodies was measured as 4-5 nm. Thus, the Fab molecules were smaller than the antibodies, and were believed to lie flat on the gate surface.

To check the density of the immobilized receptors, fluorescence measurements was performed (Typhoon 9410, GE Healthcare Bio-Sciences KK, Piscataway, NJ, USA). The density of the immobilized Fab was estimated as $1.23 \times 10^4$ molecules/μm$^2$, while that of the immobilized antibody was calculated as $0.65 \times 10^4$ molecules/μm$^2$ in a previous study [10]. Because a single antibody features two antigen-binding sites, the binding sites per unit area was estimated as $1.23 \times 10^4$ sites/μm$^2$ for the Fab-immobilized surface and $1.30 \times 10^4$ sites/μm$^2$ for the antibody-immobilized surface, suggesting that both the Fab-immobilized FET and antibody-immobilized FET have approximately the same number of binding sites on the sensing surface in this work.
Fig. 2.2.2 Atomic force microscope (AFM) images of the field-effect transistor (FET) gate surface obtained after (a) introduction of glutaraldehyde (GA); (b) immobilization of Fab; and (c) immobilization of whole antibody. Z range = 10 nm. Each image has a cross-sectional profile shown. Reprinted with permission from [26]. Open access.
2.2.3.2 Comparison of the sensitivity for protein detection between Fab-immobilized FET and antibody-immobilized FET

To examine the effect of the receptor’s size, the responses of the Fab-immobilized and antibody-immobilized FETs were compared in the presence of the target tumor marker protein, AFP. When AFP (1 μg/mL) was added onto the Fab-immobilized FET gate surface to interact with Fab, the threshold voltage shifted ($\Delta V_g$) in a positive direction by 111 mV, while the antibody-immobilized FET had a $\Delta V_g$ of 51 mV. It is clearly evident that the use of the Fab surface immobilization gave a greater FET response to AFP than the antibody immobilization. This can be attributed to the closer approach of AFP to the charge-detectable region of the FET gate surface, which is defined by the Debye length of the protein in solution. Because the Debye length at the gate/solution interface is 7.5 nm in 0.01 × PBS (pH 7.4), part of the bound AFP molecule with a size of 5 nm × 5 nm × 5 nm [21] may remain outside charge-detectable region in the case of the antibody-immobilized FET. Conversely, with the Fab-immobilized FET, a binding event between Fab and AFP is expected to occur within the Debye length where charge screening effects are minimal. As mentioned in Section 2.1.3.1, the number of the binding sites for the Fab-immobilized surface and antibody-immobilized surface were similar, suggesting that the improvement in the sensitivity of the FET-based biosensor in this study results from the reduced receptor size, rather than differences in the number of binding sites. In terms of specificity, the Fab-immobilized FET, showed essentially no shift when a non-related protein, HSA (isoelectric point = 4.7) [22], was added instead of AFP. To determine the sensitivity of the Fab-immobilized FET, I examined the response of the FET biosensor to AFP solutions ranging from 100 pg/mL to 1 μg/mL (Fig. 2.2.3). Increasing the concentration of AFP increased the amount of negatively charged AFP molecules within the Debye length, resulting in a greater shift of $\Delta V_g$. In a previous study [9], the detection limit for AFP of our antibody-immobilized FET was found to be 10 ng/mL. The use of the smaller receptor Fab increased the sensitivity by lowering the detection limit from 10 ng/mL to 100 pg/mL.
Fig. 2.2.3 Relation between α-fetoprotein (AFP) concentration and \( \Delta V_g \) magnitude for Fab-immobilized FET. Reprinted with permission from [26]. Open access.
From Fig. 2.2.4, consider an analytical model for the Fab-immobilized FET biosensor response. The relationship between $\Delta V_g$ and AFP concentration is nonlinear, therefore, a calibration scheme needs to be developed based on the adsorption mechanism to compare the sensing results across FETs. The surface density ($S_A$) of AFP molecules from the surface charge density ($\sigma_0$) and the charge of AFP were estimated. The value $\sigma_0$ was calculated from the change in the surface potential by setting the Stern potential in the Grahame equation [8] equal to $\Delta V_g$. The concentration-dependent adsorption density of the immobilized AFP molecules on the surface was in good quantitative agreement with a model based on the Langmuir adsorption isotherm for equilibrium protein binding, as shown in Fig. 2.2.4, where the best fit to the data yielded a two-component Langmuir equation. Here, the values of $S_{A_{\text{max}}}$ and the dissociation constant ($K_d$) represent the maximum surface density and affinity properties of the biomolecule interactions, respectively, for each component. Component 1 exhibits a low affinity for AFP ($K_d = 2.5 \times 10^{-9}$ M), while component 2 shows a stronger affinity ($K_d = 1.5 \times 10^{-11}$ M). Assume that the difference in affinity arises from different orientations of the Fab molecules. Component 2 may be related to Fab molecules that are vertically immobilized on the surface (i.e., end-on orientation), towards which AFP may approach more easily with reduced steric hindrance. Conversely, component 1 may be related to Fab molecules lying flat on the surface that may be expected to show lower affinity. Considering the value of $S_{A_{\text{max}}}$ for each component, the number of AFP molecules binding to Fab in a flat-on orientation is greater than that of the end-on oriented Fab.
Fig. 2.2.4 Surface density of AFP, calculated from the magnitude of $\Delta V_g$ and the charge of AFP (see text), as a function of AFP concentration. Inset shows the schematic representation of the two different orientations of Fab on surfaces and the two-component Langmuir-type adsorption model equation. Reprinted with permission from [26]. Open access.
2.2.3.3 Treatment for the blocking of nonspecific adsorption

Nonspecific adsorption limits the sensitivity of FETs when used for the detection of specific analytes in serum; therefore, a blocking treatment is necessary to reduce nonspecific protein adsorption. To find an effective blocking reagent for the Fab-immobilized surface, evaluated the surface blocking effects of BSA and ethanolamine (EA). Immobilized Fabs on the surface, and introduced the blocking reagents (1 wt% BSA or 10 mM EA). As shown in Fig. 2.2.5, the blocking treatment using BSA or EA solution both suppressed nonspecific adsorption. The EA-capped Fab-immobilized surfaces maintained the same response to the addition of AFP as the non-blocked Fab-immobilized surface, while the BSA-blocked, Fab immobilized surface showed a lower response than that of the specific adsorption experiments. BSA may hinder the attachment of target proteins because of its size (3.5 nm × 7 nm × 7 nm) [23], causing Fab binding sites to become obstructed by BSA molecules. In contrast, the Fab-immobilized surface capped with EA maintained its binding capacity for the target protein. Thus, EA was selected as an effective blocking reagent for Fab-immobilized FET.

It should be noted that, after treating the surface with blocking reagents BSA, the FET response of human serum (negative control) was positive. On the other hand, after treating the surface with blocking reagents EA, the FET response of human serum was negative. The FET response is related to the intrinsic charge of target molecules. There are both positively and negatively charged molecules in human serum. Therefore, the nonspecific adsorption of charged molecules contained in human serum onto the blocked-probe-immobilized (both blocked with BSA and capped with EA) surface may lead to a threshold voltage shift (ΔV<sub>g</sub>) in both positive and negative direction. It should be noted that, the nonspecific adsorption was minimized (near zero) by addition of blocking reagents (both BSA and EA). In another word, ΔV<sub>g</sub> values were around zero. Thus both positive and negative small ΔV<sub>g</sub> values were significant as a negative control. The difference of the direction of the shift in the threshold voltage might come from the experiment errors.
Fig. 2.2.5 Comparison of sensor response to protein addition for antibody-immobilized FET (non-blocked, bovine serum albumin (BSA)-blocked, and ethanolamine-capped). The proteins were foetal calf serum for negative control and AFP (1 μg/mL) for positive control. Reprinted with permission from [26]. Open access.
2.2.3.4 Quantitative detection of AFP in human serum using Fab-immobilized FET

After solving the nonspecific adsorption problem, investigated the quantitative detection of AFP in human serum. It is demonstrated that the Fab-immobilized FET detected AFP contained in human serum at varying AFP concentrations ranging from 1 ng/mL to 1 μg/mL (Fig. 2.2.6). This range covers most of the clinically relevant concentrations. It should be noted that the magnitude of $\Delta V_g$ at 1 ng/mL AFP in human serum was equal to that at 100 pg/mL in buffer solution, suggesting that other protein contaminate in the serum may obstruct the adsorption of AFP to the sensor surface. However, even under such unfavorable conditions, the EA-capped Fab-immobilized FET achieved detection of AFP at the low level of 1 ng/mL (signal-to-noise ratio $>$3), which is below the cut-off value for normal levels in humans (<10 ng/mL) [24]. Additionally, detection was completed within 60 min, which is much quicker than using a commercialized ELISA kit that usually takes approximately 3 h from serum sample incubation to the final test results. For the clinical application of FET biosensors in the future, the separation and concentration devices may be combined with our FET sensing device to sensitively and specifically detect the target molecules in blood samples [25].
Fig. 2.2.6 Detection of AFP in human serum by using Fab-immobilized FET with ethanolamine-capping treatment. The concentrations of AFP ranged from 1 ng/mL to 1 μg/mL. Reprinted with permission from [26]. Open access.
2.2.4 Summary

In this work, a label-free FET biosensor that allows for efficient AFP detection in human serum was successfully developed. To improve the sensitivity to AFP, probe receptors should ideally be small and fixed close to the sensor gate surface. Here, small Fab receptors were immobilized to allow antigen-antibody reactions to occur with the Debye length and improved the sensitivity of the FETs. The Fab-immobilized biosensor was subjected to a blocking treatment to avoid non-specific interactions and showed high-sensitivity and good specificity for AFP in human serum. This FET biosensor demonstrates good potential as a platform for early clinical diagnosis of tumor markers.
\textit{References}


Chapter 3

Development of FET biosensors for multiplexed detection of tumor markers for cancer diagnosis
3.1 Multiplexed detection of cytokeratin fragment 21-1 (CYFRA 21-1) and neuron-specific enolase (NSE) using multi-analyte FET biosensor for lung cancer differential diagnosis

3.1.1 Introduction

The detection of biomarkers is important for disease diagnoses [1]. ELISA is a conventional method to detect biomarkers. However, this technique requires labelling processes, which hinder monitoring of the probe/target interaction rapidly [2]. In comparison, FETs have emerged as an important new technique since they do not require any labeling [3-12] and enable high-sensitive detections, comparable to the other common label-free detection methods, including SPR [13-15] and QCM [16, 17] measurements. Furthermore, FETs are more attractive due to their small dimensions, less expensive and the possibility to integrate a large number of sensors on the same chip [3]. The FET biosensors are sensitive to the intrinsic electric charge of biomolecules. By modification of the FET gate surface with a biomolecular layer as a receptor, the changes in the gate voltage-drain current caused by the adsorption of analyte biomolecules can be detected. The immobilization of various biorecognition materials on the FET gate surface for biomarker analysis has been developed through surface modification technology. For examples: the immobilization of probes or receptors on aminosilane self-assembled monolayer (SAM)-modified Si$_3$N$_4$/SiO$_2$ gate insulator [5-7]; on a thiol SAM-modified gold electrode for extended gate [8]; on aminosilane SAM-modified SiO$_2$ gate insulator [9-12]. The FET biosensor can provide a label-free, rapid, simple and inexpensive analysis of various kinds of biomarkers such as DNA [5-7] and proteins [8, 10-12].

This work investigated the application of FET biosensors to tumor marker detection for lung cancer diagnosis. Lung cancer is the most common cause of cancer-related deaths, which can typically group into two large categories: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [18]. Cytokeratin fragment 21-1 (CYFRA 21-1) together with neuron-specific enolase (NSE) are useful tumor markers for the differentiation between two lung cancer types [18]. It is crucial for the patients that lung cancer type is classified early by detecting both two tumor markers (CYFRA 21-1 and NSE) levels, so that a specific treatment may be applied as soon as possible, resulting in the improvement of the survival rates [19]. Hence, a biosensor allowing rapid and multiplexed detection of CYFRA 21-1 and NSE is in demand. Biosensors for CYFRA 21-1 and NSE detection, including ELISA [2, 20],
radioimmunoassay (RIA) [21, 22] and chemiluminescence immunoassay (CLIA) [23, 24] were recently researched. These methods have a number of disadvantages including: ELISA implies lengthy steps and complex handling [2, 11]; RIA requires specialized equipments and long analytical time [25]; immunoassay with chromatography needs expensive instrumentation [26]. Moreover, these previous works didn’t show a capability for selective multiplexed detection of lung cancer biomarkers. To resolve previous limitations, I proposed an antibody-based multi-analyte FET biosensor to detect CYFRA 21-1 and NSE at the same time, with the potential application to lung cancer differential diagnosis. The multi-analyte FET biosensor (Fig. 3.1.1) described here consists of two transistors on the same chip. Each gate of the FET was immobilized with a different antibody and was capable of measuring a specific tumor marker. Herein, integrate two antibody types on the same chip. A sensor array for multiplexed detection of panels of tumor markers would be expected, by integrating more FETs on one chip [9]. This work provides a step towards the realization of this sensor arrays. On the other hand, the multi-analyte FET biosensor, as described herein, will help not only for the multiplexed detection of different two proteins, but will also help to save time, sample volume and financial resources.

I designed a multi-analyte biosensor based on FET and tested this sensor on the detection of two biomarkers at the same time. The probability of this sensor for the quantitative detection is demonstrated in Chapter 3.2.
Fig. 3.1.1 (a) Optical image of an FET chip and (b) schematic diagram of the multi-analyte FET biosensor for detection of multiple tumor markers. Each gate of the FET was immobilized with a different antibody; the reference electrode was Hg/Hg$_2$SO$_4$. Reprinted with permission from [32]. Copyright © 2015 Elsevier B.V.
3.1.2 Experimental

3.1.2.1 Reagents

The antigen, human CYFRA 21-1 was purchased from Acris Antibodies Inc. (San Diego, CA, USA). The antigen, human NSE was purchased from AbD Serotec (Oxford, UK). Monoclonal anti-CYFRA 21-1 antibody (mouse) and monoclonal anti-NSE antibody (mouse) were purchased from Gene Tex Inc. (Irvine, CA, USA). Human serum and 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), and all other chemicals were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). The proteins were used without further purification. Phosphate-buffered saline (PBS) (pH 7.4) was made in the laboratory and was prepared using 137 mM NaCl, 8.1 mM Na\textsubscript{2}HPO\textsubscript{4}·12H\textsubscript{2}O, 2.7 mM KCl, and 1.5 mM KH\textsubscript{2}PO\textsubscript{4}. Diluted PBS, 0.01 × PBS (0.1 mM PBS, pH 7.4), was prepared by diluting 1 × PBS (10 mM PBS) with ultrapure water. Antigen and antibody were diluted in 1 × PBS.

3.1.2.2 Preparation of antibody-immobilized FET biosensor

In this study, two types of FET biosensors were prepared: the first is a FET biosensor using a single type of antibody, hereafter called “single-analyte FET biosensor”, and the other is a biosensor composed of two FETs modified with different types of antibody separately, hereafter called “multi-analyte FET biosensor”. Both types of FET biosensor were based on the same FET chip consisting of two transistors (Fig. 3.1.1a). SiO\textsubscript{2}-gate FETs was developed with a high degree of chemical durability through surface modification technology using a SAM [9], and was successfully engaged in the technology transfer of the fabrication process to Toppan Printing Co., Ltd. (Tokyo, Japan). The size of each gate insulator is 10 μm (length) × 1000 μm (width). The modification process of the SiO\textsubscript{2} surface with APTES SAM and the cross-linker glutaraldehyde (GA) was the same as previously described [10]. Antibodies were allowed to react with each activated GA-modified FET at room temperature for 1 h. The surface of both gate insulators of the single-analyte FET biosensor was reacted with anti-CYFRA 21-1 antibody (50 μg/mL, 40 μL) or anti-NSE antibody (50 μg/mL, 40 μL). Alternatively, anti-CYFRA 21-1 antibody (50 μg/mL, 5 μL) and anti-NSE antibody (50 μg/mL, 5 μL) was deposited on each gate of multi-analyte FET biosensor, side-by-side, as shown in Fig. 3.1.1b. The residue on probe molecules immobilized FET
was washed by 0.01 × PBS. The coverage of the antibody-immobilized surface was calculated as 61% in a previous study for anti-human chorionic gonadotropin (hCG) antibody by using fluorescent measurement [10]. Considering almost the same size of antibody molecules employed in this study, the coverage would be the same degree. To avoid non-specific adsorption onto the surface of the biosensor, the antibody-immobilized surface was allowed to react with BSA (1 wt%) for 60 min.

3.1.2.3 Electrical measurements

The FET detects potential changes on its gate surface in terms of the intrinsic charge of target proteins that bind specifically to probe molecules immobilized on the gate surface, such as the threshold voltage shift ($\Delta V_g$), or the gate voltage ($V_g$)-drain current ($I_{ds}$) characteristics. The procedure for the electrical measurements was the same both for the single-analyte and multi-analyte FET biosensors. The measurements were made in the dark with a semiconductor parameter analyser (2612A, Keithley Instruments Inc.; Cleveland, OH, USA) at room temperature in 0.01 × PBS (pH 7.4) by sweeping the $V_g$ from −3 V to 0.5 V with a 0.1 V drain voltage. Hg/Hg$_2$SO$_4$ was used as a reference electrode. The relationship between the $V_g$ and $I_{ds}$ was measured. A threshold voltage shift, $\Delta V_g$ was calculated.
3.1.3 Results and discussion

3.1.3.1 Quantitative detection of tumor markers in PBS using a single-analyte FET biosensor

The response of the single-analyte FET biosensor, which uses a single type of antibody that is specific to only one tumor marker (i.e., either CYFRA 21-1 or NSE), was examined without blocking treatment. Analyzed the response of the anti-CYFRA 21-1 antibody-immobilized FET biosensor to CYFRA 21-1 (Fig. 3.1.2a), and the anti-NSE antibody-immobilized FET biosensor to NSE (Fig. 3.1.2b), with each analyte solution ranging from 1 ng/mL to 1 μg/mL. The $\Delta V_g$ was shifted in a positive direction after CYFRA 21-1 adsorption because CYFRA 21-1 (isoelectric point [pI] of 5.2 [27]) has a negative charge in PBS (pH 7.4). Similarly, the positive $\Delta V_g$ values for NSE was attributed to the intrinsic negative charge of NSE, as expected from its pI of 4.75 [28] and pH 7.4 in the present experimental condition. As shown in Fig. 3.1.2a (inset), the $\Delta V_g$ value of 0.8 ± 1.2 mV was obtained for the blank (0.01 x PBS). It was demonstrated that the $\Delta V_g$ values increased proportionally with an increase in CYFRA 21-1 concentration, from 1 ng/mL to 1 μg/mL. Compared with the magnitude of the $\Delta V_g$ observed after the addition of target protein-free PBS or the addition of 1 μg/mL non-specific analyte human serum albumin (HSA), the magnitude of the $\Delta V_g$ observed upon incubation of 1 ng/mL CYFRA 21-1 (8 ± 1.8 mV) was significant. Similarly, the magnitude of $\Delta V_g$ proportionally increased as the NSE concentration was increased from 1 ng/mL to 1 μg/mL (Fig. 3.1.2b). The specific and sensitive detection was achieved in the measurement of NSE, 10 ng/mL (13 ± 2.1 mV, see Fig. 3.1.2b, blank is 0.8 ± 0.3 mV) using the single-analyte FET biosensor. Thus, the detection limits for CYFRA 21-1 and NSE using the single-analyte FET biosensors were 1 ng/mL and 10 ng/mL, respectively. These numbers are significantly lower than the cut-off values (4 ng/mL for CYFRA 21-1 and 24 ng/mL for NSE [29]) necessary to distinguish between healthy individuals and those with lung cancer. The $\Delta V_g$ value for CYFRA 21-1 tends to be larger than that for NSE at the same concentrations. It should be noted here that the molecular weight of CYFRA 21-1 (40 kDa [27]) is smaller than that of NSE (96 kDa [30]), which leads to a greater molar concentration for CYFRA 21-1 than for NSE at the same mass concentration. In addition, the difference in the size of the target protein (tumor marker) would affect the magnitude of the FET response. Considering the size of CYFRA 21-1 (estimated to be equivalent to a sphere with a diameter of 4.5 nm [27]) and NSE (8.3 nm × 6.1 nm × 5.5 nm [28]), a portion of the NSE molecules bound to the antibody may remain outside of the charge-detectable region, because the Debye length
at the gate/solution interface is 7.5 nm in 0.01 × PBS (pH 7.4) [11], while the binding event between antibody and CYFRA 21-1 is expected to occur within the Debye length. Furthermore, the NSE molecules may approach more difficultly to the antibody on the surface due to steric hindrance. Those would cause the magnitude of $\Delta V_g$ observed.
Fig. 3.1.2 Quantitative detection of CYFRA 21-1 (a) and NSE (b) in PBS using antibody-immobilized FET without blocking treatment. Insets show the specificity of the FET biosensor, investigated using human serum albumin (HSA) as a negative control. Arrows indicate the cut-off value (4 ng/mL for CYFRA 21-1 and 24 ng/mL for NSE). The error bars show the standard deviation (n=5). Reprinted with permission from [32]. Copyright © 2015 Elsevier B.V.
3.1.3.2 Quantitative detection of tumor markers in human serum using single-analyte FET biosensor

From a point-of-care perspective, the direct and facile measurement of a tumor marker in blood serum is necessary. However, without any blocking treatment, a certain amount of response may be due to the nonspecific adsorption of other protein(s) in human serum. To reduce such nonspecific adsorption, introduce the well-known blocking reagent, BSA [10]. Fig. 3.1.3a and 3.1.3b show the response of the anti-CYFRA 21-1 antibody-immobilized FET and anti-NSE antibody-immobilized FET, respectively. In both FET biosensors, FET responses to human serum were decreased after the introduction of BSA (from 23 to 4.9 mV in CYFRA-21-1 [Fig. 3.1.3a]; from 19.3 to 1.9 mV in NSE [Fig. 3.1.3b]), suggesting that the nonspecific adsorption of other proteins in the human serum was minimized by addition of the blocking reagent. Furthermore, BSA blocking was demonstrated to affect the FET response to the target proteins in human serum (from 37.2 ± 12.8 to 44 ± 6 mV for CYFRA 21-1 [Fig. 3.1.3a]; from 32.5 ± 7.6 to 27.7 ± 4.6 mV for NSE [Fig. 3.1.3b]). The decrease in standard deviation (error bars) is suggestive of the increase of the sensitivity. It should be noted here that the FET response to CYFRA 21-1 and NSE was increased and decreased, respectively, with the BSA blocking treatment. The other (contaminating) proteins in human serum may affect responses in two ways. First, the nonspecific adsorption of charged proteins onto the GA-modified surface may lead to a shift in the \( \Delta V_g \) value. In addition, the nonspecific proteins may bind to the sensor and hide the recognition/binding site of the antibody, leading to a decrease in the response caused by the target protein. The decrease in the response to NSE after blocking may be the result of a reduction in the nonspecific adsorption onto the GA-modified surface, as observed in the response to the blank (serum only [Fig. 3.1.3b]). Although a similar decrease in response is expected for CYFRA 21-1, there was only a small increase in response observed after blocking. We hypothesize that smaller CYFRA 21-1 molecules become able to bind to their antibody without hindrance by adsorbed nonspecific proteins, while larger NSE molecules do not.
Fig. 3.1.3 Comparison of sensor responses to protein addition between non-blocked and BSA-blocked FETs. Target proteins are CYFRA 21-1 (a) and NSE (b). The error bars show the standard deviation (n=5). Reprinted with permission from [32]. Copyright © 2015 Elsevier B.V.
Next, tumor markers were quantitatively detected in human serum using antibody-immobilized single-analyte FET biosensors with BSA as a blocking reagent. It is demonstrated that the FETs quantitatively detected CYFRA 21-1 (Fig. 3.1.4a) and NSE (Fig. 3.1.4b) at varying target protein concentrations ranging from 1 ng/mL to 1 μg/mL. The magnitude of $\Delta V_g$ gradually increased as the target protein concentration was increased in the above range. The magnitude of the $\Delta V_g$ at 1 ng/mL CYFRA 21-1 (10.8 ± 2.7 mV) in human serum was greater than that of CYFRA 21-1-free human serum (4 ± 1.5 mV), which used as a blank. As shown in Fig. 3.1.4a, with the use of BSA as a blocking reagent, the single-analyte FET biosensor (anti-CYFRA 21-1 antibody-immobilized FET) achieved the detection of CYFRA 21-1 at the low level of 1 ng/mL in human serum, which is less than the clinical cut-off point [29]. However, the anti-NSE antibody-immobilized FET was faced with critical limit to detect NSE in human serum below the cut-off value (24 ng/mL [29]): the magnitude of the $\Delta V_g$ of NSE-free human serum was 2.5 ± 2.1 mV, while the magnitude of the $\Delta V_g$ at 24 ng/mL NSE in human serum would be approximately 8 mV (Fig. 3.1.4b). Here, 4 ng/mL corresponds to 0.1 nM for CYFRA 21-1, while 24 ng/mL is equivalent to 0.25 nM for NSE. As mentioned in Chapter 3.1.3.1, the size of protein would affect the magnitude of $\Delta V_g$ even if the molar concentration were the same. As assumed above, the binding of big molecule NSE to the antibody on the gate surface might be limited by steric hindrance.
Fig. 3.1.4 Quantitative detection of CYFRA 21-1 (a) and NSE (b) in human serum by using antibody-immobilized FET with BSA blocking. Arrows indicate the cut-off value (4 ng/mL for CYFRA 21-1 and 24 ng/mL for NSE). The error bars show the standard deviation (n=5). Reprinted with permission from [32]. Copyright © 2015 Elsevier B.V.
3.1.3.3 Application to multi-analyte FET biosensors for detection of multiple tumor markers

Due to the limited specificity of tumor markers, the measurement of a single tumor marker is usually not sufficient to diagnose cancer [31]. Thus, challenged to develop a multi-analyte FET biosensor in an attempt to accomplish the detection of multiple tumor markers. As mentioned in Chapter 3.1.1, to test the capabilities of a multi-analyte FET biosensor for multiplexed detection of tumor markers, firstly focused on CYFRA 21-1 and NSE, which are generally used to investigate different types of lung cancer (NSCLC or SCLC). For this purpose, immobilize the surface of each of the gate insulators on multi-analyte FET biosensors with anti-CYFRA 21-1 antibody and anti-NSE antibody separately as described in Chapter 3.1.2.2. Examine the specificity and sensitivity of the multi-analyte FET biosensor without blocking, for the detection of CYFRA 21-1 and NSE from a single droplet (20 μL) of analyte solution (made in PBS). As shown in Fig. 3.1.5, little response (0.4 mV) was obtained when the chip was exposed to PBS (blank). Moreover, chip exposure to HSA, used as a negative control, produced a negligible response (i.e., 3.2 mV for anti-CYFRA 21-1 antibody-immobilized gate; 1.9 mV for anti-NSE antibody-immobilized gate) from the multi-analyte FET biosensor. This indicates that nonspecific binding was minimal. After the addition of a solution containing a single-analyte at the concentrations below its respective cut-off value (1 ng/mL CYFRA 21-1 or 20 ng/mL NSE), an FET response with significant magnitude was observed only from the gate possessing the cognate antibody. The magnitude of the response obtained from the non-cognate gate was approximately equal to that of the negative control. When the analyte solution was a mixture of 1 ng/mL CYFRA 21-1 and 20 ng/mL NSE (multi-analyte), a significant response was obtained from both the anti-CYFRA 21-1 and anti-NSE antibody-immobilized gates. The results suggest that this multi-analyte FET biosensor has potential for the clinical diagnosis of different categories of lung cancer.
Fig. 3.1.5 Detection of target proteins at the concentrations below the cut-off value (1 ng/mL CYFRA 21-1; 20 ng/mL NSE) in PBS using a multi-analyte FET biosensor without BSA blocking. HSA was added as a negative control. Both single-analyte solution (CYFRA 21-1 or NSE) and multi-analyte solution (a mixture of CYFRA 21-1 and NSE) were measured. The error bars show the standard deviation (n=5). Reprinted with permission from [32]. Copyright © 2015 Elsevier B.V.
Detection of biomarkers in clinically relevant samples such as blood serum is required for cancer diagnosis. To this end, examine the potential of the multi-analyte FET biosensors for detecting CYFRA 21-1 and NSE in human serum, similar to the single-analyte FET biosensor examined in Chapter 3.1.3.2, with BSA blocking (Fig. 3.1.6). The values of $\Delta V_g$ to analyte-free human serum were 2.3 ± 1.8 mV and 1.6 ± 2.4 mV at the anti-CYFRA 21-1 and anti-NSE antibody-immobilized gates, respectively. These values were similar to (or slightly smaller than) those separately obtained using single-analyte FET biosensors (shown in Fig. 3.1.3). After the addition of a mixture of 1 ng/mL CYFRA 21-1 and 20 ng/mL NSE in human serum, the $\Delta V_g$ values of 9.6 ± 2.7 mV and 7.8 ± 3.5 mV were obtained from the anti-CYFRA 21-1 and anti-NSE antibody-immobilized gates, respectively. In comparison to the blank, the multi-analyte FET biosensor achieved the detection of CYFRA 21-1 in human serum at the low level of 1 ng/mL but was faced with critical limit to detect 20 ng/mL NSE in human serum. It should be noted that compared with the results using PBS (Fig. 3.1.5), the magnitude of the response to CYFRA 21-1 was similar; however, the response to NSE was decreased. This tendency following BSA blocking was similar to that observed for the single-analyte FET biosensors in Chapter 3.1.3.2 and Fig. 3.1.3. By using the mixture of 10 ng/mL CYFRA 21-1 and 100 ng/mL NSE in human serum, the multi-analyte FET biosensor showed a response to NSE at low concentration, down to 100 ng/mL, with a $\Delta V_g$ of 11.9 ± 4 mV under the present conditions. These results demonstrated that the sensitivity for two integrated antibody-immobilized biosensors is different. The limit detection of CYFRA 21-1 and NSE in human serum was found to be 1 ng/mL and 100 ng/mL, respectively. As discussed in Chapter 3.1.3.1, the difference of the sensitivity between CYFRA 21-1 and NSE might be mainly due to steric hindrance. Thus, multi-analyte FET biosensors have a potential to satisfy the need for detection of multiple tumor markers at the same time. This would lead to a decrease in measurement time and sample volume.

Detection of multiple tumor markers would be useful for quickly and easily identifying a cancerous region in the body. In this study, the proposed biosensor showed potential to determine the concentration of CYFRA 21-1 and NSE at each desired level, suggesting that it might easily identify lung cancer type. In the future, by integrating multiple sensors on one chip, the sensor system would be useful not only for lung cancer but also for other cancers or severe diseases.
Fig. 3.1.6 Detection of multiple target proteins (a mixture of CYFRA 21-1 and NSE) at low concentrations in human serum using a BSA-blocked multi-analyte FET biosensor. The error bars show the standard deviation (n=5). Reprinted with permission from [32]. Copyright © 2015 Elsevier B.V.
3.1.4 Summary

The label-free quantitative detection of CYFRA 21-1 and NSE, two lung cancer tumor markers, in both PBS and human serum using FET biosensors was demonstrated. To detect CYFRA 21-1 and NSE from one droplet of analyte solution, a multi-analyte FET biosensor was developed, which provides significant advantages over single-analyte biosensors in terms of convenience, measurement time, sample volume, and cost. This multi-analyte FET biosensor may offer an alternative to differential diagnoses of lung cancer. A multi-analyte detection of panels of other tumor markers could be expected, by assembling more FETs on one chip.
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3.2 Concentration-dependence detection of CYFRA 21-1 and AFP using multi-analyte FET biosensor for cancer diagnosis

3.2.1 Introduction

Cancer, one of the most common causes of mortality and morbidity worldwide, has more than 200 distinct types associated with it, affecting over 60 human organs. The early diagnosis combined with the effective treatment is crucial for the survival of a cancer patient. Thus, developing technologies applicable for cancer early diagnosis is an important task for cancer researchers. The detection of an effective cancer biomarker was of great significance in the early diagnosis of cancer [1-6]. Many researches on the biosensors combined with various transducers have been carried out to detect the levels of cancer biomarkers. However, a single cancer biomarker cannot provide sufficient information on cancer diagnosis, because most cancers have more than one biomarker associated with their incidence [7-9]. Therefore, the multi-analyte detection of multiple cancer biomarkers has become more and more important for cancer diagnosis. The multi-analyte biosensor for multi-analyte detection has been attracted much attention as a promising tool to improve the diagnostic value. Additionally, the multi-analyte biosensor has the advantages of short analytical time, low cost, simple procedure and small sample consumption as compared to single-analyte biosensors [9].

Many methods based on electrochemical multi-analyte immunoassay have been well developed for multi-analyte determination [10-13]. Wilson and Nie used a sensor consisted of an array of immunosensing electrodes, each electrode containing a different immobilized antigen, for the detection of seven tumor markers [12]. Wu et al. reported a screen printed array for simultaneous electrochemical determination of tumor markers [13]. However, traditional immunoassay usually requires labelling process, resulting in the lack of simpleness and rapidness [8, 9, 14, 15]. In comparison, field effect device has attracted increasing attention, among the variety of transducer concepts proposed for label-free detection of biomolecules [9, 14-24]. The biosensor based on field effect device enables high-sensitive detection, comparable to the other label-free measurements, such as surface plasmon resonance (SPR) [25, 26] and quartz crystal microbalance (QCM) [27, 28]. Additionally, these devises provide a lot of potential advantages including small size and weight, the prospect of low-cost mass production and the possibility of on-chip integration of arrays [9, 15]. Over the past decade, some multi-analyte detection methods based on field effect device have been proposed [14, 29-31]. Zhang et al. reported a work on multiplexed detection of several cancer markers.
using a silicon-nanowire (SiNW) array [14]. Jia et al. developed a multi tumor marker detection system based on light addressable potentiometric sensor (LAPS) [31]. The development of biosensor assays based on field effect device is of great significance in multi-analyte determination of biomolecules.

Interested in developing biosensors based on field effect transistors (FETs) for multi-analyte detection of cancer biomarkers, particularly in biological samples (e.g. serum) with appropriate sensitivity but are simple to fabricate and use. In Chapter 3.1, two antibody types were successfully integrated on the same FET chip as probe molecules which were capable of measuring a specific target protein [24]. As a result, two lung cancer biomarkers, cytokeratinfragment 21-1 (CYFRA 21-1) and neuron-specific enolase (NSE) were detected at the same time using our fabricated antibody-based multi-analyte FET biosensor, showing a potential of the application to lung cancer differential diagnosis. This section reports the use of this sensor for the diagnosis of two different cancer types. CYFRA 21-1, a lung cancer biomarker, and \( \alpha \)-fetoprotein (AFP), a liver cancer biomarker, were selected as the target analytes. Herein, monoclonal antibody of CYFRA 21-1 and AFP were immobilized on the different FETs respectively integrated on one chip (Fig. 3.2.1c). Furthermore, the concentration-dependence detection of CYFRA 21-1 and AFP at the same time in human serum were performed. The objectives were as follows: (1) multi-analyte detection of CYFRA 21-1 and AFP in human serum for the diagnosis of two different cancer types, (2) concentration-dependence detection of CYFRA 21-1 and AFP using the multi-analyte FET biosensor.
Fig. 3.2.1 Multi-analyte FET biosensor. (a) Schematic illustration of an antibody-immobilized FET used for cancer biomarker detection. The antibody was immobilized on the glutaraldehyde-modified gate surface of the FET as a receptor; the reference electrode was Hg/Hg₂SO₄. (b) Optical image of an FET chip. (c) Schematic showing two integrated FETs, FET A and FET B, where the gates were immobilized with different antibody receptors (A, red; B, blue). A mixture of cancer biomarkers (A, red; B, blue) that adsorbed specifically to its receptors, respectively, will produce a change in the surface-charge density only on the related FET.
3.2.2 Experimental

3.2.2.1 Reagents

The antigen, human CYFRA 21-1 was purchased from Acris Antibodies Inc. (San Diego, CA, USA). The antigen, human AFP was purchased from MP Biomedicals, LLC (City, State abbreviation if US, Country Santa Ana, CA., USA.). Monoclonal anti-CYFRA 21-1 antibody (mouse) was purchased from Gene Tex Inc. (Irvine, CA, USA). Monoclonal anti-AFP antibody (human) was purchased from Nippon Bio-test Laboratories Inc. (City, State abbreviation if US, Country Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). 3-aminopropyltriethoxysilane (APTES) and human serum were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). All other chemicals were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Phosphate-buffered saline (PBS) (pH 7.4) was made in the laboratory and was prepared using 137 mM NaCl, 8.1 mM Na$_2$HPO$_4$·12H$_2$O, 2.7 mM KCl, and 1.5 mM KH$_2$PO$_4$. Antigen and antibody were diluted in 1 × PBS. The proteins were used without further purification.

3.2.2.2 Preparation of multi-analyte antibody-immobilized FET biosensor with BSA blocking

The FET chip consists of two transistors (Fig. 3.2.1b, c). SiO$_2$-gate FETs were developed with a high degree of chemical durability through surface modification technology using a self-assembled monolayer (SAM) [20], and has engaged in the technology transfer of the fabrication process to Toppan Printing Co., Ltd. (Tokyo, Japan). The size of each gate insulator is 10 μm in length and 1000 μm in width. The modification process of APTES SAM and the cross-linker glutaraldehyde (GA) in this work is the same as the one described in our previous paper. Antibodies were allowed to be reacted with the activated GA-modified FETs at room temperature for 1 h. Anti-CYFRA 21-1 antibody (50 μg/mL, 5 μL) and anti-AFP antibody (50 μg/mL, 5 μL) was deposited on each gate of multi-analyte FET biosensor, side-by-side, as shown in Fig. 3.2.1c. The residue on probe molecules immobilized FET was washed by 1.0 mL of 0.01 × PBS five times. Then the antibody-immobilized surface was allowed to react with BSA (1 wt%) for 60 min to avoid non-specific adsorption onto the FET gate surface.

3.2.2.3 Electrical measurements
The gate voltage ($V_g$)-drain current ($I_{ds}$) characteristics were measured before and after antigen-antibody reaction with a semiconductor parameter analyser (2612A, Keithley Instruments Inc.; Cleveland, OH, USA) in the dark by sweeping the $V_g$ from $-3$ V to $0.5$ V with a $0.1$ V drain voltage. A calculated threshold voltage shift, $\Delta V_g$, will be discussed as an FET response. All measurements were made with $0.01 \times$ PBS (pH 7.4) at room temperature. Hg/Hg$_2$SO$_4$ was used as a reference electrode.

3.2.2.4. Observation of the surface morphology of multi-analyte FET biosensor

Surface morphologies before and after the immobilization of probes onto the FET surface and the after the blocking treatment by using BSA were examined using atomic force microscopy (AFM). The topographic images of the FETs gate surface were investigated by AFM (Shimadzu Co., SPM-9600) in dynamic mode. Silicon cantilever (Olympus Co., OMCL-AC240TSC2, spring constant 2 N/m, resonance frequency 70 kHz) was used in the measurement. The image size was $1 \mu m \times 1 \mu m$ with $512 \times 512$ pixels. Roughness parameters were obtained from AFM images.
3.2.3 Results and discussion

3.2.3.1 Multi-analyte detection of cancer biomarkers in human serum

The label-free detection of biomarkers in real biological samples, like serum, plasma, urineis and whole blood, is highly desirable, more practical from a point of care view. Here, the multi-analyte FET biosensor for the detection of two useful cancer biomarkers in human serum was examined. A lung cancer biomarker, CYFRA 21-1, and a liver cancer biomarker, AFP, were selected as the target analytes. Since the nonspecific adsorption of other protein(s) in human serum may affect the sensitivity of biosensors when used for the estimation of specific analytes, we introduced BSA, a well-known blocking agent to help reduce such nonspecific binding. Before the BSA blocking, anti-CYFRA 21-1 antibody and anti-AFP antibody were immobilized on the surface of each FET gate insulators separately (Fig. 3.2.1c), as described in Chapter 3.2.2.2, to capture the target analytes. Firstly, the response of the BSA-blocked antibody-immobilized multi-analyte FET biosensor to a mixture of CYFRA 21-1 and AFP (multi-analyte) in human serum was analysed. As shown in Fig. 3.2.2, a negligible response (i.e., 1.7 ± 2.8 mV for anti-CYFRA 21-1 antibody-immobilized gate; 1.8 ± 3.5 mV for anti-AFP antibody-immobilized gate) was obtained when the chip was exposed to analyte-free human serum (blank). This result also indicates that the nonspecific adsorption of other protein(s) in human serum was minimal. After the addition of a mixture of 1 ng/mL CYFRA 21-1 and 10 ng/mL AFP in human serum, a response (i.e., 10.6 ± 2.3 mV for anti-CYFRA 21-1 antibody-immobilized gate; 11.7 ± 3.1 mV for anti-AFP antibody-immobilized gate) was obtained from both FET gates. The positive $\Delta V_g$ values for CYFRA 21-1 and AFP were attributed to the intrinsic negative charge of CYFRA 21-1 and AFP, as expected from their isoelectric point [pI] (i.e., 5.2 for CYFRA 21-1 [32]; 4.9 for AFP [33]) and pH 7.4 in the present experimental condition. Compared with the magnitude of the $\Delta V_g$ observed after the addition of analyte-free human serum, the magnitude of the $\Delta V_g$ observed upon incubation of 1 ng/mL CYFRA 21-1 and 10 ng/mL AFP was significant. In another word, the multi-analyte FET biosensor achieved the detection of CYFRA 21-1 and AFP in human serum at the low level of 1 and 10 ng/mL, respectively, which have met the cut-off value for normal level (i.e., 4 ng/mL for CYFRA 21-1 [34]; 10 ng/mL for AFP [35]). The results suggest that this biosensor has potential for the practical detection of serum samples for the clinical diagnosis of cancers.
Fig. 3.2.2 Detection of multiple cancer biomarkers (a mixture of CYFRA 21-1 and AFP) at low concentrations (1 ng/mL CYFRA 21-1; 10 ng/mL AFP) in human serum using a multi-analyte FET biosensor with BSA blocking. The error bars show the standard deviation (n=3).
3.2.3.2 Concentration-dependence detection of cancer biomarkers in human serum using multi-analyte FET biosensor

Next, CYFRA 21-1 (Fig. 3.2.3a) and AFP (Fig. 3.2.3b) in human serum were quantitatively detected using the BSA-blocked antibody-immobilized multi-analyte FET biosensor. The multi-analyte samples, CYFRA 21-1 at varying CYFRA 21-1 concentrations ranging from 1 to 100 ng/mL (Fig. 3.2.3a, red diamond) with 10 ng/mL AFP (Fig. 3.2.3a, yellow square), contained in human serum were detected. From the magnitude of $\Delta V_g$ obtained from the anti-CYFRA 21-1 antibody-immobilized gate (Fig. 3.2.3a, red diamond); which can be seen that the magnitude of $\Delta V_g$ gradually increased as CYFRA 21-1 concentration was increased in the above range. In addition, the response to human serum containing 10 ng/mL AFP (blank) was $2.2 \pm 2.9$ mV, which is similar to the response to human serum without any analyte (i.e., blank, $1.7 \pm 2.8$ mV, see Fig. 3.2.2). Thus, compared with the blank, the detection limit for CYFRA 21-1 in human serum using the multi-analyte FET biosensor was found to be 1 ng/mL, whether AFP exists or not. On the other hand, the similar magnitudes of the $\Delta V_g$ were obtained from the anti-AFP antibody-immobilized gate (Fig. 3.2.3a, yellow square), showing that the effect on the existence of CYFRA 21-1 was minimal. Thus, this biosensor shows good selectivity for the multi-analyte detection. Similarly, as shown in Fig. 3.2.3b, AFP was quantitatively detected in human serum even with the existence of 1 ng/mL CYFRA 21-1 at varying concentrations ranging from 1 to 100 ng/mL. In comparison to the blank (i.e., $1.3 \pm 3.2$ mV for human serum containing 1 ng/mL CYFRA 21-1, see Fig. 3.2.3b; $1.8 \pm 3.5$ mV for human serum without any analyte, see Fig. 3.2.2), the limit detection of AFP in human serum was found to be 10 ng/mL. It is demonstrated that this biosensor achieved the quantitative detection of CYFRA 21-1 and AFP from a multi-analyte sample, at the low level of 1 and 10 ng/mL in human serum, respectively, which have met the clinical cut-off point [34, 35].

It should be noted that the $\Delta V_g$ value for CYFRA 21-1 tends to be larger than that for AFP at the same mass concentrations. This may due to CYFRA 21-1 has a greater molar concentration than AFP according to their molecular weight (i.e., 40 kDa for CYFRA 21-1 [32]; 70 kDa for AFP [36]). From the assumption, the second reason may come from the different size of CYFRA 21-1 (estimated to be equivalent to a sphere with a diameter of 4.5 nm [32]) and AFP (5 nm × 5 nm × 5 nm [37]). Compared with CYFRA 21-1 in smaller size, the adsorption of the AFP molecules onto the antibody-immobilized surface might be limited by steric hindrance, resulting in a decrease of the effective signals. On the other hand, under the same Debye length
condition (7.5 nm in 0.01 × PBS [24]), the binding event between antibody and AFP might occur outside the charge-detectable region, while the adsorbed CYFRA 21-1 is expected to inside the Debye length, leading to a difference in the magnitude of the FET response.

Compared all the data obtained from the single-analyte FET biosensor and the mulianalyte FET biosensor [52, 55], it is clear that the magnitude of the $\Delta V_g$ observed upon the incubation of the same target biomarkers for both two types of FET biosensors were similar, due to the immobilization condition of two types of FET gate surface were the same, indicating that the mulianalyte FET biosensor is comparable to the single-analyte FET biosensor for the biomarker detection. Thus, the mulianalyte FET biosensor allows selective and sensitive detection of multiple biomarkers at the same time.
Fig. 3.2.3 Concentration-dependence detection of CYFRA 21-1 (a) and AFP (b) with the existence of other cancer biomarkers, 10 ng/mL AFP (a) and 1 ng/mL CYFRA 21-1 (b), in human serum by using BSA-blocked multi-analyte FET biosensor. Arrows indicate the cut-off value (4 ng/mL for CYFRA 21-1 and 10 ng/mL for AFP). The error bars show the standard deviation (n=3).
3.2.3.3 Comparison of sensor performance between multi-analyte FET biosensor and single-analyte FET biosensor

In order to confirm the sensor performance of multi-analyte FET biosensor, the FET response obtained by using multi-analyte FET biosensor was compared with the one obtained by using single-analyte FET biosensor. The results were shown in Table 3.1. It’s clear that the limit detection of CYFRA 21-1 (1 ng/mL) and AFP (10 ng/mL) were the same by using multi and single FET biosensors. Thus multi-analyte FET biosensor has the same sensitivity with the single one. The reason might be the immobilization conditions of two sensor types were the same. It proved that the probe molecules were well immobilized on each sensing area of the multi sensor after controlling the immobilization process. The multi-analyte FET biosensor shows good selectivity for the multiplexed detection.

Then, this assumption was proved by observing the surface morphology of multi-analyte FET biosensor and comparing them with the single one using AFM. The results were shown in Fig. 3.2.4. The similar AFM images suggested that the immobilization of antibody onto each FET gate surface of multi-analyte FET biosensor was succeeded compared the surface morphology with the one of single-analyte FET biosensor. The decrease of $R_q$ (roughness) value after addition of BSA suggested that BSA molecules filled in the spaces where the immobilized antibody did not exist. It is demonstrated that the two sensors types have the same surface condition that resulting in the same capability for the biomarker detection. In another word, multi-analyte FET biosensor is comparable with single-analyte FET biosensor; in addition, it has lots of advantages over the single sensor, such as short analytical time, small sample volume and simple process.
Table 3.1 The FET response of CYFRA 21-1 obtained by using (a) single-analyte FET biosensor and (b) multi-analyte FET biosensor. The FET response of AFP obtained by using (c) single-analyte FET biosensor and (d) multi-analyte FET biosensor.

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Fig. 3.2.4 AFM images of surfaces of anti-CYFRA 21-1 antibody-immobilized (A) single-analyte FET and (B) multi-analyte FET biosensors; BSA-blocked anti-CYFRA 21-1 antibody-immobilized (C) single-analyte FET and (D) multi-analyte FET biosensors; anti-AFP antibody-immobilized (E) single-analyte FET and (F) multi-analyte FET biosensors; BSA-blocked anti-AFP antibody-immobilized (G) single-analyte FET and (H) multi-analyte FET biosensors. The roughness ($R_q$) value: A=0.934; B=0.841; C=0.896; D=0.812; E=1.146; F=0.974; G=1.056; H=0.936. Z range = 10 nm.
3.2.4 Summary

A label-free multi-analyte biosensor based on FET for the detection of cancer biomarkers is developed, by integrating two antibody types on the same FET chip as probe molecules. Application of our sensor to the label-free, multiplexed, quantitative, sensitive detection of CYFRA 21-1 and AFP in human serum, for the diagnosis of two different cancer types, is demonstrated. The concentration ranges of CYFRA 21-1 and AFP were 1–100 ng/mL with the detection limits of 1 and 10 ng/mL, respectively, which met the clinical level, showing the potential for practical detection in clinic serum samples. The proposed biosensor offers an alternative to the label-free multi-analyte quantitative detection of biomarkers, providing a step towards the realization of sensor arrays used for the evaluation of the multiple markers in serum. The probability of error, both in false negative and false positive, will be reduced by taking simultaneously exclusive biomolecule data. The biosensor based on FET has many advantages in miniaturization, standardization, and mass-production.
References

[15] M.J. Schöning, A. Poghossian, Recent advances in biologically sensitive


Chapter 4

General Conclusions
4.1 Conclusions

The early detection of biomarkers is very important to be developed for disease diagnoses. Field effect transistors (FETs) have emerged as an important new technique since they do not require any labeling and enable high-sensitive detections. Furthermore, FETs are more attractive due to their small dimensions, less expensive and the possibility to integrate a large number of sensors on the same chip. The FET biosensor can provide a label-free, rapid, simple and inexpensive analysis of various kinds of biomarkers. However, FET biosensors need to be further developed to meet the requirements of practical diagnostic applications, for examples, (1) the detection at low concentrations where the sensitivity of FET need to be enhanced; (2) multiplexed detection of different biomarkers where arrays of sensors need to be developed on the same chip.

This dissertation described an approach to overcome the above two big problems that will bring FET biosensors close to diagnostic tools in practical application.

In Chapter 2, the high-sensitive detection of biomarkers using FET biosensor was achieved by using small receptor molecules, antigen or Fab. To enhance the sensitivity, small receptors enable a binding reaction to occur within the Debye length, which is related to the detection range of the charged target protein in solution, were used. This resulting in an enhancement of sensitivity and lower detection limits of the sensing system.

Chapter 2.1 describes the use of antigen as a small receptor to improve the sensitivity of FET biosensors. In comparison with the antibody-immobilized FET, the antigen-immobilized FET exhibited a higher response to IgE, a biomarker for allergy detection, suggesting that the smaller receptor not only makes the more effective use of the charge-detectable region for the FET-based detection in terms of Debye length, but also provides more recognition sites for target molecules and greater ability to block nonspecific adsorption of non-related proteins because of the closely-packed immobilized receptors. In addition, the application of the antigen to FET biosensor gives an advantage in the identification of the specific allergen. These results show that the small receptor of antigen is more effective than the antibody in the allergy detection using FET biosensors. Chapter 2.2 describes the use of Fab as a small receptor to achieve the high-sensitive detection of liver cancer biomarker, AFP in human serum.
with BSA blocking. The small receptor Fab, immobilized on a sensing surface as small as 2-3 nm, offers a higher degree of sensitivity and a wider concentration range (100 pg/mL-1 μg/mL) for the FET detection of AFP in buffer solution, compared to the whole antibody. Therefore, the use of a small Fab probe molecule instead of a whole antibody is shown to be effective for improving the sensitivity of AFP detection in FET biosensors. Furthermore, the Fab-immobilized FET subjected to a blocking treatment, to avoid non-specific interactions, could sensitively and selectively detect AFP in human serum.

Thus, the application of small receptors, antigen or Fab, to FET biosensor shows an advantage in high-sensitive detection of biomarkers for disease diagnosis. The high-sensitive detection (met the clinical level) of a single biomarker was achieved. To obtain accurate diagnostic information, the multiplexed detection of multiple biomarkers is better.

In Chapter 3, the multiplexed detection of two biomarkers at the same time was achieved by integrating two receptor types on the same chip. A multi-analyte FET biosensor was developed. It was found that the multi-analyte FET biosensor was capable of detecting tumor markers in both phosphate-buffered saline and human serum. The multi-analyte FET biosensor, as described herein, will help for lung cancer differential diagnosis with advantages of simple and rapid detection procedures, low sample consumption, and low cost. Additionally, the probability of error, both in false negative and false positive, will be reduced by taking simultaneously exclusive biomolecule data.

Chapter 3.1 investigated the possibility of multi-analyte FET biosensor for differential diagnosis of lung cancer. Cytokeratin fragment 21-1 (CYFRA 21-1) and neuron-specific enolase (NSE), two useful tumor markers for the differentiation between two lung cancer types, were detected. The limit detection of CYFRA 21-1 and NSE in human serum were found to be 1 and 100 ng/mL, respectively. The proposed biosensor showed potential to determine the concentration of CYFRA 21-1 and NSE at each desired level, suggesting that it might easily identify lung cancer type. Chapter 3.2 investigated the possibility of multi-analyte FET biosensor for differential diagnosis of lung cancer and liver cancer. The multi-analyte FET biosensor achieved the detection of CYFRA 21-1 and AFP in human serum at the low level of 1 and 10 ng/mL, respectively, met the clinical level. This work provides a step towards the realization of sensor arrays
for multiplexed detection of panels of biomarkers. The multi-analyte FET biosensor, as described herein, will help not only for the multiplexed detection of different biomarkers, but will also provide significant advantages over single-analyte biosensors in terms of convenience, measurement time, sample volume and financial resources. In the future, by integrating more sensors on one chip, the sensor system would be useful for more other cancers or severe diseases.

FET biosensors have been attracting more and more attention and application in the biomarker detection due to the merits of label-free, fast detection and simple preparation. However, detection of electrically charged proteins using FETs is limited by ionic screening by the large probe molecules adsorbed to the transistor gate surface, reducing sensor responsiveness. In addition, the development of the multi-analyte detection of biomarkers is of particular significance because of the limited specificity of biomarkers, that the measurement of a single biomarker is usually not sufficient to diagnose disease. This work showed a method to achieve the detection of biomarkers at low concentrations in complex samples (e.g., serum) using FET biosensor. The FET biosensor immobilized by small receptors is attributed to the early disease diagnosis. On the other hand, the multi-analyte FET biosensor provide a step towards the realization of sensor arrays. It may have value in the development of sensor arrays suitable for point of care. The FET biosensor described in this dissertation will be very charming for the clinical diagnostic application after the experiment on real samples.
4.2 Future work

In this work, I used a separated glass reference electrode for the detection based on FET. Because the achievement of full-integration of sensing chip will bring FET biosensor close to point of care testing (POCT), which is an important thought for practical application. Therefore, in the future work, the integration of the reference electrode on the same chip has been taken in my consideration. At the present study, I used Hg/Hg$_2$SO$_4$ as a reference electrode, which is used in some cases where the use of chloride ions is not desirable. Actually, in the case of my work, to detect proteins in human serum, there is no effect on chloride ions. Therefore, the use of Ag/AgCl as a reference electrode would show a comparable performance. In addition, the use of mercury became less popular and the Hg/Hg$_2$SO$_4$ reference electrode is usually a glass electrode, which is a difficult part for miniaturization of the biosensor. Therefore, the integration of an Ag/AgCl reference electrode on one FET chip is desirable, that will realize fully integrated miniature biosensors.

Meanwhile, there are many aspects to the FET-based biosensors that need to be further improved for practical applications in diagnosis, such as the life-time and the stability. I predict a bright future for FET-based biosensors for clinical diagnostic of disease biomarkers because of its superior performance.
List of Achievements

1. Original Articles


2. Presentations


Biomarker’, 225th Meeting of the Electrochemical Society (ECS), May 13th, 2014, Orlando, USA.


3. Awards

‘Poster session award’, in the 6th International Workshop on Advanced Electrochemical Power Sources, Tianjin, P.R. CHINA.
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