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Development of immunoFET biosensor for detection of 3-nitrotyrosine

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ABSTRACT

3-Nitrotyrosine (3-NT) is one of the important biomarkers for the detection of excess of oxidative and nitrosative stress in the body, and derived from peroxynitrite-induced nitration of free and protein-bound at tyrosine residues. It was increased with oxidative/nitrosative stress-induced apoptosis in various tumor cells and many human diseases. Therefore, the analytical methods to detect 3-NT levels in biological samples are important for *in vitro* study of the apoptotic mechanism of the compounds and for the diagnosis of 3 NT-related diseases. In this study, an accurate, sensitive, and specific assay was developed for 3-NT based on the immunoFET biosensors and the effects of protein-A for orientated immobilization of antibody were investigated on the sensitivity of this biosensors. The comparison of sensor performance among two immobilization methods revealed that the assay sensitivity was appreciably improved and better with antibody attached covalently to the sensor surface than the immobilization method using protein-A. In addition, with this covalent immobilization technique, the output voltage shifts increased linearly (R^2 = 0.9923) across the 3-NT concentrations range from 10 to 1000 ng/mL and the limit of quantification of 10 ng/mL and the limit of detection was 0.15 ng/mL. Thus, the developed immunoFET sensors would be applicable for the detection of 3-NT in the biological samples.

Keywords: 3-Nitrotyrosine, biomarker, biosensor, immunoFET

INTRODUCTION

Oxidative stress is defined as an imbalance between free radical generation and radical sequestration, leading to cells, and tissues damage. Excessive reactive nitrogen species (RNS) and reactive oxygen species (ROS) levels can cause severe and irreversible cellular oxidative damage, leading to the development of diseases.[1] RNS, including peroxynitrite anion ($ONOO^-$) can nitrate peptides and proteins at the specific tyrosine residues, resulting in 3-nitrotyrosine (3-NT). Thus, 3-NT is a biomarker for studying the *in vivo* nitration/oxidation of protein and found to be elevated in RNS- and ROS-associated diseases such as cardiovascular diseases, immunological disorders, degenerative neurological disorders, carcinogenesis, and diabetes.[2] 3-NT that causes apoptosis in many cell types is formed as a product after modifying

protein tyrosine residues by peroxynitrite generated from the reaction of nitric oxide (NO) and superoxide.[3] Elevation of peroxynitrite levels in the mitochondria of MCF-7 breast cancer cells treated with tamoxifen leads to peroxynitrite-induced tyrosine nitration, resulting in oxidative stress and mitochondrial apoptosis.[4] In spite of being a strong oxidant and nitrating agent, peroxynitrite is not a free radical and does not cause a direct reaction with tyrosine residues.[5] Through a free radical mechanism, nitronium (NOO^+) is added to the tyrosine residues to form 3-NT through the process known as protein tyrosines nitration.[6] It is extremely difficult to measure directly the species causing tyrosine nitration in the biological system because they usually have very short half-lives. Because 3-NT is very stable, it is a suitable analyst to serve as a specific fingerprint marker for *in vivo* peroxynitrite production and NO-mediated tissue damage.[7] For this reason, detection of 3-NT during biological processes is a crucial step in diagnosis of progressive diseases as well as in the screening of the compounds with the action of oxidative/nitrosative stress-induced apoptosis and cell damage.

There are a variety of methods for detection and quantification of 3-NT in biological samples[8] such as high-performance liquid chromatography (HPLC) coupled to diode array, ultraviolet-visible (UV/VIS), LC with mass spectrometric (LC-MS) or gas chromatography with MS (GC-MS) detectors, immunological methods (e.g., enzyme-linked immunosorbent assay [ELISA], and immunohistochemistry), and Western blotting using monoclonal and polyclonal antibodies against 3-NT. Among them, MS-based methods are the most sensible in the quantification of 3-NT but necessitate a pre-analytic derivatization step. In contrast, such derivatization step is not required for HPLC that is however not as accurate as GC-MS.[8] However, for the application of the chromatographic methods, expensive large scale apparatus, and tedious and time-consuming pre-treatment of samples, and highly trained technicians are necessary.

Biosensor is a device used to measure the analyte with quantitative responses in the form of measurable electric signal through a transducer utilizing a biochemical mechanism as the recognition system. It can be used for the detection of the biomarkers such as proteins, metabolites, DNA, or RNA through the highly specific molecular recognition and immune-complexes interaction of antibodies and antigens.[9] Ion-sensitive field-effect transistor (ISFET) is a subclass of the electrochemical biosensor that is able to detect a signal resulting from the interaction of an analyte with a biological element, and transform accurately the signal into an electrical signal.[10] ImmunoFET biosensors are derived from the ISFET by replacing the ion-sensitive layer of ISFETs with the layers of immobilized antibodies[11] and can detect an immunoreaction at the dielectric interface on the basis of the differential voltage output of the antibody and the antigen/antibody complex.[12]

For the detection of 3-NT, Jin and colleagues, and He and co-workers developed a surface plasmon resonance (SPR)-based biosensor that recognizes 3-NT in human urine.[13,14] However, the SPR has such a disadvantage as the interference of non-specific bindings to the outcome signals.[15] Therefore, a simple and versatile ISFET-based immunosensors were constructed in this study for the detection of 3-NT in the biological samples. The main objectives of the present study were to develop the immunoFET biosensors with different immobilization techniques using ISFET-Si_3N_4 (silicon nitride) substrates and to evaluate immobilization techniques in term of sensitivity and specificity for detection of 3-NT.

Biomolecules (e.g., antibodies, enzymes, and DNA) can be immobilized on the ISFET gate to detect a specific antigen. There are several different approaches to correctly immobilize antibodies on different surface materials. In this case, non-oriented covalent technique and the oriented Protein A/antibody complex stabilized with crosslinking reagent were used. Covalent immobilization of antibodies on to various solid surfaces is the most intensely studied immobilization approach as it does not cause leaching of biomolecule from the substrate during use, facilitates the long-term storage, and reusability of immunosensors, and thus represents a rational and robust approach.[16] The random attachment of the antibody may occur due to its simplicity. Thus, oriented immobilization is considered to be preferable.[17] In this protocol, the antibody capturing molecule, Protein-A, is pre-activated with a bifunctional cross-linker, glutaraldehyde (GA) [Figure 1], and subsequently, any residual reagent is washed away to avoid any contact of the free crosslinking reagent with the antibody.

MATERIALS AND METHODS

Materials

Anti-3-NT antibody (SC-32757) was purchased from Santa Cruz Biotechnology. 3-aminopropyl triethoxysilane (APTES), GA, glycine, Protein-A, and 3-nitro L-tyrosine were purchased from Sigma-Aldrich. Bovine serum albumin (BSA), absolute ethanol, hydrochloric acid, hydrogen peroxide, methanol, and

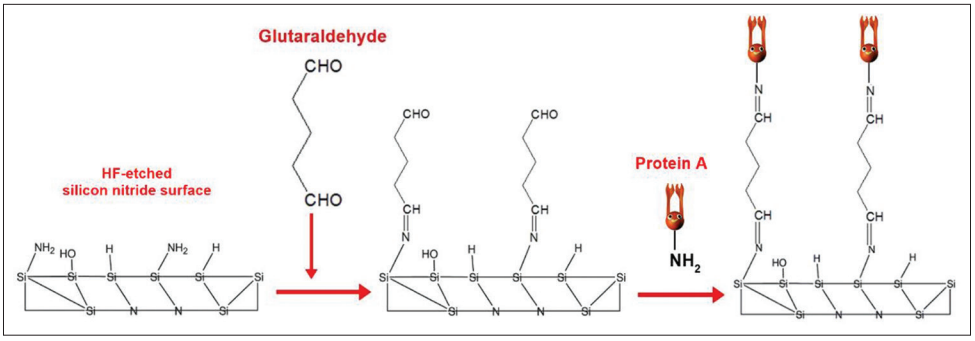

Figure 1: Schematic presentation of Protein A-immobilized surface through a crosslinker, glutaraldehyde

L-tyrosine were purchased from Merck Millipore. Hydrofluoric acid (HF) was purchased from J.T. Baker. Phosphate buffered saline (PBS) tablets was purchased from calbiochem. Sodium cyanoborohydride was purchased from Thermo Fisher Scientific. Silicon ISFETs (ISFETs, P010101) were designed and fabricated by Thai Microelectronics Center (TMEC), Thailand. The Ag/AgCl reference electrode and readout circuit were obtained from Winsense Co., Ltd (Thailand).

Electrical Measurement System Setup

The measurement system consisted of ISFET-Si_3N_4 (silicon nitride) sensing membrane, Ag/AgCl reference electrode, constant current-constant voltage readout interfacing circuit, data logger, and a computerized system with signal recording software from Winsense. All measurements were performed in a light-protective vial at a constant room temperature (RT at 25°C) to minimize the temperature and photoelectric effects. The reference electrode was immersed in the buffer solution (pH 7.4) together with ISFET sensors and connected to the interfacing circuit. The interface circuit was set to supply a drain-source voltage (V_{DS}) of 0.3 V between a constant drain and source current (I_{DS}) of 80 μA. The change in the gate potential (Δ V) in all of the experiments is referred to the charges of biomolecules interacting at the gate dielectric.

Covalent Immobilization

The immobilization of the anti-3-NT antibody onto the Si_3N_4 surfaces was carried out by minor modification of method described previously by Awsiuk *et al.*, 2010.[18] For cleaning and hydrophilization of the Si_3N_4 surfaces of ISFETs, the silicon substrates were immersed in 1:1% (v/v) solution of 1 M HCl/methanol for 1 h at RT, thoroughly rinsed with deionized (DI) water and dried under nitrogen air flow. Initially, the cleaned surfaces were functionalized using 10% (v/v) APTES in absolute ethanol (EtOH) at RT for 2 h.

Afterward, the sensors were rinsed using DI water and kept overnight in the desiccator at RT to prevent the formation of bicarbonates and then imines in the presence of heat after reaction of amines with carbon dioxide from the atmosphere.[19] Then, the silanized surfaces were reacted with 2.5% GA for 1 h at RT using sodium cyanoborohydride (160 mM in PBS) as reducing agent and washed with DI water. Then, the ISFET surfaces was incubated with different concentrations of anti-3-NT antibody (12.5, 25, 50, 100, and 200 μg/mL) for 1 h in humid condition at RT and washed with the PBS. Then, the optimal concentration of antibody was chosen.

Oriented Immobilization of Antibody

The Si_3N_4 surfaces of ISFET were first cleaned with Radio Corporation of America (RCA) cleaning process,[20] which is a 2-step wet-oxidation and complex treatment and involves standard clean-1 and standard clean-2 solutions, lasting for 15 min each and rinsing with DI water for 5 min between the two steps.

Following the RCA cleaning process, the ISFET surfaces were immersed in 1% HF solution for 3 min to create an oxide-free, hydrogen-passivated, hydrophobic silicon surface, and rinsed with DI water. Then, the GA solution (2.5% in PBS buffer solution) was deposited on the surfaces by drop coating method with two-step approach to minimize dimerization of protein[21] and dried at RT for 90 min after each deposition. After that, the gate potential was measured in 10 mM PBS buffer (pH 7.4) and set as baseline voltage ($V_{baseline}$).

On the GA-modified gate surfaces of the ISFET, 10 μL of protein-A (different concentrations: 0.25, 0.50, 0.75, 1.00, and 2.00 mg/mL) was incubated for 1 h at RT in humid condition, and the gate potential was measured. The gate potential change (Δ V) was then calculated and compared to choose the optimal concentration of Protein-A.

Data Analysis

Statistical processing of the obtained results using the program "IBM SPSS (version 21.0) statistics" was carried out. The one-way ANOVA with Bonferroni test was used in choosing an optimum concentration of antibody, and the level of significance $P < 0.05$ was adopted. The graphs were constructed using GraphPad Prism 6, Microcal™ Origin™ Software (version 5.0) and Microsoft Office Excel 2013.

RESULTS

Covalent Immobilization of Anti-3-NT Antibody on Si_3N_4 Surface

APTES has been used extensively for the functionalization of bioanalytical platforms. The APTES-amino groups also facilitate the formation of siloxane bonds with surface Si−OH. The amino-functionalized silane then forms covalent bonds with the amino (GA)-mediated reaction of biomolecules.

As shown in Figure 2, the gate potential (V_{GS}) increased significantly after the formation of self-assembled monolayer (SAM = APTES + GA) on the gate surface of ISFET on the basis of the bare surface but it was decreased by the immobilized anti-3-NT antibody.

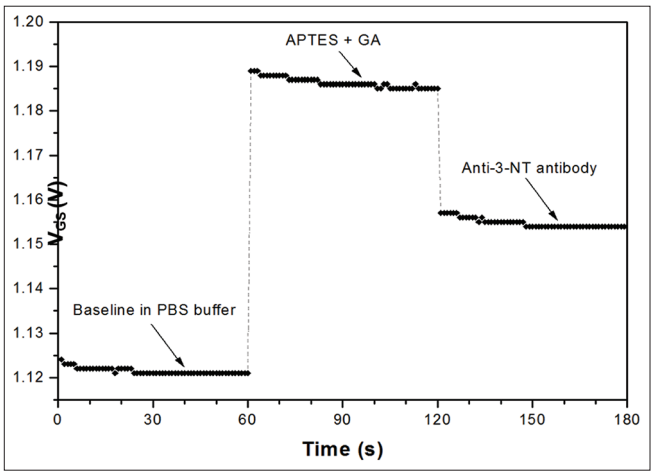

Figure 2: Typical response curve for ion-sensitive field-effect transistor biosensor before and after antibody immobilization (VGS: Gate-source voltage; APTES: 3-aminopropyl triethoxysilane; GA: glutaraldehyde)

Optimization of Anti-3-NT Antibody Concentrations

After comparing the gate potential change ($\Delta V = V_{Ab} - V_{SAM}$) among different concentrations [Table 1], the Δ V of anti-3-NT antibody at a concentration of 100 μg/mL was not significantly different from that of 200 μg/mL [Table 1 and Figure 3]. Therefore, the concentration of anti-3-NT antibody at the concentration of 100 μg/mL was chosen to use the further development of the immunosensors for the detection of 3-NT.

Comparison of Sensor Response after Blocking with BSA and Glycine

The acceptable background signal due to non-specifically bound reactants should have a strict limitation for the high sensitivity of an immunoassay and the surface should be blocked thoroughly with an inert or irrelevant protein to achieve the background signal to be low. The efficiency of blocking agents varies with their molecular weight. The pH should be considered as an important factor in choosing the blocking agent because the ISFET sensors are pH-sensitive. For this reason, BSA solution (1% w/v, pH 7.23) and glycine solution (1 M, pH 6.92), possessing an approximately neutral pH, were tested in this experiment.

The results as shown in Figure 4 confirmed the superiority of glycine as a blocking agent. After blocking with glycine, anti-3-NT antibody-immobilized surface at a concentration of 200 ng/mL produced better response to 3-NT than with BSA. In addition, using of ant-3-NT antibody at a concentration of 800 ng/mL also showed the same results compared to that of 200 ng/mL [Figure 4]. Thus, glycine solution at 1 M was chosen as a blocking agent to inactivate the residual aldehyde groups of GA.

Oriented Immobilization of Antibody

The Protein-A from *Staphylococcus aureus* was chosen to be used as an intermediate binding protein that encourages antibody immobilization through the Fc region on the sensor surface and improves the biosensor performance by orienting antibody with a tail-on direction. To choose an optimum Protein-A concentration, the gate voltage induced by protein-A ($V_{Protein\ A}$) on the crosslinked surface was measured and the gate potential change ($\Delta V = V_{Protein\ A} - V_{baseline}$) was calculated.

Optimization of Protein-A Concentrations

The gate potential change (Δ V) induced by various concentrations of Protein-A (0.25, 0.50, 1.00, and 2.00 mg/mL) was 5.93 ± 0.21, 3.65 ± 0.07, 6.40 ± 0.14, and 6.70 ± 0.85 mV [Figure 3 and Table 2]. Thus, the ΔV was gradually increased after Protein-A had been crosslinked onto the insulated gate surface of immunoFET. The Δ V of immunoFET ranged from 5.93 to 12.07 mV [Figure 3 and Table 2]. After comparing the different gate potential change (Δ V) among concentrations, 1 mg/mL concentration of Protein-A was selected to immobilize anti 3-NT antibody. After the Protein-A attachment to the sensor surface, the remaining aldehyde groups were blocked using the glycine solution to avoid unspecific adsorptions.

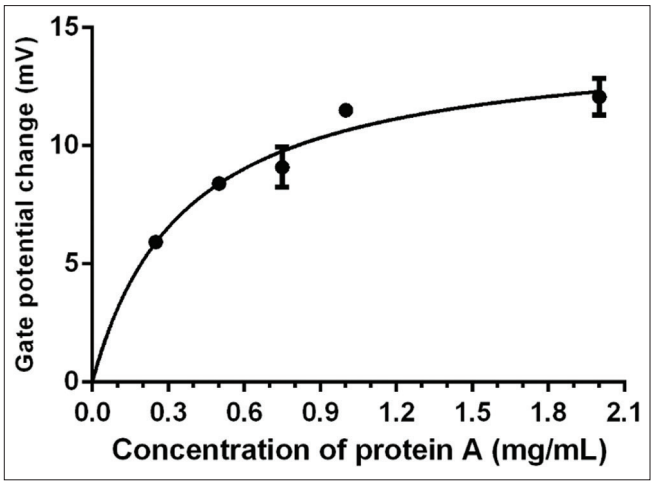

Figure 3: Gate potential change (ΔV) induced by various concentrations of Protein-A crosslinked on the surface of ion-sensitive field-effect transistor biosensor

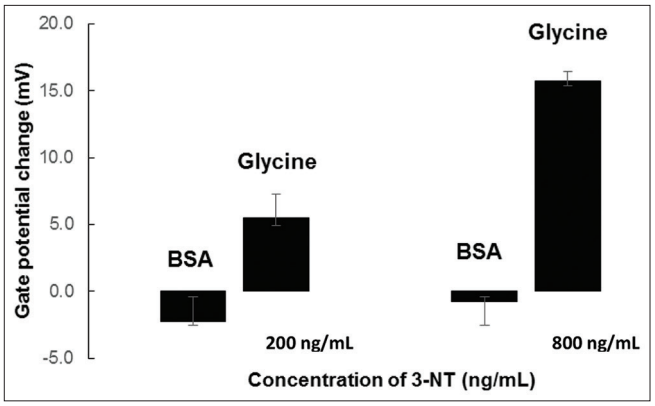

Figure 4: Response of immunoFET sensor to two different concentrations of 3-nitrotyrosine (200 ng/mL, 800 ng/mL) after blocking with BSA and glycine

Table 1: The gate potential change (Δ V) after immobilization of anti-3-nitrotyrosine antibody on silanized surface

Anti-3-nitrotyrosine antibody concentrations (μg/mL)	Δ V(mV) (mean±SD)
12.50	−3.72±0.75*
25.00	−15.96±0.77*
50.00	−19.80±1.93*
100.00	−31.82±1.67*
200.00	−35.87±1.99

* $P<0.05$ among each concentration groups

Optimum Antibody Concentration

Subsequently, immobilization assay was carried out using different concentrations of anti-3-NT antibody. The Fc region of anti-3NT antibody adhered to the top of the protein layer. The gate potential change (Δ V) of immunoFET was obtained by comparing the observed value (V_{AB}) to the baseline value of the gate voltage (V_{PrA}) of the immunoFET ($\Delta V = V_{AB} - V_{PrA}$) [Table 3 and Figure 5]. The ΔV value ranged from 9.10 to 18.73 mV that was produced by immobilization of various

concentrations of antibody through Protein-A on crosslinked surfaces.

Detection of 3-NT

The immunoFET sensors developed with oriented immobilization method through protein-A could detect the concentrations of 3-NT in microgram per milliliter range [Table 4].

The sensors developed using covalent immobilization provided the limit of quantification (LOQ) of 10 ng/mL. The limit of detection (LOD) was 0.15 ng/mL estimated from the standard deviation of the measurement signals for the blank. To confirm its activity, they were detected in different concentrations of 3-NT including the negative standard sample (triplicate sensors for each concentration) and the results are presented in Table 5. In the negative standard sample, the output voltage shifted only 1.26 ± 0.05 mV.

Calibration Curve of 3-NT Obtained in PBS Buffer

As shown in Figure 6, the graph is a linear fit within the range of 10–1000 ng/mL with a R^2 = 0.9923. There is a linear relationship between the output voltage shift and the concentrations of 3-NT. The LOD was 0.15 ng/mL estimated from the standard deviation of the measurement signals for the blank.

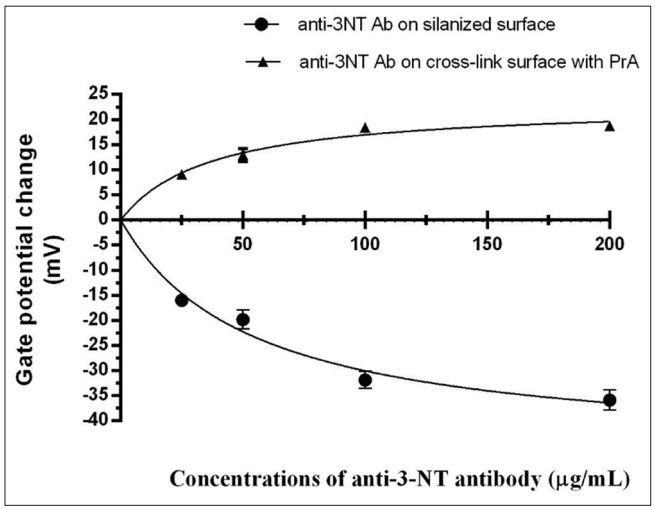

Figure 5: Comparison of gate potential change (Δ V) of ion-sensitive field-effect transistor biosensor after antibody immobilization with two different methods (Ab = antibody; PrA = protein-A)

Table 2: Comparison of gate potential change induced by Protein-A binding to the sensor surface

Protein-A concentrations (mg/mL)	Gate potential change (mean±SD)
0.25	5.93±0.21*
0.50	8.40±0.26*
0.75	9.10±0.85*
1.00	11.50±0.26*
2.00	12.07±0.78

*P<0.05 among concentration groups

Specificity of 3-NT Immuno-FET Biosensors

As tyrosine is a structural analog of 3-NT that may serve as possible interfering molecule for the sensitivity and selectivity of the developed biosensor when applied in practice, the differential output voltage induced by the binding between anti-3-NT antibody on the sensor surface against 3-NT and tyrosine was investigated and the results are shown in Figure 7. It was observed that the shift in output voltage induced by the non-specific binding between anti-3-NT antibody on the sensor surface against tyrosine was much lower than that induced by the specific binding of 3-NT.

DISCUSSION

3-NT in biological samples has been detected and quantified by a variety of methods such as immunological methods such as ELISA; LC methods such as HPLC-UV/VIS, LC-MS/MS; and gas chromatographic methods, such as GC-MS/MS. GC-based methods exhibit the highest sensibility in the quantification

Table 3: Comparison of gate potential change induced by anti-3-nitrotyrosine antibody binding to Protein-A crosslinked to the sensor surface

Anti-3-nitrotyrosine antibody concentrations (µg/mL)	Δ V (mV) (mean±SD)
25.00	9.10±0.62*
50.00	12.96±1.29*
100.00	18.43±0.16*
200.00	18.73±0.45

*P<0.05 among concentration groups

Table 4: Response of immunoFET developed with oriented immobilization after testing to increasing concentrations of 3-nitrotyrosine

3-Nitrotyrosine (µg/mL)	Gate potential change (mV) (Mean±SD)
125	2.63±0.47
250	5.17±0.96
500	7.47±0.40
1000	16.53±1.86

Table 5: The gate potential change of immunoFET developed with covalent immobilization to different concentrations of 3-nitrotyrosine

3-nitrotyrosine (ng/mL)	Gate potential change (mV) (Mean±SD)
10	2.89±0.05
100	3.92±0.12
200	5.50±0.71
400	9.74±0.42
600	12.35±0.21
800	15.75±0.35
1000	17.72±0.16

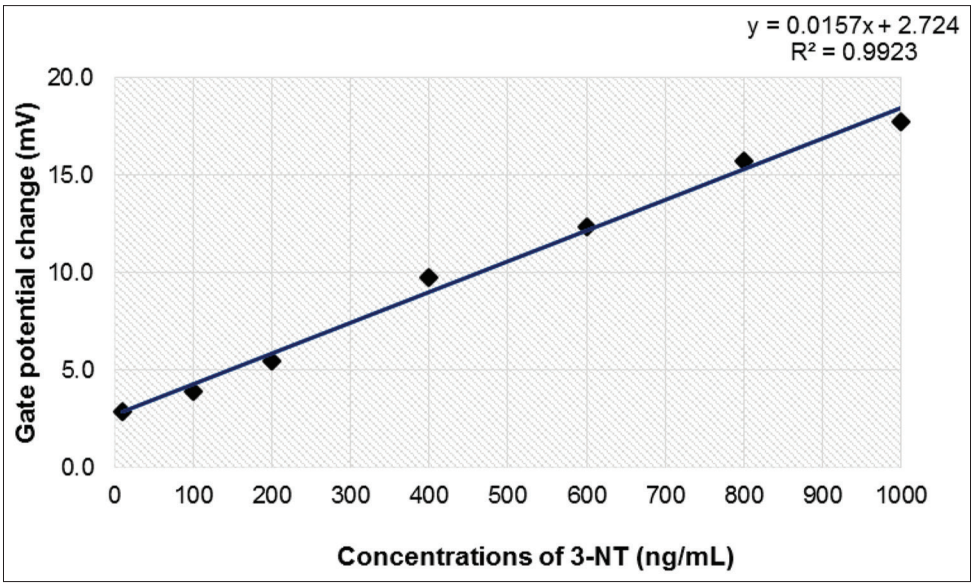

Figure 6: Standard calibration curve of the immunoFET biosensor after covalent immobilization of antibody for the detection of 3-nitrotyrosine in buffer solution (each point represents the mean value of measurement of triplicate samples)

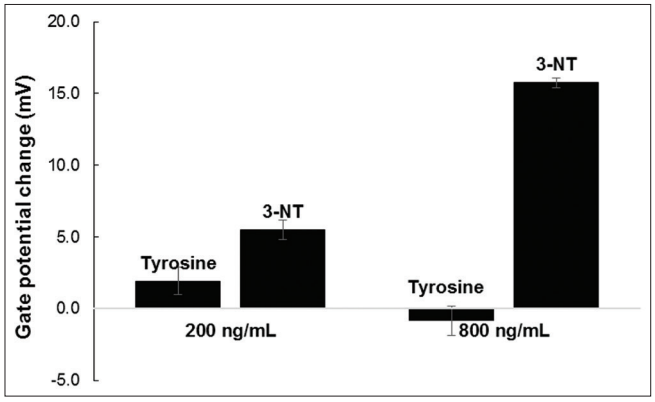

Figure 7: Specificity test of 3-nitrotyrosine immunoFET assay against two different concentrations of tyrosine and 3-nitrotyrosine

of 3-NT and more accurate than HPLC but require a prior time consuming derivatization step. Conversely, HPLC does not require such derivatization step, despite being not as accurate as GC.[8] The main disadvantage of these methods is requirement of expensive, large scale apparatus, and tedious and time-consuming pre-treatment of samples. Conventional ELISA is not economical if it is used for a small number of samples. In addition, it requires a relatively time-consuming analysis procedure, involving sample, and reagent incubation as well as washing stages preceding an indirect measuring technique.

Biosensors offer a much more rapid, cost-effective, and highly sensitive method of analyte detection. Compared with ELISA, it can provide quantitative responses through a transducer in the form of measurable electric signal. It can detect biomarkers based on the highly specific molecular recognition of antibodies and antigens whose interactions give rise to immunocomplexes[9] and have the advantages such as being cheaper, faster, and more flexible than other methods, and also allowing the use of multi-target analyses and automation.[22] For detection of 3-NT, Jin *et al.*[13] as well as He *et al.*[14] developed SPR-based biosensors for the detection of 3-NT in human urine, but alternative designs are still valuable.

The immunosensor based on the electrochemical biosensors was developed for the detection of 3-NT. The key point for electrochemical biosensors is the integration of the biological elements with suitable materials. In this study, ISFETs with silicon nitride substrate were used for developing the immunoFET biosensors and two kinds of surface modification approaches had been studied for adhesion between silicon nitride surface and biomolecules: Covalent immobilization and oriented immobilization methods. The output of the ISFET immunosensors has an optimum at pH 7.4, which is close to the acidity of human blood (pH 7.4). This makes possible the measurement of human blood samples without further adjustment of blood pH value.

Covalent Immobilization of Anti-3-NT Antibody on Si_3N_4 Surface

For the silanization of the Si_3N_4 surfaces, APTES was prepared in anhydrous ethanol to prevent APTES oligomers precipitation and thickness alteration of the final film and multilayer formation of silane.[23] During attachment of APTES, which is hydrolyzed with ethanol, to the hydroxyl groups of the hydrophilized silicon surfaces, a siloxane network terminated with amine groups pointing away from the surface (a brush-like conformation) is formed.[24] Since then, the amino-groups from aminosilane were reacted with the aldehyde groups from GA, to form secondary amine linkages. Aldehyde groups from GA are targeted to NH_2, not NH_3^+ of the antibody,[25] crosslinking the amino groups of the biomolecules to those of APTES-functionalized surfaces and forming reversible Schiff's bases.[19] Thus, sodium cyanoborohydride was used to form permanent secondary amine bonds from imine bonds

of Schiff's bases.[26] After adding anti-3-NT antibody to Si_3N_4 surfaces, a self-assembled monolayer was formed on the surfaces.

Investigation of the changes in gate potential (V_{GS}) with respect of the silanization and coupling with cross-liner, and the immobilized anti-3-NT antibody revealed that V_{GS} increased significantly after the formation of SAM on the sensor surface on the basis of the bare Si_3N_4 substrate but decreased after immobilization of anti-3-NT antibody. The previous study have been proved that chemical modification of free amino groups disposed on the antibody with a bifunctional agent produces a modified antibody with lower isoelectric point, reduced net positive charge, increased activity, and specificity.[27] The charge on the substrate surface is mainly determined by the total charge of antibody immobilized on the sensor surface. In this method, GA, a bifunctional agent, was used as a crosslinker between amino groups of APTES and antibody. Therefore, the gate potential change (Δ V) decreased after immobilization of anti-3-NT antibody on the sensor surface as a result of modification of antibody by a homo-bifunctional crosslinker, GA.

Choosing a Blocking Agent

According to the results after comparing the sensor response between two blocking agents, the sensors blocked with glycine produced better response to 3-NT than that with BSA. The reason may be that BSA hinders the reaction of antibody with the antigen, 3-NT, as it has a molecular weight of 66.5 kDa, consisting of a total of 19 tyrosine phenolate residues, 59 lysine ε-amine groups, one free cysteine sulfhydryl, and 17 histidine imidazole groups.[28] Therefore, glycine solution (1 M) was chosen to block the residual aldehyde groups of GA on the sensor surface.

Oriented Immobilization of Antibody

In this approach, GA, a homo-bifunctional crosslinker, was used to enhance the stability of protein-A on the sensor surface because it provides the aldehyde (-CHO group) for Protein-A immobilization as an appropriate linker group.[29] The cleaned Si_3N_4 surface was etched with HF before crosslinking with GA. HF-etching is a necessary step for removing the native silicon oxide from Si_3N_4 and also to enhance the N-H groups on the surface[30] since the amine groups have an advantage for N-reactivity,[31] for binding with aldehyde moiety from GA. As a consequence, the reaction for the GA attachment was carried out in aqueous media at room temperature, forming Schiff's bases with amines from the surface. After being aldehyde, Protein-A was introduced as an intermediate protein onto the surface by drop-coating method to improve the performance of the sensor by orienting antibody with a tail-on direction, and on the other hand, allow for the conjugation with the aldehyde groups from GA through their free amine groups.

Antibody attached to Protein-A on crosslinked surfaces produces the gate potential change (Δ V) ranging from 9.10 to 18.73 mV over the concentration range of 25–200 μg/mL. The Δ V was observed to be increased after immobilization of antibody on Protein-A-modified surface. This may be the reason that a more positive electric field is formed around protein A when antibody (IgG) binds to it[32] since IgG with isoelectric point greater than the solution pH, normally carries a net positive charge.[33]

Sensitivity and Specificity of 3-NT immunoFET Biosensor

To investigate the typical characteristics of signal response produced by the immunoreaction of the developed immunoFET biosensors, they were tested with 3-NT solution in PBS buffer. An additional layer caused by binding of the antigen to the antibody immobilized on the sensor surface is responsible for the change in the surface charge of the sensors, which was recorded to calculate the Δ V.

The immunoFET sensors developed with oriented immobilization method could detect the concentrations of 3-NT in microgram per milliliter range. The immunosensors developed with covalent immobilization method can detect 3-NT solution in ng/mL concentration. The observations from this study highlighted that the performance of immunosensor is largely determined by the immobilization chemistry applied on the silicon nitride surface and surface activation is required for silicon nitride substrate before immobilization with biomolecules (Protein-A or antibody). In addition, secondary functionalization through surface silanols (SiOH) had higher sensitivity to reaction conditions than through surface amines ($SiNH_2$). Thus, silanol groups might be more abundant on the silicon nitride surface than amine groups.

As described by Armbruster *et al.*,[34] the LOD was estimated from the standard deviation of the measurement signals for the blank. The biosensors developed in this study were more sensitive than that of the methods developed by Jin *et al*. (LOD = 4.7 ng/mL, LOQ = 20 ng/mL)[13] and by He *et al*. (LOD = 0.12 μg/mL).[14] The LOD difference among methods may be due to the types of biosensor and the methods used for the development of biosensors. In this method, ISFET biosensors with silicon nitride (Si_3N_4) sensing membrane were used for the development of immunoFET sensors but in other methods, SPR-based biosensors with gold surface were applied. An ISFET used in this study is an electrochemical sensor with such advantages as high sensitivity, easy miniaturization, and usefulness in turbid matrices[35] but SPR sensors have the challenges such as the high-cost platforms and components for the research and development and the commercial platforms that are not affordable to invest and do the maintenance for small research groups or points of care (PoC).[36]

The SPR-based biosensors developed by Jin *et al*. were evaluated for sensor surface specificity against three structural analogs of 3-NT: Tyrosine (TYR), p-nitrophenol (PNP), and 3-amino-l-tyrosine (3-AT)[13] and by He *et al*. against 3-chlorotyrosine, tryptophan, phenylalanine, methionine, cysteine, and glycine.[14] However, in this study, the specificity of immunoFET biosensors was tested against tyrosine that is a structural analog of –NT. According to the results, tyrosine did not interfere the detection of the immunosensors to 3-NT solution. Thus, the developed sensors should be validated in the biological and tissue samples as there are many structural analogs to 3-NT in the biological fluids such

as 3-nitro-4-hydroxyphenylacetic acid (NHPA, metabolite of 3-NT), and p-hydroxyphenylacetic acid (PHPA, and metabolite of tyrosine).[37]

CONCLUSION

In this study, two different immobilization chemistries of anti-3-NT antibody on Si_3N_4 sensing surface of ISFETs were developed and investigated, and the resulting immunoFET sensors were evaluated in term of electronic measurement. As a result of this investigation, the immunoFET sensors developed using covalent immobilization technique displayed the higher sensitivity with the detection of 3-NT in the low nanogram per milliliter range of concentration, thus exceeding that of Protein-A crosslinked immobilization method that could detect 3-NT concentration in microgram per milliliter. Therefore, it can be concluded that the immunoFET biosensors developed with covalent immobilization technique seems to be suitable for further testing for the detection of 3-NT in the biological fluids.

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