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Sensitive electrical detection of human prion proteins using field effect transistor biosensor with dual-ligand binding amplification

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ABSTRACT

Simple and accurate detection of prion proteins in biological samples is of utmost importance in recent years. In this study, we developed a label-free electrical detection-based field effect transistor (FET) biosensor using thiamine as a probe molecule for a non-invasive and specific test of human prion protein detection. We found that thiamine-immobilized FETs can be used to observe the prion protein oligomer, and might be a significant test for the early diagnosis of prion-related diseases. The thiamine-immobilized FET was also demonstrated for the detection of prion proteins in blood serum without any complex pre-treatments. Furthermore, we designed a dual-ligand binding approach by the addition of metal ions as a second ligand to bind with the adsorbed prion protein on the thiamine-immobilized surface. When the prion attached to metal ions, the additional positive charge was induced on the gate surface of the FET. This approach was capable of amplifying the magnitude of the FET response and of enhancing the sensitivity of the FET biosensor. Detection of prion proteins has achieved the required concentration for clinical diagnosis in blood serum, which is less than 2 nM. In summary, this FET biosensor was successfully applied to prion detection and proved useful as a simple, fast, sensitive and low-cost method towards a mass-scale and routine blood screening-based test.

1. Introduction

Prion proteins that can conformationally change into the aggregate form have played a vital role in several infectious and harmful prion diseases, such as the Creutzfeldt–Jakob disease and transmissible spongiform encephalopathies (TSEs). Until now, prion diseases were recognized as highly contagious and scarcely incurable (Prusiner, 1991; Collins et al., 2004; Mays et al., 2014). These diseases can easily transfer among different species by contaminated food from an infected animal and by blood transfusion from a preclinically infected individual (Yang et al., 2005; Liang et al., 2013). Such diseases have become of major concern and a critical health problem; in fact, some countries including Australia, Japan and the USA have established a nationwide surveillance system of human prion diseases (Ladogana et al., 2005; De Pedro-Cuesta et al., 2006). Thus, development of an analytical method to detect the presence of prion proteins in biological body fluid is of great importance to prevent further negative impacts of these diseases.

Currently, protein misfolding cyclic amplification (PMCA) is one of the most popular, powerful and commonly-used methods in practical tests for prion proteins and diseases (Saborio et al., 2001; Castilla et al., 2005; Chen et al., 2010; Moudjou et al., 2013). However, PMCA involves multiple steps including incubation, proteolysis and replication. These processes are time and labor consuming, suggesting that these methods are not appropriate for the use of mass-scale and routine blood screening-based tests. Over the past few years, numerous methods have been proposed to detect prion proteins, such as micromechanical resonator arrays (Varshney et al., 2009), immune-quantitative real-time PCR assays (Reuter et al., 2009), and quantum dot (QD) nanoparticles (Liang et al., 2013). Though such research demonstrated a good biosensing performance for prion detection, nevertheless most of them required complex functionalization and had technical limitations; for instance, those proposing micromechanical arrays and PCR required a labeling step and expensive instrumentation, while QD nanoparticles may have suffered difficulties when produced in stable synthesis and integrated into a miniaturized system. These issues highlight the urgency of the field and a continuous need to construct a simple, robust, rapid and low-cost detection method

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that can diagnose prion proteins with a specific and sensitive approach.

We have attempted to develop field effect transistor (FET) biosensors as a promising biosensing device that provides a low-cost and label-free electrical detection for a decade. The FET offers a type of electrical biosensor that is operated by field-effect modulation of carriers in a semiconductor substrate due to adsorbed molecules with an electric charge on the gate electrode (Schoning and Poghossian, 2006; Hideshima et al., 2012). The charged molecule that specifically interacts with a probe molecule immobilized on the gate surface of the FET device can be the target (analyte) molecule. Recently, the FET biosensor has been intensively performed for promising diagnostic applications and exhibits a remarkable performance, including detection of DNA hybridization reactions (Fu and Li, 2010), antibody–antigen reactions (Hideshima et al., 2012), glycan–protein interactions (Hideshima et al., 2013) and cell identification (Chen et al., 2011). This biosensing system offers a simple, label-free, rapid and direct electrical detection of biomolecular interactions. Furthermore, fabrication of the FET biosensor has been well established through semiconductor technology, meaning that it is suitable for mass and low-cost devices as future point-of-care technology.

To capture prion proteins, we used thiamine as a small-sized probe molecule that possesses a specific interaction with prion proteins (Perez-Pineiro et al., 2011). The thiamine molecule, which is a smaller affinity ligand than using the whole monoclonal antibody, efficiently overcomes the limitation of the FETs' performance caused by the Debye screening length. This method is in accordance with the research trend in the field of biosensors by using a small molecule, such as an aptamer (Maehashi et al., 2007), antigen binding fragment (Elnathan et al., 2012), and glycan (Hideshima et al., 2013), as a probe. Recently, we employed Congo red as a probe interacting with a cross-β structure of amyloid fibrils for the detection of a prion-inducing fragment of the yeast protein Sup35 (Sup35NM) as well as an aggregation of the amyloid β (Aβ) protein by the FET (Hideshima et al., 2014a, 2014b). Here, thiamine, which has benefits as a stable, low-cost and easy-handling reagent, was used to detect the prion protein oligomer. Such small probe molecules might provide more advantages than antibodies or enzymes towards mass fabrication of biosensor devices for practical application.

By using the thiamine-immobilized FET, we can observe the existence of the prion protein oligomer. The oligomeric forms of prions were considered to be a key state to identifying and elucidating the conformational transition of prion proteins (Solowski et al., 2003). Other research on prion diseases confirmed that the prion protein oligomer has a critical toxicity and plays a potential role in early neuronal damage (Kayed et al., 2003; Walsh and Selkoe, 2004; Simoneau et al., 2007). Those theories indicated a crucial issue in developing a technique that is able to monitor the oligomeric form of prion proteins. Hence, the thiamine-immobilized FET, which is capable of detecting the prion protein oligomer, can provide early diagnosis of the molecular states of prion molecules with a simple electrical detection.

In addition, to achieve a highly-sensitive detection of prion proteins, we also applied a signal amplification method by using a dual-ligand binding mechanism between the prion protein, thiamine and metal ion. Dual-ligand bindings are formed by a sandwich-interaction with the prion protein between thiamine and metal ion. At first, the prion protein adsorbed onto the thiamine-immobilized gate surface of the FET. Then, the addition of the metal ion subsequently attached onto the specific binding-site in the prion protein. As a result, an increase of the surface charge density on the gate surface at the solid–liquid interface was generated due to the additional positive charge of metal ions. This approach enables the FET biosensor to extend the sensitivity into the desired limit of detection (LOD) of prion proteins in blood serum.

In the present study, we demonstrate that the thiamine-immobilized surface can be successfully used as a biosensing platform for the detection of prion proteins in human blood serum. The thiamine-immobilized FET biosensor can also be used to observe the prion protein oligomer, which is an important stage of the preclinical period of the disease. Then, the quantitative performance of the FET biosensor for prion proteins in the blood serum sample was examined by the addition of metal ions via the dual-ligand binding approach.

2. Materials and methods

2.1. Reagents

The recombinant human prion protein, PrP^c (23-231), was purchased from Jena Bioscience (Germany). The prion protein was initially solubilized in 1.5 M of guanidine HCl and stored at −60 °C as a stock solution. Then, the prion protein sample solution was prepared by diluting the stock solution in a 10 mM phosphate buffer saline (PBS), with a pH of 7.4 (of at least 10-fold), followed by a pre-treatment using an ultrasonic chamber and vortex mixer. A fresh sample solution was used for each analysis of the electric measurement. Thiamine hydrochloride was purchased from Sigma-Aldrich and dissolved in 10 mM of PBS (pH 7.4). A self-assembled monolayer reagent, 3-aminopropyltriethoxysilane (APTES), and a crosslinker reagent, glutaraldehyde (GA), were purchased from Sigma-Aldrich. The organic solutions were provided by Kanto Chemical Co. Human serum albumin (HSA) and human serum type AB (male) were also purchased from Sigma-Aldrich. The chemicals of the metal ion (Copper Sulfate, $CuSO_4$; Manganese (II) Chloride, $MnCl_2$; Zinc Chloride, $ZnCl_2$) were purchased from Sigma-Aldrich and diluted using ultrapure water. A 10 mM PBS was made in-house using NaCl (137 mM), $Na_2HPO_4 \cdot 12H_2O$ (8.1 mm), KCl (2.7 mm), and KH_2PO_4 (1.5 mm), then 0.01 mM PBS for electrolytes on the electric measurement, prepared by diluting the 10 mM PBS with ultrapure water (Milli-Q, Millipore).

2.2. Preparation of thiamine-immobilized FET biosensor

A schematic image of the thiamine-immobilized FET biosensor is shown in Fig. 1. The n-type FET device, where the SiO_2 insulator layer on the Si substrate acted as the gate electrode with a size of 10 μm in length and 1000 μm in width, was fabricated by Toppan Printing Co. Ltd. (Japan). The FET devices were first cleaned with acetone and soaked for 10 min in an ultrasonic chamber to remove the layer of photoresist on the chip. The fabrication process for tailoring the nano-bio-molecular structure on the FET gate surface can be described as follows. First, the FET device was exposed with O_2 plasma at 200 W for 1 min (gas plasma device, model: PR301, Yamato Scientific Co., Japan), producing a hyrodxyl group on the gate surface. The gate surface was then incubated in a toluene solution containing a 1% (v/w) APTES reagent at 60 °C inside a glove box with an Ar atmosphere (99.99% pure). The APS-modified FET was ultrasonically cleaned in a mixture solution of methanol and toluene (1:1), then dried with a N_2 gas spray to remove the residue. The FET device was then annealed at 160 °C for 1 h.

Before electric measurement, the FET gate surface was immersed for 30 min at room temperature in PBS containing 2.5% GA, followed by washing with PBS. The fabrication of stable GA-modified aminopropylsilyl by reducing the Schiff bases was performed in the same way as the previous procedure

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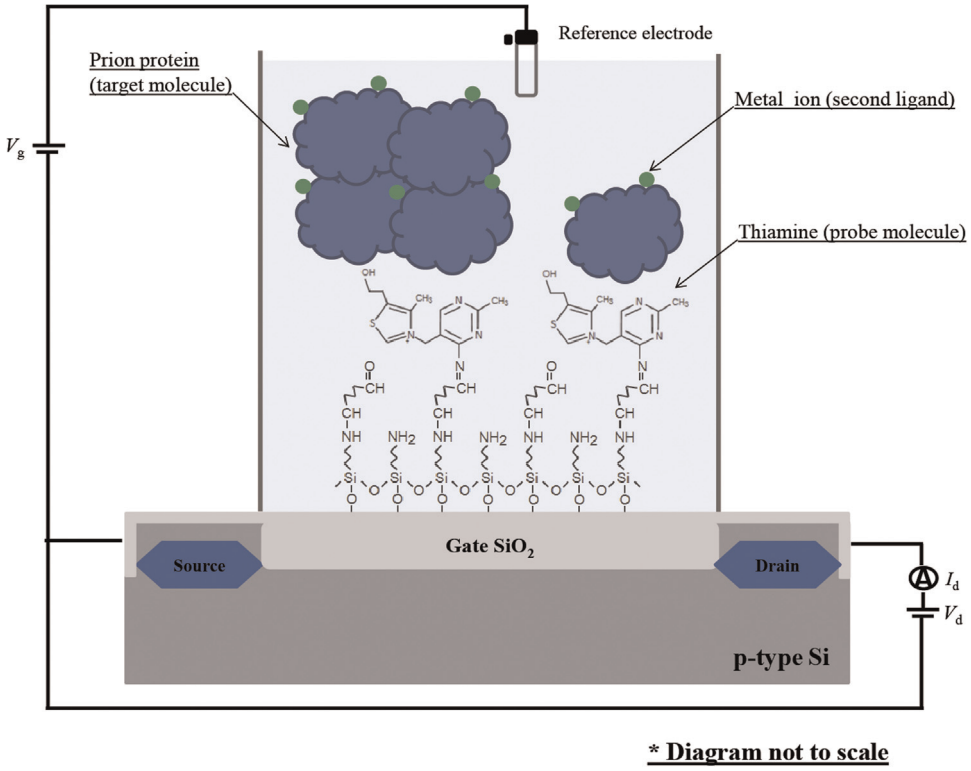

Fig. 1. Schematic illustration of a FET biosensor used for prion detection. The thiamine as a probe molecule was immobilized on the glutaraldehyde-modified gate surface of the FET device; the reference electrode was Hg/Hg_2SO_4.

(Hideshima et al., 2011). Thiamine was immobilized by immersing the FET gate surface in a 100 ng/mL thiamine solution for 1 h and then washed with PBS prior to use.

2.3. Electric measurement

The thiamine-modified gate surface was analyzed in a 0.1 mM PBS buffer with a pH of 7.4 by electrochemical measurement. The electrochemical measurements were carried out using a digital source meter (Keithley 2612) by sweeping the gate voltage (V_g) from -3 V to $+1$ V and setting a constant drain voltage at 0.5 V. The relationship between the V_g and drain current (I_d), or the V_g–I_d characteristic, was measured. The Hg/Hg_2SO_4 was used as a pseudo-reference electrode. A threshold voltage shift value, ΔV_g, was calculated.

2.4. Prion detection and addition of metal ion

Firstly, the thiamine-immobilized gate surface of the FET was incubated in a different concentration of the prion protein solution for 1 h at room temperature, and then rinsed with PBS. To amplify the FET response signal for the detection of prion proteins, the addition of 100 mM of a metal ion solution (Cu^{2+}, Mn^{2+}, Zn^{2+}) was dropped for 10 min at room temperature onto the gate electrode, which had bound to the adsorbed prion protein. After each incubation and addition of metal ion, the device was washed three times with 10 mM and 0.1 mM of PBS to reduce non-specific adsorption.

2.5. Surface characterization

X-ray photoelectron spectroscopy (XPS) was used to analyze the functionalization of the nanostructure and to confirm the protein adsorption. The XPS measurement was performed by a micro XPS instrument (PHI-5000 Versa Probe WS, ULVAC-PHI Inc.) using an Al Kα X-ray source. Charge neutralization was used for all samples, and the binding energy scale was calibrated to the charge reference of C 1s peak at 284.6 eV. In addition, the characterization of surface morphology on the FET gate was measured by atomic force microscopy (AFM) (SPM-9600, Shimadzu Co., Japan). The experiment was performed with a dynamic mode AFM using a silicon cantilever (Olympus Co., Japan). The imaging point was set up in with a size of 500 nm × 500 nm using 512 × 512 pixels.

3. Results and discussion

3.1. Characterization of thiamine-immobilized surface

As illustrated in Fig. 1, the immobilization of thiamine molecules on the FET gate surface is achieved through covalent binding between the amino group of thiamine molecules and the aldehyde-terminated surface. The amino group of thiamine is not involved in the interaction with prion proteins (Perez-Pineiro et al., 2011), whereas the GA-modified surface was recognized as being the most efficient and stable cross linker to rapidly react with the amino group, and is useful for creating a reliable and reproducible molecular structure for biosensing application (Okuda et al., 1991).

To discuss the immobilization of thiamine molecules on the surface, XPS was used to study the elemental composition of the outermost few nanometer layers of a surface prior to and after the immobilization of thiamine. In the XPS spectrum of the GA-modified surface on the SiO_2 substrate, the photoelectron peaks corresponding to the elements on the sample including carbon (C 1s), nitrogen (N 1s), oxygen (O 1s) and silicon (Si 2p) were recorded. As shown in Fig. 2, the relative intensity of N 1s and C 1s peaks increased after the sample immersion in the thiamine solution, while that of O 1s and Si 2p peaks appeared to diminish. The former, an increase in the intensity of N 1s and C 1s peaks, can

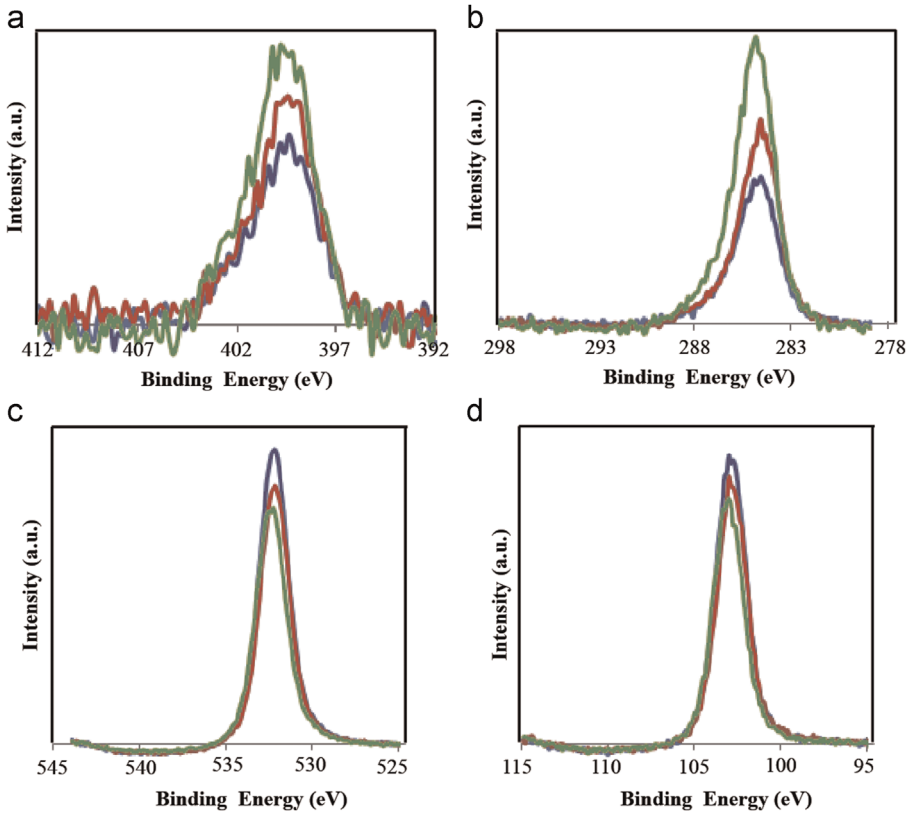

Fig. 2. XPS narrow spectra of N 1s (a), C 1s (b), O 1s (c) and Si 2p (d) for GA-modified surface (blue), thiamine-immobilized surface (red) and after adsorption of prion protein (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

be interpreted as an indirect measure of the enhancement of elemental composition and the presence of thiamine on the GA-modified surface. The nitrogen and carbon peaks in the XPS result are typically used as indicators of organic molecule attachment or protein deposition in the field of bioengineering (McArthur, 2006). The latter, a decrease in the intensity of O 1s and Si 2p peaks, is mainly due to the layer of thiamine adhering onto the sample surface and caused a higher inelastic mean free path (IMFP) of the photoelectrons. Additionally, an apparent peak was observed at the sulfur (S 2p) region after the immobilization of thiamine, which should be attributed to sulfur atom in thiamine molecule (Supplementary Fig. S1). These XPS results suggested that thiamine molecules are successfully immobilized on the gate surface.

3.2. Specificity of thiamine-immobilized FET

The FET biosensor was conveniently utilized as a promising, highly-specific and label-free semiconductor-based biosensing device by monitoring a shift in the gate voltage (V_g) vs the drain–source current (I_d). The threshold voltage shift (ΔV_g) can be measured by the change of the surface charge density on the FET gate caused by the biomolecular interaction at the solid–liquid interface. In this work, we initially investigated the specificity of the thiamine-immobilized FET to the prion protein and human serum albumin (HSA) solutions. The ΔV_g of the FET biosensor with 400 nM of prion proteins and 400 nM of HSA are shown in Fig. 3. The ΔV_g in the negative direction was significantly observed in the

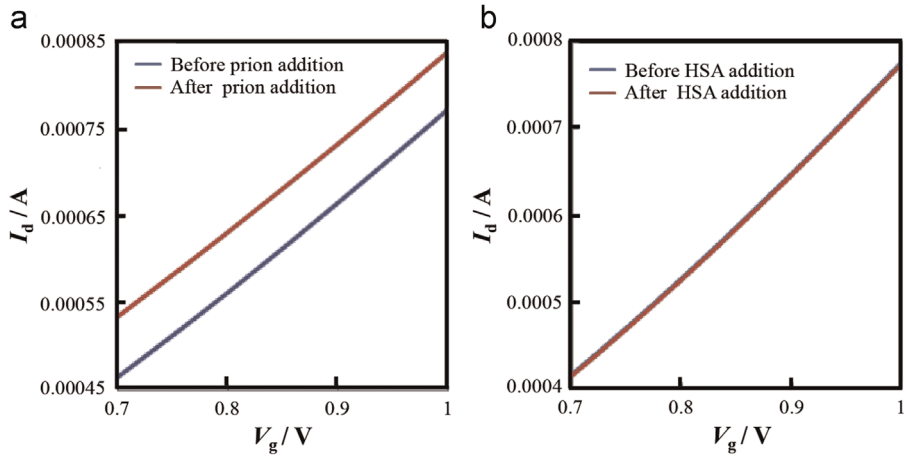

Fig. 3. Threshold voltage shift (ΔV_g) of thiamine-immobilized FETs caused by the addition of 400 nM of prion proteins as a positive control (a), and 400 nM of human serum albumin (HSA) as a negative control (b).

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presence of prion proteins (Fig. 3a). The signal confirmed that the prion protein adsorbed on the gate surface and induced a change of surface charge density. This negative ΔV_g, which is around −48 mV using an n-type FET biosensor, was attributed to the intrinsic positive charge of prion proteins (isoelectric point, pI, 9.3) (Morillas et al., 1999) under the experimental condition (pH 7.4). However, a small ΔV_g equal to 3 mV in the positive direction was observed after the addition of non-specific analyte HSA (pI 4.7) (Avalle et al., 2005), as shown in Fig. 3b, indicating that no significant interaction may take place. The obtained results suggested that the thiamine-immobilized FET possesses the ability to detect the prion protein in a specific manner. An ability to suppress the non-specific molecule was mainly suggested due to the small thiamine molecules might be densely immobilized on the surface that can obstruct any non-specific analyte. To further optimize the sensing performance of the thiamine-immobilized surface, several thiamine solutions with different concentrations were prepared for surface modification on the FET gate, and 100 ng/mL of thiamine was found as the optimal concentration, which was then used on the further experiments (Supplementary Fig. S2).

To confirm that the biomolecular interaction occurred on the FET gate surface, AFM was utilized to check the presence of adsorbed proteins. It is evident from Fig. 4b that some round-shaped particles can be distinctly observed on the FET gate surface when a prion protein was added, and there is a notable difference with only the thiamine-immobilized surface, as shown in Fig. 4a. The aggregates were measured to be 47 nm in diameter and 25 nm in height and were considered as the oligomeric form of prion proteins, which was previously discovered as existing in dynamic equilibrium with the monomeric form (Sasaki et al., 2008). The prion protein oligomer was recognized to be equivalent with 14–28 prion protein molecules and with a size around 47 nm (Silveira et al., 2005). Perez-Pineiro et al. reported in 2011 that thiamine can interact with the specific binding site on the outer structure of the prion protein monomer. In our designed biosensing surface, we suggested the thiamine-immobilized FET can provide a well-arranged probe molecule and promote a more available binding site to interact with larger forms of prion proteins such as the oligomeric state. From the viewpoint of the polymerization stage of prion proteins, the prion protein oligomer has the highest level of infectivity and cytotoxicity. Also, the polymerization will take a long time after the infection, as prion diseases can take from months to decades to appear. Therefore, identification of a prion protein oligomer with a simple FET biosensor is essential to prevent further protein formation before the clinical onset of prion diseases. In contrast, there is only an identical morphology of the FETs' gate surface after the addition of HSA, as shown in Fig. 4c. This phenomenon suggested that the thiamine-immobilized FET can detect prion protein species before they convert into the fibril form, which is useful for early diagnosis of prion diseases.

Furthermore, XPS measurement was carried out to complement the evaluation of protein adsorption on the thiamine-immobilized surface, indicating a highly specific adsorption of prion proteins, as also shown in Fig. 2. There are differences in the intensity and also chemical shifts on the N 1s peak as well as the C 1s peak. The increased intensities indicate an additional element on the top surface, and the chemical shifts to a higher binding energy reveal that the chemical bonds, having a higher binding energy, appeared, such as –C–O– and –(C=O)–. This is closely related to the presence of proteins adsorbed on the surface. On the other hand, the XPS spectra survey is relatively similar before and after the addition of HSA to the thiamine-immobilized surface, which indicated no adsorption occurred on the surface (data not shown). This result could be used as supporting data to suggest the specificity of the thiamine-immobilized surface.

3.3. Selectivity test of thiamine-immobilized FET

Selectivity is a primary indicator of the sensing performance of any biosensor. To determine the selectivity of the thiamine-immobilized FET, two sample solutions, one being 400 nM of prion mixed with 400 nM of HSA in 10 mM of PBS (pH 7.4) and the other being 400 nM of prion in blood serum, were prepared. The FET response for each sample was recorded 3 times using an electric measurement and the average response is shown in Fig. 5

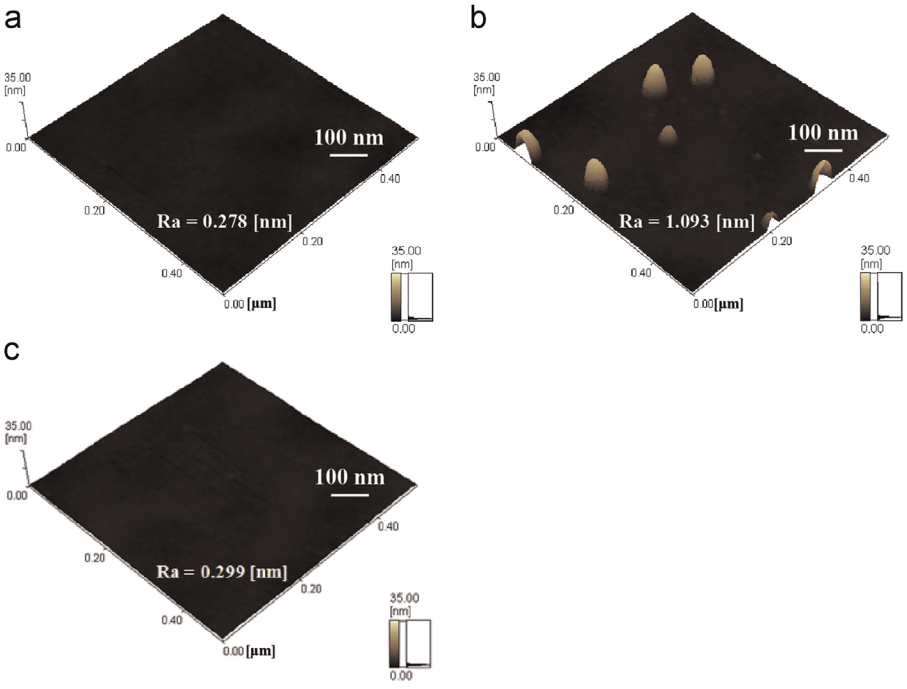

Fig. 4. Atomic force microscopy images of the gate surface for (a) the thiamine-immobilized FET, (b) after the addition of a prion protein oligomer, and (c) after the addition of HSA.

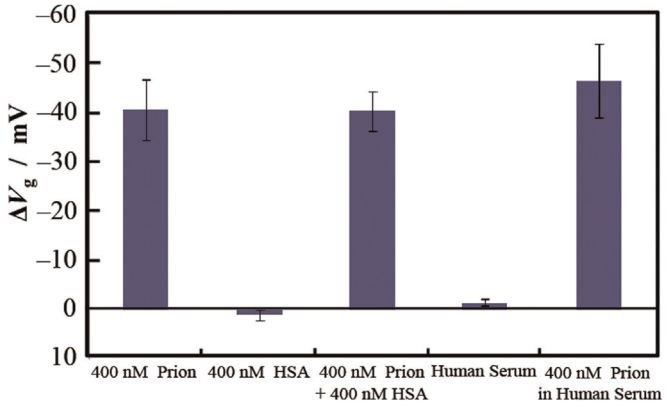

Fig. 5. Comparison of the signal of thiamine-immobilized FETs for the detection of prion in different sample solutions, including a mixed sample solution containing prion and protein contaminant, and also prion in blood serum sample.

as a histogram. The thiamine-immobilized FET shows a similar detected signal between only prion proteins ($\Delta V_g = -41$ mV) as well as in the presence of contaminating proteins ($\Delta V_g = -40.5$ mV). This result clearly exhibits that the contaminating protein (HSA) does not have any significant effect on the performance of the thiamine-immobilized FET biosensor. This suggests the advantage of such a small probe molecule (thiamine) in preventing the non-specific analyte from being adsorbed on the surface. In addition, the FET response showed a good performance even in the human serum sample. The ΔV_g was observed to be

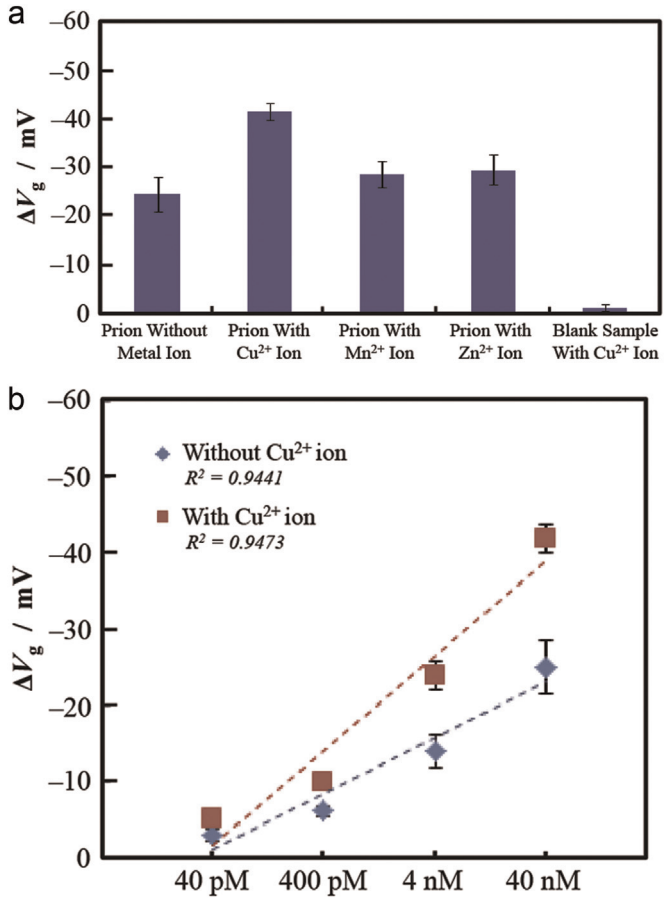

Fig. 6. Amplification of FET signal for prion detection by the addition of metal ions. (a) The effect of several metal ions added to amplify the signal of the FET device. (b) Quantitative detection of prion proteins in a blood serum sample ranging from 40 nM to 40 pM with and without the addition of Cu^{2+} ions.

equal to −47 mV. A small increase in the sensor response is suggested due to the presence of small molecules such as protein fragments, and cations, which existed in the human serum sample (Ahmad et al., 2013). These molecules were assumed to be weakly attached onto the thiamine-immobilized surface and generated a small increase in the FET response. It should be noted that the value of ΔV_g caused by addition of human serum sample only (without any analyte) as negative control was −1.5 mV, as small as the ΔV_g value of approximately 2 mV observed on human serum samples using antibody-modified FET and Fab-immobilized FET (Hideshima et al., 2011; Cheng et al., 2014). Thus, it was demonstrated that the thiamine-immobilized FET showed the response to the contaminating proteins as low as FETs with the use of antibodies or reduced antibody fragments, which indicates high sensitivity of the present system. In summary, this FET biosensor provides a potential application towards mass and routine test of prion detection by its use of a simple biosensing system.

3.4. Improvement of the sensitivity of prion detection by the addition of metal ion

We designed a dual-ligand binding to improve the sensitivity of prion detection with a simple technique. By using several metal ions (Cu^{2+}, Mn^{2+}, Zn^{2+}), here, we applied the same concentration (100 mM) of the metal solution to amplify the FET response due to the effect of the reaction between the association of metal ions and prion proteins adsorbed on the FET gate surface. Fig. 6a shows the magnitude of the voltage shift for prion detection at 40 nM with an addition of different types of metal ions. It can be observed that the Cu^{2+} ion contributed to the most improved signal, whereas the Mn^{2+} ion and Zn^{2+} ion created only a small increase in the signals, much lower than improvement by the Cu^{2+} ion. A general interaction between the metal ion and the prion protein has been widely studied and it has been argued that the Cu^{2+} ion has the strongest attraction, in principle, to the prion protein (Sigurdsson et al., 2003; Jackson et al., 2001). Moreover, in the blank sample, which did not contain any prion adsorbed on the gate surface, a very small voltage shift could be observed. It is suggested that dual-ligand binding only occurred by the presence of the thiamine-immobilized surface, and the prion and metal ions.

The typical FET response caused by adsorption of prion proteins and further addition of the Cu^{2+} ion is shown in Supplementary Fig. S3. Then, the sensitivity was examined in the range 40 nM–40 pM. Fig. 6b displays a concentration dependence of FET responses for prion detection in human serum samples, both with and without the addition of the Cu^{2+} ion. The calibration curves were drawn as straight lines obtained by the method of least squares. The concentration of the Cu^{2+} ion was 100 mM and the reaction time was 10 min, followed by rinsing with PBS after immersion. In this case, the signal enhancement ratios were 68%, 71% and 66% for 40 nM, 4 nM and 400 pM, respectively. By using this approach, the thiamine-immobilized FET can detect the prion protein in the blood serum sample at the 40 pM level, which is more sensitive than without Cu^{2+} ion addition. This result indicated that thiamine-immobilized FETs can be used for practical diagnostics in which the concentration of prion proteins in the blood serum is less than 2 nM (MacGregor and Drummond, 2001).

4. Conclusion

In this study, we proposed a new prion detection method based on a thiamine–prion protein interaction using a FET biosensor. The thiamine-immobilized FET could detect the positively charged prion protein molecules as a threshold voltage shift in the negative

direction. The immobilization of thiamine was confirmed through XPS analysis, and the specific adsorption of prion protein molecules on the thiamine-immobilized surface was confirmed by AFM analysis. The thiamine-immobilized surface of the FET specifically captured prion protein oligomers in blood serum, while suppressing the non-specific adsorption of blood related proteins. Furthermore, a dual-ligand binding approach was proposed to enhance the signal of FET detection. Thus, thiamine-immobilized FETs can provide a simple, label-free, and sensitive detection of prion proteins and open up possibilities for further study regarding practical application.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.08.028.

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