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An Aptamer-Based Electrochemical Sensor That Can Distinguish Influenza Virus Subtype H1 from H5

Jin-Moo Lee[1,2†], JunWon Kim[1†], Ilhwan Ryu[1†], Hye-Min Woo[1], Tae Gyun Lee[1], Woong Jung[3], Sanggyu Yim[1], and Yong-Joo Jeong[1*]

[1]*Department of Applied Chemistry, Kookmin University, Seoul 02707, Republic of Korea*
[2]*Division of Influenza Virus, Korea National Institute of Health, Cheongju 28159, Republic of Korea*
[3]*Department of Emergency Medicine, Kyung Hee University Hospital at Gangdong, Seoul 05278, Republic of Korea*

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*Corresponding author
Phone: +82-2-910-5454;
Fax: +82-2-910-4415;
E-mail: jeongyj@kookmin.ac.kr

†These authors contributed equally to this work.

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The surface protein hemagglutinin (HA) mediates the attachment of influenza virus to host cells containing sialic acid and thus facilitates viral infection. Therefore, HA is considered as a good target for the development of diagnostic tools for influenza virus. Previously, we reported the isolation of single-stranded aptamers that can distinguish influenza subtype H1 from H5. In this study, we describe a method for the selective electrical detection of H1 using the isolated aptamer as a molecular probe. After immobilization of the aptamer on Si wafer, enzyme-linked immunosorbent assay (ELISA) and field emission scanning electron microscopy (FE-SEM) showed that the immobilized aptamer bound specifically to the H1 subtype but not to the H5 subtype. Assessment by cyclic voltammetry (CV) also demonstrated that the immobilized aptamer on the indium thin oxide-coated surface was specifically bound to the H1 subtype only, which was consistent with the ELISA and FE-SEM results. Further measurement of CV using various amounts of H1 subtype provided the detection limit of the immobilized aptamer, which showed that a nanomolar scale of target protein was sufficient to produce the signal. These results indicated that the selected aptamer can be an effective probe for distinguishing the subtypes of influenza viruses by monitoring current changes.

Keywords: Influenza virus, hemagglutinin, aptamer, SELEX, cyclic voltammetry

Introduction

Influenza viruses cause serious respiratory diseases and are considered to be of great concern to human health. The viruses affect up to 100 million people worldwide, resulting in severe symptoms and 250,000–500,000 deaths every year [1]. The influenza genome consists of eight segments of single-stranded negative-sense RNA and belongs to the *Orthomyxoviridae* family [1]. Of the several proteins produced by influenza, hemagglutinin (HA) and neuraminidase (NA) are expressed on the virus surface and are responsible for attachment to and release from the host cell, respectively [2, 3]. Influenza viruses are classified depending on the subtypes of HA and NA. Thus far, 18 subtypes of HA and 11 subtypes of NA have been identified [4].

HA is a homotrimeric integral membrane glycoprotein on the virus surface. It is initially synthesized as HA0, and then successively cleaved into HA1 and HA2. HA1, the protein of focus in this study, is responsible for binding to the sialic acid of host cell receptors, whereas HA2 is embedded in the virus membrane [5, 6]. Therefore, HA1 is regarded as a major target protein for the development of anti-influenza agents and diagnostic tools [7, 8]. Currently, several diagnostic methods, such as enzyme-linked immunosorbent assay (ELISA) [9–12] and polymerase chain reaction (PCR)-based methods [13–16], are widely used to detect influenza antigens. To detect the viral antigens, specific molecular probes are necessary for the development of optimal devices. In this regard, aptamers have emerged as effective specific molecular probes to meet the diagnostic needs for influenza.

Aptamers are single-stranded oligonucleotides that bind

to specific targets with high affinity, and they are formed by the systematic evolution of ligands by exponential enrichment (SELEX) method [17]. In comparison with antibodies, the use of aptamers as a molecular probe to detect specific antigens has a few advantages, such as ease of modification, low cost, high stability, and low toxicity [18]. Another strength of aptamers is the ability to discern the tiny differences between target molecules. Indeed, we have previously isolated single-stranded DNA (ssDNA) aptamers that can distinguish between HA1 of subtype H1 (H1-HA1) and HA1 of subtype H5 (H5-HA1) proteins [19].

In this study, the HA1 surface proteins of influenza virus (H1-HA1 and H5-HA1) were electrochemically detected by cyclic voltammetry (CV) with cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-NANA) and a previously isolated ssDNA aptamer specific to H1-HA1 [19], in an ongoing effort to develop a biosensor for the detection of influenza subtypes. CMP-NANA, which has a sialic acid moiety in its molecular structure, acts as a HA1 probe, whereas the amine-modified (NH_2-modified) DNA aptamer serves as a H1-HA1 subtype-specific probe. The immobilization of linker materials, CMP-NANA, and NH_2-modified aptamers was determined by monitoring the changes in water drop contact angles, ELISA, field emission scanning electron microscopy (FE-SEM), and CV. In addition, the specific and selective binding of surface-immobilized ssDNA aptamers to H1-HA1 was evaluated by ELISA, FE-SEM, and CV.

Materials and Methods

Immobilization of CMP-NANA and the ssDNA Aptamer on Silicon (Si) Wafer

To immobilize the aptamer, the surface of a Si wafer was modified by the self-assembled monolayer method, as described previously with slight modifications [20]. Every step in this experiment was performed at room temperature. A silicon oxide (SiO_2) wafer (2 × 2 cm in a beaker) was washed by sonication in 20 ml ethanol for 30 min and dried with N_2 gas. Then, the cleaned wafer was exposed to UV-O_3 for 330 sec and immediately immersed in 20 ml of 2% (v/v) (3-aminopropyl)triethoxysilane (APTES; Sigma-Aldrich, USA) in ethanol for 30 min. The unbound silane was removed by washing with 20 ml of ethanol and dried. Subsequently, the silanized wafer was activated with 20 ml of 2% (v/v) glutaraldehyde (Sigma-Aldrich, USA) in H_2O for 1 h, washed with 20 ml of deionized H_2O, and dried with N_2 gas. To immobilize the aptamer on the Si wafer, 5′ NH_2-modified oligonucleotides were used; 0.5 ml of 10 μM 5′ NH_2-modified aptamers (named Ap I as described previously [19], 5′-GCAATGGTACGGTACTTCCGGGTGGGT GGGAGGGGGTGGAGGTTGGGGGTTGGACGCAGAGTGCCA AAAGTGCACGCTACTTTGCTAA-3′) in PBS was added and incubated for 1 h. After incubation, the aptamer-bound wafer was washed with PBS and dried. Then, the wafer was blocked by 0.5 ml of 5% (w/v) BSA in PBST (0.1% TWEEN-20 in PBS) for 1 h and washed with PBS. Finally, 0.5 ml of 3 μM purified GST-tagged HA1 proteins in PBS were added to the wafer and incubated for 1 h. The contact angles of the surface-immobilized aptamers were analyzed using a contact angle analyzer (Phoenix 300 System). CMP-NANA (Sigma-Aldrich, USA) was used as a positive control instead of the aptamer. Since CMP-NANA contains a sialic acid moiety, HA can bind to it.

ELISA Experiments

ELISA was performed to determine whether Ap I was bound to GST-tagged HA1 proteins. First, 0.5 ml of GST antibody-conjugated horseradish peroxidase (1:1,000 in PBST; Santa Cruz Biotechnology, USA) was added to the immobilized wafer (2 × 2 cm in a beaker) for 1 h and dried with N_2 gas. Then, the unbound antibody was removed by washing with 20 ml of PBST and dried. The color-developing reaction was initiated by addition of 1 ml of 3,3′,5,5′-tetramethylbenzidine solution (Merck, Germany) and terminated by adding 2.5 N H_2SO_4. The absorbance was measured at 450 nm using a TRIAD microplate reader (Dynex Technologies, USA).

FE-SEM Images

To obtain FE-SEM images, the Ap I-immobilized Si wafer was reacted with GST-tagged HA1 proteins and then incubated with 0.05 ml of biotinylated GST antibodies (1:1,000 in PBST, Santa Cruz Biotechnology, USA) for 1 h. Unbound antibodies were removed by washing. Then, the wafer was treated with 0.05 ml of gold-labeled streptavidin (1:1,000 in PBST; KPL; Santa Cruz Biotechnology, USA) for 1 h, washed with 20 ml of PBS, and dried. The immobilized surfaces were observed by FE-SEM (JEOL JSM-7410F).

CV Measurements

For CV measurements, an indium thin oxide (ITO)-coated glass substrate was used instead of the Si wafer. The ITO-coated glass substrate (ITO thickness: 140 nm; resistance: 25 Ω/sq; 1 × 2 cm in a beaker) was cleaned by sonication in 20 ml of ethanol for 10 min, dried with N_2 gas, and silanized using APTES and GA, as described earlier.

CV was performed in PBS containing 0.1 M KCl and 5 mM $K_3Fe(CN)_6$ as a redox probe, immobilized ITO wafer as a working electrode, Pt plate as a counter electrode, and Ag/AgCl as a reference electrode. CV measurements were conducted in the potential range of −1.0 to 1.0 V with a scan rate of 100 mV/sec. The different electrochemical properties were observed by CV using a WPG1000e potentiostat (WonATech, Korea).

Results

Immobilization of the ssDNA Aptamer on Si Wafer

The functionalization process of the surface was monitored

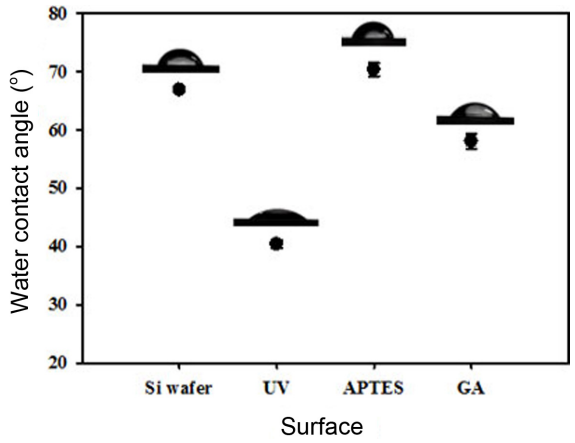

Fig. 1. Visualization of water droplets on self-assembled monolayers formed on the Si surface.
The contact angle could change depending on the functional groups on the surface.

by measuring the static contact angle. A change in contact angle values would represent a change in the functional groups on the Si wafer. As shown in Fig. 1, the contact angles could change depending on the process of surface immobilization. The contact angle of the Si wafer was 66°, which indicated the hydrophobicity of the surface, and the contact angle decreased after UV exposure because hydrophilic OH groups were introduced (Table 1). When the surface was covered with APTES and GA, the contact angles increased owing to the alkyl groups in the cross-linkers. However, the contact angles decreased in the subsequent immobilization of CMP-NANA or the aptamer, which may be attributed to an increase in hydrophilicity due to the many hydroxyl groups (Table 2). This finding indicated the successful surface immobilization of the linkers and subsequent molecular probes. After immobilization on the surface of the wafer, we also measured the contact angles to determine whether HA1 proteins were bound to the molecular probes (Table 2). In the case of CMP-NANA (known to bind to both types of HA1 proteins), the contact angle increased as expected from 47.55° to 51.14° (H1-HA1) and to 51.27° (H5-HA1). Interestingly, the contact angle increased only when H1-HA1 was added to the immobilized Ap I (from 37.95° to 54.36°). However, no changes were found when H5-HA1 was added to the immobilized Ap I, indicating that Ap I binds to H1-HA1 but not to H5-HA1.

Table 2. Measurement of the contact angles of CMP-NANA and the Ap I aptamer after HA1 protein addition.

Surfaces	No protein	H1-HA1	H5-HA1
CMP-NANA	47.55 ± 1.56°	51.14 ± 0.92°	51.27 ± 0.90°
Ap I	37.95 ± 1.07°	54.36 ± 1.30°	37.31 ± 0.86°

Each experiment was performed in triplicates and averaged.

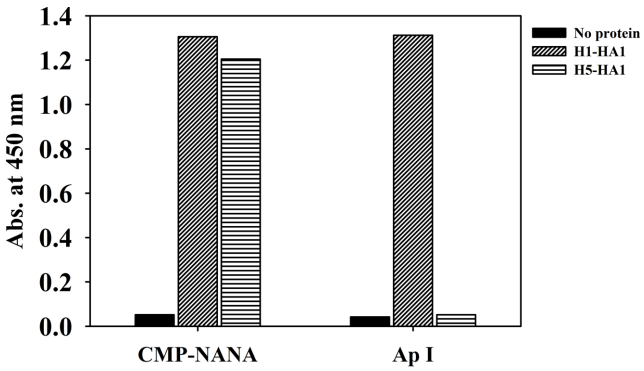

Fig. 2. ELISA experiments.
After immobilization of CMP-NANA and the Ap I aptamer on the Si surface, HA1 proteins (H1-HA1 and H5-HA1) were added. Both HA1 proteins were bound to the surface-immobilized CMP-NANA, whereas only H1-HA1 was bound to the surface-immobilized Ap I.

ELISA Experiments of the Immobilized Aptamer on Si Wafer

ELISA was performed to determine whether the immobilized Ap I could distinguish between the different HA1 proteins. Fig. 2 shows that the absorbance increased when H1-HA1 was added to the immobilized Ap I, indicating that the aptamer was bound to the H1-HA1 protein. However, the addition of H5-HA1 to the immobilized Ap I did not lead to an increase in absorbance. Control experiments with CMP-NANA showed that both HA1 proteins were bound to CMP-NANA.

FE-SEM Images

To determine whether the immobilized aptamer on Si wafer could distinguish between H1-HA1 and H5-HA1, FE-SEM images were obtained. The specific binding of Ap I to HA1 proteins can be detected through FE-SEM images using gold nanoparticles. Fig. 3 shows typical FE-SEM images of the immobilized molecular probes after the

Table 1. Measurement of the Si wafer surface immobilization-dependent contact angles.

Surfaces	Si wafer	UV	APTES	GA
Contact angle (°)	66.76 ± 0.33	40.34 ± 0.66	70.30 ± 1.13	57.96 ± 1.29

Each experiment was performed in triplicates and averaged.

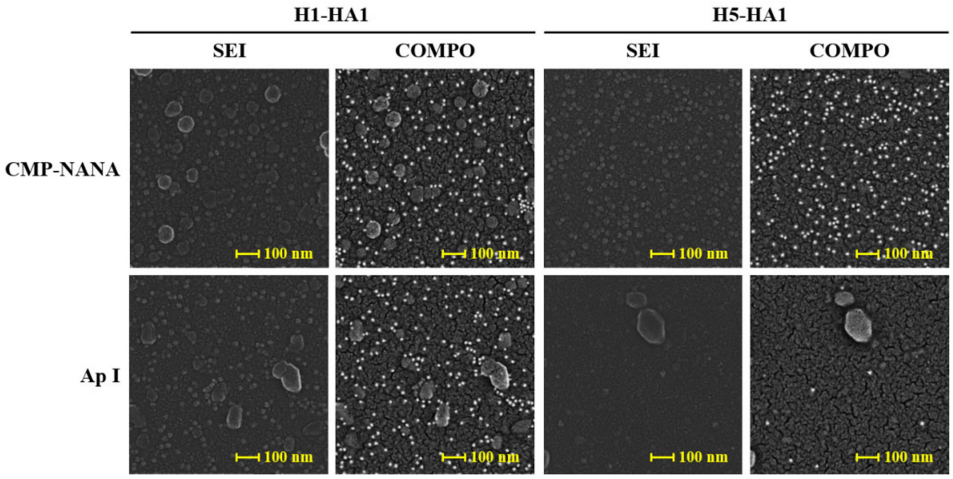

Fig. 3. FE-SEM images.
After immobilization of CMP-NANA and the Ap I aptamer on the Si surface, HA1 proteins (H1-HA1 and H5-HA1) were added. White dots represent gold-labeled streptavidin, which indicated whether the HA1 proteins were bound to the immobilized CMP-NANA or Ap I aptamer. Both HA1 proteins were bound to the surface-immobilized CMP-NANA, whereas only H1-HA1 was bound to the surface-immobilized Ap I. The scale bar indicates 100 nm.

addition of HA1 proteins. White dots represent gold-labeled streptavidin that was bound to HA1 proteins conjugated with immobilized molecular probes on the Si wafer (COMPO mode). The contrast can differ depending on the atomic number (Z) in COMPO mode, which is an appropriate method for detecting the metal element of the gold-labeled protein [21, 22]. White dots were detected only when H1-HA1 was added to the immobilized aptamer; conversely, no significant binding was detected when H5-HA1 was added. Control experiments with CMP-NANA showed that both HA1 proteins were bound to CMP-NANA.

CV Measurements

CV measurements were conducted to investigate the feasibility of the selected aptamer as an electrical sensor probe. Fig. 4A shows the cyclic voltammograms obtained from the ITO, APTES-ITO, and GA-APTES-ITO immobilized electrodes. The oxidation peak of the redox couple appeared around 0.4 V because of the presence of $K_3/K_4[Fe(CN)_6]$ in the buffer solution. The increase in oxidation current was observed after APTES was bound to the ITO electrode. Because the positive charge from the amino group of APTES was attracted to the negative charge of cyanide, there was an increase in oxidation current [23]. On the other hand, a decrease in the current was observed in the case of GA-APTES-ITO. This implied that the GA-bound electrode on the APTES surface could function as an insulating layer because of the electrostatic property of GA [24]. Fig. 4B shows the changes in the current depending on the interaction between CMP-NANA and HA1 proteins. Immobilization of CMP-NANA was confirmed by the slight increase in current due to the introduction of the NH_2 group. When the two HA1 proteins were added to the CMP-NANA-immobilized surface, a further increase in current was observed. This indicated that the two HA1 proteins were successfully bound to CMP-NANA. Fig. 4C shows the changes in the current depending on the interaction between Ap I and the HA1 proteins. Immobilization of the aptamer was confirmed by the slight increase in current due to the introduction of the NH_2 group of the ssDNA, which was similar to CMP-NANA. Then, H1-HA1 and H5-HA1 proteins were added to the aptamer-immobilized surface. A further increase in current was observed only in the case of H1-HA1. The addition of H5-HA1 did not result in changes in the current, indicating that Ap I was selectively bound to H1-HA1. The result was consistent with the FE-SEM results. Furthermore, we measured the current changes after addition of various amount of H1-HA1 to the immobilized aptamer. Fig. 4D shows the changes in the current depending on the concentration of H1-HA1 (from 0.3 to 3,000 nM). When we added 0.3 nM H1-HA1, no significant increase in current was detected. However, the increase in oxidation current was observed from 3 nM H1-HA1 addition to aptamer. This indicated that the limitation of our aptamer-based sensing system was around the nanomolar scale of H1-HA1.

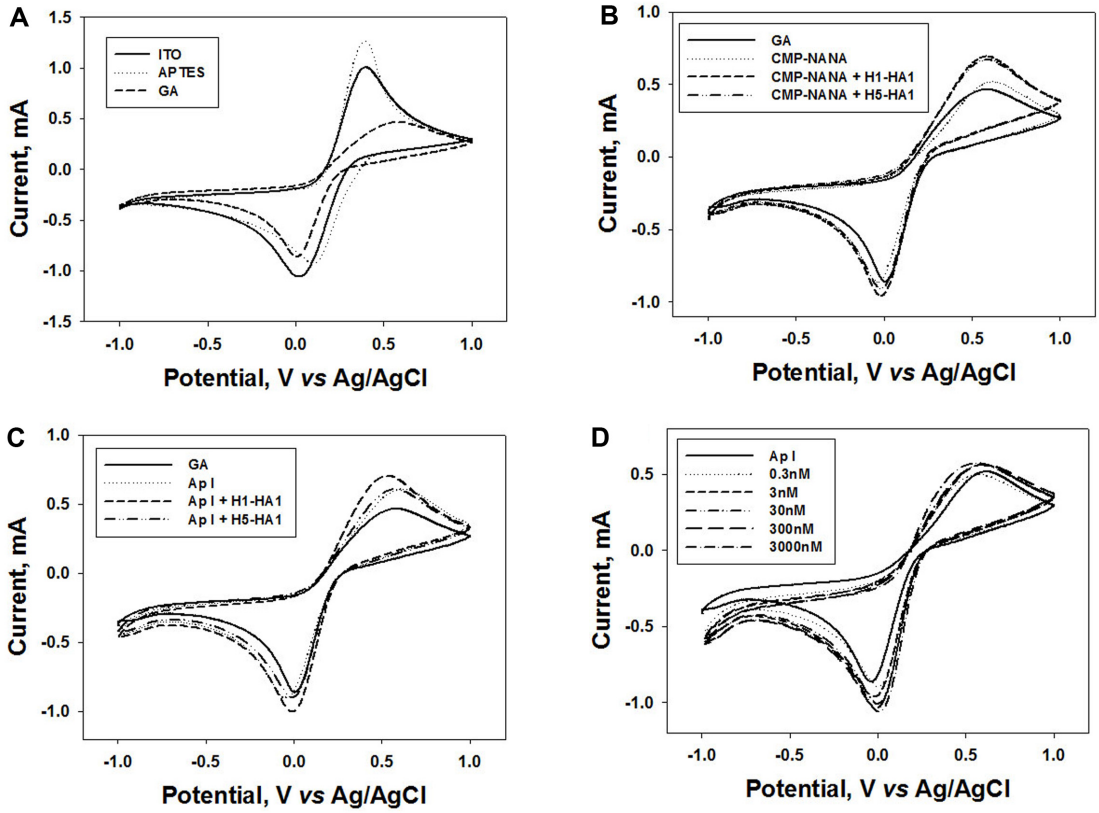

Fig. 4. Cyclic voltammograms.
(**A**) CV measurements of ITO, APTES-ITO, and GA-APTES-ITO. (**B**) CV measurements after the addition of HA1 proteins to the immobilized CMP-NANA. Similar changes in CV were observed when both HA1 proteins were added to the immobilized CMP-NANA, which indicated that both HA1 proteins were bound to CMP-NANA. (**C**) CV measurements after the addition of HA1 proteins to the immobilized Ap I. A current change was observed after the addition of H1-HA1; however, no change was observed after the addition of H5-HA1. (**D**) CV measurements after the addition of various concentrations of H1-HA1 proteins to the immobilized Ap I.

Discussion

The surface HA protein of influenza virus plays an important role in host cell receptor binding, which leads to viral infection [25]. Thus far, 18 subtypes of HA have been found; the accurate identification of HA is helpful to determine the species that are infected. Moreover, an accurate and rapid detection system is necessary to determine the type of influenza virus present in the sample. We have been attempting to develop a detection system for influenza using appropriate molecular probes. Indeed, ssDNA aptamers that can distinguish between H1-HA1 and H5-HA1 were successfully isolated by the counter-SELEX method [19]. To determine whether the selected aptamer, which is specific to H1-HA1 but not H5-HA1, is suitable as an electrochemical molecular probe, further experiments were conducted.

To immobilize the aptamer, the Si wafer surface was initially exposed to UV-O_3, which transformed the SiO_2 of the surface into hydrophilic SiOH. APTES treatment introduced the NH_2 group to the surface, and successive treatment with GA, which is a cross-linker, enabled the immobilization of the NH_2-modified aptamer on the Si wafer. To determine whether each immobilization step was successfully completed, water drop contact angles were measured. We monitored the changes in the contact angle that depended on the groups immobilized on the surface, which indicated the reaction on the surface was successful. After immobilization of the selected aptamer, the HA1 proteins were added to the surface to monitor their interaction with the aptamer. As a positive control, we used CMP-NANA in the present study, which has a sialic acid moiety and a NH_2 group in its molecular structure. Since sialic acid is known to bind to HA, and the NH_2 group is necessary for immobilization on the GA-treated surface, CMP-NANA is a suitable control probe in this

study. As expected, CMP-NANA immobilized on the Si wafer was able to bind to the HA1 proteins. However, it was unable to distinguish between H1-HA1 and H5-HA1, as shown by contact angle measurement, ELISA, and FE-SEM results. On the other hand, when CMP-NANA was substituted for the aptamer specific to H1-HA1, the immobilized aptamer was able to bind to H1-HA1 but not to H5-HA1. This indicated that the specific aptamer can be a useful molecular probe for detecting a specific antigen on the surface.

Furthermore, we performed additional experiments on the aptamer to assess its role as an electrochemical molecular probe by measuring current changes. The aptamer was immobilized on the ITO electrode, similar to Si wafer immobilization. After the addition of HA1 proteins to the immobilized molecular probes on the ITO surface, electrochemical signal responses were measured by CV. The measurements using CMP-NANA as a molecular probe showed that both H1-HA1 and H5-HA1 proteins were bound to CMP-NANA. However, only H1-HA1 was bound to the surface when we used the H1-HA1 subtype-specific aptamer as a molecular probe. We also investigated the detection limit of the H1-HA1-specific aptamer by measuring the changes of CV. Our result showed that the nanomolar range of target protein was sufficient. Therefore, the appropriate selection of an aptamer specific to an antigen will be useful for the development of an electrical sensor system.

Acknowledgments

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