

Detection of influenza virus by a biosensor based on the method combining electrochemiluminescence on binary SAMs modified Au electrode with an immunoliposome encapsulating Ru (II) complex

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Abstract Recently, point of care testing (POCT) used for diagnosis of influenza infection has a problem showing false negative diagnosis because of the low sensitivity. We would like to report detection of influenza virus A (H1N1) by an immunosensor based on electrochemiluminescence (ECL) that uses an immunoliposome encapsulating tris(2,2'-bipyridyl)ruthenium(II) complex. By using the sensor, we could detect the virus that competed with hemagglutinin (HA) peptide immobilized on self-assembled monolayers (SAMs) in immunoreaction of the antibody bound on the surface of liposome. The HA peptide was 19 mer (TGLRNGITNKNVNSVIEKAA). We demonstrated great improvement of sensitivity and accuracy by introducing binary SAMs instead of mono SAMs. The binary SAMs was prepared from 3,3'-dithiodipropionic acid and 1-hexanethiol. Use of the binary SAMs enabled to increase the SAMs coverage on Au electrode; the fact was confirmed by observation of the cathodic desorption currents. By using such an electrode, first the detection method of BSA was optimized to lower ECL background signal. Then we applied the method to the detection of influenza virus. We could successfully detect the

virus with higher sensitivity compared with that by POCT and ELISA. The detection range was from a concentration of 2.7×10^2 to 2.7×10^3 PFU/mL.

Keywords Electrochemiluminescence · Immunoliposome · Influenza virus · Highly sensitive detection · Self-assembled monolayers

Abbreviations

BSA	Bovine serum albumin
CV	Cyclic voltammetry
DPPE	1,2-Dipalmitoyl-rac-glycero-3-phosphoethanolamine
DPPC	1,2-Dipalmitoyl-rac-glycero-3-phosphocholine
DTPA	Dithiodipropionic acid
DTT	(±)-Dithiothreitol
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay
HA	Haemagglutinin
HT	Hexanethiol
NHS	N-Hydroxysuccinimide
OT	Octanethiol
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque formation unit
PSA	prostate-specific antigen
RT-PCR	real-time PCR
SAMs	Self-assembled monolayers
SPDP	N-Succinimidyl-3-(2-pyridyldithiol) propionate

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WSC Ethyl-3-(3-dimethylaminopropyl)
carbodiimide, hydrochloride

Introduction

For the past several hundred years, pandemic of influenza frequently caused a great number of deaths worldwide. The emergency of new influenza virus has been recognized as a very serious threat to human life. In order to safeguard many people from a pandemic of influenza, we must invent novel tools that show quite high sensitivity to detect a small amount of the virus as far as possible. One virus has hundreds of HA molecules as a trimer of protein on the surface of the envelope membrane. Accordingly, the HA molecules are a good mark for detection of the virus.

Virus detections are currently being achieved with several methods. The rapid diagnosis kit such as lateral-flow assay [1, 2] on the basis of immunochromatography gives such low sensitivity that it needs over 10^4 PFU/mL, though it has a big advantage in the short detection time, within 30 min [3, 4]. Conventional ELISA having the same sensitivity requires a detection time of about 2 h [5, 6]. RT-PCR method can detect viruses with high sensitivity and moreover identify the subtype, but needs a detection time of several days, expensive equipment, and skilled technicians to perform [7]. For this reason, it is very important to develop a more highly sensitive and simple method.

Recently, many researchers have proposed a variety of ECL sensors utilizing nanoparticles (NPs) or liposomes [8–10]. ECL is very attractive owing to the possibility of high sensitivity [11]. Immunosensor based on cathodic ECL using thionine-modified SiO₂ NPs has been applied to the detection of several proteins; the response range for α -fetoprotein was 7×10^{-11} to 1.4×10^{-8} g/mL [12]. A ECL sensor combining Ru complex-labeled protein has shown sufficient sensitivity for disease-derived antibody [13]. An immunosensor based on ECL has been constructed by combining the Au-NPs/MWCNT-immobilized GC electrode and dendrimer having Ru complex-modified DNA, and shows a detection limit of 4.2 fg/mL [14]. An ECL peptide-based biosensor for determination of PSA has successfully used the induced cleavage of peptide to gain high sensitivity; the lower detection limit is 8×10^{-13} g/mL [15]. Liposomes are a promising tool for enhancing the sensitivity or for affording the site of various reactions. A liposome-PCR assay using encapsulated DNA reporters has accomplished detection of a trace amount of a biological toxin (0.02 fg/mL) [16]. Antibody-modified liposomes, immunoliposomes, have been utilized to detect *E. coli* by encapsulating dyes [17]. Biotin-modified liposomes have been used to determine the concentration of a protein (100 ng to 10 μ g/mL) by combining ECL with magnetic microbeads [18]. Such methods using liposomes could be utilized to rapidly

detect very low amounts of the virus. In our previous work, an ECL sensor employing an immunoliposome encapsulating a novel Ru complex was fabricated and enabled the detection of HA in extremely low concentration of 3×10^{-14} g/mL. This method had duplicate amplifications of ECL signal by liposome and adsorption of Ru complex onto Au electrode. Therefore, the results strongly prompted us to detect the virus with many HA proteins on the surface [19].

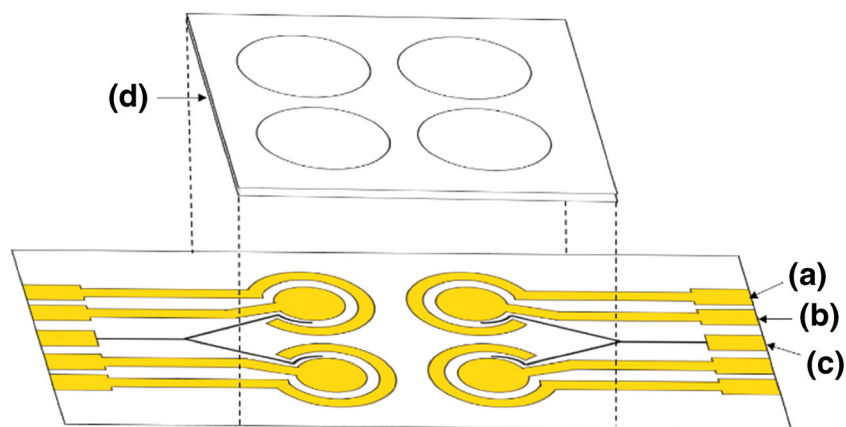
On the other hand, modification of metals with SAMs has been widely utilized for generating new functionality on the surface of materials, for example, hydrophilicity, water repellency, and sites for a variety of reactions [20]. Binary SAMs formed from two components of thiols and/or disulfides are noteworthy for easily giving higher functionality in comparison with mono SAMs. Recently, several researchers have tried to use binary SAMs that create some space for bulky enzymes or antibodies to efficiently work [21], and for electron transfer [22], in addition to immobilization of proteins [23, 24]. As far as we are aware, the application of the binary SAMs to ECL sensors has not been found in any research as yet.

Though use of the SAMs is very promising, the coating surface frequently has small defects or pinholes. The defects seriously affect background signals and signal intensities for detections. In the present paper, we aim to prepare an ECL sensor to detect the influenza virus with high sensitivity. To keep the ECL signal highly accurate, we need to decrease the troublesome background signal that may generate from adsorption of the immunoliposome onto the SAMs defect areas on the Au electrode. Thus, we try to use the binary SAMs to solve such a problem. The ingredients of the SAMs used here are DTPA and HT that have a short alkyl chain capable of advancing fast electron transfer.

Materials and methods

Tris (2,2'-bipyridyl) ruthenium perchlorate was synthesized ruthenium (III) chloride and 2,2'-bipyridyl (Kanto Chemical Co., Inc., Nihonbashi, Tokyo, Japan). DPPC and DPPE were obtained from Sigma Aldrich. SPDP was obtained from Thermo Scientific. Cholesterol was obtained from Kanto Chemical Co., Inc. DTT and NHS were obtained from Wako Pure Chemical Industries, Ltd, Osaka, Japan. WSC was obtained from Dojindo Lab. Co. BSA was obtained from Wako Pure Chemical Industries, Ltd. IgG fraction rabbit anti-bovine albumin was obtained from Inter-Cell Technologies, Inc. Jupiter, FL, USA. Influenza virus, A/Hiroshima/2001 (H1N1) was kindly donated by Dr. Shin-ichi Takao, Hiroshima Prefecture Technology Research Institute, Japan. Antigen peptide of HA was TGLRNGITNKVNSVIEKAA (19 mer, MW = 2105.30). Anti-HA mouse monoclonal antibody (antiHA-mAb) was obtained through genetic engineering as previously reported [19].

Fig. 1 Four Au electrodes and spacer. (A), counter electrode; (B), working electrode; (C), reference electrode; (D), spacer



Preparation of immunoliposome

DPPE and antiHA-mAb (or antiBSA-pAb) were reacted with SPDP to give DTP-DPPE and SPDP-HA mAb (or SPDP-BSA pAb). DPPC (10 μmol), DTP-DPPE (50 nmol), and cholesterol (2.5 μmol) were mixed in $\text{CHCl}_3:\text{CH}_3\text{OH}=9:1$ solvent. After removal of the solvent by evaporation, 5 mM Ru (II) complex solution (dissolved in 10 mM PB) was added to the lipid film. This mixture was vortexed and extruded through membrane filter with a pore size of 50 nm. The extruded solution was chromatographed on Sephadex G-50 fine column (mobile phase: 10 mM PB, pH 7.4). The liposome fractions were collected by fluorescence measurement, and concentrated by centrifugal filtration with a Centriprep YM-10 filter unit. The SPDP-antibody activated by DTT and the liposome was reacted at room temperature for 24 h. The reaction solution was

chromatographed on Sepharose 4B (mobile phase: 10 mM PB, pH 7.4). The immunoliposome fractions were collected and concentrated to 700 μL by centrifugal filtration. The immunoliposome thus obtained was stored under N_2 at 4 $^\circ\text{C}$. The diameter was determined to be 90 nm by a Zetasizer Nano (Malvern Instruments Ltd., Malvern, Worcestershire, UK).

Cyclic voltammetry measurement

Au working electrode with a diameter of 1.6 mm was cleaned by applying cyclic potential scanning from 0 V to -1.4 V in 0.1 M NaOH with a sweep rate of 0.1 V/s until a stable background peak was obtained. After polishing by alumina (particle diameters: 1000 nm and 50 nm) and ultrasonic cleaning, the electrode was immersed in DTPA/HT ethanol solution (0, 0.5, 1, 2, 5, 10 % of HT mole ratio) or DTPA/OT ethanol

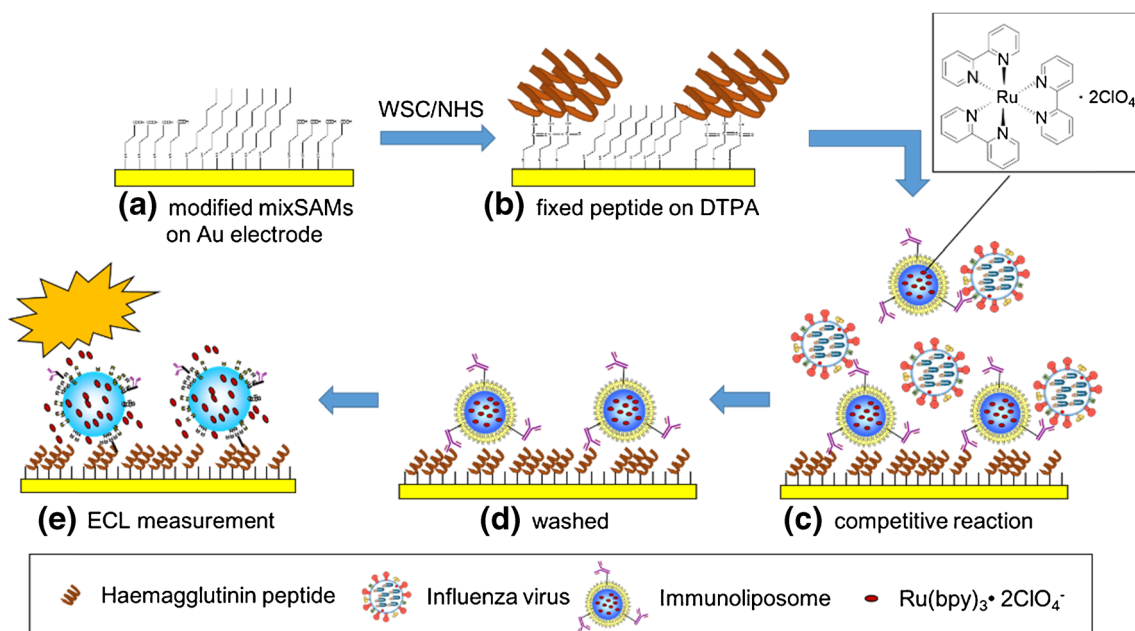
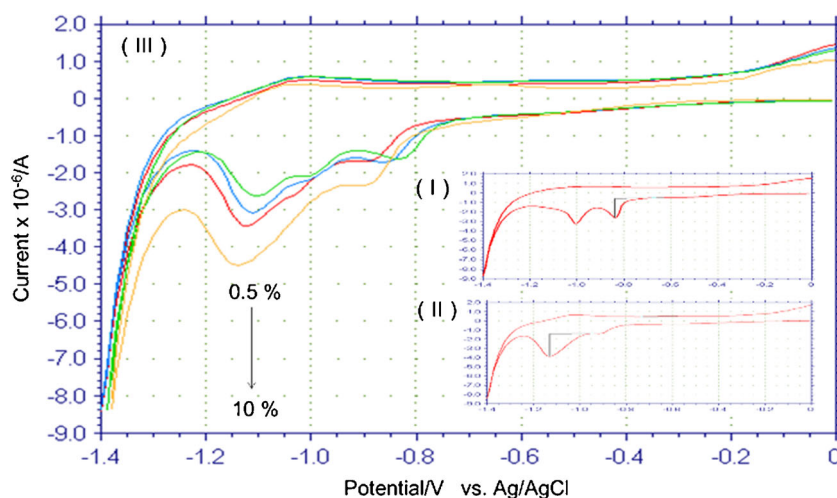


Fig. 2 Detection procedure: (a) modifying of DTPA/HT binary SAMs on Au electrode; (b) fixing of hemagglutinin peptide on activated DTPA; (c) competitive reaction of immunoliposome and influenza virus; (d) washing; (e) ECL measurement on application of potential

Fig. 3 Cyclic voltammograms for reductive desorption of binary SAMs and mono SAMs. The CV curves for each reductive desorption were analyzed in 0.5 M KOH solution at 25 °C and scan rate was 0.1 V/s. The voltammograms were (I) DTPA SAMs, (II) HT SAMs, (III) binary SAMs, respectively. Binary SAMs were prepared in 0.5–10 % of HT concentration



solution (0, 0.5, 1, 2 % of OT mole ratio) at 50 °C for 2 h, and subsequently rinsed with ethanol and water. Reductive desorption currents of the modified electrode were measured by CV in 0.5 M KOH with 0.1 V/s at 25 °C. The counter and reference electrodes were a Pt wire and Ag/AgCl electrode, respectively.

Virus and BSA detections by ECL

The detection procedure was slightly different from the one reported previously [19]. The concentration step of Ru(II) complex with bis(aminobutyl) moiety onto Au electrode was omitted and the overall detection steps using Ru(II)(bpy)₃2Cl were thus shortened. Figure 1 shows four Au electrode units formed on a glass plate (20 mm × 30 mm) for ECL measurement. The electrode cleaned by piranha solution was immersed in 10 mM DTPA ethanol solution containing 1 % HT at 50 °C for 2 h (Fig. 2a). The SAMs immobilized electrode was activated by 26 mM WSC/NHS at room

temperature for 2 h and allowed to react with 0.1 µg/mL antigen peptide (or 1 mg/mL BSA) containing 0.1 M PB (pH=7.4) at 4 °C for overnight. The electrode was then immersed in 0.1 M 2-aminoethanol at room temperature for 1 h to block activated carboxyl groups (Fig. 2b). After covering the electrode with a silicone sheet, an aliquot (50 µL) of influenza virus (or BSA) solution and the immunoliposome solution (10 µL) were added to the electrode for competitive immunoreaction at room temperature for 1 h (Fig. 2c). To the electrode washed with 0.1 M PB (Fig. 2d) was added 0.1 M PB containing 0.1 M triethylamine and 100 ppm Triton X-100. ECL was finally acquired after application of 1.3–1.4 V versus Au quasi reference electrode with a DNA Reader Pro1 (Microtech Niton Company, Ltd., Funabashi, Chiba, Japan) (Fig. 2e). ECL intensity was measured three times and averaged. In the ECL response curves, each point showed the standard deviation using (n - 1). The detection range was estimated from the value of two standard deviations away from the background.

Fig. 4 Relationship between numbers of molecules in SAMs and HT preparation concentration (0, 0.5, 1.0, 2.0, 5.0, and 10.0 %). The red and blue bars represent the numbers of DTPA molecules and total molecules (DTPA + HT), respectively

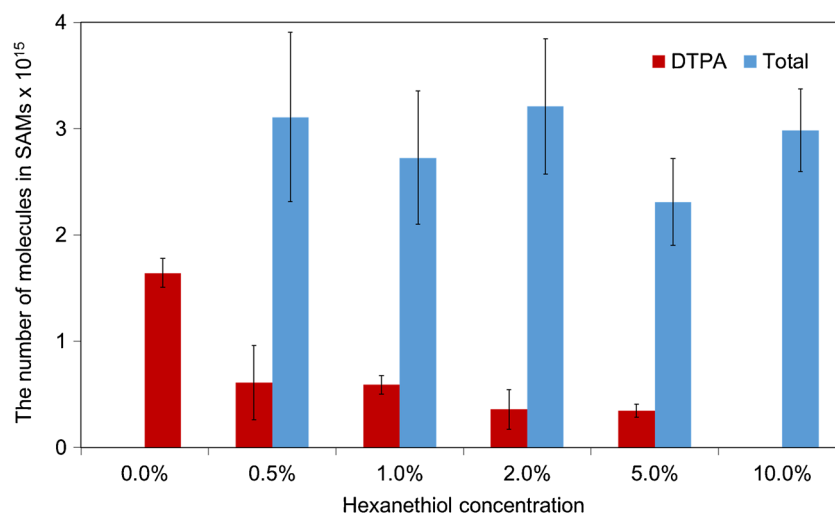
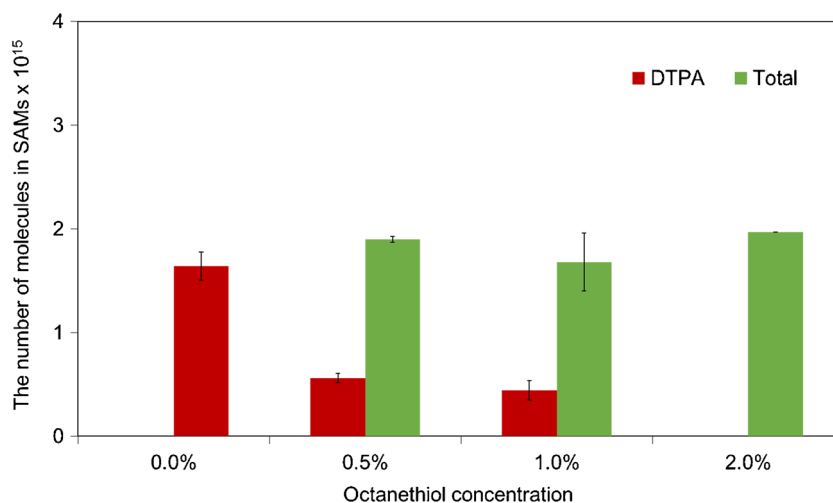


Fig. 5 Relationship between numbers of molecules in SAMs and OT preparation concentration (0, 0.5, 1.0, and 2.0 %). The red and green bars represent the numbers of DTPA molecules and total molecules (DTPA + OT), respectively



ELISA

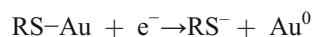
The conventional method was carried out in the same way as previously reported [19, 25].

Results and discussion

Developed bioanalytical procedure

In order to improve the coverage of SAMs on Au electrode, we first searched the composition of DTPA and HT in the binary SAMs by CV. Figure 3 shows voltammograms for reductive desorption of DTPA/HT binary SAMs on Au electrodes. The complex waves are due to overlap of reductive desorption currents of two components on the surface of polycrystalline Au. Inset (I) represents the reduction peaks for DTPA at -1.0 V and -0.85 V that can be ascribed to reductive desorption currents from (110) and (111) faces of Au electrode [26, 27]. Similarly,

inset (II) also shows the reductive peaks for HT at -1.15 V and -0.90 V that can be ascribed to reductive desorption currents from (110) and Au (111) faces, respectively [28]. Generally, the reductive desorption of alkanethiolates from Au proceeds through one-electron step as follows [20]:



Separation of the overlapped desorption currents in Fig. 3 was conducted, assuming that the shoulder around -1.0 V and the peak around -0.85 V may be mainly due to the reductive desorption current of DTPA and the contribution of HT desorption current around -0.85 V is neglected because of very small desorption current as shown in inset (II). Additionally, both reductive desorption currents of DTPA and HT per molecule can be assumed to be the same. To estimate the molecules of the binary SAMs, the base current was first subtracted from the cathodic currents. In the resulting voltammograms, the electricity calculated from the integration of the peak current from -1.25 V to -0.75 V was converted to the total molecules on

Fig. 6 A typical ECL and current profiles on potential application. ECL (orange) and current (green dotted line) were measured by application of the potential (blue) that was linearly increased up to 1.4 V in 0.4 s and then kept at 1.4 V

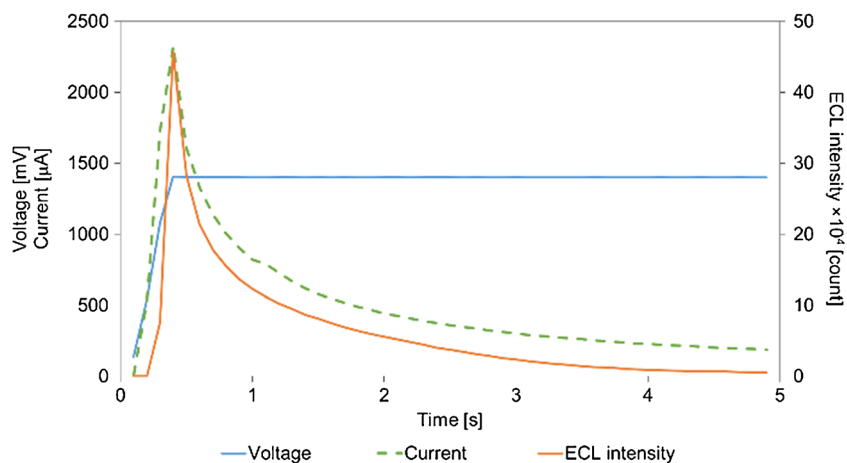
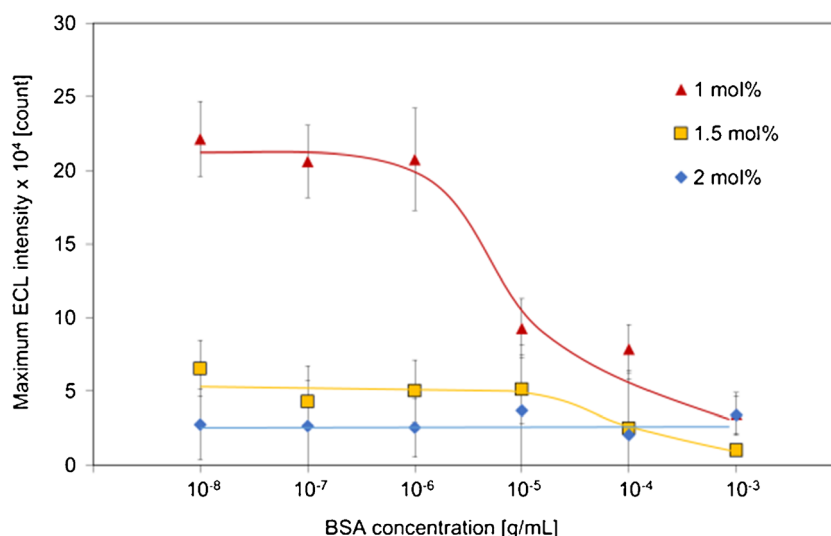


Fig. 7 Detection of BSA on Au electrode modified with differential DTPA/HT binary SAMs. Each binary SAMs was formed from the HT preparation solutions of 1.0, 1.5, and 2.0 %. Applied voltage was 1.3 V



the electrode [29]. Similarly, the molecules of the DTPA was roughly obtained from the electricity calculated from the integration of the peak current from which the leaned base current was subtracted (-1.10 V to -0.90 V) because of the location on the shoulder of the HT current; in the potential range of -0.90 V to -0.75 V, the peak current attributable to DTPA was neglected because it was inseparable from that to HT.

Figure 4 shows the total molecules (DTPA and HT) and molecules of DTPA, estimated from the quantity of the electricity, on the binary SAMs electrodes. Clearly, the total molecules of DTPA and HT in the binary SAMs are about two times larger than DTPA molecules on the mono SAMs electrode. The fact indicates that the coverage of Au electrode increased up to twice. On the other hand, with an increase in HT mole fraction in the preparation solution, the molecules of DTPA gradually decreased and disappeared at 10 % HT. The decrease of DTPA on the electrode is not a serious issue. In case of 1 % HT, the

presence of about 5×10^{14} DTPA molecules in the binary SAMs may be enough to bind with proteins, and the resulting space due to HT can afford an useful reaction site where the immunoreaction efficiently takes place [21, 24]. It is not surprising that the resulting ratio of DTPA to HT molecules in the binary SAMs is significantly different from that of the preparation solution [20]. The difference is due to more stable SAMs formation of HT [20, 30]. The binary SAMs of DTPA and OT were also pursued in the same way. Figure 5 shows the total molecules (DTPA and OT) and molecules of OT in the binary SAMs, estimated in the same way as in Fig. 4. The coverage by binary SAMs is slightly larger than that by the mono SAMs of DTPA. It should be noted that the desorption current due to DTPA disappeared at 2 % OT in the preparation solution. Thus, from a viewpoint of the large coverage, we should select the binary SAMs prepared from 1 % HT preparation solution for protein detections shown below.

Fig. 8 Comparison of one component SAMs and binary SAMs by BSA detection. Applied voltage was 1.3 V

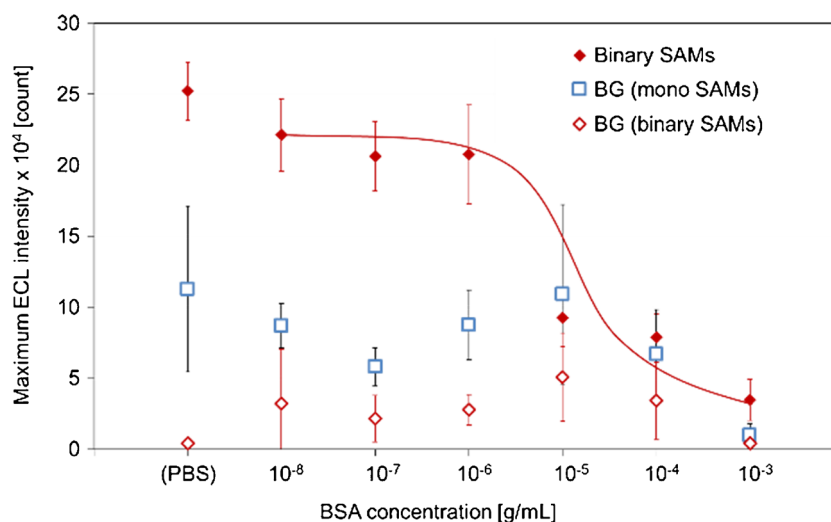
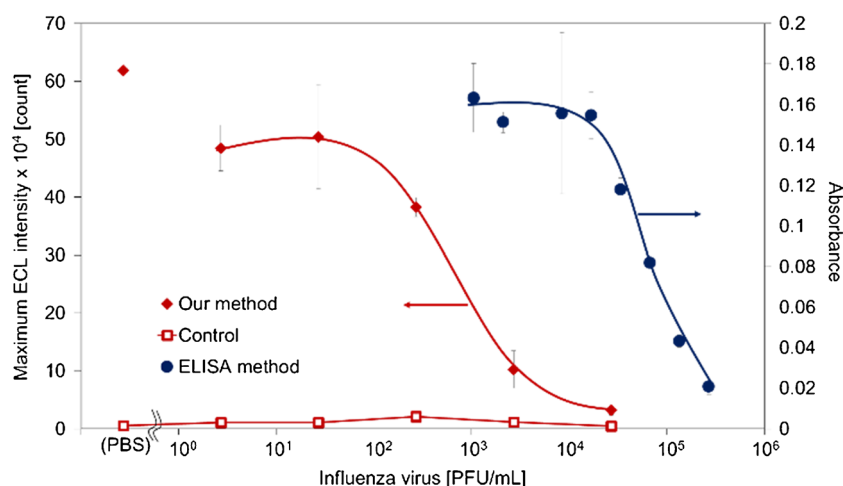


Fig. 9 Calibration curves for influenza virus (H1N1) by ECL method (red) and ELISA method (blue). Applied voltage was 1.4 V



Detection for BSA

The biosensor with the SAMs modified with Au electrodes was subsequently applied to detection for BSA as a preliminary experiment. Typical ECL and current profiles are shown in Fig. 6, indicating that the ECL and current abruptly increased to 0.4 s (1.4 V) and then decayed greatly. Figure 7 shows calibration curves for BSA with several ratios of DTPA/HT binary SAMs modified Au electrodes. At 1 % HT, the sensor shows the strongest response that is a sigmoidal curve with a little slow steep because of competitive reaction. Conversely, the ECL intensities of the responses at 1.5 and 2 % HT were considerably weaker. The weak responses may be due to the decrease in DTPA molecules that were brought about by preferential adsorption of HT. Therefore, we decided to use the 1 % HT SAMs as the optimal condition.

Lowering of background signal is crucial for high sensitive detection. As is shown in Fig. 8, in the control experiments where the fixation of BSA onto SAMs was intentionally omitted, the background ECL intensities lowered up to one-half compared with that with the one component SAMs electrode. Use of the calibration curve allows determining BSA concentrations ranging from 10^{-6} to 10^{-3} g/mL and the sensitivity is as high as that by conventional ELISA.

Detection for virus

As shown in Fig. 9, a calibration curve for influenza virus is obtained by the sensor with the binary SAMs modified Au electrode that was formed from 1 % HT preparation solution and can be used for the determination of the virus in the concentration range of 2.7×10^2 to 2.7×10^3 PFU/mL. In the control experiment where the fixation of HA peptide onto the SAMs was omitted, the curve responded little to the various concentrations of the virus. In contrast, the conventional ELISA shows the determination range from 3×10^5 to 4×10^6 PFU/mL. Clearly, the lower detection limit by the present sensor is lower

by a factor of two than that by the ELISA and commercially available POC. The high sensitivity for the virus may be partly due to numerous antibodies bound to the surface of the liposome. Recently, several researchers have tried to detect the virus by new methods. The sandwich ELISA for the MP, M, and NP1 proteins in virus has shown about four times higher sensitivity than the conventional ELISA, although it needs many antibodies [31]. The immunosensor based on electrochemical impedance spectroscopy has displayed almost the same sensitivity as the ELISA, although it can work with a simple system [32]. Additionally, lateral flow immunoassay using dye-doped silica NPs has established over eight times higher sensitivity than the ELISA [33]. It is obvious that the present sensor can detect the virus with higher sensitivity. The sensor may be a promising tool for detecting several hundred PFU/mL of the virus in about 1 h. To make the sensor suitable for practical use, the measurement time should be shortened to about 30 min.

Conclusion

We suggest detecting influenza virus by the novel biosensor combining ECL and immunoliposome. We utilized the binary SAMs modified Au electrode for improving accuracy. In the case of DTPA/HT binary SAMs, the total molecules were about two times larger than mono SAMs of DTPA. The fact indicated that the coverage of Au electrode increased up to two times. Then in the detection for BSA using the binary SAMs formed at 1 % HT preparation solution, the biosensor showed the strongest response. The background signal lowered to one-half and the sensitivity was higher than mono SAMs of DTPA. The sensor was finally applied to the detection for influenza virus (H1N1) in 1 h and the detection range was 2.7×10^2 to 2.7×10^3 PFU/mL. This result was clearly a higher sensitivity than conventional ELISA and POCT [34]. In the future, the present sensor will be applicable as a POCT giving no false negative diagnosis.

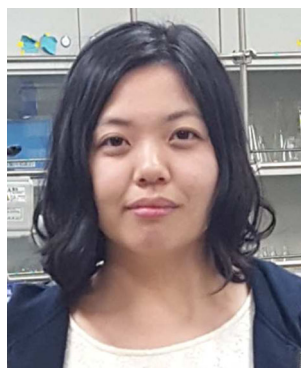
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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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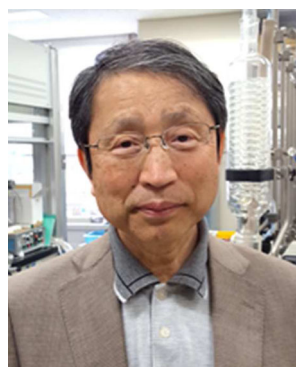
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specifically examine new dechlorination of dioxin, dioxin-like compounds, and aliphatic compounds by nanoparticles under dry or environmentally friendly conditions, but also on new solidification and immobilization method of toxic heavy metals in soil.