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Amperometric bioaffinity sensing platform for avian influenza virus proteins with aptamer modified gold nanoparticles on carbon chips

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ABSTRACT

A sandwich assay platform involving a surface formed aptamer–protein–antibody complex was developed to obtain the highly selective and sensitive amperometric detection of H5N1 viral proteins using a gold nanoparticle (NP) modified electrode. This is the first aptamer–antibody pairing reported for the selective detection of H5N1. Nanoparticle deposited screen-printed carbon electrodes were first functionalized by the covalent immobilization of a DNA aptamer specific to H5N1 followed by the adsorption of H5N1 protein. Alkaline phosphatase (ALP) conjugated monoclonal antibody was then adsorbed to form a surface bound Au NPs-aptamer/H5N1/antiH5N1-ALP sandwich complex which was further reacted with the enzyme substrate, 4-amino phenyl phosphate (APP). The current associated with the electrocatalytic reaction of the surface bound ALP with APP increased as the H5N1 concentration increased. A lowest detectable concentration of 100 fM was obtained with a linear dynamic range of 100 fM to 10 pM using differential pulse voltammetry. As an example, the biosensor was applied to the detection of H5N1 protein in diluted human serum samples spiked with different concentrations of the viral protein target.

1. Introduction

Over the last decade highly pathogenic avian influenza virus of type A of subtype, H5N1, have resulted in 668 cases of human infection (WHO, 2014) in addition to a loss of over $760 million worldwide in the poultry industry (Burns et al., 2008). Adverse effects of H5N1 on economic as well as social wellbeing necessitates the need for a rapid, field applicable, sensitive and reliable method to detect H5N1 as a precautionary step to control the spread and for diagnostic purposes (Kamikawa et al., 2010; Wei et al., 2013). Typically employed methods to detect H5N1 include serological tests (Desvaux et al., 2012), enzyme-linked immunosorbent assays (ELISAs) (He et al., 2010; Zhu et al., 2014; Lebarbenchon et al., 2012; Moreno et al., 2013), immunoblotting (Jackson et al., 2010), reverse transcription polymerase chain reaction (Dhumpa et al., 2011) and western blot assay (Uyeki et al., 2012). But these methods are associated with some disadvantages like requiring excessive reagents, long sensing time, complicated sample processing and further sophisticated tests for confirmation leading to an urge to develop a portable and robust biosensor (Kamikawa et al., 2010; Wei et al., 2013). High levels of sensitivity, cost effective fabrication and easy handling in addition to low sample quantity requirement make electrochemical biosensors one of the outstanding candidates (Lv et al., 2010; Ricci et al., 2012; Ionescu et al., 2007).

Considerable research has also been undertaken on creating a diverse range of optical and electrochemical H5N1 sensors over the past few years. For example, fluorescence biosensors based on metal organic frameworks with a detection limit of 1.6 nM (Wei et al., 2013), and a CdTe quantum dot based biosensor with a detection limit of 3 ng mL^{-1} (Nguyen et al., 2012). Quartz crystal microbalance (QCM) aptasensors and magnetic nanobeads amplified QCM immunosensors have also been reported for the detection of H5N1 protein (Brockman et al., 2013; Li et al., 2011). Alternatively, electrochemical biosensors have also been reported as a promising approach; for instance, the label-free detection of H5N1 protein using indium-tin-oxide thin film transistors showed a detection limit of 80 pg mL^{-1} (Guo et al., 2013), while a carbon nanotube network field effect transistor was reported to detect 1.25 pM H5N1 protein (Thu et al., 2013). In addition, non-faradic impedance biosensors and microelectrode based impedance immunosensors have been proved as a potential method for H5N1 hemagglutinin (Lum et al., 2012; Wang et al., 2009). Another method based on immobilization of polydiacetylene vesicles on to a polystyrene microsphere surface was also investigated and a detection limit of 1 ng mL^{-1} for H5N1 protein was achieved using

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both electrochemical and fluorescence measurements (Dong et al., 2013).

The integration of nanomaterials within electrochemical sensors can provide greater flexibility, functionalities and controlled properties within various detection format (Hayat et al., 2014). For example, the detection of H5N1 gene sequence has been reported based on electrically active magnetic nanoparticles and glassy carbon electrodes modified with carbon nanotubes-cobalt phthalocyanine-polyamidoamine dendrimer nanocomposites (Kamikawa et al., 2010; Zhu et al., 2009) achieving a detection limit of 1.4 μM and 1.0 pg mL^{-1}, respectively. In particular, Au NPs have been widely employed for developing biosensors due to their exceptional electrochemical properties, excellent catalytic behavior and providing a greater active surface area and biocompatibility such as for enzyme immobilization (Pingarron et al., 2008). We have also reported the combined use of Au NPs and surface enzyme reactions to enhance the sensitivity and selectivity of electrochemical biosensors for immunoglobulin E, phenol and catechol (Nam et al., 2012; Karim and Lee, 2013; Karim et al., 2014).

In this paper, we demonstrate a novel, portable and highly reproducible amperometric biosensing platform composed of a surface sandwich complex of H5N1 aptamer/H5N1 protein/H5N1 antibody conjugated with alkaline phosphatase (antiH5N1-ALP) formed on a gold nanoparticle modified screen printed carbon electrode (SPCE). The advantages of using aptamers when available instead of antibodies is well established (e.g. chemical stability and synthesis, reproducibility, selectivity) (Cho et al., 2009; Lee et al., 2008). We have also shown aptamer-functionalized surfaces to be superior to antibodies for bioaffinity measurements in serum both in terms of non-specific interactions and higher specificity (Jang et al., 2014). The H5N1 specific aptamer–antibody pairing introduced in this work is being reported for the first time with the electrochemical detection signal enzymatically amplified utilizing ALP to create an electroactive substrate in the presence of the protein target. Recently, Liu et al. (2011) reported the electrochemical detection of a synthetic H5N1 gene sequence via a redox active DNA intercalator using a hybrid electrode composed of Au NPs, polypyrrole nanowires and carbon nanotubes assembled on a gold surface. Our measurements were performed on a SPCE onto which Au nanoparticles (NPs) were electrochemically deposited followed by surface functionalization. Following validation of the aptamer–antibody pairing, the electrocatalytic reaction of the surface bound ALP with APP substrate was correlated to H5N1 concentration via both cyclic (CV) and differential pulse (DPV) voltammetries. In addition, the biosensors were employed for the analysis of H5N1 concentrations spiked into diluted serum samples.

2. Experimental section

2.1. Reagents and solutions

All of the chemicals listed here were used as received unless otherwise specified. Gold (III) chloride trihydrate ($HAuCl_4$, Sigma-Aldrich), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, Thermo), 3-mercaptopropionic acid (MPA, Sigma-Aldrich), 11-mercaptoundecanoic acid (MUA, Sigma-Aldrich), *N*-hydroxysulfosuccinimide (NHSS, Thermo), sulfuric acid, 95%, (OCI Company Ltd), tris(hydroxymethyl)-aminomethane (≥ 99.9%, Sigma-Aldrich), potassium chloride (Merck), magnesium chloride anhydrous (Junsei Chemical Co., Ltd), 4-aminophenylphosphate monosodium salt (APP, LKT Laboratories, Inc.), influenza A virus hemagglutinin H5 full length protein (Abcam), human serum from human male AB plasma (Sigma-Aldrich), minisart SRP 4 syringe filter (pore size=0.2 μm, Sartorius Stedim Biotech GmbH), influenza A virus hemagglutinin H5 monoclonal antibody (Abnova), H5N1 DNA aptamer sequence: 5'- TTG GGG TTA TTT TGG GAG GGC GGG GGT T-NH_2-3' (Shiratori et al., 2014) and NC4 control sequence: 5'-H_2N-GGG GCA CGT ACG GGC ATC ATA ACA ACA GGC GTG CCC C-3' were synthesized by Integrated DNA Technologies (IDT). Also, immunoglobulin G from human serum (IgG, Sigma-Aldrich), albumin from human serum lyophilized powder (globulin free, Sigma-Aldrich), brain natriuretic peptide (BNP, Tocris) and alpha-1 antitrypsin antibody (antiAAT, R&D Systems), were used along with the NC4 sequence for non-specific interaction and selectivity studies. 10 mM phosphate buffered saline (PBS, pH 7.4, Gibco) was used as a storage buffer for H5N1 protein, H5N1-aptamer and antiH5N1. AntiH5N1 was conjugated with ALP using a ALP labeling kit-NH_2 (Dojindo Molecular Technologies, Inc.) following the protocol provided by the company website (www.dojindo.com/store/p/47-Alkaline-Phosphatase-Labeling-Kit-NH2.html). The electrocatalytic reaction between ALP and APP was performed in 50 mM Tris buffer solution containing 10 mM KCl and 1 g/L of $MgCl_2$ (pH 8.5) at room temperature (Nam et al., 2012). Millipore-filtered water was used to make all aqueous solutions.

2.2. Validation of aptamer–antibody pairing

A series of surface plasmon resonance (SPR) measurements were performed to verify the antibody and aptamer bioaffinity interactions both separately and in a sandwich assay format. These measurements were also used to confirm surface activity on a gold surface and obtain binding coefficients for each probe. A Biacore 3000 at the National Nanofab Center (Daejeon) was used at a flow rate of 5 μL/min and a running buffer of PBS (pH 7.4) solution was used throughout. Further experimental details on the gold film SPR chips and data acquisition are provided in supporting information.

2.3. Electrochemical measurements

All electrochemical investigations were performed using a computer controlled potentiostat (Autolab PGSTAT128N, Ecochemie) operated by General Purpose Electrochemical System (GPES) version 4.9 software package. SPCE consisting of a carbon working electrode with a geometric area of a 28 mm^2, a Ag/AgCl reference electrode and a carbon counter electrode were purchased from The BIO Co., Ltd. SPCE's were connected with the potentiostat using a sensor connector. CV and DPV were used throughout the experiments with the condition as follows: for CV, the scan rate of 50 mV/s, while for DPV, the step potential of 20 mV/s, pulse interval of 50 ms and amplitude of 50 mV.

2.4. Electrodeposition of gold nanoparticles on SPCE

Electrodeposition of Au NPs was performed onto carbon working electrodes using a well-established method that has been previously reported (Karim and Lee, 2013; Renedo and Martínez, 2007; Barquero-Quirós et al., 2014; Alvarado-Gámez et al., 2015; Alonso-Lomillo et al., 2009). SPCEs were immersed into a 0.5 M H_2SO_4 solution containing 0.5 mM $HAuCl_4$ followed by applying a constant potential of 0.18 V (vs. Ag/AgCl) for 400 s while stirring. The Au NP deposited SPCE was then thoroughly washed with water and dried using a N_2 stream.

2.5. Biofunctionalization of gold deposited electrode surface

Au NP deposited SPCEs were soaked in 20 mM MPA solution prepared in absolute ethanol/water in a v/v ratio of 75/25 for 15 h at room temperature leading to the formation of a self-assembled carboxylic acid terminated monolayer. The bulk concentrations and timescales used for MPA coating of the electrodeposited Au NPs are