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Rapid detection of avian influenza virus H5N1 in chicken tracheal samples using an impedance aptasensor with gold nanoparticles for signal amplification

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A B S T R A C T

Highly pathogenic avian influenza virus H5N1 is a continuous threat to public health and poultry industry. The recurrence of the H5N1 led us to develop a robust, specific, and rapid detection method for the virus. In this study, an impedance aptasensor was developed for the virus detection using specific H5N1 aptamer and a gold interdigitated microelectrode. Streptavidin was immobilized on the microelectrode surface and biotin labeled H5N1 aptamer was bound to the immobilized streptavidin. The microelectrode was blocked with the polyethylene glycol and the bound aptamer captured the virus. The impedance change caused by the captured virus was measured using an impedance analyzer. To enhance impedance signal, a nanoparticle-based amplifier was designed and implemented by forming a network-like gold nanoparticles/H5N1-aptamer/thiocyanuric acid. The detection limit of the impedance aptasensor was 0.25 HAU for the pure virus and 1 HAU for the tracheal chicken swab samples spiked with the H5N1 virus. The detection time of aptasensor without employing the amplifier was less than an hour. The amplifier increased impedance by a 57-fold for the 1 HAU samples. Only negligible impedance change was observed for non-target viruses such as H5N2, H5N3, H7N2, H1N1, and H2N2. This aptasensor provides a foundation for the development of a portable aptasensor instrument.

1. Introduction

Avian Influenza A virus (AIV) is not only considered a zoonotic infection, but under certain conditions, the virus causes a serious public health threat. Highly pathogenic avian influenza A virus H5N1 (HPAI H5N1) poses a major challenge to human and avian. According to the official report of the World Health Organization (WHO, 2015), there were 844 laboratory-confirmed human cases with HPAI H5N1 infection since 2003 in 16 countries, of which 449 (case fatality rate (CFR) 53%) died with the infection. According to FAO (2012), HPAI H5N1 has caused the killing or culling of more than 400 million domestic birds across the world. This cost up to $20 billion in poultry industry. It has been impossible to fully eradicate the virus from chicken flocks, wild birds, and to prevent transmission to mammals. Therefore, the H5N1 is a persistent threat to humans and animals because the virus mutates and evolves. This gives it the ability to infect humans due to their lack of immunological defenses, and this will increase the chance of a potentially perilous pandemic (Webster et al., 1992; Guan et al., 2004; Fauci and Collins, 2012). In addition to the H5N1, the first human case with AIV H7N9 was confirmed by the WHO in 2013. The H7N9 is highly pathogenic in humans with CFR > 36% and the virus has pandemic potential (Su et al., 2015; Tanner et al., 2015).

A Rapid, specific, and sensitive detection of the virus is crucial to aid infection control and public health responses during influenza outbreaks (WHO, 2009; Dunn and Ginocchio, 2014). Current virus detection techniques are available, such as virus isolation, ELISA, and RT-PCR (Leland and Ginocchio, 2007; Ho et al., 2009; Ng et al., 2005). Virus isolation has been the "gold standard" due to the highest sensitivity and reliability, but it is a very time-

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consuming procedure (4–7 days). ELISA is a simple and rapid test in processing mass serum samples, thus it is commonly used for serum antibody detection, but not for virus detection in practice or diagnostics. RT-PCR and real-time RT-PCR methods have the advantages of high sensitivity and specificity, but possess disadvantages of requiring expensive PCR equipment and reagents, qualified laboratory facilities and well-trained technical personnel. Rapid influenza diagnostic tests (RIDTs) are simple to use and can provide results within 15 min (Bose et al., 2014). Nevertheless, RIDTs have poor sensitivity compared to the virus isolation and RT-PCR detection methods (Drexler et al., 2009; Chan et al., 2012).

A promising alternative to above detection assays is biosensors technology. Several biosensors have been developed to detect AIV. Previously Yanbin Li's group reported successful use of impedance immunosensors. Wang et al. (2009) reported the development of an impedance immunosensor based on an interdigitated array (IDA) microelectrode for H5N1 detection. Wang et al. (2011) also reported an impedance biosensor for H5N2 avian influenza detection based on a combination of immunomagnetic nanobeads, and a microfluidic chip with an IDA microelectrode. Then, Lum et al. (2012) further improved the impedance biosensor for H5N1 detection by a combination of immunomagnetic nanoparticles, a microfluidic chip, an interdigitated microelectrode and red blood cells (RBCs) for signal amplification. Other types of biosensors have also been developed for avian influenza detection, such as magnetic nanobeads amplification based quartz crystal microbalance (QCM) immunosensor for H5N1 detection (Li et al., 2011). H7N2, H7N3, and H8N4 subtypes were detected by an interferometric biosensor immunoassay through virus capture on a planar optical waveguide (Xu et al., 2007). Moreover, swine-origin influenza A (H1N1) virus (S-OIV) was detected by a paired surface plasma waves biosensor (PSPWB) in a dual-channel biosensor (Su et al., 2012). Detection methods based on biosensors are characterized by accuracy, speed, ease of use, low cost, and portability.

Aptamers are artificial nucleic acid ligands that can bind to target molecules and they are generated by an in vitro selection process called SELEX (Tuerk and Gold, 1990). Recently, our research group developed a DNA aptamer that can bind specifically to H5N1 virus with high affinity, dissociation constant (K_D) of 4.65 nM (Wang et al., 2013). Aptamers offer several advantages over antibodies. They are stable at room temperature, simple to synthesize, easily modified chemically, with low structural variation during chemical synthesis, and low cost of production (Sun et al., 2014). We have developed several aptasensors based on this aptamer. Here, three of them have highlighted: (i) the H5N1 DNA-aptamer was used as a specific recognition element in a portable Surface Plasmon Resonance (SPR) biosensor for rapid detection of AIV H5N1 in poultry swab samples (Bai et al., 2012); (ii) a quartz crystal microbalance (QCM) aptasensor was developed based on the H5N1 DNA aptamer crosslinked polymeric hydrogel for detection of AIV H5N1 (Wang and Li, 2013); (iii) a novel impedance biosensing method was developed by exploiting the enzyme catalysis in ultra-low ion strength media to induce ion strength increase in the detection of H5N1 (Fu et al., 2014). These aptasensors showed improved sensitivity in detection compared to immunosensors.

In this study, we describe an impedance biosensor based on aptamers and IDA microelectrode for rapid detection of H5N1 influenza virus. The main innovation of this study was to use a network-like thiocyanuric acid/gold nanoparticles as an amplifier for dramatically enhancing detection signal. In addition, the AIV H5N1 specific aptamer was used as affinity ligand instead of antibody, and IDA microelectrode was employed for impedance measurement. This report provides a foundation for the development of a robust and sensitive impedance aptasensor and its application for rapid detection of H5N1.

2. Materials and methods

2.1. Viruses and aptamers

The stock concentration of viruses was 128 hemagglutination units (HAU)/50 μl. The virus was inactivated by the USDA with b-propiolactone, eliminating viral infectivity while preserving hemagglutination activity (Goldstein and Tauraso, 1970). The virus was diluted in PBS (10 mM, pH 7.4) at a range from 16 to 0.125 HAU in 50 μl and only 30 μl were used for the assay on the surface of microelectrodes. Inactivated normal AIV (A/chicken/Scotland/59(H5N1)) was supplied from the USDA/APHIS National Veterinary Services Lab (Ames, IA, USA). Inactivated non-target AIV subtypes of A/H5N2/PA/chicken/85, A/H5N3/WileyLab/87, A/H7N2/PA/chicken/3779-2/97, A/H1N1/WileyLab/87, and A/H2N2/PA/chicken/1117-6/04 were from Animal Diagnostic Laboratory (ADL), at Pennsylvania State University (University Park, PA, USA). They were diluted in PBS with titer of 16 HAU in 50 μl. Because of biosecurity requirement in handling live AIV strains and the biosafety limitation of our laboratory condition (biosafety level 2), only inactivated AIV strains were used in this study. The selected aptamer with high affinity and specificity against AIV H5N1 was developed in our research group (Wang et al., 2013). Biotinylated aptamer and amine modified aptamer were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The sequences are: 5′-Biotin-GTG TGC ATG GAT AGC ACG TAA CGG TGT AGT AGA TAC GTG CGG GTA GGA AGA AAG GGA AAT AGT TGT CCT GTT G-3′ and 5′-/5AmMC6/GTG TGC ATG GAT AGC ACG TAA CGG TGT AGT AGA TAC GTG CGG GTA GGA AGA AAG GGA AAT AGT TGT CCT GTT G-3′ respectively.

2.2. The IDA microelectrodes

The microelectrode has 25 digital pairs with 15 μm digit width and 15 μm interdigit spaces. The total area of the IDA microelectrode was ~77 mm^2 with a working area of ~4.5 mm^2. It was fabricated at the State Key Lab of Integrated Optoelectronics at the Institute of Semiconductor, Chinese Academy of Science.

2.3. Preparation of gold IDA microelectrodes

First, the gold IDA microelectrodes were checked under a microscope to be sure that all the 25 paired digits were intact. Washing of the microelectrodes were done in three steps. At the beginning, 30 μl of 1 M NaOH was added to surface of the microelectrodes and incubated for 10 min at room temperature (RT). Then, 30 μl of 1 M HCl was added to the microelectrodes and incubated for 5 min at RT. Finally, they were rinsed with EtOH and softly wiped with lens paper. At the end of each step, microelectrodes were washed thoroughly with ultra-pure water and dried under a stream of N_2 gas. The electrodes were considered clean if the recorded impedance was under 200 ohm at 100 Hz frequency.

2.4. Modification of gold IDA microelectrodes

Streptavidin (Rockland, Gilbertsville, PA, USA) was immobilized on the surface of microelectrodes through physical adsorption (1 mg/ml). The microelectrodes were washed thoroughly with ultra-pure water to remove unbound proteins and dried out at RT. Then, biotinylated H5N1 aptamers were bound to the immobilized streptavidin on the microelectrodes (20 ng/μl). The microelectrodes were washed thoroughly to remove the unbound aptamers and dried out. Later, blocking of the microelectrodes was done with poly(ethylene glycol) methyl ether thiol (PEG, 0.1 mg/ml). Finally, the microelectrodes were washed and dried out at RT. Each incu-