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Specific detection of avian influenza H5N2 whole virus particles on lateral flow strips using a pair of sandwich-type aptamers

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ABSTRACT

We report a selection of a cognate pair of aptamers for whole avian influenza virus particles of H5N2 by using graphene-oxide based systemic evolution of ligands by exponential enrichment (GO-SELEX), and the application of a pair of sandwich-type binding aptamers on the lateral flow strips. The aptamers were characterized by GO-FRET assay, and Kd values of the selected aptamers were estimated to be from 6.913×10^5 to 1.27×10^6 EID_{50}/ml (EID_{50}/ml: 50% egg infective dose). Based on the evidence from confocal laser scanning microscope (CLSM), surface plasmon resonance (SPR), and circular dichroism (CD) spectrum analysis, the aptamers, J_3APT and JH_4APT, were found to be working as a cognate pair that binds to the target virus at the different sites simultaneously. This cognate pair of aptamers then was successfully applied on the lateral flow strips, clearly showing sandwich-type binding images with the presence of the certain numbers of H5N2 virus particles. On the newly developed lateral flow strips, the target virus was detectable down to 6×10^5 EID_{50}/ml in the buffer and 1.2×10^6 EID_{50}/ml in the duck's feces, respectively, by the naked eye. By using the ImageJ software, the LOD was found to be 1.27×10^5 EID_{50}/ml in the buffer and 2.09×10^5 EID_{50}/ml in the duck's feces, respectively. Interestingly, on the lateral flow strips, enhanced specificity towards the target virus (H5N2) appeared over other subtypes of H5Nx. To the best of our knowledge, this is the first report about the application of the cognate pair of aptamers for the detection of influenza virus on the lateral flow strips. This study shows the promising perspective of a cognate pair of aptamers for the on-site detection system which could be useful for rapid detection of avian influenza viruses for preventing the pandemic influenza viruses from spreading.

1. Introduction

Avian influenza viruses, which have 8 RNA segments as a genome, can infect almost every animal in the world including human beings (Capua and Alexander, 2007; Peiris et al., 2007; Song et al., 2008; Spackman, 2008). These avian influenza viruses possess pandemic potentials due to mutations which originate from their instability of RNA genomes and reassortment of their genomes (Zhou et al., 1999). Furthermore, potential pandemic risk has been increased because of the development of the poultry industry, transportation, global trades, and travel routes which makes viruses easily spread. Avian influenza viruses cause outbreaks of bird flu, which damaged the poultry industry all around the world (Chen et al., 2005). Since the outbreak of the bird flu in 1997, economic loss due to outbreaks of bird flu is reported every year (Alexander, 2007). Therefore, accurate, rapid, and early detection of influenza virus should be very important for preventing pandemic outbreaks of influenza.

There are diverse detection platforms for avian influenza virus including polymerase chain reaction (PCR) (Amano and Cheng, 2005). However, lateral flow assay, which is a paper-based platform, is considered to be suitable for the detection of the influenza viruses on site due to its rapidity and simplicity. The sandwich-type detection using lateral flow (LF) strips is most commonly used for large analytes because it can provide highly stable, sensitive, and specific results, compared to the competitive-type detection using LF strips (Mak et al., 2016; Posthuma-Trumpie et al., 2009).

One of the essential components on LF strips is the recognition element that can specifically bind to the target. There could be a variety of recognition molecules for the detection of influenza viruses, such as a genomic DNAs, glycans, antibody, aptamers (Grabowska et al., 2014; Hai et al., 2018; Sun et al., 2017; Weis et al., 1988). Aptamer, a single-stranded oligonucleotide, is a notable recognition element because it should have some advantageous features such as thermostability, low-cost, ease of modifications, and so on (Ellington and Szostak, 1990;

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Tuerk and Gold, 1990). For screening aptamers, systematic evolution of ligands by exponential enrichment (SELEX) is used. Since the 1990s when SELEX methods had reported, DNA or RNA based aptamers were developed for various targets including small molecules, protein biomarkers, bacterial cells, and human cells (Kim et al., 2014; Tan et al., 2013; Torres-Chavolla and Alocilja, 2009). Recently, there have been a few studies about the screening of aptamers binding to influenza viruses (González et al., 2016; Hmila et al., 2017; Lai et al., 2014; Lao et al., 2014; van den Kieboom et al., 2015; Woo et al., 2013) for diagnostics and anti-viral drugs. Most of these aptamers are obtained by targeting a specific protein, hemagglutinins (HA) (Misono and Kumar 2005; Park et al., 2011; Shiratori et al., 2014), while some research groups used whole virus targets including this study (Bai et al., 2018; Nguyen et al., 2016; Wang et al., 2013). However, there have been few reports to develop a cognate pair of aptamers binding to the different site of influenza viruses simultaneously. In other words, there have been few reports about the detection of viruses using aptamer-based sandwich-type binding format because a pair of aptamers has not been available (Bruno et al., 2012; Nguyen et al., 2016; Park et al., 2014). Even though these single aptamers were also applied to various types of biosensors, such as electrochemical, optical, and mass-based platforms (González et al., 2016; Hamula et al., 2011), there have been no studies on LFAs using a pair of aptamer-based sandwich-type detection of any viruses, except one case reporting no complete study (Bruno et al., 2012). As mentioned previously, the one major reason is the absence and difficulties in the screening of cognate pair of aptamers binding to the different sites of target viruses simultaneously, which allow constructing the sandwich-type binding on LF strips. Recently, our group developed immobilization-free SELEX using graphene-oxide (GO-SELEX) (Park et al., 2012). The GO-SELEX uses the characteristics of GO on that ssDNAs adsorbed on it through π-π stacking. When ssDNAs have an affinity to the target molecule, ssDNAs can be detached out from the GO by a conformational change of its structure, while other ssDNAs that do not have affinity remain adsorbed on GO. In GO-SELEX, the target molecules are not anchored to the carrier. Thus, epitopes of the target molecules can be fully exposed which increase the possibility of screening pair of aptamers that can bind to a target in a sandwich manner. Using this immobilization-free GO-SELEX method, our group has reported several pair of aptamers which binds to the target simultaneously (Ahmad Raston and Gu, 2015; Lee et al., 2019; Nguyen et al., 2016; Park et al., 2014).

Here, we report the successful GO-SELEX based development of a cognate pair of aptamers binding to whole virus particles of H5N2 in a sandwich-type, and its successful application on LF strips in a sandwich-type binding for the detection of H5N2 whole virus particles for the first time. For the characterization of the pair of aptamers and their features, the whole virus particle was used as a target, and the graphene-oxide based fluorescence resonance energy transfer assay (GO-FRET). The GO-FRET assay, which is recognized as a sensitive analytical technique, has been widely used in biological assays. The GO has been known to be an effective quencher for organic dye molecules. The fluorescence-labeled ssDNAs, aptamer, in this case, can be adsorbed to GO by π-π stacking which results in quenching of the fluorescence. The fluorescence signal can be restored when ssDNAs bound to the target and detached from GO. Thus, there have been many reports for using GO-FRET for the aptamer characterizations and aptasensing applications (Jung et al., 2013; Morales-Narvaez and Merkoci, 2012). In addition, surface plasmon resonance (SPR), confocal laser scanning microscope (CLSM), and circular dichroism (CD) spectrum analysis were used for characterization of the aptamer. For LF strip application, the gold nanoparticles (AuNPs) were synthesized according to the citrate reduction method and used as signaling labels (Liu and Lu, 2006). Implying AuNPs as a label is well-known in analytical science (Rosi and Mirkin, 2005; Zeng et al., 2011), and it is widely used in LF strip sensors due to their unique optical property that can be recognized by the naked eye (Zhan et al., 2017). The analytical performance of the successfully developed cognate pair of aptamer-based sandwich-type lateral flow strip biosensor was evaluated with buffer and duck's feces spiked samples. To the best of our knowledge, this is the first report on the development of LF strip biosensor using a cognate pair of aptamers binding to the influenza virus in a sandwich format.

2. Experimental

2.1. Materials

Gold chloride trihydrate, Trisodium citrate dihydrate, Potassium chloride, Sodium chloride, Magnesium chloride, Calcium chloride, Bovine serum albumin, Tween-20, 6-mercapto-1-hexanol (MCH), tris (hydroxymethyl)aminomethane, Streptavidin, and 3,3′-Dithiodipropionic acid (DTPA) were purchased from Sigma-Aldrich and used without any additional purification. The sample pad and the nitrocellulose membrane were purchased from Millipore. Influenza viruses were supplied from Konkuk University (H1N1, H1N2, H2N1, H3N8, H4N6, H5N2 (K09-652, K08-404), H6, H7N8, H9N2, H10N4, H12N5, infectious bronchitis virus, and Newcastle disease virus). The random library, primers, and avian influenza virus binding aptamers were synthesized from GenoTech Corp. (Daejeon, Korea) with the following sequence:

Random library: 5′-CGTACGGAATTCGCTAGC-40N-GGATCCGAGC TCCACGTG-3’.

J_3 Aptamer (J_3APT): 5′- CGTACGGAATTCGCTAGCTGATGGTGTGG CGGGGGGCG.

GCCTGGGGCGGGCCGCCGATGGGATCCGAGCTCCACGTG- 3’

JH_4 Aptamer (JH_4APT): 5′- CGTACGGAATTCGCTAGCGGTGGCTC TAGGGCCTATC.

GTTGCGCCGCGGATCCGAGCTCCACGTG - 3’

JH_2 Aptamer (JH_2APT): 5′-CGTACGGAATTCGCTAGCGGTGGCTCT AGGGCCTATC.

GTTGCGCCGACGGATCCGAGCTCCACGTG - 3’

J_{15}Aptamer (J_{15}APT): 5′- GTACGGAATTCGCTAGCTGATGGTGTGG CGGGGGCGGC.

CTGGGGCGGGCCGCCGATGGGATCCGAGCTCCACGTG - 3’

2.2. GO-SELEX for aptamer screening

To develop the avian influenza virus particle-specific aptamer, the graphene-oxide based SELEX (GO-SELEX) was used (Lee et al. 2017, 2019; Park et al. 2012, 2014). Two hundred pmoles of random library hanged with primer for PCR amplification in binding buffer (100 mM NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, pH 7.6) were incubated with 200 µl of GO (5 mg/ml) for 30 min followed by centrifuging the solution to remove unbound random library to GO. Next, 100 µl of target virus (avian influenza virus H5N2/K08-404 1×10^7 EID_{50}/ml: 50% egg infective dose) was added and incubated for 30 min to screen out the sequences that bind to the target virus. After that, supernatant which contains target binding sequences was collected by centrifuging the solution. To increase the specificity of the aptamer counter-selection was also performed using counter target mixtures including different types of avian influenza viruses (H1N1, H1N2, H2N1, H3N8, H4N6, H5N2/K09-652, H6, H7N8, H9N2, H10N4, H12N5, infectious bronchitis virus, and Newcastle disease virus). When the counter selection was performed, the random library was incubated with counter targets and then sequentially graphene oxide, and the target virus was added to the solution to get ssDNA that binds to the target specifically. After the final selection round, aptamer candidates were obtained by cloning and sequencing.

2.3. Characterization of selected aptamer

2.3.1. GO-FRET assay

The affinity and specificity of aptamer candidates were

characterized using the Graphene-Oxide based Fluorescence Resonance Energy Transfer assay (GO-FRET) (Chung et al., 2013; Wang et al., 2011). FAM-labeled aptamer candidates (4 nM) in binding buffer were incubated for 30 min with GO solution (1.6 μg/ml) to quench the fluorescence. Then, the target virus or counter target viruses were added. For affinity test, different concentrations of the target virus were incubated and recovered fluorescence was measured at 520 nm. For specificity test, various counter targets (H1N1, H4N6, H5N3, H6, H7N8, H9N2, H10N4, counter target mix) were added, and fluorescence signal was measured (SpectraMax i3x, Molecular Devices, U.S.A.).

2.3.2. SPR assay for aptamer duo screening

To screen a cognate pair of aptamers, which binds to the different site of the target, surface plasmon resonance technique (SPR) was used (Homola et al., 1999). The aptamer-modified gold chip was prepared using the streptavidin-biotin complex method. Before aptamer immobilization, the surface of a gold chip was functionalized using 50 mM of DTPA for 12 h. Then, streptavidin was covalently attached to the surface by using carbodiimide crosslinker chemistry, and unreacted active functional groups were blocked by 50 mM of ethanolamine. Next, 1 μM of biotin modified aptamers characterized by GO-FRET assay were added to the surface of the gold chip. Finally, the chip was blocked with BSA (50 μg/ml). The cognate pair of aptamers screening assay procedure was conducted as follows. Baseline was measured for 10 min by flowing the binding buffer. Then, the target viruses (1×10^6 EID_{50}/ml) were injected for 10 min, and unbound targets were washed for another 10 min. Finally, a cognate pair of aptamers candidates were injected. The flow rate was maintained 30 μl/min for all SPR assay. By analyzing the angle change which represented by a response unit, a cognate pair of aptamers was screened.

2.3.3. Imaging of a cognate pair of aptamers in sandwich formation by using confocal laser scanning microscopy (CLSM)

The confocal laser scanning microscope was used to get the image of the sandwich formation of a cognate pair of aptamers. Primary aptamer J_3APT was immobilized on the magnetic bead using EDC/NHS chemistry. The J_3APT immobilized magnetic beads in binding buffer condition were reacted with three different conditions (without the target, with the target (H5N2/K08-404), counter target (H9N2)). Next, Cy5 labeled secondary aptamer (JH_4APT) was incubated. After washing the magnetic bead, fluorescence image was taken by a confocal laser scanning microscope (LSM 700, Carl-Zeiss, Germany).

2.3.4. CD spectrum analysis

Circular Dichroism (CD) spectroscopy was used to study the changes of a cognate pair of aptamers structures when binding to the target. The CD spectrum of five combinations of samples, including each molecule as a control, were measured. 1 μM of primary aptamer (J_3APT) and secondary aptamer (JH_4APT) in binding buffer were used, and target virus (1×10^7 EID_{50}/ml) was added. All the CD spectrum measurements were done in quartz crystal cuvettes (Hellma Analytics, Germany).

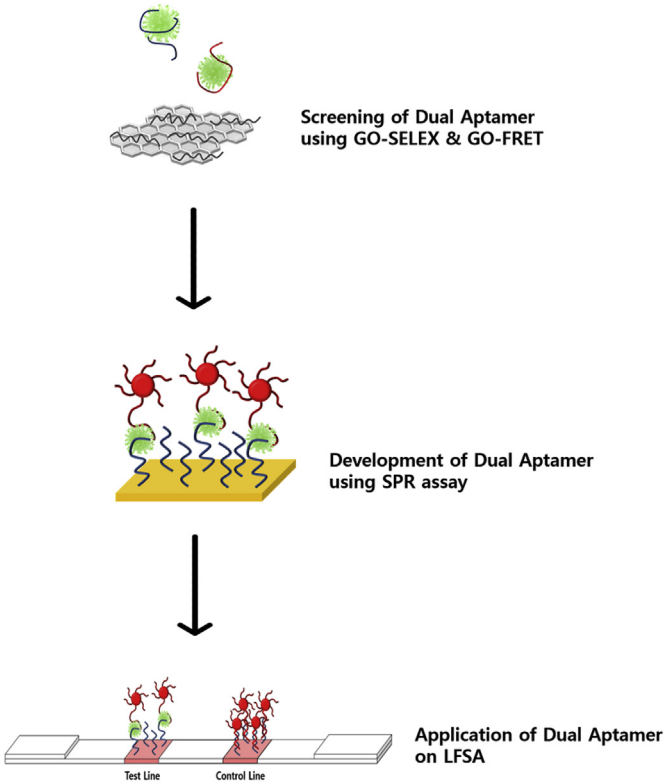
2.4. Cognate pair of aptamer-based lateral flow strip biosensor

As shown in Fig. 2a, the cognate pair of aptamer-based sandwich-type lateral flow strip biosensor was designed. For the test line, 10 μM biotin modified at 5′ of primary aptamer (J_3APT) was incubated with streptavidin (1 mg/ml) for 1 h at room temperature. Then, the solution was purified using amicon filter (100K) 2 times. For the control line, 10 μM biotin modified poly A sequence was incubated and purified using the same protocol. For the signal, the 13 nm gold nanoparticle was synthesized by citrate reduction method (Liu and Lu, 2006), and 100 μM of thiol and poly T modified at 5' of secondary aptamer (JH_4APT) was immobilized onto the gold nanoparticle solution using the previously reported protocol (Liu and Lu, 2006). The conjugation of the JH_4APT to gold nanoparticles was confirmed by UV-Vis Spectra (Ultraspec 6300 pro, Amersham Biosciences, Piscataway, NJ, USA) (Fig. S4). Test line and control line were drawn onto the nitrocellulose membrane (HF135MC100) by using the dispenser (ZX 1010 B0056, Biodot, U.S.A.). After drying, NC membrane was blocked with blocking buffer (1% BSA, 10 mM Tris, 100 mM NaCl). Next, NC membrane was washed with washing buffer (0.1% Tween20, 10 mM Tris, 100 mM NaCl) to remove excessive BSA proteins which might block the pores of the NC membrane. Finally, the sample pad and the absorbent pad was assembled onto the NC membrane, and assembled strips were stored in the 4 °C before use.

The dipstick method was used for testing all the lateral flow strip biosensor using 96 well plates. The assay was conducted in the buffer and spiked-in-duck's feces condition. In the case of the buffer condition, 50 μl of target solution was added to the sample pad and flowed for 5 min. Then, 50 μl of JH_4APT-AuNP solution flowed for 5min followed by washing for 10 min using a washing buffer. For the duck's feces condition, the target virus was spiked in 1/50 diluted duck's feces, which large debris was removed by filtering, were used as a test sample following the same procedure used in the buffer condition. The ImageJ software was used for analyzing the signal intensities of LF strips. Images were processed by measuring the total intensity of each area of the test line. For an accurate comparison, images were taken at equal light exposure conditions.

3. Results and discussion

The Scheme 1 illustrates the whole process of successful screening and characterization of a cognate pair of aptamers, and its application onto the sandwich-type lateral flow biosensor for detection of the whole virus particles, avian influenza type H5N2 (K08-404). To promote the interaction of aptamers towards to the natural virus and to increase the chance of making the cognate pair of aptamers, whole virus particles were used as a target, and the graphene-oxide based immobilization-



Scheme. 1. The schematic illustration of the whole process of the selection and application of a cognate pair of aptamers.

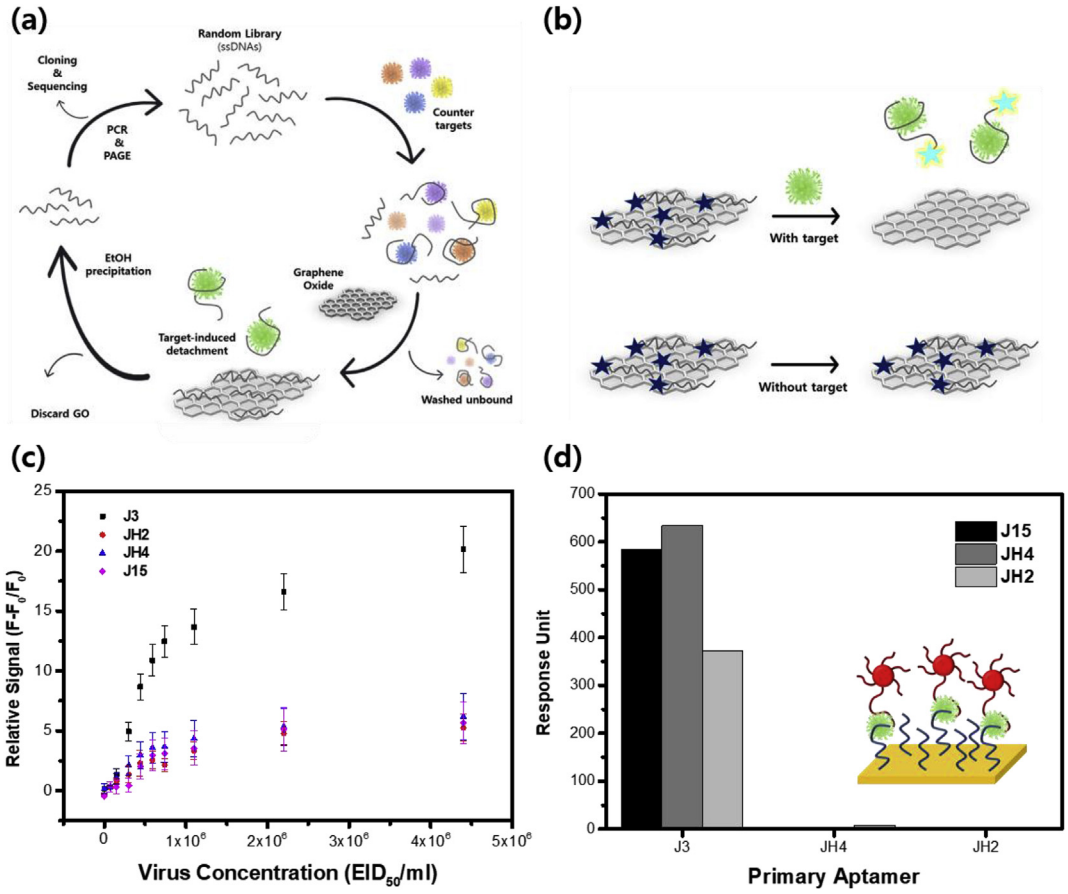


Fig. 1. (a) Diagram of the immobilization-free GO-SELEX for screening aptamer that specifically binding to the target virus particle. (b) Diagram of the GO-FRET assays for the characterization of aptamers. (c) Affinity analysis of the selected aptamers to the target virus particle (H5N2/K08-404). The saturation curves were obtained by plotting the relative signal as a function of virus concentration. (d) Screening of cognate pair of aptamers using sandwich-type SPR assays (inset). The response unit represents the intensity of secondary aptamer binding to the target captured by the primary aptamer. The concentration of the target virus was 1×10^6 EID_{50}/ml.

free SELEX methods were employed. After aptamers were characterized by the GO-FRET, screening of the cognate pair of aptamers, which could yield an elevated performance of the aptasensor, was carried out by SPR, CLSM, and CD. Finally, the cognate pair of aptamers (J_3APT and JH_4APT) were successfully applied to the lateral flow biosensor, and the analytical performance of this newly developed sandwich-type aptasensor was examined with buffer and duck's feces spiked samples.

3.1. Screening of avian whole virus-specific aptamer (SELEX & GO-FRET)

To develop ssDNA aptamers which can bind to the avian influenza virus particle, graphene-oxide based immobilization free screening method which known as GO-SELEX was used (Fig. 1a). The overall SELEX procedure was conducted for eight rounds including two negative selections with the mixture of counter target viruses to increase the specificity of the aptamer. After the last selection round, the aptamer candidates were obtained by cloning and sequencing the enriched pool.

To characterize the obtained aptamer candidates, graphene-oxide based fluorescence resonance energy transfer assay (GO-FRET) was used (Fig. 1b). Aptamer candidates were labeled with the fluorescence (FAM), and GOs were used as a fluorescence quencher. The relative signal was determined by calculating the ratio of the fluorescence, (F-F0)/F0. The FAM-labeled aptamers were adsorbed to the GO by π- π stacking, which caused the quenching of the fluorescence emitted by the FAM. The fluorescence was recovered only when the target avian virus particles were captured by the aptamer. When the counter targets were added, the fluorescence remained quenched. As a result, four sequences (JH_4APT, JH_2APT, J_3APT, J_{15}APT) of avian influenza virus particles binding aptamer, which showed specific binding to the target, were selected and characterized (Fig. S1). Moreover, binding affinities of selected four aptamers were characterized by the dose-dependency test as shown in Fig. 1c. Among four aptamer sequences, J_3APT showed the highest binding affinity represented as the lowest dissociation constant value (Kd) as 6.913×10^5 EID_{50}/ml (EID_{50}/ml: 50% egg infective dose). Dissociation constant value of other aptamers (JH_2APT, JH_4APT, J_{15}APT) were 1.112×10^6 EID_{50}/ml, 8.821×10^5 EID_{50}/ml and 1.27×10^6 EID_{50}/ml, respectively.

3.2. Screening of cognate pair of aptamers

For practical application of aptamers to the various biosensor platform, screening of aptamers that binds to the target as sandwich-type is important. In order to screen cognate pair of aptamers that bind to the different epitopes of the target virus particle, sandwich-type assays using SPR technique were performed using previously selected four aptamers as shown in the inset of the Fig. 1d. To screen cognate pair of aptamers more efficiently based on our experiences for finding a cognate pair of aptamers, we have reduced the combination to test by setting the aptamers which have high affinity towards the target as primary aptamers. As shown in Fig. 1d, the combinations of J_3-J_{15}, J_3-JH_4, and J_3-JH_2 were shown to cognate binding interactions to the target. Among the three combinations, the combinations of J_3 and JH_4 showed the highest SPR response unit compared to other combinations. Thus, J_3 aptamer as capturing aptamer and JH_4 aptamer as reporting

aptamer were used for further investigations.

Furthermore, CD spectrum analysis was performed to study the structural changes of a cognate pair of aptamers binding to the target virus. The differences in the peak are related to the structural changes of ssDNA. When the J_3APT or JH_4APT binds with the target virus, a high negative peak appeared around 220 nm, and a positive peak of aptamer itself decreased at around 287 nm. In the case of sandwich-type binding condition, a negative peak of 220 nm was decreased, and the negative peak of 245 nm was increased compared to the single aptamer binding. Moreover, the positive peak was shifted to 287 nm (Fig. S2). In consequence, we could assume that there is a specific way of interaction between the cognate pair of aptamers and its target represented as a conformation change of the aptamers.

Additionally, sandwich-type binding of the cognate pair of aptamers was visualized by confocal laser scanning microscopy (CLSM) using magnetic beads-based sandwich assay. The magnetic beads conjugated with primary capturing aptamer (J_3APT) and the fluorescence tag labeled secondary reporter aptamers (JH_4APT) were used. Although we could observe the fluorescence from the negative control, it is likely due to the non-specific adsorption of the secondary aptamers onto the magnetic beads. The fluorescence signal of the counter target sample was similar to the negative control. When the target was added, we could clearly see the fluorescence signals evenly throughout the magnetic beads which agreed well with previous SPR assay result (Fig. S3). Thus, we have confirmed that the J_3APT and JH_4APT are binding to the target virus particle as a cognate pair.

3.3. Development of a sandwich-type lateral flow strip biosensor using a cognate pair of aptamers for the whole virus

The cognate pair of aptamer-based sandwich-type lateral flow strip biosensor for whole virus particle was designed and developed as shown in Fig. 2a. The primary aptamers (J_3APT) was used as a capturing aptamer for test line, and Gold nanoparticle labeled secondary aptamer (JH_4APT) was used as a reporting aptamer. The concentration of primary aptamer (J_3APT) and the secondary aptamer (JH_4APT) conjugated AuNPs were optimized to 10 μM and 10 nM, respectively (Fig. S5). When the 2nd aptamer-conjugated AuNPs concentration was increased, the signal intensity was also increased. At the same time, however, a higher concentration of 2nd aptamer-conjugated AuNPs makes washing difficult, and it could result in high background noise. Thus, the condition (1st Aptamer: 10 μM; 2nd aptamer-conjugated AuNPs: 10 nM) was used for all our LF strip biosensor experiments. The analytical performance of the newly developed lateral flow strip biosensor for whole virus particle was studied by testing the different concentrations (1×10^7, 5×10^6, 2.5×10^6, 1.2×10^6, 6×10^5, 3×10^5, 1.5×10^5, 0 EID_{50}/ml) of the target virus (H5N2/K08-404) in the buffer. As shown in Fig. 2b, the target virus was detectable as low as 6×10^5 EID_{50}/ml, and the detectable range was 6×10^5 - 1×10^7 EID_{50}/ml by the naked eye. Further, the corresponding results were analyzed by the ImageJ software, and its resulting calibration plot is shown in Fig. 2c. The limit of detection (LOD) was estimated to be 1.27×10^5 EID_{50}/ml calculated by using blank + 3 standard deviations.

To test the applicability of the cognate pair of aptamer-based sandwich-type lateral flow strip biosensor in the field, target virus sample spiked in the duck's feces were tested. The same concentration range of target virus as buffer condition was used. As can be seen from Fig. 3a, the target virus was detectable as low 1.2×10^6 EID_{50}/ml, and the detectable range was 1.2×10^6 - 1×10^7 EID_{50}/ml by the naked eye. The Fig. 3b shows the calibration plot of duck's feces spiked sample, and the LOD was found to be 2.09×10^5 EID_{50}/ml using the same calculation method. When duck's feces were applied, we could observe the decrease in both signal intensity and sensitivity compared to the buffer condition. It is likely due to the complex matrices in duck's feces disturbed the performance of the cognate pair of aptamers.

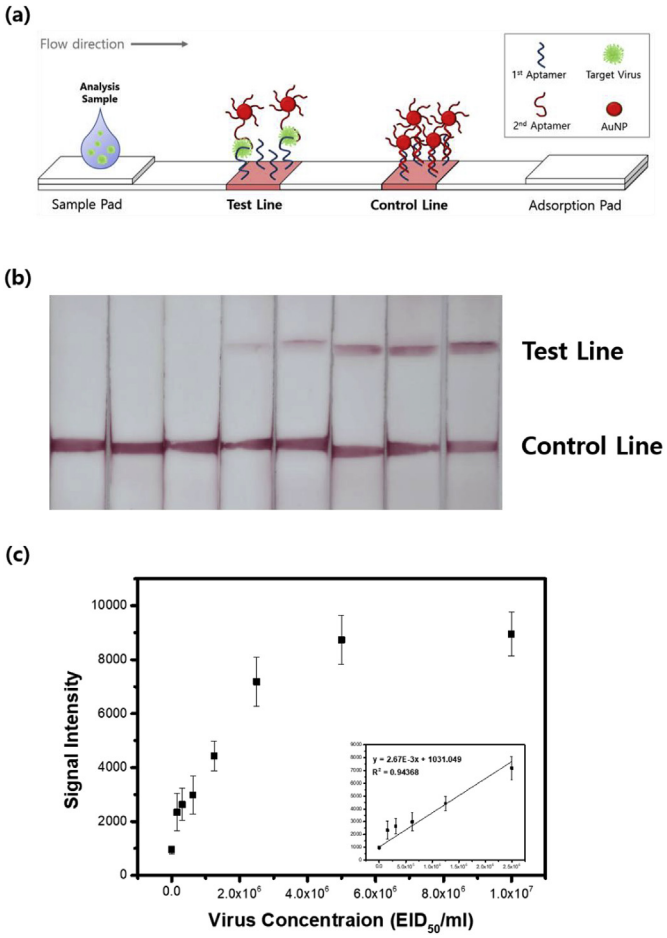


Fig. 2. (a) Diagram of whole virus particle detection system using a cognate pair of aptamer-based lateral flow strip. (b) Image of the lateral flow strip tested with different concentrations (From left to right; 0, 1.5×10^5, 3×10^5, 6×10^5, 1.2×10^6, 2.5×10^6, 5×10^6,1×10^7 EID_{50}/ml) of the target virus in buffer condition. (c) Calibration curve of the cognate pair of aptamer-based sandwich-type LFAs in buffer condition.

Additionally, the sensor performance of influenza virus detection using newly developed sandwich-type lateral flow aptasensor along with previously reported studies were compared, as shown in Table 1. This sandwich-type lateral flow biosensor using a cognate pair of aptamers showed comparable sensitivity to commercially available rapid kits detecting broad subtypes of influenza A virus. However, Further enhancements in signal intensity are still needed to increase the sensitivity of the newly developed sensor for on-site detection of influenza viruses.

The specificity of this lateral flow aptasensor was also evaluated by testing with various counter target viruses, including infectious bronchitis virus (IB) and Newcastle disease virus (ND). As shown in Fig. 4, a significant signal response was observed only in the target virus (H5N2/K08-404), while minor signals were observed for counter targets, indicating the fine specificity of this cognate pair of aptamers-based sandwich-type lateral flow biosensor for avian influenza virus (H5N2/K08-404).

Interestingly, when a cognate pair of aptamers was used, compared to the specificity of the single aptamer, we could observe the enhanced specificity. In the characterization studies for the single aptamers, J_3APT and JH_4APT, both aptamers were also found to bind to the other subtype species, such as H5N2 (K09-652) and H5N3 (Fig. S1). In the case of a cognate pair of aptamers, however, there was no significant signals for the subtype species, H5N2 (K09-652) and H5N3 (Fig. 4), which is likely due to a specific configuration of a pair of aptamers binding to the target virus particle (H5N2/K08-404) formed. However,

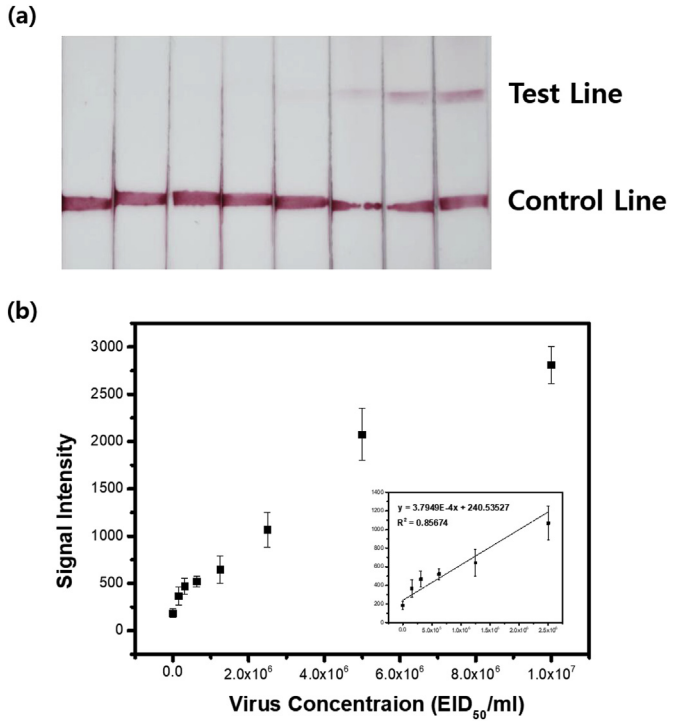


Fig. 3. (a) Image of the lateral flow strip tested with different concentrations (From left to right; 0, 1.5×10^5, 3×10^5, 6×10^5, 1.2×10^6, 2.5×10^6, 5×10^6,1×10^7 EID_{50}/ml) of the target virus spiked in the ducks' feces condition. (b) Calibration curve of the cognate pair of aptamer-based sandwich-type LFAs in the ducks' feces condition.

a cognate pair of aptamers could have unsuccessful configurations formed for the subtype counter target viruses, H5N2 (K09-652) and H5N3, which resulted in fortunate discrimination of these counter target viruses and so no signals generated on LF strip. The newly developed LF strip biosensor in this study, therefore, could be useful for on-site detection of whole avian influenza viruses.

4. Conclusion

In conclusion, we have developed a cognate pair of aptamers binding to the different sites of the whole virus particles simultaneously and demonstrated its successful application on sandwich-type LF strips for the detection of whole avian influenza virus particle (H5N2) for the first time. The cognate pair of aptamers were successfully characterized and evidenced by GO-FRET, CLSM, CD, and SPR methods. By applying the aptamer pair, this newly developed cognate pair of aptamer-based LF strip biosensor could detect the target H5N2 virus particles with high specificity, and the LOD was estimated to be 1.27×10^5 EID_{50}/ml and 2.09×10^5 EID_{50}/ml in the buffer and the duck's feces, respectively. Therefore, this cognate pair of the aptamer-based sandwich-type biosensors including SPR and LF strip could be an effective tool for the detection of whole avian influenza viruses on site.

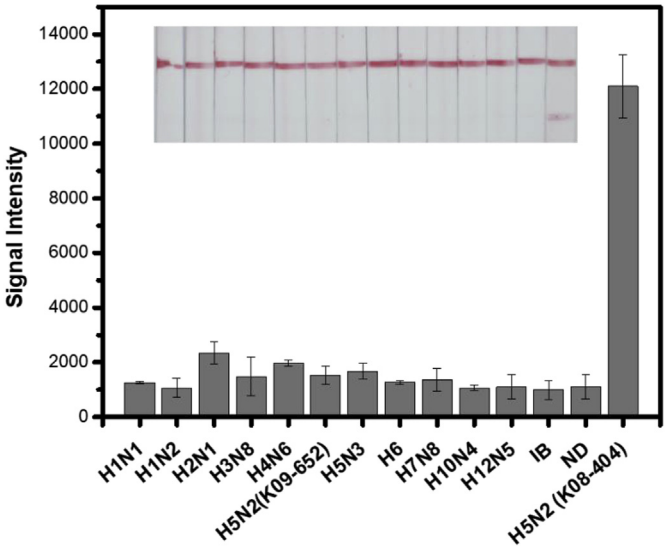


Fig. 4. (a) The specificity of the cognate pair of aptamer-based sandwich-type LFAs. From left to right, H1N1, H1N2, H2N1, H3N8, H4N6, H5N2(K09-652), H5N3, H6, H7N8, H10N4, H12N5, infectious bronchitis virus (IB), Newcastle disease virus (ND), and H5N2(K08-404) in buffer condition. The same concentration of viruses (5×10^6 EID_{50}/ml) was used for the specificity test.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Declarations of interest: none.

CRediT authorship contribution statement

Sang Hoon Kim: Conceptualization, Data curation, Methodology, Writing - original draft, Writing - review & editing. **Junho Lee:** Data curation, Methodology. **Bang Hyun Lee:** Data curation, Writing - original draft, Writing - review & editing. **Chang-Seon Song:** Resources. **Man Bock Gu:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

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Table 1
Comparison of the performance of commercial rapid kits for influenza virus detection[a].

Type	Test Kit Name	Limit of Detection (LOD)	Ref.
Rapid Kit	Synbiotics Flu Detect	10^5 EID_{50}/ml	Marché and Berg (2010)
Rapid Kit	Anigen AIV Ag	10^6 EID_{50}/ml	Marché and Berg (2010)
Rapid Kit	Remel X/pect Flu A7B	10^4 EID_{50}/ml	Marché and Berg (2010)
Rapid Kit	ImmunoAce Flu	$10^{4.9}$ EID_{50}/ml	Yamanaka et al. (2016)
Rapid Kit	Bright POC Flu	$10^{5.6}$ EID_{50}/ml	Yamanaka et al. (2016)
Rapid Kit	QuickVue	$10^{5.7}$ ELD_{50}/ml	Woolcock and Cardona (2005)
Rapid Kit	Directigen Flu A kit	$10^{4.7}$ ELD_{50}/ml	Woolcock and Cardona (2005)

[a] EID_{50}/ml = 50% egg infection dose; ELD_{50}/ml = 50% embryo lethal dose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bios.2019.03.061.

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