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Aptamer-based SPR Biosensor for Detection of Avian Influenza Virus

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of the requirements for the degree of
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By

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Abstract

Rapid and specific detection of avian influenza (AI) virus is urgently needed with the concerns over the outbreaks of highly pathogenic H5N1 avian influenza in animal and human infection. Aptamers are artificial oligonucleic acid that can specifically bind to target molecules. They show comparable affinity for target virus and better thermal stability than monoclonal antibodies. Those advantages make aptamers promising candidates in diagnostic and detection applications. The goal of this research was to use DNA-aptamer as the specific recognition element in a portable surface plasmon resonance (SPR) biosensor for detection of AI H5N1 virus in poultry.

A SPR biosensor was fabricated using the selected aptamers based on streptavidin-biotin method. Streptavidin was directly adsorbed onto the surface of a gold waveguide in the SPR biosensor. Then, biotinylated aptamers were immobilized on the sensor surface via streptavidin-biotin binding. The immobilized aptamers captured AI H5N1 virus in a sample solution, causing an increase in refraction index (RI). Performances of the aptamer-based SPR biosensor were studied in streptavidin modification, aptamer immobilization and virus detection. The optimal concentrations of streptavidin and aptamers were determined to improve the sensitivity of the biosensor. The response of the aptamer-virus interaction was shown to be virus titer-dependent, and a linear range for the titers of AI H5N1 was found between 0.128 and 1.28 HA unit. The aptamer-based SPR biosensor could detect the H5N1 virus at a titer greater than 0.128 HA unit within 1.5 h. No significant interference was observed from non-target subtypes such as AI H7N2, H9N2, H2N2, H1N1 and H5N2. The aptamer-based SPR biosensor was further evaluated for detection of AI virus in poultry swab samples. All of the AI viruses used in this study were killed ones to ensure biological safety.

Keywords: Aptamer, Surface Plasmon Resonance, Biosensor, Avian Influenza Virus

This thesis is approved for Recommendation

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Chapter 1 Introduction

Avian influenza (AI), also called “bird flu”, is an infectious disease of birds caused by type A strains of the influenza virus, causing huge economic losses in the world (WHO, 2012). The infection can cause a wide spectrum of symptoms in birds, ranging from mild illness, which may pass unnoticed, to a rapidly fetal disease that can cause severe epidemics (WHO, 2012). Generally, avian influenza viruses only infect birds, and do not normally infect humans (WHO, 2012). However, recently, continuous outbreaks of avian influenza virus infection of humans have been reported in many countries since 2003: like the Republic of Korea, Viet Nam, Japan, Thailand, Cambodia, Lao People’s Democratic Republic, Indonesia, and China in Asia (WHO, 2012). The first human case of avian influenza virus H5N1 infection was reported in Hong Kong in 1997 (WHO, 2012).

Due to the different pathogenicity avian influenza can be divided into high-pathogenicity avian influenza (HPAI), low-pathogenicity avian influenza and non-pathogenic avian influenza (HDYK, 2005). Avian influenza virus has many serotypes among which H5 and H7 can cause human infection (HDYK, 2005).

HPAI is a great threat not only for the poultry industry but also for humans, leading to high morbidity and mortality rate. There were a total 584 confirmed human cases and 345 deaths reported by WHO from 2003 until Feb. 8, 2012 (WHO, 2012). These events established that AIV H5N1 was considered a risk to public health. Also, high mortality rate and worldwide spread give humans much more concerns on AIV H5N1.

The raised interest in the detection of AI H5N1 in humans as well as animals has been stimulated by increasing emergence of the infectious influenza diseases. A variety of technologies of

diagnosing AIV infection have been reported, including in vitro virus isolation by culture, serologic assays, enzyme-linked immunosorbent assays (ELISA), and polymerase chain reaction (PCR)-based assays. To some extent, they are useful methods; however, many disadvantages make them less than ideal. For example, in vitro virus isolation by culture is time-consuming and needs about 10 days; the criteria for serologic detection of influenza virus, the hemagglutination inhibition (HI) assay, have been proved to have low sensitivity and cannot detect the kind of antibody that is against diverse avian influenza viruses (Rowe et al., 1999; Katz, 2003).; PCR-based assays are more sensitive but plenty of mismatches between the primers and AIV sequences can happen and those assays cannot distinguish the live viruses from inactivated viruses (Fouchier et al., 2000; Suarez et al., 2003). Moreover, virus isolation, serological methods and PCR-based assays often require the lab workers to be highly trained, are time-intensive procedures as well as require highly sterile experimental environment (Amano et al., 2004; Chen et al., 2008). Dot-ELISA alone can only be used for qualitative rather than a quantitative detection. Therefore, in this research an aptamer-based SPR biosensor was studied for rapid detection of AIV H5N1 in poultry swab samples.

Chapter 2 Literature Review

2.1 Overview of Avian Influenza

2.1.1 Avian Influenza

Influenza, or flu, is caused by RNA viruses of the Orthomyxoviridae family, infecting birds and mammals. Influenza can cause acute upper respiratory tract infection and is airborne through sneezes or coughs. Periodic pandemics around the world are often severe and occur rapidly, leading to considerate deaths of birds ranging from 250,000 to 500,000 annuly (WHO, 2009). Incidentally, viruses are transmitted from the wild waterfowl to other species and may then cause devastating outbreaks in domestic poultry or give rise to human influenza pandemics (Klenk et al., 2008).

Influenza virus is a representative of Orthomyxoviridae, including human influenza virus and animal influenza virus. In virus taxonomy human influenza viruses are divided into three categories: influenza A virus, influenza B virus, and influenza C virus. Of the three viruses, type A viruses possess the strongest virulence and highest pathogenicity, causing the most severe disaster. Also, influenza A virus antigenically mutates easily, resulting in a number of worldwide pandemics, and it has a wide host range, including humans, pigs, birds and others, affecting very young, old and sick members of a population. Influenza B virus has low pathogenicity, and the host range is slightly narrower than that of Influenza A, covering humans and birds. While influenza C virus only causes asymptomatic or slight upper respiratory tract infection, hardly causing worldwide pandemic, and the host range is relatively narrow only involving in humans.

Influenza A viruses are actually avian viruses (Hay et al., 2001). Wild waterfowl provide natural hosts for many kinds of influenza A viruses. Influenza A viruses are subdivided into a number of different serotypes based on distinct combinations of hemagglutinin (HA) and neuraminidase (NA) that are the two major glycoproteins on the surface of the viral particles (Hay et al., 2001). Until today, 16 HA and 9 NA subtypes of influenza A viruses have been reported (Jong et al., 2006). Theoretically, there may be 144 (16×9) subtypes of influenza A viruses.

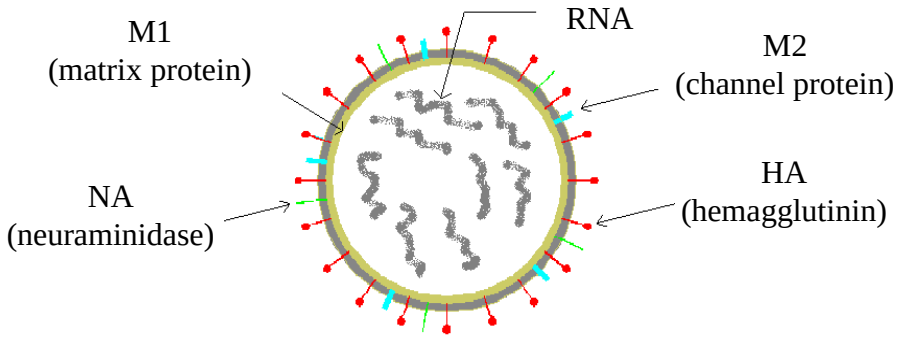


Figure 2. 1 Schematic diagram of influenza virus A.

2.1.2 Avian Influenza Virus

The HA protein is a homotrimeric integral membrane glycoprotein that plays a vital role in binding of the virus to target cells and penetration of the viral genome into the target cell during the process of virus infection. Influenza virus specificity for the host is mediated by the viral surface glycoprotein HA, which binds to receptors containing glycans with terminal sialic acids. Sialic acid is found on the surface of target host cells. The molecular-recognition process leads to the host cell-virus adhesion stage (Paulson et al., 1985). The sialic acid receptor of various influenza virus strains differs in affinity to sialic acids that are terminally linked either in α (2, 3) - or α (2, 6)-position to the galactose (Gal) residues. Human influenza A virus preferentially

recognize α (2, 6)-linkage, while avian influenza virus has a preference for α (2, 3)-linkage (Rogers and Paulson, 1983).

The HA protein is cylindrical and 13.5 nanometers in length. In order to fuse the endosomal and viral envelop, HA must be cleaved by proteinases from host cells to be active as a fusion protein (Flutrackers, 2007). HA is made up of three identical polypeptides. HA can be divided into two parts: HA1 and HA2 subunits after cleavage by cellular proteases. HA1, the globular head, can be found on the surface of the virus particle while HA2 is anchored in the viral envelope membrane. Although cleaved by cellular enzymes, HA1 and HA2 are still together on the viral surface. After cleavage, a very hydrophobic portion called fusion peptides is involved in the new N-terminal of HA2 caused by cleavage (Flutrackers, 2007). This so-called "fusion peptide" acts like a molecular grappling hook by inserting itself into the endosomal membrane and locking on.

When infecting host cells, firstly, the HA1 subunit recognizes the monosaccharide sialic acid receptor on the surface of viral particles and binds to it. Then the viral particle will be engulfed to form an endosome. At that time, the cell tries to digest the content inside the endosome by decreasing the pH. However, when the pH value inside the endosome is reduced from 7.0 to 5.5, the globular head of the viral HA1 subunit unfolds backward to virion, releasing the very hydrophobic portion of HA2. Then, the hydrophobic portion—the fusion peptide—tightly inserts itself into the endosomal membrane like a sharp hook. HA2 generates a conformational change and draws the endosomal membrane close to the viral envelope resulting in the fusion of the viral and endosomal membranes.

While HA is involved in binding to sialic acid groups from glycoproteins and entry into host cells, NA functions in release of progeny virus from infected cells by cleaving sialic acid groups that bind the mature viral particles and is responsible for virus replication. Otherwise, the release of the progeny virus particle is suppressed by the presence of HA-sialic acid binding. Therefore,

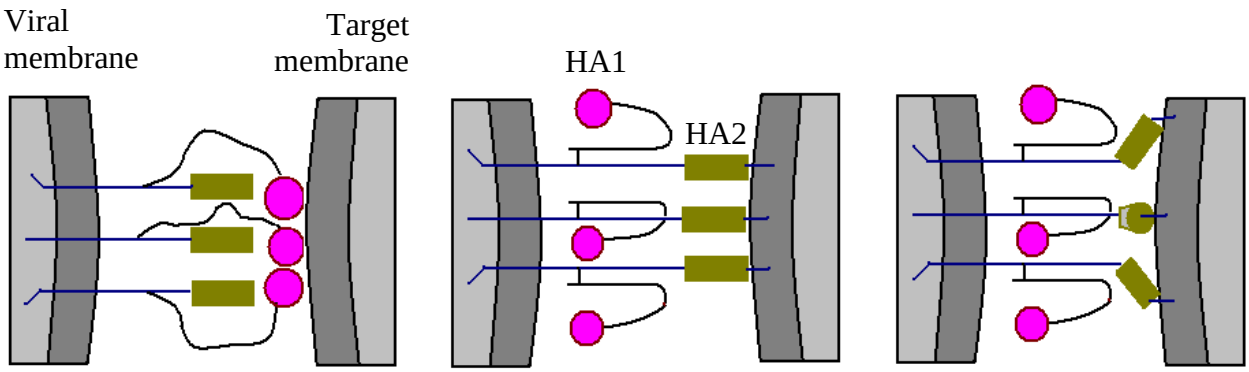


Figure 2. 2 Influenza virus attachment and penetration.

NA must cleave sialic acid groups from glycoprotein which is found on the host cell surface. The performance of NA's function stimulates the release of the progeny virus into the infected host cell and promotes the viral replication in host cell.

Additionally, an integral membrane protein also called M2 ion channel protein that is located in the surface of Influenza A virus is a channel protein which can select positive ions from the endosome (Sakaguchi, 1997; Jason and James, 2008). The M2 ion channel protein is made up of four identical molecules to form a homotetramer. Low pH before HA mediated fusion results in the activation of M2 protein (Sakaguchi, 1997; Jason and James, 2008). This channel protein plays an important role in the viral life cycle. It contributes to the entry of hydrogen ions from the endosome to viral particle and therefore lowers the pH inside viral particle, which leads to

the HA's conformational changes and degradation of matrix protein (M1) promoting the fusion of the viral and endosomal membranes. The mechanism of the anti-influenza A virus durgs amatidine and rimantidine is that the drug molecules bind to the transmembrane region of the virion and thus block the M2 ion channel, inhibiting the function of this protein and prevents uptake and uncoating of viral particle. Thus, the drug should be taken early in infection.

For the influenza viruses, two processes contribute to the changes of antigens on the surface of virus particles: antigenic drift and antigenic shift. Antigenic drift is the progressive accumulation of mutations within one subtype. During this process RNA encoding HA or NA proteins mutates leading to a new codon which will be translated into a new amino acid and further a new or mutated protein. Although the mutation is quite minor, it will prevent the vaccine against the previous virus from recognizing the antigen on the viral surface. Antigenic shift is described as the process in which two or more different strains of a virus, or strains of two or more different viruses, combine to form a new subtype having a mixture of the surface antigens of two or more original strains. Antigenic shift often occurs in any multiply-infected host, and causes immediate change in antigenic properties. For instance, birds and humans have α (2, 3) - and α (2, 6) - linkage to the galactose (Gal) residues respectively, while pigs have both of them. That means the viruses that are able to infect birds and humans can infect pigs simultaneously. When viruses replicate inside pig cell, pseudorecombination or reassortment can happen. A mixture of both genomes is produced. When antigenic drift and antigenic shift happen, people who have had the illness in the past will lost their immunity to the mutated subtype and vaccines against the original virus will also become less effective.

The World Health Organization (WHO) published the formal nomenclature for the isolation of influenza viruses. The complete and correct nomenclature should include the type of influenza virus (A, B or C), the original host excluding human, the geographic origin of isolation, the strain number, the year of isolation (4-digit year if the influenza virus was isolated in 2000 or later; 2-digit year if virus was isolated during the 1900s), and the subtype (16 HAs and 9 NAs) (CDC, 2010).

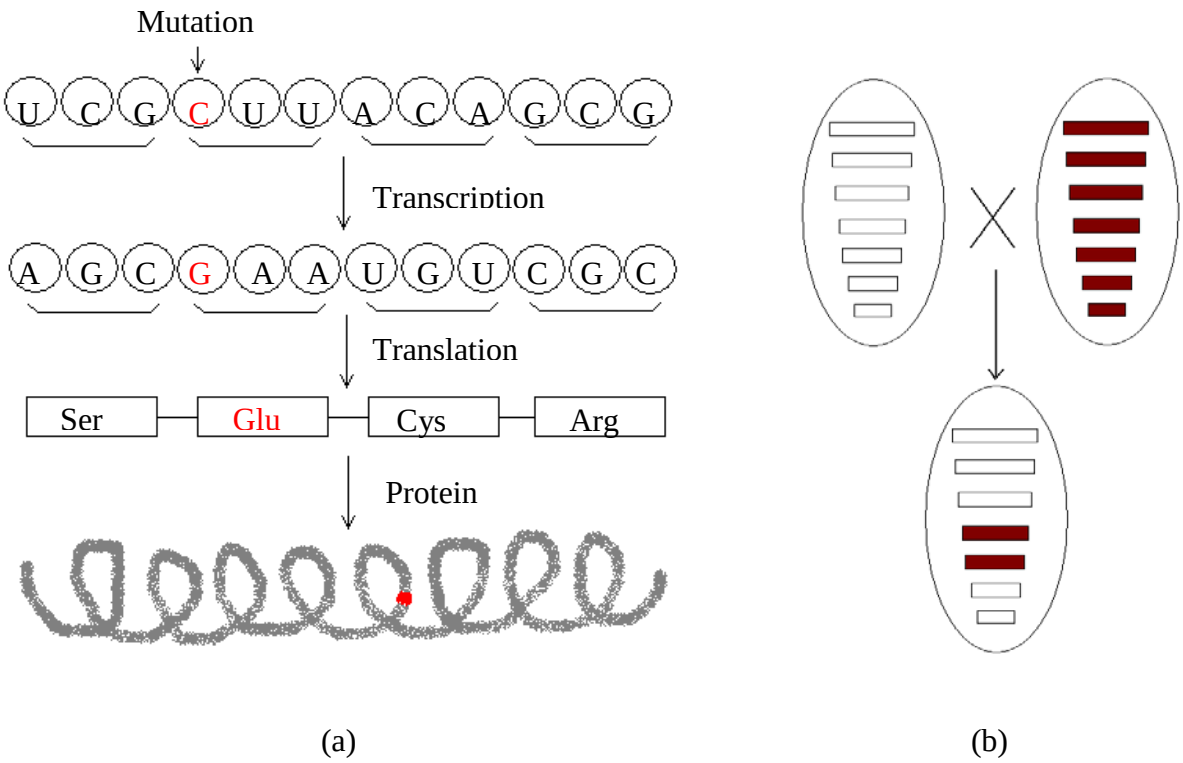


Figure 2. 3 (a) Schematic diagram of influenza virus antigenic drift. RNA encoding HA or NA proteins mutates leading to a new codon which will be translated into a new amino acid and further a new or mutated protein. (b) Antigenic shift.

For instance, a type A influenza virus that was isolated in 1997 from a chicken in Hong Kong with a stain number of 32 and an H5N1 subtype is named

Influenza A/chicken/Hong Kong/32/97 (H5N1).

If the virus was isolated from human, the name should be

Influenza A/Hong Kong/32/97 (H5N1).

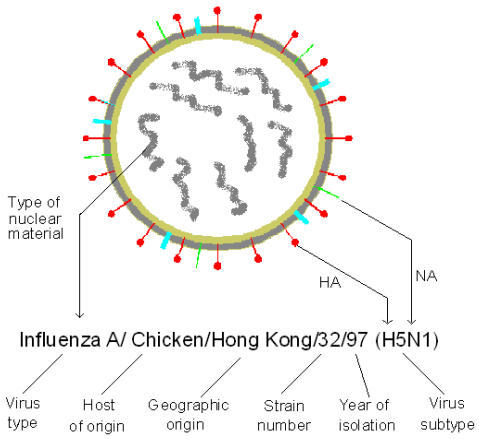


Figure 2. 4 Nomenclature of avian influenza virus.

2.1.3 Viral Inactivation

Viral inactivation renders viruses inactive, or unable to infect. Many viruses contain lipid or protein coats that can be inactivated by chemical alteration. Viral inactivation is different from viral removal because, in the former process, the surface chemistry of the virus is altered and in many cases the viral particles remain in the final product. Rather than simply rendering the virus inactive, some viral inactivation processes actually denature the virus completely. Viral inactivation is used widely in the blood plasma industry.

In order to achieve inactivation of the viruses in the sample, it is necessary to perform "special" purification processes that will chemically alter the virus in some way. Some of the more widely used processes are as follows: solvent/detergent inactivation; pasteurization (heating); acidic pH inactivation.

In some cases viral inactivation is not a viable removal alternative because even the denatured or otherwise inactivated viral particles can have deleterious effects on the process stream or the product itself.

2.1.3.1 Solvent/Detergent (S/D) Inactivation

This process is the most widely used viral inactivation method to date. It is predominantly used in the blood plasma industry (Mast et al., 1999). This process is only effective for viruses enveloped in a lipid coat, however (Horowitz et al., 1998). The detergents used in this method interrupt the interactions between the molecules in the virus's lipid coating (Mast et al., 1999). Most enveloped viruses cannot live without their lipid coating, so they die when exposed to these detergents. Other viruses may still live, but they are unable to reproduce, rendering them non-infective. The solvent creates an environment in which the aggregation reaction between the lipid coat and the detergent happen more rapidly. The detergent typically used is Triton-X 100.

This process has many of the advantages of the "traditional" removal techniques. This process does not denature proteins, because the detergents only affect lipids and lipid derivatives (Horowitz et al., 1998). There is a 100% viral death achieved by this process and the equipment is relatively simple and easy to use. Equipment designed to purify post-virus inactivated material would be necessary to guard against contamination of subsequent process streams.

S/D treatment utilizes readily available and relatively inexpensive reagents, but these reagents must be removed from the product prior to distribution which would require extra process steps. Because this process removes/inactivates the lipid coating of a virus, viruses without any sort of

lipid envelope will be unaffected. There is also no inactivation effect by the buffers used in this process.

2.1.3.2 Pasteurization

Inactivation of viruses by means of pasteurization can be very effective if the proteins that you are trying to protect are more thermally resistant than the viral impurities with which they are in solution. Some of the more prominent advantages of these types of processes are that they require simple equipment and they are effective for both enveloped and non-enveloped viruses (Schlegel et al., 2001). Because pasteurization involves increasing the temperature of solution to a value that will sufficiently denature the virus, it does not matter whether the virus has an envelope or not because the envelope alone cannot protect the virus from such high temperatures. However, there are some proteins which have been found to act as thermal stabilizers for viruses. Of course, if the target protein is not heat-resistant, using this technique could denature that target protein as well as the viral impurity. Typical incubation lasts for 10 hours and is performed at 60°C (Schlegel et al., 2001).

2.1.3.3 Acidic pH Inactivation

Some viruses, when exposed to a low pH, will denature spontaneously. Similar to pasteurization, this technique for viral inactivation is useful if the target protein is more resistant to low pHs than the viral impurity. This technique is effective against enveloped viruses (Nicola et al., 2003), and the equipment typically used is simple and easy to operate. This type of inactivation method is not as effective for non-enveloped viruses however, and also requires elevated temperatures.

So in order to use this method, the target protein must be resistant to low pHs and high temperatures (Scholtissek, 1985) which is unfortunately not the case for many biological proteins. Incubation for this process typically occurs at a pH of 4 and lasts anywhere between 6 hours and 21 days.

2.1.3.4 Ultraviolet (UV) Inactivation

UV rays can damage the DNA of living organisms by creating nucleic acid dimmers (Kumar et al., 2004). However, the damages are usually not important due to low penetration of UVs through living tissues. UV rays can be used, however, to inactivate viruses since virus particules are small and the UV rays can reach the genetic material, inducing the dimerisation of nucleic acids. Once the DNA dimerised, the virus particules cannot replicate their genetic material which prevent them from spreading (Goodrich et al., 2010).

UV light in combination with riboflavin has been shown to be effective in reducing pathogens in blood transfusion products (Ruane et al., 2004). Riboflavin and UV light damages the nucleic acids in viruses, bacteria, parasites, and donor white blood cells rendering them unable to replicate and cause disease (Kumar et al., 2004; Goodrich et al., 2010).

2.2 Current Diagnostic Methods

2.2.1 Embryonated Egg Culture

Virus isolation in the amniotic cavity of embryonated hens' eggs is a classical method for the direct recovery of influenza virus from a clinical specimen (Oxford et al., 1991). Embryonated eggs are among the most useful and available forms of living animal tissue for the isolation and identification of animal viruses, for titration viruses, and for quantity cultivation in the production of viral vaccines (Joseph, 2008). The embryo proper, chorioallantoic membrane, yolk sac, allantoic sac, or amniotic sac may be inoculated in hen eggs of various ages, so that a wide choice of types of tissue is available to fit the characteristics of the virus under study or for special studies. The chorioallantoic membrane is frequently used; in some infections, such as smallpox, vaccinia, and herpes simplex, characteristic lesions are produced which in some cases may resemble those in the natural host (Joseph, 2008). For example, smallpox virus when cultured on the chorioallantoic membrane produces pocks and typical inclusions within the infected cells. When the embryo is inoculated, characteristic skin eruptions appear. Influenza virus, however, when inoculated into the amniotic cavity, does not give rise to pathology like that of the natural infection (Joseph, 2008).

The growth of influenza virus in the allantoic cavity of embryonated eggs is probably the best-known use of eggs for animal virus culture. After inoculation into the allantoic cavity, the number of virus particles decreases exponentially, with almost 50% adsorbed to cells within 1 h. After adsorption the virus particles disappear (presumably by penetration of the cells), and an "eclipse period" of about 4 h follows. Evidence of viral multiplication first appears in the middle of the eclipse period, when soluble complement-fixing antigen (noninfective and nonhemagglutinating) is detectable. Hemagglutinating antigens can be found 3–4 h following inoculation, but in no greater amount than in the original inoculum; increases in hemagglutinating activity are seen only after 4 h or more. Infective virus is detectable 5–6 h after

the allantoic cavity is inoculated; it is released almost at once into the allantoic fluid. Infective virus particles thus released then infect other cells of the allantoic sac, and virus continues to accumulate in the allantoic fluid for about 48 h, after which the system is exhausted (Joseph, 2008).

Embryonated egg culture is a classical method for virus, but a couple of disadvantages cannot be ingored. Growth of human influenza virus in eggs can lead to the selection of variants that differ antigenically from the original seed virus (Hickling and D'Hondt, 2006). Individual virus isolates may exhibit different growth characteristics in eggs, thereby affecting the yield of the viruses (Hickling and D'Hondt, 2006).

2.2.2 Hemagglutination Inhibition (HI) Assay

In order to understand the HI assay well, the hemagglutination assay should be introduced first. Hemagglutinin, or HA is an envelope protein on the influenza virion, which is able to bind to the sialic acid receptors found on the surface of host cell. The virus can also bind to the N-acetylneuraminic acid on the surface of erythrocytes (red blood cells), which results in agglutination of red blood cells (Amano and Cheng, 2005). This process is named hemagglutination and forms the foundation of a rapid assay to determine the concentration of influenza virus in a sample. For HI assays, a virus dilution (e.g. two-fold serial dilution) will be mixed with a specific amount of red blood cells, and then added to the wells of a plastic 96-well plate for 30-min incubation. The red blood cells will precipitate at the bottom of the well if they do not bind to the influenza virus, forming a red circular dot, while the red blood cells that are

bound by influenza virus will agglutinate to form a lattice like a film covering the bottom of the well (Virology blog).

Take the test below as an example: eight different influenza viruses (A to H) are two-fold serially diluted, applied to a chicken red blood cell solution, and placed into the wells of a 96-well plate. The results can be detected after 30-min incubation. Sample A doesn't cause red blood cell precipitation up to the 1: 256 dilution, thus the HA titer of this virus stock is 256. No virus can be detected in sample B and C. Sample D has an HA titer of 512.

HA assay is the foundation of hemagglutination inhibition assay. Antibodies against the viral protein responsible for hemagglutination can inhibit binding of virus to red blood cells and hemagglutination cannot occur. The advantage of HA-HI assay is that currently it is applied by WHO to detect the influenza viruses globally, and can detect all influenza virus isolates and viral infection and antibody present in the serum (Amano and Cheng, 2005). HI assay is relatively easy and inexpensive to perform. However, its disadvantages cannot be neglected. HI has been shown to be less sensitive for the detection of antibodies induced by avian influenza viruses (Rowe et al., 1999). Accurate reading skills are also required for the complicated assay (Amano and Cheng, 2005).

If the antibodies against the viral protein are included in the serum, the hemagglutination will not happen until the serum is diluted sufficiently. On the contrary, the hemagglutination will be detected if the serum doesn't contain antibodies preventing hemagglutination (Amano and Cheng, 2005).

2.2.3 Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISA) were introduced in the 1970s. ELISA also known as enzyme immunoassay (EIA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a given sample (Crowther, 2001). A kind of antibody specifically against a particular antigen is required in an ELISA. For traditional ELISA, a 96-well polystyrene microtiter plate is used as a solid vector for immobilization of a given sample with an unknown amount of antigen. After that, a detectable antibody as primary antibody is added, causing specific antigen-antibody reaction. A secondary antibody that biologically conjugates with an enzyme is added, covalently binding to the primary antibody. After every addition of sample, the free antibodies should be removed by washing with a mild washing solution. In the final step, an enzymatic substrate is applied to the plate, and a visible signal can be showed approximately indicating the quantity of antigen in the given sample (Crowther, 2001). Commonly in ELISA there are two antigen-antibody reactions which occur just after the addition of primary antibody and secondary antibody, respectively. Antigen-antibody reaction occurs under certain temperature. The incubation temperature is 37℃ for ELISA.

Due to the disadvantages of traditional ELISA such as too much false positive, more reagent used, and results that cannot be saved, Dot-ELISA technique was developed. Dot-ELISA utilizes nitrocellulose (NC) membrane as solid vector which has stronger absorption to proteins than polystyrene. A little amount of unknown antigen is added on the NC membrane, followed by the addition of primary antibody and secondary antibody. Finally, signal substrate that can generate

visibly insoluble precipitation with the secondary antibody is applied onto the NC membrane. The room temperature is suitable for Dot-ELISA.

In short, Dot-ELISA has the same principle as traditional ELISA (Belo et al., 2010). However, Dot-ELISA has more advantages than traditional ELISA, such as better sensitivity, simplicity of performance, no need for complex laboratory equipment, and fast speed (Belo et al., 2010).

2.2.4 Polymerase Chain Reaction (PCR)

2.2.4.1 Traditional Polymerase Chain Reaction

The polymerase chain reaction (PCR) was developed in 1980s by Mullis (Bartlett et al., 2003). PCR is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR has played a very essential role in medical and biological research for a variety of applications since it was developed. PCR technique is applied in DNA cloning for sequencing, functional analysis of genes, diagnosis of hereditary diseases, the identification of genetic fingerprints and the detection, diagnosis of infectious diseases, etc.

PCR is used to *in vitro* duplicate DNA strand that is complementary to the template DNA with the help of enzymatic DAN polymerase and specific primer as the start point of extension. PCR requires thermal cycling, including circular procedure of heating and cooling of the reaction in denaturation, annealling, and extension. Several components and reagents are utilized in PCR process covering template DNA with the target, primer, four deoxynucleoside triphosphates

(dNTPs), and DNA polymerase. The primer is actually a piece of DNA fragment with known sequence that can bind to the complementary region in DNA template, and performs as a binding site of DNA polymerase to direct DNA replication.

PCR has higher sensitivity than ELISA and relatively shorter performance time than viral culture (24 to 36h versus 2 to10 days) (Robert et al., 1996; Joanna et al., 2002). The disadvantages of PCR involve in cost, false positive, intricate procedure, and possibility of contamination (Joanna et al., 2002). Additionally, to evaluate the amplicated products during PCR, laborious post-PCR handling steps are required, that is to say, much more time is needed (Lan et al., 2002). Moreover, EB may cause cancer due to its inhibition of RNA, DNA and protein synthesis (NTP, 2005). EB should be avoided to touch directly.

2.2.4.2 Reverse Transcription PCR

With increasing development of PCR technique, many variations on the basic PCR technique have gradually been introduced into the research fields. Reverse Transcription PCR (RT-PCR) is a good example. Most recently, PCR, especially RT-PCR has been greatly developed as an extremly effective technique for DNA amplification, viral detection, typing and subtyping (Zhang et al., 1991). During RT-PCR, a single-strand RNA is reversely transcribed into a complementary DNA (cDNA) by the reverse transcriptase. Then a new DNA strand complementary to cDNA is synthesized directed by the deoxynucleotide primer and DNA polymerase. Exponential growth is developed with every cycle. The previous single-strand RNA is degraded by RNase H, leaving cDNA.

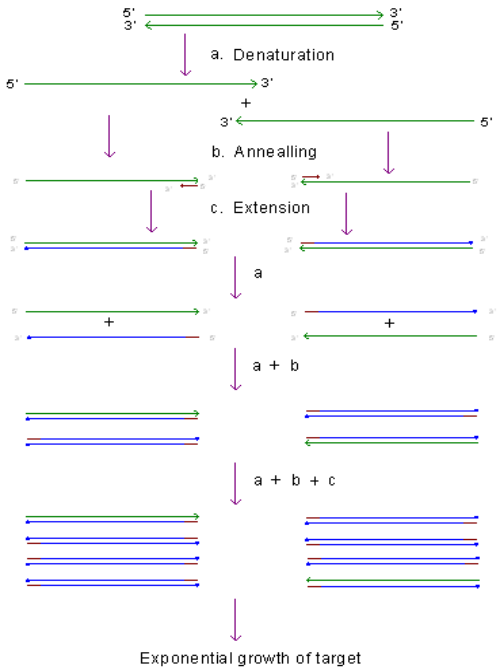


Figure 2. 5 Schematic diagram of the PCR cycle. A single copy of a piece of DNA was amplified to several orders of magnitude through denaturation, annealing, and extension of PCR.

2.2.4.3 Real Time RT-PCR

Sometimes RT-PCR refers to the real-time PCR. To distinguish it from reverse transcription PCR, real-time PCR is commonly called quantitative PCR, or real-time quantitative PCR, or abbreviated to RTQ-PCR, Q-PCR, or QRT-PCR. Real-time PCR is a process by which the increase of DNA can be detected with the ongoing amplification cycles with the help of a fluorescent reporter molecule. The amount of specific product depends on the intensity of the fluorescence, predicting the initial amount of target gene without product isolation.

There are two major kinds of fluorescent reporters used in real time RT-PCR, which are general non-specific DNA binding dyes such as SYBR green and TaqMan Probes. As for the former, excessive SYBR green is added to the PCR reaction system, and fluorescent signal is emitted if

SYBR green specifically binds to the double-strand DNA, while SYBR green cannot generate fluorescent signal if it doesn't bind to DNA strand to ensure simultaneous increase of fluorescent signal and PCR product. To the latter, a specific TaqMan probe is applied to the PCR system at the same time of addition of primers. The TaqMan probe is an oligonucleotide labeled at both ends with fluorophore and quencher. Fluorescent signal generated by fluorophore is absorbed by quencher if the probe is complete. During PCR amplification, TaqMan probe is enzymatically degraded by Taq polymerase, separating fluorophore and quencher. Therefore, fluorescent signal can be detected. Every formation of new DNA strands brings a fluorescent signal, fulfilling the simultaneous increase of fluorescent signal and PCR product.

The fluorescent signal is continuously determined in real time during the amplification processes, and can form a curve with time going on. During the early phase of PCR, the thermal cycler cannot distinguish the fluorescence signal from the background noise, and later, the curve of dynamic process shows an exponential growth of fluorescence signal. However, actually the reaction doesn't always show a standard exponential curve but a "S" curve due to the inactivation of DNA polymerase, depletion of dNTP and primer, and the resistance of by-product as the cycles increased. The linear phase and plateau of fluorescence signal are followed by the exponential growth phase. Consequently, amplicons can be determined at one point within exponential growth phase due to excellent reproducibility in this phase so that the initial amount of template will be obtained.

Quantitative PCR technique makes it possible to detect gene amplicons during PCR taking place in real time. To meet the need of experimental purposes, very often real time PCR and reverse

transcription PCR are integrated to detect specific target gene using trace amount of template RNA in real time. The combination of both PCR is called quantitative RT-PCR (qRT-PCR).

PCR techniques have considerably contributed to the viral detection especially the influenza virus detection. However, PCR cannot separate the live virus from the inactivated virus, causing a false positive result (Suarez et al., 2003). Moreover, conventional PCR is a time-consuming technique with improtant limitations when compared to real-time PCR technique due to the requirement of post-PCR (Lan et al., 2002).

2.3 Aptamers

Aptamers are artificial oligonucleic acid or peptide molecules that can bind to a specific molecule with high affinity and selectivity, such as viruses. Aptamers were developed in 1990 within two independent labs: the Gold lab (Tuerk and Gold, 1990) and the Szostak lab (Ellington and Szostak, 1990) in the USA. In Gold lab, high-affinity nucleic acid ligands for a protein were isolated by a procedure that depends on alternate cycles of ligand selection from pools of variant sequences and amplification of the bound species, and multiple rounds exponentially enrich the population for the highest affinity species that can be clonally isolated and characterized (Tuerk and Gold, 1990). Subpopulations of RNA molecules that bind specifically to a variety of organic dyes isolated from a population of random sequence RNA molecules in Szostak lab (Ellington and Szostak, 1990). Aptamers are usually created by selecting them from a large random sequence pool. They can be applied for basic research as well as clinical purposes. More specifically, aptamers can be classified as DNA or RNA aptamers which consist of (usually short)

strands of oligonucleotide, and peptide aptamers including a short variable peptide domain. Nucleic acid aptamers are usually investigated.

2.3.1 Selection of Aptamers

Nucleic acid aptamers have been developed through an *in vitro* selecting technique called systematic evolution of ligands by exponential enrichment, abbreviated as SELEX, and the resulted oligonucleotides are referred to as aptamers, derived from the Latin *aptus* meaning 'to fit' (Sumedha, 1999). The SELEX process begins with a random sequence library consisting of randomly generated sequences of fixed length flanked by constant 5' and 3' ends that serve as primers and the library is obtained from combinatorial chemical synthesis of oligonucleotides (Ciara, 2001). Consequently the very combinatorial libraries of oligonucleotides are screened by a repeated process of *in vitro* selection and amplification (Ciara, 2001). Each member in a library is a linear oligomer of a unique sequence and the molecular diversity is dependent on the number of randomized nucleotide positions (Ciara, 2001).

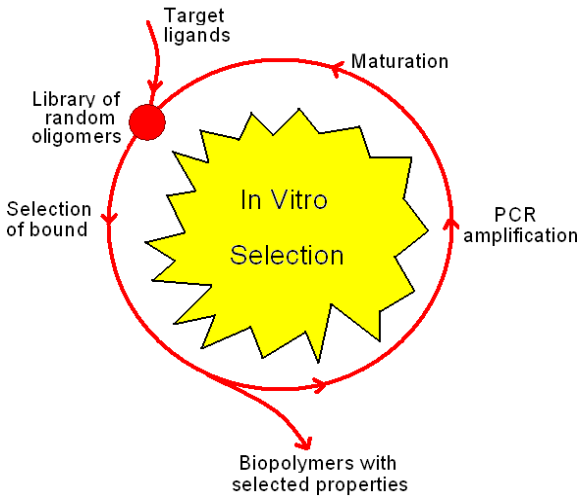


Figure 2. 6 Schematic indicating steps involved in aptamer selection using the SELEX process starting with target ligands. The SELEX process begins with a random sequence library. The very combinatorial libraries of oligonucleotides are screened by a repeated process of *in vitro* selection and PCR amplification.

In the screening process, a random sequence oligonucleotide library is incubated with a target of interest in a buffer of choice at a given temperature. During the cycles of selection, a very small fraction of individual sequences bind with the target, and these sequences are separated from the rest of the library by some physical separation techniques such as affinity chromatography or filter binding (Burke and Gold, 1997). The population of sequences bound to the target is isolated and amplified to obtain an enriched library to be used for the following selection/amplication cycle. The efficiency of enrichment of high-affinity binders is governed by the stringency of selection of each round, and the number of cycles required for aptamer identification is usually dependent on the degree of stringency imposed at each round as well as the nature of the target (Sumedha, 1999). The progress of the enrichment of high-affinity binders can be determined by carrying out binding analysis of enriching populations against the target. When affinity saturation is reached after several rounds of selection/amplification, the enriched pool is cloned and sequenced to obtain the sequence information (Sumedha, 1999). Individual

sequences are further characterized on the basis of their ability to bind to the target. In the end, the counter selection is employed to effectively discard ligands that have the ability to bind the closely related structural analogs of the target, resulting in aptamer sequences with high specificity (Jenison et al., 1994). Aptamers can then be truncated as desired, eliminating the fixed primer sequences and the nucleotide stretches that are not important for direct interaction with the target or for folding into the structure that facilitates target binding (Sumedha, 1999). When the desired sequence has been identified the aptamer can be produced in sizeable quantities by chemical synthesis.

2.3.2 Comparison between Aptamers and Antibodies

Nucleic acid aptamers are often considered as artificial antibodies. However, when compared to antibodies, aptamers have considerable advantages that make them more conspicuous and more potential in analytical and diagnostic application than antibodies. The main advantage of aptamers is that they can be selected *in vitro*, while the antibodies have to be obtained *in vivo* through induction of an immune response in a biological system. Antibodies function only under physiological conditions, which limit the application and function of antibodies. The immune system couldn't respond to the targets or toxic compounds which have similar structure to the endogenous protein but are harmful to the biological system. Otherwise, aptamers can be specific against any target molecules and applied for any condition not only *in vivo* but also *in vitro*. Moreover, aptamers are more stable at high temperature, and can stand boiling for several minutes but doesn't denature, resulting in a longer shelf life than antibodies.

The SELEX selection process usually takes about 8 weeks for the development of an aptamer and while antibody selection will have to take up to 6 months. Therefore, a plenty of time will be saved when compared to the *in vivo* selection process for antibodies.

Aptamers can be manipulated to bind different region of the target in different conditions. Therefore, high-quality aptamers can be obtained after they are synthesized chemically and then purified to a very high degree by eliminating the batch-to-batch variation which can be found for antibodies, leading to good stability, strong affinity and high specificity of aptamers. The comparison of properties of antibodies and aptamers are listed in the table below.

Table 2. 1 Comparison of the properties of antibodies and aptamers.

Antibodies	Aptamers
Biological system required	In vitro selection process
Animals required	No need of animals
Target for immunogenic proteins	Target for any proteins
Limited modifications	Chemical modifications result in high specificity.
Unstable, poor high-temperature resistance	Stable, excellent heat resistance
Short shelf life	Unlimited shelf life
Time consuming and expensive	Short selection time
May undergo denaturation after high temperature.	Denatured aptamers can be regenerated within minutes.
Antibodies often suffer from batch to batch variation.	Aptamers are produced by chemical synthesis resulting in little or no batch to batch variation.

2.3.3 Aptamers Against AI

The raised interest in the detection of AI in humans as well as animals has been stimulated by increasing emergence of the infectious influenza diseases and also the development of various aptamers. A variety of aptamer-based technologies have been used to detect AI. Choi et al. (2011) selected DNA aptamers against the receptor binding region of hemagglutinin and those aptamers

can prevent avian influenza viral infection. In this study, they cloned the cDAN fragment which encoded the whole globular region of hemagglutinin of the H9N2. As we know, the entrance of influenza virus into host cells is facilitated by the attachment of the globular region of viral hemagglutinin to the sialic acid receptors on host cell surfaces (Choi et al., 2011). To identify specific DNA aptamers with high affinity to H9 peptide, they conducted the SELEX method. 19 aptamers were isolated, and a random mixture of these aptamers showed an increased level of binding affinity to the H9 peptide (Choi et al., 2011). The sequence alignment analysis of these aptameres revealed that 6 aptamers have highly conserved consensus sequences. Among these, aptamr C7 showed the highest similarity to the consensus sequences. This aptamer showed strong binding capability to the viral particles which suggest that this apatmer can recognize the hemaglutinin protein of AIV and inhibit the binding of the virus to target receptors (Choi et al., 2011).

Another paper by Park et al. (2011) reported that RNA aptamer candidates targeting the biologically active HA protein were selected after 15 rounds of interative SELEX. The selected RNA aptamer HAS 15-5, which specifically binds to HA1 that is the receptor binding domain of HA protein, exhibited significant antiviral efficacy according to the results of a hemagglutination inhibition assay using egg allantoic fluids harboring the virus (Park et al., 2011). Thus, the RNA aptamer HAS15-5, which acts by blocking and inhibiting the receptor-binding domain of viral HA, can be developed as a novel antivial agent against type H5 AIV (Park et al., 2011). Gopinath et al. (2005) isolated aptamers specific for hemagglutinin protin of human influenza virus B/Johannesburg/05/1999. The selected aptamers, Class A-20, bind specifically to hemagglutinin of B/Johannesburg/05/1999 and able to discriminate it from hemagglutinin of human influenza A virus (Gopinath et al., 2005).

In Dr. Yanbin Li's lab in University of Arkansas, another type of DNA aptamer against H5N1 was selected using an in vitro SELEX. In this study, the mixture of DNA pool and H5N1 viruses passed through a nitrocellulose filter (Zhao, 2011). The aptamers with the affinity and specificity to H5N1 kept on the filter surface, and the rest of the pool were washed away. Then, the DNA aptamer candidates were amplified by PCR. Single-stranded DNA aptamers were obtained through λ digest of a dsDNA stock. Lambda exonuclease that is a 5' to 3' exodeoxyribonuclease is able to digest the 5'-phosphorylated strand of dsDNA (Zhao, 2011). The ssDNA was applied for the next cycle, and ssDNA aptamers with higher affinity and specificity were obtained through interative separation-amplification cycles (Zhao, 2011).

2.4 Biosensors for Detection of Avian Influenza

Worldwide, there are many strains of avian influenza virus that cause varying degrees of clinical symptom and illness. In the United States, outbreaks of the disease – primarily spread by migratory aquatic birds – have plagued the poultry industry for decades with millions of dollars in losses. The only way to stop the spread of the disease is to destroy all poultry that may have been exposed to the virus. Quick identification of avian influenza infection in poultry is critical to controlling outbreaks, but current detection methods can require several days to produce results. Current interest has focused on developing new sensors for quick and reliable testing for influenza with minimum sample handling and laboratory skill requirement. In recent years, various biosensors have been studied as an alternative to conventional methods for detection of influenza viruses.

2.4.1 Introduction of Biosensors

A biosensor is an analytical device which converts a biological response into an electrical signal. Biosensors can provide reliable, real-time, on-field, user-friendly, and inexpensive analysis with improved or equivalent sensitivity, specificity and reproducibility of culture-based tests (Alocilja et al., 2003). A successful biosensor must possess at least some of the following beneficial features:

The biocatalyst must be highly specific for the purpose of the analyses, be stable under normal storage conditions and, except in the case of colorimetric enzyme strips and dipsticks, show good stability over a large number of assays (i.e. much greater than 100).

The reaction should be as independent of such physical parameters as stirring, pH and temperature as is manageable. This would allow the analysis of samples with minimal pre-treatment. If the reaction involves cofactors or coenzymes these should, preferably, also be co-immobilised with the enzyme.

The response should be accurate, precise, reproducible and linear over the useful analytical range, without dilution or concentration. It should also be free from electrical noise.

If the biosensor is to be used for invasive monitoring in clinical situations, the probe must be tiny and biocompatible, having no toxic or antigenic effects. If it is to be used in fermenters it should be sterilisable. This is preferably performed by autoclaving but no biosensor enzymes can presently withstand such drastic wet-heat treatment. In either case, the biosensor should not be prone to fouling or proteolysis.

The complete biosensor should be cheap, small, portable and capable of being used by semi-skilled operators.

There should be a market for the biosensor. There is clearly little purpose developing a biosensor if other factors (e.g. government subsidies, the continued employment of skilled analysts, or poor customer perception) encourage the use of traditional methods and discourage the decentralisation of laboratory testing.

A biosensor is an analytical device for the detection of an analyte that combines a biological component with a physicochemical detector component. It consists of 3 parts: The sensitive biological element (biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.): a biologically derived material or biomimic component that interacts (binds or recognises) the analyte under study. The biologically sensitive elements can also be created by biological engineering.

The transducer or the detector element (works in a physicochemical way; optical, piezoelectric, electrochemical, etc.) that transforms the signal resulting from the interaction of the analyte with the biological element into another signal (i.e., transducers) that can be more easily measured and quantified;

Biosensor reader device with the associated electronics or signal processors: this part is primarily responsible for the display of the results in a user-friendly way. This sometimes accounts for the most expensive part of the sensor device. However it is possible to generate a user friendly

display that includes transducer and sensitive element. The readers are usually custom-designed and manufactured to suit the different working principles of biosensors.

In general, the application of the transducer in the analytical system separates the biosensors from conventional assays. Depending on the transduction mechanism, the biosensor includes electrochemical, optical and piezoelectric biosensors.

2.4.2 Biosensors for Detection of Avian Influenza

Influenza is an acute respiratory disease caused by the influenza virus. The disease occurs annually, causing fatality in the elderly and children and billions of dollars loss in business and productivity. Although traditional viral detection methods are effective, these methods generally involve labor-intensive laboratory procedures and often require trained personnel to carry them out. The development of biosensor technologies will enable rapid and specific disease diagnosis on-site so that a clinician can quickly determine whether treatment is needed. Biosensors will have an impact on people's daily lives in the not too distant future, since they can fill very real need in health care, environmental monitoring and control, agriculture and chemical industries.

There is much interest in developing new influenza sensors for quick and reliable testing for influenza with minimum sample handling and laboratory skill requirement. One of the strategies is to develop single step direct sensing methods that eliminate separation, incubation or use of any signal-reporting agents. In recent years, non-labeling techniques such as surface plasmon resonance (SPR) and quartz-crystal microbalance (QCM) have shown high promise in sensor research and gained momentum in detection of viral samples. Detection limits of QCM and SPR

are both around the sub-nanogram region. Because the molecular weight of influenza A/B virus is about 2.5×10^6, the detectable number of virions is considered to be around 10^6 (Yoshihisa and Quan, 2004).

For example, the impedance biosensor is a class of electrical biosensors that show promise for point-of-care and other applications due to low cost, ease of minizturization, and label-free operation (Daniels and Pourmand, 2007). Impedance biosensors include two electrodes with applied altemating voltage, amplitudes from a few to 100 mV are used (Pohanka and Skladal, 2008). There have been some promising approaches of impedance biosensors. Hybridization of DNA fragments previously amplified by PCR has been monitored by an impedance assay (Pohanka and Skladal, 2008). A portable impedance biosensor for detection of avian influenza virus was also evaluated by Wang et al. (2011). In the impedance biosensor system, the magnetic nanobeads were coated with AIV subtype-specific antibody for immobilization of a target virus, and a microfluidic chip with an interdigitated array microelectrode was used for transfer and detection of target virus (Wang et al., 2011). The impedance of the bio-nanobeads and AI virus complexes in a buffer solution can be measured based on a combination of magnetic nanobeads and a microfluidic chip (Wang et al., 2011).

Additionally, surface plasmon resonance is a direct optical-sensing technique that measures the refractive index change due to biospecific interactions occurring in the vicinity of a thin metal film surface. Its label-free detection scheme together with very high sensitivity offers enormous opportunity and flexibility to biosensing assay development. Judging from the increasing number of publications, it is clear that surface plasmon resonance has become one of the most attractive techniques for detection of various biochemical analytes. Surface plasmon resonance has been

used for detection of influenza virus and the study of interactions that involve viral proteins and receptors. The first use of SPR in influenza virus detection was reported by Schofield and Dimmock (Schofield and Dimmock, 1996). The sensor chip was coated with carboxylated dextran polymer matrix, on which monoclonal antibody HC10 for capture of influenza virus was coupled. Influenza virus was injected into the flow system, and the binding affinity with the surface antibody was monitored. This study demonstrated that the feasibility of affinity measurements and detection for large virus particles by SPR.

2.4.3 Aptamer-based Biosensors (Aptasensor)

The nucleic acid aptamers are the one type of synthetic ligands that have the potential for various biosensing applications. Aptamers are single strand nucleic acid ligands, and the ability of aptamers to bind their target molecule with high specificity and other advantages make them a good tool for therapeutic, diagnostic and analytical application, such as mediation for tumor cell lysis (Jing et al., 2010; Boltz et al., 2011), cancer detection or therapy (Medley et al., 2011; Rialon and White, 2011; Zhu et al., 2010), nanoparticle-based detection using aptamer (Song et al., 2011; Wang et al., 2011; Medley et al., 2011; Chen et al., 2011), and aptamer-based biosensors (Ma et al., 2010; Sassolas et al., 2011; Liu et al., 2010; Qureshi et al., 2010). They are especially well suited as biosensors.

Aptamer-based electrochemical biosensor was applied to detect the interferon gamma (IFN-γ) produced by a number of immune cell types including T-helper (CD4) cells and cytotoxic T-cells by Ying et al. (2010). In this research, IFN-γ binds to the DNA aptamer, consequently forming the aptamer-IFN-γ complex. Then, methylene blue (MB) redox tag was conjugated with the

aptamer- IFN-γ complex. By self-assembly technique, the aptamer-IFN-γ-MB compounds are immobilized on the gold surface of the electrode. The DNA aptamers unfold due to the IFN-γ binding, and so the MB redox molecules are forced to move away from the electrode, which lowers the electron-transfer efficiency and subsequently the redox current is changed (Ying et al., 2010). An electrochemically voltammetric biosensor is used to measure the change in redox current highly proportional to IFN-γ concentration. The minimum limited concentration of IFN-γ for this aptasensor was 0.06 nM. Moreover, regeneration of the same aptasensor is performed by breaking down the bound aptamer-IFN-γ complex in urea buffer, and the sensor can be reused multiple times (Ying et al., 2010).

The aptamer has already been applied in capacitive biosensor for the detection of C-reactive protein by Anjum et al. (2010). C-reactive protein (CRP) is a biomarker for cardiovascular disease risk (CVR) and can be biorecognized by the RNA-aptamer in this research. Anjum et al. used a RNA-aptamer-based biosensor for measuring transducing aptamer-CRP recognition events based on charge distribution under the applied frequency by non-Faradaic impedance spectroscopy (NFIS) (Anjum et al., 2010). RNA aptamer is immobilized in the thin layers on the gold interdigitated (GID) electrode. Consequently, the charge distribution changes with the formation of RNA-CRP complex when CRP binds, following the signal generation in terms of capacitance. Previously, antibody-based capacitive biosensor for CRP detection has been reported, but antibodies show inactivation over a period of time (Quershi et al., 2009; Anjum et al., 2010). Therefore, the utility of aptamer in this study overcomes the drawback of antibodies.

Also, the aptamer was applied as a molecular recognition element in a fluorescent biosensor for measurement of potassium ion using a pyrene-labeled molecular beacon (Chao et al., 2010). The

pyrene is used to label the 5' end and 3' end of complementary oligonucleotide with the aptamer. When K^+ is added the complementary oligonucleotides will move away from the aptamers. The two pyrene moieties are drawn very close to each other due to the self-hairpin structure of the complementary oligonucleotide, resulting in the release of pyrene fluorescence (Chao et al., 2010). The fluorescence intensity of pyrene is proportional to the concentration of K^+. However, only monomer (the complementary oligomer conjugated with aptamers) is produced in the absence of K^+.

Several aptamer-based biosensors for detection of various viruses have been reported including those specific to the HIV-1 Tat protein (Tombelli et al., 2005), hepatitis C virus (Seram et al., 2007), herpes virus (Garai-lbabe et al., 2011), and plant virus (Gergely et al., 2010). For instance, aptamer-based piezoelectric QCM biosensor is investigated for detection of the protein trans-activator of transcription (Tat) of HIV-1. In this work, a RNA aptamer as bio-recognition element for the HIV-1 Tat protein was immobilized on the gold electrode of the crystal, binding with streptavidin previously attached on the electrode surface (Minunni et al., 2004). The interaction with aptamer after addition of the Tat protein is monitored by the changes of the oscillation frequency of the crystal. To compare with immunosensors, this aptamer-based biosensor demonstrates excellent sensitivity, selectivity and reproducibility (Minunni et al., 2004).

2.4.4 Surface Plasmon Resonance (SPR) Biosensor

The application of surface plasmon resonance (SPR) has been used almost three decades since 1982 (Jiri et al., 1999), and SPR sensing has received continuous attention from the scientific

community since the first application. SPR-based optical biosensors are now being used extensively to define the kinetics of a wide variety of macromolecular interactions and high- and low-affinity small molecule interactions (Priyabrata, 2005). SPR-based biosensors significantly affect both basic and applied research.

2.4.4.1 Principle of Surface Plasmon Resonance (SPR)

Surface plasmons are actually a phenomenon of surface electromagnetic waves that occurs on interface of a metal and the external medium. The wave is very sensitive to any change of the interface, such as the adsorption of molecules to the metal surface.

The surface plasmon resonance is from the stimulation of surface plasmon by light. To generate resonance by stimulating the surface plasmons, an electron or light beam is required (Priyabrata, 2005). Generally, the p-polarized light is used because it can be passed through a layer of glass to increase the wave number and obtain the resonance at a given wavelength and angle. Silver and gold are the metals typically used for surface plasmons.

Specifically, SPR is capable of detecting changes in refractive index (RI) occurring near the surface of a metal (within ~200 nm) (Sigal et al., 1997). Refractive index refers to the speed with which light passes through a material compared to the speed with which it passes through air. Its most elementary occurrence is in Snell's law of refraction (Figure 2.7),

$$\sin \theta_1 \, RI_1 = \sin \theta_2 \, RI_2 \qquad (1)$$

where θ_1 and θ_2 are the angles of incidence of a ray crossing the interface between two media with refractive indices RI_1 and RI_2. For example, light travels more slowly through glass than it does through air, therefore glass has a higher refractive index than air (RI_{glass} = 1.5-1.6, RI_{air} = 1) (Foster, 1997). A simple SPR instrument set-up generally consists of a light source, a glass prism with a high refractive index RI, a thin metal film placed in contact with the bottom of the prism and a optical detector (Figure 2.8 a). The molecular layer of interest can be coated onto the thin metal film may be coated with a molecular layer of interest on the side opposite the prism.

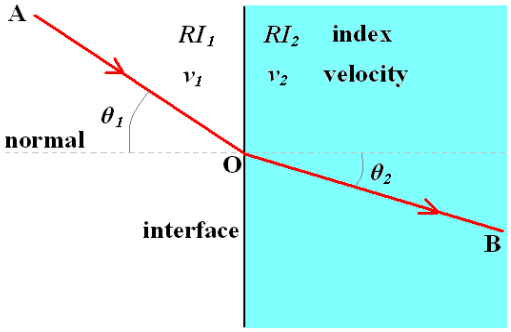


Figure 2. 7 Refraction of light at the interface between two media.

Surface plasmon waves (SPWs) can be generated at the interface between the conductive metal film and the insulating molecular layer by striking the metal sensor with a particular type of light (Caruso et al., 1998; Homola et al., 1999). At the same time that SPWs are generated, light is also reflected off of the metal surface. Past a specific incident angle (Figure 2.8), and only in the presence of the highly refractive glass prism, all the energy from the incident light wave will be transferred to the reflected light wave (total internal reflection) (Fishbane et al., 1993). However, at a very specific angle past the point of total internal reflection (the SPR angle), a majority of the incident light energy that would have typically been transferred to the reflected light wave will instead interact with the generated SPWs, resulting in a phenomenon called resonance

(Levesque et al., 1997; Stenberg et al., 1991). At resonance, a minimum in reflected light intensity will be observed, and the SPR angle can thus be determined by measuring the intensity of the reflected light (via optical detector), and plotting it as a function of incidence angle (see Figure 2.8 a) (Stenberg et al., 1991).

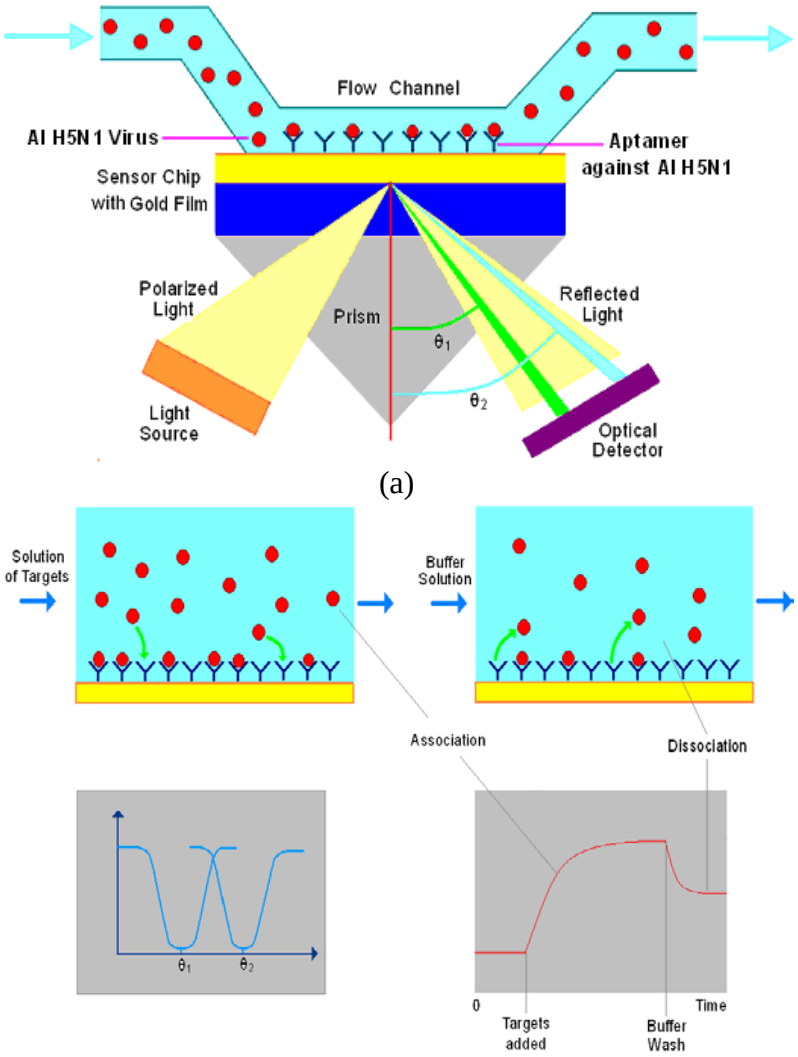


Figure 2. 8 The configuration for measuring surface plasmon resonance: (a) Schematic diagram of SPR.When the incident light was used to the prism of SPR sensor, an angle of θ_1 was formed which can be observed by the optical detector. Then, the targets were applied to the gold surface of the sensor chip and they specifically bound to the probes or aptamers in this research which resulted in the change of thickness of the gold surface. When the light source was used at that time, a different SPR angle θ_2 was formed. The difference between both angles is highly related with the properties of the targets. (b) Target binding. After targets were applied to the gold surface, they bound to the ligands on the surface with high or low affinity. The binding with low

affinity or nonspecific binding were washed away through using buffer solution, only leaving the binding with higher affinity.

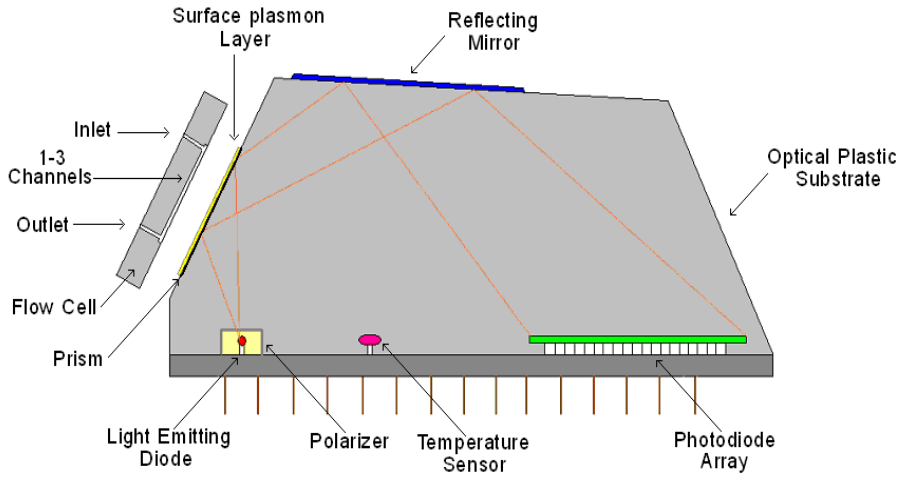


Figure 2. 9 Schematic diagram of SPR chip. Before and after the targets were used to the flow cell, the light formed different refractive angles due to the change of thickness of the gold surface.

2.4.4.2 Kinetic Analysis of Surface Plasmon Resonance

Surface plasmon resonance is a charge-density oscillation that may exist at the interface of two media with dielectric constants of opposite signs, for instance, a metal and a dielectric (Homola et al., 1999). The charge density wave is associated with an electromagnetic wave, the field vectors which reach their maxima at the interface and decay evanescently into both media (Homola et al., 1999). A SPW is a transverse-magnetic (TM) wave (magnetic vector is perpendicular to the direction of propagation of the SPW and parallel to the plane of interface) and is an electromagnetic wave which propagates along the boundary between a dielectric and a metal (Homola et al., 1999; Homola, 2003). A SPW is characterized by the propagation constant and electromagnetic field distribution. The propagation constant of a SPW, β, can be expressed as:

$$\beta = k \sqrt{\frac{\varepsilon_{\mathrm{m}} RI^2}{\varepsilon_{\mathrm{m}} + RI^2}} \qquad (2)$$

where k is the free space wave number, ε_m is the dielectric constant of the metal, RI is the refractive index of the dielectric (Homola et al., 1999). At optical wavelengths, this condition is fulfilled by several metals of which gold and silver are the most commonly used (Ordal et al., 1983).

Due to the face that the vast majority of the field of a SPW is concentrated in the dielectric, the propagation constant of the SPW is extremely sensitive to changes in the refractive index of the dielectric (Homola, 2003). This property of SPW is the underlying physical principle of affinity SPR biosensors - biomolecular recognition elements on the surface of metal recognize and capture analyte present in a liquid sample producing a local increase in the refractive index at the metal surface. The refractive index increases give rise to an increase in the propagation constant of SPW propagating along the metal surface which can be accurately measured by optical means (Homola, 2003).

2.4.4.3 Application of Surface Plasmon Resonance

The SPR technique was used in the development and characterization of ultra-thin films (Yuan et al., 2007). Over the last decade, the SPR biosensor technique has received great research interest. SPR biosensors have become an established method for measuring molecular interactions. The best indicator of optical biosensor technology is the growing number of commercially available instruments.

The optical system, the sensor system and the detection system together build the whole SPR sensor system. The optical system includes the light source and the optical path used to produce the incident light fit for the performance requirements. The sensor system is applied in the transformation of sensitive information to the change of refractive index. Lastly, the detection system measures the intensity of the reflected light, and the position of the resonance adsorption peak is recorded for the further analysis (Yuan et al., 2007). Therefore, SPR biosensor experiments include the immobilization of one reactant on the metal surface and detection of its interaction with a second component in terms of the refractive index change near the sensor surface (Rebecca et al., 2000). The SPR biosensors can monitor the interactions between ligands and targets in real-time without labeling requirements. Subsequently, they can be used to detect the binding reactions of any biological system from proteins, oligonucleotides, oligosaccharides, and lipids to small molecules, phage, viral particles and cells (Rebecca et al., 2000).

The SPR phenomenon has been known for a long time. However, the application of SPR-based biosensors is relatively new. Currently, SPR biosensor technology has obtained rapid development and improvement since the first use of SPR for biosensing purposes, being an effective alternative for analyzing biological interactions. There are a large number of papers reported the application of SPR biosensors, such as protein immobilization (Park et al., 2011; Oliveira et al., 2011), antibody selection and detection (Razavi et al., 2011; Nosach et al., 2009), bacteria immobilization (Arya et al., 2011), papillomavirus genotype (Wang et al., 2010), diagnosis of hepatitis B virus and dengue virus (Zheng et al., 2010; Kumbhat et al., 2010). A few studies on influenza virus detection using SPR biosensors have been reported, like avian influenza DNA hybrization (Kim et al., 2010), adamantane binding sites in the influenza A M2

ion channel (Rosenberg and Casarotto, 2010), and influenza virus hemagglutinin monitoring (Mandenius et al., 2008).

Chapter 3 Objectives

Highly pathogenic avian influenza virus H5N1 has been a great threat for the poultry industry as well as for humans, causing high morbidity and mortality rate. In addition to containment procedures, sensitive detection assays for early diagnosis are important to reduce the chances of spread and lower the risk of development into an epidemic. However, current detection methods are either time-consuming, or expensive, or require complex techniques. A rapid and specific detection method of avian influenza virus H5N1 is needed and will become increasingly important and urgent in the face of concerns over the outbreaks of highly pathogenic avian influenza virus H5N1 and the cases of human infection. Here we developed a new technique to specifically detect the AI H5N1 in which an aptamer-based SPR biosensor was used as a transducer and the aptamer used was sequenced in our lab and specifically against AI H5N1.

The specific objectives of this project were as follows:

1. To optimize the parameters:

In order to get the best data using minimum amount of material, incubation time and concentrations of streptavidin and aptamers were optimized.

After SPR device was pretreated and set up, an aliquot of 250 μl of 0.025 mg/ml streptavidin was applied into the SPR flow cell and incubated for 25 min. After streptavidin immobilization, 1 ml of 10 mM PBS was applied to wash away the excess streptavidin and then the test was paused. The response data was collected through the software of Spreeta 5. Then, another new test for concentration optimization of streptavidin was started. In this research, the concentration of streptavidin used was 0.35, 0.20, 0.15, 0.10, 0.05, and 0.025 mg/ml, respectively. All procedures

are the same in the concentration optimization except the concentration of streptavidin. Similarly, the concentration of aptamer was also optimized.

After SPR pretreatments and set up were performed, streptavidin as a constant was injected into the SPR flow cell to incubate for 10 min. After streptavidin immobilization, PBS was applied to wash away the excess streptavidin and then the test was paused. The response data were collected. Then, another new test for time optimization of streptavidin was started. In this research, the streptavidin incubation time periods tested involved in 10 min, 15 min, 20 min, 25 min, and 30 min, respectively. All procedures are the same in the time optimization except the streptavidin incubation time. Similarly, the incubation time of aptamer was also optimized.

2. To determine the specificity and sensitivity of the SPR biosensor:

In this research, both the targets against avian influenza H5N1 and not-targets were detected using SPR biosensor to obtain the response values. Through the comparison of the response values between targets and not-targets, the specificity of the aptamer could be determined.

During this study, the aptamer-based SPR biosensor for detection of the avian influenza virus H5N1 was performed as well as Dot-blot for virus detection. Comparable study showed that the developed SPR biosensor was comparable to Dot-blot and had better sensitivity than Dot-blot.

3. To evaluate the SPR biosensor for detection of avian influenza virus H5N1 in poultry swab samples.

Chapter 4 Materials and Methods

4.1 Materials

4.1.1 AI Virus

Killed AIV H5N1 was provided by USDA-APHIS National Veterinary Services Laboratories (NVSL) in Ames, Iowa. The virus was inactivated by the USDA lab using β-propiolactone. The H5N1 virus used in the tests was isolated from chickens in Scotland in 1959. The stock concentration of the virus was 107 EID50/ml or the equivalent 128 HA titer. All dilutions were done using PBS (pH7.4).

Killed non-target avian influenza viruses were obtained from Animal Diagnostic Laboratory (ADL) at Pennsylvaina State University, University Park, PA for use in specificity tests. Subtypes were picked for overlapping antigenic properties with AIV H5N1. Avian influenza virus subtypes H1N1, H2N2, H5N2, H7N2 and H9N2 were used.

4.1.2 Aptamers

The biotin-aptamers developed by Li et al. (2010) are specific against H5N1. They were synthesized by Integrated DNA Technologies (IDT), Coralville IA. Aptamers were dissolved to an appropriate dilution for use in the tests. In our study, we selected DNA aptamers rather than RNA aptamers due to several reasons. First, DNA aptamers are more stable in harsh conditions and can be used easily in the field. Second, they could function better without further modifications in detection and diagnostic assays where aptamers may have a contact with different biological samples for a short period of time.

4.1.3 Biological and Chemical Reagents

Streptavidin was bought from Rockland, Gilbertsville, PA. Biotin-aptamers were specific against H5N1 and synthesized by Integrated DNA Technologies (IDT), Coralville IA. Streptavidin-AP was supplied by Sigma-Aldrich (St. Louis, MI). It also needed to be diluted with 10 mM PBS to 1: 500. Bovine serum albumin was purchased from Sigma-Aldrich (St. Louis, MI), and it was dissolved in 10 mM PBS to a concentration of 0.5% and stored at 4 ℃ for use in the experiments. Tween^{20} was from Mallinckrodt (Hazelwood, MO) and was diluted with 10mM PBS to form a solution called PBST as washing solution, remaining a concentration of 0.25% and stored at 4 ℃ for use in the tests. BCIP/NBT was supplied by Kirkegaard and Perry Laboratories, Inc. (KPL, Gaithersburg, MD). as a substrate in Dot-ELISA tests and stored in a brown bottle. Phosphate buffered saline (PBS, 10X) was obtained from Sigma-Aldrich (St. Louis, MI) and diluted with Milli-Q (Milli-Q, Bedford, Massachusetts) pure water to 10 mM (pH 7.4) for the usage in the whole research. Sodium hydroxide (NaOH, solid) and hydrochloric acid (HCl, 36-38%) were all purchased from Sigma-Aldrich (St. Louis, MI) and then diluted with Milli-Q (Milli-Q, Bedford, Massachusetts) pure water to 1 M for the pretreatment of SPR electrode. Ethanol was supplied by Sigma-Aldrich (St. Louis, MI) for the pretreatment of SPR electrode. All the water used in the research was obtained from the Millipore water purification system (resistivity 18.2 MΩ cm; Milli-Q, Bedford, MA).

4.1.4 Poultry Swab Samples

Poultry swab samples were obtained from Poultry Health Lab, University of Arkansas, Fayetteville, AR. The swabs were obtained from the saliva in the chicken throat. Twelve tubes

with swabs were prepared in each of which two birds were used as well as 1 ml of PBS. First, each tube with swabs was mixed sufficiently using a votex mixer. After mixing, each swab was pressed against the tube wall several times and then discarded. All tubes of the solution, then, were combined into one tube. Finally, the solution was filtered using syringe filter (0.45 μm) and was spiked with avian influenza A virus H5N1 for further use. The original avian influenza H5N1 virus titers were diluted to different concentrations in the poultry swab solution. Pure swab solution without spiking with avian influenza A H5N1 virus was used as a control.

4.2 Apparatus

Nitrocellulose membrane was purchased from Schleicher & Schuell BioScience Inc. (Keene, NH) and then cut by clean scissors to prepare 60 mm X 12 mm test strips for five specimens. 1 ml-syringeS were purchased from Sigma-Aldrich (St. Louis, MI). Spreeta SPR detector was purchased from Texas Instruments, Dallas, TX, and the system includes SPR sensor, integrated multichannel flow cell, and 12-bit interface box; DELL laptop, Latitude D610 or 630. Several petri dishes were needed in the Dot block tests. The Vasi-Mix was used for laying the petri dishes and allowed nitrocellulose membrane strips to react sufficiently.

4.3 Experimental Methods

4.3.1 Surface Plasmon Resonance (SPR) Tests

4.3.1.1 Pretreatments and Set Up

The Au surface of SPR biosensor was pretreated with 300 μl of 1 M NaOH for 20 min and 300 μl of 1 M HCl for 5 min in order to get rid of any irregularities and obtain a clean Au surface (Su and Li, 2004; Autolab SPR). After pretreatment, the crystals were rinsed by spraying ethanol and water successively, and dried in a stream of nitrogen. The SPR sensor was then installed in the flow cell, initialized in the air and calibrated in deionized water. 1 ml of 10 mM Phosphate buffered saline (PBS, pH 7.4) was injected into the flow cell. After PBS injection, the change of refraction index can start to be monitored, and the first baseline in PBS can be obtained within 3 to 5 min.

4.3.1.2 Aptamer Immobilization

The streptavidin was used to immobilize biotin-aptamer onto the cleaned Au surface due to strong affinity of streptavidin to the cleaned Au surface through physical adsorption. An aliquot of 250 μl of 0.2 mg/ml streptavidin was applied to the flow cell and incubated for 25 min. After streptavidin immobilization, 1 ml of PBS was used to wash the excess streptavidin to get the second PBS baseline within 3 to 5 min. An aliquot of 300 μl of 2.04 μg/ml biotin-aptamer was injected into the flow cell to bind to the binding sites on the streptavidin and incubated for 25 min. The excess aptamers were rinsed off using PBS, resulting in the third PBS baseline within 3 to 5 min.

4.3.1.3 Virus Detection

After rinsing with PBS to remove the excess aptamers, an aliquot of 300 µl of avian influenza A virus H5N1 diluted with PBS was then spread over the channel of Au surface and incubated for 25 min. Every time the materials were immobilized, the excess was removed by rinsing with PBS, and so does the virus. Therefore, the forth PBS baseline can be received within 3 to 5 min.

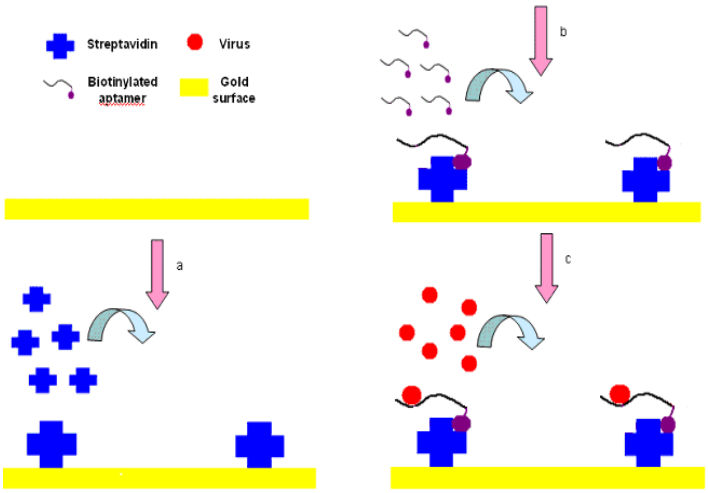


Figure 4. 1 Principle of SPR biosensor for detection of AIV H5N1.

4.3.2 Dot-blot Test

First of all, the nitrocellulose membrane was cut by clean scissors into 60 mm X 12 mm test strips for use in the tests. Vinyl gloves must be worn at all times when handling the nitrocellulose (NC) membrane. An aliquot of 5 µl of test viruses (H5N1, H9N2, H5N2, H2N2, H5N3, H5N9, H7N2, and H1N1) was added on the NC strips and allowed to air dry, using pencil to mark the upper side of the membrane. The strips were then transferred to a clean Petri dish and layed out one by one. Strips with different test specimens can be put together in the Petri dish but should not overlap. An aliquot of 0.5% BSA as a blocking solution was poured into the petri dish and cover every strips. The strips were allowed to react for 15 – 30 min on the shaker. After the

reaction, the strips were removed and lay out on chromatography paper to air dry, and then transferred into a new petri dish. An aliquot of 1: 1000 aptamers were applied to the strips and cover the entire strips surface (do not need to pour, only add 150 µl on every strip) and incubated for 45 min. The strips were then washed with a PBST solution three times and each wash requires a 5 min soak period on the shaker. After wash, strips were taken out from the dish and allowed to air dry.

The dried strips were consequently transferred into another new Petri dish. An aliquot of 200 µl of 1: 500 streptavidin-AP was applied to conjugate aptamers, allowing the reaction to go for 25 min. The strips were then washed with a PBST solution three times and each wash requires a 5 min soak period on the shaker. After wash, strips were taken out from the dish and allowed to air dry.

Finally, the strips were transferred into another new Petri dish. The BCIP/NBT substrate was added to the strips, remaining the substrate and the strips in the dark to allow for color development. A purple color was evident within 5 min. In order to avoid the strong background, the color developing time shouldn’t last too long. The color development can be stopped by immersing the strips in water for 10 – 20 s. The strips were kept in the dark to air dry, and then stored in sealed plastic and kept in the dark to prevent the color from fading.

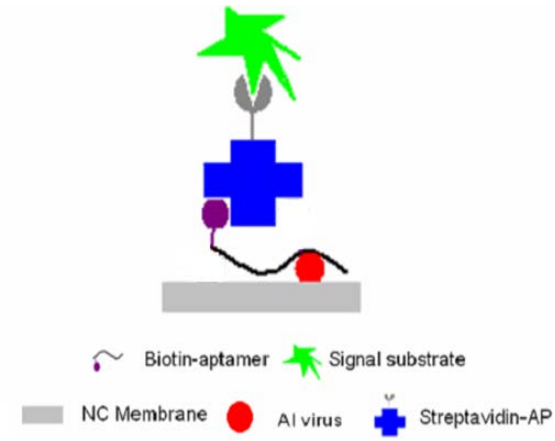


Figure 4. 2 Principle of Dot-blot analysis for detection of AI virus.

4.4 Data Collection and Statistical Analysis

All the SPR data were recorded using the software of Spreeta 5. Microsoft Excel was used for statistical analysis of all data, including determination of means and standard deviation. The data were used to plot in a figure with figure title, x and y axis titles and units. Arrows and words were used to indicate where the streptavidin, aptamers and H5N1 as well as washing were started. The response values caused by immobilized streptavidin, aptamers and captured H5N1 were calculated and listed with a table title. The factors that can affect the performance of the SPR immunosensor in virus detection were discussed based on the data collected. Some measures to improve the lower detection limit of this SPR biosensor in general applications were suggested.

Chapter 5 Results and Discussion

5.1 Optimization of Parameters

5.1.1 Streptavidin Optimization

5.1.1.1 Streptavidin Concentration

An aliquot of 5 mg of streptavidin powder was dissolved with 1ml of 10 mM PBS. Then the 5 mg/ml of streptavidin solution was packed into 1.5-ml tube with 200 μl /tube and stored in the -20℃ for the future tests. As discribed in 4.3.1, after SPR device was pretreated and set up, an aliquot of 250 μl of 0.025 mg/ml streptavidin was applied into the SPR flow cell and incubated for 25 min. After streptavidin immobilization, 1 ml of 10 mM PBS was applied to wash away the excess streptavidin and then the test was paused. The response data was collected through the software of Spreeta 5. Then, another new test for concentration optimization of streptavidin was started. In this research, the concentration of streptavidin used was 0.35, 0.20, 0.15, 0.10, 0.05, and 0.025 mg/ml, respectively. All procedures are the same in the concentration optimization except the concentration of streptavidin. The sorted data was listed in the table below. The histogram for concentration optimization of streptavidin was also shown. Based on data shown in that table and the histogram, the response values from 0.025 to 0.2 mg/ml were increased gradually, but after 0.2 mg/ml, the values tend to become more stable. Therefore, a conclusion can be made that the optimal concentration for streptavidin is 0.2 mg/ml in SPR tests.

Table 5. 1 Optimization of streptavidin concentration.

Con. of Streptavidin (mg/ml)	Refractive Index (RI) * 10^6			Mean ± Std (RI*10^6)
0.25	414	529	582	508 ± 86
0.05	743	969	693	802 ± 147
0.1	978	879	1174	1010 ± 150
0.15	1319	1428	1291	1346 ± 72
0.20	2047	2022	1911	1993 ± 72
0.35	1924	2212	1907	2014 ± 171

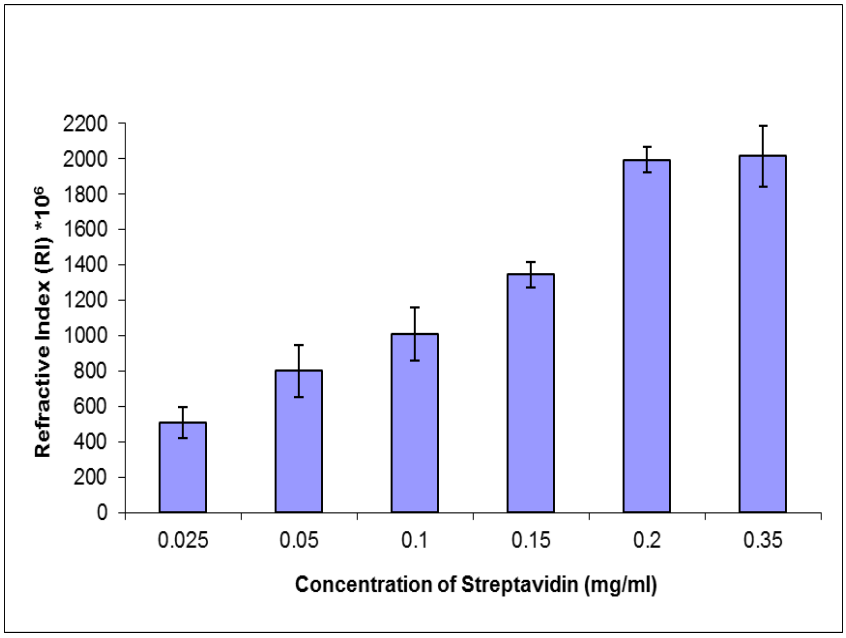


Figure 5. 1 Optimization of streptavidin concentration in SPR biosensor. Six different concentrations of streptavidin (0.025, 0.05, 0.1, 0.15, 0.2, and 0.35 mg/ml) was used to the SPR biosensor. The streptavidin was incubated for 25 min. Three repeated tests were performed. Means and standard deviations were calculated.

5.1.1.2 Streptavidin Incubation Time

As described in 4.3.1, after SPR pretreatments and set up were performed, an aliquot of 250 µl of 0.2 mg/ml streptavidin as a constant was injected into the SPR flow cell to incubate for 10 min. After streptavidin immobilization, 1 ml of 10 mM PBS was applied to wash away the excess streptavidin and then the test was paused. The response data were collected by the software of

Spreeta 5. Then, another new test for time optimization of streptavidin was started. In this research, the streptavidin incubation time periods tested involved in 10 min, 15 min, 20 min, 25 min, and 30 min, respectively. All procedures are the same in the time optimization except the streptavidin incubation time. The sorted data were listed in the table below. The histogram for time optimization of streptavidin was also shown. Based on that table and the histogram, the response values from 10-min to 25-min periods were increased gradually, but after 25-min period, the values tend to become more stable. Therefore, a conclusion can be made that the optimal incubation time for streptavidin is 25 min in SPR tests.

Table 5. 2 Optimization of streptavidin incubation time.

Time (min)	Refractive Index (RI) * 10^6			Mean ± Std (RI*10^6)
10	546	746	853	715 ± 156
15	1142	989	1072	1068 ± 77
20	1700	1273	1326	1433 ± 233
25	2126	1956	2267	2128 ± 158
30	1552	2526	2219	2099 ± 498

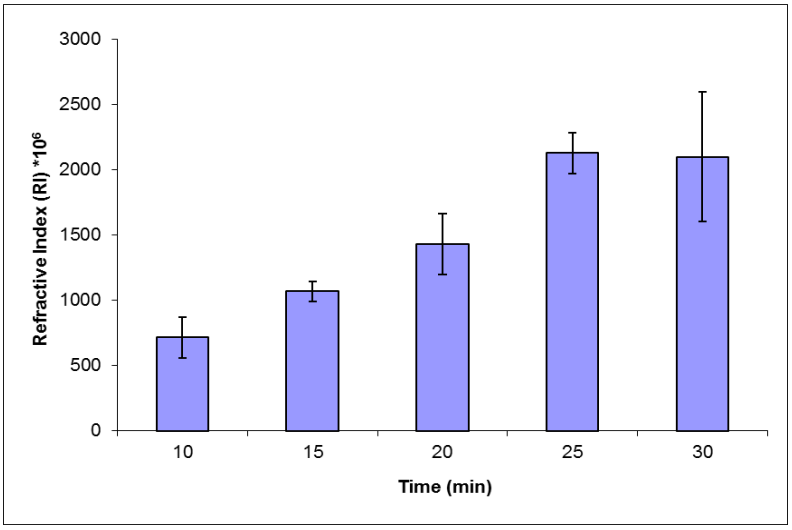


Figure 5. 2 Optimization for streptavidin incubation time in SPR biosensor. Five different time periods (10, 15, 20, 25, and 30 min) were used for streptavidin incubation. The concentration of streptavidin was 0.2 mg/ml. Three repeated tests were performed. Means and standard deviations were calculated.

5.1.2 Aptamer Optimization

5.1.2.1 Aptamer Thermal Treatments

As mentioned in 4.3.1.2, biotinylated aptamer immobilization is followed just after streptavidin immobilization. Before aptamers were injected into the flow cell, they were thermally treated in order to unfold the aptamer DNA strand making the biotin label at the 5' end available for the interaction with streptavidin on the electrode surface (Tombelli et al., 2005). In this research, the biotinylated aptamers were thermally treated by three methods: **a**. the original aptamer solution without any thermal treatments, **b**. the aptamers were boiled for 5 min, and then cooled down for 30 min in room temperature, **c**. the aptamers were boiled for 5 min, and then cooled down in ice for 1 min in order to stabilize the DNA in its unfolded structure. Aptamer **a** were injected into the SPR flow cell after streptavidin immobilization. Then aptamer **b** and **c** were also applied in another two new tests. Based on the data collected, aptamer **a** and **b** didn't show any readable signal, but aptamer **c** gave identifiable signal change. Thus, method **c** was used for aptamer thermal treatment in the whole research.

5.1.2.2 Aptamer Concentration

As shown in 4.3.1.2, aptamer was injected following streptavidin immobilization. After that, 1 ml of 10mM PBS was applied to wash away the excess aptamer and then the test was paused. Then, another new test for concentration optimization of aptamer was started. In this research, the concentration of aptamer used was 1.2, 1.6, 1.8, 2.0, and 2.5 μg/ml, respectively. All procedures are the same in the concentration optimzation except the concentration of aptamers.

The sorted data was listed in the table below. The histogram for optimization of aptamer concentration was also shown. Based on the data shown in the table and the histogram, the response values from 1.2 to 2.0 μg/ml were increased gradually, but after 2.0 μg/ml, the values tend to become more stable. Therefore, a conclusion can be made that the optimal concentration for aptamers is 2.0 μg/ml SPR tests.

Table 5. 3 Optimization of aptamer concentration.

Con. of aptamer (μg/ml)	Refractive Index (RI) * 10^6			Mean ± Std (RI*10^6)
1.2	62	75	96	78 ± 17
1.6	158	149	241	183 ± 51
1.8	294	247	352	298 ± 53
2.0	444	569	481	498 ± 64
2.5	382	509	499	463 ± 71

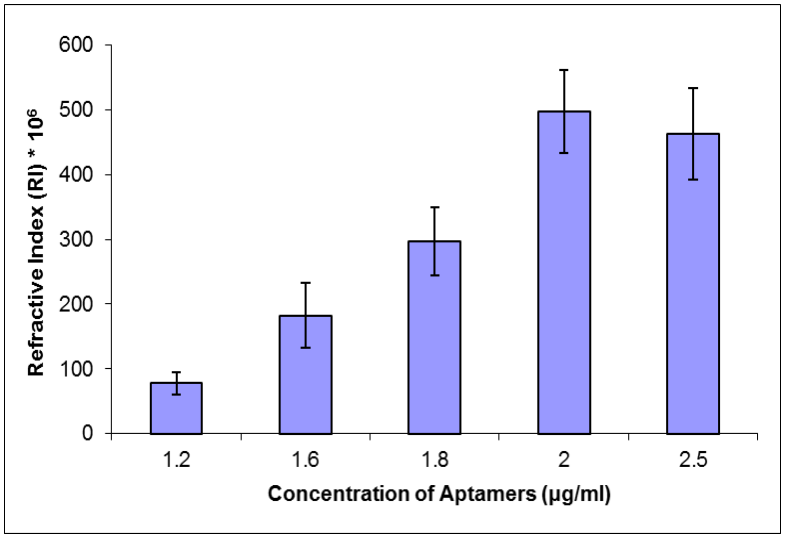


Figure 5. 3 Optimization of aptamer concentration in SPR biosensor. Five different concentrations of aptamers (1.2, 1.6, 1.8, 2.0, and 2.5 μg/ml) was used to the SPR sensor. The aptamers were incubated for 25 min. Three repeated tests were performed. Means and standard deviations were calculated.

5.1.2.3 Aptamer Incubation Time

Based on the optimization of streptavidin, 0.2 mg/ml of streptavidin to be incubated for 25 min was used for streptavidn immobilization in the future tests. For the concentration of biotinylated aptamer, 2.0 μg/ml of it was applied in the future tests as mentioned above. As described in 4.3.1.2, after streptavidin was immobilized and biotinylated aptamers were thermally treated, an aliquot of 300 μl of 2.0 μg/ml of aptamer thermally treated as a constant was injected into the SPR flow cell to incubate for 10 min. After aptamer incubation, 1 ml of 10 mM PBS was applied to wash away the excess streptavidin and then the test was paused. The response data were collected by the software of Spreeta 5. Then, another new test for time optimization of aptamer was started. In this research, the aptamer incubation time periods tested included 10 min, 15 min, 20 min, 25 min, and 30 min, respectively. All procedures are the same in the time optimzation except the aptamer incubation time. The sorted data was listed in the table below. The histogram for time optimization of aptamer was also shown. Based on the data in that table and the histogram, the response values from 10-min to 25-min periods were increased gradually, but after 25-min period, the values tend to become more stable. Therefore, a conclusion can be made that the optimal incubation time for aptamer is 25 min in SPR tests.

Table 5. 4 Optimization of aptamer incubation time.

Time (min)	**Refractive Index (RI) * 10^6**			**Mean ± Std (RI*10^6)**
10	263	207	217	229 ± 30
15	207	412	367	329 ± 108
20	405	231	295	310 ± 88
25	481	509	541	510 ± 30
30	503	439	587	510 ± 74

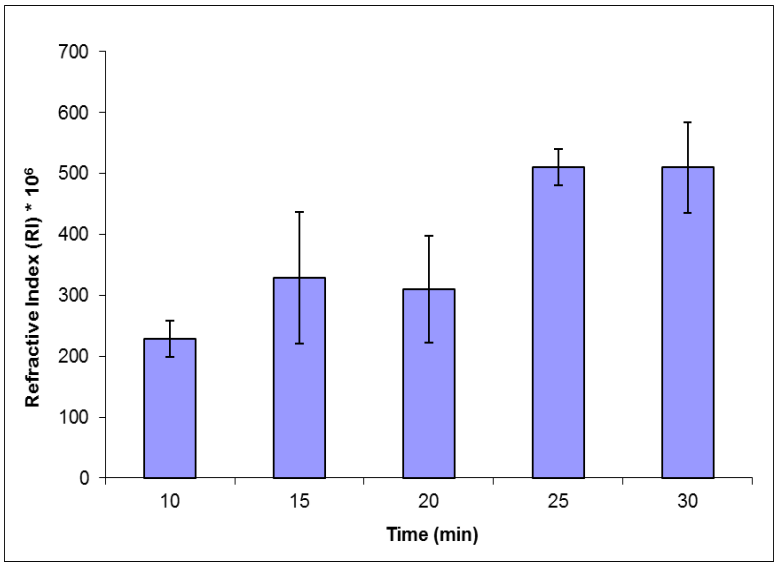


Figure 5. 4 Optimization of aptamer incubation time in SPR biosensor. Five different time periods (10, 15, 20, 25, and 30 min) were used for aptamer incubation. The concentration of aptamer was 2.0μg/ml. Three repeated tests were performed. Means and standard deviations were calculated.

5.2 Detection of Avian Influenza Virus

5.2.1 AI Virus in Pure Culture

After parameters optimizations, avian influenza virus H5N1 was detected as described in 4.3.1. A typical curve of AIV H5N1 detection using SPR biosensor is shown in Figure 5.6. The first blue line is PBS baseline. Then, streptavidin was immobilized on the gold surface of SPR chip, causing the refractive index changes and forming the second blue line. In order to wash away the excess streptavidin, PBS was applied to the chip surface, resulting in the second PBS baseline. The difference between the first PBS baseline and the second PBS baseline was the signal change of the streptavidin in terms of refractive index (0.001816585 RI shown in Figure 5.6. Similarly, the signal change of aptamers can be obtained by subtracting the value of the second

PBS baseline from the value of the third PBS baseline. The refractive index change of AIV H5N1 is equal to the difference between the values of the fourth and the third PBS baseline.

Based on the collected data (the data weren't shown on this paper), the developed SPR biosensor cannot read the refractive index of AIV H5N1 with the titer of 0.0128 HAU. From 0.128 HAU to 1.28 HAU, the refractive index showed a great linear correlation with the R^2 of 0.99 (Figure 5.5). After 1.28 HAU, the signal tended to become stable (Figure 5.5). Therefore, the detection limit was 0.128 HAU (minimum) and 1.28 HAU (maximum). The calibration curve and regressive curve of H5N1 detection was shown in Figure 5.5.

Table 5. 5 Result of the tests for detection of AIV H5N1 in pure culture.

Con. of H5N1 (HAU)	Refractive Index (RI) * 10^6			Mean ± Std (RI*10^6)
0.128	91	28	147	89 ± 60
0.32	276	315	196	262 ± 61
0.64	823	518	577	639 ± 161
1.28	1156	1090	1165	1137 ± 41
3.2	1012	910	1446	1127 ± 285

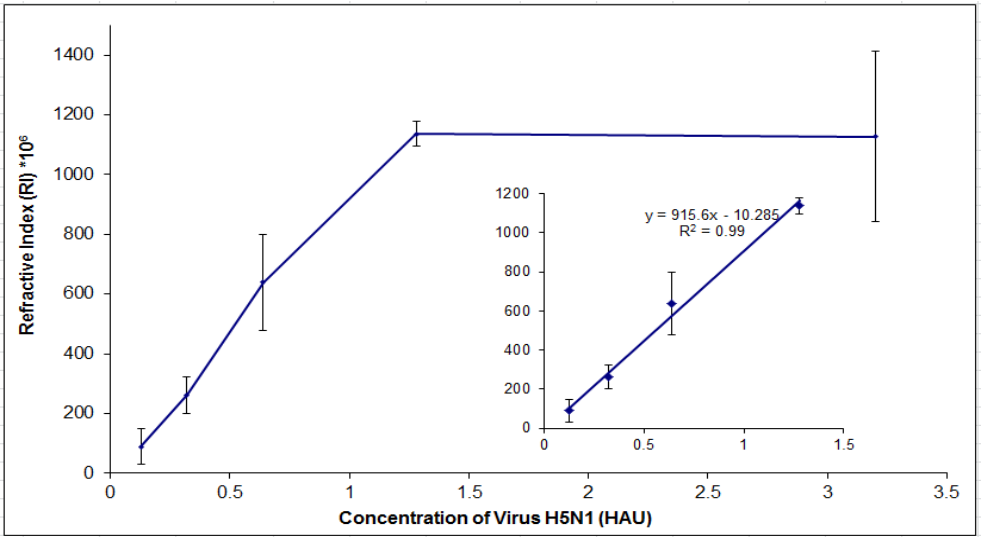


Figure 5. 5 Calibration curve and regression curve for detection of AIV H5N1.

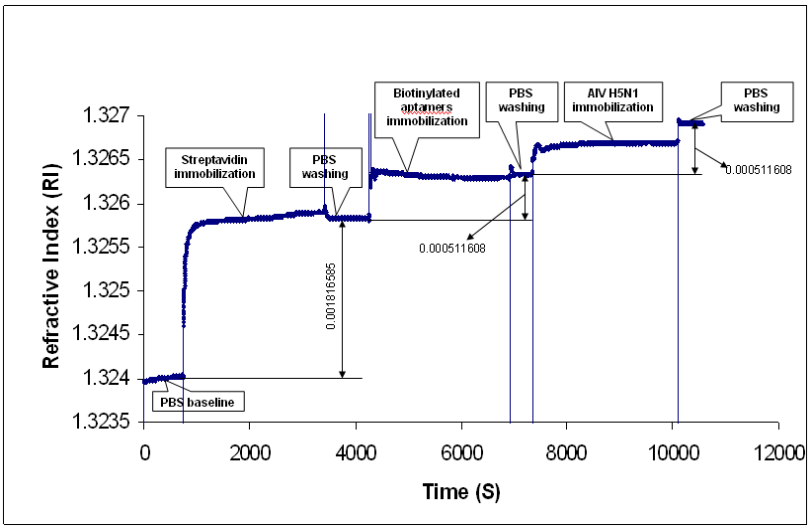


Figure 5. 6 A typical response curve of aptamer-based SPR biosensor to the surface modification and AIV H5N1 detection.

5.2.2 AI Virus in Poultry Swab Samples

The procedure for detection of poultry swab sample was the same as that for detection of AIV H5N1 except virus dilution. The AIV H5N1 was dissolved with poultry swab sample instead of PBS which was used in the previous detection of pure virus. With the increase of the virus titer, the value of refractive index gradually ascended. Due to the impurities and/or other materials like other proteins in the swab sample which might have a negative impact on the binding of virus to the aptamer, the number of virus particle that were responsible for the detection signal may be less than that of virus diluted with PBS. Therefore, no platform of signal can be found in the detection of poultry swab sample.

Table 5. 6 Result of the tests for detection of AIV H5N1 in poultry swab samples.

Con. of H5N1 (HAU)	Refractive Index (RI) * 10^6			Mean ± Std (RI*10^6)
Control	8	-7	1	1 ± 8
0.0128	145	96	109	117 ± 25
0.32	257	198	175	210 ± 42
0.64	511	259	535	435 ± 153
0.96	710	514	599	608 ± 98
1.28	875	707	1028	870 ± 161
12.8	812	1067	899	926 ± 130

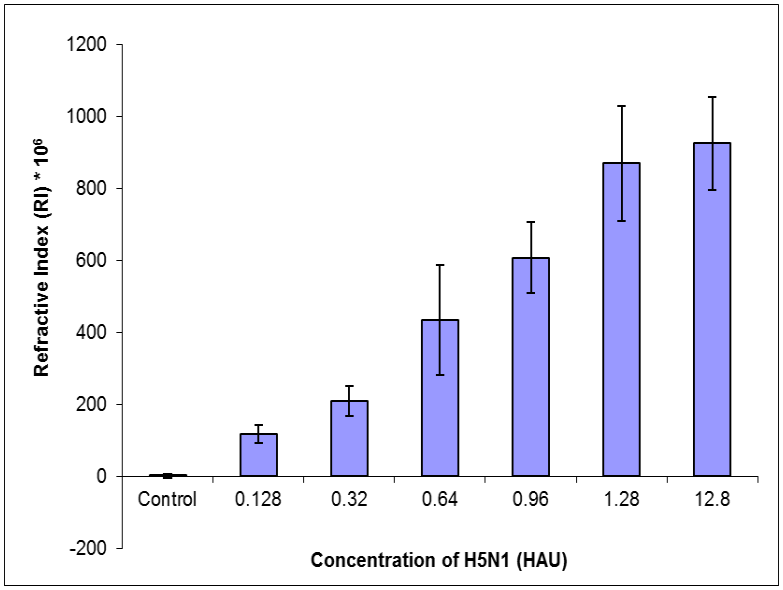


Figure 5. 7 Aptamer-based SPR biosensor for detection of AIV H5N1 in poultry swab samples. Six different concentrations of avian influenza virus H5N1in poultry swab samples (0.128, 0.32, 0.64, 0.96, 1.28 and 12.8 HAU) were applied to the flow cell of SPR biosensor. PBS was used for the control instead of H5N1. Three repeated tests were performed. Means and standard deviations were calculated.

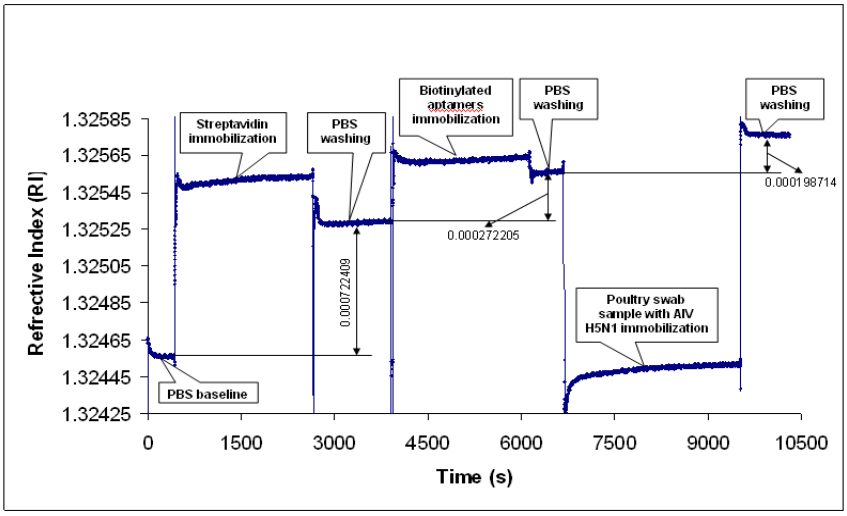
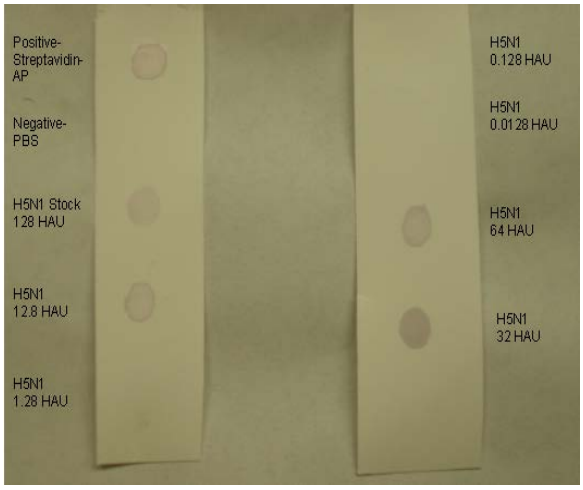


Figure 5. 8 A typical response curve of aptamer-based SPR biosensor to the surface modification and detection of AIV in poultry swab samples.

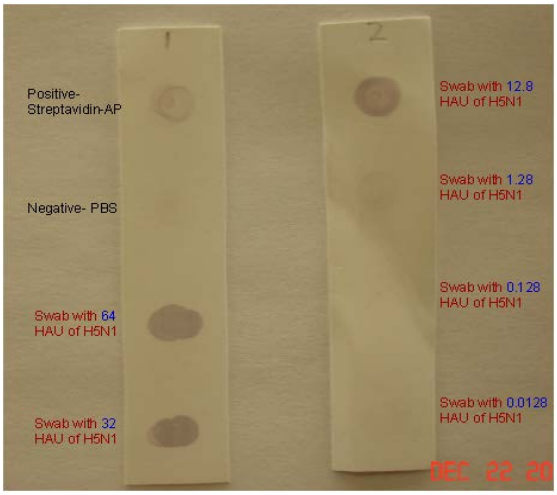
5.2.3 Comparison between Biosensor and Dot-blot Tests

As described in 4.3.2, Dot-blot experiments were performed. The experimental data are shown in Figure 5.9. Based on the data in Figure 5.9 a which was Dot-blot for detection of AIV H5N1 with different dilutions**,** the minimum detection limit that was able to read in Dot-blot experiments was 12.8 HAU of AIV H5N1 while the detection limit tested using developed SPR biosensor was 0.128 HAU (Shown in Figure 5.5). Therefore, the developed SPR biosensor has better sensitivity than Dot-blot.

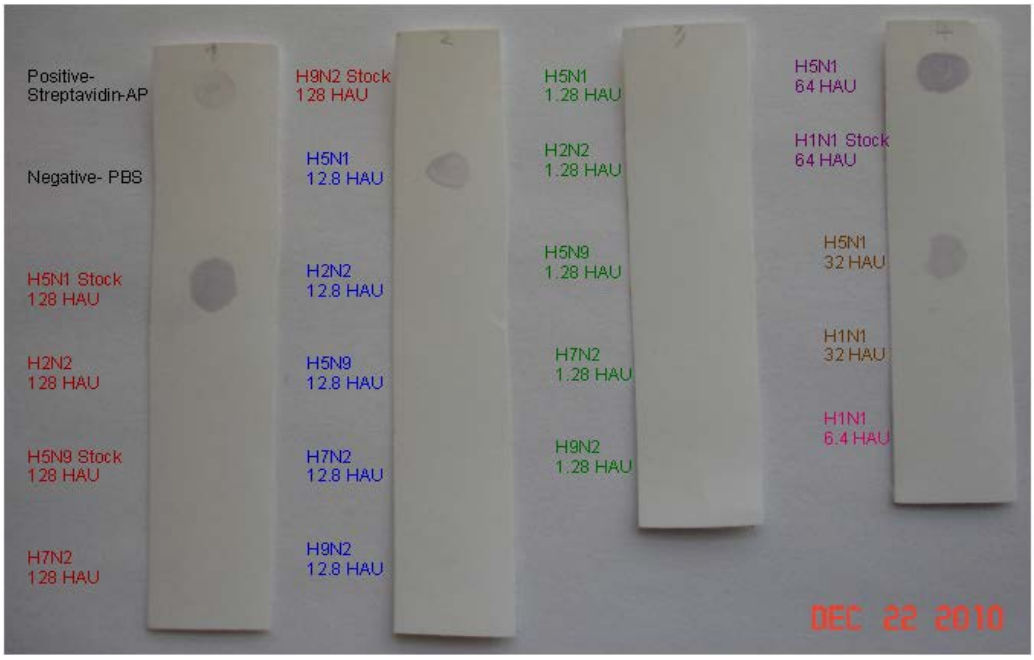
As shown in Figure 5.9 c that was the comparison experiment between H5N1 and not-targets, only AIV H5N1 were responsible for the purple dots on the stripes, while non-target subtypes didn't show any symbol. Consequently, the comparison study between AIV H5N1 and non-targets using Dot-blot illustrated that the biotinylated aptamer had higher specificity for H5N1 than the non-targets.



(a)



(b)



(c)

Figure 5. 9 Results of Dot-blot tests: (a) Detection of pure AIV H5N1; (b) Detection of AI H5N1 in Poultry swab samples; (c) comparison between AIV H5N1 and non-targets of other AIV subtypes.

Based on the Figure 5.9 b, Dot-blot was also able to detect the poultry swab samples, and the minimum detection limit was 1.28 HAU, while the detection limit of SPR biosensor was 0.128

HAU (Shown in Figure 5.7). The comparison study also showed that the developed SPR biosensor has better sensitivity than Dot-blot when detecting AIV in the poultry swab samples.

Chapter 6 Conclusions

An aptamer-based SPR biosensor was designed, fabricated, and tested for the detection of AIV H5N1 in poultry swab samples. The optimum parameters were obtained for the concentration and incubation time of streptavidin (0.2 mg/ml, 25 min) and aptamers (2.0 µg/ml, 25 min). A good correlation (R^2=0.99) was found between AIV concentration in the range of 0.128 to 1.28 HAU and the refractive index. The specificity of aptamer-based SPR biosensor was confirmed by comparison of AIV H5N1 with other non-target AIV subtypes such as AIV H1N1, H2N2, H5N2, H7N2, and H9N2, which showed that no interference was observed from these non-target subtypes. The developed SPR biosensor was able to detect AIV H5N1 in the poultry swab samples with a lower detection limit of 0.128 HAU in 1.5 h. The aptamer-based SPR biosensor has potential to provide the poultry industry a new method for in-field detection of avian influenza in poultry.

Chapter 7 Recommendations for Future Research

For future improvement of this SPR biosensor, several issues can be addressed:

1) In this research , the streptavidin was immobilized through physical adsorption which is relatively weak. In order to firmly immobilize the material on the sensor surface, self-assembled monolayer is suggested to replace the physical adsorption. A self assembled monolayer (SAM) is an organized layer of amphiphilic molecules in which one end of the molecule, the "head group" shows a specific, reversible affinity for a substrate. SAMs also consist of a tail with a functional group at the terminal end. Adsorbate molecules adsorb readily because they lower the surface free-energy of the substrate (Love et al., 2005) and are stable due to the strong chemisorption of the "head groups." These bonds create monolayers that are more stable than the physisorbed bonds of Langmuir–Blodgett films (Madou and Marc, 2002; Kaifer and Angel, 2001).

2) The poultry swab samples used in this study were pretreated using 0.45 μm syringe filter. There might still be some impurities or other proteins that may affect the detection of virus. To prepare better testing sample from the poultry swab samples, magnetic separation method may be considered for use in the future research. Magnetic separation is a process in which magnetically susceptible material is extracted from a mixture using a magnetic force. Small super-magnetic particles or beads coated with ligands against specific targets will be used and efficient for the isolation of targets from non-targets.

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