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Biosensors for Rapid Detection of Avian Influenza

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Abstract

The scope of this chapter was to review the advancements made in the area of biosensors for rapid detection of avian influenza viruses (AIVs). It is intended to provide general background about biosensor technology and to discuss important aspects for developing biosensors, such as selection of the suitable biological recognition elements (anti-AIV bioreceptors) as well as their immobilization strategies. A major concern of this chapter is also to critically review the biosensors' working principles and their applications in AIV detection. A table containing the types of biosensor, bioreceptors, target AIVs, methods, etc. is given in this chapter. A number of papers for the different types of biosensors give hints on the current trends in the field of biosensor research for its application on AIV detection. By discussing recent research and future trends based on many excellent publications and reviews, it is hoped to give the readers a comprehensive view on this fast-growing field.

Keywords: biosensor, avian influenza virus, rapid detection, bioreceptor, nanobiosensor

1. Introduction

Influenza viruses, which belong to the *Orthomyxoviridae* family, are classified as A, B, and C based on antigenic differences in their nucleoprotein (NP) and matrix (M_1) protein [1]. All avian influenza viruses (AIV) are classified as type A. Type A viruses are further subtyped on the basis of antigenic differences of the surface glycoproteins, the hemagglutinin (HA), and the neuraminidase (NA) proteins [2]. So far, seventeen HA (H1 through H17) and ten NA (N1 through N10) subtypes have been identified [3]. **Figure 1** presents a schematic diagram of influenza A virus, and **Figure 2** shows a transmission electron microscopy photograph. Influenza A virus is an enveloped RNA virus approximately 80–120 nm in diameter [1, 2]. There are two major

surface glycoproteins (HA and NA) and a small number of M2 protein, which has ion channel activity. The ratio of HA and NA is approximately 4:1. The genome of type A virus consists of eight segments of negative-sense single-stranded RNA, which are associated with many NP and the transcriptase complex (RNA polymerase components PB1, PB2 and PA) to form ribonucleoproteins (RNPs). The matrix protein M1 has an interaction with the RNPs and is under the lipid bilayer. NS₁ is found only in infected cells and is not thought to be a structural component of the virus, but small amount of NS₂ are present in purified viruses.

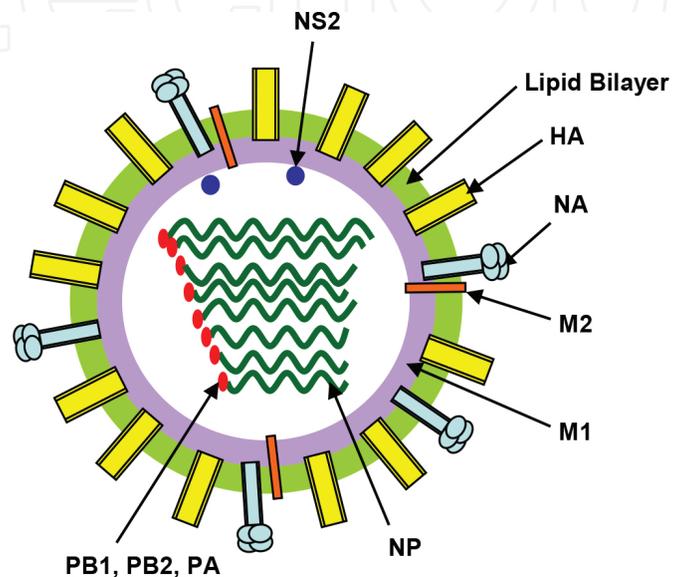


Figure 1. A schematic diagram of the structure of the influenza A virus particle.

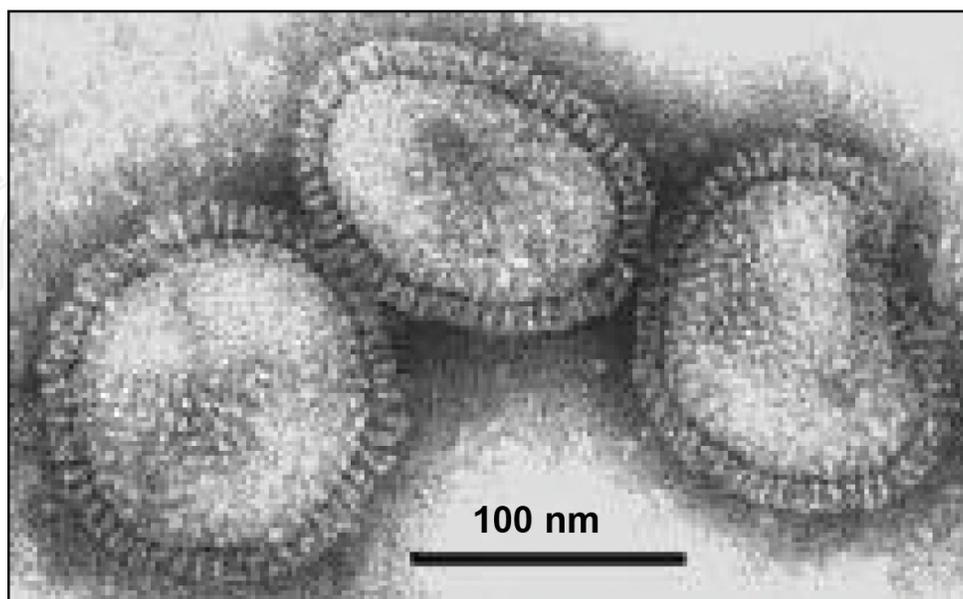


Figure 2. A transmission electron micrograph of influenza A virus particles.

AIVs have a large impact on the poultry each year and also represent a threat to human health. The highly pathogenic H5N1 avian influenza (HPAI H5N1), which originally emerged in Southeast Asia in late 1990s, cost the poultry industry an estimated \$10 billion between 1997 and 2008 [4]. HPAI H5N1 has also caused global concerns for public health and continues to spread throughout the world. Since 2003, a total of 62 countries (regions) have been affected by HPAI H5N1 and new human and animal cases are continuously being confirmed and reported [5]. The link between human and AI has raised concern among public health authorities and the scientific community about the prevalence and pandemic potential of AI virus. The H5N1 virus has caused 449 deaths of 846 people infected since 2003, according to the World Health Organization [5]. Therefore, advanced technologies for more rapid and sensitive AIV detection are needed for better surveillance and control of the outbreaks.

Current virus detection techniques are available, such as virus isolation, immunofluorescence antibody (FA) test, immunohistochemistry (IHC), and ELISA as well as rapid procedures based on molecular tools, such as polymerase chain reaction (PCR)-based assays, gene sequencing, and microarrays. Among these test methods, virus isolation has been the “gold standard” as its highest sensitivity and reliability, but it is a very time-consuming procedure (4–7 days). ELISA is a simple and rapid test in testing mass serum samples; thus, it is commonly used for serum antibody detection, but not used for virus detection in practice or diagnostics. Other immunoassays of FA or IHC can be used as a direct virus detection test from tissue specimens, but they either require labels or have limitations of low sensitivity and cross-reactions in certain circumstances. The molecular assays of RT-PCR and real-time RT-PCR methods have the advantages of high sensitivity and good specificity, but possess disadvantages of requiring expensive PCR equipment and reagents, and well-trained skillful technical personnel. Therefore, in order to minimize the social and economic costs, the development of rapid detection methods is essential. These rapid detection methods should meet the following requirements: (1) high throughput, (2) possibility of multiple target detection, (3) high sensitivity, (4) good specificity, (5) high speed, (6) simple operation, (7) suitability for on-site and/or in-field use, and (8) less cost. Biosensors have shown a great potential to meet such criteria.

The biosensor research began in the early 60 s of the twentieth century, when a glucose biosensor was proposed by Clark and Lyons [6] at Children’s Hospital in Cincinnati. From then on, biosensor applications have expanded throughout the medical diagnosis field and also into the fields of environmental monitoring, agricultural production, food safety, pharmaceutical screening, and biodefense. A biosensor can be defined as a “compact analytical device or unit incorporating a biological or biologically derived sensitive recognition element integrated or associated with a physiochemical transducer” [7]. This device is composed of a biological recognition element (often called bioreceptor) and directly interfaced signal transducer (**Figure 3**). The selective and reversible process of interaction between the analyte and bioreceptor is transduced into a measurable signal, for example, electrical signal, which is proportional to the concentration or activity of an analyte in any type of samples. Biosensors can be classified based on the transduction element or the biological element. According to the basic principles of signal transduction, there are three main biosensor types, which have been

studied for use in AIVs detection: piezoelectric, optical, and electrochemical biosensors. If we follow the types of biological element or bioreceptors, AIV biosensors can be classified into immunosensors, aptasensors, DNA or RNA-probe sensors, and more. In this chapter, we focus on these types of biosensors studied and developed recently for rapid detection of AIVs. It offers a survey of the principles, fabrication, operation, and the most popular types of biosensing devices in research or application today for AIVs detection. By discussing recent research and future trends based on many excellent publications and reviews, it is hoped to give the readers a comprehensive view on this fast-growing field.

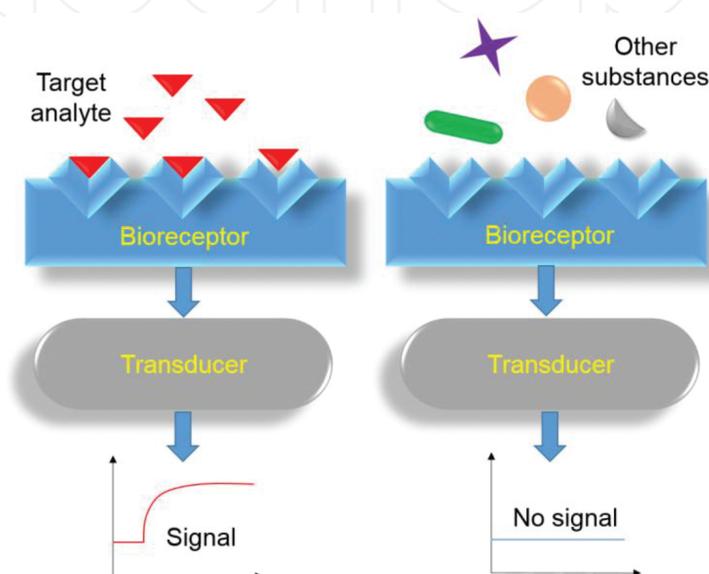


Figure 3. A schematic diagram of biorecognition and signal transduction in a typical biosensor.

2. Biosensors for detection of AIVs

As shown in **Figure 4**, the number of papers published in the field of biosensor research for detection of AI has kept increasing from year 2001 to 2015 based on PubMed. Biosensors offer the advantages of simple operation, rapid response, low cost, portability, automation, and easy to integrate with nanomaterials/nanostructure, Micro-Electro-Mechanical System(MEMS)/ Nano-Electro-Mechanical System(NEMS), biotechnology, Global Position System(GPS)/ wireless, and image technologies. One of the major requirements in developing a biosensor for AIVs is the need for a sensitive analytical device that can easily go down to very low detection levels without significant changes in selectivity. AIVs will spread rapidly through a community before any symptoms appear for the identification. A biosensor that can rapidly, sensitively and selectively detect target virus will be invaluable. In addition, a simple, robust, rapid, cost-effective, and potable biosensor, suitable for use in the field, is urgently needed. While a variety of biosensors have been developed for use in different applications, three types of biosensors have been mainly studied for AIV detection: piezoelectric, optical, and electrochemical biosensors.

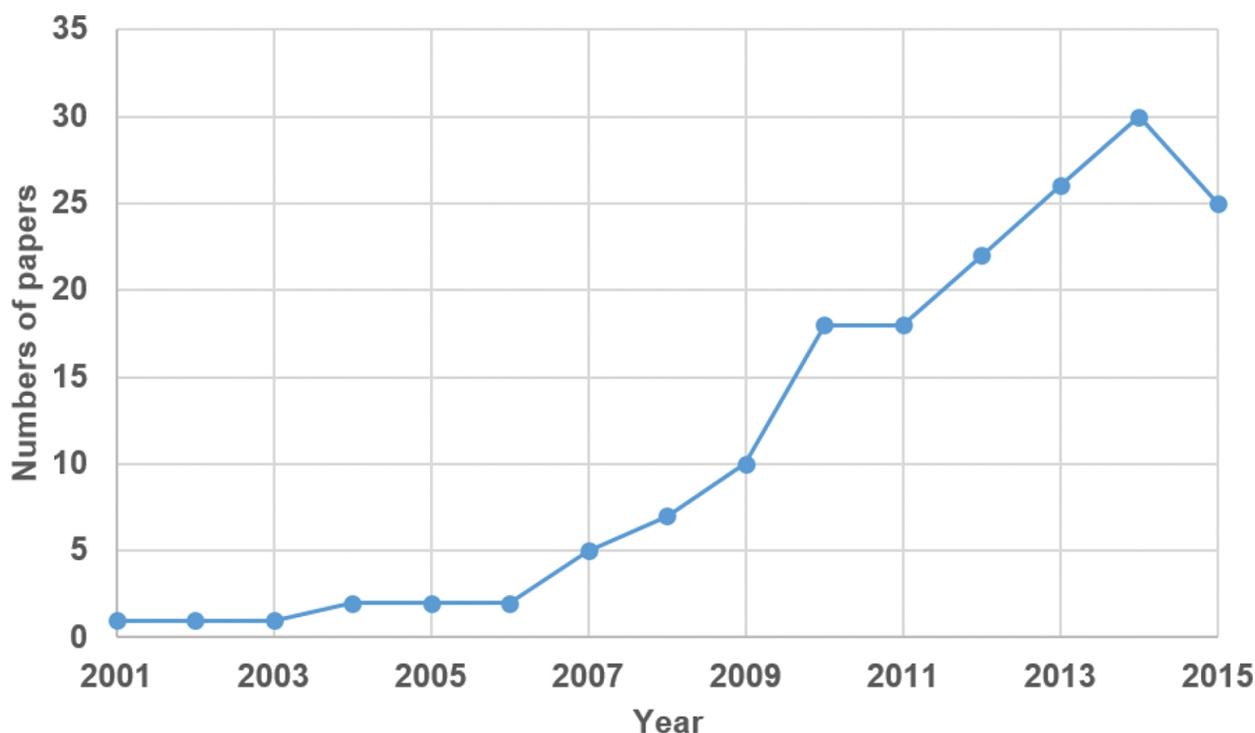


Figure 4. Numbers of papers published in the field of biosensor research for detection of AIVs between year 2001 and 2015.

2.1. Piezoelectric biosensors

Piezoelectric biosensors utilize crystals capable of generating a piezoelectric field to detect mass changes in the sensing environment [8]. The crystal is sandwiched between two excitation electrodes, which apply an electrical field that causes the crystal to undergo dimensional changes, or oscillations, at the crystal's natural resonant frequency. An increase in the mass on the surface of the crystal, such as antibody immobilization or capture of antigen, decreases the resonant frequency. Piezoelectric biosensors are useful because they are low cost, label free, sensitive, and have extremely low detection levels [8]. The most intensively studied piezoelectric biosensor is the quartz crystal microbalance (QCM), which uses a thin wafer of quartz as the transducing crystal. Quartz crystals have the advantages of being widely available, relatively inexpensive, durable, direct detection, and real-time output. Other techniques are often coupled with QCM to increase the performance and capability of the biosensor. QCM biosensors have attracted interest in applications for AIVs detection. **Table 1** summarizes the main analytical features of QCM biosensors for detection of AIVs. Different studies used different concentration units for AIV. It would be helpful to know the comparable relationship between them. The common concentration units for AIV contain EID_{50} (50% egg infectious does), ELD_{50} (50% egg lethal does), PFU (plaque-forming units), and HAU (hemagglutination unit). $1 \times 10^3 EID_{50} ml^{-1}$ equals to $\sim 0.0128 HAU/50 \mu l$ [9], $1 \times 10^{6.2} ELD_{50} ml^{-1}$ equals to $128 HAU/50 \mu l$ [10], and $1 \times 10^5 PFU$ equals to 1 HAU [11].

Influenza subtype	Target	Bioreceptor	Sensitivity	Detection range	Detection time	Labelling method	Reference
H3N2	Virus	Antibody	4 virus particles/ml	0.02–3 HAU	–	Label-free	[12]
H1N1	Virus	Antibody	10 ⁴ pfu/ml 10 ³ pfu/ml	10 ³ –10 ⁷ pfu/ml	45 min 105 min	Label-free Label with 13 nm gold nanoparticles	[13]
H5N1	Virus	Antibody	0.0128 HAU	0.128–12.8 HAU	120 min	Label with 30 nm magnetic nanobeads	[14]
H5N1	Virus	Aptamer	1 HAU	1–4 HAU	60 min	Label with 30 nm magnetic nanobaeds	[22]
H5N1	Virus	Aptamer hydrogel	0.0128 HAU	0.0128–0.64 HAU	30 min	Label-free	[9]
H5N3	HA	Glycan	K_d 1.44 × 10 ⁻⁸ M	–	–	Label-free	[26]
H5N3	Virus	N-Acetylglucosamine (GlcNAc)	K_d 2.03 × 10 ¹⁰ M	–	–	Label-free	[27]
H5N1			K_d 4.35 × 10 ¹⁰ M				
H1N3			K_d 2.56 × 10 ¹² M				

Table 1. QCM biosensors for detection of AIVs.

Antibodies are the most common bioreceptor of choice for sensing, and they are generated by immunizing an animal system with antigen. The interaction between antibody and antigen can be transduced into measurable signal changes. Early studies focused on the development of QCM immunosensor based on antibody-antigen interaction for influenza virus detection [12–14]. For example, the characterization of a QCM biosensor for the direct detection of influenza A viruses was reported by Owen et al. [12]. Self-assembled monolayers (SAMs) of mercaptoundecanoic acid (MUA) were formed on QCM gold electrodes for the immobilization of anti-influenza A antibodies, and the limit of detection was estimated to be 4 virus particles/ml. QCM immunosensor has been used also to detect avian influenza label free in nasal washings with a lower detection limit of 10⁴ pfu/ml, although, with the addition of a gold nanoparticle conjugate, the detection limit was reduced to 10³ pfu/ml, which is comparable to

the sensitivity and specificity of viral isolation techniques [13]. Li et al. [14] reported a nano-beads amplified QCM immunosensor with polyclonal antibody as the recognition ligand for detection of AIV H5N1.

In recent years, aptamers have been investigated as an alternative of sensing elements, which have the potential to replace the antibodies. This is possible due to the unique features of aptamers (sensitivity, specificity, reusability, stability, nonimmunogenicity), which can be easily exploited in biosensor technology. Aptamers are single-stranded RNA or DNA oligonucleotides which rely on hydrogen bonding, electrostatic and hydrophobic interactions rather than Watson-Crick base pairing for recognition to their target. Aptamers can fold into distinct secondary and tertiary structures, bind to their targets with high affinity (dissociation constants on the order of nano- to picomolar), and recognize their targets with a specificity that challenges antibodies and other biological ligands. They are selected *in vitro* through systematic evolution of ligands by exponential enrichment (SELEX). The selection procedure involves the iterative isolation of ligands out of the random sequence pool with affinity for a defined target molecule and PCR-based amplification of the selected RNA or DNA oligonucleotides after each round of isolation. As biorecognition ligands, aptamers possess numerous advantages, including small size, rapid and reproducible synthesis, simple and controllable modification to fulfill different diagnostic and therapeutic purposes, slow degradation kinetics, nontoxicity, and a lack of immunogenicity.

The majority of aptamers developed for AIVs have focused on inhibition of the hemagglutinin protein preventing viral infection. For example, a DNA aptamer was developed by Jeon and co-workers [15]. RNA aptamers that inhibit membrane fusion of AIV H3N2 and influenza B virus hemagglutinin also were developed [16, 17]. DNA and RNA aptamers that target HA1 proteins of influenza virus hemagglutinin subtype H5 were investigated in two different studies [18, 19]. An aptamer that binds efficiently to the HA of highly pathogenic AIV H5N1 and H7N7 was studied by Suenaga and Kumar [20], which inhibits HA-glycan interactions. These aptamers were developed to inhibit function of the hemagglutinin protein and prevent or treat influenza infection. Based on the function and likely binding sites of these aptamers, it seems unlikely these aptamers would be optimal sequences to use in a biodetection assay. A DNA aptamer for avian influenza H5N1 was developed by Wang et al. [21] using a combination of protein and whole virus targets. It is unique in its binding affinity in that it specifically targets the H5N1 subtype, having no binding activity with other H5 or N1 subtype viruses. This suggests that the binding site be at an intersection of the H5 and N1 proteins.

Aptamer-based QCM biosensors, also called QCM aptasensors, have been developed for the detection of AIVs. Brockman et al. [22] studied a QCM aptasensor for detection of AIV H5N1 using magnetic nanobeads labels as mass amplifiers. The biosensor was found to have a lower detection limit of 1 HAU, although the detection time was reduced by half compared to a similar QCM biosensor using antibodies [14]. A hydrogel-based QCM aptasensor (**Figure 5**) was able to detect AIV H5N1 at 0.0128 HAU in 30 min [9]. The QCM aptasensor was based on swelling of the hydrogel after AIV H5N1 capture due to dissolution of the cross-linked aptamers and ssDNA in the hydrogel polymer. The developed aptasensor was both rapid and sensitive.

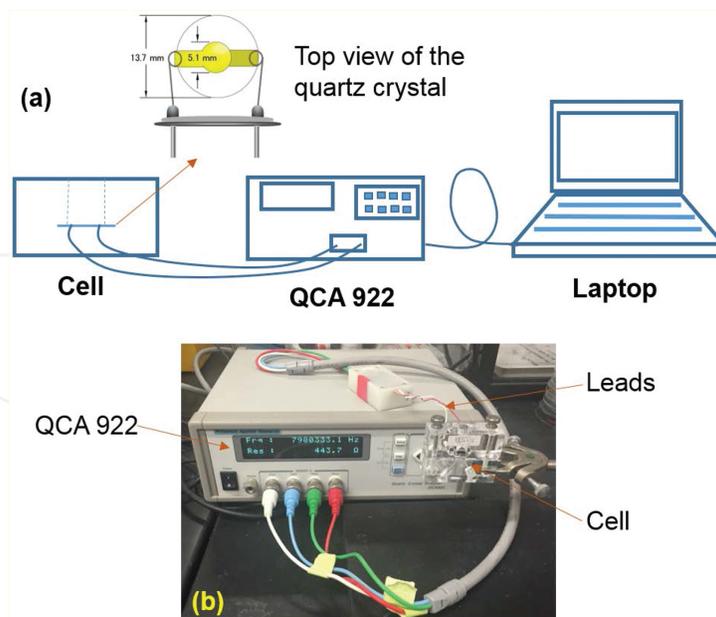


Figure 5. (a) Schematic of the QCM aptasensor; (b) a photograph of the QCM aptasensor setup. The aptamer hydrogel is immobilized on the gold electrode surface. The quartz in the electrode acts as a transducer, converting the mass and viscosity change to a frequency signal.

Influenza virus specificity for the host is mediated by the viral surface glycoprotein hemagglutinin (HA), which binds to receptors containing glycans with terminal sialic acids. This molecular recognition process leads to the host cell-virus adhesion stage [23]. The sialic acid receptor of various influenza virus strains differs in affinity to sialic acids terminally linked either in $\alpha(2,3)$ or $\alpha(2,6)$ position to the galactose (Gal) residues. Human influenza A viruses preferentially recognize the $\alpha(2,6)$ -linkage, while avian viruses have preference for the $\alpha(2,3)$ -linkage [24, 25]. Each monovalent sialic acid-binding site of HA is weak, with dissociation constants in the millimolar range, while the high virus-cell affinity is due to multivalency.

Takahashi and co-workers [26] demonstrated kinetics of HA binding to sulfatide and ganglioside GD1a by QCM analysis. QCM analysis showed that the HA bound with the K_d of 1.44×10^{-8} M to sulfatide immobilized on a sensor chip, which indicated that sulfatide directly binds to the ectodomain of HA with high affinity. N-Acetylglucosamine (GlcNAc) is a natural ligand and is part of the oligosaccharide ligand responsible for the influenza virus binding first step. In a report by Wangchareansak et al. [27], GlcNAc was employed as bioreceptor immobilized on QCM gold sensor surface for detection of influenza A virus (H5N3, H5N1, H1N3), which displayed high binding affinity with K_d values of 2.03×10^{10} , 4.35×10^{10} , and 2.56×10^{12} M, respectively.

QCM biosensor has advantages, such as simplicity, cost-effectiveness, real-time output, and direct detection. But there also exists some disadvantages, such as the lack of stability, difficulty with sensor surface regeneration, loss of the immobilized ligands after multiple washing and regeneration, nonspecific binding of other nontarget biomaterials, and relatively long incubation time. Further improvements are required to address these limitations before the QCM biosensor technology can be routinely employed for AIVs detection.

2.2. Optical biosensors

Optical biosensors rely on visual phenomena to detect the interaction between the biological element and the target analyte. Examples of optical biosensors include surface plasmon resonance (SPR), absorption, luminescence, and fluorescent sensors. Detection by optical biosensors can occur in two ways: by the analyte directly affecting the optical properties of the sensing environment, such as in SPR or absorption methods, or by the analyte being tagged with a label that produces an optical phenomenon, such as in fluorescence methods. Optical biosensors, which are sometimes referred to as “optodes,” have received considerable interest in the detection of AIVs. The main analytical features of optical biosensors for detection of AIVs are summarized in **Table 2** [3, 28–38]. The target “virus” in **Table 2** is the entire virus particle and target “HA” is the HA protein or recombinant HA protein of virus.

Influenza subtype	Target	Bioreceptor	Sensitivity	Sensor	Labelling method	Reference
H1N1	Virus	Glycan	45.6×10^{-13} M	SPR	Label-free	[29]
H3N2	HA	Antibody	1 nM	Waveguide	Dye	[32]
H5N1	HA	Glycan	K_d 1.6×10^{-9} M	SPR	Label-free	[30]
H5N1	Virus	Aptamer	0.128 HAU	SPR	Label-free	[31]
H1N1	HA	Aptamer	K_d 67 fM	SPR	Label-free	[28]
H3N2	Virus	Antibody	0.2 HAU/ml	Prism-free SPR	Fluorescent	[33]
H3N2	Virus	Antibody	–	Waveguide	Gold nanoparticles	[3]
H9N2	Virus	Antibody	8.94 ng/ml	Nanobioprobe coupled with immunomagnetic beads, Fluorescent		[36]
H7 subtype	HA gene	Oligonucleotide probe	7 pM	Luminescence resonance energy transfer (LRET)		[37]
H1N1	Virus	Antibody	1.0×10^{-13} g/ml	Silver nanoparticles labeled antibodies with indirect fluorescence		[35]
H5N1	HA	Aptamer	2 ng/ml (buffer); 3.5 ng/ml (human serum)	Silver nanoparticles; Fluorescent		[34]
H1N1 H3N2	Virus	Antibody	0.1 pg/ml 50 pfu/ml	Gold nanoparticle-decorated carbon nanotubes; photoluminescence		[38]

Table 2. A list of optical biosensors reported for the detection of AIVs.

Various modes of optical measurement exist (i.e., absorption, reflection, fluorescence, chemiluminescence, and phosphorescence); however, biosensors based on surface plasmon resonance (SPR) and fluorescence principles are the most common and promising methods for AIVs detection.

The Kretschmann configuration [39] is the most popular optical setup for SPR applications. SPR biosensors measure the change in refractive index due to binding of biomolecules near the sensor surface. Refractive index is defined as the ratio of the speed of light in vacuum and the phase velocity of light in the medium. SPR is one of very few techniques that are able to provide noninvasive, real-time kinetic data on association, and dissociation rates, along with equilibrium binding constants for receptor or ligand systems. Estmer-Nilsson et al. [40] were able to utilize SPR to quantify influenza virus for vaccine production via an antibody inhibition assay using HA proteins immobilized on the sensor surface. Some research showed SPR biosensors for avian influenza DNA hybridization [41], adamantane binding sites in the influenza A M2 ion channel [42], influenza virus hemagglutinin monitoring [43], and binding kinetics study [44]. Studies by Gopinath et al. [28] revealed that SPR-based biosensor is a useful tool for detection of human and avian influenza viruses.

SPR-based Biacore technology has been designed to investigate biomolecular interactions, which was initiated in 1984. The commercial available systems include Biacore 1000, Biacore 3000, BIAlite, and Biacore T100. Biacore is the dominant SPR technique used for AIVs detection. The main advantage of this technology is its capacity to monitor weak macromolecular interactions that cannot be detected by other sensors and its subject ability to automation. SPR was used to evaluate the binding of influenza A virus H1N1 directly to a neomembrane of bovine brain lipid or an egg yolk lecithin fraction [29] and to monitor the interactions between influenza HA and glycan [30] or aptamers [28]. However, Biacore instruments are expensive and need proper maintenance. Bai and co-workers [31] have shown that a portable hand-held SPR-based biosensor (Spreeta™, Texas Instruments, Dallas, TX, USA) (**Figure 6**) can be employed for the detection of AIV H5N1. The fabrication of the SPR biosensor was based on the streptavidin-biotin binding. The streptavidin was directly adsorbed on the gold surface, and then, biotinylated ssDNA aptamers were immobilized. Target AIVs were captured by the immobilized aptamers and resulted in an increase in the refraction index. It was able to detect AIV H5N1 in poultry swab samples with a lower detection limit of 0.128 HAU in 1.5 h.

The working principle of a waveguide mode sensor is similar to that of a SPR sensor. The only difference is that the measurement is conducted using a waveguide mode rather than a surface mode [32]. Based on the properties of light-guiding and dimensions, there are two general classes, fiber optical waveguides and planar waveguides, which can be further divided into single mode and multimode [45]. Waveguide mode biosensors were developed using antibody as bioreceptor for the detection of HA from H3N2 [32] and H3N2 virus [3].

Of the optical methods requiring labels for detection, fluorescence is the most widely studied [34–36, 46, 47]. Fluorescence measurements are of particular interest in biosensor systems due to their high sensitivity. Commonly used labels in fluorescent biosensors are dyes, quantum dots (QDs), and fluorescent proteins, with the latter two becoming more popular as they are further researched [46]. A fluorescent aptasensor was developed by Pang and co-workers [34]

for detection of AIV H5N1. The Ag@SiO₂ core-shell nanoparticles were coated with the anti-HA aptamers. The binding of aptamer-HA protein formed a G-quadruplex complex, which captured thiazole orange (TO) and reported the fluorescent signal of TO. Moreover, it caused a surface plasmon resonance enhancement and performed as a metal-enhanced fluorescence sensing. The detection of HA protein of the AIV H5N1 could be operated both in aqueous buffer and human serum with the detection limit of 2 and 3.5 ng/mL, respectively. The total detection time was only 30 min. Using antibodies as bioreceptors, Li et al. [35] developed a highly sensitive fluorescent immunosensor for detection of H1N1, which was based on Ag autocatalysis. It had a detection range of 1.0×10^{-12} to 1.0×10^{-8} g/ml with a detection limit of 10^{-13} g/ml.

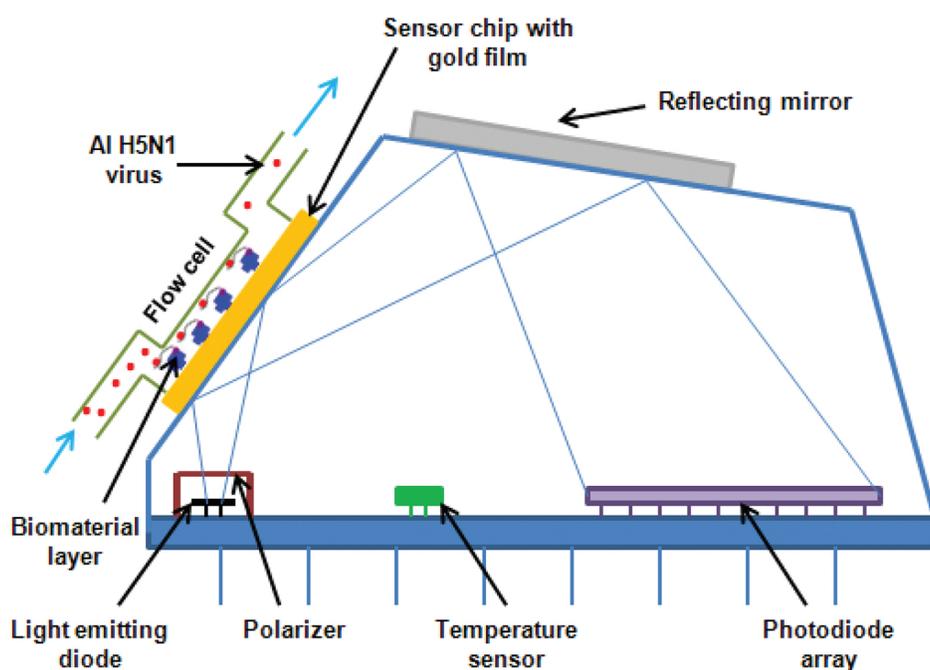


Figure 6. Configuration for measuring AIV H5N1 using the portable SPR aptasensor (with permission from MDPI).

Fluorescence resonance energy transfer (FRET) is used in the design of a biosensor, which is based on the energy transfer between two light-sensitive molecules, a donor and an acceptor chromophore. In the nucleic acid-based FRET method, a reporter and a quencher are conjugated at the terminals of a nucleic acid probe. Without the target, the duplex is formed, bringing these two molecules in close proximity, which results in fluorescence quenching. The presence of a target can cause the conformational change of the probe, which changes the position of the reporter and quencher and emits fluorescence. A QD-induced FRET system was developed by Chou and Huang [47] using two oligonucleotides. These two oligonucleotides were designed to specifically recognize two regions of the AIV H5 sequences and were employed as the capturing and reporter probes, respectively. They were conjugated to QD655 (donor) and Alexa Fluor 660 dye (acceptor), respectively. At target concentrations ranging from 0.5 nM to 1 μM, the QD emission decreased at 653 nm and dye emission increased at 690 nm. Another luminescence resonance energy transfer (LRET)-based biosensor was developed by Ye et al.

[37] for rapid and ultrasensitive detection of AIV H7 subtype. In this work, BaGdF5:Yb/Er upconversion nanoparticles (UCNPs) and gold nanoparticles (AuNPs) were used as the pair of donor and acceptor. The oligonucleotides with H7 HA gene sequence were conjugated with thiol and then were assembled on the AuNPs surfaces. The complementary sequence probes were modified with amino group and then were covalently bond to poly(ethylenimine) modified BaGdF5:Yb/Er UCNPs. The hybridization process brought these two molecules in close proximity, resulting in fluorescence quenching.

QDs are a type of semiconductor with a diameter typically between 2 and 10 nm whose excitons are confined in three spatial dimensions, giving them properties of both unconfined semiconductors and discrete molecules. In comparison with organic dyes and fluorescent proteins, QDs have unique optical and electronic properties including size-tunable light emission, improved signal brightness, high resistance against photobleaching, and simultaneous excitation of multiple fluorescence colors. QD-based bioconjugates used for optical sensing could dramatically increase the sensitivity that would permit simultaneous measurement of several targets. Lee and co-workers [38] developed a plasmon-assisted fluoro-immunosensor by the conjugation of antibody onto the surface of cadmium telluride QDs and the Au nanoparticle-decorated carbon nanotubes (AuCNTs) for the detection of H1N1 and H3N2. A detection limit of 0.1 pg/ml and 50 pfu/ml was obtained for H1N1 and H3N2, respectively.

Fluorescent biosensors are simple and show improved sensitivity and reduced detection time. But they have a drawback of requiring relatively expensive reagents. In order to apply the fluorescent biosensor for detection of real samples with complex background, such as tracheal and cloacal swabs from birds, further work would be required to combine an effective sample pretreatment method with the fluorescent biosensor for the detection of AIVs in the field.

2.3. Electrochemical biosensors

More than half of the biosensors used for the detection of AIVs are based on electrochemical transducers. Electrochemical biosensors use changes in the electrical properties caused by biochemical reactions to detect an analyte [48]. Electrochemical transducers offer several advantages: low cost, high sensitivity, ease of miniaturization, independence from solution turbidity, low power requirements, and simplicity of use [49]. These characteristics make electrochemical detection methods highly attractive for field monitoring of infectious diseases and biological warfare agents. Electrochemical biosensors can be divided by the electrical parameter that they measure: amperometric, potentiometric, conductimetric, and impedimetric. Amperometric biosensors work by applying a constant potential across an electrode and measuring the current associated with either the reduction or oxidation of an electroactive species created by the interaction of the biological element and the analyte. Amperometric biosensors are often used with an enzyme capable of catalyzing the production of an ionic product and increasing the selectivity and the sensitivity [50]. Potentiometric biosensors gather data by converting a biological reaction into a potential signal with the use of ion-selective electrodes [51]. Conductimetric biosensors simply measure the conductivity change in a medium caused by the analytes. Impedimetric biosensors measure a combination of the

resistive and capacitive or inductive properties of a material in response to a small amplitude sinusoidal excitation signal [52].

A detailed review of the electrochemical biosensors for detection of AIVs before year 2014 can be found in the paper by Grabowska et al. [53], which covered the electrochemical genosensors and electrochemical immunosensors for detection of AIVs. A total of 69 references were cited in their review paper. Within the context of this chapter, the electrochemical biosensors reported prior to 2014 are referred to the review paper by Grabowska et al. and the electrochemical biosensors reported in the years 2014 and 2015 plus some impedance biosensors not mentioned by Grabowska et al. are reviewed in detail.

Impedance biosensors are a class of electrical biosensors that measure the electrical impedance of an interface in AC steady state with constant DC bias conditions. This is accomplished by imposing a sinusoidal voltage at a given frequency and measuring the current. This measurement can be done over a range of frequencies or at a given frequency [54]. Many impedance biosensors utilize a capture probe on the detecting surface to hold the target molecule, thereby stabilizing the point of detection. Due to the ease of miniaturization, low energy usage and relatively low cost, impedance biosensors show promise for AIVs applications. Impedance biosensors can use a variety of biological sensing elements but the most commonly used are antibodies. When using antibodies as the biological element the biosensor is often referred to as an impedance immunosensor. Impedance immunosensors rely on the interaction of the antibody and the antigen to generate a detectable signal for the transducing element. This allows the immunosensor to detect either indirect or direct impedance measurements. Direct impedance measurement, or label-free detection, is dependent on monitoring the changes in the electrical properties of the sensing environment caused by the antibody-antigen interaction. A label-free detection method has several advantages over an indirect detection method, including reduced detection time, lower cost, and simpler detection protocol.

There are two general classes of impedance biosensors, Faradic and non-Faradic impedance biosensor, which have been used for the detection of AIV H5N1 or subtype H5. A Faradic impedance biosensor was developed by Wang and co-workers [55] using an interdigitated array microelectrode. Polyclonal antibody against H5 was oriented immobilized on the gold surface through protein A to capture target virus, and red blood cells (RBCs) were used as biolabels for signal amplification. The biosensor had a lower detection limit of 10^3 EID₅₀ ml⁻¹. Wang et al. [10] also reported a non-Faradic impedance biosensor for H5N2 avian influenza detection, which was based on the combination of an immunomagnetic nanobeads separation and a microfluidic chip with an interdigitated array microelectrode. A lower detection limit of 10^3 EID₅₀ ml⁻¹ was achieved with detection time less than 1 h. Then, Lum et al. [56] further improved the non-Faradic impedance biosensor for H5N1 detection by a combination of immunomagnetic nanoparticles, a microfluidic chip, an interdigitated array microelectrode, and RBCs for signal amplification. The specificity of the biosensor was improved due to the use of anti-H5 as a capture antibody and anti-N1 as a detection antibody.

A miniaturized biosensor was developed by Diouani et al. [57] for the detection of H7N1. Polyclonal antibodies against AIV H7N1 were attached to a gold electrode using a self-assembled monolayer. The impedance measurement was carried out in the presence of a redox

probe and inside a Faraday cage. The lower detection limit of this biosensor was determined to be 5 µg/ml of specific antigen. Hassen et al. [58] developed an impedance biosensor capable of quantifying influenza A virus in a media comprised of numerous other proteins and viruses. The biosensor had a lower detection limit of 8 ng/ml, even in the presence of nonspecific viruses and proteins.

In **Table 3**, the electrochemical biosensors developed for the detection of AIVs in year 2014 and 2015 are summarized [59–79].

Influenza subtype	Target	Bioreceptor	Sensitivity	Detection range	Reference
H1N1	Virus	Antibody	1 pfu/ml	1–10 ⁴ pfu/ml	[65]
H5N1	HA	Antibody	2.2 pg/ml	4–20 pg/ml	[66]
H1N1	PB1-F2 protein	Antibody	0.42 nM (monomeric); 16 nM (oligomeric)	5 nM–1.5 µM (monomeric); 5 nM–0.5 µM (oligomeric)	[60]
H5N1	Virus	Antibody	2 ⁻¹⁵ HAU/50 µl	2 ⁻¹⁵ –2 ⁻⁸ HAU/50 µl	[67]
H5N2, H7N1, H1N1	M1 protein	Antibody	20 pg/ml (80–100 virus/µl)	–	[59]
H1N1	PB1-F2 protein	Antibody	0.42 nM	5 nM–1.5 µM	[61]
H5N1	Antibody	HA	2.1 pg/ml	4–20 pg/ml	[63]
H5N1	Antibody	HA	2.4 pg/ml	4–100 pg/ml	[64]
H7N9	Virus	Antibody	6.8 pg/ml	0.01–20 ng/ml	[68]
H5N1	Virus	Antibody	2 ⁻¹ HAU/50 µl	2 ⁻¹ –2 ⁴ HAU/50 µl	[62]
H5N1	Complementary DNA (20-mer); RNA (280-mer)	Complementary sequences probe	73 pM (DNA 20-mer); 0.87 pM (RNA 280-mer)	–	[71]
H5N1	Full length H5 gene	DNA probe	fM level	–	[69]
H5N1	Full length H5 gene	DNA probe	0.03 fM (20- mer ssDNA); 0.08 fM (ds DNA)	–	[70]
H7N9	A fragment of the HA gene sequence	DNA probe	100 fM	–	[72]
H7N9	A fragment of the HA	Primer and DNAzyme	9.4 fM	50 fM–100 pM	[73]

Influenza subtype	Target	Bioreceptor	Sensitivity	Detection range	Reference
	gene sequence				
H5N1	Virus	Aptamer	8×10^{-4} HAU/ 200 μ l	0.001–1 HAU	[74]
H5N1	Virus	Aptamer	0.0128 HAU	0.0128–12.8 HAU	[78]
H5N1	Viral proteins	Aptamer- antibody pair	100 fM	100 fM–10 pM	[75]
H5N1	Virus	Aptamer	2^{-9} HAU	0.001–4 HAU	[77]
H5N1	HA	Glycan	–	–	[76]
H5N1	HA	Glycan	aM level	140 aM–14 nM	[79]

Table 3. A list of electrochemical biosensors reported in 2014 and 2015 for the detection of AIVs.

For the past two years, the immunosensors have been the dominant electrochemical biosensors for detection of AIVs [59–68]. For example, universal anti-M1 antibodies, which allow detection of all serotypes of influenza A virus, were used for the development of an universal immunosensor for detection of the influenza A virus. It showed similar sensitivity (80–100 virus particles/ μ l) to molecular methods [59]. Miodek and co-workers [60, 61] developed an electrochemical immunosensor, which was based on conductive polypyrrole modified with ferrocenyl groups as a redox marker for the detection of PB1-F2, a nonstructural accessory protein of influenza A virus. Lin et al. [62] demonstrated that an impedance immunosensor, which was based on low-cost microelectrodes and specific monoclonal antibodies for rapid detection of AIV H5N1 in chicken swabs. Studies by Jarocka and co-workers [63, 64] revealed that the immobilization of the recombinant His-tagged HA was able to detect antibodies against AIV H5N1 in hen serum.

Although electrochemical biosensors for avian influenza detection have mostly been investigated for whole virus or virus protein detection, some groups have developed biosensors for the detection of avian influenza nucleic acid sequences [69–73]. These biosensors are based on the use of nucleic acid probes, which bind to specific sequence in the influenza genome. Several genosensors have been studied for the detection of the full length H5 gene of AIV H5N1. In the study of genosensors by Grabowska et al. [69, 70], redox active compounds (such as cobalt porphyrins or 3-iron bis(dicarbollide)) were conjugated with the DNA probes, which were very close to the electrode surface. These developed genosensors showed a sensitivity in the fM range. Malecka and co-workers [71] reported an AET/Phen-Epoxy/Fe(III)/(Phen-Epoxy-NH₂-NC₃)₂ genosensor, which has a working principle similar to this already reported by Grabowska et al. [69]. The hybridization process caused the formation of the duplex structure on the electrode surface, which resulted in the thickness changes of the double layer at the interface between the electrode and solution.

A new trend in the development of biosensors is the use of nanomaterials which exhibit unique and attractive chemical, physical, and electronic properties. Various nanomaterials have been used as an electrode platform in highly sophisticated electroanalytical biosensing devices. The

working electrodes (actual physical transducers) upon modification with these materials gain large effective surface area, high catalytic capabilities, and high conductivity. Thus, these transducers could act as effective mediators and facilitate electron transfer between an active site on the receptor and the electrode surface. Nanoparticles [74, 75], carbon nanotubes [65], magnetic nanobeads [68, 74], or quantum dots [76] enhance sensitivity and selectivity of the electrochemical detection. Among the variety of metal nanoparticles, gold nanoparticles (GNPs) have been extensively utilized in recent years, mainly because of their nanoscopic size, good conductivity, and biocompatibility. One of the most commonly used is single-walled (SW) or multiwalled (MW) carbon nanotubes (CNTs) as well as graphene and graphene nanosheets. Thanks to their fast electron transfer ability, mechanical strength, chemical stability, catalysis effect, and thermal and electrical conductivity, they are attracting greater interest than other applied technologies.

A magnetic nanobeads-based impedance aptasensor was designed and developed by Fu and co-workers [74] (**Figure 7**). Briefly, H5N1 aptamers were coated on the magnetic nanobeads (MBs) for specifically capturing target AIV H5N1. Then, bionanocomposites (BNCs) were added using Au nanoparticles (AuNPs) as carriers, which were conjugated with concanavalin A (ConA) and glucose oxidase (GOx). The BNCs attached on the captured target virus through ConA-glycan interaction formed a sandwich complex. Finally, the sandwich complex was transferred to a glucose solution, resulting in an efficient enzymatic reaction and the impedance decreased correspondingly on a screen-printed interdigitated array electrode. This method took advantages of the high efficiency of enzymatic catalysis and the high susceptibility of electrochemical impedance on the ion strength and endowed the aptasensor with high sensitivity.

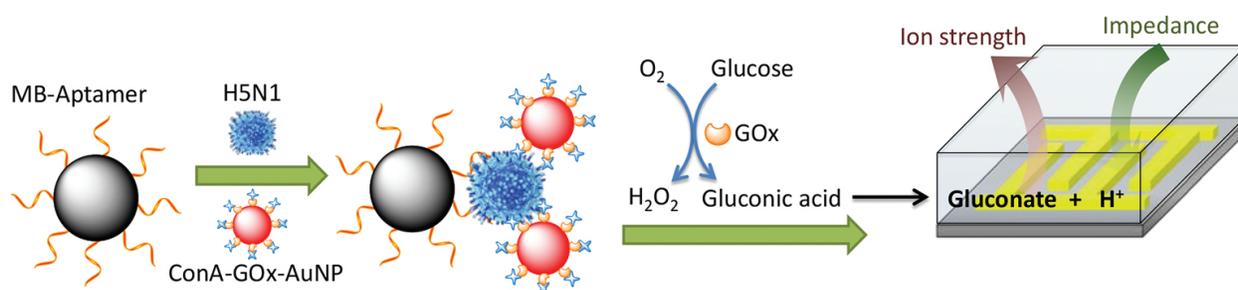


Figure 7. Illustration of the aptasensing mechanism and construction of the aptasensor (with permission from ACS).

Wang and co-workers [77] developed an aptamer-based bifunctional bionanogate, which could selectively respond to target molecules and control enzymatic reaction for electrochemical measurements (**Figure 8**). A nanoporous gold film with a pore size of ~ 20 nm was prepared by a metallic corrosion method and then was functionalized with two types of thiol-modified single-stranded oligos (SH-ssDNAs) by means of Au–thiolate bonding. The bases of the two immobilized SH-ssDNAs were selected to be partly complementary to that of the two ends of an aptamer, respectively. Then, aptamers were added and hybridized with the two immobilized SH-ssDNAs, resulting in the nanopore covered with aptamers, a “closed” bionanogate. Finally, the aptamer covered nanopore film was placed onto an enzyme pre-coated glassy

carbon electrode to form an aptasensor. The working principle of the bionanogate-based aptasensor for detection of AIV H5N1 is illustrated in **Figure 8**. Initially, without the target AIV, the bionanogate was kept “closed,” which isolated coenzymes and substrates in the testing solution from the enzymes immobilized on the electrode so that the enzymatic reaction was restricted (**Figure 8a**). Upon the target virus binding, the aptamers dissociated into solution from their ssDNAs and formed aptamer–virus complexes, which triggered the bionanogate “open” (**Figure 8b**). This “open” state allowed coenzymes and substrates to diffuse freely through the opened nanopore and to create contact with the immobilized enzymes, resulting in an efficient enzymatic reaction (**Figure 8c**).

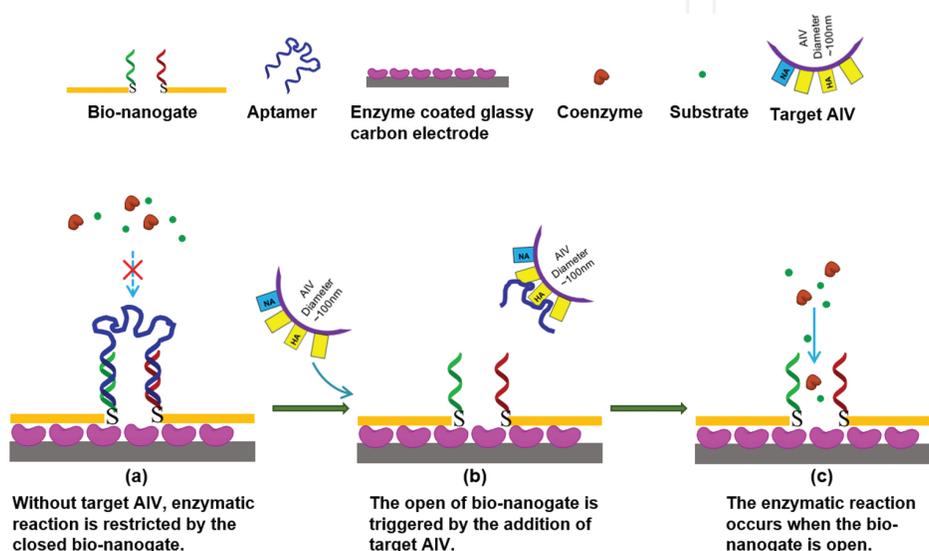


Figure 8. Principle of the bionanogate-based aptasensor for detection of AIV H5N1 (with permission from Elsevier).

Despite the remarkable sensitivity, rapid response, miniaturization capability, and low cost of the electrochemical biosensors for AIV detection, there are still problems with long-term stability and selectivity in real samples with complex background. Obviously, further work would be required to demonstrate that the electrochemical biosensors do not suffer from stability and selectivity problems when handling real samples in the field.

3. Conclusion

Biosensors have attracted tremendous interest in AIVs detection. They are characterized by good selectivity and sensitivity with a wide dynamic range from subfemtomolar to nanomolar, easy and rapid experimental protocol, reasonable cost, and usage of the sample volume in a μl range. Rapid development of nanotechnology has opened a new way for design and construction of biosensors with even better features. However, many contributions to the field of biosensors for AIVs detection still are at the “proof-of-concept” stage. Thus, authors hope that this chapter will promote lively and valuable discussions in order to generate new ideas

and make new approaches toward the development of innovative biosensors for applications in AIVs detection.

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