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Immunosensor-based label-free and multiplex detection of influenza viruses: State of the art

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ABSTRACT

The ability of influenza viruses to rapidly evolve has caused significant challenges in viral surveillance, diagnosis, and therapeutic development. Molecular sequencing methods, though powerful tools for monitoring influenza evolution at the genetic level, are not able to fully characterize the antigenic properties of influenza viruses. Understanding influenza virus antigenicity is critical to vaccine development and disease prevention. Traditional immunoassays which have been widely used for evaluating influenza antigenicity have limited throughput. To alleviate these problems, new bioanalytical tools to investigate influenza antigenicity by measuring antibody-antigen binding are an active area of research. Herein, we review immunosensor technologies from the aspects of various sensing principles, while highlighting recent developments in multiplex, label-free detection strategies. Highlighted technologies include electrochemical immunosensors relying on impedimetric detection; these demonstrate simple design and cost effectiveness for mass production. Antibody arrays implemented on an optical interferometric sensor system allow systematic characterization of influenza antigenicity. Quartz microbalance immunosensors are highly sensitive but have yet to be explored for multiplex sensing. Immunosensors made on lateral flow strips have shown promise in rapid diagnosis of influenza subtypes. We anticipate that these and other technologies discussed in the review will facilitate advances in the study of influenza, and other viral pathogens.

1. Introduction

Infectious influenza is a long-running scourge on human health, with devastating societal consequences (World Health Organization, 2018; Taubenberger and Morens, 2006; Kilbourne, 2006; Centers for Disease Contr, 2018a). The human toll related to influenza virus infection includes costs associated with treatment and work lost (Molinari et al., 2007; Bridges et al., 2000), in addition to the virus' direct impact on human quality of life. Influenza virus is an enveloped negative-sense RNA virus with single stranded gene segments encoding the proteins associated with the virus' structure and functions (Enami et al., 1991). Hemagglutinin (HA), a surface glycoprotein, plays an essential role in the infection of animal host cells by influenza. Another surface viral protein, neuraminidase (NA), mainly functions as a virion releaser by cleaving virus-bound carbohydrates (sialic acids) from the cell surface (Webster et al., 1992). Since HA binds to specific sialic acids of receptors on host epithelial cells, and these sialic linkages change based on species, species isolation of influenza virus infection is common (van Riel et al., 2007). Influenza viruses are classified into A (infecting a wide range of species including humans, pigs, horses, sea mammals, and birds), B (mostly infects humans), C (almost exclusively infect humans), and D (only infect cattle) types. Among these, influenza A viruses are the most serious, and are responsible for seasonal epidemics (Taubenberger and Morens, 2008). Currently, there are eighteen HA and eleven NA subtypes identified to date, and different combinations of the two (plus minor variants i.e. point mutants) form the complete reservoir of influenza A viruses in all species (Centers for Disease Contr, 2018b). However, frequent immune selection, mutation, and re-assortment of HA genes tends to produce new strains leading to inter-species transmission. This process may sometimes cause influenza pandemics, with severe illness and fatalities in human populations (Kilbourne, 2006; Neumann et al., 2009; Smith et al., 2009a). Definitively diagnosing an infection or understanding that a pandemic event has occurred is only possible if technology is available to rapidly detect and analyze the influenza virus. Therefore, rapid and sensitive sensors and diagnostics are critical tools in responding to and preventing individual infections, epidemics, and pandemics.

Extensive efforts have been made in influenza detection and

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identification through health organizations, government agencies, academia, and independent laboratories worldwide. These efforts over the past few decades are beginning to drive a shift in strategy from culture-based serological assays to genetic characterization methods and new optical and electrical biosensors. Among genetic methods, the polymerase chain reaction (PCR) and related techniques including reverse transcription PCR and real-time PCR (rtPCR) (Yuen et al., 1998; Lee et al., 2001; Spackman et al., 2002) have been widely applied in biomedical laboratories for detecting viruses and measuring influenza virus gene expression. In addition, whole genome sequencing methods have been used to expand information on viral genetics (McGinnis et al., 2016). These molecular detection methods are sensitive, and allow rapid and specific detection of genetic variations among many different influenza viruses. In the context of viral surveillance for vaccine development, although thousands of influenza virus samples from patients are collected and analyzed during year-round surveillance for influenza (Ginsberg et al., 2009; Smith et al., 2009b; Nelson et al., 2008; Ghedin et al., 2011), genetic information does not always enable a complete understanding of influenza virus antigenicity and its evolution. This is because genetic mutations may alter virus recognition by components of the immune system in ways that are challenging to predict (Glaser et al., 2005; Smith et al., 2004). As a result, selection of the appropriate vaccine candidates frequently results in suboptimal protection against circulating viral strains (Hilleman, 2002; Salzberg, 2008; Center for Disease Contro, 2005–2018). For example, overall vaccine effectiveness was reported to be only 19.8% for the influenza A H3N2 subtypes circulating in the 2014–2015 season (Zimmerman et al., 2016). Efficacy during the 2017–2018 season was even worse, dropping as low as 13% and resulting in the highest spike in influenza associated deaths and hospitalizations across the United States in recent years (Flannery et al., 2018).

Due to these issues, alternative high throughput methods for evaluating influenza virus antigenicity that do not rely on sequencing are a critical need. Serological immunoassays, including hemagglutinin inhibition (HAI), micro-neutralization (MN), and the enzyme linked immunosorbent assay (ELISA), all described in more detail in section 2.1 below, are commonly used in biomedical laboratories for measuring antigenic responses of serum antibodies to influenza antigens. While such experiments can provide the best correlation of protection against influenza (Ohmit et al., 2011), they require extensive labor for the production and optimization of needed reagents when working with antigenically distinct or uncharacterized viruses (Easterday et al., 1997). In addition, these traditional methods are technically laborious, time consuming, complex in work flow, and the requirement for multiple components including antisera and live virus as a component of the assays places constraints on the laboratory environments where these methods may be executed (Katz et al., 2011; Lebarbenchon et al., 2012; Pedersen, 2014). As an alternative, immunosensor-based assays are being developed as fast, inexpensive, and multiplex tools. These have considerable promise for measuring both the host immune response, and the antigenicity of the influenza virus itself. As such, these assays can contribute in useful ways to the ongoing process of influenza surveillance and vaccine development. This review focuses on immunosensor-based label-free approaches to influenza-targeted detection. Most of the methods we describe can also be employed in the future in a high-throughput format.

2. Principles of immunoassays and immunosensor-based technologies for influenza detection

2.1. Significance of immunoassays for determining influenza antigenicity

Immunoassays are binding assays that use specific antibodies or antibody-like reagents as the binding substance to determine and quantify antigens. “Antigens” in this context, are molecular structures present on the surface of viruses that can be recognized by the immune system to produce specific antibodies. The glycoproteins hemagglutinin (HA) and neuraminidase (NA) on the surface of influenza viruses (discussed above), which are responsible for binding the virus to host cells, are major influenza antigens (Chen and Deng, 2009). Antibody molecules produced by the host (infected) organism consist of specific binding domains that target the antigenic determinant (or “epitope”) of the antigen, which is encoded by unique HA or NA gene segments. Since HA genes undergo the highest mutation rate due to frequent immune selection, the epitope mutants tend to escape immune defense due to the loss of binding affinity (Hensley et al., 2009). Such escape usually begins with small variations in HA genes, and gradually produces closely related strains sharing similar antigenic properties. This slow accumulation of gene mutation is called “antigenic drift”, and over time can result in a distinguishable antigenic variant. Generally, antigenic drift does not fully alter the viral antigenicity; however, the 2013 avian H7N9 influenza virus was able to switch its host species to humans by two single-point mutations in HA genes (Gao et al., 2013). Another type of escape comes more abruptly, and involves the mixing of genes from different host species (Olsen, 2002). This process, described as “antigenic shift”, can result in totally new strains that may be highly infectious to humans, causing an influenza pandemic. As an example, Fig. 1 provides a schematic of antigenic evolution of human influenza H3N2 viruses. Gene reassortment of avian H3N2 strains and human H2N2 viruses yielded the “mother clone” of influenza A H3N2 in Hong Kong (1968) (antigenic shift) (Cox and Subbarao, 2000; Wiley et al., 1981). This pandemic strain continues to evolve (via antigenic drift), resulting in antigenically distinguishable seasonal H3 strains (Russell et al., 2008). Both types of evolutionary pathways could lead to severe consequences without timely monitoring: accurate and early detection of newly emerging strains is critical for the rapid initiation of vaccination and therapeutics (Webster and Govorkova, 2014). In general, influenza antigenicity is not predictable from genetic sequence (Smith et al., 2004), thus demonstrating the importance of conducting immunoassays to characterize viruses.

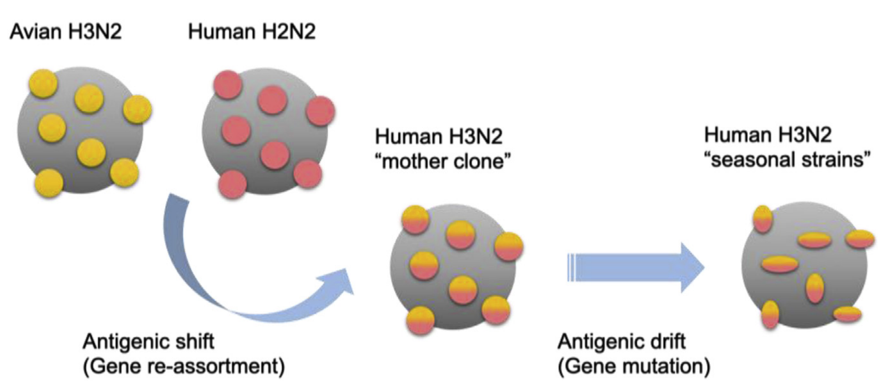

Fig. 1. Modes of influenza antigenic evolution.

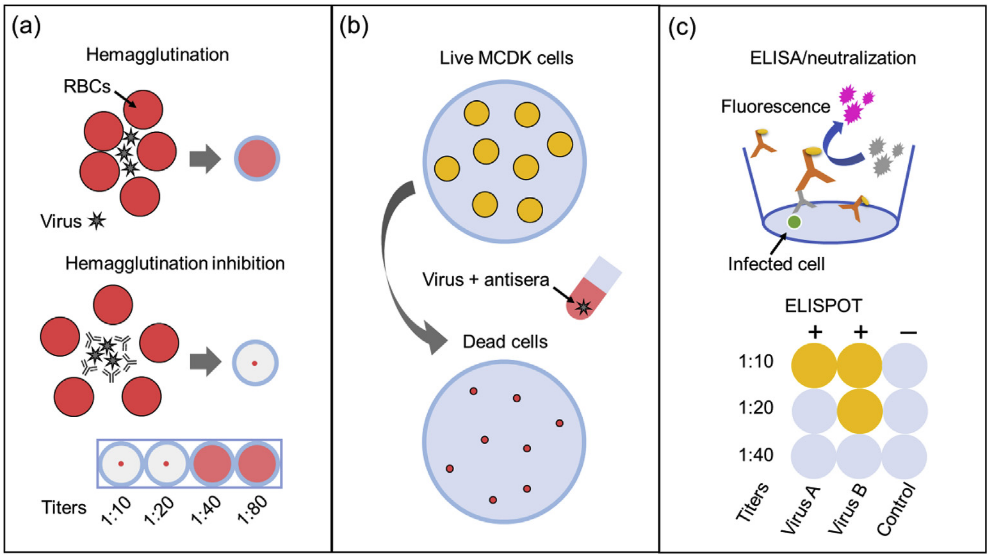

Fig. 2. Traditional immunoassays. (a) Hemagglutination inhibition (HAI); (b) Virus neutralization (VN or SVN); (c) Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot assay (ELISPOT).

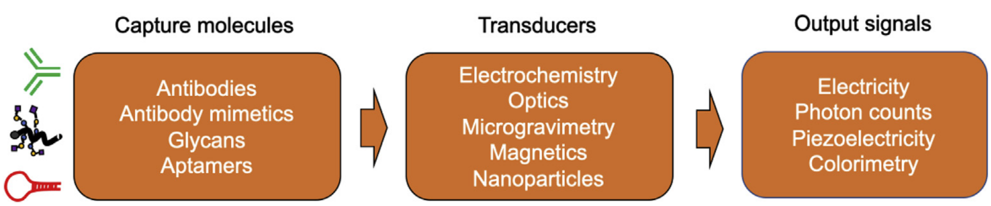

Fig. 3. Components of label-free immunosensor design.

Currently, the standard immunoassays used to characterize influenza antigenicity rely on serological methods involving live cells, viruses, and specific serum reagents. Hemagglutination and hemagglutination inhibition (HAI) assays are the most commonly used immunoassays to detect influenza viruses and determine antigenicity by quantifying the specific interaction of HA with the host cell surface glycan. Agglutination of red blood cells is used as an indication of HA mediated crosslinks, or the amount of specific antibodies against the virus that are present in the sample (Spackman, 2014). The reciprocal of the lowest concentration that results in complete agglutination is defined as the HAI titer, as shown in Fig. 2 (a). HAI assays are also performed in clinical laboratories as a pivotal method for probing the human immune response against influenza viruses due to the assay's simplicity, speed, and ability to be visualized by the naked eye. However, the sensitivity of this type of assay may be compromised especially when mutation occurs in the receptor-binding site of the HA (Chandrasekaran et al., 2008; Nobusawa et al., 2000; Xiong et al., 2013). As an alternative serological tool to HAI assays, the serum virus neutralization assay (SVN, Fig. 2(b)) has been widely used for clinical purposes to evaluate the inhibition of virus infectivity in the presence of neutralizing antibodies obtained from human or animal sera. This approach visualizes the number of plaques that have survived the antibody neutralization process in cell culture. Similar to HAI titer, the virus neutralization titer is expressed as the reciprocal of the lowest concentration at which the infection is completely blocked. Although such conventional neutralization assays are considered highly sensitive for early detection, the process of cell culture is always laborious and slow. As an alternative, the Enzyme-Linked Immunosorbent Assay (ELISA, Fig. 2(c)) has been combined with microneutralization assays (MN) in microwell plates to detect the presence of influenza infected cells. Further, array assisted enzyme-linked immunospot (ELISPOT) methods have been developed to facilitate multiplex detection (Czerkinsky et al., 1983; Tanguay and Killion, 1994). However, this approach still suffers from workflow complexity due to labeling, especially since reagents need to be optimized to allow detection of multiple antigens. Therefore, utilizing advanced label-free technologies to replace traditional immunoassays is key to accelerate the accurate determination and study of influenza antigenicity.

2.2. Design and assembly of influenza immunosensors

In general, biosensors are tools used to detect analytes that selectively interact with specific biomolecules (the “probe” or “capture molecule”) immobilized on the sensor (Turner et al., 1987). Immunosensors, depicted in Fig. 3, are a major type of biosensor widely applied for biomedical research and clinical use. These detect antibodies or antigens in a complex background medium such as serum (Lazcka et al., 2007). The probe molecules used in immunosensors are specific antibodies or antigens that bind corresponding targets via antibody-antigen interactions. Traditional serology immunoassays mostly rely on polyclonal antibodies due to a number of factors including ready availability from commercial suppliers, and a rapid and cheap mass production process compared to traditional methods of generating monoclonal antibodies (Clough and Hauer, 2005). However, polyclonal antiserum contains a heterogeneous mixture of antibodies with different affinities for a heterogeneous set of antigenic epitopes, and thus the effect of a single or a few changes are not significant (Lipman et al., 2005). Therefore, polyclonal antibodies are less sensitive or specific for fully characterizing virus antigenic properties than monoclonal antibodies, especially for systematic studies of analytes with minor differences. Recently, progress in the rapid production of monoclonal antibodies has revolutionized immunoassays for influenza antigenicity (Wrammert et al., 2008; Henry et al., 2016). Many studies have also shown human monoclonal antibodies with neutralization ability and diverse reactivity for subtyping influenza HA proteins or whole virus molecules (Wrammert et al., 2011; Henry et al., 2015; Fu et al., 2016;

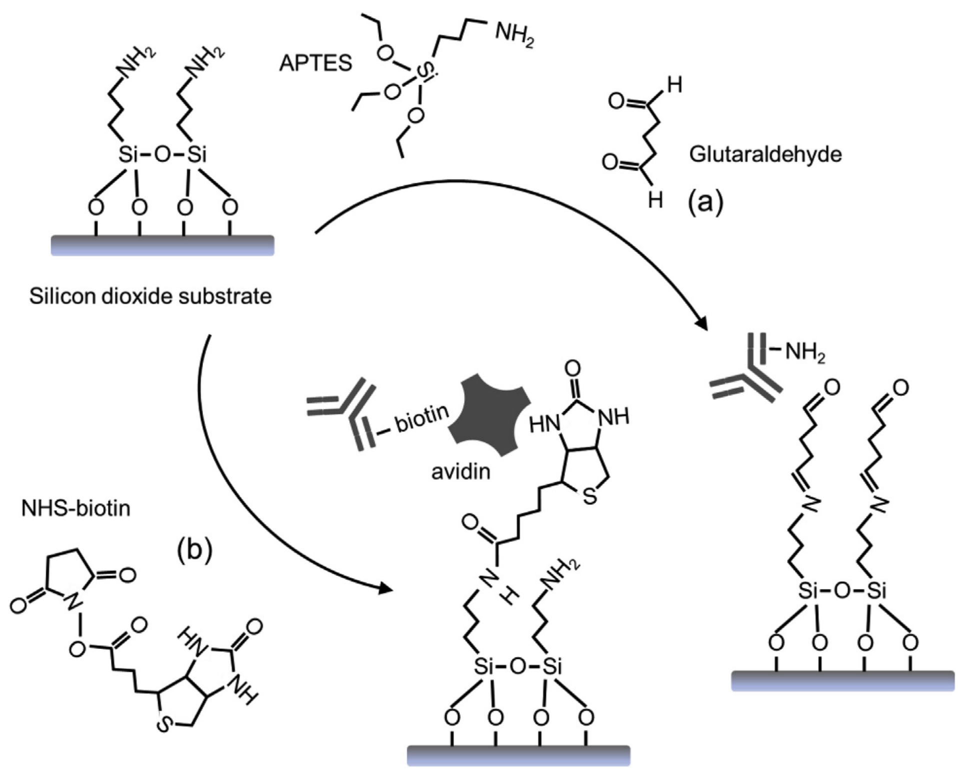

Fig. 4. Examples of surface functionalization strategies for capturing antibody probe molecules. (a) APTES/glutaraldehyde; (b) APTES/NHS-biotin.

Feng et al., 2018). These developments in the production and use of monoclonal antibodies have contributed to the generation of improved immunosensors to facilitate influenza surveillance, therapeutics, and research towards the development of new vaccines. Although the resulting devices are not "immunosensors" in a strict sense since they do not use antibodies, alternative capture reagents for influenza sensors include molecular imprinted polymers (MIP, synthetic structures intended to mimic the molecular recognition characteristics of natural antibodies) (Vlatakis et al., 1993), aptamers (artificial DNA or RNA segments with high affinity to targets), and receptor binding analogues (sialic acid moieties on the host cells for HA binding). MIPs and aptamers have advantages over natural antibodies in terms of mass production and ease in modification. However, MIPs may not have sufficient specificity to be effective in complex clinical samples. Recently, development of chemical and enzymatic-based synthesis of carbohydrate-based receptor analogues in different formats has led to the extensive applications in characterizing specific influenza HA proteins for different species, indicating that these synthesized molecules are promising probe reagents for the study of human adaptation of avian sourced influenza viruses (Stevens et al., 2006).

For the past few decades, the development of biosensor technologies has intensively focused on increasing the sensitivity and specificity for the detection of target molecules in a label-free format, and this review will highlight such technologies. However, directly detecting biological targets according to their physical properties (such as size, mass and charge) without signal amplification is extremely challenging, especially at ultralow concentration of the target molecules. Therefore, most current biosensor technologies usually exploit a specific 'reporter molecule' with high affinity towards the target to enable an indirect detection and quantification of a sample. For example, in the case of influenza HA protein detection, this reporter molecule typically consists of a broadly reactive anti-influenza antibody raised against the specific HA protein antigen, with an enzyme (e.g. alkaline phosphatase) conjugated with the antibody for signal amplification. Following binding to the target molecule, the reporter molecule can thus be visualized with a 'label' (enzyme-substrate reaction) that produces a colorimetric response which is easy to measure and quantify. This method provides a detection limit within the femtomolar range for HA protein molecules such as seen with an ELISA assay (Chappell et al., 2014). Although such high sensitivity is useful for influenza virus detection, especially for early stage infections, the use of fluorescent labels or enzyme conjugation can interfere with or alter the specificity of binding processes, a critical issue in the systematic characterization of influenza viruses. In addition, the introduction of labels in the detection system may increase nonspecific binding effects. These can interfere with distinguishing closely related target influenza viruses. These issues have been part of the driving force behind development of biosensor transducing systems based on label-free technologies capable of direct characterization of binding. Such systems have enabled many research investigations and have played a key role in fundamental biological discoveries such as protein binding kinetics for understanding of influenza virus antigenicity. Therefore, three major types of label-free detection scenarios and their potential use for multiplex sensing application will be the primary focus of this review: electrochemistry, optics, and microgravimetry. Electrochemical interactions and piezoelectricity produce small electrical signals that can be easily monitored. Optical transducers rely on the interaction of light with a responsive surface, producing a variety of output formats such as a spectrum, a spectral shift, or an image. Although these major direct detection methods hold the advantage of simplicity for the development of immunosensors, other unique approaches regarding magnetic effects and colorimetric strategies will also be discussed in this review.

In order to achieve the highest possible performance for an immunosensor, the transducer surface must be further modified to enable efficient immobilization of probe molecules. Typically, noncovalent physical adsorption is the simplest option, as it allows attaching molecules directly to the transducer surface via electrostatic forces or hydrophobic interactions (Wiseman and Frank, 2012). However, this strategy tends to result in loss of effective probes due to either surface-induced denaturation or weak adsorption of probe molecules at the sensing surface (Lu et al., 1998). In contrast, surface modification relying on chemical reaction is more efficient and specific in producing uniform and densely-packed layers of probe molecules. Since free amines of amino acid residues are abundant over the entire antibody structures, the most common immobilization process involves the formation of covalent bonds between primary amines on the surface of antibodies and functional electrophilic groups on the sensing substrate. For example, the attachment of aminoalkoxysilanes (such as aminopropyl triethoxysilane, APTES) to the surface of silicon-based sensors in Fig. 4 (a) enables direct immobilization of free amines following modification of the APTES surface with glutaraldehyde (Yadav et al., 2014). Like physical adsorption, this chemical reaction still results in probe molecules on the transducer surface having a random orientation. We discuss antibody probes as an example. The structure of each antibody molecule contains two identical variable regions called fragment antigen binding (Fab) domains, and a shared tail region called the fragment crystallizable (Fc) domain. Since the isoelectric point of a Fab domain is often larger than that of an Fc domain in an intermediate pH environment (a preferred condition to mimic clinical or physiological effects), the Fab domain is always positively charged while the Fc domain is negatively charged (Davies and Cohen, 1996). This characteristic of antibody molecules results in preferred deposition orientations to different transducer surfaces (Trilling et al., 2013). As such, the impact of random orientation on probe activity needs to be considered. In Fig. 4 (b), a biotin-avidin binding complex is established using NHS-biotin compounds. As a site directed strategy for antibody probes, NHS-biotin compounds are also extensively used as a universal platform for synthetic probes such as MIPs, aptamers, and receptor glycans for covalent immobilization to different transducer surfaces to facilitate the exposure of expected binding domains. In addition, these surfaces can be further exploited by conjugating macromolecules onto the probes to increase the sensitivity of the immunosensor (Chaki and Vijayamohanan, 2002a). For example, hydrogel nanoparticles and protein A/G can be used as probe carriers due to high compatibility with both sensing surface and probe molecules (Tavakoli and Tand, 2017; Welch et al., 2017). Other approaches are possible, including functionalization of gold or other noble metals (Chaki and Vijayamohanan, 2002b).

3. Recent advances in immunosensor-based label-free detection of influenza viruses

3.1. Electrochemical immunosensors

Electrochemical biosensors use changes in electrical properties such as voltage and current, caused by biochemical interactions, to detect an analyte. A typical electrochemical biosensor system consists of a sensing electrode and a reference separated by an aqueous electrolyte to complete the circuit for current flow. This conceptually simple design can be implemented in principle at low cost and with high energy efficiency, because inexpensive electrodes can be easily integrated with low power electronic systems to perform rapid and real-time measurements in miniaturized formats (Hammond et al., 2016). These characteristics make electrochemical biosensors highly attractive for applications in medical diagnosis and infectious disease monitoring (Campuzano et al., 2017). For many years, most electrochemical biosensors for influenza virus detection have focused on nucleic acid detection (Grabowska et al., 2014). The hybridization process causes formation of a duplex structure of nucleic acid probes and targets on the electrode surface, in turn changing the thickness of the layer between the electrode and electrolyte. Like sequence-based electrochemical biosensors (Grieshaber et al., 2000), electrochemical immunosensors can also be classified into three groups based on electrical transducing parameters illustrated in Fig. 5 with characteristic response curves. Potentiometric approaches convert the binding interaction into a potential change on the electrodes (Koncki, 2007). The built-up complex at the electrode surface changes the electrode potential proportionally to the concentration of the analytes. Recent developments in the production of field-effect transistor (FET) devices allow miniaturized and integrated arrays of potentiometric transducers, as well as enhancement of the output signals (Fig. 5 (a)). The potential change (V_g) detected at the electrode gate controls and amplifies the signal (V_{ds}) across the FET. This type of device is simple, but the sensitivity may be adversely affected by nonspecific binding of sample constituents on the electrodes (Bhattacharyya, 2019). In contrast, amperometric methods (Fig. 5 (b)) apply a constant potential across an electrode and measure the current associated with either the reduction or oxidation of an electroactive species created by the interaction of the biological element and the analyte (Lojouand Bianco, 2006). The signals generated are specifically from redox components in an electrochemical process, and thus they are more resistant to nonspecific binding. However, as most antibody-analytes for immunosensors are not able to induce electrochemical reactions themselves, labels of electrochemical reagents or products from enzymatic reactions are required for sensing (Miodek et al., 2014; Buozis et al., 2018; Qatamin et al., 2019; Lee et al., 2019). Therefore, applications in label-free detection are limited.

As impedimetric techniques detailed in Fig. 5 (c) measure electron transfers from the analyte complex in response to a small sinusoidal voltage excitation, sensitive and direct detection of analytes without using labels or redox couples can be realized (Prodromidis, 2010). Many impedimetric immunosensors, based on high-affinity antibody-antigen interactions, achieve a comparable limit of detection to genetic sequencing for the detection of influenza viruses. For example, Lin et al. demonstrated use of specific monoclonal antibodies for rapid detection of influenza A H5N1 in swabs obtained from chickens (Lin et al., 2014). For their assay, H5 specific monoclonal antibodies were immobilized on the Protein A modified surface of the electrode. This method enables the direct orientation of Fc domains of the anti-H5 monoclonal antibodies on the sensor electrode to enhance the exposure of Fab binding domains. Thus, this immunosensor achieved as high a sensitivity as PCR in measuring a low concentration of influenza in the sample. In contrast to direct immobilization of antibodies for virus detection, Jarocka et al. developed an impedimetric immunosensor with immobilized recombinant HA proteins that enabled pg/mL sensitivity for antibodies against influenza A H5N1 viruses in hen serum, better than the ng/mL detection limit they were able to obtain via ELISA (Jarocka et al., 2014). Both immunosensors provided very low limits of detection; however, extension of these methods to a multiplex format has yet to be reported. To expand the range of detection, polyclonal antibodies against M1 protein, a universal biomarker for influenza virus, were used for the detection of all subtypes of influenza A viruses via electrochemical impedance analysis (Nidzworski et al., 2017; Siuzdak et al., 2019). A nano-scale boron-doped diamond electrode surface was functionalized with polyclonal anti-M1 antibodies, and then exposed to M1 proteins of H1N1 and H3N2 viruses in buffered saliva. This immunosensor achieved a sensitivity corresponding to 5–10 viruses per sample, as low as traditional plaque assays can detect. The reliability of this technique has been also confirmed by testing M1 proteins of different influenza subtypes. Another example of sensitive detection and selective discrimination among influenza subtypes is a glycan-based impedimetric immunosensor that is able to specifically detect intact influenza A H3N2 subtypes with a low limit of detection (around 10 viral particles per microliter) (Hushegyi et al., 2016). Since glycans are host receptors responsible for virus binding during influenza infections, immunosensors of this type are potentially useful in the study of viral host adaptation and transmission properties.

Overall, impedimetric immunosensors have shown several advantages in label-free detection of influenza viruses relative to potentiometric and amperometric approaches. Further modification of the design of the sensing electrode may allow improvement in detection sensitivity, while providing flexibility for integration into microsystems. One example is the development of large-scale interdigitated array (IDA) microelectrodes as shown in Fig. 6 (a) (Yang et al., 2004). This design enhances the signal-to-noise ratio by dissecting the

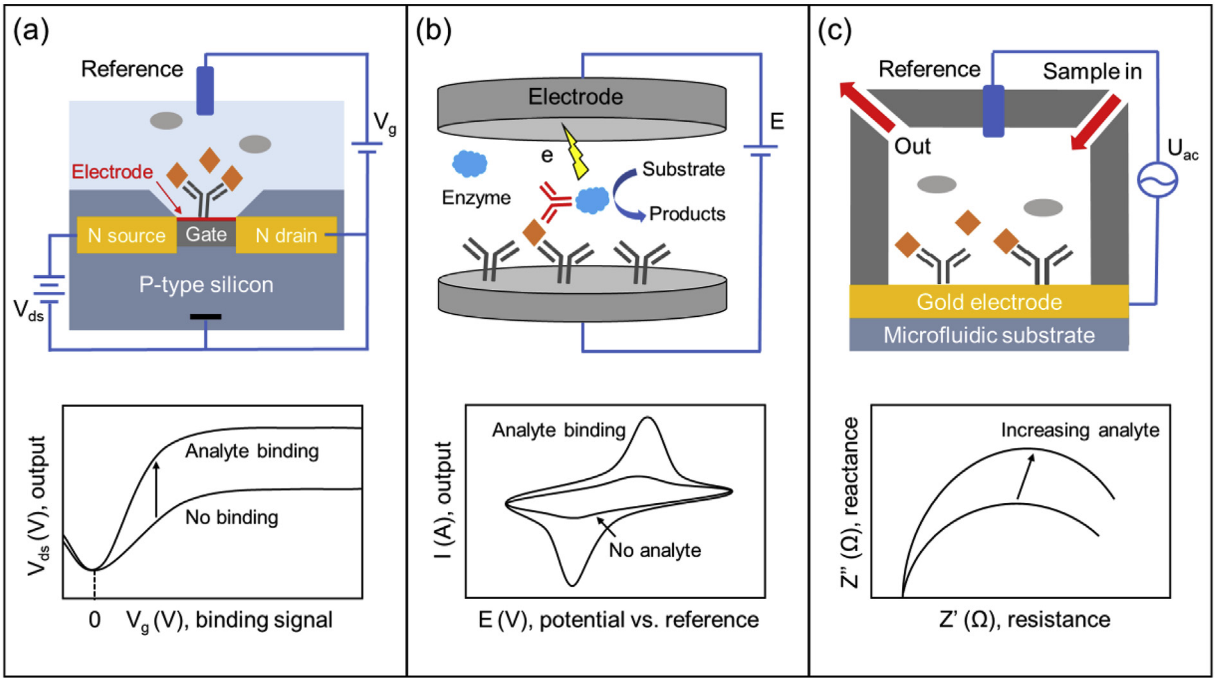

Fig. 5. Schematics and characteristic response demonstration of (a) FET integrated potentiometric immunosensor; (b) enzyme labeled amperometric immunosensor; (c) microfluidic integrated impedimetric immunosensor.

conventional electrode into a series of microelectrode/reference pairs that facilitate a rapid and efficient cycling of analytes at different locations without the impact of a slow diffusion-controlled reaction. However, utilizing multiple capture probes on different IDA microelectrodes is still a challenge for multiplex and high-throughput sensing due to the intrinsic detection principle that an individual pair of microelectrodes is not able to work independently (Varshney and Li, 2009). Recently, a multifunctional CMOS (complementary metal–oxide–semiconductor) based microelectrode array (MEA) system was developed for performing separate impedimetric measurements simultaneously. An SEM image of the bio-compatible array shown in Fig. 6 (b) shows a detail of a larger sensor chip consisting of tens of microelectrodes arranged in multiple units that are able to perform different measurements in parallel (Dragaset al., 2017). The microelectrode system contains multiple functional units that conduct impedimetric measurements on the electrochemical properties of the bioanalytes. The array surface is bio-compatible for saline buffers and cell culture medium, and furthermore well separated from the base metal layers to ensure the functions of the sensor. For biosensing application, the electrode array architecture utilizes a switch matrix to connect any electrode in the array for performing different measurements in parallel. Based on such feature, this technique has significant potential in promoting multiplex sensing for influenza detection.

3.2. Optical immunosensors

Because of their ability to directly generate binding-induced signals, nondestructive interaction with the sample (helping to ensure that molecules of interest retain their original form when sensed), and high resistance to electromagnetic noise compared with other transducer techniques, optical biosensors have become particularly widely studied devices for bioanalytical analyses. The introduction of optical fibers, waveguides, and optoelectronic systems has also provided versatile options for integration of optical sensors into a broad range of system configurations. Label-free optical biosensors have shown the ability to efficiently detect viral proteins, nucleic acids, and whole virions (Fan et al., 2008).

The detection principles of optical label-free immunosensor

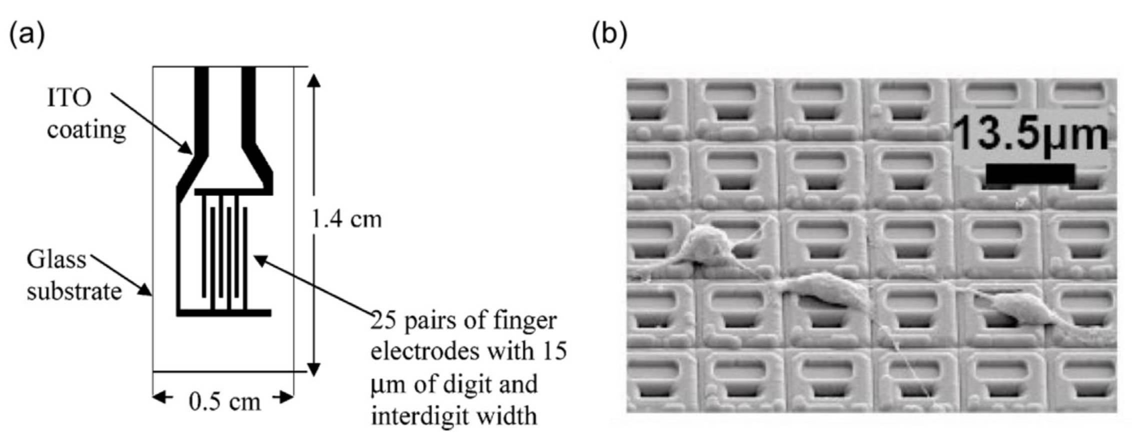

Fig. 6. (a) Schematics of interdigitated array microelectrodes (IDA) for impedance measurements (Yang et al., 2004); (b) SEM images of partial layout of multi-functional microelectrode arrays (MEAs) (Yang et al., 2004).

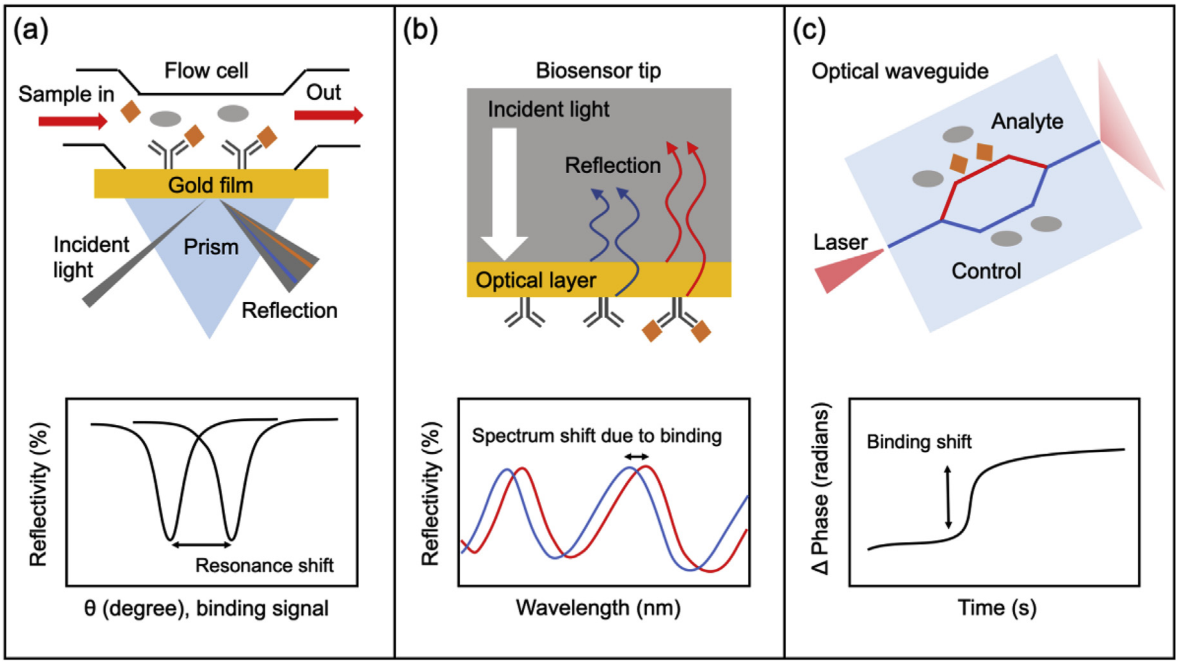

Fig. 7. Schematics and signal demonstrations of (a) SPR; (b) BLI; (c) Mach-Zehnder interferometric waveguide.

technologies most commonly rely on refractive index (RI) changes induced by antibody-antigen binding on a surface. Detection technologies include surface plasmon resonance (SPR), interferometer-based methods, optical ring resonators, photonic crystals, and others. For example, SPR (Liedberg et al., 1983) based detection has been extensively studied, in part because of its status as one of the most widely available commercial label-free sensor technologies. In SPR, signal is produced by a binding-dependent refractive index change near the surface of the gold substrate. This is measured by observing resonance features of coupled light, typically as a function of resonance angle, as shown in Fig. 7 (a) (Homola et al., 1999). In 1996, Schofield and Dimmock provided the first demonstration of detection of influenza A viruses via SPR, and measured binding constants to IgG antibody Fab domains (Schofield and Dimmock, 1996). Although their work is fundamental to the development of immunosensors for influenza virus detection, extension of SPR to routine clinical use has proven challenging. Research use of single-channel and imaging SPR includes antigenic printing of antibody response in human sera in exposure to emerging influenza virus (Khurana et al., 2016) and developing cross-reactive antibodies for influenza universal vaccines and therapeutics (Krammer, 2016).

Other plasmonic biosensors such as optical resonators and photonic chips have gained increasing interest in the application for influenza detection because their main advantages are high sensitivity, fast read out, small size, low cost mass production, and scalable for point-of-care applications. For example, quantum dot conjugated aptamer beacons were used for point-of-care detection of H1N1 influenza virus based on three-dimensional photonic crystals. Owing to the enhancement of output signals in the photonic structures, visualization of virus detection can be easily realized by a smartphone, and sensitivity comparable to the detection limit of SPR based techniques may be achieved (Lee et al., 2018). Resonator based structures can achieve even higher sensitivity. For example, Vollmer et al. reported a high-Q (where Q is the "quality factor", a description of the efficiency of the resonator defined by the full width half maximum of the resonance divided by the resonance wavelength) microsphere resonator that was able to detect single influenza viral particles within a concentration of femtomolar range depending on discrete resonance shift of a whispering-gallery mode induced by the binding of influenza virions on the surface of microspheres (Vollmer et al., 2008). This frequency shift strongly depends on the radius of the microspheres, and thus reducing the dimension of the microspheres can further increase sensitivity. As such, in comparison to other single molecule detection strategies, the measured resonance shift also enables the determination of the size and mass of influenza virus particles.

Interferometric approaches have achieved success in label-free detection due to the simplicity and robustness of measurements. Bio-layer interferometry (BLI), shown in Fig. 7 (b), analyzes the interference pattern reflected from the tips of optical fibers to quantify binding of attached biomolecules. Xiong at el. used BLI to measure the binding affinity of human H7N9 and avian H7N3 viruses to immobilized receptor analogues (Xiong et al., 2013). Although BLI suffers from a lower sensitivity (Yang et al., 2016) than SPR, its simplicity and high stability are ideal for high-throughput sensing.

Mach-Zehnder interferometers constitute a second method for interferometric sensing. These may be implemented in an integrated photonic format by measuring the phase change of interference patterns (Fig. 7(c)). Using this approach, Xu et al. reported detection of whole intact avian influenza virus with a sensitivity of 5×10^{-4} HA units/ml (1 HA unit corresponds to a number of viral particles ranging from 10^4 to 10^7) (Desselberger, 1975) that indicates the detection of a relatively small number of viruses (Xu et al., 2007). In addition, Sakamoto et al. prepared a sol-gel surface on a Mach-Zehnder optical waveguide with densely immobilized H1N1 specific antibodies, achieving detection of intact H1N1 influenza virus at a concentration of 100 μg/ml in solution (Sakamoto et al., 2016). Ymeti et al. also reported discrimination of different types of avian influenza virus using a Young interferometer sensor, in which each waveguide channel was coated with a different capture probe.

Approaches using Mach-Zehnder or Young interferometers could be extended to building a useful platform for multiplex detection of influenza viruses (Ymeti et al., 2007). Theoretically, more waveguide channels could be introduced into this sensor architecture to generate interference patterns allowing expansion of the number of identifiable viruses. However, in practice this is limited by the physical dimensions of the device. Therefore, spectral interferometry has been considered as a more efficient candidate for optical immunosensors to rapidly and sensitively characterize antibody-antigen interactions in a high-

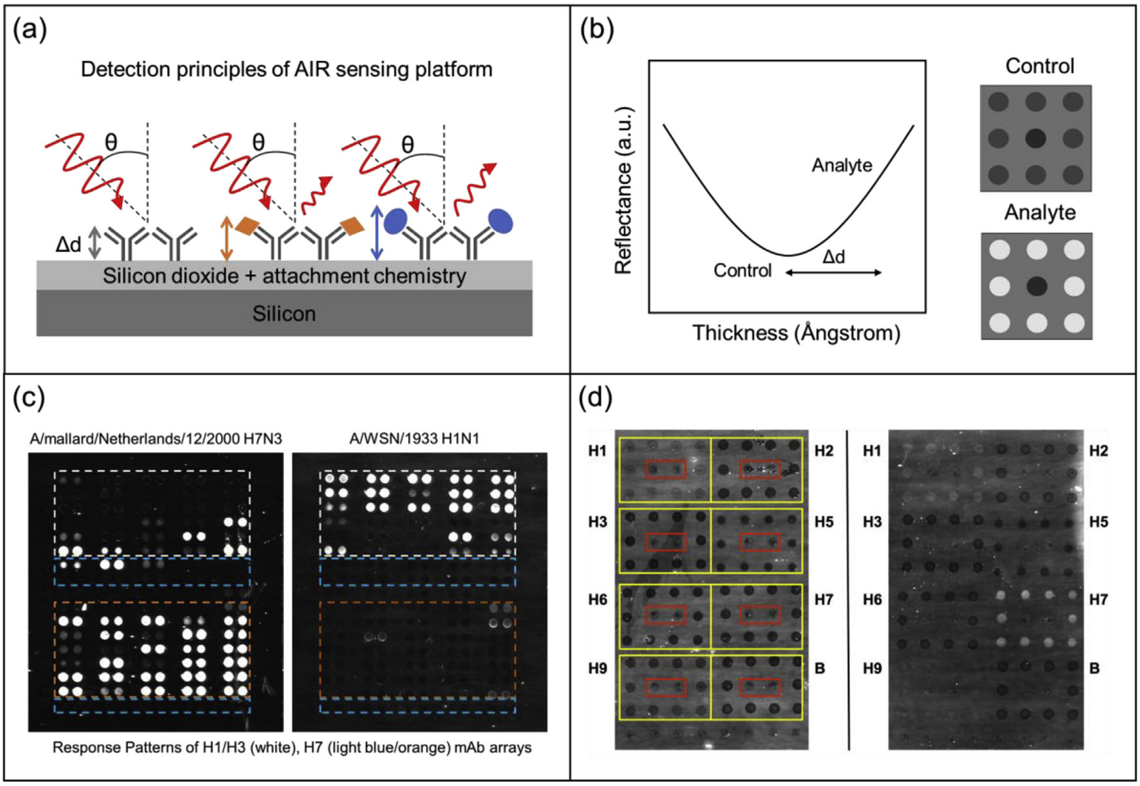

Fig. 8. Schematics of (a) AIR and (b) binding signal demonstration; (c) response patterns of human monoclonal antibody arrays against H1 and H7 influenza viruses (Zhang et al., 2018); (d) response patterns of influenza HA arrays against antiserum (Bucukovski et al., 2015).

throughput manner (Gauglitz et al., 1993). Spectral interferometry measures the interference pattern caused by the superposition of two reflected partial beams at parallel interfaces. Spectral reflection depends on the refractive indices of the sensing materials, incident wavelength, and angle of incidence. The adsorption of target molecules at the probe layer changes the optical path length of the beam, and thus any refractive index difference will change the intensity of the reflected beam. As a result, the reflection of each small area on the sensing surface can be captured by a CCD camera, and the output reflection-based imaging patterns can be related back to accumulation of mass at different locations. For assembling a multiplex influenza immunosensor, various antibody probes can be immobilized in a sequence of small independent sensing areas with known specificities. The averaged reflection signal of each individual area corresponds to the 1-plex detection of antibody-antigen interaction.

Based on this concept, we have for the first time demonstrated a multiplex, label-free microarray sensing method for rapidly serotyping influenza viruses. The sensing platform, known as Arrayed Imaging Reflectometry (AIR), (Fig. 8 (a)) has been described in detail elsewhere (Mace et al., 2006). AIR relies on the creation, and target binding-induced disruption, of a near perfect anti-reflective condition due to the destructive interference of light reflected from the sensing surface (a silicon chip). Pure s-polarized 632 nm light from a HeNe laser source incident on a chip with a SiO_2 coating of the appropriate thickness, at an incident angle (θ) of approximately 70°, produces an antireflective condition that can approach 1 part in 10^8 (Fig. 8(a)). Capture of biological targets is via capture molecules (antibodies, antigens, or other molecules able to specifically bind a biological target) immobilized at specific locations on the chip. Capture of a target biomolecule on any of these locations produces a change in the optical thickness of the anti-reflective film, resulting in light leaking out from that area of the chip in a manner that quantitatively maps to the amount of material captured. The imaging system has no moving parts due to a non-scanning design for imaging, and a temperature-controlled stage is also not required (unlike techniques such as surface plasmon resonance, or many other photonic sensing systems) (Ozdemir and Turhan-Sayan, 2003; Amrita Kumar et al., 2017). As data acquisition simply involves producing an image of light reflected off the chip surface, total measurement time may be 100 ms or less (although total experimental time is determined by binding kinetics of material to the surface). Thus far, AIR has been successfully demonstrated for sensitive detection of pathogenic bacteria (Horner et al., 2006), cytokines and inflammatory biomarkers (Mace et al., 2008; Carter et al., 2011), small molecule pollutants (Carter et al., 2016), and human autoimmune disease antigens (Bucukovski et al., 2018). The technique has also been validated via comparisons to reference methods including SPR and spectroscopic ellipsometry (Sriram et al., 2011). Using this technology, we demonstrated that arrays of human monoclonal antibodies to influenza subtypes could be employed in a pattern-recognition approach to expedite influenza virus serology, and to study the antigenic evolution of newly emerging viruses via antigenic cartographies determined by principle component analysis (PCA), a general method for mapping the relations between patterns of multiplex response data (Zhang et al., 2018). These human monoclonal antibody arrays readily discriminated among various subtypes of influenza viruses, including H1, H3 seasonal strains, and avian-sourced human H7 viruses (Fig. 8 (c)). Array responses also allowed the first determination of antigenic relationships among influenza vaccine strains directly from hmAb responses. Using this platform with HA arrays for detecting serum influenza antibodies has also shown advantages in multiplex sensing for avian influenza surveillance and serology (Bucukovski et al., 2015; Mace et al., 2011), as shown in Fig. 8 (d).

3.3. Quartz crystal microbalance immunosensors

The final label-free detection method we will discuss that has been applied to influenza virus immunosensing is microgravimetry or piezoelectric quartz crystal microbalance (QCM). The QCM utilizes a thin

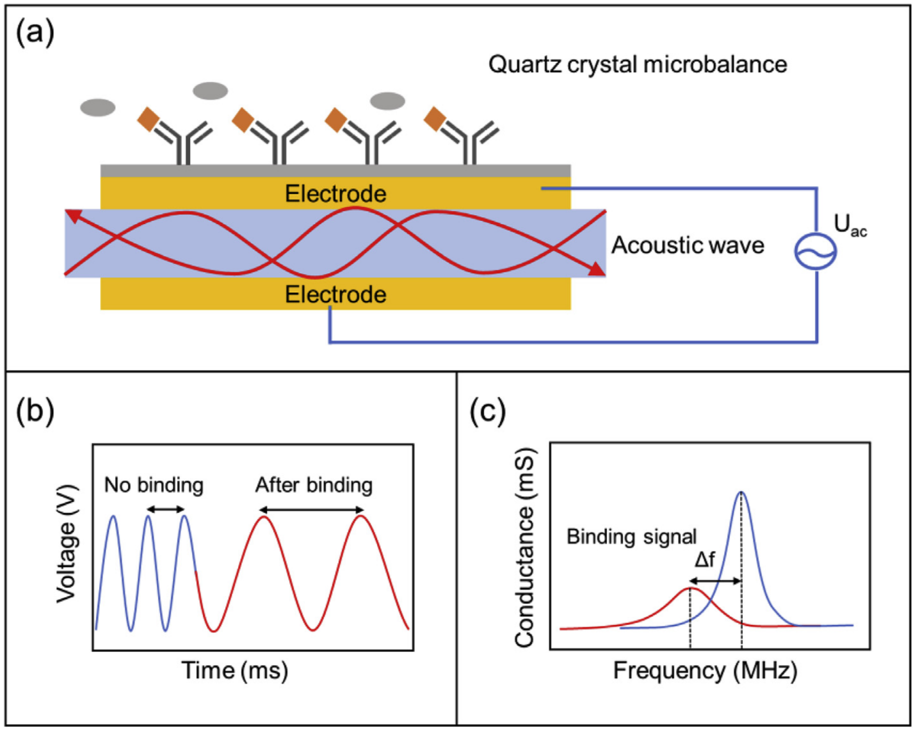

Fig. 9. Schematics of (a) quartz crystal microbalance immunosensors; (b) Real-time detection signal; (c) Binding induced resonance frequency shift for quantitative analysis.

wafer of quartz as the transducing media in a piezoelectric field to detect mass changes in the sensing environment. Sauerbrey et al. first pioneered bioanalytical applications of QCM sensors by calculating the frequency changes of the device as a function of mass adsorbed or deposited on the surface of the crystal (Sauerbrey, 1959). In a typical QCM immunosensor construction shown in Fig. 9 (a), two metal electrodes on the sides drive the QCM to undergo dimensional changes or resonate at a characteristic resonant frequency. The acoustic wave that oscillates inside the bulk of the QCM generates a transverse evanescent field in the perpendicular direction to the quartz plane. Changes of the mass on the electrode surface within the evanescent field lead to a time dependent frequency shift of the acoustic wave shown in Fig. 9 (b). Similar to the SPR technique, QCM immunosensors generate an evanescent acoustic wave to measure the resonance of quartz crystal substrates for sensing. Changes in physical properties within the evanescent field lead to a shift of resonance. A reduction in the frequency (Δf) of the resonance thus corresponds approximately linearly to the amount of adsorbed materials for quantitative analysis, as indicated in Fig. 9 (c). The major advantages of these devices are their simple assembly, label-free detection capability, and real-time output. The transducing materials, quartz crystals, have a high chemical stability, biocompatibility, and are resistant to extreme pH and temperatures (O'Sullivan and Guilbault, 1999). However, it is difficult to distinguish nonspecific interference effects (as usually seen in other label-free immunosensor systems) from real binding events since incorporating a proper reference in the sensor device has proven challenging. Therefore, magnetic resonance techniques and magnetic nanomaterials have been coupled with QCM to increase sensitivity and specificity, since magnetic nanoparticles can inherently enhance the piezoelectricity of the device meanwhile concentrating the immobilized capture molecules. Further, these conjugated particles can be easily regenerated and reused (Tang et al., 2007; Jin et al., 2009).

QCM immunosensors utilizing antibody-antigen interactions have also been widely applied for rapid identification of influenza viruses. For example, the first QCM-based immunosensor for the real-time and direct detection of aerosolized influenza A H3N2 virus was reported by Owen et al. (2007) In their work, self-assembled monolayers were used to immobilize influenza H3N2 polyclonal IgG antibodies on QCM electrodes, and the limit of detection was found to be four viral particles per milliliter (comparable to the sensitivity of plaque assays). Later, Li et al. fabricated a QCM immunosensor using H5 specific polyclonal antibodies for the detection of avian influenza H5N1 virus (Li et al., 2011). Here, in addition to having antibodies coated on the sensor surface, the antibodies were also attached to magnetic nanobeads via biotin-streptavidin binding. The additional layer of antibody-nanobead complexes that bound to captured virus further amplified the binding signal and increased the sensitivity in a similar mechanism to using magnetic nanoparticles as previously discussed. Although this method has shown significant effect in signal enhancement for QCM immunosensors, multiplex sensing using various labeled nanoparticles would be challenging due to the cross reactivity of conjugated antibodies and complexity of labels. Integrated microelectrodes for large scale multiplex sensing potentially addresses this issue. For example, a multielectrode QCM sensor shown in Fig. 10 (a) (Latif et al., 2011), was proven successful for simultaneously detecting various ionic analytes via imprinting specific polymer layers. Three electrodes were functionalized with different imprinted ionic species, while one electrode was left uncoated and used as the reference. This unique design could be implemented for simultaneous recognition of diverse analytes through immobilizing different probe materials. However, potential interference of electrical signals generated by multiple electrodes limits the application for multiplex sensing. Recently, a QCM sensor array illustrated in Fig. 10 (b) (Deng et al., 2018), was reported as being able to conduct measurements on individual units modified with different porous materials. Although this strategy has shown high accuracy in discrimination of various chemical groups (Vaughan et al., 2018), the difficulty in fabricating a large number of high-quality units still limits its utility for high-throughput detection, and its application in biosensing regime is yet to be explored.

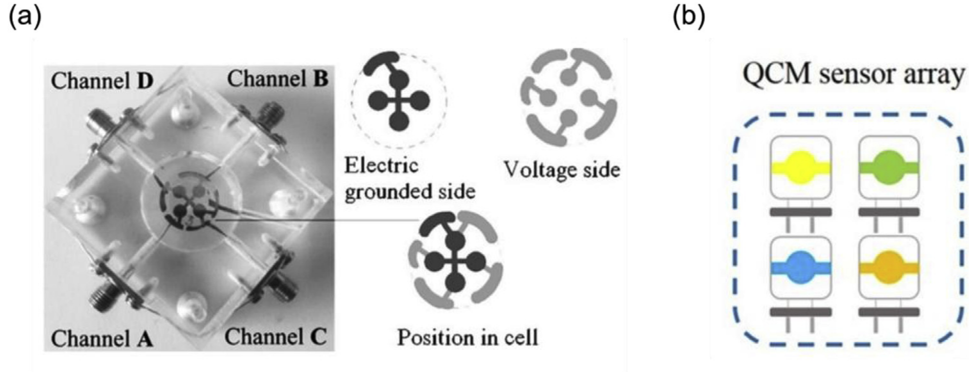

Fig. 10. Demonstration of (a) setup image of multielectrode QCM sensor (Latif et al., 2011); (b) Array layout of independent QCM units for multiplex detection (Deng et al., 2018).

3.4. Colorimetric immunosensors

Development of simple and easily accessible immunosensors for influenza virus detection is highly important for immediate and effective influenza outbreak prevention and disease control. Colorimetric immunosensors are widely used for rapid influenza diagnostics, striking a balance between sensitivity and efficiency. The most commonly used colorimetric method is the lateral flow immunoassay test, also known as the lateral flow immunochromatography test (ICT) (Posthuma-Trumpie et al., 2009). This is designed to detect the presence of a color-coded complexes for point-of-care (POC) diagnostic purposes, especially in infectious disease diagnostics including virus detection (Cazacu et al., 2004; Hara et al., 2008). In detail, a lateral flow strip consists of a series of pads that are capable of transporting the sample fluid spontaneously and sequentially, shown in Fig. 11 (a). The sample solution that contains target analyte is injected from the sample pad. The absorbent pad on the other end applies a capillary force, drawing the sample solution through the strip. After the analyte solution mixes and interacts with color-coded reporter molecules at the conjugate pad, all of the conjugated complexes will reach the test spot, where corresponding detection antibodies will capture the analyte and result in the accumulation of sandwiched color-coded analyte complexes. Unbound reporter conjugates will continue to flow through a control line consisting of pre-immobilized secondary antibodies; these will bind to the reporter conjugates to indicate the end of the flow process. Both the test spot and control line will light up to demonstrate a positive pattern; a negative sample will produce no binding at the test spot, but a response on the control line to indicate that the assay was correctly performed (Fig. 11 (b)). The material from which the strip is made varies depending on the specific purpose of the device. For example, nitrocellulose and polyvinylidene difluoride (PVDF) membranes are commonly used, because these materials are inexpensive to make, flexible in textures, and most importantly provide high affinity to antibodies or probe biomolecules. In addition, a well-designed filtration system controlled by the pore size of the strips can improve the efficiency and sensitivity of the detection by ensuring uniform flows interact with the test spot. Lateral-flow ICTs typically provide a direct signal readout within minutes, making them ideal for point-of-care diagnostic applications in which quantitation is not critical (Son, 2019; Bhardwaj et al., 2019).

Modification of the reporter conjugates with nanoparticles has been used to facilitate detection and indirect quantitative analysis with enhanced sensitivity via the absorbance of light at a certain wavelength. A detailed discussion of this strategy can be found elsewhere (Banerjee and Jaiswal, 2018). For example, gold nanoparticle (AuNP) conjugated colorimetric strategies have been developed in which control of the assembly and disassembly of the AuNP conjugated complexes allows for influenza virus detection. The key principle is that the presence of the probe-analyte complex yields reduced repulsion among AuNPs, resulting in their aggregation. Aggregation generates a shift in the absorption spectrum as a color change that can be directly visualized. Recently, Hwang et al. demonstrated a novel lateral flow immunosensor using an AuNP-conjugated neuraminidase enzyme inhibitor as the probe to measure concentrations of influenza analytes based on the gradual change in the color (Hwang et al., 2018). Although nanoparticle conjugation strategies can greatly enhance sensitivity, multiplex sensing on a single flow pad is challenging due to the confined

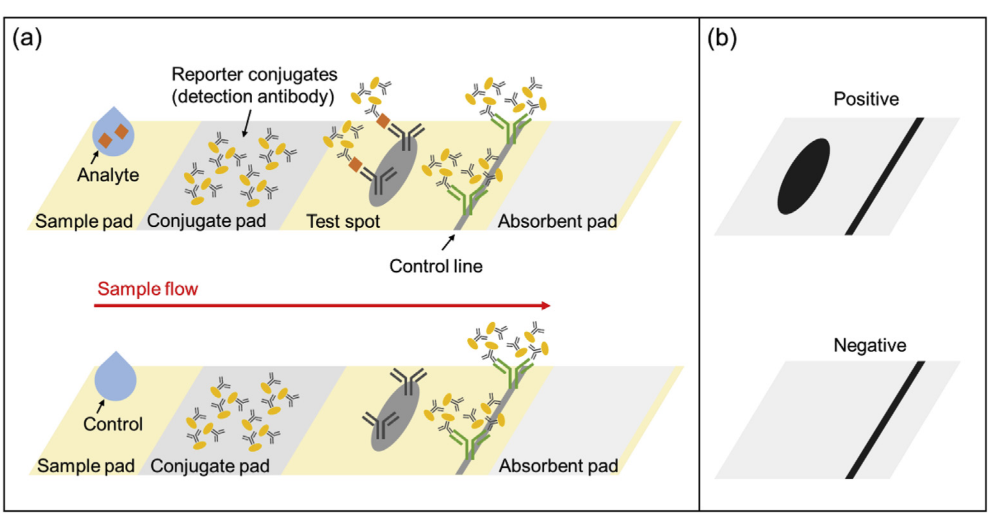

Fig. 11. Schematics of (a) detection principles for lateral flow immunosensors; (b) Test results indicated by response patterns on the lateral flow strip.

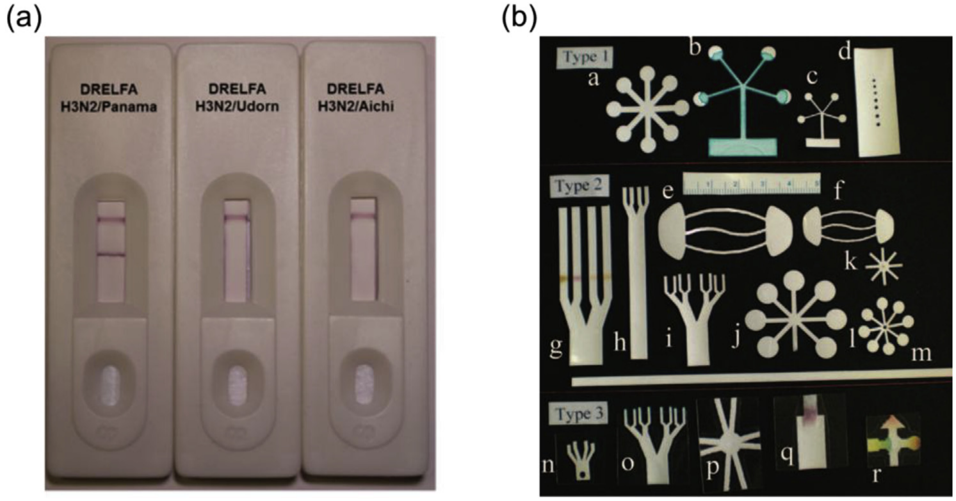

Fig. 12. (a) Dual recognition element lateral flow assay (DRELFA) demonstrates the ability of differentiating three H3N2 subtypes (Le et al., 2017); (b) Three types of two-dimensional shaping design of multiplex lateral flow strips on porous medium (Fenton et al., 2009).

reactivity of probe antibody complex on AuNPs for recognizing influenza subtypes (Hanafiah, 2017). To address this issue, Le et al. demonstrated a dual recognition element strategy to replace the capture antibodies with biotinylated aptamers. Reliance on two recognition events potentially overcomes the limitations of antibody cross-reactivity for multiplex detection of influenza strains within the same subtype shown in Fig. 12 (a) (Le et al., 2017). It also allows the discrimination of various strains among H3N2, H5N1, H7N1, and H9N2 subtypes with a high efficiency. Utilizing the design of patterned and multilayer flow substrates could also benefit multiplex detection from a single test such as the devices illustrated in Fig. 12 (b) (Fenton et al., 2009). In this work, the authors explored fabricating the lateral flow strip based on porous materials in different two-dimensional shapes and compared their performance in multiplex lateral-flow assays. However, designs with a long flow path may result in unevenly distributed analyte content due to the evaporation of the flow, thus resulting in degraded sensitivity and specificity.

3.5. Other novel label-free immunosensors

Other label-free techniques have also been reported for detection and characterization of influenza viruses, and show the potential of being developed for multiplex detection and point-of-care diagnostics. For example, a novel method for early influenza point-of-care diagnosis utilizes NMR-based magnetic-relaxation switches (MRSw). Switching occurs when antibody-antigen binding induces the aggregation of magnetic particles (Fig. 13 (a)). The transverse relaxation time (T_2) associated with the aggregation of magnetic particles changes in response to magnetic field when target HA molecules bind to the antibodies (Koh et al., 2008). In detail, HA-Tag peptide (a general epitope of HA which is widely used in expression vectors) from the influenza HA protein molecules was conjugated to magnetic nano and microparticles and exposed to target antibodies which will induce the aggregation. Such method is also promising in multiplex detection by conjugating influenza HA molecules with magnetic particles in different sizes. Each size of magnetic particles will generate a unique response T_2 for differentiating influenza subtypes. Another sensitive magnetic technique utilizing monoclonal antibodies against nucleoprotein (NP) of influenza viruses in combination with magnetic nanoparticles has enabled a large-scale real-time detection of swine influenza A H3N2 virus on a giant magnetoresistance (GMR) immunosensor (Krishna et al., 2016). The detection principle is based on the principle that stray field (magnetic field induced by the magnetization) from antibody magnetic nanoparticles that bound on sensor surface will alter the magnetization in the free layer, thus changing the resistance of GMR immunosensors. The number of targets bound to GMR sensors per unit area generate a detection signal that is proportional to the concentration of the target samples. This technique allows the detection of influenza viruses with a higher sensitivity than traditional ELISA immunoassays. Furthermore, arrays of GMR sensors facilitates the multiplex detection shown in the Fig. 13 (b). Except for the new detection methods, novel materials have been extensively developed to assemble the influenza immunosensors due to their unique materials properties. For example, single-walled carbon nanotubes (SWCNTs) with excellent electrical properties (low resistance comparable to metals) are widely used as a lab-on-a-chip sensing platform. Binding of antibody probes and influenza viruses on the surface of nanotubes changes the conductance of underlying SWCNTs thin film and thus, the resistance shifts after the target binding. The low-cost fabrication of carbon nanotubes allows the large-scale microfabrication and leads to simple and integrated device for the applications as the point-of-care on-site diagnostics (Lee et al., 2011).

4. Conclusions

Human influenza is an acute respiratory disease which is highly contagious, sometimes with continuous person to person transmission ability. Rapid detection and characterization of influenza viruses via modern sensing techniques is an important component of influenza surveillance and disease control. Rapid access to antibodies or other probe molecules that are able to differentiate various subtypes of influenza viruses is critical for the detection of newly emerging influenza strains and characterization of antigenicity for vaccine development. While current sensing techniques have achieved considerable progress, there is still an urgent demand for simple, rapid, and high-throughput methods especially in characterizing influenza antigenicity. In particular, as the number of influenza samples needing characterization continues to grow, the need for efficient methods for characterizing influenza antigenicity likewise grows. This is important for effective disease surveillance and risk preparedness, as well as diagnosis. Label-free, multiplex detection methods have demonstrated advantages in rapid and large-scale detection, and will continue to be a focus of the field. Multiplex immunosensors that are capable of mapping influenza epitopes will be of great help in understanding influenza infection, transmission, and evolution. A significant gap in current capability is that existing sensors measure binding, not neutralizing ability: immunosensors that are able to quickly measure the neutralizing ability of influenza antibodies against multiple strains would have profound

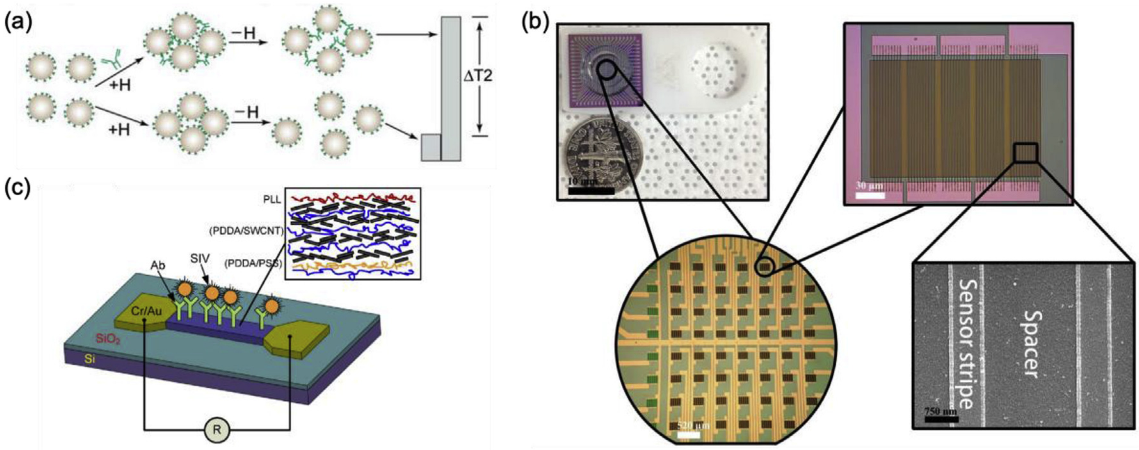

Fig. 13. (a) Magnetic field enhanced NMR-based magnetic-relaxation switches induced by the aggregation of magnetic particles due to antibody-antigen binding (Koh et al., 2008). (b) Images of large-scale arrays of magnetoresistance immunosensors for multiplex detection (Krishna et al., 2016). (c) Schematic illustration of electric immunosensors built with SWCNTs (Lee et al., 2011).

significance for influenza immunology research, vaccine development, and other clinical applications. It is worth noting that development of diagnostic assays based on immunosensors should also aim at commercializing affordable and reliable point-of-care devices to facilitate the application of biomedical care in developing countries. Finally, new and emerging immunosensors for influenza detection and antigenic characterization will have a large impact not only in influenza, but also in our response to other viral pathogens. As such, these will benefit public health and prosperity by reducing the burden of viral disease.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: B. L. Miller is a founder, shareholder, and board member of Adarza BioSystems, a company commercializing the Arrayed Imaging Reflectometry technology discussed in part of this manuscript.

CRediT authorship contribution statement

Hanyuan Zhang: Writing - original draft. **Benjamin L. Miller:** Writing - original draft.

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