

Current aspects in immunosensors

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ABSTRACT

Sensing applications can be used to report biomolecular interactions in order to elucidate the functions of molecules. The use of an analyte and a ligand is a common set-up in sensor development. For several decades, antibodies have been considered to be potential analytes or ligands for development of so-called “immunosensors.” In an immunosensor, formation of the complex between antibody and antigen transduces the signal, which is measurable in various ways (e.g., both labeled and label-free based detection). Success of an immunosensor depends on various factors, including surface functionalization, antibody orientation, density of the antibody on the sensor platform, and configuration of the immunosensor. Careful optimization of these factors can generate clear-cut results for any immunosensor. Herein, current aspects, involved in the generated immunosensors, are discussed.

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1. Introduction

Elucidating biomolecular interactions is necessary to understand the events of disease development and gene regulation. Finding techniques to report data about biomolecular interactions and to discriminate between similar biomolecules is important in the field of bioscience. In the past, probes were developed to discriminate between closely related molecules such as proteins (Doudna et al., 1995), peptide enantiomers (Michaud et al., 2003),

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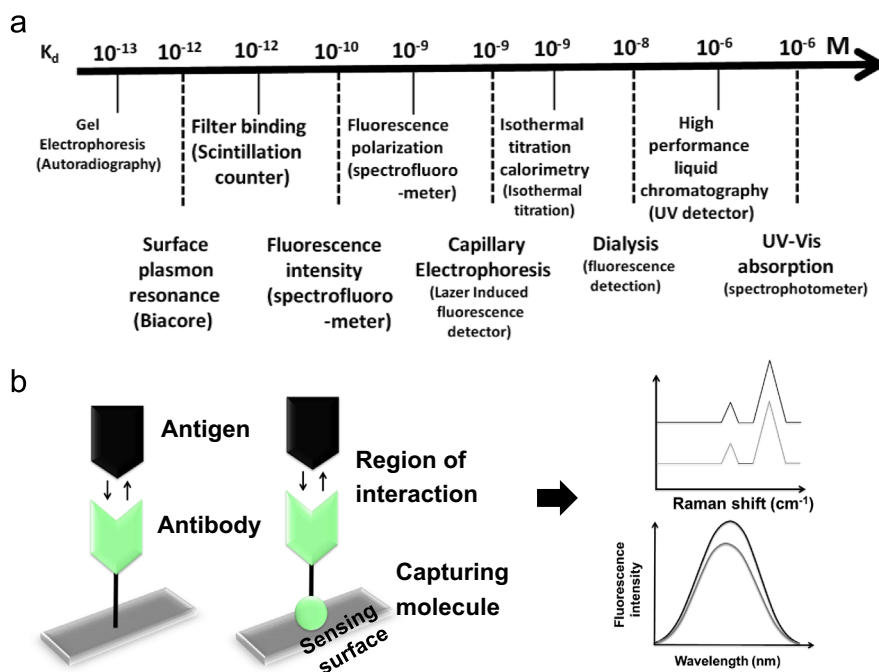


Fig. 1. (a) Dissociation constants of different sensing system (Jing and Bowser, 2011) ranging from highly sensitive systems to those with lower sensitivity. (b) General scheme for Ab-Ag interaction. Ab can be immobilized directly on the sensor surface or through the capturing molecule. Interaction of the Ab with the Ag results in the shift of the peak, for example in the Raman shift (as in surface enhanced raman scattering measurement) or fluorescent intensity shift.

and phosphorylated and non-phosphorylated forms of molecules (Seiwert et al., 2000). Current immunological detection systems include enzyme-linked immunosorbent assay (ELISA) (Watcharatanyatip et al., 2010), immunoblotting (Bahgat et al., 2009), immunosensor-based methods (Wang et al., 2009), interferometry (Gopinath et al., 2009), fluoroimmunoassay (Chen et al., 2010), methods based on surface plasmon resonance (SPR) (Gopinath et al., 2006), and immunochromatography (Watanabe et al., 2009). The major prerequisite of an effective sensor is detection and measurement of a specific target in a sample containing a mixture of compounds without compromising convenience and sensitivity. Sensitivity, which varies depending on the detection system and various test parameters, is the determining factor for the quality of the sensor (Jing and Bowser, 2011; Fig. 1a). For immunological detection systems, the crux of the sensor is the antibody (Ab) or immunoglobulin, which is the vital probe that interacts with antigen (Ag). The Ab plays an important role in the development of label-based and label-free biosensors in the pharmaceutical, biochemical, imaging, and diagnosis fields and in other fields closely related to biology.

Humans and higher animals produce natural Abs that are present in serum. In 1891, Paul Ehrlich first used the term “antibody” in his article titled “Experimental Studies on Immunity,” and it was followed by various discoveries of Abs (Lindenmann, 1984). For example, based on a large number of bovine sera samples collected in Chile and analyzed using the immune-diffusion test, the presence of Abs against bluetongue virus was revealed for the first time (Tamayo et al., 1985). Abs for use in the bioscience fields can be produced in various ways. Production of polyclonal Abs occurs in vertebrates as a part of their defensive immune response, whereas monoclonal antibodies are produced by hybridoma technology. The discovery of hybridoma technology revolutionized the production of several monoclonal antibodies for use in clinical research (Kohler and Milstein, 1975). Recent advancements in the understanding of Ab production has expedited the production of bi-specific antibodies by co-culturing bacteria that express two distinct half-antibodies. This approach, which can quickly produce a large amount Abs that can engage two different targets

simultaneously, has a potential role in Ab-based therapeutics (Spiess et al., 2013). Engineered Abs have also been introduced and investigated (Hudson and Souriau, 2003). These developments have led to the generation of several Ab-based sensors. Ab-based sensing systems have been developed with wide range of sensitivities for use in biotechnological and pharmaceutical research for target validation and disease diagnosis (Luppa et al., 2001; Ricci et al., 2007).

2. Immunosensors

An immunosensor uses Ab as the probe to detect the corresponding Ag; the Ab provide information about the interaction with the Ag based on measurable changes in the system (Fig. 1b). An Ab has different isotypes with an amine (NH_2) terminus at the Ag binding regions and the carboxyl (COOH) terminus at the stem (Fig. 2a–c). Yalow and Berson (1959) first introduced the idea of using Abs in immunosensors, described as immunoassay. Immobilization of the Ab on the solid surface was initially reported by Catt and Niall (1967). Different sensing strategies then were formulated using Ab as the probe to detect Ag (Hock, 1997 and references therein).

Table 1 summarizes the range of immunosensor available. ELISA is a sensing system that involves Ag-Ab interactions, and it is applied widely in the field of parasitology (Engvall and Permman, 1971; Voller et al., 1975). It is considered to be a gold standard assay for several infectious diseases, including HIV and influenza (de la Rica and Stevens, 2012; Schmeisser et al., 2012). Recently, Zhao et al. (2013) used ELISA to show the specificity of subtype-specific Abs against the hemagglutinin 1 (HA1) fragment of European porcine influenza viruses. The first detection of whole influenza virus as the target Ab using the SPR platform was achieved by immobilizing specific antibodies on a carboxymethylated dextran covalently attached to gold (Au) surface of a Biacore system (Schofield and Dimmock, 1996). Biacore is a SPR-based system that is widely used for immunosensing. For example, it has been used to detect the complex between HA-tagged scFv Ab

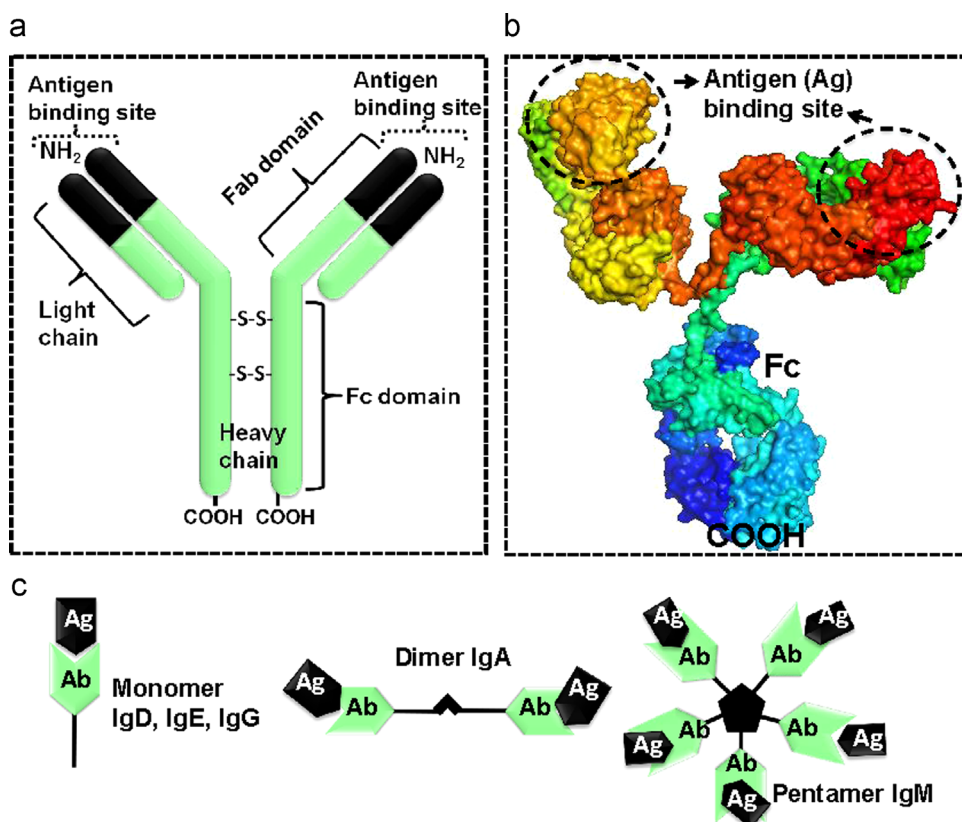


Fig. 2. Structure of an antibody: (a) diagrammatic representation of an Ab with different regions displayed; (b) three-dimensional view of an Ab (PDB code: 1IGT); (c) isotypes of an Ab. Monomers (IgD, IgE, and IgG), a dimer (IgA), and a pentamer (IgM) are shown. Circles indicate the region of interaction between the antibody and the antigen.

fragments and anti-HA Ab (Leonard et al., 2011). As an optical-based detection system, Biacore monitors the change in the angle of reflectance that occurs in close proximity to the metal surface when the immobilization of ligands and analytes take place. Another SPR-based system is Autolab, which has been applied to monitor the interaction between Ab immobilized on the Au surface and the target protein cancer antigen 125 (Dutra et al., 2007). Nomura et al. (2013) used a new prism-less SPR sensing system equipped with CCD capturing (newly designed fluorescence sensing system with V-trenched fluidic channels operates based on surface plasmon) to study the interactions of anti-influenza Ab against influenza virus and reported the sensitivity to be as low as 0.2 HA units/ml. Influenza viruses have several proteins and RNAs, therefore the expression units for detection varies with methods. Based on PCR assay the detection limit was shown to be 10^2 PFU per reaction (Poon et al., 2005). Chan et al. (2009) determined the detection limit for Swine influenza [based on tissue culture infectious dose 50 (TCID₅₀)] as log₁₀ of 3.3–4.7. With the high-speed PCR assays, the diagnosis of influenza A with an analytical sensitivity of 200-genome copies/μl was achieved (Wernike et al., 2013). Recently, Gopinath et al. (2013a) used a waveguide-mode sensor and anti-HA Ab to detect intact influenza viruses or HA of human and bird influenza viruses. Waveguide-mode sensor works in a similar manner to a SPR sensor, except that the measurement is a waveguide mode rather than a surface mode, while light intensity distribution or phase velocity is maintained. However, immobilization or interaction of the molecules with its target alters the phase velocity as a result of changes in the dielectric environment, which can be measured with high sensitivity. Dostálek et al. (2001) analyzed the interactions between human chorionadotropin and its Ab using a SPR biosensor based on an integrated optical waveguide. In other

studies using the integrating waveguide biosensor, 40 pg/ml of mouse IgG was detected (Ligler et al., 2002).

Holford et al. (2012) summarized the recent trends in Ab-based sensors, including those for electrochemical, optical, piezoelectric, and magnetic based sensors. Some of the highlights include Kee et al. (2013) use of a pan-specific antibody for direct detection of protein histidine phosphorylation in native cell lysates (using mass-spectral analyses and supported by basic immunoassays). Surface enhanced Raman scattering (SERS) garners information about the vibrational, rotational, and lower frequency transitions that occur during the process of inelastic scattering when the immobilization and interaction of biomolecules further enhance the scattering. As the proof-of-principle, interaction between anti-influenza-H1/anti-p53 Abs and the corresponding target were carried out using SERS. This multiplex detection was made possible within a single laser spot (Kho et al., 2012). For high-throughput analyses of Ab–Ag interactions on a single platform, the interferometry-based platforms BioCD and BioDVD can be used to accommodate several thousand samples at a time (Gopinath et al., 2009; Wang et al., 2011). These systems operate based on superimposition of electromagnetic waves with similar or different phases whereby similar in phases yields constructive interference, while superimposition of those with different phases results in destructive interference. Immobilization or interaction between any biomolecules affects the interference, whereby information extracted and indicates the interaction (Hariharan, 2007). Wang et al. (2011) immobilized an array of 96-spotted anti-prostate specific Ag–Abs on the surface of BioCD and showed that up to 4 ng mL^{-1} of prostate specific membrane antigen could be detected in the serum. BioDVD is also suitable for high-throughput immunosensing applications. Gopinath et al. (2009) immobilized the Ab against HA of the influenza A virus on the Au

Table 1
Characteristics and applications of immunosensors.

Target	Sensor	Substrate	Label	Sensitivity	Application	References
Label-free strategies						
Au – substrate						
Anti-capsid protein VP 1 Ab	Impedance spectroscopy	Au	Label-free	–	Detection of anti-capsid protein VP 1 Ab	Knichel et al. (1995)
Bovine insulin	SPR	Au	Label-free	100 ng mL ^{−1}	Detection of bovine insulin	Lee et al. (2005)
Cancer antigen 125 (CA 125)	SPR	Au	Label-free	–	Detection of CA 125	Dutra et al. (2007)
Influenza virus	BioDVD	Au	Label-free	10 nM	Typing and sub-typing	Gopinath et al. (2009)
Fibrinogen	Nanostructure	Au	Label-free	0.1 ng mL ^{−1}	Ag–Ag interactions	Hiep et al. (2010)
Enzyme alanine aminotransferase (ALT)	Quartz crystal microbalance (QCM) and electrochemical impedance spectroscopy (EIS)	Au	Label-free	QCM-60 ng/mL, EIS-92 ng/mL	Detection of ALT	Wu et al. (2011)
Cardiac troponin BSA	SPR	AuNP	Label-free	0.1 ng mL ^{−1}	Detection of cardiac troponin	Li et al. (2013)
	Piezoelectric- cantilever	Borosilicate	Label-free	1 pg mL ^{−1}	Sensing Ab–Ag interaction	Maraldo and Mutharasan (2007)
IgG	Quartz crystal microbalance	Magnetic nanocomposite	Label-free	15 ng mL ^{−1}	Ag–Ag interactions	Zhang et al. (2008)
PSMA	BioCD	Silica	Label-free	4 ng mL ^{−1}	Detection of PSMA	Wang et al. (2011)
Hemagglutinin (HA)	SPR	Dextran	Label-free	–	Detection of HA	Leonard et al. (2011)
Palytoxin	SPR	Dextran	Label-free	0.52 ng mL ^{−1}	Detection of Palytoxin	Yakes et al. (2011)
Glycosylated haemoglobin (HbA1c)	Electro- chemical	Glassy carbon	Label-free	4.5%	Detection of HbA1c in Serum	Liu et al. (2012)
Influenza-H1/p53 protein	SERS	SAM of 4-aminothiophenol 67	Label-free	–	Multiplex detection of influenza-H1/p53 protein	Kho et al. (2012)
Anti-hepatitis C virus (HCV) NS5A protein	Amperometric measurement	Silk fibrion	Label-free	28 μ A μ g ^{−1} mL	Detection of Anti-hepatitis C virus (HCV) NS5A protein	Moraes et al. (2013)
Strategies with labeling or tags						
Au – substrate						
IgG	Impedance spectroscopy	Au	Biotin	2 μ g mL ^{−1}	Detection of IgG	Pei et al. (2001)
IgG	Sandwich SPR	Au	Au	1 ng mL ^{−1}	Detection of IgG	Wang et al. (2009)
IgE	SPR	Au	Au	1 fM	Sensitive detection of IgE	Kim et al. (2010)
IgE	Electrochemical	Au	Au and methylene blue	0.52 ng mL ^{−1}	Detection of IgE	Wang et al. (2010)
Benzo[a]pyrene (BaP)	Amperometric immunosensor	Au	Enzymatic	5.6 ng mL ^{−1}	Detection of BaP in H ₂ O	Ahmad and Moore (2012)
Bovine leukemia virus antigen gp51	Surface enhanced Raman scattering	Magnetic AuNP	Au	0.95 μ g mL ^{−1}	Detection of gp51 in milk	Baniukevic et al. (2013)
Influenza virus	Prism-free SPR	Au	Fluorescent	0.2 HAU/ml	Detection of influenza	Nomura et al. (2013)
Anti-Brucella sp. antibodies	Fluorescence	Glass	Cyanine-5	0.005 mg mL ^{−1}	Detection of anti-Brucella sp. antibodies	Silva et al. (2004)
IgG	BioCD	Silicon	Fluorescent	30 pg mL ^{−1}	Ag–Ag interactions	Wang et al. (2008)
C-reactive protein (CRP)	Sandwich	AR chips Epoxy	Dye	10 μ g mL ^{−1}	CRP detection in serum	Pultar et al. (2009)
HA	Waveguide	SiO ₂	Dye	1 nM	Detection of influenza	Gopinath et al. (2010)
Carcinoembryonic antigen	Chemiluminescence	Mesoporous silica nanoparticles	HRP	0.1 ng/mL	Detection of Carcinoembryonic antigen	Chen et al. (2011)
Iduronate-2-sulfate sulfatase	ELISA	Polystyrene	Enzymatic	8 ng mL ^{−1}	Immuno-quantification	Sosa et al. (2011)
IgG against Helicobacter pylori	Chemiluminescence	3-aminopropyl-modified controlled pore glass (AP-CPG)	Alkaline phosphatase (AP)	0.17 U mL ^{−1}	Detection of IgG against Helicobacter pylori in serum sample	Seia et al. (2012)
Human chorionic gonadotrophin antigen (HCG-Ag)	Fluorescence	Nanoporous PtRu	Quantum dot (QD) CdTe	–	Detection of HCG-Ag	Zhang et al. (2012)
HA	Waveguide	SiO ₂	Au	8 $\times$ 10 ³ pfu/ml	Detection of influenza	Gopinath et al. (2013b)
Chloramphenicol	Lateral flow immunoassay	Nitrocellulose	Europium	0.25 ng mL ^{−1}	Detection of chloraphenicol	Xia et al. (2013)

surface of the BioDVD and estimated an equilibrium dissociation constant value of 12 nM. The primary prerequisite for any type of sensor is the immobilization of the Ab on the surface of the sensor platform.

3. Surface functionalization of Abs on the sensor surface

Molecules to be analyzed can be immobilized directly on the sensing surfaces either by physical adsorption or by chemical means. Physical adsorption can be performed by attaching molecules directly to the surface or by forming a complex with other particles. Adsorption of Ab on the surface is accomplished via van der Waals force, hydrophobic interaction, electrostatic interaction, and hydrogen bonding (Yoshimoto et al., 2010). One common example of physical adsorption is the immobilization of the Ab on the surface of microtiter plate (as in ELISA). Surface functionalization is the most important criterion for the development of faithful outcome on any sensing plate, but in most cases physical adsorption is considered to be non-specific, as no reaction occurs between residues.

Chemical immobilization can be more specific, due to the reaction between different residues of interacting molecules. The general chemical immobilization method involves immobilization of Abs or other proteins based on the reaction between amino residues on the protein and groups on the sensing surface that can react with amines such as N-hydroxysuccinimide, periodate, glutaraldehyde, isothiocyanate, and active ester (Yoshimoto et al., 2010). A common platform for sensor development is Au, whereby Au atoms can form semi-covalent bonds with the thiol-modified Ab. Hence, immobilization of Abs on the Au surface can be achieved by attaching thiol groups to the Abs (Gopinath et al., 2009). A variety of sensor plates use alkyl silanes with reactive groups such as amines, thiols, epoxides, carboxylic acids, and aldehydes (Mukundan et al., 2009). Because silicon materials are commonly used to make sensors, amine coupling remains a

popular functionalization strategy (Awazu et al., 2007; Kang et al., 2007; Wolter et al., 2007). An amine-functionalized surface can be linked with aldehyde groups, such as glutaraldehyde (Glu). Glu has two aldehyde groups that interacts with amines to form a peptide bond, which leads to immobilization of protein on the amine-containing surface (Gopinath et al., 2010).

A Glu-modified surface is recommended for smaller sized molecules due to its optimal surface chemistries that favor most of the biomolecules, as shown by atomic force microscopy images (Gopinath et al., 2012). Recently, Lakshmi Priya et al. (2013) used polyethylene glycol (PEG) polymers on a Glu-modified surface to develop a strategy with high sensitivity and specific detection. Self-assembled monolayers (SAMs) have also been shown to be important for surface modification and highly beneficial for higher specific reactions on the sensing surface to capture Ab (Iyer et al., 2004; Nomura et al., 2013). SAMs are formed by spontaneous absorption of macromolecules containing the functional groups on the surface, especially on the Au surface. The functional groups (e.g., COOH) can interact and ensure the immobilization of Ab onto the surface. Application of a SAM can maintain the surface properties (such as surface charge) so as not to disrupt the orientation of the immobilized Abs (Chen et al., 2003). Nomura et al. (2013) used COOH-SAMs to immobilize anti-influenza Abs on an Au surface generated for prism-free SPR-sensor surface. The suitability of surface modifications for different molecules was evaluated using different sizes of gold nanoparticles (AuNP) conjugated with streptavidin. Among the different surface chemistries tested, *N,N'*-carbonyldiimidazole (CDI) was found to be best for molecules with higher molecular weight (more than 20 nm). Due to the suitability of the CDI surface for larger sized molecules, influenza viral (100 nm in size) detections have been performed using Ab (Gopinath et al., 2013a, 2013b). Although different surface chemistries have been proposed, the sensitivity of Ab-based sensors is highly dependent on the orientation of immobilized antibodies.

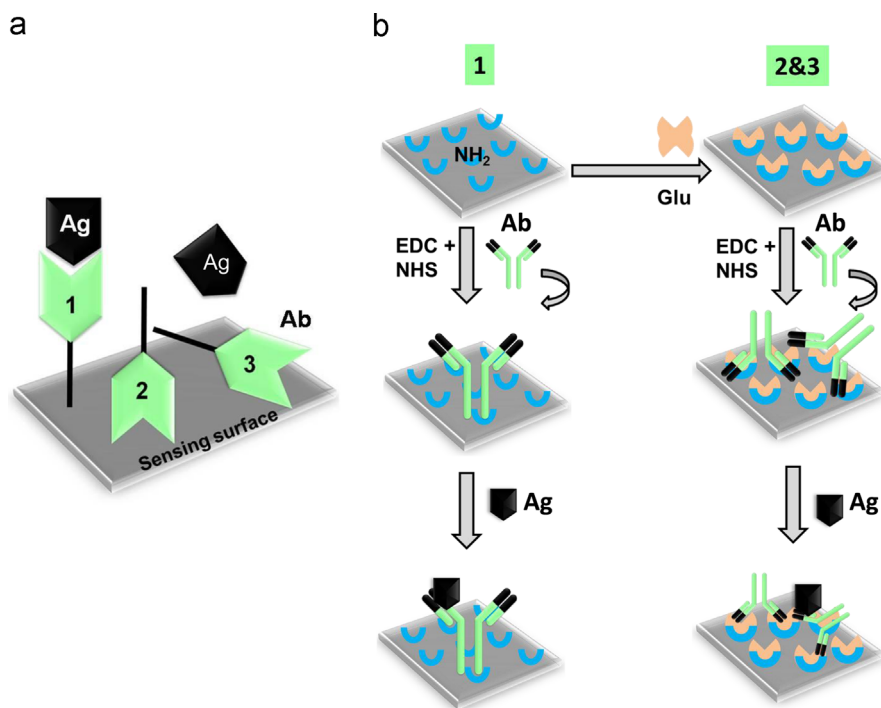


Fig. 3. Orientation of antibodies on the sensor surface: (a) possible orientation of Abs on the sensing surface are (1) totally exposed for Ag binding, (2) non-exposed for Ag binding, and (3) partially exposed for Ag binding; (b) Activation of the antibody with EDC and NHS results in the formation of NHS ester intermediate. This NHS ester intermediate is highly reactive and reacts with amines of antibody, which promotes its immobilization. In the presence of glutaraldehyde (Glu), the amine group of the antibody that reacts with Glu leads to antibody immobilization. However in this type of immobilization, the immobilized antibodies are partially exposed or non-exposed for antigen binding.

3.1. Orientation of immobilized Abs on a solid surface

Very few commercial applications of immunosensors have been implemented due to unresolved issues regarding immobilization, quantity, conformation, density, and orientation of the Abs to achieve stable interaction with Ag (Luppa et al., 2001). These factors depend on the immobilization technique, surface characteristics of the Ab and the sensing surface (Yoshimoto et al., 2010). Proper orientation of Ab or other ligands immobilized on the surface is expected to yield better performance for any given sensor (Chen et al., 2003). Extensive analyses of the proper immobilization and orientation of Ab are available in the literatures (Jung et al., 2008; Trilling et al., 2013). Generally, three possible ways of Ab immobilization in terms of orientation on solid sensing surface viz., (1) total exposure of the fragment antigen binding region (Fab); (2) non-exposure of the Fab; and (3) partial exposure of the Fab (Fig. 3a). An amine-containing sensing surface often is used to immobilize the Abs with proper orientation through linkers, such as *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride, via linkage to the carboxyl-terminus of the Abs. EDC–NHS based coupling method converts antibody into NHS ester intermediate. This NHS ester intermediate is highly reactive, forming peptide bond with amine-functionalized surface, which facilitates Ab immobilization. The scheme for this site-specific chemical reaction was also shown by Vashist (2012). With these linkers, the Ag binding region of the Ab is exposed, leading to highly specific and sensitive binding. This can be evaluated on Ab-immobilized amine or Glu surfaces followed by the interaction with the antigen (Fig. 3b). For example, Trummer et al. (2001) analyzed the interactions of bovine serum albumin (BSA) and its Ab on amine surfaces of integrated optical waveguide mode sensors.

Another way to achieve the proper orientation is by capturing the fragment crystallizable (Fc) region of the Ab using an aptamer or another probe on the sensing surface (Hiep et al., 2010). Similarly, protein A and G can be used as the capturing agent to correctly orient the Abs, as these protein bind the Fc region of the Abs (Saha et al., 2003). Chen et al. (2003) analyzed the orientation of immobilized antibodies on amine- (positively charged) and carboxyl- (negatively charged) terminated SAMs and concluded that IgG1 had better orientation on amine-terminated SAMs. However, they have also found that IgG2 had equal adsorption on both surfaces, thus concluded that this variation was due to surface charges of the Ab whereby the charges controlled the orientation of the immobilized antibodies. Due to variations in the charges, IgY was found to have better orientation on the negatively charged polymers (Davalos-Pantoja et al., 2001). Ventura et al. (2011) used UV irradiation with the antibody (i.e., light-assisted antibody immobilization) to demonstrate that tryptophan residues absorb irradiation strongly and disrupt the nearby disulfide bridges that form thiol groups. These thiol groups interact with the Au surface, which leads to the proper orientation of Ab on the surface. Beside, polymers such as dextran and PEG also play a major role in properly orienting molecules. By blocking the polymers [poly(ethylene glycol)-*b*-poly(acrylic acid) (PEG-*b*-PAAc) and pentaethylenhexamine-terminated PEG (N6-PEG)] on the SiO₂ surface, proper alignment of biomolecules was actualized, and this was accompanied by improvement of sensitivity up to 1000-fold (Lakshmi Priya et al., 2013).

3.2. The role of polymers in minimizing biofouling of immunosensors

A good immunosensor should minimize biofouling in order to achieve high performance and enhance the signal to noise ratio. Currently, several blocking agents are available to improve specificity via proper alignment of the ligand on the sensing surface

(as described above). However, common blocking agents such as BSA, ethanolamine, and casein are not suitable for all sensing surfaces, especially the Au surface. As an alternative, several polymers can be used to alleviate non-specificity. For example, (Nagasaki, 2011, and references therein) described synthetic polymers that reduce non-specific binding on sensing surfaces. Densely packed PEG tethered-chain surfaces have been shown to have high resistance to biofouling (Kamimura et al., 2008; Yuan et al., 2009; Yoshimoto et al., 2009; 2010). Furthermore, the combination of long and short PEG dual chains greatly reduces non-specific adsorption of proteins onto the sensing surface (Uchida et al., 2005). This characteristic was evident with the upsurge in the sensitivity shown for Abs co-immobilized on the mixed-PEG chain surface (Nagasaki et al., 2007; Yoshimoto et al., 2009). These researchers have designed different PEG block copolymers, such as PEG-*b*-polycations, PEG-*b*-polyanions, and PEG-*b*-hydrophobic polymers, that have proven to be versatile materials for immunosensing applications. Using streptavidin-installed ytterbium and erbium ion-doped Y₂O₃ with co-immobilization of PEG polymer, Kamimura et al. (2008) monitored specific interactions with biotinylated Abs. Due to bio-compatibility, chemically inert, dispersible, flexibility and hydrophilic nature of PEG polymers are highly suitable for non-fouling interaction of whole or partial fragments of Abs not only on ytterbium and erbium ion-doped Y₂O₃, but on the sensing surfaces.

4. Signal enhancements

4.1. Emission assisted signaling

Common ways of augmenting sensitivity involve the use of fluorescent and chemiluminescent labeling (Fig. 4a). Fluorescence is the most common method, and widely used because it is sensitive and non-destructive. Fluorescence derives from fluorophores such as fluorescein isothiocyanate, rhodamine, coumarin, and cyanine, which permit real-time monitoring of Ab–Ag interaction. An immune-homogeneous assay with fluorescent-labeled Abs (with fluorescence energy resonance transfer) was described by Zeng et al. (2012). Using mouse monoclonal anti-goat IgG conjugated to cyanine-5, IgG was detected using optical fibers. As the proof-of-principle, the authors further demonstrated that anti-*Brucella* sp. antibodies could be detected in the range of 0.005–0.11 mg ml^{−1} (Silva et al., 2004). Using alkaline phosphatase enzyme-conjugated secondary antibodies and laser-induced fluorescence, quantitation of the IgG antibodies against *Helicobacter pylori* (the causative agent of peptic ulcers and gastritis diseases) was achieved. The portable system has a detection limit of 0.17 U mL^{−1} (Seia et al., 2012). Another portable system is based on chemiluminescence, in which the substrate luminol is catalyzed by the enzyme horseradish peroxidase (HRP) to form 3-aminophthalate. If a system with a longer fluorescence lifetime, higher and near uniform fluorescence quantum yield, and less susceptibility to environmental quenching is needed, Quantum Dot (QD) is a better alternative than systems based on fluorophores (Bogomolova and Aldissi, 2011). QD are characterized by their long fluorescent lifetime, resulting in narrow emission spectra with broad excitation range with high and near uniform fluorescence quantum yield.

4.2. Nanoparticle assisted signal amplifications

Among the different metal nanoparticles (NPs) available, AuNP and AgNP (silver nanoparticles) are commonly used in immunosensor development (Fig. 4b). An extensive overview of AuNP-conjugated Ab-based sensors was published recently (Omidfar et al., 2013). NP-conjugated immunosensing includes

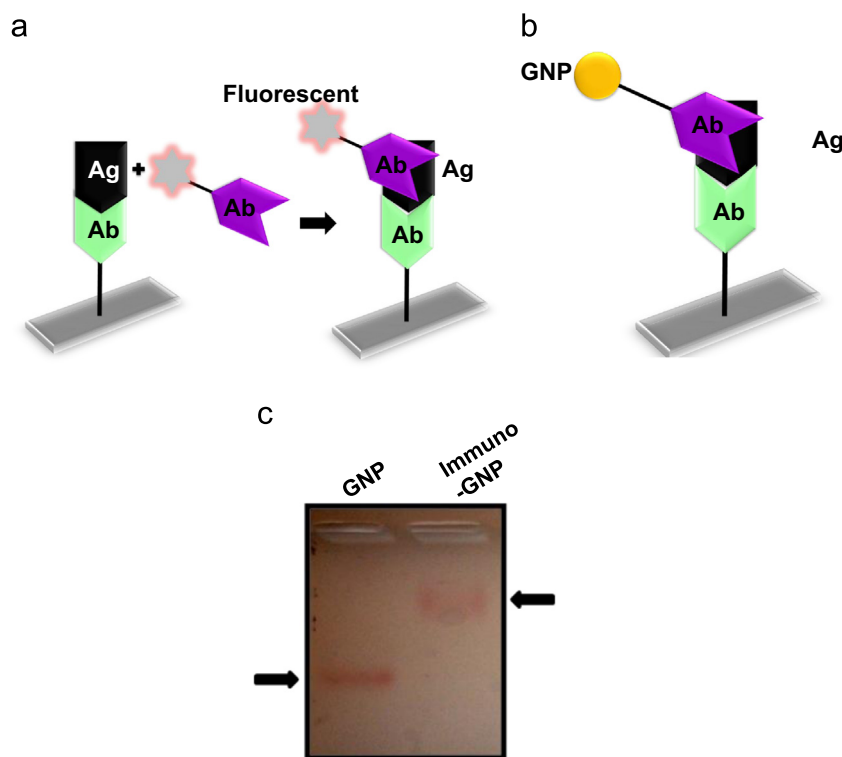


Fig. 4. Signal amplifications on immunosensor: (a) signal enhancement with fluorescent labeled Ab. Addition of the fluorophore-labeled Ab that reacts with antigen results in the fluorescence emission; (b) signal enhancement with AuNP-conjugated Ab; and (c) mobility of AuNP and immune-AuNP on 1% agarose gel (arrows indicate band position).

Au (Chen and Tang, 2007; Ko et al., 2009; Gopinath et al., 2013a), inorganic NPs (Guo and Dong, 2009), platinum NPs (Zhang et al., 2010), europium NPs (Xia et al., 2013), and AgNPs (Dawan et al., 2011). Increasing the particle size increases the surface area to accommodate more Abs, which ultimately improves sensitivity (Chen et al., 2009). Lou et al. (2012) analyzed Ab attachment to different sizes of AuNPs (14, 16, 35, and 38 nm) and found that a lower amount of Ab was required to coat the smaller AuNP (16 nm). Furthermore, the molecular size of the AuNP-conjugated Ab is usually higher compared to that of the bare AuNP, and the former migrates more slowly than the latter (Fig. 4c). Surface enhanced infrared absorption on AuNP films deposited on SiO₂/Si and shown with enhanced signal for Ab–Ag reaction (Enders et al., 2006). Using AuNP conjugates, a 10⁶-fold increase in sensitivity was recorded for the interactions of aptamer–IgE–Abs (Kim et al., 2010). Influenza viruses or viral proteins with 10 nm AuNP-conjugated Abs have been used to analyze subtypes of influenza viruses, which were detected at a concentration of 8×10^3 pfu/ml (Gopinath et al., 2013b). In one investigation, HRP and Ab against carcinoembryonic antigen were immobilized on the surface of mesoporous silica nanoparticles, and this system enabled the detection of the Ag at up to 0.1–40 ng/mL (Chen et al., 2011). To detect cardiac troponin, biotinylated Ab against this protein was immobilized on the surface of luminol functionalized AuNP (Li et al., 2013). The streptavidin coated AuNPs on the modified Au electrodes formed a linkage with the biotinylated Ab, which subsequently interacted with cardiac troponin, and the detection limit reached up to 0.1 ng mL^{−1} (Li et al., 2013). Although metal NPs with higher diameter are expected to improve the sensitivity of the system, Gopinath et al. (2013b) demonstrated that larger sized AuNPs are more prone to non-specificity compared to their smaller counterparts. Lou et al. (2012) also reported that high non-specificity was associated with larger sized AuNPs.

This usage of metal NPs is the basis of the immunochromatography test (ICT), which is a rapid diagnostic system for direct

visualization (Sun et al., 2013). In this system, binding of the Ag to the Ab-conjugated metal NPs results in signal amplification, which is accompanied by production of colored bands. ICT is used commonly in home diagnostic tests. For example, the first ICT-based home pregnancy test, which was commercialized in 1960, was designed to detect human chorionic gonadotrophin (HCG) (Kohn, 1968; Holford et al., 2012). Recently, europium NP-based lateral flow immunoassay was proposed for the sensitive detection of chloramphenicol (Xia et al., 2013). For enhanced signal amplification, QD CdTe was immobilized on the surface of nanoporous PtRu alloy together with Ab against HCG–Ag. The Ab was linked with Glu for immobilization on the surface of chitosan-coated Fe₃O₄ magnetic NPs. The high magnetic property of the NPs permitted the assembly and separation of the Ab on the surface of indium tin oxide glass; the signal amplification conferred by QD was approximately 4.67-fold higher than that of the signal from the unamplified QD (Zhang et al., 2012).

5. Different sensing mechanisms

5.1. Sandwich and competitive immunoassays: efficient configuration of immunosensors

Sandwich immunoassays are currently the most commonly and successfully used, mainly due to their high sensitivity and minimized background signal. Sandwich immunoassays can be performed on any kind of sensing surface. The main criterion of these assays is the availability of two probes with different binding sites on the Ag. With this criterion, it is important to choose these probes from totally different sources such as (i) Abs developed from different host animals (mouse and rabbit) for a single Ag; (ii) Abs of different serotypes (IgG and IgY) for the same Ag (Fig. 5a); (iii) a combination of an Ab and another probe, such as an aptamer

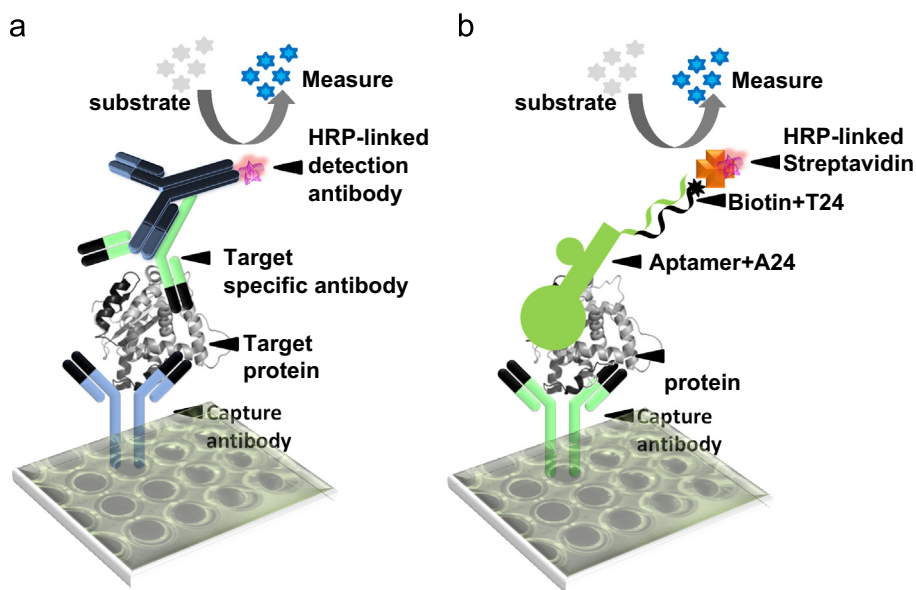


Fig. 5. Strategies for sandwich assay: (a) Ab–Ag–Ab sandwich on ELISA and (b) Ab–Ag–aptamer sandwich on ELISA. The complete procedure using different molecules is shown.

(Fig. 5b); or (iv) a receptor protein and an Ab for a single Ag. In a sandwich immunoassay, a specific capture Ab against the Ag to be assayed is bound to a sensing surface. A second labeled Ab then is used for detection of the Ag; this Ab is specific for a different epitope on the Ag, which enhances the overall specificity of the assay. As an example, Sosa et al. (2011) developed an immune quantification assay for human and recombinant iduronate-2-sulfate sulfatase using rabbit IgG and chicken IgY as the capture and detection antibodies, respectively.

In a different scenario, Pultar et al. (2009) used an aptamer–Ab sandwich immunoassay to detect C-reactive protein in human serum samples. Dubbed chemical antibodies, aptamers are ssDNA/RNA that can bind to a wide variety of targets with high binding affinity. Because aptamers and Abs behave in very similar way, these two molecules can complement each other in the development of the sandwich pattern of target detection. Wang et al. (2009) developed an Ab–Ag–aptamer sandwich assay using the SPR platform. Sensitivity lower than 1 fM was reported for the interactions of aptamer–IgE Ab-conjugated with AuNP on the Au surface of the SPR platform (Kim et al., 2010). Recently, we developed the sandwich Enzyme-Linked Aptamer Assay for Erythropoietin (EPO) using both the anti-EPO Ab and the RNA aptamer against EPO; the detection limit of this assay was 0.29 nM (unpublished data). By applying PEG/Ab co-immobilized magnetic beads combined with alkaline phosphatase-assisted fluorescent system, a sandwich ELISA pattern was developed using alpha-fetoprotein as the Ag (Nagasaki et al., 2007). In another study using SERS, the sandwich immunoassay was demonstrated to detect bovine leukemia virus antigen gp51 at a concentration of $0.95 \mu\text{g mL}^{-1}$ (Baniukevic et al., 2013). These sandwich patterns are more applicable for competitive immunoassays for screening of better molecules. Several biological ligands impart functions by the specific interactions with other molecules. Compounds that are able to dissociate the complexes (antibody–antigen) by competition can act as potential molecules to block the interactions of the antigen/antibody. This strategy is useful to find the suitable inhibitory molecules from the library of molecules that can have better affinity against the target compared to the originally bound ligands. Moreover, high-throughput analyses can be designed with library of molecules with competitive immunoassays. Competition between antibody and small molecule will generate an effective

competitive assay, due to differences in sizes of the molecules. Modeling which represents fractional sensitivity analysis of a competitive microfluidic heterogeneous immunoassay for drug phenytoin, a small molecule was shown (Fu et al., 2009). The fractional sensitivity analysis can be used to identify system parameters, which is vital for the control and quantification of the variability between assays. Another application of fractional sensitivity is for the identification of operating ranges for the parameters that enhances sensitivity. Sandwich and competitive heterogeneous assay formats were outlined by Zeng et al. (2012). Recently, Wang et al. (2013) have formulated indirect competitive immunoassay for the detection of vitamin B12.

5.2. Ab fragments in immunosensors

Abs made up of heavy and light chains have different regions such as Fc and Fab. These regions can be fragmented from the Ab by proteolytic enzyme digestion (pepsin or papain) and dithiothreitol-mediated reduction of the disulphide bridge in the Fab region (Holford et al., 2012). Several immunosensing strategies have been developed that harness these fragments obtained from different Abs (Nissim et al., 1994; Toth et al., 1999; Charlton et al., 2001). Both Fc and Fab regions represent the most common regions for developing immunosensors, and they are used to immobilize Abs exposed to Ag binding regions (Fig. 6). Capturing these Abs using an aptamer, protein A, or protein G that can target the Fc region of the Ab is also feasible (Saha et al., 2003; Hiep et al., 2010). Hiep et al. (2010) generated a RNA aptamer that can bind the Fc region of an Ab and further used this aptamer to interact with the Ab on an Au-capped NP. Abs contains glycosylation sites on the Fc domain that can be easily oxidized and subsequently biotinylated with biotin hydrazide (Yoshimoto et al., 2010). These biotinylated antibodies can readily react with streptavidin. Splitting of the Ab fragment produces native sulfanyl (SH) groups, which readily react with the Au surface or any SH reactive linker terminated substrate. The SH group and Ag binding region on the Fab are situated structurally on the opposite side, which leads to highly orientated immobilization of the Ab (Yoshimoto et al., 2010). Covalent attachment of the SH group of the Fab fragment to the SH reactive linker terminated substrate was accomplished in

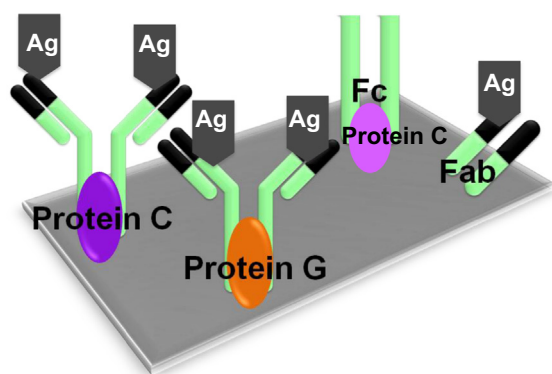


Fig. 6. Strategies to capture sensing Abs on the sensor surface. Antibody capturing by protein C or G is shown. Fc and Fab are immobilized, which places them in the proper orientation to capture Ag.

the past, substrate includes silica (Lu et al., 1995) and Au (Harada et al., 2000; Kim et al., 2007) as demonstrated by atomic force microscopy (Harada et al., 2000). Yoshimoto et al. (2010) performed expanded analyses of the interactions of C-reactive protein with the Fab fragment of an Ab immobilized on an Au surface (with time-dependent changes) in the presence of polymers on the SPR platform. Lee et al. (2005) detected bovine insulin using Fab on the Au surface of the SPR chip with a sensitivity of 100 ng mL^{-1} .

Polyclonal Abs can be purified using a matrix immobilized with Protein A or G, as these proteins can interact with the Fc domain of the Abs (Fig. 6). Chien et al. (2007) analyzed the interactions of protein G and an Ab using a coupled waveguide SPR biosensor with sub-wavelength grating. They obtained a sharper resonance, which implies that the sensor provides precise measurements. The resolution achieved was up to 0.01 nm , which is even better than that demonstrated by Cunningham et al. (2002) who applied two-dimensional diffraction grating structure in detecting biochemical molecules with a resolution of 0.1 nm for protein thickness measurement. Bae et al. (2005) studied the orientation of immunoglobulin G on a protein G layer, and Huang et al. (2006) used a miniaturized germanium-doped silicon dioxide-based SPR waveguide sensor to study for the interactions of protein A, monoclonal Ab, and Ag (avian leucosis virus). In another approach, Torrance et al. (2006) used the single chain variable fragment (ScFv) for an immunosensor based on the SPR platform. ScFv was developed in 1980 as an alternative to whole natural Abs. It can be engineered with metal binding amino acids in the peptide linker and with positively charged amino acids so that it can be easily immobilized on sensing surfaces with negative charges, such as SAMs (Zeng et al., 2012). Zeng et al. (2012) described the development of immunosensors designed using the heavy chain variable region of Abs.

5.3. Peptide-based immunosensors

Short peptides that are also specific against the target Ag/Ab can be another alternative to Ab fragments. Knichel et al. (1995) carried out one of the earliest investigations of this premise; they immobilized a peptide of the amino acid (position 135–154) of the capsid protein VP 1 functionalized with alkanethiol on the surface of Au electrodes. Subsequent interaction with the target protein was monitored via impedance spectroscopy (Knichel et al., 1995). It measures the dielectric properties of a medium as a function of frequency, based on the interaction of an external field with the electric dipole moment of the sample containing interactive molecule. Another immunosensor was developed using peptide fragment NS5A-1 of the hepatitis C virus (HCV) NS5A protein

(Moraes et al., 2013). Immobilization of the peptide on the surface of silk fibroin films was monitored via UV-vis-absorption measurement. Interaction with the target protein (anti-HCV Ab) was achieved via amperometric measurement with the sensitivity of detection reaching up to $28 \mu\text{A } \mu\text{g}^{-1} \text{ mL}$ (Moraes et al., 2013). Recently, Shi et al. (2013) developed a peptide antigen-based multicolor fluorescent AuNP immunosensor. In this study, three peptides against the target (leucine-enkephalin, prion protein and human cardiac troponin I) that were labeled with three different fluorophores were allowed to react with Abs conjugated to AuNPs. In the presence of the target, the peptide antigens were released due to competition, resulting in stronger fluorescence readout for sensitive detection. This approach is simple and can provide highly sensitive data for multiplex detection at a low cost. Via a process known as peptide phage display (Clackson and Lowman, 2004), a peptide fragment that has affinity against a target protein can be produced on the surface of a bacteriophage. Phage display is one of the widely used techniques for the study of protein–protein, protein–peptide, and protein–DNA interactions. In this method, gene that encodes the protein of interest is inserted into the phage coat protein gene, which causes the phage to “display” the protein of interest on its surface. These displaying phages can then be screened against library of proteins, peptides or DNA sequences. For example, a peptide fragment that is specific against the enzyme alanine aminotransferase (ALT), a biomarker of hepatotoxicity, was selected by M13 phage display (Wu et al., 2011). This peptide fragment (ALT5–8) was conjugated to cysteine and was immobilized on the Au surface of the quartz crystal. The interaction of the peptide fragment with the target protein ALT was monitored by quartz crystal microbalance (QCM; measures a mass per unit area by relying on the alteration of the frequency of a quartz crystal resonator) and electrochemical impedance spectroscopy (EIS). The limit of detection for QCM and EIS was 60 ng/mL and 92 ng/mL , respectively (Wu et al., 2011).

Peptide aptamers (peptamers) are short peptide sequences can be selected from an initial library of molecules containing different peptide sequences. Like Abs, peptamers bind the target protein with high affinity and specificity. In one study, a peptamer against cyclin-dependent protein kinases that was immobilized on the surface of Au electrodes enabled the detection of the target protein in whole cell lysate (Evans et al., 2008). Peptamers can specifically distinguish isoforms in an array format based on changes in electrical measurement. As each of the microelectrodes can be individually conjugated with these peptamers, change in the electricity is distinct for each of the peptamer-isoform interactions, which permits differentiation of the isoforms. The interaction of the individual peptamer with its target is based on the subtle alteration in the electrical impedance measurement. As the proof-of-concept, lysates containing CDK2 and CDK4 which belong to the human cyclin-dependent protein kinases (CDKs), were distinguished by electrical impedance measurement with the values of 11 and 12.5 mA when bound by the peptamers, respectively.

6. Future perspectives: use of immunosensors for point-of-care diagnostics

Immunosensors have been applied in biotechnological and pharmaceutical research for target validation and disease diagnosis. As stated by Holford et al. (2012), for any immunosensor to play a significant role in monitoring analytes, it must be of low unit cost, subjectable to automation, reusable, and easy to use. Moreover, immunosensors, which are portable, should work in tandem with the Point-of-care testing (POCT) scheme that enables patients to be diagnosed anywhere from bedside to the playing field. Portable immunosensors such as ICT are the core of POTC, as

diagnosis can be performed in rural areas where resources are scarce. Results interpreted based on color changes, such as AuNP-based colorimetric assay (controlled assembly and disassembly of antibody conjugated AuNP, upon binding of antigen) or immuno-chromatography test (lateral-flow of antibody conjugated AuNP and its interaction with antigen), can prompt decisions to be made instantly, thereby allowing immediate administration of appropriate treatment. In addition, enhanced sensitivity is of paramount importance, and this criterion can be met by using aptamers, which are alternative probes that can complement Abs in sandwich-based detection systems. Other alternatives to Abs, such as Ab fragments and peptide aptamers, are also paving the way for efficient sensor development.

Acknowledgments

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