Pankaj Suman
Pranjal Chandra *Editors*

Immunodiagnostic Technologies from Laboratory to Point-Of-Care Testing

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Biosensors for Clinical Samples: Consideration and Approaches

Mihaela Tertis, Oana Hosu, Anca Florea, and Cecilia Cristea

Abbreviations

Ab	Antibody
AD	Autoimmune diseases
Ag	Antigen
AGA	Antigliadin antibodies
anti-CPP	Anti- citrulline peptide
anti-tTG	Anti-transglutaminase antibodies
AFP	Alpha fetoprotein
AChE	Acetylcholinesterase
AP	Alkaline phosphatase
APTES	3-aminopropyltrietoxy silane
Apt	Aptamer
ASV	Anodic stripping voltammetry
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
c-Myc	Myelocytomatosis oncogene
cTnT	Troponin-T
cTnI	Cardiac Troponin-I
CA125 (MUC16)	Cancer antigen 125
CA 19-9	Carbohydrate antigen 19-9
CEA	Carcinoembryonic antigen
CA125	Carcinoma antigen 125
CK-MB	Creatine kinase MB

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CK-MM	Creatine kinase MM
CNT	Carbon nanotubes
CSF	Cerebrospinal fluid
CRP	C-reactive protein
CV	Cyclic voltammetry
CVD	Cardiovascular diseases
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assay
DDAB	Didodecyldimethylammonium bromide
Den	Dendrimer
DPV	Differential pulse voltammetry
EGFR (ErbB-1; HER1)	Epidermal growth factor receptor
EIS	Electrochemical impedance spectroscopy
GADA	Glutamate decarboxylase antibody
GCE	Glassy carbon electrode
GO	Graphene oxide
hCG	Human chorionic gonadotropin
HER-3	Human epidermal growth factor receptor 3
HE4	Human epididymis-specific protein 4
HIgG	Human immunoglobulin G
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IgB	Immunoglobulin B
IgG	Immunoglobulin G
IL-8 (CXCL-8)	Interleukin 8
IL-17	Interleukin 17
ITO	Indium tin oxide
LF	Lateral-flow
LFTSs	Lateral-flow test strips
LSW	Linear sweep voltammetry
MBP	Myelin basic protein
MBs	Magnetic beads
MBP	Myelin basic proteins
MDM2	Murine double minute 2
MH	Mercaptohexanoic acid
MIF	Macrophage migration inhibitory factor
MPR	Myeloperoxidase
MS	Multiple sclerosis
MUC4	Mucin 4
Myo	Myoglobin
MWCNT	Multi-walled carbon nanotubes
ND	Neurological disorders
NSE	Neuron-specific enolase
NT	Neurotransmitters
PB	Prussian blue

PEI	Polyethylenimine
PMMA	Polymethylmethacrylate
POC	Point-of-care
POD	Peroxidase
PSA	Prostate specific antigen
PtNPs	Platinum NPs
QD	Quantum dot
RA	Rheumatoid arthritis
RF	Rheumatoid factor
rGO	Reduced GO
RIA	Radioimmunoassay
SAM	Self-assembled monolayer
SCCA	Squamous cell carcinoma antigen
SPE	Screen-printed electrode
SCC-Ag	Squamous cell carcinoma antigen
SPR	Surface plasmon resonance
SWV	Square wave voltammetry
SWSV	Square wave stripping voltammetry
TMB	3,3,5,5-tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
tris(Ru(bpy))	Tris (4,4′-dicarboxylicacid-2,2′-bipyridyl) ruthenium(II) dichloride
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
uCTX-II	Collagen type II
β-CD	β-cyclodextrin
μPAD	Microfluidic paper analytical device

1 Introduction

Biosensors are sensing devices made up of a recognition element for the target compound and a transducer. From this category of devices, immunosensors are probably the most reported type of biosensors. In this case, antibodies are the biorecognition elements, while the detection is related to the interactions between antibodies and their specific antigens.

In the case of electrochemical immunosensors, the highly sensitive and selective biological signal is converted and measured at the electrochemical transducer level. Low cost, easy manipulation and handling, simple instrumentation, fast response, easy miniaturization and integration into portable platforms, as well as the possibility to be applied for multiple target detection are some of their advantages (Chandra 2016). These important properties qualify electrochemical immunosensors for

analytical applications in biomedical (pharmaceutical and clinical), environmental, food and forensic fields. Electrochemical detection may be performed both directly and indirectly usually by the means of labels. Such examples are redox enzymes that have been intensively used as catalysts for the electrochemical reactions at the electrode. These compounds are expensive and poorly stable, thus are being replaced lately in sensors with other electroactive compounds or catalytic active materials with improved stability.

The technological development recorded in the last decade has determined explosive development in the field of biosensors and immunoassay technology, particularly progress being registered in clinical diagnosis and disease monitoring. Currently, the information used both in doctors' offices and in hospitals for diagnostic is obtained mainly using the facilities of centralized and specific laboratories. However, future plans of decentralization and simplification of the diagnostic process are desperately needed, which is to be achieved with the so-called point-of-care (POC) testing systems. These devices must present some important features such as small size, easy operation system, fast response time for qualitative and quantitative (semi-quantitative) signal evaluation, high portability and the possibility of being used at the patients' bedside or outside of clinics. The worldwide demographic expansion, the global trend and the continuous progress in medicine determined the continuous development in POC. Thus, huge efforts have been made in this direction and the World Health Organization (WHO) has introduced the so-called ASSURED criteria (A = Affordable; S = Sensitive; S = Specific; U = User-friendly; R = Rapid and Robust; E = Equipment-free and D = Deliverable to end-user) (Zhu et al. 2019).

One important topic in electrochemical immunosensor's field is the use of nanomaterials and nanocomposites with improved properties that can increase the stability and provide a bigger active surface with increased sensitivity due to the higher density of antibody molecules at the sensor surface. The biocompatibility of the system is also increased. Several materials were applied for the development of electrochemical immunoassay, some suggestive examples are: carbon nanotubes (CNTs), gold nanoparticles (AuNPs), platinum nanoparticles (PtNPs), Au-Fe_3O_4 NPs, silica NPs, graphene oxide-chitosan, ionic liquid-CNTs and silver nanoparticles (AgNPs) (Suresh et al. 2018).

Within this chapter, the most recent progress in the electrochemical immunosensors field of research was highlighted, especially those with implications in autoimmune diseases, cancer, cardiovascular and neurological disorders. Therefore, two important common points were followed in the study of the research articles selected for this paper: the use of antibodies for immunosensors elaboration and the use of electrochemical detection technique in the above mentioned bioclinical applications. Hence, immunosensors and electrochemical theoretical principles, immobilization strategies and detection methods are presented.

1.1 Principles for Immunoassays and Immunosensors Development

1.1.1 Strategies for Antibody Immobilization

An important issue in designing effective immunosensors is the choice of immobilizing the antibody in order to preserve its reactivity by avoiding bad orientation, steric hindrance or even, denaturation (Hosu et al. 2018a). Currently, for accurate antibody immobilization, few procedures could be chosen such as electrostatic interactions, specific interactions between biomolecules (biotin–avidin, protein A or G, etc.), chemisorption by thiol groups, covalent coupling with modified electrode surfaces. The nature of the working electrode surface could affect the procedure immobilization considering that metals or different forms of carbon based materials are used. For clinical application screen-printed electrodes (SPEs) or wearable electrodes printed on different surface are also used (Ciui et al. 2018a). Irrespective of the nature of the electrodes, the use of solid phase for antibody immobilization can lead to hindrance in electron transfer, reduce voltammetric signal (and a loss of sensitivity), passivation or fouling, etc. To overcome those problems and to improve the sensitivity of the system, the usage of nanoparticles (metallic or magnetic) for bioelements immobilization or the immunoreactions has been intensively used (Săndulescu et al. 2015). The main advantages of the micro and nanoparticles are represented by their large surface area which increases biomolecule loading.

1.1.2 Types of Immunosensor Format

Two types of immunosensor formats are currently used: labeled and non-labeled or label free. In the first case, the assays are classified into competition, sandwich and displacement formats. The main difference consists in the immobilized species, such as antibody, antigen or hapten conjugate and the number of layers to be used. In order to choose the optimum approach is important to consider all the parameters that influence its analytical performances: the nature of the analyte, availability of antigen and antibody for the required sensitivity.

In the case of label-free immunosensors their simplicity gains over the loss of sensitivity. Their rapidity in response as well as the type of immunological reaction that takes place combined with the response time attracted the researchers and represents good promises for clinical applications.

When the molecular target has multiple binding sites capable of binding several antibodies at the same time, the sandwich assay is preferred. In a direct sandwich assay, a primary antibody is immobilized on a solid phase, and then the antigen is added. Thereafter, the solid phase is washed to remove the unreacted components and then incubated with a secondary-labeled antibody, to induce a second affinity reaction with the antigen. After the washing step, the amount of secondary antibody which was immobilized on the solid phase is detected being directly correlated with the antigen amount in the sample (Hosu et al. 2018b).

When the target has only one binding site the competitive assay is generally used, this approach is based on a competition reaction between two reagents for a third.

In an indirect competitive assay, the immobilization of a spacer-linked antigen is used for the solid phase modification, and then the labeled antibody and the free antigen are added. The solid phase bound and free antigens present in the tested solution compete for the antibody-binding site. The extent of the affinity reaction is detected by adding a secondary-labeled antibody able to bind the antibody immobilized at the solid phase. When adding increasing analyte concentration more available active sites are being occupied, therefore less labeled antibody can bind to the immobilized solid phase antibody. Therefore, when the analyte concentration increases, a decrease in the signal is obtained.

In a direct competitive assay, after modifying the solid phase through the immobilization of the specific antibody, the addition of free and labeled antigen is performed. The concentration of the labeled antigen is kept constant.

As it can be noticed, most of the electrochemical immunosensors use enzyme-linked immunosorbent assay (ELISA) principles, where antibodies or antigens are labeled with an enzyme that generates an electroactive product, which can be detected at the transducer. This combination of high enzyme activity and selectivity with the sensitive methods of electrochemical detection provide a basis for the development of immunosensors.

Several enzymes were intensively used as labels for a plethora of substrates, such as alkaline phosphatase (AP) and horseradish peroxidase (HRP). To increase the sensitivity of immunosensors and prevent the disadvantages of using enzymes as labels (inactivation in the case of a small variation of experimental conditions), metal nanoparticles (i.e. gold, silver, quantum dot, etc.) have also been applied (Cristea et al. 2017).

Compared to the standard assays (i.e. ELISA), which are sensitive but rather suffer from reagent consumption, being a serious drawback when important samples are analysed, the electrochemical immunoassays in large-scale studies offer a fast, sensitive, specific, low cost and ease of use alternative.

1.1.3 Detection Methods

The role of the transducer in the sensor design is to convert a measurable electrical output from the products of the biorecognition reaction, this signal being in direct or inverted relationship to the analyte concentration (Thévenot et al. 2001).

The signal from the biorecognition reaction that undergoes transduction may likely to be *electrochemical* (current, potential, conductance, impedance), *piezoelectric* (mass) or *optical* (absorbance, chemiluminescence, fluorescence, surface plasmon resonance (SPR)).

The most common *electrochemical techniques* applied in electrochemical sensors include *potentiometry, amperometry, voltammetry, conductometry* and *electrochemical impedance spectroscopy* (*EIS*) (Brett and Oliveira Brett 1998). Potentiometric transducers evaluate the change in the voltage at zero current between two electrodes related to analyte activity. By contrast, amperometric procedures measure the current

generated at a fixed potential, by means of the reduction or oxidation of the electroactive species in solution. If the current is recorded when varying the potential, the technique can be referred to as voltammetric, like cyclic, differential pulse or square wave voltammetry. In conductometric biosensors, the transducers measure the modification of the solution electrical conductivity when applying a constant potential difference; this process usually occurs within biochemical reactions which cause an alteration in the ions content. Capacitance changes, owing to changes in the interfacial characteristics, of an electrochemical system can also be measured. Impedimetric technique has attracted much attention in the last decade due to its high applicability in the design of label-free biosensors. In EIS, the sinusoidal current response is measured as a function of small (<10 mV amplitude) sinusoidal voltages. By applying different frequencies of the sinusoidal perturbation, the electrode surface impedance can be calculated.

For most *electrochemical quartz crystal microbalance* (EQCM) sensors, mass changes correspond to the deposition or loss of material at the working electrode. There is simultaneous monitoring of the mass change and the electrochemical response. For immunosensors, the advantages of using QCM transducers are real-time monitoring of target antigens by non-tagged immunosensors. However, there are some disadvantages such as the lack of specificity and sensitivity, and the difficulty in drawing a calibration plot. For this reason, piezoelectric immunosensors have not received much attention and are considered inferior to electrochemical and optical ones (Marrazza 2014).

During the last two decades, *optical* methods attracted more and more attention owing to their fast response and high sensitivity. The best-described optical methods are based on *fluorescence, chemiluminescence* and *SPR* detection. Fluorescence and chemiluminescent detection systems involve the use of an entity which has fluorescent or chemiluminescent properties during immunosensor's development (Wang and Zhao 2018). SPR technique is based on physical optics phenomena produced by thin metal films. Some issues in the system sensitivity can occur when working with small molecular weight targets as a result of the small change in the refractive index corresponding to the binding event (Hoa et al. 2007).

1.1.4 Clinical Samples

When developing a method or device for the detection of biomarkers, it is imperative to consider their practical applicability. Therefore, it is necessary to identify the type of real samples for which the analytical technique or the sensing device will be developed for.

The detection and quantification of biomarkers in biological fluids (e.g. whole blood, plasma, serum, urine, saliva, sputum, tears, sweat, cerebrospinal fluid, faeces, tissue, cells, etc.) have also found important clinical applications. Some examples can be mentioned here, such as diagnosis, prognosis and therapy monitoring for severe affections, including cancer, neurological disorders and cardiovascular affections. It is worthy to mention that the sensing process will depend on the nature, composition and pretreatment of samples.

Blood and blood-derived samples are usually used for sensing biomarkers with clinical implications. However, recent trends suggest that saliva has received increased attention, this being mainly due to its facile collection and ease handling and conservation compared to blood or serum (Gug et al. 2019). Furthermore, the detection of various biomarkers in biological fluids that can be collected in a non-invasive manner such as urine and saliva has gained increased popularity. Furthermore, after saliva sampling, no anticoagulation treatment is usually required (Prasad et al. 2015). Thereby, the most important advantage is related to the sampling procedure that should be (1) non-invasive; (2) unstressed; (3) easy; (4) cost-effective. In these matrices there are fewer interfering proteins, thus being a relatively clean medium and there are fewer risks for transmitting diseases compared to blood. Some particular conditions should be considered for technologies in the case of applications in clinical practice: (1) the sample collection should be fast, simple, non-invasive (or minimum invasive) for patients and it must involve low costs ; (2) the existence of biomarkers that could be directly and specifically associated with the disease or its absence; (3) the technology needed should be simple and easy to use, portable and with accurate information for diagnosis and suitable for health and disease monitoring. It is well known that the majority of biomarkers are usually found in saliva and urine in traces, thus being difficult or impossible to be detected.

New, sensitive and accurate methods are thus required for the detection and quantification of salivary and urinary biomarkers even if they are found in traces in this complex matrix. Another interest relies on the compatibility of these strategies with POC applications, for improved clinical diagnosis and treatment. Sensing biomarkers in tissues samples provide contextual information and the possibility to assess the interaction between these markers and different cells. The implementation of biomarker evaluation in tissue in clinical practice ends with a laboratory developed test that validates in accordance with regulation. This test is suitable for a wide variety of compound and can be relatively simple or rather complex when dealing with multiple analytes simultaneous detection (Carvajal-Hausdorf et al. 2015).

1.2 Immunosensing Approaches for Bioclinical Applications

1.2.1 Autoimmune Diseases

Autoimmune diseases (AD) are heterogeneous conditions that arise when the intrinsic tolerance to self-antigens is lost. There is limited knowledge on the disease pathogenesis, but it is postulated that the complex interplay of environmental factors, genetics and epigenetics have a contribution to the disease onset in genetically susceptible individuals. Their diagnosis is usually achieved based on the symptoms combined with laboratory analyses like levels of autoantibodies in serum and complement proteins (Wu et al. 2018). Identifying specific and sensitive biomarkers for AD in early stages is of utmost importance to hinder the appearance of symptoms and diminish organ damage (Cipriani et al. 2014; Szentpétery et al. 2017).

AD comprise over 100 diseases with a high number of genetic and serologic biomarkers, therefore this chapter only covers a few examples recently reported in

literature, to demonstrate the usefulness of immunosensors for diagnosis and activity monitoring of AD. A number of immunosensing devices for diagnosis and monitoring of various AD have been reported recently.

Celiac disease is a commonly investigated AD with an increasing prevalence, from 0.03% in the 70s to 1% in 2010 (Tack et al. 2010). Celiac disease is an enteropathy sensitive to gluten determined by the ingestion of gluten which produces autoantibodies for gliadin and tissue transglutaminase. Thus, antigliadin antibodies (AGA) and anti-transglutaminase antibodies (anti-tTG) can be found in the blood and small intestine of patients with celiac disease (Briani et al. 2008; Rosales-Rivera et al. 2011). A novel portable diagnostic device for celiac disease was developed and validated, combining SPEs and remote-controlled IoT-Wi-Fi acquisition board which permits real-time data sharing to inform users. AuNP-modified SPEs were used as an electrochemical platform for immunosensor elaboration and acquisition of the enzymatically generated signal (Giannetto et al. 2018).

Rheumatoid arthritis (RA) is a multi-factorial AD with a prevalence of 0.5–1%, characterized by synovial chronic inflammation and destruction of the joints (Kurkó et al. 2013). Rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibody are biomarkers used for the clinical detection of RA, anti-CPP having a higher specificity than RF (Raptopoulou et al. 2007). Anti-CPP is made in the mucosal tissues such as lungs. It is also produced locally where inflammation takes place. Levels of CPP found in serum and synovial fluid are indicators of RA onset and of the level of severity as well (Snir et al. 2010). Human immunoglobulin G (HIgG) is part of the immune system and also a non-specific biomarker in various AD such as RA (Zhang et al. 2016; Thunkhamrak et al. 2017).

Multiple sclerosis (MS) is another AD which causes slow damage to the nervous system by damaging the myelin sheaths around the nerve axons.

Although MRI still remains the most reliable tool for MS diagnosis (Alshayeji et al. 2018), MS biomarkers are commonly used to predict how disability progresses, and to evaluate the response to treatment (Ziliotto et al. 2018). A common biomarker used for the detection of MS is the antibody against myelin basic proteins (MBP) which is present in the early stages of the disease (Derkus et al. 2013).

A series of other biomarkers are present in AD such as insulin autoantibody (IAA), glutamate decarboxylase antibody (GADA) or protein tyrosine phosphatase antibody (anti-IA_2) which are common biomarkers found in the blood of patients with autoimmune diabetes (Valdez et al. 2001). Pro-inflammatory cytokines such as interleukins or tumour necrosis factor (TNFα) are also important biomarkers for various inflammation-based conditions. For example, interleukin-17 (IL-17) is secreted by activated T-cells and is present in a high level in inflammatory events and AD, thus serving as a non-specific biomarker for autoimmune conditions (Zhong et al. 2012; Beringer and Miossec 2018).

A very sensitive immunosensor for the detection of IL-17 was reported recently based on the voltammetric detection of cadmium immobilized on polystyrene beads and used as a label for an anti-IL-17 antibody.

Primary anti-IL-17 was immobilized onto graphene electrodes for a sandwich immunoassay showing a high sensitivity in the range of concentrations of fg/mL was

obtained (Jiang et al. 2013). Overexpression of another interleukin, IL-12, has been correlated to the diagnosis of MS. Joshi's group reported a label-free immunosensor able to detect IL-12 based on electroplated printed circuit board electrodes which serve as a platform for the immobilization of anti-IL-12 antibodies. The novel sensor is able to detect IL-12 at levels lower than <100 fM (Bhavsar et al. 2009), higher levels than this cut-off value defining the disease onset.

Table 1 presents various examples of immunosensors aimed at detecting biomarkers of AD. Most immunosensors are coupled with electrochemistry as detection method due to its high sensitivity.

1.2.2 Cancer

The most common definition of *cancer* refers to the abnormal and uncontrolled nature of cell evolution and growth. This phenomenon can be attributed to the independent accumulation of genetic and epigenetic defects of cells. It should be mentioned that during the last decades, cancer was the major threat to human health, and the dominant cause of human mortality. Biomarkers can be found both intracellular and extracellular; those in the first category usually require sampling and preconcentration steps in order to be detected since their concentration is usually very low (Chandra et al. 2017).

Tumour biomarkers represent important tools for cancer diagnosis at the beginning of its development, and also provide important information for the evaluation of the therapy efficacy and for monitoring the disease evolution. These compounds can be present in various real samples collected from patients suffering of cancer, namely blood and its derivatives (serum or plasma), urine, tears, saliva or tissues. There are many biomarkers which have proven to be useful in cancer investigation, the most frequently ones found in the literature are listed below:

- *carcinoembryonic antigen* (CEA)—a glycoprotein biomarker found at the surface of the cells(200 kDa) was found useful in clinical tests for the detection of colon, breast, ovarian, gastric, pancreatic and lung tumours; the level of CEA in adults ranges from 2.5 to 5.0 ng/mL, increased levels indicating cancer progression or recurrence;
- *prostate-specific antigen* (PSA)—another glycoprotein biomarker consisting of one chain (32–33 kDa) was the first biomarker with the clinical application for diagnosing and screening of prostate tumours; the level of PSA in healthy subjects is under 4 ng/mL and its level was found significantly increased for patients suffering from prostate cancer;
- *α-1-fetoprotein* (AFP)—another important glycoprotein biomarker consisting of one chain with 590 amino acids (70 kDa); the blood level of AFP in healthy adult subjects is around 20 ng/mL, with increased levels in hepatocellular carcinoma, neuroblastoma and liver carcinoma;
- *carbohydrate antigen 19-9* (CA 19-9)—tumour biomarker for colorectal, gastrointestinal, hepatic cancers and pancreatic and biliary cancer;
- *cancer antigen CA15-3* (Mucin1)—a breast-associated protein and biomarker for carcinoma patients;

Table 1 Immunosensors for diagnosis and monitoring of AD: examples and comparison of analytical parameters

Target	Type of assay	Detection method	LOD	Sample	Ref
IgA AGA IgG AGA IgA anti-tTG IgB anti-tTG	Indirect immunoassay; secondary Ab labeled with AP	Electrochemical[a]	3.16 U/mL 2.82 U/mL 2.45 U/mL 2.95 U/mL	Serum	Neves et al. (2013)
IgA anti-tTG IgB anti-tTG	Indirect immunoassay; secondary Ab labeled with AP	Electrochemical[b]	3.2 AU/mL 1.4 AU/mL	Serum	Giannetto et al. (2018)
	Indirect immunoassay; secondary Ab labeled with AP	Electrochemical[a]	9.1 U/mL 9.0 U/mL	Serum	Neves et al. (2012)
	Indirect immunoassay; secondary Ab labeled with HRP	Electrochemical[a]	1.7 AU/mL 2.7 AU/mL	Serum	Giannetto et al. (2014)
IgA anti-tTG	Indirect immunoassay; secondary Ab labeled with HRP	Electrochemical[c]	Not reported	Serum	Pividori et al. (2009)
	Direct immunoassay; Ag immobilized onto transducer	Electrochemical[b]	20 fg/mL	Serum	Gupta et al. (2017)
Anti-tTG	Indirect immunoassay; secondary Ab labeled with AuNPs	Piezoelectric	1.3 μg/mL	Serum	Manfredi et al. (2014)
	Indirect immunoassay; secondary Ab labeled with POD	Electrochemical[d]	Not reported	Serum	Jhumka (2010)
	Indirect immunoassay; secondary Ab labeled with CdSe QDs	Electrochemical[b]	7 U/mL	Serum	Martín-Yerga et al. (2014)
AGA	Indirect immunoassay; secondary Ab labeled with HRP	Electrochemical[c]	46 ng/mL	Serum	Rosales-Rivera et al. (2011)
GADA	Direct immunoassay; Ag immobilized onto transducer	ECL[e]	0.10 ng/mL	Serum	Ma et al. (2018)
HIgG	Direct immunoassay; anti-hIgG Ab immobilized onto transducer	Electrochemical[a]	1.70 ng/mL	Urine	Thunkhamrak et al. (2017)
	Sandwich immunoassay Secondary Ab labeled with AgNPs/carbon nanocomposite	Electrochemical[b]	0.001 ng/mL	Serum	Zhang et al. (2016)
IL-17	Sandwich immunoassay Secondary Ab labeled with cadmium-polystyrene beads	Electrochemical[f]	50 fg/mL	Serum	Jiang et al. (2013)

(continued)

Table 1 (continued)

Target	Type of assay	Detection method	LOD	Sample	Ref
Anti-CCP	Direct immunoassay; Ag (CCP) immobilized onto transducer	Chemiluminescence	0.2 pg/mL	Serum	Zhao et al. (2018)
MIF	Direct immunoassay; anti-MIF Ab immobilized onto transducer	Electrochemical[b]	0.7 ng/mL	Serum	Li et al. (2008)
Anti-MBP	Direct immunoassay Ag immobilized onto transducer	Electrochemical[d]	0.15 ng/mL	Serum CSF	Derkus et al. (2013)
TNFα	Direct immunoassay; anti-TNFα Ab immobilized onto transducer	Electrochemical[d]	3.7 fg/mL	Serum	Aydın et al. (2017)
	Direct immunoassay; anti-TNFα Ab immobilized onto transducer	Photoelectrochemical	3.33 fg/mL	Serum	Hu et al. (2018)
	Sandwich immunoassay Secondary Ab labeled with magnetic particles	Piezoelectric	1.62 pg/mL	Serum	Pohanka (2018)

[a]CV
[b]DPV
[c]Chronoamperometry
[d]EIS
[e]ECL
[f]SWV

- *cancer antigen 125* (CA125 or Mucin16)—a glycoprotein situated on membranes (200 kDa), with applications in the epithelial ovarian cancer detection and monitoring;
- *mucin 4* (MUC4)—a marker that was found to be relevant for endometriosis and pancreas, oesophagus and breast cancers;
- *neuron-specific enolase* (NSE)—was found useful as a biomarker for small-cell lung carcinoma, neuroblastoma and neuroendocrine tumours;
- *murine double minute 2* (MDM2)—biomarker amplified and overexpressed in sarcomas, lymphomas, breast, lung and testicular germ cell carcinomas, as well as in glioblastomas and astrocytomas, and oral squamous cell cancers;
- *squamous cell carcinoma antigen* (SCCA)—biomarker for squamous cell cancer;
- *myelocytomatosis oncogene* (c-Myc)—an oncoprotein involved in the transcription of genes that are responsible for growth, differentiation and apoptosis of healthy cells, with an increased level in breast and lung tumours, in melanoma and lymphoblastic leukaemia;
- (EGFR, ErbB-1; HER1 in humans)—a glycoprotein biomarker that can be found at the surface of the cells. It is a trans-membrane protein that was found overexpressed in non-small cell lung cancer, in head, neck and oesophageal cancers, as well as in breast, gastric, colorectal, prostate, bladder, renal, pancreatic and ovarian carcinomas;
- (SCC-Ag)—glycoprotein biomarker (45–55 kDa) that can be found in cervical carcinoma and other types of cancers (e.g. lung, head and neck and hepatocellular tumors); in cervical cancer can diagnose recurrence at an early stage;
- *human chorionic gonadotrophin* (hCG)—a biomarker with clinical relevance in diagnostic of pregnancy related carcinomas; levels of this marker are increasing in several conjunctures such as abnormal placental invasion or placental immaturity;
- *human epididymis-specific protein 4* (HE4)—ovarian marker with increased levels determined especially in neoplastic tissue and serum of patients; HE4 was also found overexpressed in early stages of cancer and recurrences;
- (HER-3)—biomarker involved in the activation of intracellular signalling, and in the response of the organism to extracellular stimuli. This marker overexpression is related to breast, ovarian and pancreatic cancers progression and recurrences, with gastric carcinoma, malignant melanoma and metastases, and head and neck squamous cell cancer;
- *vascular endothelial growth factor* (VEGF)—biomarker that was found involved in the angiogenesis and vascular permeability regulation, mitogen for endothelial cells and marker of unfavourable prognosis and metastasis;
- *interleukin 8* (IL-8 or CXCL-8)—biomarker with high potential as neutrophil chemoattractant, involved in the regulation of several biological processes in human body. It is a pro-inflammatory cytokine with several functions that were found overexpressed in melanoma and several other cancers, a marker for progression in urogenital carcinomas, and for diagnostic in oral cancer (Hasanzadeh and Shadjou 2017; Felix and Angnes 2018; Aydın et al. 2018).

Several immunoassay methods have been tested for the detection of tumour biomarkers with high accuracy, sensitivity and precision. An interesting review (Bahadir and Sezgintürk 2015) was published recently summarizing in an interesting way the latest research in the field of electrochemical biosensors for detection and quantification of biomarkers for cancer and heart diseases. These biosensors were comparatively presented revealing their analytical performance, namely stability in time, reproducibility, selectivity for the target, precision and the possibility of multiple use and regeneration (Felix and Angnes 2018).

Different strategies based on biochemistry, immunology and molecular biology have been adopted to date and applied for the determination of cancer biomarkers; in most cases, they were tested only in serum as biological fluid. Immunoassay-based methods such as radioimmunoassay (RIA), ELISA, fluoroimmunoassay, chemiluminescent and electrochemiluminescent (ECL) immunoassays, with increased specificity for molecular recognition, have been also applied in clinical trials for cancer biomarkers detection and quantification.

For example, PSA was detected using an electrochemical immunosensor starting from an SPE decorated with AuNPs and chitosan. The protocol used for immunosensor fabrication implies the anti-PSA primary antibody binding directly on the surface of AuNPs/CHI/SPE, followed by the subsequent immobilization of PSA antigen and HRP-tagged secondary antibody. H_2O_2 was added as HRP substrate, while methylene blue was used as a redox mediator for signal amplification. This immunosensor proved to be stable, sensitive and reproducible and was applied for PSA detection in human serum (Suresh et al. 2018).

A novel strategy has been implemented for electrochemically amine derivatization of MoS_2 nanostructures, which promoted the immobilization of antibody molecules through EDC/NHS chemistry. The optimized immunosensor was characterized with good detection limit and a wide dynamic concentration range for PSA sensing in serum samples of healthy volunteers. The characterization of the NH_2-MoS_2 probes reveals the formation of spherical entities of 2-ABA in the top of the MoS_2 film, an observation confirmed by FE-SEM microscopic characterization (Fig. 1). The polymerization of the 2-ABA on the MoS_2 determined apparent changes and revealed the globular-type structured polymer, while the EDX tests proved the purity of the generated MoS_2 probe (Kukkar et al. 2018).

An electrochemical immunosensor with multiple uses, based on temperature-responsive polymeric structures, was implemented and tested for repeated and simultaneous amperometric sensing of three important cancer tumour biomarkers such as CEA, PSA and CA125. Furthermore, the analytical signal was greatly amplified by using HRP enzyme and antibody-labeled NPs. The desorption of biorecognition elements was performed easily by a temperature gradient, therefore keeping their biological activity and showing high potential for industrial and clinical applications (Hong et al. 2016).

The first electrochemical immunosensor for IL-13 receptor Rα2 (IL-13Rα2), that was selected as a biomarker for metastatic colon carcinoma, was reported by Valverde et al. Sandwich immunocomplex strategy was followed based on captured antibody and biotinylated secondary antibody labeled with streptavidin-horseradish

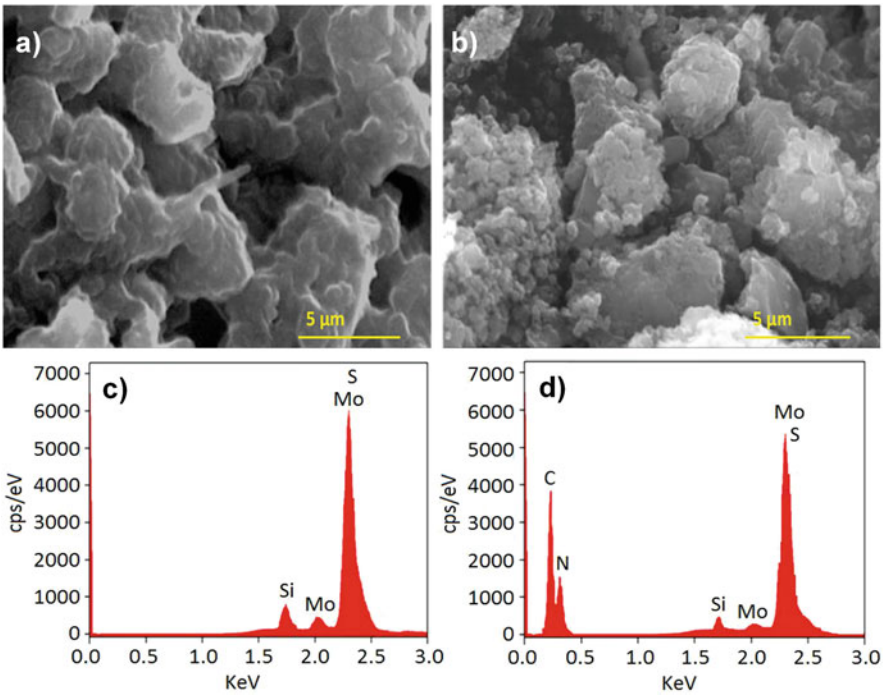

Fig. 1 FE-SEM images of: (**a**) MoS_2 nanosheets; (**b**) MoS_2 nanosheets modified with 2-ABA. EDX data of: (**c**) MoS_2 nanosheets; (**d**) MoS_2 nanosheets modified with 2-ABA. Reprinted with permission from (Kukkar et al. 2018)

peroxidase (Strep-HRP) polymer. The biocomposite materials were immobilized through amidic bonds using the carboxylic acid functionalities generated on the surface of magnetic microbeads (HOOC-MBs) and amperometric detection was then performed using carbon SPEs (Fig. 2). This immunosensor presents excellent selectivity against other proteins and was applied in lysates of colon carcinoma cells (used in their raw form) and to reveal the risk of metastasis of intact cells based on the recognition event of this target extracellular receptor (Valverde et al. 2018).

Two electrochemical systems were developed for fast and sensitive detection of MUC1 using MBs and coupling screen-printed arrays of sensors. It is about sandwich-type immunoassays based on aptamers or antibodies immobilized on streptavidin and protein G-modified MBs, respectively. These immunoassays were applied to capture MUC1 from real samples (serum from cancer patients) and tested by DPV. A limit of detection of 0.19 nM was obtained by using the antibody-assay, while the aptamer assay have provided a much lower LOD (0.07 nM). Thus, the sensor with aptamer presented increases selectivity for MUC1 in complex matrices and provided promising prospects for clinical applications (Marrazza et al. 2015). Another two EIS based sensors based on aptamers were elaborated and tested for the detection of MUC1 tumour biomarker exploiting its specific recognition provided by

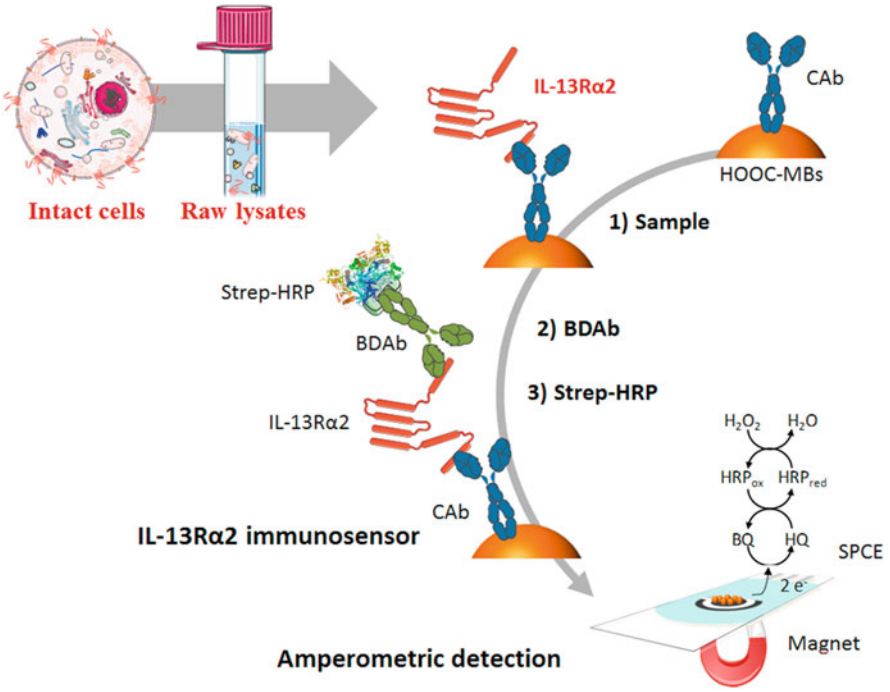

Fig. 2 Schematic representation of IL-13Rα2 sandwich immunosensor based on functionalized MBs and the reactions implied in the amperometric process. Reprinted with permission from (Valverde et al. 2018)

thiolated aptamers bounded on AuNP-modified carbon and gold SPEs with excellent analytical parameters for the quantification of MUC1 marker (Florea et al. 2013).

The first immunosensor based on electrochemical detection of MUC54 has been elaborated and tested, using carbon-based SPEs functionalized through diazotization. Thus, phenylacetic moieties that enabled the immobilization of the antibody molecules that are highly specific for the target were used, this step is followed by the affinity reaction with protein biomarker. Several parameters involved in the immunosensor elaboration protocol were optimized, the results being encouraging for further biomedical and clinical tests (Hosu et al. 2017a).

The design and development of a colorimetric smartphone-based immunosensor was elaborated for CA125 cancer biomarker after the fixation of the primary antibody on a nitrocellulose membrane. The AuNPs functionalized with antibody units were immobilized onto immunospots after the incubation with CA125 solutions. The silver deposition was induced from a silver enhancer solution, this step having as the result the formation of gold-silver NPs of grey colour dots. The intensity of grey colour for these spots proved to be dependent on the CA125 concentration. The image was acquired coloured with a smartphone camera and after data handling it was determined that this protocol allowed to obtain a detection

limit of 30 U/mL for CA125. This biosensor was used also in human serum spiked with a known amount of CA125 with promising results (Hosu et al. 2017b).

Several reviews have been published about tumour biomarkers detection with biosensors. Thus, herein only a few examples were presented and discussed, and the critical clinically important parameters, such as detection limit and the configuration of bioassay, all being presented in Table 2.

1.2.3 Cardiovascular Diseases

The diagnosis of *cardiovascular diseases* (CVD) is of paramount importance due to the high mortality and morbidity associated with those diseases. WHO stated that every year, an estimated 17 million people globally die of CVD, particularly heart attacks and strokes, occurring almost equally in men and women. CVD are referring to disorders of the heart and circulatory system including coronary heart diseases, cerebrovascular diseases, rheumatic hearts diseases and other heart conditions (WHO 2011).

Several sensitive biomarkers for myocardial inflammation, damage and cell death have been established and used in clinical analysis. Several proteins like Cardiac Troponin-I (cTnI) and troponin-T (cTnT), C-reactive protein (CRP), tumour necrosis factor TNF-α, myoglobin (Myo) and several enzymes: myeloperoxidase (MPR), creatine kinase MM (CK-MM) and creatine kinase MB (CK-MB) could be detected from biological fluids by using immunosensors (Hosu et al. 2018a). Cardiac troponins are specific biomarkers for myocardial injury compared with other proteins like CRP, interleukins, TNF-α or troponin. For instance, nowadays troponin measurement has largely replaced other analysis in many hospitals. Several examples of biosensors for cardiac biomarkers detection are presented in Table 3. Recently, the interest in lab-on-chip sensors equipped with smartphones and microfluidic devices has been growing due to the fact that could lead to POC devices capable of fast data acquisition and reporting. Those attributes are extremely relevant when dealing with CVD, increasing patient survival rates.

1.2.4 Neurological Disorders

Neurological disorders (ND) are dysfunctions registered in the functioning of the brain, of spine and nerves that connect each other. There are different abnormalities of the nerves (e.g. biochemical dysfunction, structural or electrical degradations, etc.) that can determine severe symptoms such as paralysis, muscle weakness, confusion, pain or altered levels of consciousness. According to data provided by WHO, over 600 diseases affecting the nervous system were reported, such as tumours, epilepsy, Parkinson's and Alzheimer's diseases, stroke or dementia. The WHO also estimated that ND and their direct consequences affect around one billion people worldwide and identified ND as one of the tremendous threats to public health because there is also associated discrimination to these diseases (WHO 2012).

Neurotransmitters (NTs) are endogenous chemicals that enable neurotransmission and play an important role in some critical brain functions, namely behaviour, cognition, muscle tone, heart rate, sleeping, memory, consciousness and appetite (Hasanzadeh and Shadjou 2017). The selection of the most relevant and specific

Table 2 Immunosensors for diagnosis and monitoring of biomarkers involved in cancer

Target	Type of assay	Detection method	LOD	Sample	Ref
CEA	Sandwich immunoassay; primary Ab bounded on paper electrode functionalized with nanoporous Au and chitosan; secondary Ab labeled with green-luminescent graphene QD functionalized Au@Pt core-shell NPs	ECL[a]	0.6 pg/mL	Serum	Hasanzadeh et al. (2016)
	Label-free immunoassay; Ab fixed on a AuNPs/graphene/chitosan/silver/Au electrode	Electrochemical[b]	0.2 fg/mL	Serum	
	Sandwich-type immunoassay; detection Ab fixed on the nano-Au, SiO_2/thionine nanocomposition and nano-Au self-assembled sandwiched layers	Electrochemical[c]	0.34 ng/mL	Serum	
	Sandwich-type immunoassay; Secondary Ab fixed on a 3D structured membrane of AuNPs/chitosan-MWCNT labeled with PB	Electrochemical[c]	0.1 ng/mL	Serum	
	Sandwich-type immunoassay; secondary Ab immobilized on Ni hexacyanoferrates NPs/AuNPs/ GCE	Electrochemical[d]	0.1 ng/mL	Serum	
	Sandwich immunoassay; primary Ab fixed on a long-chain polythiol with AuNPs labels	Electrochemical[c]	0.015 fg/mL	Serum	
	Label-free immunoassay; secondary Ab fixed at AuNPS/thionine/rGO/GCE	Electrochemical[e]	4 pg/mL	Serum	
	Sandwich-type immunoassay; secondary Ab immobilized on QD labels	Fluorescence	0.02 ng/mL	Serum	Hong et al. (2016)
	Sandwich-type immunoassay; primary Ab immobilized on MBs; HRP functionalized Au labeled secondary Ab	Electrochemical[c]	10 pg/mL	Serum	
	Sandwich-type assay; secondary HRP-labeled Ab	Electrochemical[c]	0.2 ng/mL	Serum	
	Sandwich-type immunoassay; secondary Ab immobilized on Cd-Te QD label	ECL[a]	0.01 ng/mL	Serum	
	Sandwich-type immunoassay; secondary Ab fixed on HRP-labeled polypyrrole NPs	Electrochemical[c] colorimetric	0.8 pg/mL 0.6 pg/mL	Serum	

PSA	Sandwich-type immunoassay; primary Ab fixed on AuNPs	SPR[f]	1 ng/mL	Serum	Hong et al. (2016)
	Sandwich-type immunoassay; primary Ab fixed on AuNPs	Optical	1 fg/mL	Serum	
	Sandwich-type immunoassay; secondary Ab labeled with AP	Electrochemical[e]	1.4 ng/mL	Serum	
	Sandwich-type immunoassay; primary Ab immobilized on MBs HRP-labeled secondary Ab	Electrochemical[c]	0.5 ng/mL	Serum	
	Sandwich-type immunoassay; Secondary Ab labeled with tris (Ru(bpy))	ECL[a]	40 pg/mL	Serum	
	Sandwich-type immunoassay HRP functionalized MWCNT labeled secondary Ab	Electrochemical[c]	4 pg/mL	Serum	
	Sandwich-type immunoassay Secondary Ab immobilized on HRP-labeled polypyrrole NPs	Electrochemical[c] Colorimetric	0.7 pg/mL 0.8 pg/mL	Serum	
CA125	Sandwich-type immunoassay Primary Ab immobilization on colloidal AuNPs/GCE; HRP-labeled secondary Ab	Electrochemical[c]	1.73 U/mL	Serum	Hasanzadeh et al. (2016)
	Sandwich-type immunoassay; Protein immobilized on porous carbon membrane; HRP-labeled secondary Ab	Electrochemical[c]	1.8 U/mL	Serum Saliva	
	Sandwich-type immunoassay; Primary Ab immobilized on Au hollow microspheres and porous polythionine/GCE	Electrochemical[c]	1.3 U/mL	Serum	
	Sandwich-type immunoassay; Primary Ab immobilized on MWCNT/PEI/GCE; Secondary Ab labeled with ferrocene carboxylic acid encapsulated liposomes	Electrochemical[g]	0.5×10^{-3} U/mL	Serum Saliva	
	Label-free immunoassay Primary Ab immobilized on amine functionalized CuO nanoflakes	Electrochemical[b]	0.77 U/mL	Fetal bovine serum	
	Sandwich-type assay; Secondary Ab immobilized on HRP-Labeled polypyrrole NPs	Electrochemical[c] Colorimetric	5×10^{-3} U/mL 6×10^{-3} U/mL	Serum	Hong et al. (2016)
	Sandwich-type assay; Secondary Ab labeled with tris(Ru (bpy))	ECL[a]	0.6 U/mL	Serum	

(continued)

Table 2 (continued)

Target	Type of assay	Detection method	LOD	Sample	Ref
CA 15-3	Sandwich-type assay; capture aptamer/Ab fixed on MBs functionalized with Protein-G and Streptavidin; Detection aptamer/AP-labeled Ab	Electrochemical[e]	0.07 nM (aptasensor) 0.19 mM (immunosensor)	Serum	Marrazza et al. (2015)
	Label-free immunoassay Aptamer fixed on carbon and Au SPEs functionalized with AuNPs	Electrochemical[b]	3.6 ng/mL	Serum	Florea et al. (2013)
	Label-free immunoassay Aptamer fixed on carbon and Au SPEs functionalized with AuNPs	Electrochemical[c]	0.95 ng/mL	Serum	
	Label-free immunoassay Ab immobilized on ZnO nanorods using APTES and glutaraldehyde	Piezoelectric	–	–	Rauf et al. (2018)
	Label-free immunoassay Ab immobilized on ZnO/Au nanocomposite film	SPR[f]	0.025 U/mL	Pleural fluid	
CA 15-3	Sandwich-type immunoassay; secondary Ab immobilized on a composite material based on PtNPs decorated with L-Lys/Mn–ZnO nanorods modified with tris(Ru(bpy))	ECL[a]	0.014 U/mL	Serum	
	Sandwich-type immunoassay; primary Ab fixed on GCE modified with CNT, core-shell organosilica@chitosan nanospheres, Pt nanoclusters and glucose oxidase.	Electrochemical[c]	0.04 U/mL	Serum	
	Sandwich-type immunoassay Primary Ab immobilized on PMMA beads with CA15-3 Secondary Ab labeled with fluorescence label	Fluorescence	0.21 U/mL	Serum	
	Sandwich-type immunoassay; Primary Ab immobilized on graphene/SPE; methylene blue-labeled secondary Ab	Electrochemical[e]	0.04 U/mL	Serum	

	Label-free immunoassay; Primary Ab immobilized onto N-doped graphene/GCE.	Electrochemical[e]	0.012 U/mL	Serum	Marques et al. (2018)
	Sandwich-type immunoassay; Primary Ab immobilized nanoporous Au/graphene/GCE; Secondary Ab labeled with HRP encapsulated liposome.	Electrochemical[e]	5×10^{-6} U/mL	Serum	
	Label-free immunoassay; primary Ab attached onto N-doped graphene/GCE	Electrochemical[e]	0.012 U mL	Serum	
	Sandwich-type immunoassay Primary Ab immobilized on GO/Au electrode; A bioconjugate Ab/MWCNT/ferritin was used for detection	Electrochemical[e]	5×10^{-3} U mL	Serum	
	Label-free immunoassay; A bioconjugate Ab/MWCNT/ferritin; Ab immobilized on ferrocene/rGO/AuNPs/ GCE	Electrochemical[e]	0.015 U mL	Serum	
	Sandwich-type immunoassay; primary Ab fixed on AuNPs-graphene/GCE; Secondary Ab labeled with Ru(II) luminophore	ECL[a]	3×10^{-3} U mL	Serum	Marques et al. (2018)
	Sandwich immunoassay; primary Ab fixed on GO/SPE Secondary Ab labeled with composite based on peroxidase-like magnetic silica NPs and GO	Electrochemical[e]	2.8×10^{-4} U mL	Serum	
CA 15-3 HER2-ECD	Sandwich-type immunoassay; primary Abs fixed on bi-SPE-AuNPs; Detection based on AP used as label	Electrochemical[h]	5.0 U/mL 2.9 ng/mL		
CA15-3 CA 125 CEA	Sandwich-type immunoassay; primary Ab fixed on grapheme/SPE; Secondary Ab labeled with mesoporous PtNPs	Electrochemical[e]	1×10^{-3} U/mL	Serum	
	Sandwich immunoassay; primary Ab immobilized on AuNPs-graphene/SPE; Au clusters and AP-labeled secondary Ab	Electrochemical[h]	1.5×10^{-3} U/mL	Serum	
CA15-3 CA 125 CA19-9	Sandwich-type immunoassay; primary Ab immobilized on MBs; PAMAM dendrimer-metal sulfide QD-labeled secondary Ab	Electrochemical[i]	5×10^{-3} U/mL	Serum	

(continued)

Table 2 (continued)

Target	Type of assay	Detection method	LOD	Sample	Ref
HER2-ECD	Label free; affibody immobilized on AuNPs/SPE	Electrochemical[b]	6.0 ng/mL	Serum	
	Sandwich-type immunoassay; primary Ab adsorbed on AuNPs/SPE; AP-labeled secondary Ab	Electrochemical[h]	4.4 ng/mL	Serum	
	Sandwich-type immunoassay; primary Ab coupled by protein A modified MBs; Secondary Ab labeled with AP	Electrochemical[e]	6 ng/mL	Serum	
	Sandwich-type immunoassay; nanobodies labeled with HRP for detection	Electrochemical[c]	1 µg/mL	Serum	
	Label free; Au electrode covered with Ab immobilized on iron oxide NPs	Electrochemical[d]	0.995 pg/mL	Serum	
	Sandwich immunoassay; primary Ab immobilized on AuNPs/GCE Detection using a conjugate of hydrazine, AuNPs and aptamer	Electrochemical[i]	0.037 pg/mL	Serum	
	Label free; Primary Ab fixed on AuNPs/HDT/AuNPs@MWCNT-CIL surface	Electrochemical[b]	7.4 ng/mL	Serum	

[a]Electrochemiluminescence
[b]EIS
[c]Chronoamperometry
[d]CV
[e]DPV
[f]SPR
[g]SWV
[h]LSW
[i]SWSV

Table 3 Examples of immunosensors for cardiac biomarkers detection

Target	Type of assay	Detection method	LOD	Sample	Ref
cTnT	Graphene SPE	Electrochemical[a]	0.005 ng/mL	Serum	Bakirhan et al. (2018)
	Streptavidin polystyrene microspheres on SPE	Electrochemical[b]	0.2 ng/mL	Serum	
	MIP/Au electrode	Electrochemical[a]	9 pg/mL	Serum	
cTnI	GNPs deposited ITO	Electrochemical[c]	100 ng/mL	Serum	
	Au/MHA/TMB/Den/anti-TnI/Au electrode	Electrochemical[c]	2 pg/mL	Serum	
	Anti-cTnI/AuNP/DDAB/SPE	Electrochemical[d]	0.1 ng/mL	Plasma	
TNF-α	*Fullerene-functionalized CNTs/ionic liquid*-butyl-3-methylimidazolium bis (trifluoromethyl sulfonyl) imide	Electrochemical[a]	2.0 pg/mL	Serum	Mazloum-Ardakani et al. (2015)
	PB functionalized ceria NPs Primary anti-TNF-α antibody onto AuNPs-modified CNT	Electrochemical[e]	2 pg/mL	Serum	Li et al. (2012)
	Apt-Ag@Pt-GRs/SPE	Electrochemical[a]	1.64 pg/mL	Serum	Bakirhan et al. (2018)
CRP	RNA aptamer coupled to a monoclonal antibody and AP immobilized on MBs and carbon-based SPEs	Electrochemical[a]	–	Serum	
	ITO electrode modified with educed graphene oxide nanoparticles	Electrochemical[c]	1 ng/mL	Serum	
	ZnO nanotubes anti-CRP glutaraldehyde	Electrochemical[f]	1 ng/mL	Serum	
Myo	SAM/Au modified with Anti-MYO/4-ATP	Electrochemical[c]	5.5 ng/mL	Serum	
	Anti-MYOIgG/MWCNT/SPE	Electrochemical[c]	0.08 ng/mL	Serum	

[a]CV
[b]DPV
[c]Potentiometry
[d]EIS
[e]Chronoamperometry
[h]Adsorptive stripping voltammetry

biomarkers for NTs and their monitoring is the demanding step for the elaboration of reliable diagnostic approach and treatment schemes. These are also critical tasks for the assessment of potential new drug therapies. Another important challenge is the extremely low concentrations and small volumes of probes (μL to nL) available for the most important biomarkers. Moreover, the onset of irregularities also determines minor changes in biomarker amounts (Ribeiro et al. 2016). Many methods for

analytical characterization of real samples have been proposed and tested for neurological biomarkers with clinical relevance detection and quantification, such as chromatography, capillary electrophoresis (CE), flow injection analysis (FIA), HPLC-MS and electrochemical sensors. To date, there are three strategies frequently used to detect NT biomarkers: (1) the use of electrochemical methods combined with other methods such as HPLC or FIA, (2) the improvement of the sensing properties by functionalization of the electrode and (3) in vivo testing of NTs by using FSCV at carbon fibre microelectrodes.

The developed approaches proved to be suitable for accurately detection these analytes at concentration levels with clinical relevance, but the challenge is to be able to detect them in a complex matrix, in the presence of other compounds (e.g. ascorbic acid as interfering species that is present in real samples at more than 100 times higher concentration than NTs) (Ribeiro et al. 2016). The development of simple, fast and sensitive methods and sensors for the detection of NTs could replace traditional methods used for diagnosis (e.g. pen-and-paper questionnaire), while mobile devices could become the next generation of approaches to diagnose, personalized treatment and efficient monitoring (Hasanzadeh and Shadjou 2017). The huge number of ND and biomarkers and the importance of this topic have led to numerous studies in the field and implicitly to a large number of publications, impossible to centralize in a single study. Thus, only a few examples of biosensors will be summarized here since there are several review-type articles dealing with this subject (Ribeiro et al. 2016).

An enzymatic biosensor was proposed for dopamine sensing starting from a composite platform based on reduced graphene oxide (rGO) modified with β-cyclodextrin (β-CD) and tyrosinase. This composite layer was deposed and fixed at the surface of a GCE using polyethylenimine and allowed the sensitive and selective detection of dopamine in pharmaceutical formulation, urine and serum, respectively (Fritea et al. 2015b). Another biosensor was developed and tested for dopamine starting from 12-(pyrrol-1-yl) dodecyl] triethylammonium tetrafluoroborate, an amphiphilic pyrrole used for the entrapment of tyrosinase together with a composite material between rGO and β-CD-pyrrole. The polymerization process leads to the formation of a graphene framework with functionalized with β-CD units and allowed the enzyme immobilization without losing its activity. This biosensor was applied for amperometric determination of dopamine and catechol in urine and serum (Fritea et al. 2015a).

Interleukin 6 (IL-6) is an inflammation biomarker overexpressed in inflammations and infections. Furthermore, there is a correlation between its concentration tested in sweat, saliva and also in plasma. Recently it was observed that increased levels of the inflammatory biomarkers, including IL-6 in plasma, are correlated with major depression and with depressive symptoms in subjects diagnosed with bipolar disorder (Tertiş et al. 2017). Thus, an electrochemical aptasensor, elaborated starting from an SPE functionalized with a nanocomposite layer of polypyrrole and AuNPs, was elaborated and successfully tested for IL-6 determination in human serum. A limit of detection below the physiological level of

this protein in serum was obtained, which allows evaluation of the protein in serum with good recoveries (Tertiş et al. 2017).

1.2.5 Biosensors Based on Unconventional Detection Strategies

POC testing allows assays in the field for more accurate and time-effective data collection and dissemination that could be performed by any user with little knowledge of operation systems. Therefore, POC diagnosis tools represent ideal candidates to ensure medical assistance in remote areas and first aid centres.

On this hot topic, smartphones as detection elements have become a new trend in the nowadays research. As reported in a recent review (Quesada-González and Merkoçi 2017), biosensors coupled with mobile phone are considered "an emerging diagnostic and communication technology". Some features as fast worldwide connection and distribution of data, remote internet access and high throughput out accessibility, made these devices being called "smart". Hence, one would only see major benefits in introducing smartphones in medical research as mobile health tools. Smartphone-based biosensors have great potential as POC and point-of-need tools for healthcare, food safety, environmental monitoring and biosecurity of most predisposed areas being well positioned for commercialization (Hosu et al. 2017b). Thus, smartphone-mediated detection strategies might allow for easier and faster diagnosis with little use of complicated instruments.

Another rather unconventional immunosensing strategy is represented by paper-based microfluidics. Since the first paper-based microfluidic system was developed for chemical testing in 2007, the idea became a hotspot in the analytical chemistry field and has extended to biomedical designs, more precisely to immunoassays (Zhu et al. 2019). Moreover, due to the simplicity, fast and cost-effective features of in vitro lateral flow strips which resemble of immunochromatography principle, more research have been focused in this direction for various analytical applications (Kanchi et al. 2018).

A short overview of smartphone- and paper-based immunosensors is presented in Table 4, considering the materials used, detection sensing system and their clinical applications.

The development of wearable sensors highlights the possibility of personalized diagnosis tools with applications in healthcare, food safety, environment, entertainment. Therefore, screen-printing technology has been mostly employed to develop low-cost wearable sensors on various platforms like PET, rings, gloves, temporary tattoo, textiles or mouthguards for non-invasive biomarker detection in fluids as saliva, tears or sweat (Cristea et al. 2015; Ciui et al. 2018b). To this, much attention must be focused on the specified applicability of the sensor as within the manufacturing process the non-planarity and mechanical properties of the human body should be considered. Because the term "wearable sensors" is linked to skin/cavities-mounted devices, biocompatible properties of these devices are a must. Therefore, physical properties as thicknesses, mechanical properties and thermal masses of the wearable devices are usually required to be close to those of the human skin, and implicit to enable compliant and robust contact upon attaching on the human body. Nevertheless, wearable sensing devices must have other two important

Table 4 Short overview of different smartphone-mediated and paper-based detection systems from the literature

Immunosensing principle	Detection method	LOD	Target	Sample	Ref.
LFTSs	Integrated digital camera	0.1×10^{-3} U/mL	AP	Milk	Kanchi et al. (2018)
96-well microtiter plate	Flash LED[a]	70 µg/mL	CRP	Whole blood and plasma Clinical plasma	
Nitrocellulose membrane	Flash LED[a]	30 U/mL	CA125	Serum	
Immunoblotting assay	Illumination sensor	0.2 ng/mL	uCTX-II	–	
LF immunoassay	Chemiluminescence	0.3 ng/mL	Salivary cortisol	Saliva	
Immunoassays	Fluorescence and electrochemical	12.3 pM	Hepatitis C virus core antibody	–	
Screen-printed carbon electrodes and interdigital gold electrodes	Electrochemical[d]	1.78 µg/mL 2970 µg/mL	BSA and thrombin	–	
Paper-based bipolar electrode	ECL	1.75 µM	Glucose	Artificial urine	
Chloridometry	Fluorescence	800 µM	Chloride—cystic fibrosis biomarker	Sweat	
Fluoropolymer microcapillary film	Flash LED[a] and fluorescence	0.08 ng/mL	PSA	Whole blood	
µPAD	Flash LED[a]	20 µM 60 µM	Glucose Lactate	Cell culture medium	
	Colorimetric	10 CFU/mL	*E. coli* *N. gonorrhoeae*	Urine	
	Electrochemical[b]	0.36 mIU/mL	HCG	Serum	Zhu et al. (2019)
	Electrochemical[b]	10 pg/mL	17β-estradiol	Serum	
	Electrochemical[c]	0.1 U/mL	AChE	Whole blood	
	Chemiluminescence	0.8 pg/mL	PSA	Serum	
	ECL	4.3 mU/mL	CA125	Serum	
	ECL	6.4 pg/mL	IgG	Serum	
	Fluorescent	0.4 pg/mL	AFP	Serum	
	Flash LED[a]	2.6 pg/mL	CEA	Serum	

[a]Colorimetric
[b]DPV
[c]CV
[d]EIS

features: (1) to bend in accordance with the structure of the skin, but also (2) to stretch to overcome the strains resulted from body motion.

Ciui et al. developed a bandage and a microneedle-based wearable electrochemical sensor for tyrosinase detection. It is considered to be the first wearable sensor developed for tyrosinase monitoring toward future decentralized screening of melanoma. The results achieved within the use of bandage and microneedle wearable sensors present good analytical performance and possible application for assessing the different stages of skin cancer as an alternative to current invasive screening procedures (Ciui et al. 2018a).

Some examples of glove-based electrochemical sensing devices are exemplified in a recent review (Hubble and Wang 2018); the importance of these devices for many areas including clinical field is highlighted.

Thus, two printed chemical sensors have been integrated on to a laboratory glove for the simultaneous and fast voltammetric detection of *Pseudomonas aeruginosa* by means of monitoring its two major virulence factors: pyocyanin and pyoverdine. The sensor was successfully tested on surfaces like furniture, medical scalpels or moist sinks. Prior to the on-site detection, the finger screen-printed sensors were firstly coated with a conductive hydrogel matrix for electron-conducting purposes (Ciui et al. 2018c).

A very interesting study has aimed the simultaneous monitoring of several markers in foodstuffs and beverages. The concept is based on the use of a robotic hand covered by a glove having three different electrochemical sensors printed on three different fingers. An integrated wireless electronic interface was used as transducer and enabled the fast and reliable discrimination of sweet, sour and spicy taste, by determining the presence of glucose, ascorbic acid and capsaicin, respectively (Ciui et al. 2018b).

A novel cavitas-printed wearable sensor was developed for the direct electrochemical detection of N^{ϵ} (Carboxymethyl)lysine from saliva samples. The electrochemical cell was incorporated at the mouthguard surface in order to test the analyte in the human oral cavity. This non-invasive sensor proves its efficiency in monitoring the analyte concentration in raw, untreated human saliva; this proof-of-concept represents a promising tool for the healthcare strategies in improving the management and monitoring of chronic diseases (Ciui et al. 2019).

The chemical signals from clinically relevant biomarkers can be related to the signals from the human body, thus, there is only a matter of time until wearable chemical sensors for these analytes will be available on the market (An et al. 2017).

1.3 Conclusion and Future Trends

The development of biosensors and bioassays has focused recently on small, easy to use in a decentralized and portable manner and low-cost analytical devices for sensing and quantitative determination of biomarkers. Special attention has been given to biomedical applications for improving global health. A hot topic among researchers is represented nowadays by the finding of technical solutions for the

improvement of the detection platforms through their functionalization. Furthermore, smartphones can be coupled with some detection methods (e.g. optical or electrochemical) to achieve fast, compact, portable and user-friendly sensing systems.

Biosensors provide many benefits compared with the currently used analytical methods, such as high sensitivity and increased selectivity when applied in complex samples. Another important advantage is related with their suitability for miniaturization, portability and possibility of on the field testing and monitoring. Moreover, the possible applications are diverse, starting with bioprocess control, food quality tests, as well as evaluation of contaminants and other compounds in agriculture, security and medical fields, with minimum or no sample pretreatment. Immunosensors were heavily used during the last decades since they combine the specificity of the immune reaction between antibody and its specific antigen with the sensitivity provided by the detection platform. More recently, aptamers (synthetic oligonucleotides) are widely used for the design of biosensors due to the improved specificity and stability in comparison to the antibody-based sensors.

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Immunodiagnosis by Electrochemical Multiplexing in Clinical Samples

S. Campuzano, P. Yáñez-Sedeño, and José M. Pingarrón

1 Introduction

Nowadays earlier and more reliable clinical diagnosis requires rapid, sensitive, selective multiplexed determination of certain protein biomarkers to minimize false negatives and positives likely to occur when a single molecule is measured and translate to personalized diagnostics leading to a more efficient therapy (Munge et al. 2016; Wan et al. 2013; Yáñez-Sedeño et al. 2018). Indeed, reliable, fast, and early diagnoses of diseases are crucial especially for patient survival but also for saving costs and not consuming excessive time (Bahadır and Sezgintürk 2015).

The National Cancer Institute (NCI) defines biomarker as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease." Biomarkers existing at different molecular levels include DNA, RNA, or proteins and must ideally possess certain characteristics: (a) both clinical specificity and high sensitivity, (b) prompt blood releasing to enable early diagnosis, (c) stay in the blood for long time at high levels, and (d) ability to be determined quantitatively (Bahadır and Sezgintürk 2015). Since different diseases alter the concentrations of characteristic biomarkers, the required methods for their determination and follow-up should be selective, sensitive, and ideally fast and simple (Bahadır and Sezgintürk 2015).

A variety of biochemical, immunological, and molecular biology-based methods have been reported and used for determining protein biomarkers in clinic. Immunoassay techniques, including radio-, fluoro-, chemiluminescent, and electrochemiluminescent immunoassays, and enzyme-linked immunosorbent assay (ELISA), based on schemes highly specific for molecular recognition are commonly employed in quantification of clinical biomarkers. However, despite their sensitivity, precision, and selectivity, these techniques have radiation exposure hazards, require

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sophisticated instrumentation and qualified personnel, are expensive, and require long time. Among them, ELISA is by far the most popular immunoassay technique. The commercial availability of antibodies for almost all types of molecules (e.g., biomacromolecules, nucleic acids, toxins, haptens, viruses, and cells), the high sensitivity due to the enzymatic amplification (for example, HRP can produce 10^7 molecules per one minute; Kudr et al. 2018), the high throughput (96-well plate), and the simplicity of handling are among the most important reasons that justify the ELISA technique's success and long adoption. Furthermore the variety in the assay format (i.e., direct, sandwich-type, or competitive) makes this technique suitable for both antigens and antibodies determination (Ricci et al. 2012). However, this technique possesses some practical constraints such as time-consuming operation, applicability just for the determination of one protein at a time, high reagents cost, and assay workflow complexity. Other limitations derived from using optical transduction include the requirement of expensive and noneasy miniaturized instrumentation, signal potentially false because of colored or turbid samples, and the required minimum volume of sample and path length to achieve certain actions because of the Lambert–Beer law. All these limitations hinder the application of ELISA techniques in POC testing. Consequently, there is a growing demand for developing faster and more affordable methods compatible with portable instrumentation for on-site multiplexed determinations to amend a reliable and early personalized clinical diagnosis and, subsequently, improved therapy efficiency. The reason why, despite requiring similar incubation and washing steps, immunosensors with electrochemical transduction are widely utilized nowadays in clinical diagnosis is due to their operational simplicity, inherent selectivity and high sensitivity, fast analysis, low cost, large-scale production, compatibility with measurement in scarce sample volumes (nL-μL) and in turbid and colored samples, disposable use, microfabrication technology, miniaturization, portability, automation, simultaneous multitarget analysis, decentralized determinations, and high-throughput analysis (Ricci et al. 2012; Bahadır and Sezgintürk 2015; Justino et al. 2016a; Pingarrón et al. 2018; Felix and Angnes 2018). These features make electrochemical immunosensor to be uniquely positioned to provide diagnostic devices for POCT, with which diagnosis can be performed outside centralized clinical laboratories, at the bedside, in the clinic, or at limited resource settings.

However, adequacy of an ELISA method to an electrochemical assay is not a direct operation and, although the assay configuration and the steps involved in the overall analysis usually remain the same, several important aspects such as the suitable electrode surface utilized as solid support instead of the ELISA plate, the enzymatic substrate, the required electrochemical technique (instead of a simple optical absorbance), and the needed instrumentation should be carefully selected in order to get comparable better performances, if possible, than those offered by the commercially available ELISA methods (Ricci et al. 2012).

Although both the application of immunosensors in clinical diagnosis (Bahadır and Sezgintürk 2015; Justino et al. 2016b; Laocharoensuk 2016; Pingarrón et al. 2018) and the relevance of nanomaterials in the preparation of electrochemical immunosensors (Ronkainen and Okon 2014; Yañez-Sedeño et al. 2015; Addorahim

et al. 2016; Cho et al. 2018) have been reviewed nicely, this chapter describes the potential of immunosensing devices designed for multiplexed determination in clinical diagnosis by highlighting recent advances and key points to satisfy the current POC immunodiagnosis demands (high sensitivity, multitarget detection, and integration). Unresolved challenges, perspectives, and commercialization opportunities are also discussed.

2 Electrochemical Immunodiagnosis

Electrochemical immunosensors are analytical tools designed to detect electrochemically the binding between an antigen (Ag) and its specific antibody (Ab) leading to the formation of a complex with a high stability by immobilizing either Ab or Ag (Fig. 1). This type of electrochemical affinity biosensors combines the high selectivity and sensitivity that characterize the antigen–antibody reactions and the particular advantages offered by the electroanalytical methods. Electrochemical immunosensors imply measurements of an electrical signal (voltammetric, potentiometric, amperometric, conductometric, or impedimetric) derived from the immunoreactions and produced on electrochemical transducers, which are generally compact, mass produced in a robust and inexpensive way, have fast response times,

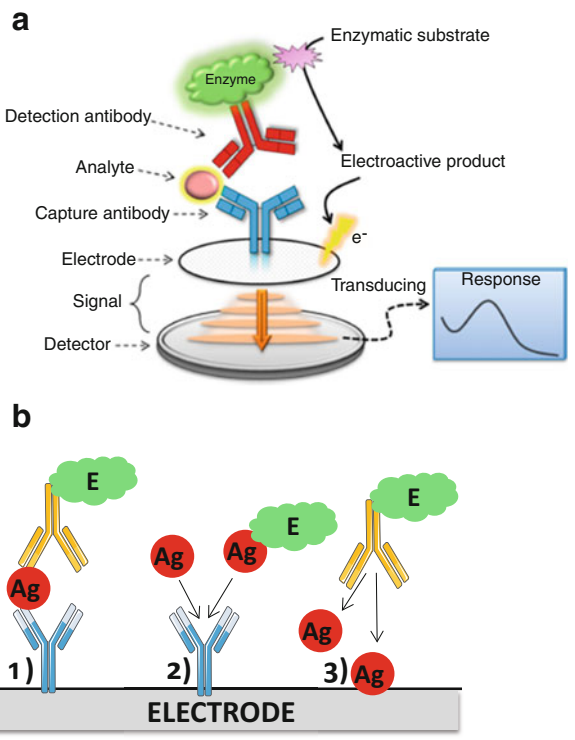

Fig. 1 Schematic display of an electrochemical immunosensor (**a**) and of the main immunosensors formats (**b**): sandwich (1), direct competitive (2) and indirect competitive (3). Reprinted from (**a**) (Cho et al. 2018) with permission

and require small analyte volumes (Felix and Angnes 2018). The signals usually come from labeled molecule acting as the signal tracer and linked in some way with the detection antibody (Fig. 1) or directly from the antigen–antibody interaction (label-free approaches) (Cho et al. 2018).

These affinity biosensors are constructed by immobilizing on the electrode surface the antigen or the antibody, which will act as the recognition element, followed by the careful selection of the most appropriate immunoassay configuration and electrochemical technique to perform the transduction. Regarding the immunoassay format, both sandwich and competitive configurations can be used to develop electrochemical immunosensors (Fig. 1b). Furthermore, two types of competitive immunosensors may be considered: one of them involves reaction of free antigens in competition with labeled antigens with the immobilized antibodies (direct competitive, 2 in Fig. 1b), while the other implies immobilized antigens and labeled antibodies (indirect competitive, 3 in Fig. 1b), which prevent problems associated with inappropriate antibody orientation during immobilization. In a sandwich-type assay, after binding immobilized antibodies to free antigens, labeled antibodies (generally interacting with a different binding site of antigen) are used to "sandwich" the antigen between the two antibodies (1 in Fig. 1a) (Ricci et al. 2012).

Electrochemical immunosensors are considered nowadays fundamental devices for diagnosis of important human diseases including tropical, bacterial, and viral infections, neurodegenerative diseases, metabolic disorders, cardiovascular, cancer, or autoimmune diseases through accurate determination of characteristic biomarkers. As it is known, considerable attention has recently been paid to perform POC diagnosis at the bedside or in the clinic, which requires apart from sensitive, selective, and rapid detection, affordable, integrated, and small devices. The inherent benefits provided by electrochemical immunosensors with regard to sensitivity, moderate cost, low-power expenditure, and amenability to automation make these devices promising for POC testing (Wang et al. 2014). However, various demands must still be attained such as high sensitivity, multianalyte detection, and integration. As it will be seen in the following section, two key points for sensitivity improvement are discussed in detail: the used ways for immobilizing the recognition element onto the electrodes surface and for amplification of the electrochemical response related to the event of antibody–antigen binding (Wang et al. 2014).

2.1 Improving Sensitivity in Electrochemical Immunodiagnosis

Immobilization is a key step because it has a significant effect in the stability and sensitivity of the developed immunosensor. In electrochemical immunosensors, biomolecules should be immobilized on the electrode surface in large quantities, in a stable conformation, maintaining its bioactivity and with the appropriate orientation to allow high efficiency in immunological recognition reactions. This has been achieved mainly by using a plethora of (nano)materials as electrode modifiers (Cho et al. 2018) or applying attractive surface chemistries, such as the diazonium salts 1 (Yáñez-Sedeño et al. 2018), to modify the surfaces (Pingarrón et al. 2018).

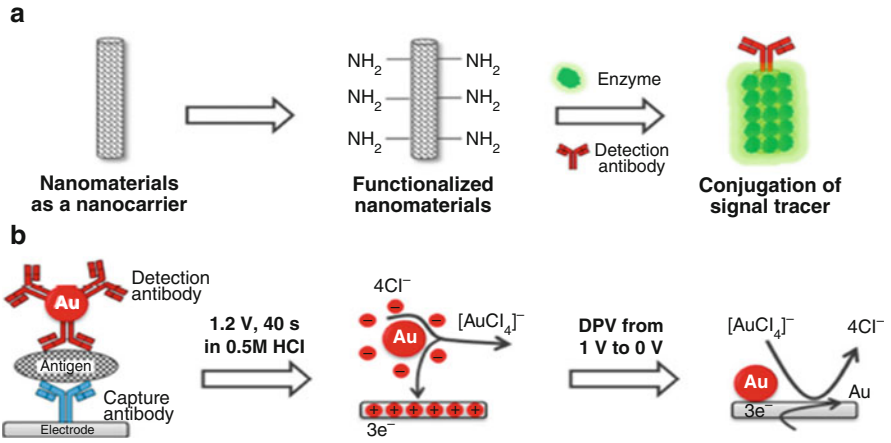

Fig. 2 Schematic display of labeling approaches involving nanomaterials in electrochemical immunosensors for amplification of the current response. Nanomaterials are employed as carriers of enzyme tags (**a**) or as electroactive nanotracers (**b**). Reprinted and adapted from Cho et al. (2018) with permission

The use of nanomaterials, mostly metal-based nanoparticles and carbon nanomaterials, in the construction of electrochemical immunosensors has been widely exploited with the aim of enhancing their analytical performance. In these devices, nanomaterials can serve as: (1) electrode surface modifiers to improve the conductivity and provide large area, biocompatible and/or high electrocatalytic surfaces, for the attachment of large amounts of immunoreagents (Cho et al. 2018), (2) nanocarriers of enzyme labels (Fig. 2a) (Pei et al. 2013; Sánchez-Tirado et al. 2017a; Cho et al. 2018), (3) electrochemical nanotracers (Fig. 2b) (Cho et al. 2018), and (4) seeds for metal deposition (Kokkinos et al. 2016; Justino et al. 2016a). In order to enhance sensitivity and reduce the limits of detection, many immunosensing strategies merge the use of nanostructured electrodes with multilabel bioconjugates.

Different nanomaterials have been used as modifiers of the electrode surface or nanocarriers in electrochemical immunosensors, including metal and silica nanoparticles, mesoporous metal structures, carbon nanomaterials (graphene, carbon nanotubes [Sánchez-Tirado et al. 2017b], graphene quantum dots [Mollarasouli et al. 2018], and nanohorns [Ojeda et al. 2014a; Sánchez-Tirado et al. 2017c; Yánez-Sedeño et al. 2016]), hybrid nanostructures (Felix and Angnes 2018), quantum dots (QDs), and magnetic nanoparticles. However, due to their chemical stability, biocompatibility, and high surface area-to-volume ratio, metal-based nanoparticles (NPs) and QDs have widely been used as electrochemical nanotracers to develop ultrasensitive immunosensors, overcoming some of the drawbacks related to the use of enzymes (such as scarce stability and low signal amplification capacity attributed to the low ratio between redox active sites and volume) (Kokkinos et al. 2016). Label metal NPs are usually quantified through their acid dissolution and determination of the released metal ions by amperometry, voltammetry, or stripping voltammetry.

In particular, AuNPs are the most extensively used NP-based labels. Their detection can be accomplished by: (1) (electro)chemical oxidation in acid media followed by stripping voltammetry (SV); (2) reduction of released Au (III); and measuring the electrochemical reduction of $AuCl_4$ (generated by peroxidation of AuNPs) to metallic gold by DPV (Fig. 2b) or SWV. Other interesting detection methods include the direct electrochemical detection of AgNPs labels, which is performed through a solid-state Ag/AgCl process related to the presence of AgNPs in an aqueous solution containing KCl, or using NPs as nucleation sites for deposition of a second metal (Ag, Cu) over AuNPs, and further used as the electroactive label (Kokkinos et al. 2016).

Hybrids composed of metal NPs and different types of microbeads, such as carbon microsphere/AuNPs (Xu et al. 2012) and polystyrene microbeads/AgNPs (Lou et al. 2014), have also been utilized with excellent results as labels in electrochemical immunosensors. However, the relatively close stripping peaks of usually employed metal NP labels (Au, Ag, Cu) make it difficult for their application in multiplexing detection schemes.

In this context, the use of QD-based labels constitutes an interesting alternative mostly in sandwich-type assays, due to the differences in the oxidation potentials of the diverse QDs. When using as labels, QDs are oxidized in acid media (HCl or HNO_3) and the respective cations, mainly Cd^{2+}, Pb^{2+} and Zn^{2+}, are released and transferred to an electrochemical cell where they are measured with a high sensitivity. Immunoassays involving QDs are usually developed at gold, bare, or modified carbon surfaces and on different types of electroplated metal electrodes (film electrodes, FEs) prepared with mercury, (MFEs), bismuth (BiFEs), tin (SnFEs), or antimony (SbFEs) and more recently on electrodes produced in massive way using sputtered/photolithographic as well as screen-printed electrodes modified with these metal precursors (Kokkinos et al. 2016).

However, despite the great advantages that nanomaterials offer, they are pretty expensive, (above all if they are bought from specialized companies) and their modification protocols often involve complicated and long processes, of difficult implementation as an industrial process or in sensors mass production. All these drawbacks may explain why nanomaterials are not usually employed in prototypes or commercialized instruments. However, it is worth to note that a company leader in the fabrication of screen-printed electrodes (Metrohm-DropSens) provides them already modified with single or hybrid nanomaterials (graphene and graphene derivatives, single and multiple-walled carbon nanotubes, AuNPs), which have already demonstrated good applicability in the preparation of electrochemical multiplexed immunosensors applied to clinical diagnosis (Escamilla-Gómez et al. 2009; Ricci et al. 2012; Neves et al. 2013).

Other attractive electrochemical immunosensing strategies involve the use of integrated designs by taking advantage of rational chemistry for surface functionalization using grafted diazonium salts (Moreno-Guzmán et al. 2012; Martínez-García et al. 2016; Yáñez-Sedeño et al. 2018; Campuzano et al. 2018), or the use of conducting polymers with a high number of functional groups (Serafín et al. 2014, 2017a; Martínez-Periñán et al. 2018). These versatile, simple, and fast

chemical alternatives have demonstrated a great potential to covalently immobilize in a stable, reproducible, and functional way both immunoreagents (Guerrero et al. 2015) and nanomaterials (Serafín et al. 2018). Indeed, some attractive immunosensing strategies developed for clinical diagnosis combine the advantages of using nanomaterials and this particular chemistry (Fig. 3) (Guerrero et al. 2015; Serafín et al. 2018). Moreover it is worth to mention the easiness, rapidity, and versatility offered by the electrografting methodology to create functions individually onto each electrode platform, which opens up very interesting chances for the mass manufacturing of integrated multiplexed electrochemical biosensors (Yáñez-Sedeño et al. 2018).

Apart from regeneration, which is considered a key factor in the application of immunosensors in clinical diagnosis (Bahadır and Sezgintürk 2015), the requirements of several steps or the difficulties involving direct immobilization of the recognition element onto the electrode surface make them to be not always adequate for mass production. Another drawback affecting integrated immunosensing surfaces is that the use of the electrode during the whole immunological chain may cause the surface passivation/poisoning by nonspecific adsorptions of sample species, worsening considerably the analytical performance of the immunosensor. Moreover, the many washing steps usually required by the immunological assays may create defects on the immunorecognition layer compromising the resulting reproducibility. In addition, the surface confinement of the recognition element may limit both the kinetic of the affinity reaction and the loading of immobilized biomolecules (Ricci et al. 2012; Kokkinos et al. 2016).

An appealing alternative to overcome all these drawbacks is the utilization of magnetic nanobeads or microbeads (MBs) as solid support where the whole immunoassay is carried out (Yánez-Sedeño et al. 2016; Kudr et al. 2018). MBs are particles consisting of a dispersion of magnetic material (such as magnetite, Fe_3O_4, and its oxidation product maghemite, γ-Fe_2O_3) covered with a thin shell of polymer that protects them from aggregation and serves as a suitable surface for coupling a large variety of molecules (see Fig. 4a). It is worth mentioning that MBs are much more widely used than magnetic nanoparticles in the preparation of electrochemical immunosensors because of the much more difficult magnetization of the latter due to the smaller size as well as their higher trend to form particle agglomerates and suffer from matrix effects (Serafín et al. 2017b). MBs functionalized with streptavidin (Esteban-Fernández de Ávila et al. 2013), tosyl groups (Sarkar et al. 2008), carboxylic groups (Ojeda et al. 2014b), bacterial antibody-binding proteins (Eguílaz et al. 2010; Moreno-Guzmán et al. 2010; Martínez-García et al. 2015), and tetradentate metal chelator (Campuzano et al. 2014) have been used for fast and efficient immobilization of antibodies.

Moreover, the working protocol with MBs implies that the whole immunoassay is performed by successive incubation steps in microtubes using small solution volumes and a rotation sample mixer, followed by washing steps where the MBs are concentrated by placing the microtubes in a magnetic separator. Therefore, this protocol allows fast immunoreactions and easy separation after application of washing and reaction steps.

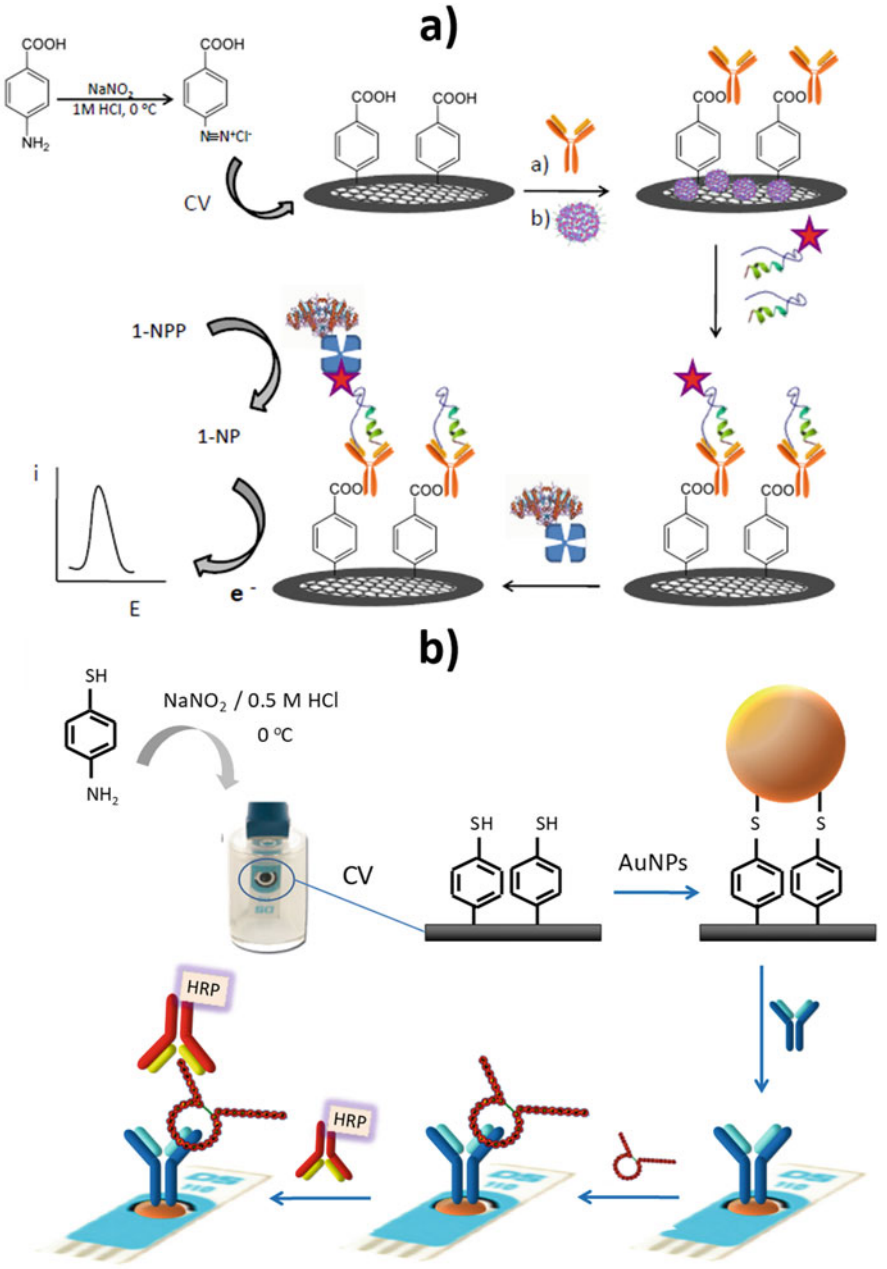

Fig. 3 Scheme of the steps involved in the fabrication of integrated electrochemical immunosensors: for anorexigen peptide YY (PYY) prepared by grafting of 4-ABA diazonium salt onto GCE modified with rGO-modified GCE and covalent immobilization of captured antibody (**a**) and for brain natriuretic peptide (BNP) by captured antibody immobilization onto a SPCE modified with AuNPs grafted through 4-aminothiophenol diazonium salt (**b**). Reprinted and adapted from (**a**) Guerrero et al. (2015) and (**b**) Serafín et al. (2018) with permission

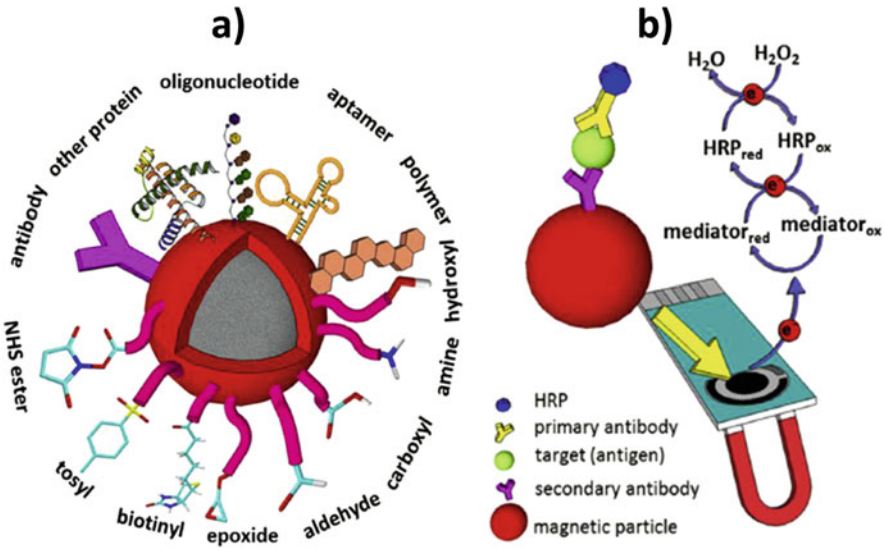

Fig. 4 Schematic display of the most common MB functionalizations (**a**) and of an electrochemical MB-based sandwich immunosensor involving the H_2O_2/HRP system (**b**). Reprinted and adapted from Kudr et al. (2018) with permission

An additional important practical advantage of using MBs is that large amounts of MBs can be modified with the immunorecognition element and stored during various weeks with no loss of activity. This storage ability until the determination is required is particularly interesting for reducing the analysis time and in the cases where there is a need to analyze a large number of samples. Moreover, since this technology makes possible the easy separation of the conjugate MB–analyte complex from the sample, and the electrode is used only in the transduction step, passivation and electrochemical interference events are minimized. This increases the assay selectivity and leads to an appropriate biosensing behavior in scarcely diluted/treated challenging samples. Upon modification, MBs can be magnetically captured onto the surface of magnetized electrodes or planar SPEs by means of specially designed supports with neodymium magnets embedded to be positioned exactly below the working electrodes. This procedure guarantees the stable and reproducible capture of the magnetic bioconjugates as well as the close contact of the enzymatic tracer with the electrode surface (Fig. 4b) (Serafín et al. 2017b).

Label-free electrochemical immunosensors are particularly attractive because they enable simplified and low-cost onsite, reagentless measurements (labeled secondary antibody or enzymes are not required) (Fig. 5), providing increased scope for commercial exploitation. Conversely to that occurring in immunoassays performed with labeled immunoreagents, where the electrochemical readout is obtained by measurement of the current, potential, or charge variations in the tagged species or the resulting immunocomplex (usually the electrochemical signal increases with the concentration of the target protein), in these label-free approaches

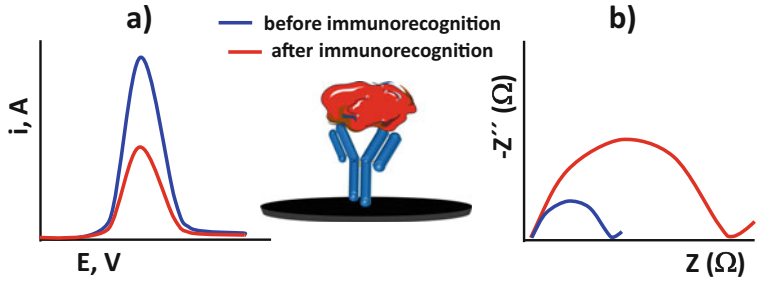

Fig. 5 Illustration of the signals obtained from a label-free immunosensing approach involving voltammetric (**a**) and impedimetric (**b**) transduction

the event of immunocomplexation can be monitored by measuring the decrease in the electrochemical (mainly voltammetric) response (Fig. 5a) of an electroactive probe (typically $[Fe(CN)_6]^{4-/3-}$) or the rise in the magnitude of the electron transfer resistance (R_{et}) measured by EIS (Fig. 5b) (Pingarrón et al. 2018).

2.2 Multiplexing and Integration in Electrochemical Immunodiagnosis

When facing POC clinical diagnosis, electrochemical immunosensors need to overcome several challenges including multiplexing detection and integration in handheld analyzers (Wang et al. 2014).

The association of protein biomarkers with various diseases and the changes in the expression of a single biomarker among populations make compulsory the determination of markers panels ("specific signatures") to enhance the diagnostic accuracy by minimizing false positive and negative results found when a single biomarker is used (Wang et al. 2014; Munge et al. 2016; Rusling et al. 2010). Furthermore, to date it has not been confirmed that an individual molecular biomarker is capable of accurately providing enough information for diagnosis. Therefore, a specific panel comprising information regarding a variety of biomarkers will provide more information in less time and more reliable diagnostic tests (Guo and Wu 2016; Rusling et al. 2010; Laocharoensuk 2016). However, the wide range of concentrations clinically relevant for different biomarkers (usually from pg mL^{-1} to ng mL^{-1} or above) in addition to the possibility that they are at a different molecular level makes multiplexed electrochemical detection particularly challenging. Thus, methods to determine simultaneously both elevated and low concentrations of diverse biomarkers in a panel related to the same sample and in the absence of interference from many other nontarget molecules (many of them at much larger concentration than that of analyte) are highly demanded (Pingarrón et al. 2018). Among other analytical technologies, electrochemical immunosensors exhibit inherent advantages for the multiple determination of biomarker panels due to the production by screen-printed technology of affordable, rapid mass-produced

electrode arrays with great versatility in both ink-printed and substrate type used (Wang et al. 2014).

Another challenge that POC systems face is the integration of electrochemical immunosensors into an instrument with analytical application. Although some approaches have been realized directly to the integration of electrochemical immunosensors with flow-through devices to develop automated configurations (Tang et al. 2010), these systems show drawbacks for further miniaturization. Therefore, integration of electrochemical immunosensors into microfluidic devices has been explored, providing exciting challenges for miniaturization, automation, and high-throughput detection (Wang et al. 2014).

Multiplexed immunosensors, which offer the abovementioned benefits of more accurate diagnosis, shorter analysis time, smaller volume of sample, and lower costs, have more complex technical requirements for application and acquisition of multiple response signals (Kokkinos et al. 2016). Current multiplexed electrochemical measurements can be made making use of two different approaches: with arrays of multielectrodes, where each single electrode has a distinct antibody/antigen immobilized, or using a barcode strategy with reporting antibodies labeled with different labels (nanomaterials such as NPs, QDs, or dissolvable metals, enzymatic and redox electroactive labels) able to generate distinguishable electrochemical signals on a single electrode and scan (Kokkinos et al. 2016).

In the first case, the spatial distance of neighboring electrodes should be long enough to avoid "cross-talking" effects. Although multielectrode arrays, usually consisting of various sensing surfaces with their own reference and auxiliary electrodes, require more complex electrochemical instrumentation (an independent multichannel electrochemical workstation), they offer greater flexibility and have been used more widely than the "barcode" approach due to the limited number of labels available so far to generate different electrochemical responses in a single sweep (Kokkinos et al. 2016).

Multiplexed electrode arrays are normally constructed by means of screen-printing techniques, which allows fast, cost-effective, and mass production of sensors with good electrical properties, great versatility to be easily customized in different geometries, materials and diverse formats (arrays of 4, 8 electrodes, and/or the 96-well electrochemical plate marketed in recent years), and compatible in size with small pocket-sized devices (Felix and Angnes 2018). Moreover, these disposable electrodes can be utilized through specific cable connectors both with bench-top and portable instruments. Although the bench-top instruments are more flexible and can generally apply a broader range of electrochemical techniques, their cost is higher (>20,000 €) and, at least for some electrochemical biosensing applications, they offer comparable performances to those of portable instruments (Serafín et al. 2019). In this connection, different companies, such as DropSens, PalmSens, and Uniscan, supply low-cost (2000–4000 €), friendly-to-user, and precise platforms able to implement the usual techniques employed in electrochemical immunosensors. These portable instruments can work with multielectrode arrays, and be also customized quite easily, and are provided with USB plugs for simple joining to everyday electronic devices (Ricci et al. 2012).

Electrochemical immunosensors have been successfully applied to the single determination of a variety of diagnostic targets including biomarkers of cardiovascular, cancer and infectious diseases, metabolic disorders, and inflammation processes, as well as hormones, neurotransmitters, bacteria, viruses, and cancer cells (Yánez-Sedeño et al. 2017). However, for multiplexed purposes, they have been mostly utilized for determining cancer and cardiovascular biomarkers both at the regulatory (proteins and autoantibodies) and genetic (microRNAs and methylated DNAs) levels. Regarding the type of analyzed sample, electrochemical immunosensing has been widely applied to the determination of circulating protein antigens in biofluids (mostly serum but also saliva [Martínez-García et al. 2015] and urine [Ojeda et al. 2014a, b]) and, more recently, to the determination of emerging relevance biomarkers such as serum autoantibodies (Garranzo-Asensio et al. 2016; Zhao and Liu 2016) and extracellular protein receptors in cells (lysed or whole) (Eletxigerra et al. 2015; Valverde et al. 2018a) and tissues (both fresh and paraffin-embedded) (Valverde et al. 2018b; Muñoz-San Martín et al. 2018). It is also important to point out that immunosensing platforms have shown usefulness to perform the simultaneous determination of biomarkers with clinical ranges differing in more than three orders of magnitude (Esteban-Fernández de Ávila et al. 2014). Moreover, the emerging relevance of other biomarkers besides proteins has led to the use of immunosensors for the multideterminations of immunogenic molecules such as miRNAs (Torrente-Rodríguez et al. 2016a, b) and methylated DNAs (Povedano et al. 2018a, b, 2019) in cells and paraffin-embedded tissues. Although most of the reported strategies have been applied to dual determinations, there are some electrochemical immunoplatforms able to carry out the determination of 3 or more biomarkers. Table 1 summarizes the relevant features of representative electrochemical immunosensing platforms reported recently for the multiplexed determination of at least three different biomarkers. They are classified according to the approach used for multiplexing (multielectrode arrays or barcode strategy) and type of biomarker/analyzed sample.

Table 1 indicates that, to date, electrochemical multiplexed immunosensors have been applied mostly to the determination of biomarkers related to cancer and cardiovascular diseases. The most commonly used immunoassay configuration is the sandwich type, which generally provides the best selectivity and sensitivity (Xia et al. 2018). The lack of multiplexed immunosensors involving competitive configurations and the development of label-free formats using direct immunoassays (Gupta et al. 2016; Zhang et al. 2011; Baraket et al. 2014) should be pointed out. In addition, the approaches involving electrode arrays used commonly screen-printed substrates, while those based on the barcode strategy are implemented on conventional electrodes (generally GCE). As it was already mentioned, the use of nanomaterials is a common strategy to construct immunosensors fulfilling the sensitivities required for clinical diagnosis. Graphene, AuNPs, and rGO have been used as electrode modifiers, whereas AuNRs, AuCs/G, CGN, and GS/AuNPs (as carriers of enzyme or redox labels) and M-PtNPs and QDs (as electrochemical nanotracers) have been employed as labels. The most commonly used electrochemical techniques are DPV, SWV, CV, LSASV, and chronamperometry (using enzyme/

Table 1 Multiplexed electrochemical immunosensors capable of the simultaneous determination of at least three different biomarkers

	Electrode	Immunoassay format	Label	Biomarkers/ disease	Electrochemical technique	Linear range	LOD	Sample	Ref.
Electrode Arrays	SPAuEs (with 4WEs)	Sandwich	AuNRs-HRP	Phospho-$p53_{392}$, phospho-$p53_{15}$, phospho-$p53_{46}$, and total p53/cancer	SWV (Thi/H_2O_2)	0.01–20 nM ($p53_{392}$) 0.05–20 nM ($p53_{15}$) 0.1–50 nM ($p53_{46}$) 0.05–20 nM (total p53)	5 pM ($p53_{392}$) 20 pM ($p53_{15}$) 30 pM ($p53_{46}$) 10 pM (total p53)	–	Du et al. (2011)
	Flexible PDMS slice deposited with 8 × 8 nano-Au film electrodes	Sandwich onto MBs	AuNRs-HRP	PSA, PSMA, IL-6/cancer	CV (H_2O_2)	0.1–10 ng mL^{-1} (PSA) 0.005–1 ng mL^{-1} (IL-6) 0.8–400 (PSMA)	0.1 ng mL^{-1} (PSA) 0.005 ng mL^{-1} (IL-6) 0.8 ng mL^{-1} (PSMA)	Serum	Liu et al. (2014)
	G/SPCEs (with 3WEs)	Sandwich	M-PtNPs	CA125, CA153, and CEA/cancer	DPV (H_2O_2)	0.05–20 U mL^{-1} (CA125) 0.008–24 U mL^{-1} (CA153) 0.02–20 24 U mL^{-1} (CEA)	0.002 U mL^{-1} (CA125) 0.001 U mL^{-1} (CA153) 7.0 pg mL^{-1} (CEA)	Serum	Cui et al. (2014)
	AuNPs/G/SPCEs (with 3WEs)	Sandwich	AP-AuCs/G	CA153, CA125, and CEA/cancer	LSASV (3-IP/ Ag deposition)	0.005–50 U mL^{-1} (CA153) 0.001–100 U mL^{-1} (CA125) 0.004–200 U mL^{-1} (CEA)	0.0015 U mL^{-1} (CA153) 0.00034 U mL^{-1} (CA125) 0.0012 U mL^{-1} (CEA)	Serum	Ge et al. (2012)
	CNF microE chip (3 × 3)	Direct	–	CRP, cTNI, and Mb/cardiovascular diseases	DPV (label-free, $Fe(CN)_6^{4-}$)	–	–	Serum	Gupta et al. (2016)
	SiNW array	Direct	–	cTnT, CK-MM, CK-MB/ cardiovascular diseases	Resistance changes caused by specific binding	1 pg mL^{-1} to 10 ng mL^{-1} (three biomarkers)	1 pg mL^{-1} (three biomarkers)	Blood	Zhang et al. (2011)

(continued)

Table 1 (continued)

	Electrode	Immunoassay format	Label	Biomarkers/ disease	Electrochemical technique	Linear range	LOD	Sample	Ref.
	BioMEMS based on silicon substrate (with 8WEs)	Direct	–	IL-1, IL-10, IL-6/ cardiovascular diseases	EIS (label-free, $Fe(CN)_6^{3-/4-}$)	1–15 pg mL^{-1} (IL-10)	–	–	Baraket et al. (2014)
	Screen-printed carbon electrodes array (with 8WEs)	Direct	Strep-HRP	miRNA-21, miRNA-31, and miRNA let-7a/ cancer	Chrono-amperometry (H_2O_2/HQ)	1.2–100 pM (miRNA-21)	0.66 pM (miRNA-21)	RNA_t extracted from cancer cells and cervical precancerous lesions	Jirakova et al. (2019)
Barcode approach	G/Au/GCE	Sandwich + HCR	THI (or Co or Fc or AQ)-strept-biotin-dsDNA/ strept-biotin-Ab_2/AuNPs/ SiO_2/Fe_3O_4	CEA, CA125, PSA, and AFP/cancer	DPV (THI, Co, Fc, AQ)	0.2–600 pg mL^{-1} (CEA), 0.2–1000 pg mL^{-1} (CA125), 0.2–800 pg mL^{-1} (PSA), 0.2–800 pg mL^{-1} (AFP)	48 fg mL^{-1} (CEA), 77 fg mL^{-1} (CA125), 60 fg mL^{-1} (PSA) 62 fg mL^{-1} (AFP)	–	Zhu et al. (2015)
	AuNPs/GCE	Sandwich	PVP/GS/ PtPdNPs/ HRP/AQ (or THI)	AFP, APT; DCP, AFP-L3, γ-GT, and AFU/cancer	DPV (AQ or Thi)	0.025–5.0 ng mL^{-1} (AFP), 0.024–9.6 ng mL^{-1} (APT), 0.032–3.2 UL^{-1} (DCP), 0.024–2.4 ng mL^{-1} (AFP-L3), 1.0–9.5 U L^{-1} (γ-GT), 1.2–9.0 U L^{-1} (AFU)	0.008 ng mL^{-1} (AFP), 0.0082 ng mL^{-1} (APT) 0.01 U L^{-1} (DCP), 0.008 ng mL^{-1} (AFP-L3), 0.33 U L^{-1} (γ-GT), 0.4 U L^{-1} (AFU)	Serum (spiked)	Yang et al. (2014)
	GCE (Hg)	Sandwich onto MBs	PAMAM-CdS (or ZnS, PbS)	CA125, CA15-3, and CA19-9/cancer	SWASV (Cd, Zn, and Ag)	0.01–50 U mL^{-1} (three biomarkers)	0.005 U mL^{-1} (three biomarkers)	Serum	Tang et al. (2013)

	GCE	Sandwich onto MBs	Envision™-CdS (or PbS or AuNPs)	AFP CEA and CA19-9/cancer	DPASV, DPCSV (Cd, Pb, and Au)	0.001–50 ng mL^{-1} (AFP and CEA) 0.005–100 ng mL^{-1} (CA19-9)	0.02 pg mL^{-1} (AFP) 0.05 pg mL^{-1} (CEA) 0.3 pg mL^{-1} (CA19-9)	Serum (real and spiked)	Wang et al. (2014)
	IL/rGO/PSS/GCE	Sandwich	CGN-THI CGN-DAP CGN-Cd^{2+}	CEA PSA and AFP/cancer	SWV (THI, DAP, Cd^{2+})	0.01–100 ng mL^{-1} (three biomarkers)	2.7 pg mL^{-1} (CEA) 4.8 pg mL^{-1} (PSA) 3.1 pg mL^{-1} (AFP)	Serum	Xu et al. (2015)
	Protein A/Nafion/GCE	Sandwich	GS/AuNPs-THI (or $Co(bpy)_3^{3+}$, Fc)	AFP CEA and SS2/cancer	DPV (THI, $Co(bpy)_3^{3+}$, Fc	0.016–50 ng mL^{-1} (AFP) 0.010–50 ng mL^{-1} (CEA) 0.012–50 ng mL^{-1} (AFP)	5.4 pg mL^{-1} (AFP) 2.8 pg mL^{-1} (CEA) 4.2 pg mL^{-1} (AFP)	–	Zhu et al. (2013)

AFP alpha fetoprotein, *AP* alkaline phosphatase, *AuCs* Au cluster, *AuNRs* gold nanorods, *CA125* carbohydrate antigen 125, *CEA* carcinoembryonic antigen, *CNF* carbon nanofiber, *CGN* carbon-gold nanocomposite, *CV* cyclic voltammetry, *DPV* differential pulse voltammetry, *EIS* electrochemical impedance spectroscopy, *Fc* ferrocene, *GCE* glassy carbon electrode, *G* graphene, *HCR* hybridization chain reaction, *HRP* horseradish peroxidase, *IL* interleukin, *3-IP* 3-indoxyl phosphate, *HCR* hybridization chain reaction, *MBs* magnetic beads, *LSASV* linear sweep-anodic stripping voltammetry, *M-PtNPs* mesoporous platinum nanoparticles, *NPG* nanoporous gold, *PDMS* polydimethylsiloxane, *PSA* prostate-specific antigen, *PSMA* prostate-specific membrane antigen, *rGO* reduced graphene oxide, *SPAuEs* screen-printed gold electrodes, *SPCEs* screen-printed carbon electrodes, *Strept* streptavidin, *SS2 Streptococcus suis* serotype 2, *TB* toluidine blue, *THI* thionine, *TMB* 3,3′,5,5′-tetramethylbenzidine

redox label carriers as labels), SWASV, DPASV, and DPCSV (using electrochemical labels as QDs), and EIS in label-free approaches. Although most of these multiplexed immunoplatforms are prepared by integrated formats, in some of them MBs are used as solid supports for the immobilization of sandwich immunoconjugates (Tang et al. 2013; Liu et al. 2014; Wang et al. 2014). As expected, most of the immunoplatforms have been applied to the determination of protein biomarkers. However, recently, immunoplatforms for the multidetermination of biomarkers at different molecular level such as miRNAs have been also reported (Jirakova et al. 2019). Further development in this direction will be determined by the production and commercial availability of antibodies specific for nonprotein targets. Regarding the type of sample analyzed, the approaches described to date for the multiple determinations of three or more biomarkers have been utilized only in applications for analyzing human serum. Only very recently, an electrochemical immunoplatform has been developed for the determination of RNA_t extracted from cells and samples collected using cervical brushes (Jirakova et al. 2019), despite the great progress demonstrated recently to their successful application in the single or dual determination of target proteins (Eletxigerra et al. 2015, 2016a, b; Torrente-Rodríguez et al. 2017), miRNAs (Torrente-Rodríguez et al. 2016a, b; Jirakova et al. 2019), methylated DNAs (Povedano et al. 2018a, b, 2019), or multilevel molecular biomarkers (Valverde et al. 2018a, b) in cells (lysed or intact) and tissues embedded in paraffin. It is worth to highlight the development of a flexible multiplexed substrate (Liu et al. 2014) (Fig. 6) and immunoassays coupled with enzyme-free and isothermal hybridization chain reaction (HCR) for amplification purposes (Zhu et al. 2015).

3 Electrochemical Immunosensing Platforms from Laboratory to Commercial *Point-of-Care* Testing

The all-embracing need of effective POCT grows continuously. Currently, POCT dominates as an end-user application in the clinical field, and it is predicted that the market for POC biosensors in 2027 will be around $33 billion (Pereira da Silva Neves et al. 2018). Such global need is due both the new discoveries in the diagnosis field and the global trends as world demographic growth and population aging in developed areas. Therefore, tremendous efforts must be realized to supply analytical solutions with affordable cost for early and reliable diagnosis even in the settings with most limited resources, where instrument maintenance is not possible, making ideal the equipment-free POCT systems (Zhu et al. 2019).

Despite the research done in laboratories and the important achievements reached in developing highly sensitive and selective multiplexed electrochemical immunosensors (Pereira da Silva Neves et al. 2018), there are just a few examples of systems commercially available for POC use. These are the i-STAT analyzer, http://www.poct.co.uk/index.cfm, electro-immunointerfaces and surface nanobiotechnology, the ELISHA system, http://www.immunosensors.com, and the Ask-lepiossystem (http://genefluidics.com/products_asklepios.php#). Some reasons

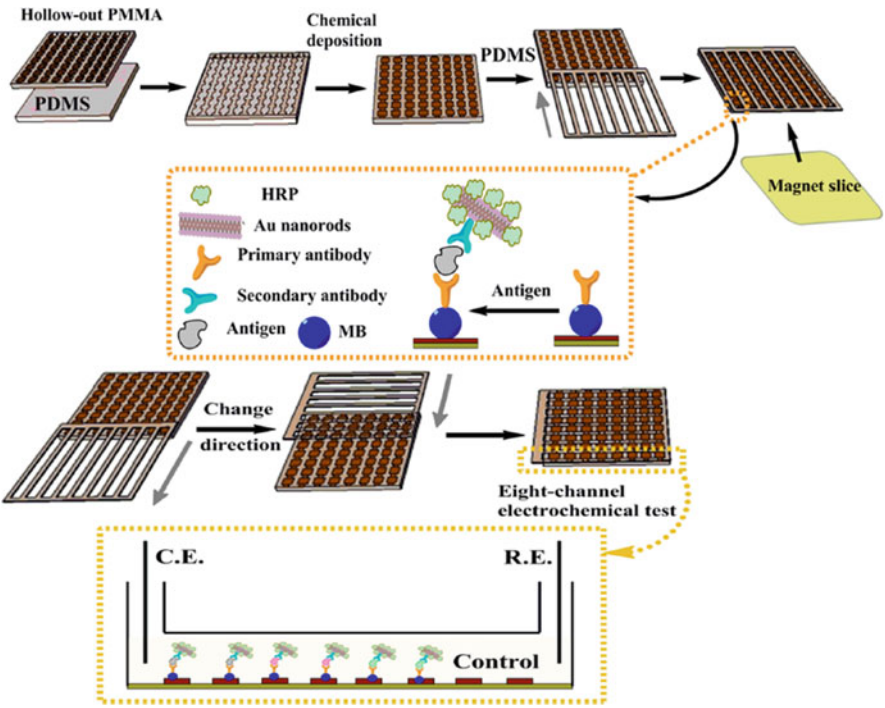

Fig. 6 Schematic display of a flexible gold electrode array for multiplexed electrochemical immunosensing of three protein biomarkers related with prostate cancer (IL-6, PSA, and PSMA). Reprinted and adapted from Liu et al. (2014) with permission

that may explain this insufficient representation of electrochemical immunosensors in the market include (1) the long and sometimes elaborate procedures required for the incorporation of nanomaterials in the immunosensors together with their relative high cost; (2) the difficult replacement of established methods, the high costs of innovative R&D, and some legal aspects such as regulatory approvals or clinical trials; (3) the large gap existing between academic research and industry, and the lack of: (4) adequate internal validation detailing the procedures, general application requirements, characteristics of fundamental performance, and applicability (selectivity, truthfulness, and precision); (5) comparison studies performed with well-established methodologies, and (6) realistic solutions to tackle analytical drawbacks related to nonspecific binding, reproducibility, long-term stability, and transportation capabilities of the immunosensors. Work in coordination to overcome these major issues is expected to give electrochemical immunosensors a leading position in the global market for immunodiagnostics (Kokkinos et al. 2016).

Future POC systems are anticipated to become as common cellular phones, personal computers, or integrated into small household devices. This means that additional research efforts are needed to develop POC systems with the required features for automation, miniaturization, full integration, and reduced costs toward

marketization (Wang et al. 2014). Related to this, the use of smart materials (paper, flexible, and wearable substrates), the development of microfabrication technologies and of microfluidic systems, and the employment of systems more autonomous and handheld readout have nowadays attracted a great attention.

In the last years, miniaturization and integration of electrochemical cells together with the advances in techniques, materials, and electronics for microfabrication have made easier the fabrication of sensors portable, cheaper, and smaller, overcoming the limitations of large-scale production. In addition, microfluidic methodologies (lab-on-a-chip and micro-total analysis systems, μTAS) have enabled the implementation in the form of automated steps, those involved in the inherently elaborate workflow assay such as labeling, separation, washing, incubation, and response amplification. Additional advantages of microfluidic immunosensors over conventional immunoassay methods include affordable fabrication materials, faster analysis by speeding up the antibody–antigen interaction (due to the smaller length scales and the high surface area-to-volume ratios), lower amounts (often nL) of immunoreagents and samples, and integration with other elements such as pumps, valves, mixers, and detectors to achieve POC demands (Han et al. 2013). Therefore, electrochemical immunoassay integration into microfluidic devices of low cost, ideally able to be fully fabricated by emerging 3D printing methods, can yield portable, versatile, and affordable tools for clinical diagnosis by means of in-field measurements of various biomarkers in clinical actionable times and with minimal reagents and sample amounts required (Kokkinos et al. 2016; Felix and Angnes 2018; Pingarrón et al. 2018).

The design of MB-based immunosensors embedded into microfluidic systems is particularly attractive for POC devices for diagnosis, where the magnetic particles, magnets, and screen-printed electrodes are integrated within the microfluidic system allowing automated measurements to be made in the flow mode and substantially reducing both reagent consumption and manipulation by the end users (Fig. 7) (Medina-Sanchez et al. 2014; Kokkinos et al. 2016).

Paper-based electrochemical immunosensors (e.g., microfluidic analytical devices made of cellulose/nitrocellulose paper or lateral flow strips) offer important advantages in clinical diagnosis because they are affordable, lightweight, and disposable multiplexable POC platforms. Furthermore, they are easy to use and possess capacity to function without the need of external pumps although sometimes sacrificing sensitivity (Fig. 8). These interesting merits make paper-based devices, although still at their early development stages, to envisage an outstanding position in the advancement of commercially available POC (Kokkinos et al. 2016; Pereira da Silva Neves et al. 2018; Zhu et al. 2019; Lee et al. 2018). Its recyclability, regeneration, and easy printing and coating with chemical reagents or biomolecules make paper the ideal substrate for microfluidic-based sensors (μPADs), which are being able to move slowly from research to industry since their early beginning for the opportunity they offer to facilitate multiplexed testing by integrating different kinds of electrochemical sensors. More recently, polymeric substrates have attracted also great attention as smart and flexible materials avoiding the limitations of common rigid surfaces and allowing the development of sensors with improved mechanic

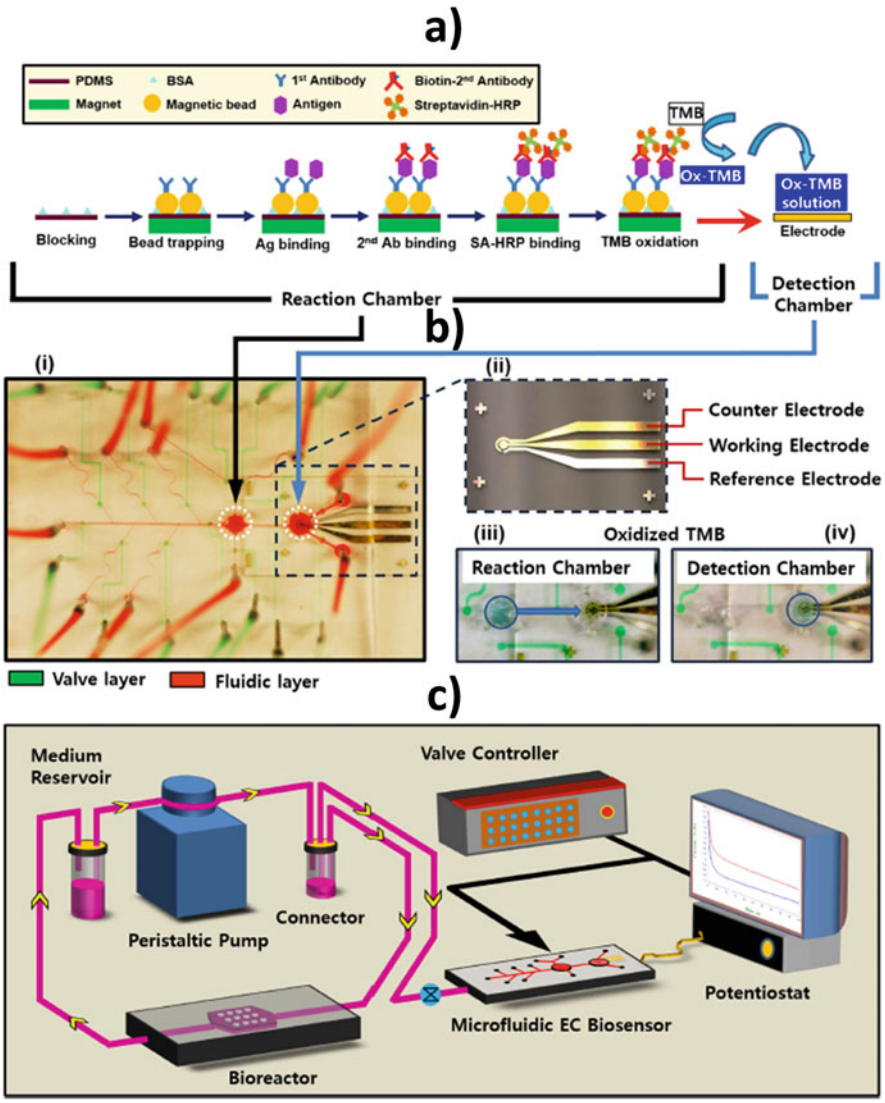

Fig. 7 Automated microfluidic system using electrochemical immunosensors based on MBs and integrated with a bioreactor for continuous monitoring of biomarkers secreted from cells. Illustration of the electrochemical immunosensing platform (**a**). Design of the microfluidic sensor chip (photographs of the microfluidic chip with an integrated microelectrode (1), microelectrode (2), reaction chamber containing oxidized TMB (3), and oxidized TMB transfer to the detection chamber (4) (**b**). Scheme of the whole automated microfluidic platform (**c**). Reprinted and adapted from (Riahi et al. 2016) with permission

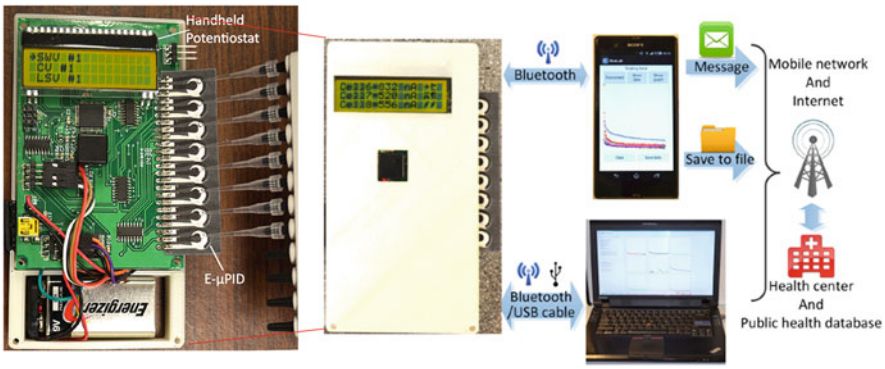

Fig. 8 A paper-based portable platform for diagnosis with a handheld multichannel potentiostat integrating a microfluidic immunosensor array (E-μPID) (left). Wireless data transmission from the potentiostat through the diagnostic device to a smartphone or PC and then to a remote site (right). Reprinted and adapted from Zhao and Liu (2016) with permission

resistance, better conformal adjustment in implantable/wearable devices, and lower mechanic stress (Pereira da Silva Neves et al. 2018).

Although nonwearable biosensors still account for major market share, it is expected that wearable devices begin to progressively gain ground. The development of wearable noninvasive electrochemical biosensors, already implemented in objects of daily use (mouth guards, bandage, contact lenses, eyeglasses, rings, skin tattoos, and textile materials), hopefully will allow replacing the invasive gold-standard blood analysis by painless analysis in other biofluids (sweat, saliva, and tears). Wearable perspiration sensors have been applied in personalized or sports medicine but also in clinical diagnosis or drug abuse control. It is expected that the combination of wearable electrochemical sensors with automated wireless data communication systems for continuous monitoring of relevant biomarkers brings new market possibilities to the immunodiagnosis field (Pereira da Silva Neves et al. 2018).

Moreover, the implementation of more autonomous handheld readout systems will also contribute definitely to the market growth of electrochemical immunosensors and in the concept of out-of-lab analysis. In this context, acquisition of real-time data combined with the ubiquitous availability and portability of smartphones, and other remote base stations, usually through bluetooth or near-field communication, has been exploited to achieve lab-on-smartphone applications (see Fig. 8). The ultimate goal is to achieve full portability by constructing robust and reliable self-powered biosensors, which in turn requires major efforts to avoid obstacles such as the biocomponents instability and the short lifetime as power supply (Pereira da Silva Neves et al. 2018).

Fundamental and applied research and the acceptance by the clinical community will be determinant also to ensure efficient transference of this promising technology from academia to industry. Assuming all these realities, the development of POC

diagnosis devices to routinely measure multiple biomarkers is a daunting challenge but realizable in the coming future.

4 Outlook: General Conclusions, Challenges, and Prospects

The impressive evolution that immunosensors have undergone in recent years, reflected by the intensive scientific production, suggests that they will play a key role in multiplexed POC clinical diagnosis by providing expedient solutions to the most contemporary demands. Nevertheless, we must be aware that there is still a long way toward the commercialization and widespread POC use of electrochemical immunosensing technology especially for multiplexed determinations.

Indeed, electrochemical immunosensors are the type of immunosensors most used in clinical diagnosis as POC devices because of their portability, simplicity, cost, and disposability in most cases. The nanotechnology applied to electrochemical immunosensing has demonstrated to maximize its detection capabilities by improving both the analytical performance and stability leading to the detection of diagnosis biomarkers at very low concentrations. However, it should be noted that, so far, most of the research efforts have been focused on the preparation of the immunosensors and their characterization using standards rather than the real application. Accordingly, they have been mostly reported as prototypes and tested under laboratory conditions and for a limited number of biomarkers. Moreover, applicability of the developed immunosensors has been limited in most cases to doped biological samples or, in the best cases, to a low number of real samples not enough to ensure a reliable validation. Therefore, future research should be focused to identify and clinically validate new biomarker signatures, so that electrochemical immunosensors can find wide applicability in other prevalent diseases, apart from cancer and cardiovascular diseases, such as the autoimmune and degenerative ones. Optimization of storage, stability, and transportation conditions of immunosensors as well as ensuring appropriate functioning in scarcely treated/diluted complex samples will be the challenges to face up to construct commercial devices for multiplexing analysis of biomarkers even with high differences in their threshold clinical levels (Justino et al. 2016b).

In summary, despite the numerous and important challenges to address, the unique features offered by electrochemical immunosensors make them particularly promising analytical tools for speeding up and make accessible to everyone the diagnosis of diseases, providing results at decentralized settings within few minutes, thus allowing clinical decisions to be made more quickly and reliably with the consequent reduction in patient stress and healthcare costs. Major efforts and funding are now being devoted to innovative research and development and productive collaboration between universities, research centers, companies, and end users of the technology to face up the continuous market demands on simpler, faster, cheaper, automated, and decentralized diagnosis applications while ensuring the availability of samples supply for large case studies in order to boost the commercialization of these technologies. The anticipated development of these systems will

ultimately lead to their continuous growth in the coming years, making them take their rightful place in the market as reliable tools for routine clinical diagnosis (Pereira da Silva Neves et al. 2018).

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Principle and Applications of Immunodiagnostics Using Radioisotope as Tracers

Shishu Kant Suman, Rohit Sharma, and Chandan Kumar

1 Introduction

The words "Anu" and "Paramanu" are the original Sanskrit text meaning "smallest particle" and "atom," respectively, have been documented thousands of years back. Ancient sage of India "Acharya Kanad" coined the term "Anu," while "Paramanu" is described in Brahma Samhita (written 5000 years before) Chap. 5 text 35 (Bhaktisiddhanta 1932). Around 500 BC Greek philosopher predicted the existence of the atom and named it "atomos" meaning "indivisible" and thus get recognition to coin the term. Several centuries have passed, and in 1803, John Dalton (1766–1844) described "atom" commonly known as Dalton's atomic theory. This theory is widely accepted by the modern science. However, after the mid-nineteenth century, there is a chain of discoveries leading the path of nuclear science of modern age (Kumar et al. 2013).

2 History of Radiation and Radionuclides

Wilhelm Roentgen discovered the "mysterious rays" later known as the X-ray. This electromagnetic radiation that was used to make visible the unseen organs of the body by creating image was a major revolution in the history of medicine in 1895. Another accidental but remarkable discovery by Antoine Henri Becquerel in March 1896, opens a new path of avenue the phenomena of radioisotope notably known as radioactivity, which was later described by Marie Curie (1898). Ernest Rutherford's (1911) experiments where the positively charged α-particles were bombarded to the thin gold foils were the first to conclude that there would be empty space in an atom. Because most of the particles passed through the foil undisturbed while a very small

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number of α-particles "bounced back" almost at 180°, confirmed that the atom must be made of a heavy central positive nucleus. Artificial production of radioactivity reported by Irene-Joliot-Curie and Frederic Joliot, in the year 1934, opens a new avenue for the production of several radioisotopes initiated by bombarding with alpha particles and later with neutrons. Radiotracer applications in industry and biology are possible by the pioneering work of George Hevesy (1943) that resulted in the modern day application of radioimmunoassay in medical sciences and tracer based assay in industry and hydrology.

3 Production of Radioisotopes

Radioisotopes of an element have unstable nuclear configurations. During the course of attaining nuclear stability, nucleus undergoes re-configuration and in that process several energetic radiations such as alpha (α), beta (β), or gamma (γ) rays are emitted, thus resulting in an isotope of a different element; such phenomena commonly known as radioactivity.

Radioisotopes are classified as natural and manmade (artificial). Natural radioisotope present in nature since time immemorial. The stable nuclides can be transformed into the radioisotopes (unstable nuclides) of the same or different elements through nuclear reactions well known as artificial radioactivity. This nuclear reaction can be possible in two systems, viz. nuclear reactor and cyclotron. Although cyclotron was the first route for the production of radioisotopes where target nuclides bombarded with charged particles (proton, deuteron, etc.), in contrary to the nuclear reactors where projectile is neutron. Radioisotopes used for vivid applications are mostly reactor produced since it offers a large volume irradiation, thus an economically viable production of various radionuclides could be possible.

In a nuclear reactor, neutrons are produced in abundance due to the chain reaction of fission of uranium. Stable nuclides are exposed to the neutron flux for an appropriate duration of time and thus become unstable and thereby following different routes such as (n, γ), (n, β), (n, p), (n, α), and (n, f), to attain stability. The radionuclides continue emitting different types of radiation till it become stable nuclei. In due course of time unstable radionuclides may transform into different radionuclides to achieve stability. The time duration in which one radionuclides may change to other radionuclides/stable nuclides is termed as half-life. Radioisotopes produced in nuclear reactor are generally rich in neutron. In general, reactor produced radioisotopes are decayed by beta emission followed by the emission of gamma rays. The rate of radionuclides productions from the nuclear reactor is determined by its neutron flux, purity of the target materials, and the cross section for the desired reaction.

In accelerators (cyclotron or linear accelerator), proton (p), deuteron (d), alpha (α), $^{3}He^{2+}$, etc., are used as projectiles to bombard the target nuclides. Thus, such radionuclides are in general neutron deficient and often decay through positron emission (β^{+}) or electron capture. It is a preferred route for the production of several medically important radioisotopes; however, cyclotron produced radioisotopes are

more expensive than the reactor produced radioisotopes due to the sophisticated technology of accelerator.

4 Characteristics of Diagnostic Radionuclides

On the basis of the emitted radiation, radionuclides are used for diagnosis and therapy. Alpha and beta emitting radionuclides are used for therapy since these particulate emissions are most effective for killing affected cells/tissues. While, the non-particulate emissions such as gamma and positron are used for diagnosis. Gamma emitter used for the SPECT (Single Photon Emmission Computed Tomography) based imaging, while positron emitters are used for the PET (Positron Emmission Tomography) imaging. For diagnosis, radionuclides of shorter half-life are most preferred. Radionuclides such as ^{99m}Tc, ^{111}In, ^{123}I, ^{201}Tl, ^{67}Ga, ^{131}I, ^{153}Sm, ^{166}Ho, $^{186/188}$Re, etc., are used for SPECT, while ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{68}Ga, etc., are used for the PET. Selection criteria for good diagnostic radioisotopes are as follows:

- There should be no particulate emission from the radioisotope.
- Mode of decay of radioisotope should be by gamma, positron or electron capture.
- Energy range for the gamma/SPECT imaging radionuclides should be in between 80 and 300 keV.
- There should be high photon yield for the radioactive decay of isotope used in imaging.
- Half-life of radioisotope should be optimum to avoid the burden of radiation dose to the patient.

5 Antibody and Radiolabeled Antibody

The history of antibody dated back soon after the invention of "hybridoma" technology, which opens a new avenue for the production of varieties of "monoclonal" antibodies (Köhler and Milstein 1975). OKT3 was the first murine monoclonal antibody against anti-CD3 used for treatment in 1986. However, OKT3 induced severe human anti-mouse antibody (HAMA) response in patients. Hence, to reduce the severity of HAMA response, chimeric antibodies were developed by genetic engineering (Morrison et al. 1984). Chimeric antibody consists of a constant region of human origin while variable regions are of mouse region. The first chimeric Fab, ReoPro, was introduced in 1994, and since then, several chimeric antibodies have been used in clinics. Although human anti-chimeric antibody responses were observed in clinics but it dominated the market for some decades. Further, antibody engineering tools were improved and able to produce humanized antibodies where mouse counterpart was minimized significantly except its complementary determining region (CDR), which binds to antigen (Jones et al. 1996; Dall'Acqua et al. 2005). The first humanized antibody marketed in 1997 was Zenapax (Tsurushita et al.

2005). One major disadvantage with the antibodies for diagnosis is the large size (MW 150,000 Da), owing to which, it takes a long time to reach and accumulate in the target tissues. Hence, efforts have been made to reduce the size of an antibody by fragmenting in F(ab')2, Fab, and ScFv (single chain variable fragment). Fragmented antibodies have improved pharmacokinetics and most suitable for immunoscintigraphy. The strategy to use antibody for targeted delivery of radionuclide came in realization after 5 years of the invention of hybridoma technology. The first monoclonal antibody was labeled with iodine-131 (DeNardo et al. 1980). Later Carrasquillo et al. (1984) used the fragmented radiolabeled antibody for diagnosis and therapy of solid tumors. These antibodies are radiolabeled and used for effective cancer management. Hence, radiolabeled antibodies emerge from "magic bullets" (Ehrlich 1960) to the "radioactive magic bullet" (Chamarthy et al. 2011) for cancer management. Zevalin (^{90}Y-ibritumomab tiuxetan) and Bexxar (^{131}I-tositumomab) are the only two FDA approved radiolabeled antibodies for the treatment of NHL of which Bexxar is out of market (Sharkey et al. 2005; Chinn et al. 1999). Additionally, several radiolabeled antibodies are approved by FDA for immunoscintigraphy. Radionuclides coupled to antibodies are undergoing clinical trials for different cancers (Goldenberg 2003; Milenic et al. 2004; Govindan et al. 2005; Ng 2006; Pohlman et al. 2006; Boswell and Brechbiel 2007). In general, 24–48 h are required for full radiolabeled antibody for maximum uptake in the targeted site. The physical half-life of the radionuclide must be enough for its localization in tumor for scintigraphy.

6 Methods of Radiolabeling of Antibody

The attachment of a radioactive atom to an antibody is called radiolabeling of antibody. There are various methods for radiolabeling of antibody which are summarized below.

6.1 Radioiodination of Antibody

One of the most easy and simple methods of radiolabeling of antibody is the introduction of radioiodine in the antibody moiety. Radioiodination involves incorporation of radioiodine in the tyrosyl and histidyl residues of the antibody by various oxidizing agents. Thus, there is a chance of compromised immune reactivity due to the steric effects of introduced radioiodine atoms on amino acids essential for binding. Oxidative damage (thiol, disulfide, thioether, and indole group of the antibody) during the course of radiolabeling and the change of overall charges after substitution of radioiodine molecules in the antibody may also play important role in the immune reactivity. There are various oxidizing agents available for the radioiodination of antibody. However, it is important to mention that antibody structure should not be altered extensively due to radiolabeling; otherwise its

immune reactivity and stability become compromised. Few important methods of radioiodination are listed below.

6.1.1 Chloramine-T method

In the year 1962, Hunter and Greenwood described the Chloramine-T method of radioiodination of proteins/antibodies. Chloramine-T introduces iodine onto tyrosyl and to a lesser extent histidyl and sulfhydryl residues of proteins, through cationic iodine intermediate. Chlorine is more electronegative than iodine and it converts iodine to a positively charged form that is active in electrophilic substitution reactions. Although chloramine-T is a harsh oxidizing agent, the labeled proteins may undergo substantial denaturation; however, protein damage can be controlled by carefully adjusting the radioiodination conditions.

6.1.2 Lactoperoxidase Method

This is a very safe method of radioiodination of the antibody that uses an oxidizing enzyme, lactoperoxidase, obtained from unpasteurized bovine milk. Iodination takes place principally on tyrosyl residues, although histidyl and sulfhydryl groups may also participate. The reaction involves three substrates—peroxide, iodide, and the phenolic target that is being iodinated. The selectivity of lactoperoxidase iodination may be an important advantage with an antibody that is unusually subjected to damage during chloramine-T iodination. Enzymatic iodination may also be useful in the iodination of histidyl residues.

6.1.3 Iodogen

Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) is most widely used since it is insoluble in water, can be immobilized on the wall of the reaction vessel, thus direct exposure of the antibody to the oxidant is minimized. Iodo-gen is about 2.6 times more potent than chloramine-T in terms of iodinating efficiency.

6.2 Labeling of Antibody with Bifunctional Chelating Agent

Bifunctional chelating agents (BFCA) are complex molecules of which one functional group shares with macromolecule (Protein, MAb) and other functional group is capable to bind radionuclide. There are several BFCA such as HYNIC, DOTA, NOTA, NOGDA, etc., commercially available in the kit form for the radiolabeling of an antibody. In general, BFCA must be conjugated with antibody and then it was labeled with radionuclides. The typical structure of antiboy-BFCA and 177Lu is depicted in Fig. 1.

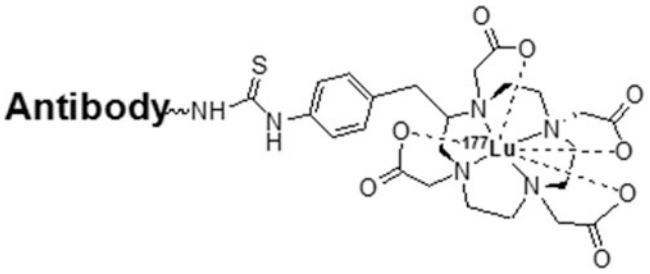

Fig. 1 Typical structure of ^{177}Lu-DOTA-antibody

7 Quality Control of Radiolabeled Antibody

Radiolabeled antibodies are intended for human administration, so it is necessary to have quality control measures after preparation. Quality control of labeled antibody includes methods to ensure the purity, binding specificity, and biological safety of the preparation. These ensure the safety of radiolabeled antibody preparation for human administration. These quality assurances are broadly classified as physico-chemical tests and biological tests.

7.1 Physico-Chemical Tests

Different in vitro physico-chemical tests are carried out to determine the purity and integrity of the radiolabeled antibody (Sood et al. 2000).

Physical Characteristics The physical appearance like color, colloidal formation, and turbidity which can be assessed by naked eyes. In addition to this pH, ionic strength, specific activity, and radioactive concentration should also be checked prior to use in the clinics.

Radionuclide Purity The amount of radioactivity present in the form of radionuclides in a given radiopharmaceutical is known as radionuclide purity. It arises from extraneous isotopic impurities in the target materials which increases undue exposure to patients and interfere with scintigraphic images. Radionuclide purity measurement is mostly done by gamma ray spectrometry using a HPGe detector. Beta-spectrometer or liquid scintillation counter is used to measure impurities due to pure beta emitters.

Radiochemical Purity The amount of radioactivity present in the form of radionuclides in a given radiopharmaceutical is known as radionuclide purity. It arises during the preparation or due to decomposition on storage by the action of solvent, temperature, pH, presence of oxidizing and reducing agents, and radiolysis.

Radiochemical impurities lead to accumulation of the radiolabeled product in non-targeted organ. Different methods like paper chromatography, TLC, HPLC, solvent extraction, paper and gel electrophoresis, etc., are in use to determine radiochemical impurity.

Chemical Purity It is defined as the fraction of antibody in the desired chemical form present in the given labeled antibody. It arises from the breakdown of antibody during the preparations. The presence of undesired chemical form may result in undesired labeled molecules and may also cause toxic effects.

Biological Tests These quality control tests are to ensure the sterility, apyrogenicity, antibody binding specificity and toxicity of the radiolabeled antibody preparation before human administration.

Sterility Tests The tests are performed to check the presence of any viable bacteria or microorganism which may cause infection in patients leading to several complications. The sterility testing is carried out by incubating the preparation either in fluid thioglycollate medium at 30–35 °C or in soybean-casein digest medium at 20–25 °C for 2 weeks and kept under observation for any growth of organisms. Since, the antibody is heat labile, so membrane filtration is the suitable method for antibody preparation which removes various microorganisms by sieving mechanisms.

Apyrogenicity Tests These tests are performed to ensure the pyrogen free antibody preparation. Pyrogens are mostly bacterial/microorganism cell debris which are composed of protein and/or polysaccharides which can cause fever, headache, pain in joints, malaise, and leucopoenia when administered in patients. Traditionally pyrogenicity of radiolabeled antibody preparation is carried out by injecting it into rabbits and observing any increase of body temperature within few hours to days after administration. Recently more rapid methods like limulus amebocyte lysate (LAL) test by gel clot method or bacterial endotoxin test (BET) is mostly employed for detection of pyrogens or endotoxin in antibody preparation. Endotoxin concentration should not exceed 25 EU/ml (EU: Endotoxin unit) in all radiopharmaceutical preparations.

Toxicity Test For using the radiolabeled antibody in humans, safe dose and its toxic dose needs to be determined. Safe dose or lethal dose and side effects are determined by in vitro study at the cellular level and in vivo studies in different animal models.

Determination of Antibody Binding to the Antigen To determine whether the specificity of an antibody has changed after radiolabeling, binding and inhibition study (competitive binding in the presence of unlabeled antibody) should be performed.

Immunoscintigraphy Study These studies are carried out in animal models to ensure the desired pharmacokinetics and distribution of radiopharmaceutical preparation when administered in humans. The radiolabeled antibody is injected in mice or rats and imaging studies, at different time intervals using Gamma camera or PET or SPECT should be carried out. Images at different time intervals are analyzed to look for the biodistribution and kinetics of radiopharmaceutical in animal models.

8 Application of Radionuclides in Healthcare

Radioisotope has been used in the healthcare dated back to 1940, where ^{131}I used for the thyroid treatment (Hertz and Roberts 1940). It also served as a diagnostic radionuclide, especially for thyroid imaging for several decades until ^{99m}Tc took over. But, over the past several decades, different radioisotopes have been used both for in vitro as well as in vivo for immunodiagnostic purposes.

8.1 In Vitro Application of Radioisotopes in Immunodiagnostic

Isotope dilution law is used to measure the concentration of unknown chemical substances in a sample by addition of known amounts of same substance labeled with active isotope to the analyzed sample. Using this law radiotracer based detection can be used to quantitatively estimate hormones, cancer related antigens, secretary proteins, toxins, drugs, etc., in biological samples such as blood, urine, etc. RIA (Radioimmunoassay) and IRMA (Immunoradiometric assay) are the major immunodiagnostic based radiotracer applications in health care

8.1.1 Radioimmunoassay (RIA)

RIA is an indirect method to measure the concentration of unknown antigens in samples by the use of antibodies. It is based on the principle of competitive binding which is as specific as ELISA but comparatively more sensitive. RIA is developed by Rosalyn Yalow and Solomon Aaron Berson (Yalow and Berson 1960). In the same year, Ekins and Slater (Ekins and Slater 1960) estimated total exchangeable sodium and potassium in human, independently using this method. In this method, analytes/biomolecules are labeled with a radioisotope (^{131}I, ^{125}I, tritium, etc.) and used as a tracer to estimate analytes/metabolites in given samples. In this, a fixed quantity of radiolabeled antigen (tracer) mixed with unlabeled antigen and incubated with a limited amount of antibody. Thus radiolabeled antigen competes with unlabeled antigen for antibody binding. So, higher the amount of unlabeled antigen in the mixture, there is a higher chance of free radiolabeled antigen (not bound to antibody) in the mixture. RIA is a comparatively sensitive assay which can measure up to femtogram of analytes per milliliter of samples; however, it lacks a direct linear relationship between analyte concentration and signal response in the form of radioactivity measurement.

8.1.2 Immunoradiometric Assay (IRMA)

IRMA is a modification of RIA which is fortified with high sensitivity and specificity, due to the use of a pair of specific antibodies. Despite this it is very simple, inexpensive, and easy to perform. Immunoradiometric assay is a noncompetitive assay in which excess amount of radiolabeled antibody are used. The antigen bound radiolabeled antibody is a direct measure of antigen concentration. Two-site immunoradiometric assays are variant of IRMA where two monoclonal antibodies against different epitopes of same antigen are being used, where one antibody binds to solid phase while other is labeled with a radiotracer. The antigen unbound radiolabeled antibodies are discarded and the radioactivity in the antigen bound fraction is able to measure which is the direct function of the antigen concentration. It shows high sensitivity and specificity due to use of two monoclonal antibodies to capture the antigen.

8.2 In Vivo Application of Radioisotopes in Immunodiagnostic

Monoclonal antibodies being specific towards the target proteins/receptors have been in use for radioimmunodiagnosis of different tumors and diseases, when labeled with a suitable diagnostic radionuclide. Approx. 5–25 mCi of radiolabeled antibody preparations are used for dosimetry of different tumors depending upon their effective half-life. Subsequently, for diagnosis, it required around same amount of radioimmunoconjugate. The radiolabeled fragmented antibody is preferable over the radiolabeled full antibody in radioimmunodiagnosis due to their small size and absence of the Fc region. This leads to its faster pharmacokinetics, high tumor/blood ratio, low risk of humoral effect, and convenient imaging points suitable for radioimmunodiagnosis. The use of radioactive preparations for immunodiagnostics as an injectable product has been established since several decades. Antibody and its fragments {Fab and F(ab')2} are labeled with diagnostic radionuclides and used in nuclear medicine for radioimmunoscintigraphy. There are two kinds of diagnostic imaging that can be possible with PET and SPECT: (a) Static imaging for obtaining anatomy/morphology of tissues/organs and (b) dynamic imaging for detecting functions of tissues/organs. Diagnostic radiopharmaceuticals are used in very low concentrations (10^{-6} to 10^{-8}M) to image the morphology as well as the functions of the organs and are not intended to have any pharmacological effects.

8.2.1 Non-invasive Immunodiagnosis by SPECT

Radionuclides that emit electromagnetic radiations such as γ or X-rays, deposit their energy over a long range, have a comparatively shorter half-life and do not emit particulate radiations, suitable for diagnostic applications. The invention of gamma camera by Anger in 1957 was an important milestone that accelerated the growth of diagnostic nuclear medicine. The gamma cameras that are capable of reconstructing 3-D images from acquired data of attenuated single energy photons are called SPECT. Several radionuclides such as ^{99m}Tc, ^{67}Ga, ^{81m}Kr, ^{82}Rb, ^{111}In, ^{123}I, ^{133}Xe, and ^{201}Tl that emit gamma rays with single energy are suitable for SPECT imaging.

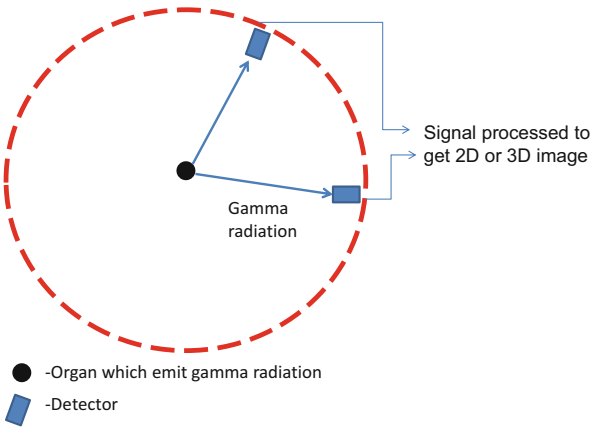

Fig. 2 Typical cross section diagram of gamma camera and SPECT imaging

However, the commercial availability of ^{99}Mo-^{99m}Tc generators and a variety of kits for preparation of ^{99m}Tc-radiopharmaceuticals to image several organs of the body made ^{99m}Tc the "work-horse" of diagnostic nuclear medicine. The typical cross section diagram of gamma camera and/or SPECT is illustrated in Fig. 2.

8.2.2 Non-invasive Immunodiagnosis by PET

The use of positron emitters, especially Fluorine-18, has increased enormously in the past 10–15 years. The positrons when emitted, immediately combine with electrons and annihilate each other resulting in the emission of two 511 keV photons in opposite (at 180° apart) directions. The two photons akin to gamma rays emitted simultaneously in opposite directions are recorded in a coincidence manner to construct a very good quality image with high resolution. Such imaging is known as PET, which also gives a 3-dimensional image even more resolved than SPECT. Although the concept of PET imaging existed since the 1950s, positron imaging made an entry into the clinics in the 1970s with the synthesis of ^{18}F-FDG at the Brookhaven National Laboratory, USA. PET imaging advanced with the development of BGO (bismuth germanium oxide) scintillation detectors and proper computations, where advance designing of coincidence circuits and a circular array of scintillation detectors efficiently detect the 511 keV photons emitted in opposite directions. The typical cross section diagram of PET imaging is depicted in Fig. 3.

9 Future Prospects of Radioimmunodiagnosis

Radioimmunodiagnosis using radiolabeled antibody and its fragments have been used for grading, staging, and prognosis of tumors during the course of treatment. Positron and γ-emitting radionuclides have been linked to antibodies or its fragments either using bifunctional chelator or directly by radioiodination. These radiolabeled antibodies or its fragments, when injected to patients travel through the blood and,

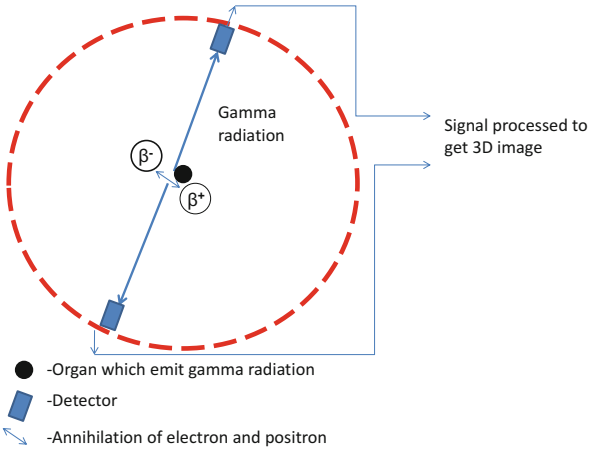

Fig. 3 Typical cross section diagram of PET imaging

reach to the target site and bind specifically which are imaged either with SPECT or PET camera. There are several FDA approved radiolabeled immunodiagnostic agents available in the market. Of which satumomab pendetide, acritumomab, nofetumomab merpentan, and capromab pendetide are used for cancer diagnosis. Satumomab pendetide is a ^{111}In-labeled IgG against tumor-associated glycoprotein-72 used for immunodiagnosis of ovarian and colorectal cancer, acritumomab is a ^{99m}Tc-labeled Fab' against carcino-embryonic antigen used for imaging of colorectal cancer, nofetumomab merpentan is a ^{99m}Tc-labeled Fab against epithelial cell adhesion molecule used for immunodiagnosis of small cell lung cancer, and capromab pendetide is a ^{111}In-labeled IgG to prostate specific membrane antigen used for prostate cancer imaging.

These diagnostic mAbs have been used in clinics mainly for the staging and grading of tumors, but overall its clinical success is limiting. Radiolabeled antibody shows slow pharmacokinetics and high blood retention due to its large size which are not preferable for radioimmunodiagnosis in vivo. So, fragmented and smaller mAb formats directed against appropriate targets in combination with sophisticated radioimmunodiagnostic instruments might give a boost to radioimmunodiagnosis using radiolabeled mAb in clinics.

Different receptors such as EGFR, CD20, CD25, HER2, etc., have been majorly targeted for radioimmunodiagnosis. EGFR or HER1 is the major tumor specific receptor overexpressed in varieties of tumors such as renal cancer, ovarian cancer, breast cancer, head and neck cancer (H&N), non-small-cell lung cancer (NSCLC), and colon cancer. Panitumumab, cetuximab, and nimotuzumab are HER-1 specific monoclonal antibodies used in clinics (Rivera et al. 2008). Nimotuzumab has been shown to be labeled with theranostic radionuclides ^{131}I, ^{177}Lu, and ^{188}Re for therapy and diagnosis of EGFR expressing tumors (Torres et al. 2008; Kameswaran et al. 2015; Pandey et al. 2019). Fragments of nimotuzumab (Fab-nimotuzumab) was labeled with ^{99m}Tc using HYNIC as a

bifunctional chelator (BFC) where as ^{68}Ga and ^{90}Y have been conjugated with antibody using DOTA-NHS-ester (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono N-hydroxysuccinimide) for immunodiagnosis of EGFR expressing tumors (Calzada et al. 2016; Alonso et al. 2013). Cetuximab also labeled with ^{64}Cu using DOTA, ^{68}Ga using NOTA, ^{86}Y using CHX-A″-DTPA have been studied as imaging probes against different tumors expressing EGFR (Sihver et al. 2014). ^{111}In-Labeled Cetuximab-F(ab′)2 was also used to image HER1 expression by SPECT/CT in Head and Neck cancer patient (van Dijk et al. 2013). ^{44}Sc–CHX-A″-DTPA–Cetuximab-Fab which was also used as PET tracer for HER1 expressing in mice bearing human glioblastoma (U87MG) tumor (Chakravarty et al. 2014). Panitumumab radiolabeled with ^{86}Y and ^{89}Zr has also been evaluated for immuno-PET imaging of the EGFR expressing tumors (Nayak et al. 2011; Chang et al. 2013). The panitumumab F(ab′)2 fragment conjugated with the CHX-A″-DTPA and radiolabeled with ^{111}In/^{86}Y showed promising results for immunoscintigraphy of HER1-positive cancers (Wong et al. 2011). ^{64}Cu-panitumumab F(ab′)2 fragments specifically localized in pancreatic cancer xenografts in mice, visualized by micro PET/CT (Boyle et al. 2015).

CD20 is a transmembrane antigen overexpressed on more than 95% of B-cell NHLs (non-Hodgkin's lymphoma). It is a promising target for therapy and diagnosis of NHL. Rituximab is a FDA approved chimeric monoclonal antibody against CD20 (Kaminski et al. 2000). Rituximab labeled with diagnostic radionuclide such as ^{99m}Tc as a radioimmunoscintigraphic agent for NHL was studied in detail (Camacho et al. 2013; Fontan et al. 2015; Stopar et al. 2008). But due to inherent problem of slow pharmacokinetics and non-specific interaction with antibody, Rituximab fragments {F(ab′)2 and Fab} were labeled with ^{99m}Tc using Hynic and ^{68}Ga using NOTA and evaluated for its potential in radioimmunodiagnosis of NHL (Suman et al. 2019a, b).

10 Conclusion

Radioimmunodiagnosis is an emerging branch of nuclear medicine and there is tremendous scope for the development of newer immunodiagnostic agents which cater to the demand of continuously growing burden of cancer and other diseases. This branch will promise the early detection and efficient management of the several diseases.

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Redox Cycling Technologies for Point-of-Care Immunodiagnostics in Various Matrices

Gorachand Dutta, Despina Moschou, and Riddhipratim Mandal

1 Introduction

Point-of-care tests are simple medical tests for bedside diagnosis (Chen et al. 2017; Lillehoj et al. 2013). But in many cases, the point-of-care simplicity was not achieved. Recently, various biosensors which are fabricated on chip-based are illustrated and up to now the know-how for electrochemical intensification upon a chip is continually counted on high-priced micro- and nanofabrication know-hows for example optical and e-beam lithography (Sandison and Cooper 2006). This method has several disadvantages even when it yields dependable research outcomes. Experimental analysis is mostly performed in centralized research laboratories where high technology tools are available and assays are performed by trained personnel in an almost unspoiled condition. Though, a huge portion of the inhabitants in developing nations do not get hold of the up-to-date diagnostic methodologies. Practical applications in point-of-care diagnosis require complex and heterogeneous sample handling (i.e., blood, or contaminated body fluids). Because of complicated sample handling procedures, the available diagnostic techniques still need benchtop methods (Shaw 2016). In addition, due to the lack of liquid reagent pre-storage, most of the diagnosis devices need additional external handling steps and as a result, there is no novel point-of-care diagnosis device available in the market that requires minimal handling. Therefore, most of the sample-to-answer systems to date are not suitable for point-of-care usage by non-experts due to the necessity for such sample pre-treatment prior to testing.

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Immunosensors are analytical devices that can detect the specific analyte of interest by forming antigen–antibody complexes by means of a transducer which can be processed, recorded, and displayed (Dutta et al. 2015, 2017; Zhu et al. 2013). However, the signal-to-background ratios by the simple immunosensor reactions are not that much high to accomplish ultrasensitive detection of biomarkers which is indispensable for beforehand diagnosis of disease. The experimental procedures are, in many cases, very complicated to perform the ultrasensitive detection of immunosensor; therefore they are inappropriate for point-of-site application. To overcome this limitation, an auxiliary amplification method (i.e., redox cycling) was combined with the simple immunosensor reaction (i.e., enzymatic reaction) (Yang 2012). The Redox cycling process can continually generate or consume signaling species (electrons or molecules) during the existence of reversible redox species (Akanda et al. 2011; Singh et al. 2013). There are many ways to generate the oxidative and reductive species in redox cycling, i.e. enzymatically, chemically, or electrochemically. Electrochemical–electrochemical (EE) redox cycling, electrochemical–chemical (EC) redox cycling, chemical–chemical (CC) redox cycling, or electrochemical–chemical–chemical (ECC) can regenerate the signaling species in situ and offer a reproducible electrochemical signal for ultrasensitive biomarkers detection (Yang 2012).

This chapter emphasises using different redox cycling techniques aimed at the ultrasensitive detection of biomarkers for point-of-site application. Different matrices will be discussed that was used for over the past few years for redox cycling immunodiagnostics. Also, the integration of redox cycling techniques to the portable devices will be discussed in the commercial arena.

2 Redox Cycling Technologies for Biomarkers Detection in Different Matrices

Over the past two decades, redox cycling technologies have seen an unprecedented development for biomarkers detection (Jagotamoy Das et al. 2007). The electrochemical–chemical–chemical (ECC) redox cycling where two chemicals are used for cycling of electrochemically redox species. It was conjoined with an enzymatic reaction of an enzyme label for accomplishing high signal amplification and sensitivity (Akanda et al. 2013). The electrode delivers a solid support for immobilizing capture biomolecules (namely, antibody) as well as electron transfer process from the biological/chemical reaction in the redox cycling, performs a vital role for ultrasensitive biomarkers detection for Point-of-Care Testing (POCT) (Chandra et al. 2011, 2013; Zhu et al. 2014). Hence, the selection of an appropriate electrode accompanied by proper surface modifications is a vital stage to develop an immunosensor.

Indium-tin oxide (ITO) electrode was used widely in biosensor for attaining low and reproducible capacitive background current/charge (Törer et al. 2018). ITO is one of the most used transparent conducting oxides in redox cycling based biosensor

because of its two main characteristics; first, its electrical conductivity and optical transparency, and second, it can be deposited as a thin film (Khan 2018).

Scientists and researchers have shown an increased endeavor towards the technical development of immunosensors for the preceding ten years (Cho et al. 2018). The aim of a POCT immunosensor is to develop a disposable test for rapid and early HIV/AIDS, tuberculosis, malaria, dengue like deadly biomarkers detection at a very early stage (Zhou et al. 2016). Electrochemical detection combination with redox cycling can perform a vital role in the microchip-based biosensor because it facilitates a miniscule sensing system. It finds application to a compact biosensing device meant for point-of-care testing (POCT) (Wan et al. 2013). Recently many immunoassays which make use of affinity binding among antibody and antigen is developed but the majority of them suffer from shortcomings in terms of rapidness, cost-effectiveness, simplicity, and ultrasensitivity as they necessitate several steps of washing prior to the actual sensing measurement and for that reason these immunoassays are considered inapplicable in the field of point-of-care diagnosis (Mani et al. 2009). A washing-free immunoassay technique was developed for minimizing the washing steps, a significant simplification of detection method and the assay time was reduced. Dutta et al. (2014) developed a washing-free immunoassay which permits quick, sensitive, and single-step detection of prostate-specific antigen (PSA) in serum with low interference using electrochemical–enzymatic (EN) redox cycling (Fig. 1). The test sample was mixed with a solution that involves the enzymatic reactions; no operation was needed before electrochemical measurement. The signal difference between the background and target was attained with a single enzyme-based proximity-dependent electron mediation: a bound labeled probe permitted faster electron mediation among an enzyme label and an electrode as compared to an unbound labeled probe. The range of detection was 1 pg/mL to 10 ng/mL. Moreover, the limit of detection was 10 pg/mL in serum and 1 pg/mL in phosphate-buffered saline (PBS).

ITO electrodes are less electrocatalytically active and help the electrochemical detection of biomarkers present in biological samples containing very low interfering species effect such as L-ascorbic acid (AA), uric acid (UA) (Dutta et al. 2015). An electrochemical–chemical–chemical (ECC) and electrochemical–chemical (EC) and redox cycling for sensitive and selective detection of trypsin was reported in human serum (Park and Yang 2014). Using redox cycling and ITO electrodes there was no interference effects of electroactive species, for instance, L-ascorbic acid and uric acid. Similarly, sensitive electrochemical detection of Vaccinia virus in a solution containing a high concentration of L-ascorbic acid was reported using reduced graphene oxide modified ITO electrode and EC redox cycling technique (Park et al. 2015). An applied potential of −0.05 V was applied for obtaining a low as well as reproducible background currents and a slow O_2 reduction. This reported redox cycling based immunosensor was promising for sensitive detection of bacteria, proteins, and viruses in solutions having large concentrations of ascorbic acid.

To prepare the sensing surface of immunosensor most cases a thick protein layer such as antibody layer is used on the top of the electrode. As a result, the electron

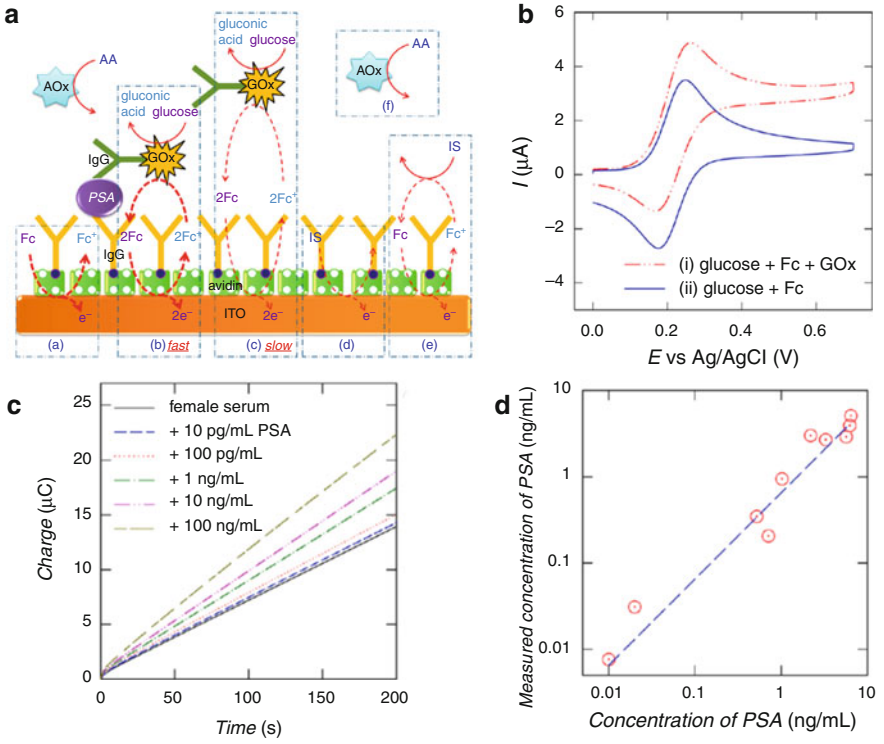

Fig. 1 (**a**) Schematic of a washing-free immunosensor using proximity-dependent electron mediation and the reactions involved. (**b**) Cyclic voltammograms obtained at ITO electrodes in (1) PBS containing 5.0 mM glucose, 100 μM ferrocene methanol (Fc), and 100 μg/mL glucose oxidase (GOx), (2) PBS containing 5.0 mM glucose and 100 μM Fc, (**c**) Chronocoulogram recorded at immunosensing electrodes for detecting different prostate-specific antigen (PSA) concentrations in real samples. (**d**) A comparison graph between washing-free immunosensor and a commercial instrument for 10 actual clinical serum samples (Reprinted with permission from Dutta et al. 2014. Copyright (2014) American Chemical Society)

transfer rate of the electrode is decreased dramatically. ITO electrodes can be modified with extraneous materials such as reduced graphene oxide (rGO) for increasing their electrocatalytic activities. An electrochemical redox cycling based protease sensor which allows the highly sensitive as well as simple detection of the botulinum neurotoxin type E light chain (BoNT/E-LC) was reported (Fig. 2), which uses ITO electrode improved by using reduced graphene oxide (i.e., rGO) (Park et al. 2016). The low nonspecific adsorption, high signal-to-background ratio, and EC redox cycling help to detect BoNT/E-LC down to 5 fg mL^{-1} in phosphate-buffered saline (PBS). The detection limit was 1 ng mL^{-1} during an incubation time of 15 min in commercial bottled water.

Currently, researchers have shown concern in developing enzyme-free immunosensor owing to their capability for enhanced stability and reproducibility in comparison to enzymatic immunosensors (Sun et al. 2015). Enzyme-free

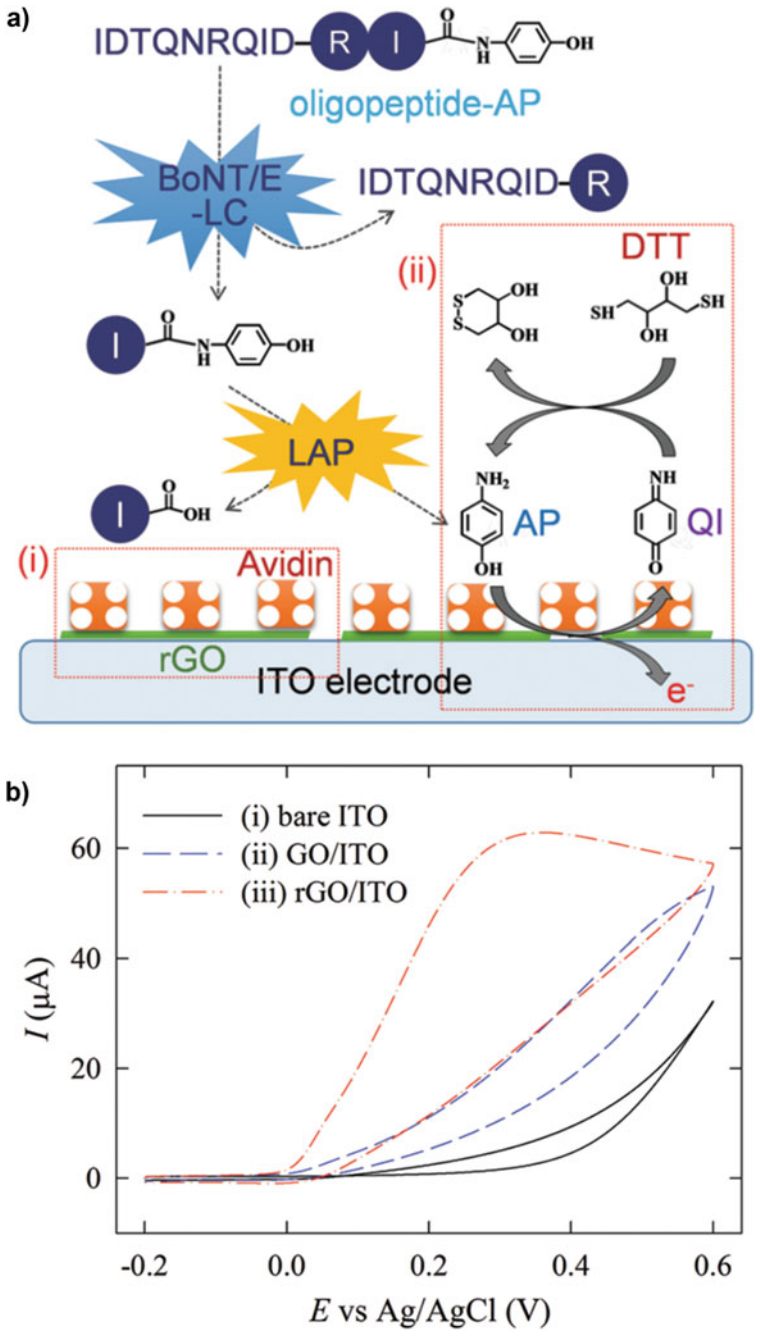

Fig. 2 (**a**) Schematic of botulinum neurotoxin type E light chain (BoNT/E-LC) detection using EC redox cycling. (**b**) Cyclic voltammograms recorded at (1) bare ITO electrode, (2) GO/ITO electrode, rGO/ITO in a PBS solution containing 0.1 mM AP (Reprinted from Park et al. 2016. Copyright (2016), with permission from Royal Society of Chemistry)

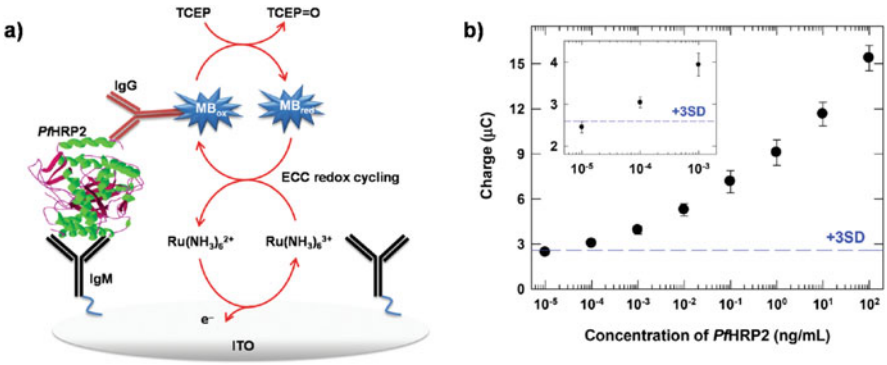

Fig. 3 (**a**) Schematic of the ECC redox cycling based immunosensor on ITO electrode using Ru $(NH_3)_6^{3+}$ and TCEP in the presence of biomarker and MB-labeled secondary antibody. (**b**) Calibration plot for *Pf*HRP2 detection in real samples (Reprinted from Dutta and Lillehoj (2017). Copyright (2017), with permission from Royal Society of Chemistry)

immunosensors enable high sensitivity sensing measurements in short duration without the limitations which are associated with enzymatic immunosensors. To overcome the limitation of enzymatic detection methods (signal-to-background ratios by enzymatic reactions are not adequately high for obtaining very sensitive biomarkers detection at an early stage. Also, the stability of enzyme is a big issue concerned with the reliability of medical devices), a non-enzymatic detection protocol was conjoined with an additional amplification procedure (i.e., redox cycling). An ultrasensitive enzyme-free immunosensor which uses methylene blue (MB) as a redox indicator was developed (Fig. 3) for the detection of *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2) in plasma as well as whole blood samples (Dutta and Lillehoj 2017). A typical ECC redox cycling scheme enhanced the electron transfer process for outer-sphere to inner-sphere redox cycling, in this manner rising the detection signal. Different redox cycling schemes were assessed by cyclic voltammetry and chronocoulometry on unmodified and antibody modified ITO electrodes. ITO electrode helps to detect the *Pf*HRP2 in human plasma as well as whole blood samples down to 10 fg mL^{-1} and 18 fg mL^{-1}, respectively. Moreover, this ITO based immunosensor reveals higher selectivity, outstanding reproducibility, and decent stability, making this immunosensor a favorable platform aimed at a point-of-care testing (POCT), particularly to detect very low concentrations of the biomarker in raw bio samples.

Carbon electrodes provide advantages of reasonable cost and wide applicability, specifically in electrochemical detection; the glassy carbon electrode was recognized promising matrices for early stage disease detection (Chikkaveeraiah et al. 2012). Various surface chemistry and modification processes were used upon glassy carbon (GC) electrodes to set up an immunosensor which is suitable for ultrasensitive biomarker detection (Wang et al. 2017). A ferritin-triggered redox cycling based immunosensor was developed on a GC electrode to detect *E. coli* antigen as a model

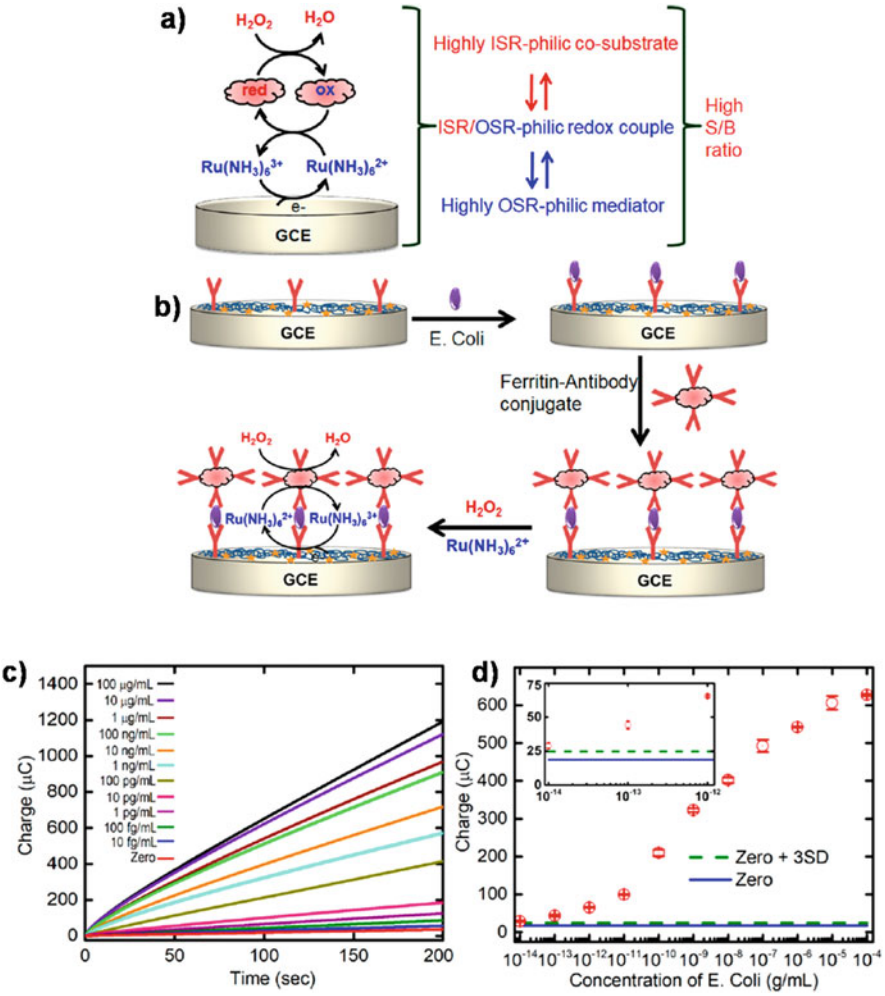

Fig. 4 Schematic of (**a**) ECC redox cycling based immunosensor mechanism, and (**b**) preparation of the immunoassay procedures, (**c**) chronocoulograms of different concentration of *E. coli* in 10 mM PBS (pH 7.4). (**d**) Calibration plot of the *E. coli* detection (Reprinted with permission from Akanda and Ju (2018). Copyright (2018) American Chemical Society)

analyte (Fig. 4) (Akanda and Ju 2018). In this scheme, $Ru(NH_3)_6^{3+}$ was used as an OSR-philic redox mediator and H_2O_2 was used as an ISR-philic oxidant. This immunosensor was resilient of the mediator counter to radical oxygen species, as a result showed very negligible background. This redox cycling based immunosensor on GC electrode showed a significant electrochemical signal amplification and excellent linearity across the concentration varying from 10.0 pg/mL to 0.1 μg/mL with 10.0 fg mL^{-1} limit of detection.

Many attempts have been tried to improve the redox cycling immunodiagnostic properties of GC electrode via employing various nanomaterials (Chikkaveeraiah et al. 2012). Nanomaterials can increase the electrochemical active surface areas, which can also help to increase the loading capacity and the mass transport of reaction molecules and amplify the detection signal. Kavosi et al. (2014) developed a sensitive electrochemical immunosensor based on enzymatic redox cycling meant for the detection of prostate-specific antigen (PSA) (Fig. 5). The anti-PSA and redox mediator (thionine) were covalently immobilized on this platform. Gold nanoparticles-incorporated polyamidoamine (AuNPs–PAMAM) dendrimer and multiwalled carbon nanotubes/ionic liquid/chitosan nanocomposite (MWCNTs/IL/Chit) were used to develop the sensing surface. AuNPs–PAMAM dendrimer on GC electrode not only raised the quantity of thionine and PSA antibody but also the electron transfer process was enhanced by encapsulated gold nanoparticles (AuNPs). The limit of detection for PSA was 1 pg mL^{-1} in the human serum sample.

Gold electrodes are widely used in redox cycling based immunosensor due to its exclusive redox property and the significant affinity of thiol compounds for its surface makes these electrodes appropriate in the sphere of point-of-care (POC) immunodiagnostics (Dutta and Lillehoj 2018; Ignatov et al. 2002). Xiang et al. described a redox cycling amplified highly sensitive sandwich-type immunosensor aimed at tumor marker detection, α-fetoprotein (AFP) on an electrodeposited nano-gold electrode (Fig. 6) (Xiang et al. 2018). This immunosensor was made by employing polydopamine-detection antibody nanoparticles (PDANPs-Ab2) as selective redox cycling based signal amplifiers and the nano-gold electrode performed a platform for accelerating electron relocation besides immobilizing capture antibody (Ab1). The reported redox cycling based immunosensor offered a wide linear range which stretches from 1 pg mL^{-1} to 50 ng mL^{-1} with a 0.3 pg mL^{-1} limit of detection. The biosensor exhibited excellent stability, reproducibility, and selectivity on a gold electrode.

A ECC redox cycling based immunosensor conjoined with immunomagnetic separation and pre-concentration was reported for sensitive as well as rapid detection of Salmonella on a gold electrode (Fig. 7) (Wang et al. 2016). Magnetic beads modified with anti-Salmonella antibodies were incorporated for separation and pre-concentration of Salmonella from phosphate buffer saline (PBS) and agricultural water. ECC redox cycling expedited regeneration of ascorbic acid on the gold electrode which resulted in an intensified signal. The detection limit was ca. 7.6 × 102 CFU/mL in PBS buffer and 6.0 × 102 CFU/mL in agricultural water.

3 Commercial System for Point-of-Care Immunodiagnostics

Various materials and methods have been exploited to prepare commercially available system aimed at the point-of-care immunodiagnostics. Screen-printed (SP) technology offers a simpler and cost-effective way compared to conventional electrodes (Wan et al. 2015; Parkash et al. 2014; Zhu et al. 2014). The screen-printed electrodes (SPE) are normally disposable after using only one or several

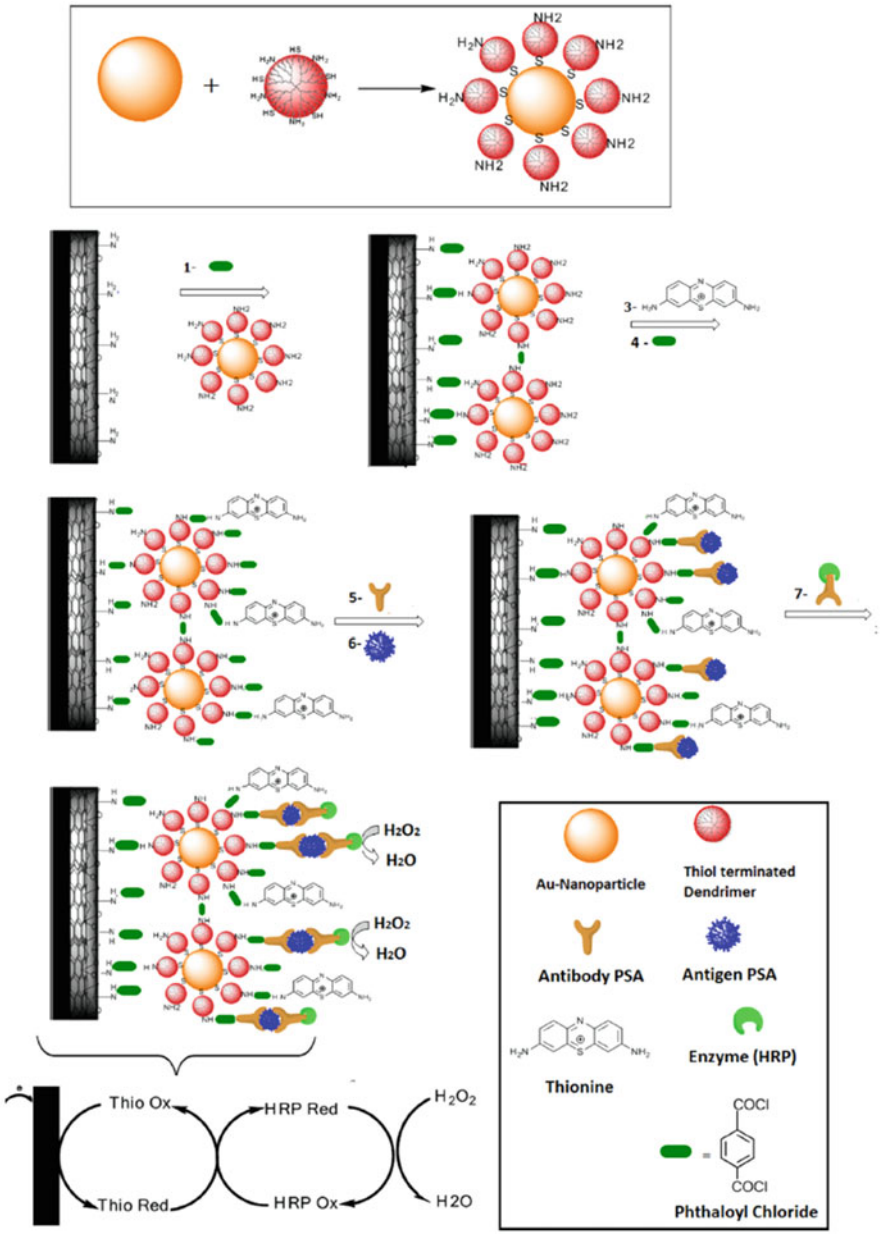

Fig. 5 Schematic illustration of the PSA redox cycling immunosensor fabrication process on GC electrode (reprinted from Kavosi et al. (2014). Copyright (2014), with permission from Elsevier)

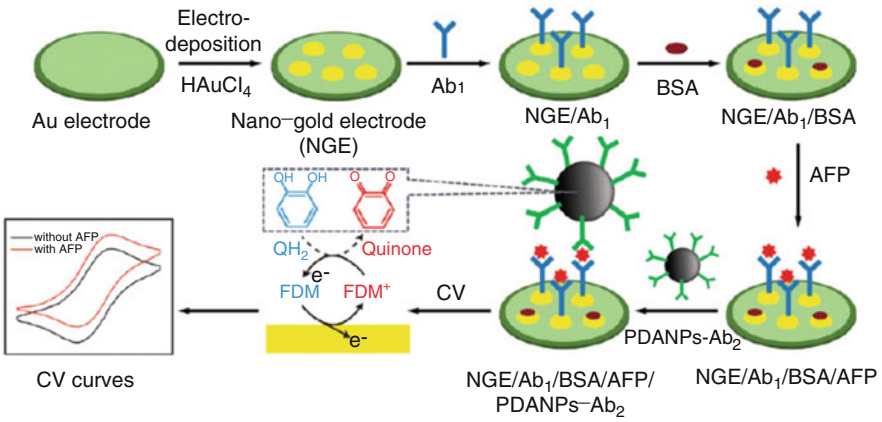

Fig. 6 Schematic of the redox cycling based immunosensor preparation for detecting α-fetoprotein (AFP) on an electrodeposited nano-gold electrode (Reprinted from Xiang et al. (2018). Copyright (2018), with permission from Royal Society of Chemistry)

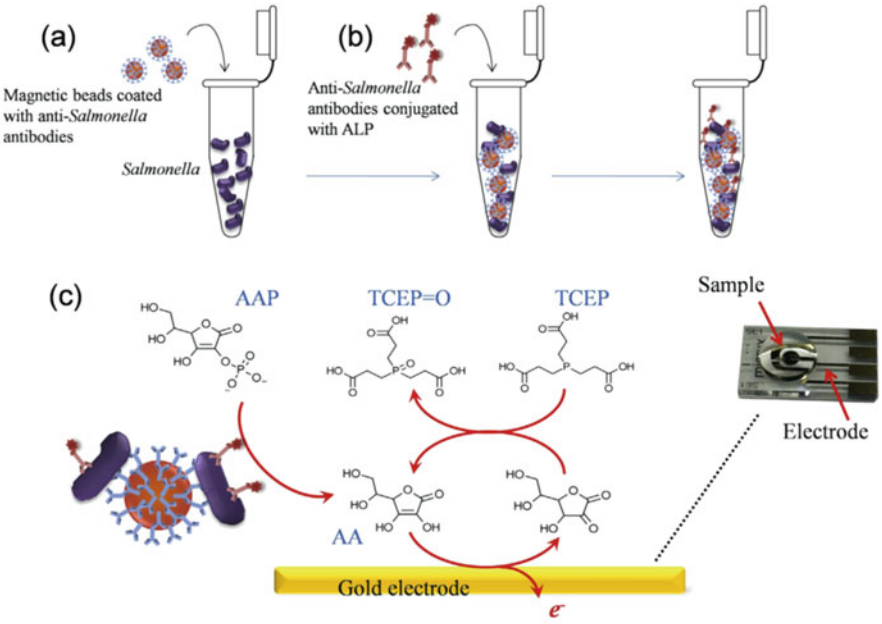

Fig. 7 Schematic illustration of the immunomagnetic pre-concentration and electrochemical detection based on redox cycling on gold electrode (Reprinted from Wang et al. (2016). Copyright (2016), with permission from Elsevier)

times and offer high reliability as well as reproducibility reliability for electrochemical analysis. The most important benefit of printing is expected to facilitate widespread, very low-cost electronics for applications such as portable devices and

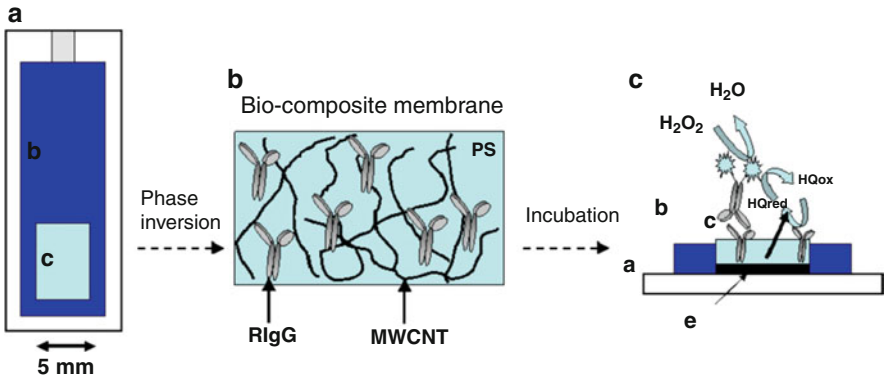

Fig. 8 Schematic illustration of screen-printed redox cycling based immunosensor using carbon nanotube/polysulfone screen-printed electrode. (**a**) The top view of screen-printed electrode; (**b**) schematic drawing of MWCNT/PSf/RIgG composite; (**c**) cross section of the detection area (Reprinted from Sanchez et al. (2007). Copyright (2007), with permission from Elsevier)

wearable sensors. Screen-printed electrodes (SPE) are produced by printing diverse electroconductive and insulative inks on different rigid or flexible substrate (i.e., plastic or ceramic substrate), thereby causing wide flexibility and versatility of these electrodes for practical application. Many related studies have reported that SPE electrodes with fabrication of foreign materials (i.e., nanoparticles) with redox cycling can produce a significantly enhanced electrochemical performance. S'anchez et al. reported a redox cycling based carbon nanotube/polysulfone screen-printed electrochemical immunosensor (Fig. 8) (Sanchez et al. 2007). The detection method was based on the competitive assay between free and labeled anti-RIgG for the available binding sites of immobilized rabbit IgG (RIgG). Electrochemical–enzymatic (EN) redox cycling was introduced using horseradish peroxidase (HRP) as an enzyme label and hydroquinone as electron mediator.

Selecting appropriate substrate material is one of the most important stages in the analytical devices. Mostly the fabrication was made on silicon (Si) or glass as substrates for the microfluidic motherboards (Regiart et al. 2017). Also other materials, i.e. plastic/polymer-based motherboards were also used in the analytical platform. Polymethamethylacrylate (PMMA), polyethylene, poly (dimethylsiloxane) (PDMS), polyimide, or polycarbonate were used because of their eclectic range of chemical and physical material parameters. Polymers have high benefit towards low cost, simple fabrication process, and rapid prototyping. One major disadvantage, however, is the lack of batch techniques for complex Lab-on-a-Chip (LoC) integration (microfluidics, sample preparation, biosensors, heaters) and the lack of such large industrial level fabrication facilities which would facilitate mass production and lower costs in order to gain industry acceptance. Fortunately, the electronics industry now has its disposal at a large industrial base for fabricating printed circuit boards at enormously high volumes and at marginal production costs (Dutta et al. 2018a, b; Moschou and Tserepi 2017). In order for an instrument to be

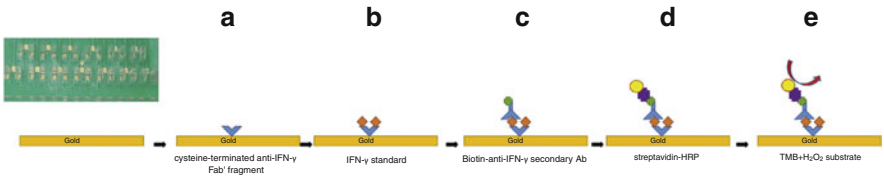

Fig. 9 Schematic of IFN-γ detection on PCB surface (reprinted from Moschou et al. (2016). Copyright (2016), with permission from Elsevier)

truly portable, it should be first and foremost lightweight, low-power in order to be able to be powered by a battery, and finally to include all functions necessary to perform its task without requiring additional instruments or procedures. Moschou et al. reported amperometric IFN-γ immunosensors which were profitably fabricated on printed circuit board sensing electrodes (Fig. 9) (Moschou et al. 2016). Enzymatic reaction was used to detect IFN-γ on a viable printed circuit board electrochemical biosensor. The commercially obtainable ELISA successfully detected the enzyme product TMB/H_2O_2 by means of amperometry. Similar enzymatic reaction can be combined with an electrochemical redox cycling (i.e., enzymatic redox cycling reaction) for ultrasensitive detection. This was a perfect example of the integration of a disposable, mechanized, electronic ELISA Lab-on-PCB diagnostic platform (Table 1).

4 Conclusions and Future Outlook

In this chapter, we described the recent technological progress in the field of redox cycling in various matrices. We mainly focused on electrochemical immunodiagnostic devices which are suitable for point-of-site detection such as medical diagnostics, biological research, environmental monitoring, and food analysis. The main driving force was to bring the test methods conveniently and immediately to the patient for early stage biomarkers detection. Indium-tin oxide (ITO), graphene, nanotubes, metallic nanoparticles, metallic electrodes were used as the transducer with redox cycling for higher signal-to-background ratios.

Point-of-care testing (POCT) of biomarkers in clinical samples is of great importance for rapid and cost-effective diagnosis. However, up to now it is extremely challenging to develop a POCT technique retaining both simplicity and very high sensitivity. The printing technology on flexible substrate and PCB could open up new opportunities for the development of bioelectronics toward practical applications in the future. The PCB can be integrated with a disposable, compact, self-contained Lab-on-Chip cartridge performing whole blood-to-result system. The complete, autonomous (reagent storage, pumps/valves integrated on chip) Lab-on-Chip cartridge is highly applicable for bedside application. The major challenge is primarily because of difficulties of integration of highly sensitive biosensor to mini or micro portable devices. Furthermore, the actual measurements should be carried

Table 1 Specification of redox cycling based immunosensor

Method	Detection platform	Biomarker	Sample medium	Limit of detection (LOD)	Reference
Wash-free electrochemical–enzymatic (EN) redox cycling	ITO	Prostate-specific antigen (PSA)	Serum	10 pg/mL	Dutta et al. (2014)
Electrochemical–chemical–chemical (ECC) and electrochemical–chemical (EC) redox cycling	ITO	Trypsin	Tris buffer	1 ng mL^{-1} (ECC redox cycling) 5 ng mL^{-1} (EC redox cycling)	Park and Yang (2014)
Electroreduction-based electrochemical–chemical (EC) redox cycling	Reduced graphene oxide (rGO)-ITO	Vaccinia virus	50% mandarin juice	4×10^3 PFU mL^{-1}	Park et al. (2015)
Electrochemical–chemical (EC) redox cycling	Reduced graphene oxide (rGO)-ITO	Botulinum neurotoxin type E light chain (BoNT/E-LC)	PBS Commercial bottle water	5 fg mL^{-1} 1 ng mL^{-1}	Park et al. (2016)
Enzyme-free electrochemical–chemical–chemical (ECC) redox cycling	APTES-ITO	*Pf*HRP2	Serum Blood	10 fg mL^{-1} 18 fg mL^{-1}	Dutta and Lillehoj (2017)
Ferritin-triggered electrochemical–chemical–chemical (ECC) redox cycling	Glassy carbon (GC) electrode	*E. coli*	PBS	10 fg mL^{-1}	Akanda and Ju (2018)
Electrochemical–enzymatic (EN) redox cycling	(AuNPs–PAMAM) and multiwalled carbon nanotubes/ ionic liquid/chitosan nanocomposite on GC electrode	Prostate-specific antigen (PSA)	Serum	1 pg mL^{-1}	Kavosi et al. (2014)
Electrochemical–chemical (EC) redox cycling	Electrodeposited nano-gold electrode	α-fetoprotein (AFP)	Serum	0.3 pg mL^{-1}	Xiang et al. (2018)

(continued)

Table 1 (continued)

Method	Detection platform	Biomarker	Sample medium	Limit of detection (LOD)	Reference
Electrochemical–chemical–chemical (ECC) redox cycling	Gold electrode	Salmonella	PBS Agricultural water	7.6×102 CFU mL^{-1} 6.0×102 CFU mL^{-1}	Wang et al. (2016)
Electrochemical–enzymatic (EN) redox cycling	Carbon nanotube/polysulfone screen-printed carbon electrode (SPE)	Rabbit IgG	PBS	1.66 µg mL^{-1}	Sanchez et al. (2007)
Electrochemical enzymatic detection	Printed circuit board (PCB)	IFN-γ	Serum	126.75 pg mL^{-1}	Moschou et al. (2016)

out in real physiological sample because real sample is far more complex and will definitely introduce a range of interfering and fouling effects.

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Next-Generation Immunosensing Technologies Based on Nano-Bio-Engineered Paper Matrices

Kuldeep Mahato, Budhhadev Purohit, Ashutosh Kumar, Ananya Srivastava, and Pranjal Chandra

1 Introduction to Low-Cost Immuno-Technologies

In the conventional healthcare systems, the identification of pathogens (Bhatnagar et al. 2018; Kashish et al. 2017), toxins (Valera et al. 2019), metabolites (Garcia-Garcia et al. 2016; Kumar et al. 2018; Purohit et al. 2019b) of clinical relevance plays an important role to diagnose the diseased conditions (Chandra et al. 2013b; Mahato et al. 2017a; Ricci et al. 2012), where various standard methods and technologies have been used (Chandra 2016; Mahato et al. 2018b). Among various such detection techniques, immunodiagnoses have found special attention for developing the analyses due to their highly selective nature towards the target molecules of clinical/biomedical importance (Chandra et al. 2017; Choudhary et al. 2016; Purohit et al. 2019a). The conventional immunodiagnoses use a number of sophisticated techniques including, quantitative immunoassay, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), immunoprecipitation assay, immunoelectrophoresis, immunofluorescence, immunohistochemistry, immunoblotting, etc. (Akhtar et al. 2018; Kindt et al. 2007; Kumar et al. 2019e; Mahato et al. 2016). These techniques require highly skilled personnel, dedicated laboratory spaces, and

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high-end instruments (Baranwal and Chandra 2018; Kumar et al. 2019d; Mahato et al. 2018b, c). The diagnoses using these conventional immuno-techniques are not only time taking but also become costlier affairs, especially when there is a requirement of high-frequency determinations/intermittent monitoring (Kumar et al. 2019a). These have driven the researchers and clinicians for the development of various such modules that can be capable of delivering undiluted sensitivity to detect the clinically important bio-analytes in cheaper cost. This also led to the foundation of a number of sensing strategies by using various kind of nanomaterials and nanocomposites, viz. AuNPs (Chandra et al. 2013b, 2015; Kumar et al. 2019c; Mahato et al. 2019a), dendrites (Purohit et al. 2019b), graphene oxide (Mahato et al. 2019b), polymers (Baranwal et al. 2018; Zhu et al. 2013), metal oxide (Kadian et al. 2018), etc. By harvesting the unique features of such nanomaterials/nanocomposites, a number of sensors have been developed in the recent past for the detection of clinically relevant biomarkers (Chandra et al. 2013a; Chung et al. 2018; Koh et al. 2011; Kumar et al. 2019b; Maurya et al. 2016; Noh et al. 2012; Pallela et al. 2016; Verma et al. 2019; Yadav et al. 2013), however, among all, the immunosensors have been found to have great attention (Mahato et al. 2017b). The immunosensors are based on the immunocomplexation reaction mediated sensing of target analytes in a robust device often used for rapid diagnostic tests (Mahato and Chandra 2019a). These devices are comprised of immunosensing matrix that interacts with the target analytes (antigen) and generates analytical signals (Chandra et al. 2012; Cheng et al. 2010). Thereafter, this analytical signal is transferred to the transducer unit of biosensor, which converts it into the measurable quantity (Mahato et al. 2018a; Mahato and Chandra 2019b). A representative schematic depiction of the working principle of immunosensors has been shown in Fig. 1 (Mahato et al. 2018b).

In the recent past, the shift in healthcare focus towards point of care detection of the diseases apart from in centralized clinics has created the demands of various rapid diagnostic test/devices which can deliver the onsite detection (Mahato et al. 2018c; Purohit et al. 2020). So far, numerous devices have been reported including immunosensors, which have been developed for the detection of clinically important molecules including cancer biomarkers, toxins, whole cells, pathogens, and other various indicators of metabolic disorders, etc. These immunosensors have been reported in various formats, viz. electrochemical and colorimetric by using nanocomposite matrices, which are relatively expensive in nature. Thus, the paper has introduced new methodologies for cheaper cost of fabrication of such biosensors, the paper has been widely accepted due to its capability of providing the flanking groups for anchoring the antibodies onto the sensing zone and adequacy/abundancy which attracts the cheaper fabrication costs. Thus, paper has found a great attention for developing the next-generation immunosensor, fulfilling the ASSURED criteria proposed by the World Health Organization, which describes about the parameter to be considered for the diagnostic devices, i.e. the module should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, deliverable to end-user. Various research activities and developments have been studied for the paper-based sensor following ASSURED guidelines, where colorimetric based diagnostics have found great attention due to their non-requirement of the accessories/essential instrumentation.

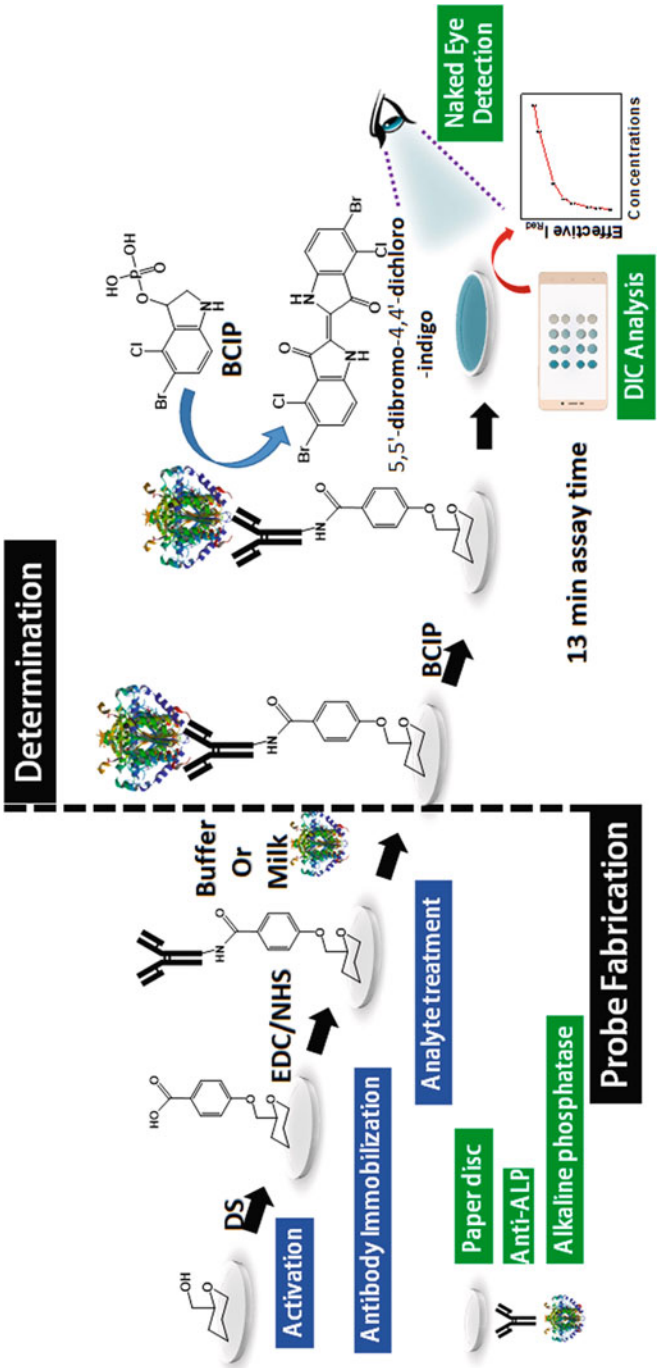

Fig. 1 Schematic representation of the paper-based immunosensor (sensing probe), its development and the detection principle for the detection of ALP and biofluid (milk) (adopted from Mahato et al. (2019a, b), copyright, Elsevier)

2 Paper as a Compatible Substrate for Clinical Diagnoses

Being an adequate and inexpensive substrate for the fabrication of immunosensors, during detection apart from capillary pumping of fluids it also offers abundant flanking groups for immobilizing antibodies to construct the immunosensor (Mahato and Chandra 2019a). Historically, the paper has been used for the diagnostic purpose by Martin and Synge, which led them to the Nobel Prize in 1952 (Parolo and Merkoçi 2013). Thereafter, the paper has been widely explored as analytical platforms. Later, down the decades the paper has been used for building the complex chips for diagnostics purposes by the group of Prof. Whitesides at Harvard's university, where they have widely explored the various aspects of paper substrate for building immunosensors based on ELISA (Verma et al. 2018).

For the purposes of the fabrication of immuno-diagnostics devices lignin-free high-quality paper is used as the presence of impurities may introduce interferences in the sensing signals (Ahmed et al. 2016). Thus, lignin-free higher grades have been adopted for the development of sensitive biosensors. Furthermore, in order to achieve greater flexibility in immobilizing the antibodies onto such sensing surfaces, a number of modification strategies have been employed using higher grades cellulosic paper substrates (Mahato et al. 2017b). In one of the example, Mahato et al. have utilized nitro flanking group based addition of antibodies (Mahato et al. 2019a, b). The paper substrate supports immunosensor by providing greater surface of immobilization and excellent wicking (capillary action) of the sample/reagent for achieving better detection, where the pore size, porosity, and the thickness of the paper play an important role for the flow control. However, such factors crucially decide the performances of the developed immunosensors as there exist several anomalies to the paper matrices including non-uniform thickness and the impurity level, these eventually make the selection of grade much challenging. In addition, the commercially available analytical paper substrates have been graduated with various numbers of indices according to the surface area available per volume, where the index between 50 and 200 has been accepted for the sensing purposes (Millipore 2013). In addition, the thickness of the paper substrate helps to arrest the bed volume by maintaining its tensile strength and enhancing the signal visibility in the colorimetric immunosensors, where empirically the greater thickness of the paper substrate introduces the less visibility of the signal at the sensing zone and lesser thickness shows the high visibility. However, when it comes to the manufacturing aspects the thicker substrates are preferred due to their higher tensile strength than the less thicker paper substrate, which are more prone to get damaged during the batch fabrications. Therefore, the optimal thickness has been preferred for attaining both manufacturing feasibility and better signal visibility (Millipore 2013). Apart from the colorimetric based sensing systems, electrochemical formats have also been reported using the paper substrate, where the paper has been impregnated with conducting inks, nanomaterials, and composite consisting of both. These additions not only make the paper conducting but also offer abundant flanking groups for the antibody immobilization.

3 Detection Approaches Using Paper-Based Immunosensors

The immunosensors based on paper platforms have been reported with various strategies, which are broadly studied under (1) colorimetric, (2) fluorometric, and (3) electrochemical modes. These have been described in this section.

3.1 Colorimetric Detection

Colorimetry based immunosensors are developed by coupling the sensing of analytes with chromogenic reactions, mediated either by the direct generation of the color-forming compounds or by the indirect mechanism. The evaluation of such immunodetection is commonly employed by naked eye determination or digital image colorimetry (DIC) (Mahato and Chandra 2019a). For example, a paper-based immunosensor has been reported for the detection of cancer based on carcinoembryonic antigen determination by using functionalized $ZnFe_2O_4$@MWNTs, and H_2O_2-HRP detection system, which have been developed on a paper-based platform. This immunosensor has successfully shown the analytical performances with the linear dynamic range (LDR) of 0.005–30 ng/mL and the limit of detection (LOD) of 2.6 pg/mL (Liu et al. 2014) (shown in Fig. 2I). In another variant of such paper-based immunosensors, multiple layered design for vertical flow coupled with the DIC technique has been used for the determination of *influenza* virus, where the LDR and LOD of 0–10,000 PFU/mL and 3.3 PFU/mL, respectively, have been obtained (Bhardwaj et al. 2019) (shown in Fig. 2II).

3.2 Fluorometric Detection

Fluorescence-based biosensors have been explored in various biomedical/clinical domains, viz. clinical diagnostics, environmental-monitoring drug-delivery, and food-safety applications. Fluorescence-based immunosensors have been developed by exploiting the fundamental properties associated with the parameters such as quenching efficiency, decay time, fluorescence intensity, energy transfer (radiative or non-radiative), and quantum yields. For instance, a fluorescence-based paper immunosensor has been reported for the detection of myocardial infarction marker, viz. cardiac troponin I using the 2-D paper chromatography coupled with fluorimetry. The reported immunosensor shows an excellent LDR of 0.01–0.1 ng/mL with the LOD of 0.05 ng/mL (Cho et al. 2014) (shown in Fig. 3I). In another research report, Ge et al. have fabricated a disposable biosensor for the detection of the tumor marker based on the sandwich immuno-reaction based on the fluorescence resonance energy transfer. They have successfully detected the alpha-fetoproteins (AFP), CA 125, CA 153, and CEA with the lower LOD of 3.0×10^{-4} ng/mL, 6.1×10^{-5} U/mL, 2.9×10^{-4} U/mL, and 1.4×10^{-3} ng/mL, respectively (Ge et al.

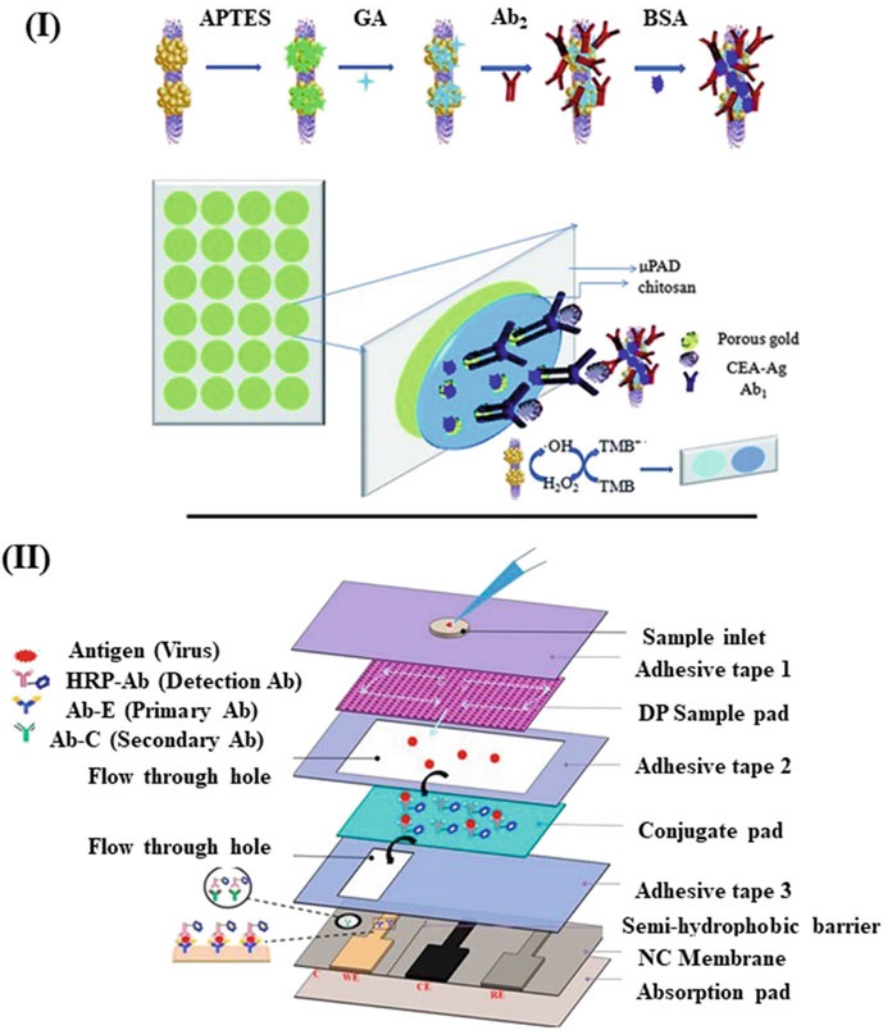

Fig. 2 Illustrative representation of the colorimetric immunosensor fabrication (**I**) shows the sandwich type immunosensor for the detection of carcinoembryonic antigen (cancer biomarker) (reprinted with the permission of Liu et al. (2014), copyright The Royal Society of Chemistry); (**II**) shows the immunosensor for the detection of influenza virus (reprinted with the permission of Bhardwaj et al. (2019), copyright Elsevier)

2013) (shown in Fig. 3II). In another example, the fluorescence-based inkjet-printed immunosensing devices have been developed by Guo et al. for the detection of the immunoglobulin G, which showed an excellent LOD of 0.4 ng/mL (Guo et al. 2019).

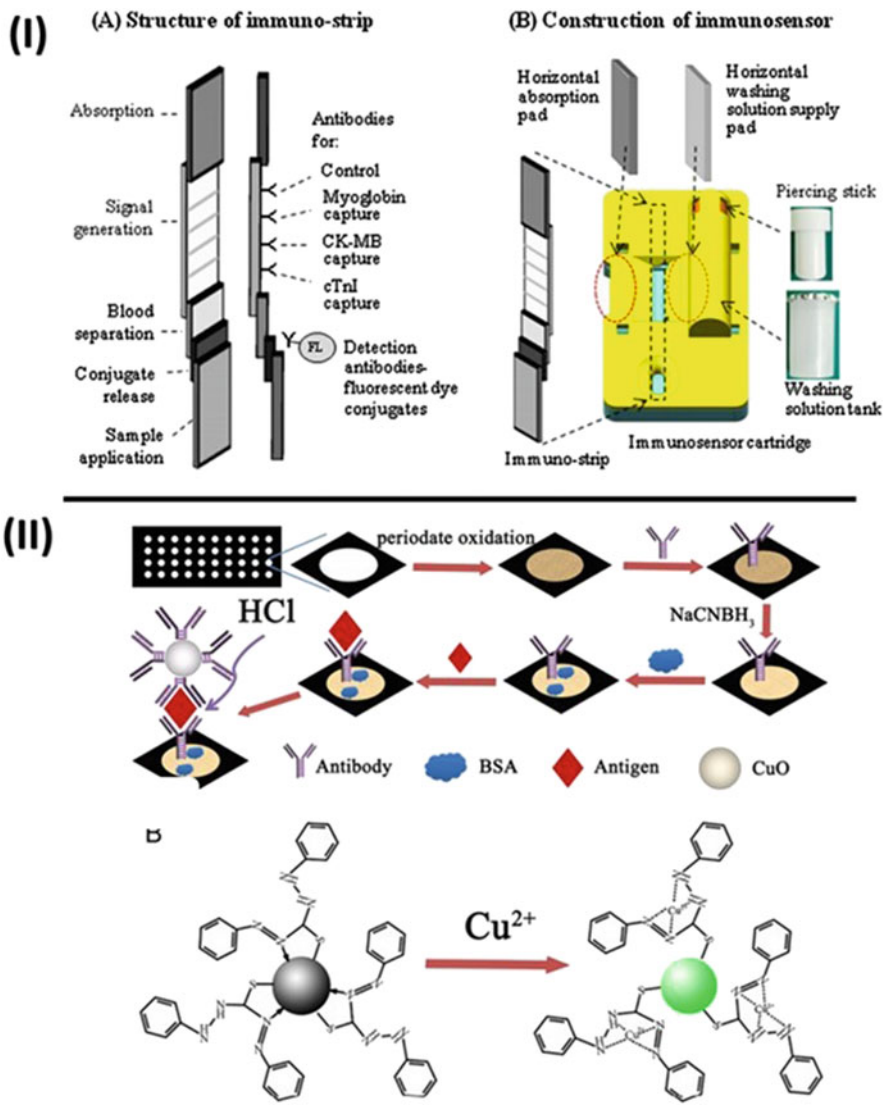

Fig. 3 Illustrative representation of the fluorometric immunosensor fabrication (**I**) shows the fluoro-immuno-strip for the detection pf cardiac troponin detection (reprinted with the permission of Cho et al. (2014), copyright Elsevier); (**II**) shows the fluorescence-based immunosensor for the detection of cancer biomarkers AFP, CA 125, CA 153, and CEA (Reprinted with the permission of Ge et al. (2013), copyright Elsevier)

3.3 Electrochemical Detection

The electrochemical paper-based immunosensors have also been used for the accurate detection of the clinically important molecules. Fundamentally, the electrochemical formats consist of the three-electrode system (viz. working electrode, reference electrode, and counter electrodes) printed on the paper-based surface with the conducting circuits. These conducting circuits are fabricated using conducting inks (Silveira et al. 2016; Tavares et al. 2016) or the nanomaterials impregnated nano-papers (Fan et al. 2019). In recent developments the paper-pencil (graphite) has been used for developing paper-based low-cost immunosensor chips (Devarakonda et al. 2017). For instance, nano-paper-based biosensors have been developed by Wang et al., where they have used the multi-walled carbon nanotube, gold nanoparticles (AuNPs), and thionine based nanocomposite for the fabrication of the electrochemical nano-chip to deliver the point of care detection of the 17-beta-estradiol. The sensor developed is capable of delivering the LDR between 0.01 and 100 ng/mL with the detection limit of 10 pg/mL (Wang et al. 2018) (shown in Fig. 4I). In a similar way, others have developed an immunosensor for the detection of cancer antigen 125 using the reduced graphene oxide, thionine, AuNPs-based nanocomposites for printing the electrochemical circuits. The immunosensor has successfully detected cancer antigen 125 between 0.1 U/mL and 200 U/mL, with the LOD of 0.01 U/mL (Fan et al. 2019) (shown in Fig. 4II). In addition to these, the antibody and single walled carbon nanotubes-based bio-conjugate have been used for the electrochemical sensing to determine the foodborne pathogen *Staphylococcus aureus*. The sensor shows the wide LDR of 10–10^7 colony forming units (CFU)/mL with the LOD of 13 CFU/mL (Bhardwaj et al. 2017).

4 Types of Paper-Based Immunosensors

In paper platforms, the detection devices have been fabricated under various formats, such as lateral flow assay, etc which broadly categorized under the dipsticks, lateral flow assays, and microfluidic based paper diagnostics assays. In the dipstick format, unlike in the molecular sensing, the immunosensor is not widely accepted due to the various limitations such as non-guided flow of samples, which causes fainted/non-perceivable signal. However, the other formats have been widely explored in the recent past for immunosensor developments. The commonly used format for the development of immunosensors has been detailed below.

4.1 Lateral Flow Assay (LFA)

Another format of the paper-based immunosensors is lateral flow-based, where the reagents are pre-deposited in the paper matrices according to the adopted reaction mechanism. These types of immunosensors work by exploiting the power of self-wicking of the paper for transferring the liquid sample/reagents. In this format, the

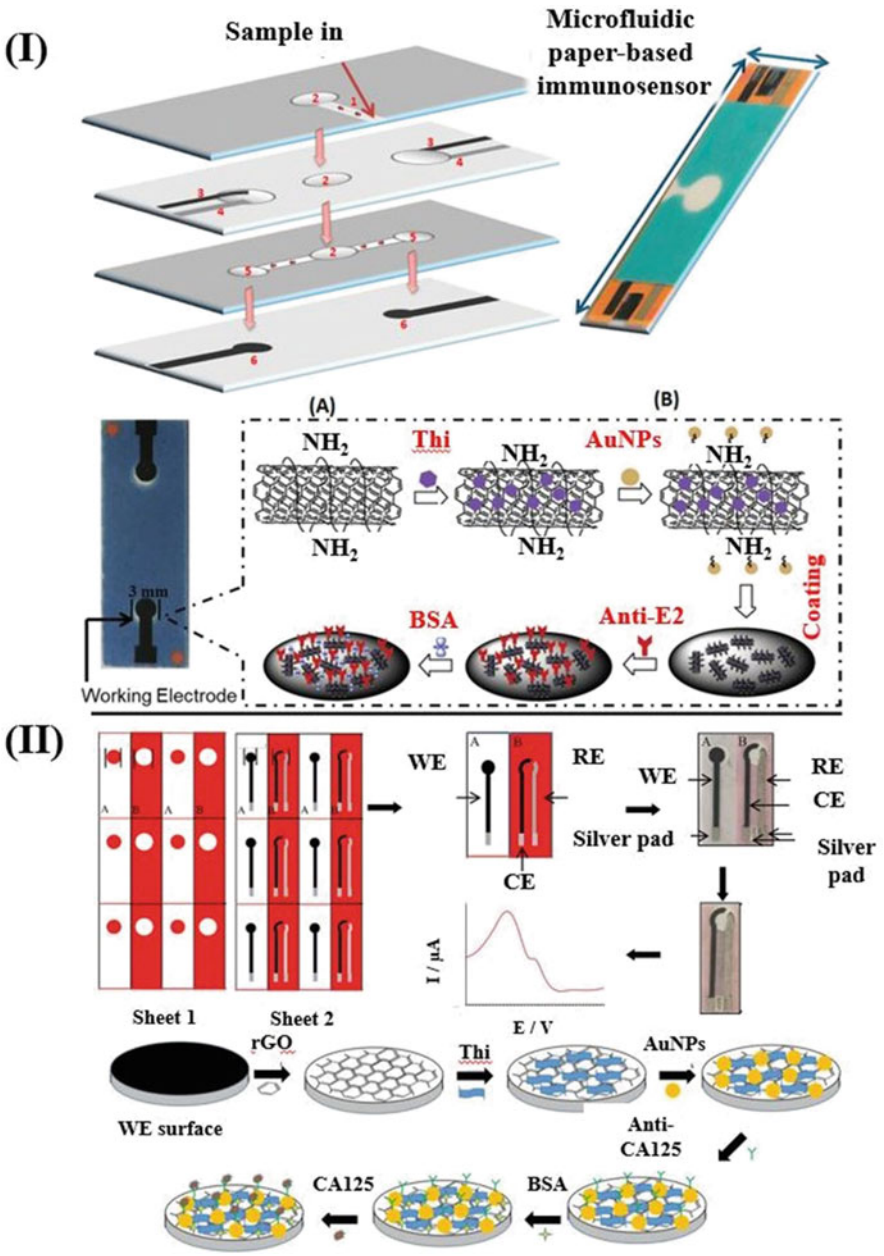

Fig. 4 Illustrative representation of the electrochemical immunosensor fabrication (**I**) shows the paper-based electrochemical chip for the determination of 17-beta-estradiol, lower panel shows the working electrode fabrication steps (Reprinted with the permission of Wang et al. (2018), copyright Elsevier); (**II**) shows the electrochemical based immunosensor for the detection of cancer biomarkers CA 125, lower panel shows the working electrode fabrication steps (reprinted with the permission of (Fan et al. 2019), copyright Elsevier)

samples are flown through different zones in order to complete the detection, and, thus, the lateral flow assays are comprised of four different parts including sample pad (sample treatment), conjugation (reagent storage) pad, detection (zone of sensing), and absorbent parts (enables the flow) (Mahato et al. 2017b). Based on LFA immunosensors, various biomarkers/diseases causing agents have been detected in recent days, viz. cancer, neurodegenerative disorders, etc. The major limitation to the conventional LFA is their incapability for delivering multiplexed, multistep, and quantitative analyses. However, in recent advancements in the precise fabrication technologies, the LFA based immunosensor has been reported with such qualities. For instance, Gerbers et al. has introduced valves to LFA platform for the controlled autonomous handling of fluids to obtain a multiplexed detection by controlling the mixing timing for the sample and various reagents. Thus, this advanced LFA platform supports the multistep process for biomolecular detection (Gerbers et al. 2014). Similarly, the LFA has also been reported with the multiplexed detection of the analytes. The quantitative analyses have also been realized in LFA based platforms. In one of the example, Zhu et al. have developed a colorimetric LFA based fast detection of 8-OHdG, where the researcher has coupled the colorimetric mechanism with the image analysis module to quantify the sensing (Zhu et al. 2014) (shown in Fig. 5I–II). In addition to the optical formats (colorimetry/fluorescence), the electrochemical LFA has also been reported, where the lateral flow assays have been combined for the precise determination. In one such report, cardiac Troponin T, a biomarker for heart injury has been detected using the voltammetric technique. This device has detected the cardiac Troponin T within 20 min and shows the LDR between the concentration of 0–700 ng/mL with the detection limit of 0.15 ng/mL (Dempsey and Rathod 2018) (shown in Fig. 5III–IV).

4.2 Microfluidic Paper-Based Analytical Device (μPAD)

Although the LFA based modules are widely explored by adding various advancements in it, these are inherently vulnerable especially when it involves sensing mechanisms with multistep processes. In order to resolve these technological issues, microfluidic channels have been used to guide the flow for precision reagent/sample handling, which not only offers the sensing with multistep reactions but also provides sensitive detection and robust modules for easy handling (Mahato et al. 2017b). The common features of these paper-based microfluidic platforms are added using various design/technological operations, including shaping, cutting, porosity blocking, wax patterning, etc. (Liu et al. 2015; Xia et al. 2016; Yetisen et al. 2013). A schematic representation of the simplistic μPAD has been illustrated the Fig. 5 (I). In addition to these standard methods, few innovative approaches have also been attempted in the recent past, where the crayons-pencils, home-made stencils have been used for the fabrication of μPAD devices (Yang et al. 2014).

Alizadeh et al. have reported paper-based microfluidic immune device based on sandwich immunoassay, where the peroxidase mimicking $Co_2(OH)_2CO_3$-CeO_2 nanocomposite is coupled with the capture antibody. In presence of the target analyte

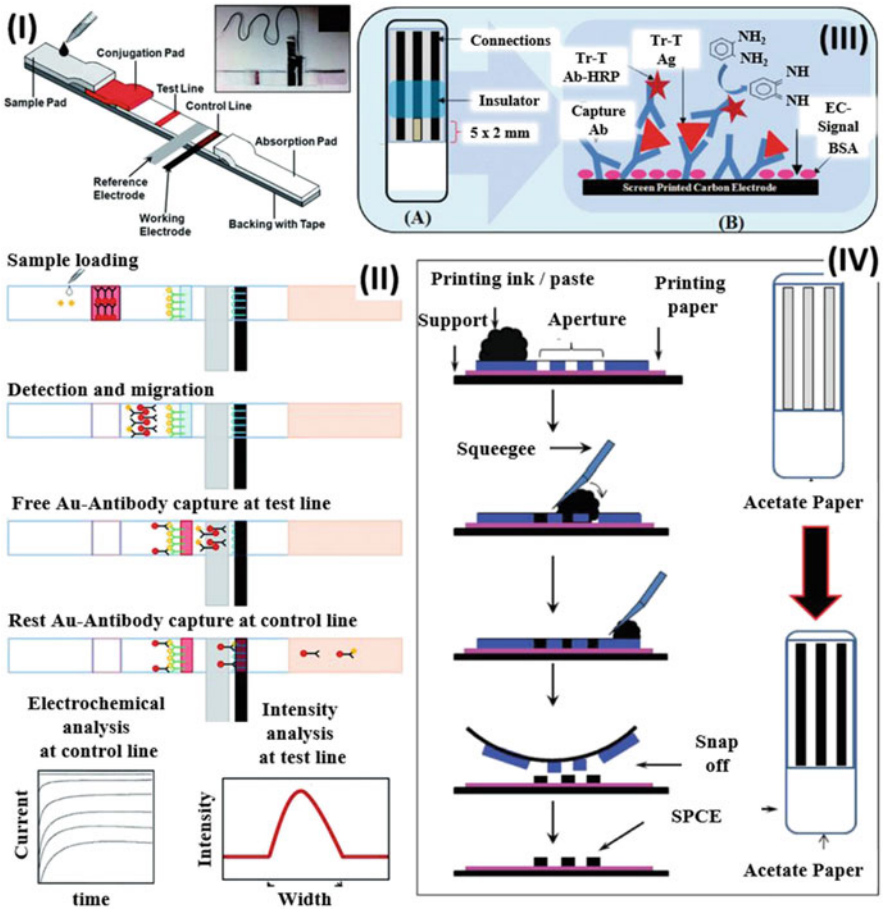

Fig. 5 Representative illustration of the different formats of LFA immunosensors used for the clinical/biomedical application (**I**) shows the colorimetric lateral flow assay based immunosensor for the detection of 8-OHdG, (**II**) shows the detailed working principle of sensor module (reprinted with the permission of Zhu et al. (2014), copyright The royal Society of Chemistry), (**III**) shows electrochemical LFA for the determination of cardiac troponin T protein, (**IV**) shows detailed description of the working principle (reprinted with the permission of Dempsey and Rathod (2018), copyright Institute of Electrical and Electronics Engineer)

carcinoembryonic antigen the coupled capture antibody gives rise to the colorimetric signal by TMB-H_2O_2 chromogenic system. The color changes have been quantified by using a smartphone assisted DIC method. This immune device has successfully determined the concentration ranges of 0.002–75.0 ng/mL with the LOD of 0.51 pg/mL (Alizadeh et al. 2018) (shown in Fig. 6I–II).

The microfluidic immune devices have also been developed by adopting the electrochemical format of immunosensing. In the recent past, various such

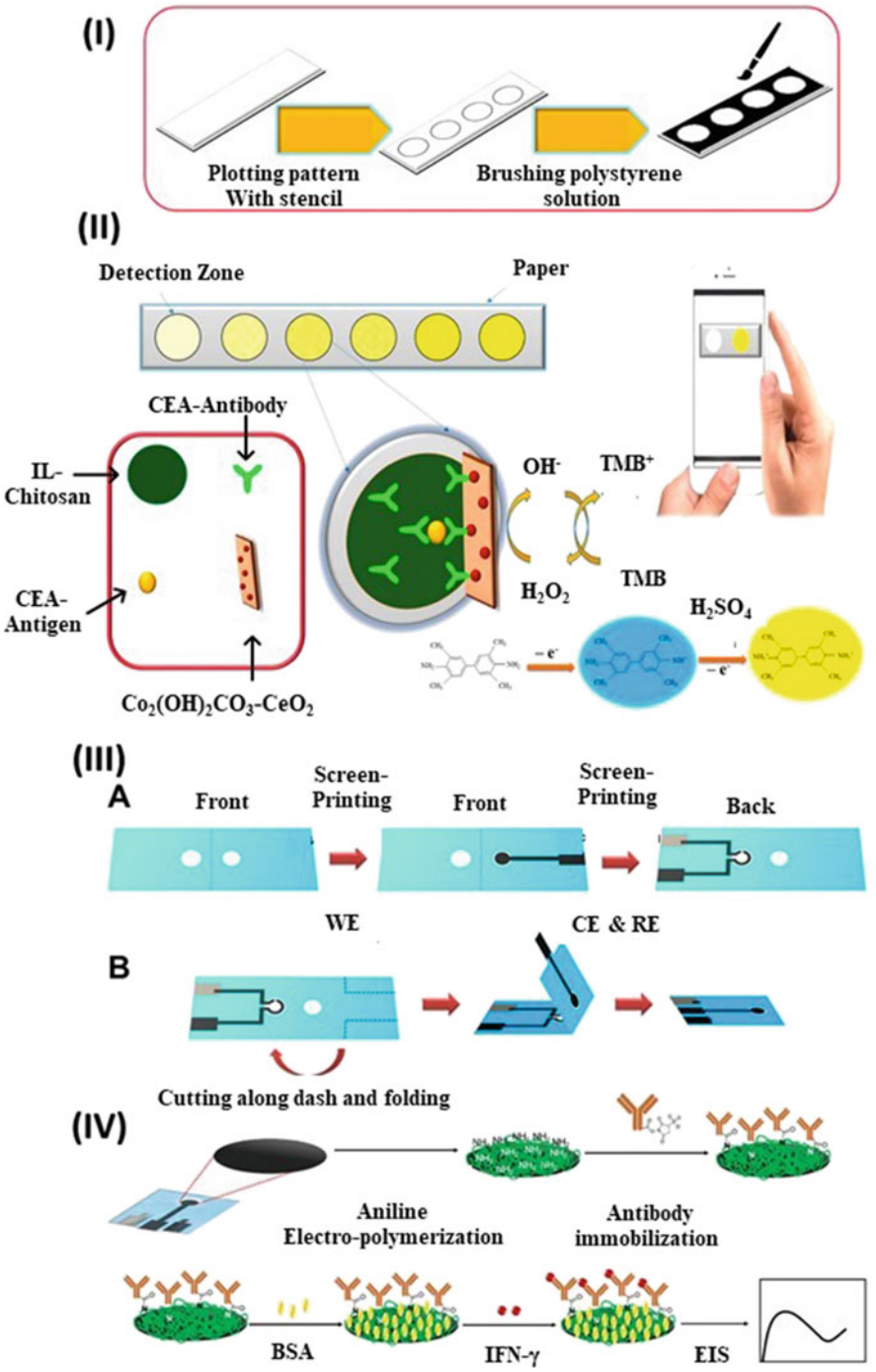

Fig. 6 Pictorial representation of the different formats of the paper-based μPAD immunosensing devices used for the clinical/biomedical application (**I–II**) shows the fabrication and detection

electrochemical immune devices have been reported for various biomarkers of clinical importance including carcinoembryonic antigen, human chorionic gonadotropin, alpha-fetoprotein, human interferon-gamma, etc. For instance, Ruecha et al. have developed a paper-based label-free electrochemical immune device for the detection of human interferon-gamma by measuring the impedance. For developing the detection chip, the graphene electrode has been printed and polyaniline has been deposited followed by the coupling of antibody interferon-gamma over it to build the sensing matrix. This developed immunosensor successfully detected the concentration ranges of 5–1000 pg/mL with the LOD of 3.4 pg/mL (Ruecha et al. 2019) (shown in Fig. 6III–IV). In another report by the Cao et al. immunofixation analysis of human chorionic gonadotropin has been reported. This microfluidic immune device has been developed by using photolithography and screen-printing. The detection of the biomarker is obtained by sandwich method, where the electrode surface has been biofunctionalized with the primary capture antibodies and the AuNPs conjugated primary detection antibody was employed. To get the analytical signal ALP-conjugated secondary antibody has been used. The analytical signal was obtained in presence of the analyte, which facilitates the formation of immunocomplex. This immunosensor has shown the LDR between 1.0 mIU/mL and 100.0 IU/mL with the LOD of 0.36 mIU/mL (Cao et al. 2017).

5 The Emerging Developments in the Paper-Based Immunosensors

Apart from the conventional immunosensing techniques, the engineering and shaping of the paper have been incorporated to build various next-generation immunosensing devices targeting clinically important biomarkers. In recent advancements the paper art-based techniques origami (folding based art) and kirigami (cutting based art) have been employed to create unique devices in order to build the advanced paper-based modules. For instance, an origami immunosensor module has been reported for colorimetric determination. In one of such unique modules in paper-based immunosensing origami crafted platform, prostate-specific antigen has been detected. This device has been fabricated by sequential deposition of the AuNPs and manganese oxide nanowires at the sensing surface of free-standing three-dimensional origami paper. This device has detected a wide LDR of the prostate-specific antigen between 0.005 ng/mL and 100 ng/mL with the LOD of 0.0012 ng/mL (Li et al. 2014). In another example, Sun et al. have reported a paper origami-based immune device for the detection of CEA in the clinical samples. The device has been developed by the seed-mediated grown flower-like AuNPs

Fig. 6 (continued) principles of the colorimetric μPAD based immunosensor for the detection of cancer biomarker CEA (reprinted with the permission of Alizadeh et al. (2018), copyright Elsevier); (**III**–**IV**) shows fabrication and detection principle of the electrochemical μPAD for the determination of interferon-gamma (reprinted with the permission of Ruecha et al. (2019), copyright Elsevier)

nanocomposite onto the paper working electrode which was then used for the immobilization of the capture antibodies. Following the sandwich immunosensing strategies the detection antibody coupled with the Au–Ag bimetallic has been employed for sensing, where the presence of target antigen gives rise to the immune complex. This immune complex catalyzes the H_2O_2 for obtaining electrochemical analytical signals. This immune device has shown excellent analytical capability with the LDR between 0.001 and 50 ng/mL with 0.3 pg/mL (Sun et al. 2014).

Further advances in paper-based immunosensors have been witnessed in form of device coupled self-powered units for electrochemical detection platforms also called self-powered paper-based immunosensing devices. As an example, Li et al. have developed a paper-based biosensing platform for the determination of carcinoembryonic antigen. This device has been fabricated by incorporating the enzymatic biofuel cell in the microfluidic paper-based immunosensing module. The device shows excellent analytical performance with the LDR between 1 pg/mL and 0.5 μg/mL with a LDR of 0.7 pg/mL (Li et al. 2015).

6 Conclusions

Paper is an inexpensive, adequate, and biocompatible substrate that found great attention for the fabrication of disposable biosensing modules. It has been employed for the fabrication of the yes/no based qualitative determination of the bio-analytes. Further exploration of the paper-based substrate has led development of immunosensors for a wide range of biomedical/clinical applications. This chapter provides a comprehensive description of paper-based immunosensor development. This chapter summarizes various types of immuno-techniques for clinical diagnoses and their limitations in current health care systems. After this, we have described the quality of paper for the reliable detection of clinically important biomarkers associated with various diseases/disorders. Followed by these, we have summarized various paper-based techniques used for the development of immunosensors. Additionally, this chapter also describes various recent trends of paper-based technologies adopted for the development of the immunosensors/immune devices of biomedical/clinical applications.

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Alternative Analyte-Binding Compounds for Immunosensor-Like Point-of-Care Application

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1 Introduction

Rapid, efficient, and consistent devices for dependable read-outs of diseased condition with the feasibility of easy operation in resource limited settings are required. The biosensor research developed and progressed through the amalgamation of chemistry, biology, and engineering sciences. Cammann coined the term biosensor (Cammann 1977) and the definition was introduced by IUPAC, as analytical devices that read biological response and transmit electrical output (Thévenot et al. 2001). Clark and Lyons reported measurement of glucose by potentiometric measurement in 1962 (Wang 2008). Different types of biosensors have been reported such as enzyme-based (Akyilmaz et al. 2010), tissue-based (Gough et al. 2010), immunosensors (Luppa et al. 2001), DNA biosensors (Wang 1998), thermal (Van Herwaarden and Sarro 1986), piezoelectric (Skládal and Hianik 2013), magnetic (Haun et al. 2010), and optical biosensors (Baird and Myszka 2001). Biosensor is mainly an integrative assembly of (1) bioreceptor to bind analyte which generates a biological signal, (2) transducer to convert the chemical signal to electrical signal, and (3) signal processor for processing the electronic read out. Thus, the complex-formation generated information signals are converted by means of a transducer and observed by a signal processing device in a model biosensor unit (Kirsch et al. 2013).

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Antibodies (proteins) can bind in specific manner to respective antigens with high affinity. Transducers could read the reaction between antigen–antibody efficiently leading to the development of immunosensors. Spectroscopy, microgravimetry, electrochemical impedance, and SPR methods are some of the direct detection methods in which the change or variation in physicochemical properties is monitored. For the indirect analysis the antibody or antigen is labeled depending on the immunoassay format (sandwich-type, capture, or competition). Signals are generated via different mechanisms such as optical-fluorescence, chemiluminescence, electrochemiluminescence, absorbance or change in mass-properties or electrochemical mode in different detection systems (Luppa et al. 2001; Gopinath et al. 2014). Research for high-performance systems developed high-throughput analytical devices with integration of micro-/nanotechnologies with microfluidics. Mainly electrochemical, optical, and piezoelectric mechanisms support the transduction platform in conventional sensors. The electrochemical transducers are amperometry-current measurement, potentiometric-potential/voltage difference measurement, and conductometric-conductivity/resistance measurement. The formation of antigen–antibody complex makes a difference in the electrical signal measurement, which is further observed, recorded, and analyzed. In case of optical transducers, optical signal such as color or fluorescence is generated and measured (Medvedev et al. 2008; Aizawa 2011). For piezoelectric transducers, quartz crystal balance or cantilever device are used to monitor the mass variations (Janshoff et al. 2000; Janshoff and Steinem 2004).

Vast expansion has been seen in the technological development of immunosensors exploiting different strategies for monitoring the antigen–antibody reactions in body fluid. After three decades of extensive research for antibody immunosensors some challenges could be observed. The traditional problems related to the methodology were observed and overcome by limited use of reagents, speeding and simplifying the process. One of the major setbacks was the immobilization and orientation of the antibodies because of large size. Several strategies were developed to overcome the complications of antigen–antibody interactions. The other problem was the unavailability of antibodies for emerging small/low molecular weight biomarkers. Thus, antibodies presented several limitations which drifted the attention of researchers towards alternative analyte-binding compounds, aptamers, to improve the sensing technology. In this review, we will discuss about aptamers, recent developments of aptamer-based sensors, and a detailed account of developed peptasensor for rapid diagnostic applications.

2 Aptamer as an Alternative Analyte-Binding Compounds

Proteins are indicative of a particular clinical state and hence helpful in understanding of the biological mechanisms at molecular level. A study in 1980 indicated that RNA encoded by viruses can bind to proteins with high affinity, specificity, and further regulate biological mechanisms such as replication and translation. For example, RNA ligand TAR (trans activation response) binds the Tat protein and

promotes activation and replication in HIV and short RNA aptamer regulates translation in adenovirus (Dollins et al. 2008; Sullenger et al. 1990; O'Malley et al. 1986; Burgert et al. 2011). In 1990, three different studies were reported discussing identification of binding ligands for different targets by a similar selection methodology. The respective groups and their contributions are mentioned as follows: (1) Joyce's group at Scripps Research Institute isolated RNA enzymes with mutation, selection, and amplification technique in vitro, (2) at University of Colorado, Larry Gold's group identified particular 8-base region (randomized) of RNA molecule that interacts with T4 DNA polymerase and patented this technique as SELEX—Systematic Evolution of Ligands by Exponential Enrichment and (3) Szostak and Ellington at Massachusetts General Hospital, Boston reported the specific and rare folding of RNA ligand molecule isolated from large, diverse pool of sequences to fit the target-binding region for target organic dyes and mentioned one-in-millionth molecule developed such structural conformation. Such recognition molecules came to be known as aptamers, combination of terms "aptus"—to fit and "meros"—part (Robertson and Joyce 1990; Ellington and Szostak 1990; Tuerk and Gold 1990). They are small size peptide or oligonucleotide biomolecules with inherent function to identify the target molecule with high binding strength and specificity. The interactions between ligand–target can be understood on the basis of different contributing forces such as hydrogen bonding, van der Waals force, and others (Keefe et al. 2010). The selection methods are continuously evolving with remarkable speed, thus a number of bio-probes have been reported for various molecules such as ions (Li 2002), small molecules (Ellington and Szostak 1990; Huizenga and Szostak 1995), peptides (Hogquist et al. 1994), single proteins (Gold et al. 2002; Roberts and Szostak 2002), organelles (Ringquist et al. 1995), viruses (Misono and Kumar 2005), and even entire cells (Gold et al. 2002).

Ligand molecules are selected from a random diverse pool through an *in vitro* cyclic process. The selection cycles mainly include three steps: (1) binding, (2) partitioning for bulk separation, and (3) amplification of selection product. Firstly, a random library comprising of approximately 10 (Aizawa 2011) unique sequences is constructed with conserved primer sites flanked on both ends of variable region. The constant region is important for amplification of binding ligands after selection cycle by annealing primers. Primarily, the target molecules are incubated with the random pool to allow for molecular interaction in a chemical environment for suitable time. Following this, efficient partitioning methods are employed for separation of non-binding or weak-binding ligands. The selected pool is then eluted and amplified for next cycle of selection. With each selection cycle, the stringency pressure is elevated for successful isolation of best candidates. The candidates in the last selection pool are further screened for their kinetic properties via SPR and other methods. However, SELEX is based on a theoretical hypothesis without monitoring of molecular interactions at each step. Hence, the outcome cannot be pre-determined, and a particular experiment may or may not have positive result. In the successful experiments, characterization of the aptamer and strategies to improve its functional properties, post-SELEX have been reported. Peptide molecules other than DNA/RNA oligomers have also been put to use for their binding and recognition

properties. Structurally, peptide aptamers are short combinatorial peptide molecules inserted within scaffold protein with specific binding to targets isolated in intracellular conditions. They are outstanding as compared to DNA/RNA aptamers because of enhanced binding characteristics due to their structural properties, although the significance of DNA aptamers cannot be ignored considering their stability (Zhang et al. 2011).

Aptamers, in the field of sensing and detection have shown tremendous progress over the years with several reports. High affinity aptamers were reported for molecules such as dopamine, ATP in the μM range, and proteins VEGF, KGF in nM-pM range (Kiga et al. 1998; Sassanfar and Szostak 1993). Also, aptamers have been reported that can discriminate between closely related structures and show high molecular discrimination as compared to antibodies, hence proving their specific nature (Paborsky et al. 1993; Jenison et al. 1994; Goicoechea et al. 1999). These bioprobes can be generated for any possible target and thus mark the potential for detection of biomarkers undetectable in the present clinical situation. Also, the edge these molecules have over the antibodies characterizes their significance. Such as the of antibody generation process is neither simple nor time effective. The dependency on the host animal makes it even more unreliable for a range of toxic and low molecular weight compounds that can be harmful and generate immunogenic response. The process of purification of the antibodies and processing is a lot more tedious as compared to aptamers. Comparing the two, aptamers are selected in vitro with the SELEX process, which is a simple and inexpensive process, independent of host. For the selection of aptamers with desirable traits, the selection conditions can be pre-designed and applied to the procedure. The selected aptamer can then be produced in large numbers through chemical synthesis (Conrad et al. 2004; Kulbachinskiy 2008; Bouchard et al. 2010). Other than ease of production, the strong viability of aptamers under extreme temperature and physiological conditions marks them superior to antibodies that can be denatured easily and lose functionality if subjected to extreme conditions (Jayasena 1999).

There is a significant collection of identified aptamers, thus indicating a linear increase in the number of studies reported with progressing time (Lee 2003). The importance of aptamers in diagnostics and therapeutics can be easily demonstrated with several reported aptamers in various stages of clinical testing (see, Table 1). Aptamers are active in blocking receptor sites and inhibiting protein activity indicating therapeutic potential, a clear example of which is Macugen (OSI Pharmaceuticals, Melville, NY), drug approved by FDA for treating macular degeneration caused with aging. Another example of therapeutic aptamer in clinical testing is an anti-VEGF pegylated aptamer, EYE001. The production of VEGF is increased in the condition termed as Von Hippel–Lindau syndrome, causing abnormal growth of blood vessels (Ng et al. 2006). Aptamer EYE001 has inhibitory effect on production of VEGF, thus showing optimistic effect in vision of patients in this study. In another study, healthy volunteers and patients with colorectal cancer (CRC) were studied for the effect of 68Ga labeled Sgc8, a 41-nucleotide ss-DNA specific for target protein tyrosine kinase-7 (PTK7) selected by cell-SELEX. PTK7 overexpresses in different cancers and in the TNM stage of CRC patients, tumor

Table 1 Developed aptamers advantageous for in vivo diagnostic applications in clinical trials[a]

Aptamer/ drug	Form	Target	Condition/disease	Phase
68Ga-Sgc8	DNA	Protein tyrosine kinase-7 (PTK-7)	Colorectal cancer	Early phase 1
EYE001	RNA	VEGF (decreases production)	Von Hippel–Lindau (VHL) disease	Phase 1
ARC1779	DNA	A1 domain of activated vWF (von Willebrand factor)	Purpura, thrombotic thrombocytopenia, Von Willebrand disease type-2b	Phase 2
NOX-A12	RNA	CXCL 12 Mobilization of HSC (hematopoietic stem cells)	Hematopoietic stem cell transplantation	Phase 1
REG-1	RNA	Coagulation factor IXa	Anticoagulant for conditions such as heart attack and other coronary artery disease	Phase1
E10030	DNA	Platelet-derived growth factor (PDGF) (anti-PDGF pegylated aptamer)	Age-related macular degeneration	Phase 1
ARC1905	DNA	Complement factor C5 (anti-C5 aptamer)	Age-related macular degeneration	Phase 1

[a]This data has been collected from the site of clinicaltrials.gov

differentiation, lymph node metastasis, distant metastasis can be correlated to its overexpression. NOX-A12, a second generation, 45-nucleotide aptamer, PEGylated for longer half-life is in phase-2 of clinical-study for its effect on the mobility of hematopoietic stem cells. This aptamer was administered with and without Filgrastim for testing its effect along with safety and tolerability in patients. In several clinical situations such as acute coronary syndrome, von Willebrand's diseases, and vWF-related platelet disorder such as Thrombotic Thrombocytopenic Purpura (TTP), second-generation aptamer ARC-1779 is known to show antithrombotic effect. ARC-1779 targets the A1 domain of activated von Willebrand factor (vWF) and the response generated can be altered by binding to complementary sequence (Cosmi 2009). Also, a combination therapy of ARC1905, an anti-C5 aptamer with Lucentis® 0.5 mg/eye-multiple doses or with one induction dose was tested in patients with subfoveal choroidal neovascularization secondary to age-related macular degeneration. For improving the control of blood thinning due to anticoagulation in patients with risk of heart attack, the REG-1-aptamer-RNA anticoagulation system was designed and evaluated. One part of the system stops the activity of factor IX (protein that helps blood clot) (Benedict et al. 1991) while the other part of the system (the antidote) inactivates the drug and stops the thinning process.

3 Aptasensor

In addition to therapeutics, bio-imaging and drug discovery applications, biosensors were developed with use of aptamers as detection molecules and came to be known as aptasensors. Based on the number of reports of aptamer-based sensors, an enormous amount of progress has been made in the respective field. In 1990, the first aptasensor was reported, wherein interaction between human-neutrophil elastase coated beads and fluorescent aptamers was captured on an optical detection system. Other reports of SELEX-derived fluorescent tagged ligand in an enzyme-linked sandwich assay and radiolabeled ligand for protein kinase C isozymes have been reported (Davis et al. 1996; Conrad and Ellington 1996; Drolet et al. 1996). High sensitivity, compatibility, miniaturization, and low-cost are attractive factors for the designing and functionality of aptasensors. For this optical, electrochemical, and mass-sensitive strategies have been employed. The initial reports of electrochemical sensor came around 2004 with the Ikebukuro group's contributions and efficient technologies such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), electrochemiluminescence (ECL), and electrochemical impedance spectroscopy (EIS) based on electrochemical analysis developed (Lim et al. 2009; Xu et al. 2005; Numnuam et al. 2008; Wang et al. 2007; Ikebukuro et al. 2005; Cho et al. 2010). Several applications and successful studies based on these sensors have become widely known in a variety of relevant areas such as environment, clinical, and point-of-care testing (Thévenot et al. 2001; Han et al. 2010; Thevenot et al. 1999). Amidst several publications of aptasensors with a variety of transduction platforms, the most effective are the electrochemical sensors with effective miniaturization-low-cost production, convenient and reliable processing, and high sensitivity.

Such novel technologies are increasingly being developed for the critical threat posing agents to human health with high priority to meet the needs of advancing healthcare. Biosensors for detection of peptide and/or protein biomarker show more advantage over the conventional methods of detection and identification of causative agent. The primary target is to develop aptasensors that can be easily available to the end user in low-resource settings. The following table (see, Table 2) lists some of the recent developments of aptasensors for disease targets with clinical applications showing detection limits in low range, followed by a discussion. The analysis of biomarkers in body fluids can be helpful in detection of the clinical condition of the patients and/or normal and can also benefit to monitor the disease-stage and progression for accurate treatment.

In present situation, there are several widespread diseases, cancer being one of the highly reported one. Due to delayed diagnosis, it is the second leading cause of loss of life after AIDS. Observation and recognition of the formation of tumor by biomarker appearance/disappearance monitored through sensitive and specific methods is required. A study comparing the aptasensor and immunosensor for the detection of prostate specific antigen (PSA) for prostate cancer was conducted showing similar results for limit of detection in range of 0.14 ng/mL. Three techniques CV, DPV, and EIS on graphene quantum dots-gold nanorods

Table 2 Recent reported studies of aptamer-based sensors for clinical point-of-care testing application

Biomarker	Condition	Aptamer	Detection	Transduction	LOD/range	Ref
Prostate specific antigen (PSA)	Prostate cancer	DNA	GQDs-AuNRs	CV, DPV, EIS	0.14 ng mL^{-1}	Srivastava et al. (2018)
Osteopontin	Breast cancer	RNA	μ-AU electrode	CV	8 nM	Meirinho et al. (2015)
CCRF-CEM cell	Leukemia	Sgc8	GO-apt-FAM	FRET	10 cells mL^{-1}/10^2 to 1×10^7 cells mL^{-1}	Tan et al. (2018)
C-reactive protein	Cardiac disease	RNA	SiMSs-Au NPs	SWV	0.0017 ng mL^{-1}/ 0.005 ng mL^{-1} to 125 ng mL^{-1}	Wang et al. (2017)
Vascular endothelial growth factor-165 ($VEGF_{165}$)	Cancer angiogenesis		Nanoplasmonic	Optical	25 pg mL^{-1} to 25 ug mL^{-1} (comparable to ELISA)	Cho et al. (2012)
Hemagglutinin protein (HA)	H1N1 flu	aHP (DNA)	GO/KF-polymerase strand displacement	Fluorescence	(HA) 2.5 μg mL^{-1}/ (H1N1 virus) 1×10^2 TCID50	Keyi Liu (2015)
AIV H5N1	Avian flu	DNA	Au-stav-bio-apt	SPR	0.128–1.28 HAU	Bai et al. (2012)
Human immunodeficiency virus type 1 trans-activator transcription protein (HIV-1 Tat)	HIV	RNA	Diamond-FET	Potentiometric		Rahim Ruslinda et al. (2013)
Hepatitis C virus (HCV)	Hepatitis		GCE-GQDs	EIS, CV, DPV	3.3 pg mL^{-1}	Ghanbari et al. (2017)
Plasmodium falciparum glutamate dehydrogenase (*Pf*GDH)	Malaria	DNA	aptaFET/IDμE (interdigitated gold micro electrodes)	Potentiometric	48.6 pM/100 fM to 10 nM	Singh et al. (2019)
Murine norovirus (MNV)	Viral gastroenteritis	AG3 (DNA)	GNPs-SPCE	SWV	180 virus particles	Giamberardino et al. (2013)
Glycated human serum albumin (GHSA)	Diabetes mellitus	DNA	GO-G8apt	Fluorescence	50 μg mL^{-1}	Apiwat et al. (2016)

(GQDs-AuNRs) modified screen printed electrodes were used simultaneously to investigate the analytical performance of both; however, aptamer-based detection is simpler, more stable, and inexpensive (Srivastava et al. 2018). In a similar study for protein Osteopontin (OPN), biomarker for breast cancer, the developed aptasensor showed selective detection of target in the presence of other interfering proteins except thrombin, thus showing a possible application for breast-cancer prognosis in future (Meirinho et al. 2015). In this work, a previously developed RNA aptamer for target OPN was used for detection with help of CV and square-wave voltammetry (SWV) techniques. Several studies of fluorescence aptasensor based on graphene oxide (GO), carbon nanomaterial because of their fluorescence quenching property and interaction with aptamers by π–π stacking have been reported (Song et al. 2011; Chen et al. 2015; Gao et al. 2015). The detection of CCRF-CEM cells for leukemia in the range of 1×10^2 to 1×10^7 cells/mL with a LOD of 10 cells/mL in a fluorescence aptasensor based on GO-apt complex has been developed (Tan et al. 2018). The change in the fluorescence signals can be measured significantly between presence and absence of Sgc8 target cell CCRF-CEM. The aptamer–target–immunoprobe chemistry has broad application in development of sandwich aptasensors. In one study, functionalized silica microspheres were used as immunoprobes in an electrochemical sandwich aptasensor for detection of C-reactive protein (CRP) in the range of 0.005–125 ng/mL and detection limit 0.0017 ng/mL via SWV. Firstly, the silica microspheres were synthesized, functionalized with gold nanoparticles (AuNPs) for immobilization of signal molecules (zinc ions, Zn^{+2}) and antibodies (Ab). RNA aptamer linked to modified AuNPs via gold-sulfur affinity was used as detection molecules, showing promising result for serum samples with CRP (Wang et al. 2017). The interesting features of gold nanoparticles have been utilized for sensing and measurement, reporting a single-step optical biosensor for detection of $VEGF_{165}$ in the range of 25 pg/mL to 25 μg/mL with inactivation of surface plasmon enhancement of fluorescent probe attached to target-binding ligand. This aptasensor showed results resembling the ELISA test for serum and saliva samples and no cross-reactivity with alternative targets—PDGF-BB, OPN, $VEGF_{121}$ present in sample, thus establishing a simple and inexpensive testing method (Cho et al. 2012).

Field effect transistors (FET), the advanced nano-electronic biosensing devices have also been studied for application as aptasensors. In a study, the effect of surface charge density on the real sample of HIV-1 Tat protein was reported. This aptamer-FET used diamond interface owing to its chemical stability and stable RNA aptamers bonded to carboxyl-terminated terephthalic acid covalently (Rahim Ruslinda et al. 2013). The detection of plasmodium falciparum glutamate dehydrogenase (PfGDH), biomarker for malaria, life threatening and endemic disease in developing countries in range of 100 fM to 10 nM and limit of detection of 16.7 pM in spiked buffer and 48.6 pM in serum samples is another example of FET-based aptasensor (Singh et al. 2019). The rapid diagnostic test available currently is non-quantitative. In this sensor, interdigitated gold micro electrodes (IDμE) were used. The ssDNA aptamer (90 bp-NG3) was immobilized on IDμE with gold–thiol chemistry and the net surface charge on captured target causes change in gate

potential, used as measure of concentration of target. The surface plasmon resonance (SPR) based sensors are also effective to study biological interactions. Several reports have been published for SPR biosensors for different strains of Influenza virus. A SPR biosensor for the detection of avian influenza virus (AIV) H5N1 in range of 0.128–1.28 HAU with nil signals for six non-target subtypes has been reported (Bai et al. 2012). In this sensor, aptamer was attached to gold surface via streptavidin–biotin chemistry and show increase in refractive index (RI) with capture of target.

4 Peptasensor (Peptide-Based Aptasensor) for POC Testing Application

Peptide aptamers offer a promising alternative as compared to antibodies and nucleic acid aptamers in terms of their sensitivity and specificity. Other than the ease of methodology for identification and characterization through in vitro selection technology, their structural conformation allows for accurate interaction with binding region of target biomarker. Such clearly defined interaction thus results in lower background signals (noise) generated due to non-specific interaction and binding parameters indicating higher strength and value could be obtained. The integrated unit, peptasensor can be developed with combined mechanism of selected peptide aptamer with advanced sensing technology.

The electrochemical sensors are most recommended for recording the binding event for point-of-care applications because of their significant sensitivity. Also, device miniaturization with the development of the screen-printing technology has further increased the employability of these sensors for multiple-assays (Chu et al. 2017; Tung et al. 2017; Keyi Liu 2015; Ghanbari et al. 2017). Recently, DEPSOR (*d*isposable *e*lectrochemical *p*rinted sen*sor*) has been introduced and developed under the Japan-based startups BioDevice Technology, Ltd. and BioSeeds Corporation and India-based startup Biyani Biosolutions Pvt. Ltd. These startup companies are engaged in setting up the platform for rapid on-site diagnostic sensors to meet all-round challenges presented by newly appearing infective target molecules. PEP-on-DEP concept (see, Fig. 1) reported in 2015 was based on integration of in vitro molecular evolution technology with sensing via DEP-chip (So et al. 2005). The peptide aptamers were selected with the cDNA display technology (Nanjappa et al. 2014) for target renin with K_D of μM-order. The selected aptamer probes were then attached to gold nanoparticles for detection of renin (300 ng mL^{-1}) via redox signals. This report describes a promising approach for the detection of unreachable biomarkers with low-cost and rapid on-site diagnosis.

The most essential requirements for an efficient biosensor are sensitivity and selectivity. The carbon nanotube field effect transistors (CNT-FETs) demonstrate sensitivity to minor variations in environment (Liu and Guo 2012; Maehashi et al. 2004; Jocson et al. 2014). However, due to selectivity issue it is challenging to operate them on field (Nanjappa et al. 2014). In this work, peptide aptamer modified SWCNTs FET sensor was developed for the label-free detection of Cathepsin E (Cat

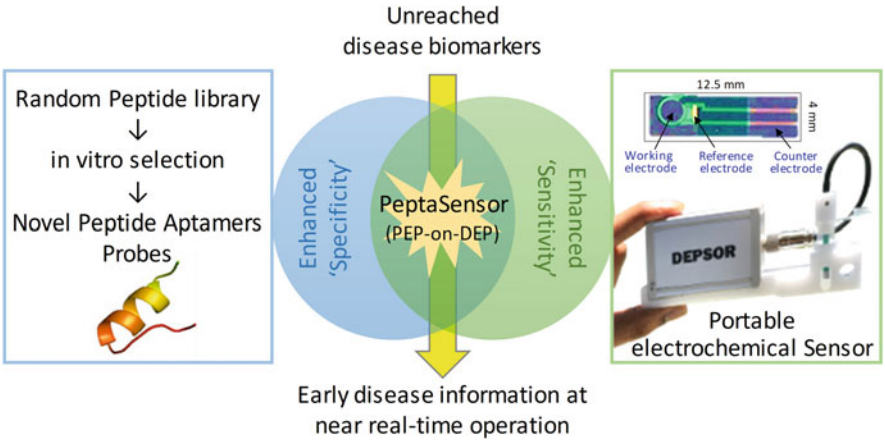

Fig. 1 Schematic of the peptide aptamer-based electrochemical (PEP-on-DEP) sensor concept. The integration of a novel peptide aptamer with DEPSOR (disposable electrochemical printed sensor) to achieve highly selective and sensitive biosensing of unreached biomarkers for point-of-care application

E) at 2.3 pM in phosphate-buffered saline (PBS) and 0.23 nM in human serum. No reactivity was observed with targets such as bovine serum albumin (BSA) and cathepsin K (CatK), thus indicating high selectivity, a major aspect for clinical application of aptasensors. A peptide scaffold was attached to the peptide aptamer selected for target Cat E via systemic in vitro evolution with 0.954 nM K_D (Kitamura et al. 2012). This complex was grafted on the SWCNT for sensitive detection with non-covalent interaction. The single-walled carbon nanotubes (SWCNTs) were used to be able to lower the noise ratio and hence this system showed improved LOD for Cat E in human serum when compared with conventional ELISA (Biyani et al. 2011). The small size of the peptide binding molecules makes them fit for detection with such a device (within the electrical double layer) over the conventional large size antibodies with size >10 nM (So et al. 2005; Chu et al. 2017). Henceforth, this report introduces novel portable sensor that can be applied in future for detection of other potential biomarkers for point-of-care testing application (Tung et al. 2017).

5 Conclusion

The science of aptamers is continuously evolving with a large number of publications being reported since the first evidence. The previous limitations in detection methods can be improved with unique properties of aptamers. Recent reports of aptamer-based sensors set grounds for improving the current detection methodologies to the next level. Based on the considerable amount of research in the field of aptamers, with some in clinical trials, development and commercialization of aptamer-based sensors for rapid on-site diagnostics is also expected.

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Bead-Based SELEX for Aptamers Selection and Their Application in Detection of Diverse Antigens

Devika N. Nagar, T. Yathirajarao, Pankaj Kumar, Pooja Kushwaha, and Pankaj Suman

1 Introduction

Systemic evolution of ligands by exponential enrichment (SELEX) is a repetitive and revolutionary in vitro process for generation of a high-affinity aptamer for small target molecules and is an alternate to antibodies. SELEX holds several advantages like it is eliminating the use of animals, reducing the production time, labour and purification steps (Bruno and Kiel 2002). Typically SELEX starts with single-stranded oligonucleotide (DNA or RNA) library comprising up to 10^{15} different oligonucleotides in the pool of random sequences flanked by a conserved region of about 20 or more bases (Yiyang Dong et al. 2018). Since 1990, when SELEX was first used for generation of aptamers, now over the years several modifications in the protocol have been made to make it better and user friendly (Ellington and Szostak 1990; Tuerk and Gold 1990). Aptamers are being used for both as detection probe (Drolet et al. 1996) and as therapeutic agent (Bruno and Kiel 2002). A typical SELEX process involves initial binding step, followed by elution and further amplification of the selected oligonucleotides. To enrich the poll of selected oligonucleotides, the whole process is repeated several times. Though the process seems very simple but a little variation in the selection protocol leads to huge impact on generation of aptamers (Yiyang Dong et al. 2018; Fig. 1), Thus in this chapter, we have focussed on to elaborate on few improvised simplified combinatorial bead-based SELEX protocols for aptamer selection against small target molecules. Applications of aptamers for diagnosis, biomarker discovery, imaging, therapeutics have also been summarized at the end.

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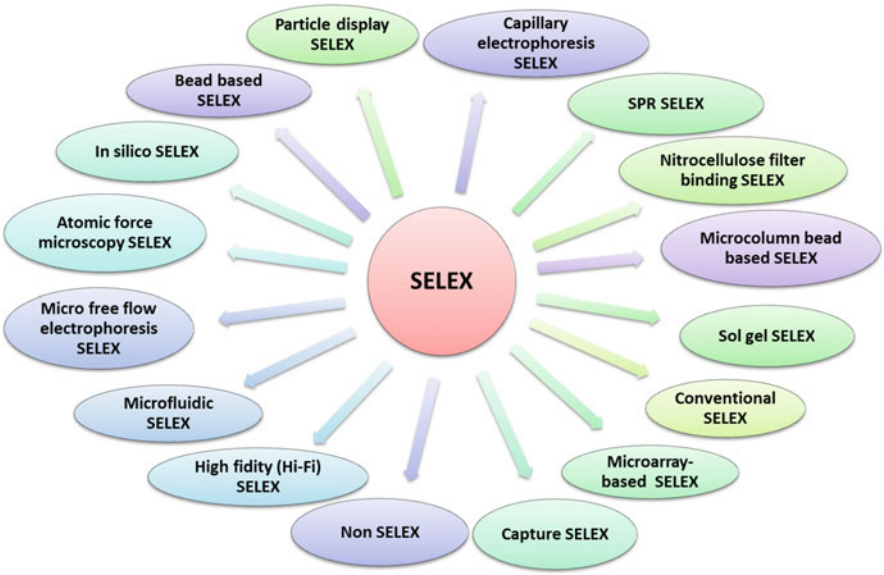

Fig. 1 Systemic evolution of ligands by exponential enrichment (SELEX) strategies

2 Systemic Evolution of Ligands by Exponential Enrichment (SELEX)

Conventionally, SELEX requires generation of chemically synthesized functional library of single-stranded oligonucleotides. Selection steps in SELEX have been modified as per the nature of the target molecules. After several rounds of selection, the obtained oligonucleotides are sequenced and their affinity for the target is determined by various methods (Jing and Bowser 2011).

3 Classical Bead-Based SELEX

In this method, the target molecules are first immobilized on a solid support like agarose, sepharose, sephadex, magnetic beads, etc. Immobilization of the target molecules can be achieved with help of tags like streptavidin–biotin pair. These tags consist of functional groups for high-affinity binding with target molecules thus providing a convenient method for aptamer selection for small target (Meral et al. 2015; Zhou et al. 2016; Bayat et al. 2018).

After immobilization of the target on solid support, they are incubated with the aptamer library to fish out specific aptamers (Fig. 2). Solid support plays a vital role

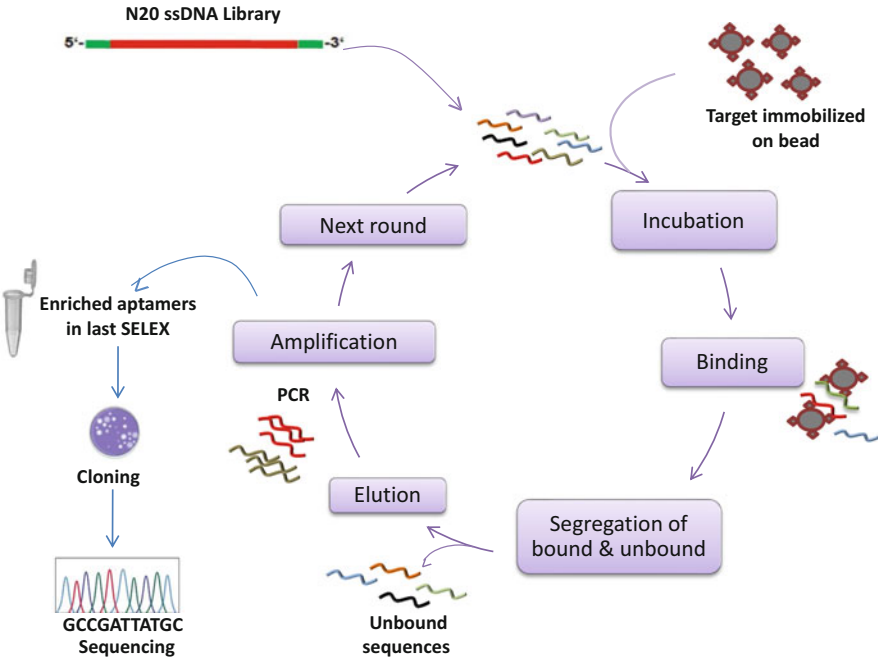

Fig. 2 Classic bead-based SELEX: The SELEX process includes incubation, binding, separation and amplification steps. During incubation period, the target molecules bind to the oligonucleotide from the random library. At the separation step, the bound sequences are isolated and PCR amplified for enrichment of target-binding sequence pool for the next round of SELEX. After several sounds of SELEX, the enriched pool is cloned and sequenced to identify the specific oligonucleotide sequence

for enrichment of high-affinity aptamer against small target molecules; as high concentration of target molecules results in cooperative binding leading to low affinity and non-specific binding of unwanted ligands. Excessive retention of undesired aptamers leads to poor selection of desired aptamers in subsequent rounds of selection (Ozer et al. 2013). Following this approach, high-affinity aptamers have been generated for a tumour biomarker, programmed death-ligand 1(PDL-1) after follwoing 10 or more rounds of SELEX. The aptamers selected against them had very high specificity and sensitivity to the extent that it can be considered for the detection of tumour cells with very low concentration, i.e. as low as 10 tumour cells/ml (Bayat et al. 2018). Bead-based SELEX has several advantages over other methods in its convenience to perform, applicability to wide range of targets and easy enhancement of selection procedures by simply altering incubation parameters and flow rate (Bayat et al. 2018).

4 Magnetic Bead-Based SELEX

A magnetic bead-based SELEX method employs magnetic beads to immobilize the target protein for binding with the oligonucleotide library, resulting in formation of aptamer–target complex. The specific oligonucleotides are separated after each round of SELEX using a magnetic field and thereby eliminating the undesired non-specific oligonucleotides (Fig. 3).

The bound oligonucleotides are used to generate ssDNA to use as pool of oligonucleotide for the subsequent cycles of selection. This way enrichment of high-affinity aptamers against the target protein takes place. These aptamers are cloned, sequenced and affinity is determined following standardized methodologies.

This method has been employed for selection of aptamer specific to target molecules such as staphylococcal enterotoxin B (SEB) and whole cholera toxin (Bruno and Kiel 2002). Aptamers generated were used to develop sensitive electrochemiluminescence assay with a detection limit of less than 10 pg. Another report suggests the selection of oligonucleotide capable of binding and detection of non-pathogenic strain of *Bacillus anthracis* (anthrax) spores (Bruno and Kiel 1999). As this is one of the convenient methods, it has also been used in

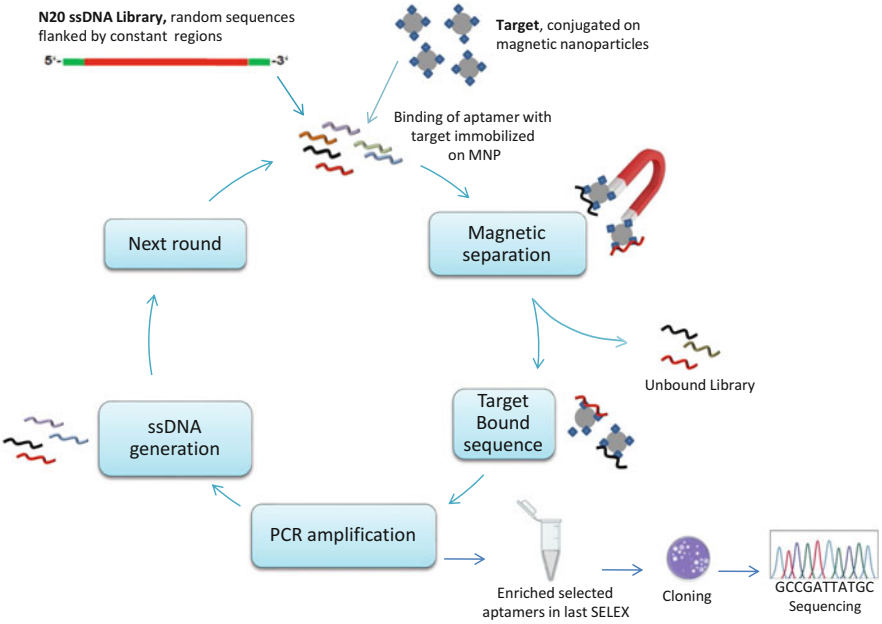

Fig. 3 Magnetic bead-based SELEX: magnetic beads are used to immobilize the target molecule. Upon incubation of the oligonucleotide library with the magnetic bead, target complex, separation of aptamers is done under the influence of magnetic field. The bound sequences are separated and amplified by PCR for the next round of selection. Selected pool of oligonucleotides from the last SELEX cycle are cloned and sequenced for identification of specific sequences recognising the target molecules

immunofluorometric assay which illustrates aptamers as diagnostic reagents against their target (Rye and Nustad 2001). Aptamers with high affinity and specificity for anabolic androgenic steroid target molecules like testosterone were also selected by employing this magnetic bead-based SELEX (Skouridou et al. 2017).

5 Microcolumn Bead-Based SELEX

In this bead-based method, the target molecule is immobilized on to the resin loaded onto the microcolumn and the random aptamer library is allowed to bind in the column. In this method, selection cycles can be automated by means of centrifuge and/or pumps. The unbound aptamers are removed and bound ones are used for the next round of SELEX. It is a modified version of affinity chromatography-based SELEX that reduces non-specific binding and dead volume, thus increasing the efficiency of aptamer selection and enrichment by minimizing the amount of resin and aptamer required. For example, aptamers selected against Negative Elongation Factor—E(NELF-E) (Pagano et al. 2014) and for Heat Shock Factor (HSF) by using microcolumn filled with GFP-immobilized resin (Latulippe et al. 2013) showed high affinity in nanomolar range only after 5–6 rounds of selection. In order to perform simple and versatile multiplex selections using different microcolumns, an improved scale-up device called Microplate based Enrichment Device Used for the Selection of Aptamers (Szeto et al. 2014). It is a 96-microcolumn device designed for high-throughput aptamer selection, process characterization and to perform any number of modified combination of serial and parallel processing. In this method, enrichment of aptamers gets significantly affected by the density of target molecules (Szeto et al. 2014; Latulippe et al. 2013). Therefore, to achieve enrichment of high-affinity aptamer, it is recommended to optimize the density of target molecules (Bayat et al. 2018).

6 Particle Display SELEX

Particle display was developed as an alternative method to overcome the limitations of most of the SELEX methods, like need of several rounds (10–15) of selection to obtain desired aptamers (Bayat et al. 2018), retainment of low-affinity aptamers (Irvine et al. 1991) and at times loss of sequence variants (Djordjevic 2007).

In contrast to bead-based methods of SELEX that require immobilization of target molecules on beads; in particle display, emulsion PCR (ePCR) is used for synthesis of each aptamer particle, where up to 10^5 copies of single amplified clones of ssDNA oligonucleotides are immobilized on each bead (Fig. 4). After this, these are denatured by NaOH treatment in order to release the reverse strands and are collected with the help of a magnet. The aptamer immobilized beads are then incubated with target molecules tagged with fluorescent molecule to sort with the help of fluorescence-activated cell sorting. The aptamers generated through this process has direct correlation of binding affinity with the fluorescence intensity generated.

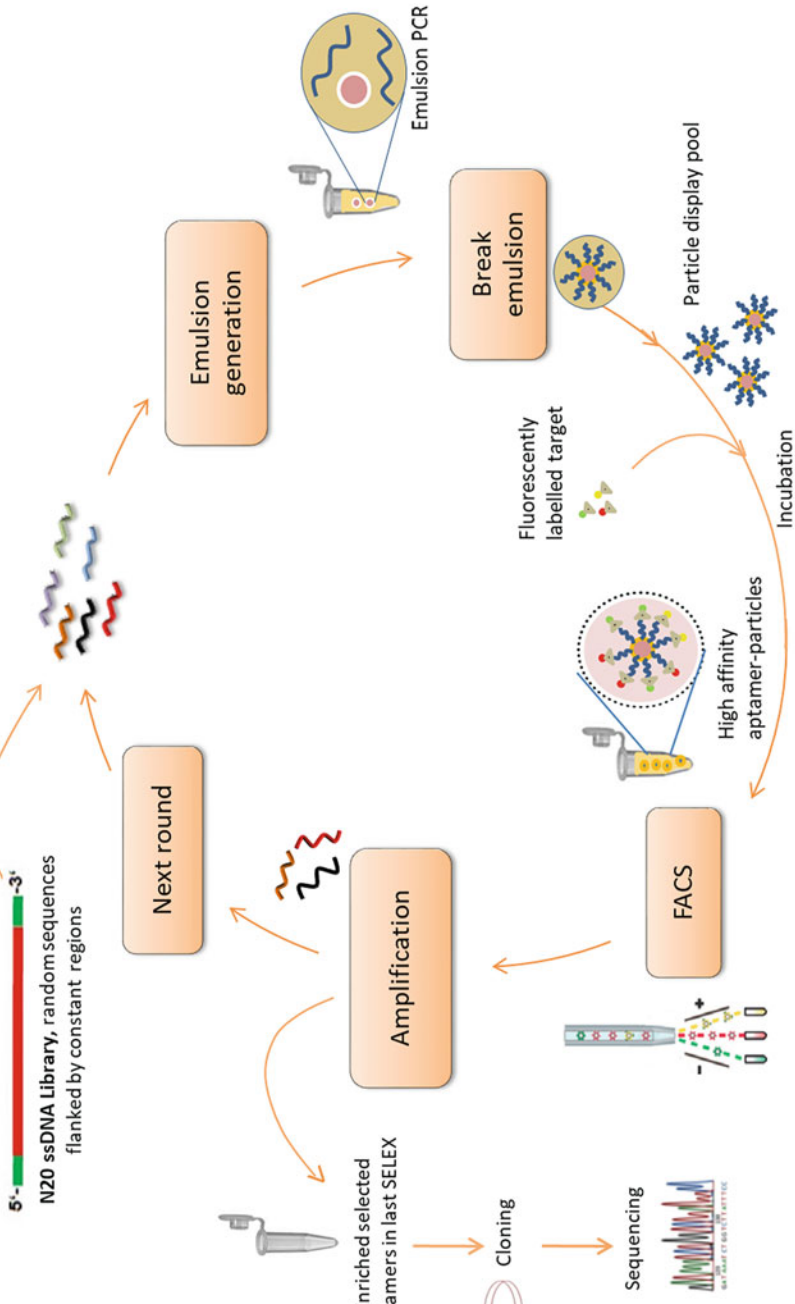

Fig. 4 Particle display SELEX: the oligonucleotide particles are generated by emulsion PCR where droplets are made such that they contain unique aptamer sequence and forward primer attached to the magnetic bead. At the end of emulsion PCR several copies of aptamer sequences are formed that is released upon breaking the emulsion. To measure the aptamer affinity, the aptamer particles are incubated with fluorescently labelled target molecules to get analysed by fluorescence-activated cell sorting (FACS)

Thus this technique allows quantitative identification and sorting of aptamers with high affinity using FACS. This method holds advantage over other conventional SELEX methods as better differentiation can be achieved between aptamers having similar affinity. Additionally, it allows selection of desired aptamers even after fewer rounds of selection. Use of FACS helps in removal of low-affinity aptamers as they have low fluorescence (Bayat et al. 2018) and enables quantitative screening of aptamers (Wang et al. 2014). The aptamers obtained by particle display method show considerably high affinity. For example, aptamers developed for thrombin (Tasset et al. 1997) and ApoE exhibited affinities far better than previously reported aptamers. High-affinity aptamers against PAI-1 and 4-1 BB have also been generated following this method as previous attempts of selection via other methods could not give desirable outcomes (Wang et al. 2014).

7 Microfluidic SELEX (M-SELEX)

M-SELEX has been developed to speed up the aptamer selection process through introduction of stringent selection parameters and application of very less amount of target molecules (Qian et al. 2009) to overcome the intensive labour and longer time required by most of the other SELEX methods. In this process, the user can get rid of from the non-specific aptamers by changing the flow rate with minimum loss of the target molecule. Micromagnetic beads have been used in one of the method of this kind to immobilize the target and it led to identification of aptamer with ~three- to four-fold higher affinity and ~two- to- four-fold higher specificity within 1–3 rounds of selection (Cho et al. 2010). The process of aptamer selection involves incubation of random oligonucleotide library with the target immobilized on to the magnetic beads. Continuous-flow magnetic activated chip-based separation (CMACS) device helps in separation of bound aptamers from unbound under the influence of magnetic field (Lou et al. 2009). During the course of advancement, micromagnetic separation (MMS) microfluidic device was developed that offers substantial improvement over the CMACS device (Qian et al. 2009) (Fig. 5).

Using the aforementioned technique, only single round of selection led to generation of aptamers against Botulinum Neurotoxin Type A light chain (BoNT/A-rLC) (Lou et al. 2009). With the aim to accelerate the selection of high-quality aptamer sequences, a new strategy termed as the Quantitative Parallel Aptamer Selection System (QPASS) was devised (Cho et al. 2013). Upon integration with a microfluidic selection and in-situ synthesized aptamer arrays next generation (NGS) sequencing, QPASS can simultaneously measure the affinity and specificity of thousands of aptamers. The potential of QPASS in generation of aptamer was explored for human cancer biomarker like angiopoietin-2 (Ang-2). Four rounds of M-SELEX were performed to select aptamer against angiopoietin immobilized on magnetic beads and selected aptamers were analysed for sequence homology and copy number by NGS data analysis. This methodology produced six choices of aptamers against angiopoietin with an affinity less than 30nM and high specificity towards the target sequence (Cho et al. 2013).

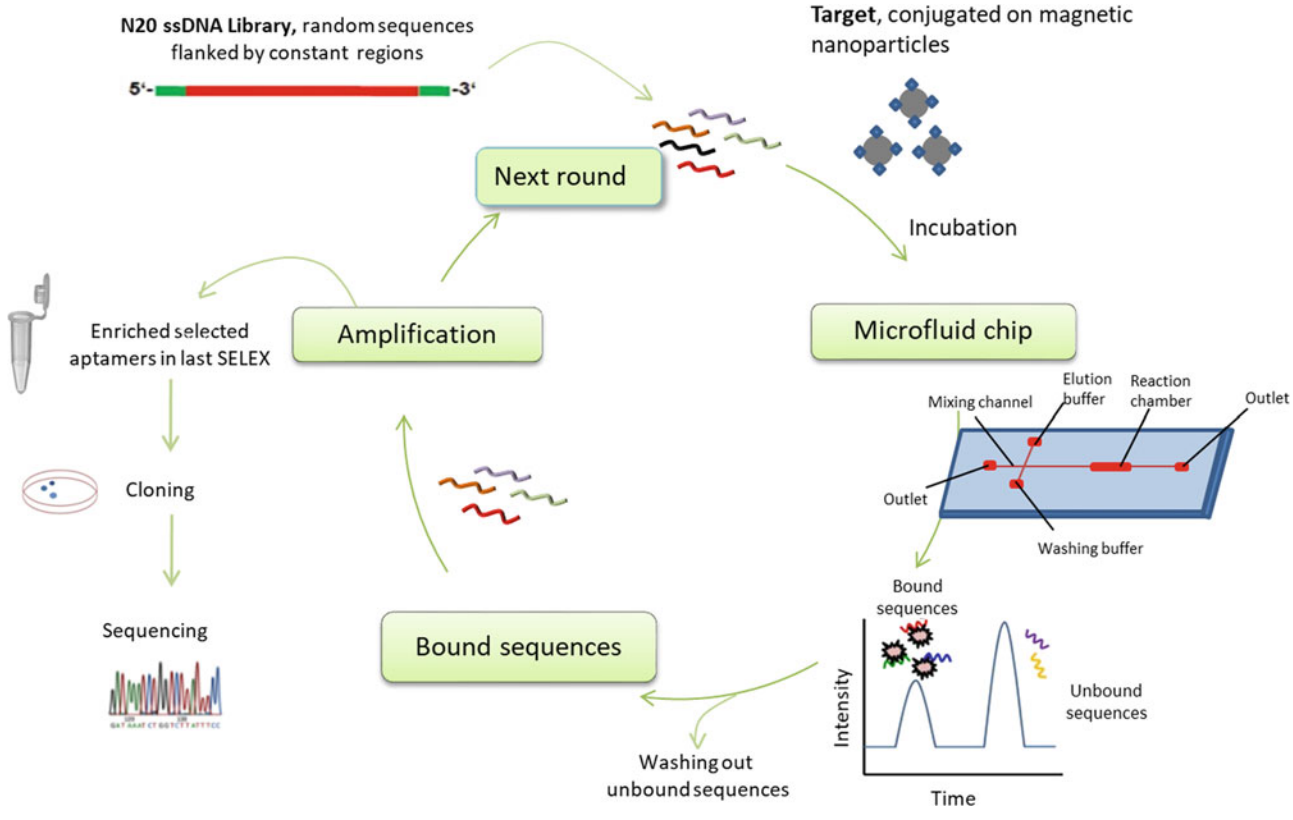

Fig. 5 Microfluidic SELEX: In this method, oligonucleotide library is incubated with target molecules immobilized on magnetic beads. After this, a continuous laminar-flow, magnetically activated, chip-based separation (CMACS) device helps in isolation of oligonucleotides. Under the influence of magnetic field, the oligonucleotides bound to magnetic bead-target complex move at the centre of chip and are eluted through middle outlet, whereas unbound oligonucleotides are eluted through the side waste outlet. Then, the bound sequences are amplified by PCR and used for the next round of selection

8 Library Immobilization Based SELEX (Capture-SELEX)

For a small molecule, the major difficulty in aptamer selection is to develop highly specific aptamer against the target molecule. Most of the methods for aptamer selection are based on target molecule immobilization on a solid support, thus during the immobilization process, an important part (epitope or functional group) of the target becomes concealed upon conjugation. So, due to the lack of epitope or functional group on target molecules, the specificity of aptamer decreases in real experimental sample as it lacks specificity for whole target molecules. Hence, library immobilization method of SELEX has higher specificity than other methods (Liu et al. 2018; Wu et al. 2014; He et al. 2011). In this method single stand, random oligonucleotide library is immobilized onto the magnetic nanoparticles where the biotinylated primer acts as a linker. Then it is incubated with streptavidin-conjugated magnetic nanoparticles and the target molecule (Fig. 6). It is anticipated that in the presence of a target molecule, the conjugated oligonucleotide sequence present in the library will detach from the complementary primer sequence attached to the magnetic nanoparticle. Thus, the target specific ssDNA will come into the supernatant when kept in the influence of strong magnetic field. Here it requires multiple

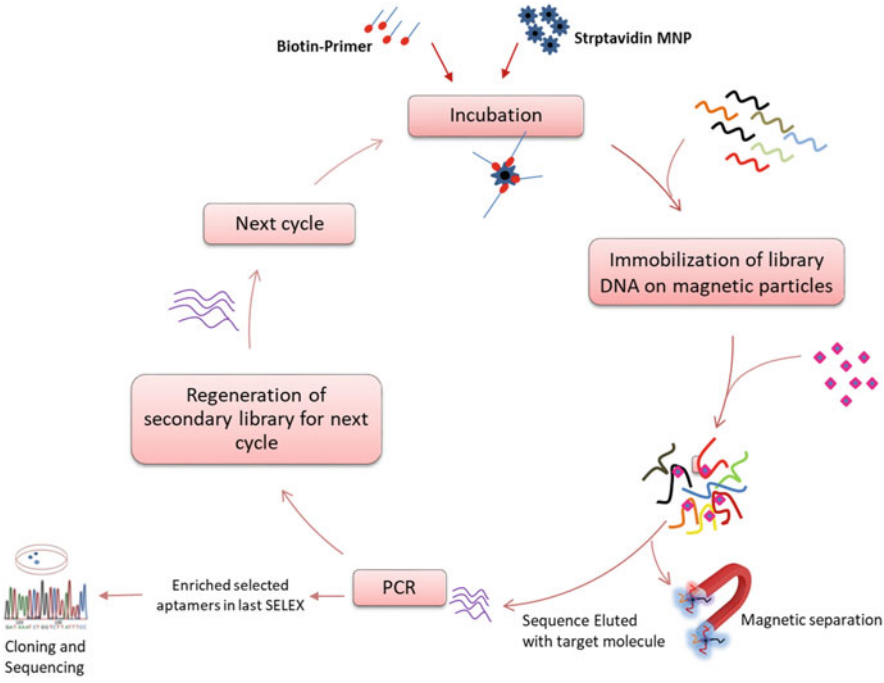

Fig. 6 Capture-SELEX: The Library is immobilized on the magnetic beads with the help of covalent or non-covalent interactions. After incubation of target with the library, the partitioning is done with the aid of a magnet. The eluted sequences are amplified to generate secondary library for next round of selection

rounds of selection to enrich the specificity and stringency of the aptamers. This procedure was employed in aptamer selection for clenbuterol and it is also reported as the first study to use multiple combination of selections and identification strategy including Q-PCR monitoring, high-throughput sequencing and library fixation (Liu et al. 2018). Recently, magnetic beads have been used to immobilize the ssDNA library and it could generate nine different families of aptamers after sixteen rounds of selection (Duan et al. 2017). Using the above-mentioned method, DNA aptamer for the aminoglycoside antibiotic kanamycin A was selected; wherein it led to selection of high-affinity aptamers for small organic molecules. Thus, this modified bead-based SELEX method involves immobilization of random DNA library with a defined docking sequence in the random region of the library on to the beads, which makes library conjugation on beads easy (Stoltenburg et al. 2012). Indeed, it is very significant that via capture-SELEX method only those aptamers are isolated and selected which on binding to target molecules undergo conformational changes, i.e. the aptamers which will have a greater affinity for target molecules can only be released.

All the reported methods for SELEX hold unique features that can be exploited as per the need. These advantages for most commonly utilized SELEX have been summarized in Table 1.

9 Applications of Aptamers in Diagnosis and Detection

Advent of aptamers has revolutionized the field of diagnostics and therapeutics by providing potential alternative to existing classical methods of detection and prophylaxis (Tan et al. 2011). Receptor–ligand binding interaction is an important application in detection assays; the major focus is designing a receptor with high specificity. Aptamers are the new class of receptors, showing specific binding with superior affinity to virtually any kind of target molecule (Ellington and Szostak 1990; Nimjee et al. 2005). Unique features of aptamers have been summarized in Table 2. Mostly antibody-based diagnostics are used in disease diagnosis, but for some diseases, it has remained a challenge to develop a simple and specific antibody-based detection test. Thus, in such cases, aptamers-based detection tests might prove to be specific, robust, rapid and more accessible. In comparison with antibodies, aptamers are not only applicable in molecular diagnostic and therapeutic, but also be screened against non-immunogenic and toxic targets like metal ions (Ciesiolka et al. 1995), organic dyes (Babendure et al. 2003) and other small organic molecules (Mann et al. 2005; Bayat et al. 2018).

One of major defining feature of aptamer is its ability of conformational change upon target binding that can be used for designing structure switching aptamers which upon binding to target generate signals like fluorescence, electrochemical, etc. (Wu et al. 2015). These qualities make them suitable for development of sensitive and rapid diagnosis for identification of target in complex samples and can be used in development of aptamer-based biosensors called ‘aptasensors’ (Chandola et al. 2016). There are many reports mentioning about aptamer being a potential

Table 1 Advantages of above-mentioned bead-based SELEX methods and few other widely used SELEX methods

S. No.	Types of SELEX	Advantages
1.	Classical bead-based SELEX	Relevant for selection of aptamers for most target molecules Convenient in fine-tuning the selection process
2.	Magnetic bead-based SELEX	Convenient in segregation of unbound from bound aptamers Relevant for selection of aptamers for most of the small target molecule
3.	Microcolumn bead-based SELEX	Target and aptamer amount required is minimal Reduces non-specific binding and dead volume
4.	Particle display SELEX	Requires fewer selection rounds It employs FACS, thus enables determination of aptamer affinity threshold in reproducible and quantitative manner
5.	Microfluidic SELEX	Amount of target molecule required in very less Requires only few selection rounds (1–6) for isolation of high-affinity aptamers
6.	Library immobilization based SELEX (capture-SELEX)	Applicable for targets that are hard to get immobilized onto the solid support Applicable for parallel selection of aptamers against more than one target molecule
7.	Capillary electrophoresis SELEX	Analyte required in very less quantity High-speed segregation of bound aptamers from unbound ones
8.	Micro free flow electrophoresis	Short separation time during electrophoresis Isolation of aptamers in few SELEX rounds (2–4)
9.	SPR-SELEX	Real-time evaluation of enriched aptamers in the course of selection Evaluation of binding and selection of aptamers are done simultaneously
10.	Microarray-based SELEX	It does not require the aptamer amplification, cloning and sequencing Can be used for large-scale analysis
11.	In silico SELEX	Provides high-performing computational docking tools for virtual screening of Library, enrichment of sequences and high-affinity aptamers.
12.	Atomic Force Microscopy (AFM) SELEX	It provides in depth analysis of the aptamer affinity as well as the features that leads to higher affinity at the molecular level
13.	High fidelity (Hi-Fi) SELEX	It retains mostly the specific aptamers and simple in operation

alternative diagnostic tool that are faster, more sensitive, simple, stable and field-applicable (Tan et al. 2011). These features make aptamers as potential agents for diagnosis of cancers and infectious diseases (Vallian and Khazaei 2007).

Table 2 Strengths of aptamer technology

Properties of aptamers	References
• Aptamers show high binding specificity and affinity to desired target molecule	Nimjee et al. (2005, 2017)
• The specificity and stability of aptamers can be enhanced by structurally modifying them	Virgilio et al. (2018), Ni et al. (2017)
• These show no immunogenic responses and are non-toxic	Nimjee et al. (2017)
• They show thermo stability	Ni et al. (2017)
• Aptamer development requires less-time and its chemical synthesis is inexpensive	Nimjee et al. (2017)

10 Aptamers for Diagnosis of Cancer

Cancerous cells have propensity to undergo metastasis if not controlled at the early stage. Thus, early detection allows better and early treatment.

Aptamers isolated against the particular cancerous cells have greater sensitivity in detecting and targeting cancer at early stages regardless of their low expression levels which conventional diagnostic methods fail to detect (Levy-Nissenbaum et al. 2008; Zhang et al. 2010). Therefore, aptamers are considered as best potential candidates for diagnosis of cancer cells, cancer cell biomarkers, hormones and growth factors (Levy-Nissenbaum et al. 2008; Molefe et al. 2018). With the aim to develop aptamers-based diagnosis for cancer, a specific aptamer for CCRF-CEM acute leukaemia cells was reported by Zhang and co-workers (Zhang et al. 2010). These aptamers were selected by a cell-SELEX method which is an in vitro process involving conjugation of aptamer specific to CEM cells on gold nanoparticles (AuNPs) and detection of cancer by using colorimetric analysis (Molefe et al. 2018). In another study CD133 protein and epithelial cell adhesion molecule (EpCAM) specific fluorescent labelled aptamer were generated (Shigdar et al. 2011, 2013). CD133 has been widely used as cell surface marker in different types of cancers, the aptamers generated against it showed high sensitivity, superior penetration and retention when compared to antibodies (Shigdar et al. 2013). Similarly, aptamer against EpCAM (Epithelial Cell Adhetion Molecule) which gets upregulated in gastric, breast, colorectal cancer has been selected and characterized and their interaction with EpCAM showes successful receptor mediated endocytosis, investigated by Flow cytometry and confocal microscopy (Shigdar et al. 2011), thereby proving its potential as molecular imaging tool for cancer diagnosis and therapeutics. There have also been reports of aptamer-based detection of cancer biomarkers such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiogenin and thrombin (Molefe et al. 2018). They are vital for control and treatment of cancer, as these are potential early cancer biomarkers for diagnosis.

11 Aptamers for Infectious Diseases Diagnosis

Rapid increase in infectious diseases and their manifestation as multi-drug resistant strain has made their characterization very crucial. Since, aptamers exhibit high affinity, and specificity, field-based detection of target molecules, as well as for the whole cell is possible. Thus, it can be used as an innovative and potential tool for detection of pathogens expressing different biomarkers. In case of *Staphylococcus aureus* infection, an aptamer-based detection assay was developed which is cost effective and ultrasensitive that detects a single cell of *S. aureus* within 1.5 h, unlike conventional detection process that combined with culturing takes days and are expensive (Chang et al. 2013). Likewise, luminesce-based aptasensor was developed for simultaneous detection of *Salmonella typhimurium* and *S. aureus* with high sensitivity as it employs two kinds of coloured aptamers attached to the magnetic particles (Duan et al. 2012). Similarly, there have been reports of identification for aptamers against viruses like Ebola, Influenza H5N1, HCV, Chikungunya, Dengue, HPV, Vaccinia, West Nile, Zika virus, etc. (Molefe et al. 2018). This information forms the basis for designing diagnostic tool for detecting these infections. This way aptamers can prove as better alternate to antibody absed diagnostics for infectious disease diagnosis as the treatment of these diseases depends on the early detection of infection (Wandtke et al. 2015).

12 Conclusion

SELEX method has gained focus for development of aptamer due to their advantage over antibodies as these are inexpensive, simple, have high stability, better affinity for target and low to no immunogenicity. Hence, keeping in mind the problems with available diagnostic tools, aptamers can be used as a diagnostic tools to ensure early and specific diagnosis of diseases. However, establishing successful universal SELEX method to isolate better and desired aptamers is painstaking. The limitations also with the need for further development of aptamers with increased efficiency and ability to detect target in clinical samples. Selected aptamers should be tuned in a way that they can detect the antigen even at low concentrations and with impurities in the samples. With the necessity of more effective and broad-spectrum aptamers for developing biosensors there is need to evolve the SELEX process that will be universal and more target oriented. However, promising result has been seen and the progress is expected in aptamer field, indicating appearance of more aptamers in biomarker discovery, in diagnostics and for more effective drug delivery.

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Changing Trends in Immunosensing Technologies and Their Commercial Aspects in Animal Health and Welfare

Sherin Kaul, Rajni Singh, Sharanya Kamaraju, and Pankaj Suman

1 Introduction

With the advancements prevalent in the field of animal biotechnology, detection and diagnosis of diseases in large animals still remain one of the biggest concerns for of the scientific community. Undetected diseases or inefficient prophylaxis of the diseased condition are the most common cause of decreased productivity of livestock, ultimately leading to massive economic losses. The continual evolution from serological, plate-based classical detection methods to more sensitive DNA-based sophisticated techniques followed by a rapid and efficient paper-based diagnosis of pathogens infecting poultry and livestock besides general animal health present a window of opportunity; thorough exploration of which is the need-of-the-hour. The traditional methods, though sensitive are tedious while being time and labor-intensive. The advent of sophisticated technologies led to a reduced on-field application with the requirement of dedicated instruments and specific handling conditions.

To combat the limitations of pre-existing methods, biosensors have emerged as a potential solution for speedy analysis of the case under observation with a simplistic and real-time approach (Vidic et al. 2017). An assembly, where the interaction of a biomarker with a bioreceptor (coupled with a transducer) can be converted, amplified, recorded, and reported as an electrical signal, on-site, with minimal labor and resource utilization forms the basic structure of a biosensor (Table 1). These are primarily designed on the basis of specific biochemical interactions of the target biomarker with an extensive range of bioreceptor molecules (monoclonal antibodies, aptamers, glycan, lectin, whole cell, etc.) (Fig. 1). For enhancement of the signal received, advanced biosensor models have been tagged with special label molecules such as fluorophores, enzymes, or biofunctionalized nanoparticles, which

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Table 1 Components of a biosensor and their functions

Components	Functions
Analyte	To interact with the specific target molecule
Transducer	To convert the biological response recorded into an electrical signal
Detector	Signals from the transducer arc detected here
Reporter	As a part of the electronic system, used for processing the data recorded and displaying the final result
Amplifier	As an axillary part of the electronic system, wherever required it can be used to enhance the signal for improved sensitivity

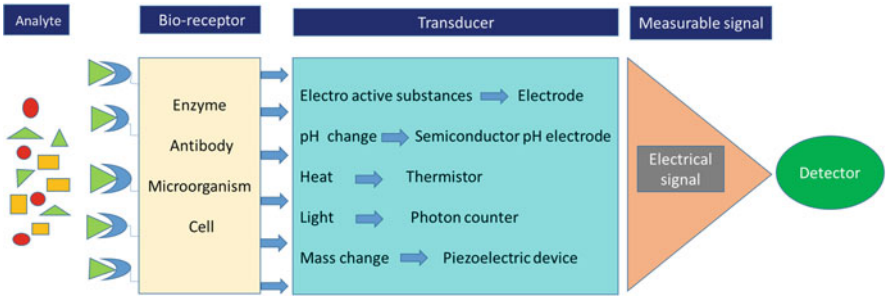

Fig. 1 Schematic diagram of a biosensor and its components

can then be detected by sandwich-type immunoassays for effective and efficient detection.

Currently, the biosensors are widely available for a range of pathogenic organisms (specific strains of *E. coli*, certain viruses, *Mycoplasma*, *Clostridium* sp., etc. to name a few), with the diagnostic method emphasizing on ensuring the capture of prime strain from a given complex (biological/environmental) sample. The crucial factor in designing of a biosensor, nevertheless, lies in the sensitivity of detection of the component of interest besides maintaining the robustness against miscellaneous interferences (Sin et al. 2014). Thus, the shift from existing strategies to biosensor-based kits is marked by advancing micro- and nano-level technologies. They enable rapid detection and analysis while multiplexing with high specificity and sensitivity. Minimal manipulation of sample volume, minimum time, and energy consumption with high portability and system integration potential are the major highlights of this progress (Table 2).

The task at hand remains the development of more such diagnostic sensors or kits, coupled with new techniques which yield portable, cost-effective, sensitive, swift, and user-friendly techniques which can be adaptable to the on-site complications. Rigorous testing and development will ultimately lead to quicker test results with reduced errors. Such advanced techniques enhance the sustainable productivity of each targeted animal leading to the development of a process known as Precision Livestock Farming. The primary goal of this technique is acquisition of data,

Table 2 Scope of immunosensor applicability in animal health management

Role	Utility
Innovation and development of new approaches	Improvement in the productivity and well-being of the animals by devising techniques that measure the dynamic changes in the physiological as well as metabolic pathway
Real time data acquisition and analysis	Help in continual investigation of different aspects of animals flexibility and adaptability to stressors and other extrinsic or intrinsic regulatory factors
Rapid characterization of food and feed	In respect to nutrient content characterisation of dietary inputs to increasing productivity Helps in formulating strategies of altering the composition and dose of feed
Animal trait analysis and selection of robust breeds	Enables selection of stark breeds on the basis of their genotype and response to the environmental factors
Statistical analysis and algorithm development	Help in development of mathematical algorithms based on acquired data for enhanced productivity of animals, besides tranquilizing the potential negative environmental impact

analysis through AI-based algorithms, and generation of results that can be employed for addressing the farmer's concern to improve animal welfare.

2 Advancement and Commercialization of Immunosensors

Traditional methods of pathogen or disease detection, in animals, relied majorly on ELISA- and PCR-based assays which involves a multi-step, labor- and time- intensive protocol. Biosensor-based assays now developed have shifted their focus from such protocols to more efficient, rapid, and advanced techniques such as PCR, chemiluminescence, nanoparticles, surface plasmon resonance, and quartz microbalance system, etc.

In the urge to translate biosensing devices from laboratories to on-site applications, a few such devices have already undergone a commercialization and paved their way to the markets. This market forms a multicomponent structure which caters to various factors involved in the field of animal healthcare and general welfare.

2.1 Organism-Based Detection

A major class of biosensing strategies primarily aims at the detection of specific causative agents of the most widespread diseases hampering the health and productivity of animals. Detection of these organisms can act as a preliminary test for assessing the probable cause and possible prophylaxis of the diseased animal.

2.1.1 *E. coli* Detection

Virulent strains of *E. coli* are a well-known causative agent for several infections in livestock as well as poultry. *E. coli* infections, such as gastroenteritis, meningitis, septicemia, to name a few reduce the growth rate, deplete production of eggs, increase mortality; all of which lead to severe economic losses. The main challenge lies in differentiation between the phylogenetically close strains of pathogenic and non-pathogenic *E. coli* (Hobson et al. 1996; Fournier-Wirth et al. 2006). Novel devices for *E. coli* detection have been reported which functions on recognition by specific biochemical reaction. The device targets at (1) capture of the bacteria from any given sample and (2) identification and characterization of the bacterial subtype. In recent times, *E. coli* specific detection devices are developed based on specific, high-affinity antibodies tagged with labels (such as fluorophores), which are capable of signal amplification. One example of such a biosensor is an anti-LPS antibody detector, coupled with magnetic nanoparticle using a graphite capture electrode for Gram negative species of bacteria (El Ichi et al. 2014). This biosensor allows real-time detection of bacteria using conductometric measurements for a sensitivity range $1–10^3$ CFUs/mL of *E. coli* culture.

2.1.2 Avian Influenza Virus (AIV)

The main reservoir of AIV, i.e., the aquatic birds can potentially lead to threat to the animal and poultry industry worldwide (Olsen et al. 2006). The highly pathogenic strains are known to cause high mortality rate with low pathogenic strains causing mild respiratory or gastrointestinal symptoms leading to death due to multiple infections in poultry (Morse et al. 2012). Avian influenza viruses, based on their antigenicity, are categorized as hemagglutinins and neuraminidases with certain subtypes such as H5N1, causing millions of birds to die globally. Ten major proteins encoded by the viral genome, and their corresponding RNA sequences, are all potent biomarkers (Vasin et al. 2014). Mutations are a frequent event of the viral genome, thereby inducing continual changes in antigenic properties of these biomarkers. Traditional tests for influenza detection predominantly include hemagglutination test, hemagglutination inhibition test, neutralization test, and virus propagation studies which require special sampling techniques and significant amount of viral particles for analysis. There exists a plethora of extrinsic and intrinsic factors which make development of advanced diagnostics for influenza virus cumbersome (Chang et al. 2009; Chiou et al. 2010; Tseng et al. 2014).

Extensive analysis of the influenza hemagglutinin has revealed striking binding differences between sialic acid glycan residues and surface proteins. Utilizing these differences, a differentiating biosensor has been formulated for H1 vs H5 influenza virus with its sensitivity up to attomolar range (Hideshima et al. 2013). Binding of such interactive molecules to gold nanoparticles can allow for faster detection by a colorimetric assay without pre-treatment or amplification (Lee et al. 2013; Fig. 2). Most other influenza detection devices developed lately are microarray- or lateral flow-based assays (Fig. 3; Dinh et al. 2014). Integration of nanoparticles and quantum dots technology with the lateral flow assay has improved the assay sensitivity significantly (Sajid et al. 2015).

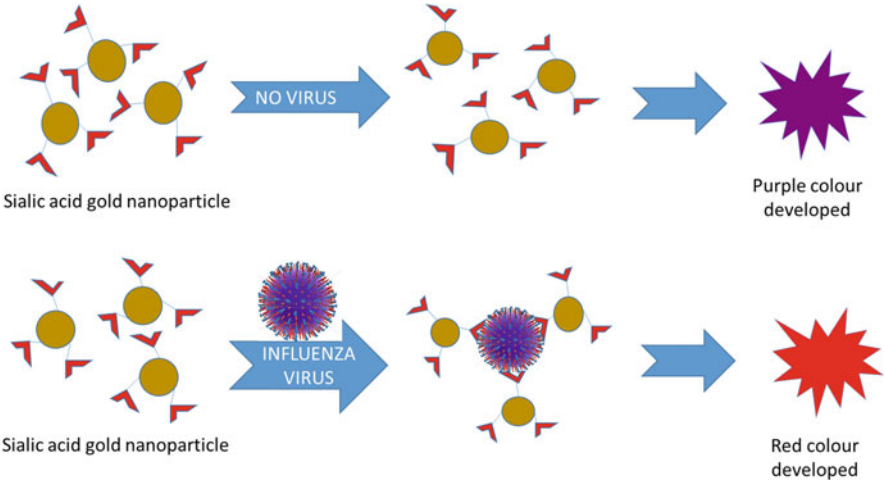

Fig. 2 Sialic based colorimetric sensor for detection of influenza virus: sialic acid particles when conjugated with gold nanoparticles specifically bind to the protein on viral surface. In the absence of the virus, the sialic-GNP conjugate shows absorbance at 510 nm and gives a purple color. When the virus is present, the whole complex gives a red color with absorbance at 600–610 nm

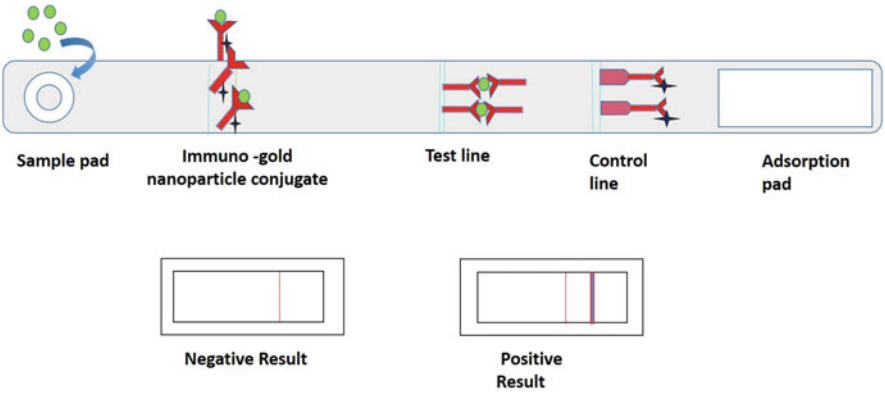

Fig. 3 Lateral flow assay strip for detection of influenza virus: The sample when added to sample pad moves by capillarity of the nitrocellulose membrane. On binding to the conjugate, the sample moves through test line and the control line. In presence of the target molecule (here, virus particle), sample binds to both test and control line. On the contrary, in absence of the target, a single line clearly depicts a negative result

Another sensitive technique developed for detection of these viral particles is the plasmon-assisted fluoro-immunoassay. It can detect viruses by use of gold nanoparticles conjugated with anti-M1 antibodies. Use of antibodies is now being replaced by the use of aptamers which bear the capacity to differentiate between several serotypes (Miodek et al. 2014a, b). Aptamers, generated by Systemic

Evolution of Ligands by EXponential enrichment (SELEX), have varying binding affinities with higher sensitivity as compared to their corresponding antibody alternatives. Subsequently, a pool of such aptamers have been generated for detection of broad range of viral subtypes and some with relatively strict specificity. These aptamers can be used for sialic acid detection as well as infectivity attenuation (Fu et al. 2014a, b). Various other kind of biochip devices have also been reported for detection of viral subtypes using fluorescent or electrochemical read out mechanisms.

2.1.3 Mastitis Causing Pathogens: The Most Pathogenic Causative Agent for Mastitis

Mycoplasma is resistant to most of the antibiotics. Besides being the prime causative agent of critical diseases such as arthritis, pneumonia, and mastitis, they act as a secondary causative agent by exacerbating pre-existing diseases. *Mycoplasma* infection has been known to be tested by conventional culture-based, fluorescent antibody dependent and serological tests. Preliminary detection of infection by these techniques is not feasible due to insufficient titer development for initial weeks of infection. If undetected in early stage, the infection aggravates to a stage where the treatment cost increases drastically (Sargeant et al. 2010; Moon et al. 2007). Initial development in the field of on-site detection kits of *Mycoplasma* started with the use of somatic cell counters. Owing to low sensitivity and specificity of these tests, the urgency of diagnostic facilities for *Mycoplasma*-induced diseases led to development of advanced in-field kits. Majority of these biosensors are designed to detect NAGase (Pemberton et al. 2001) and haptoglobin (acute inflammation phase biomarkers) using carbon electrodes. Using these markers, surface plasmon resonance based biosensor has been developed that detects the change in protein complex formation (Åkerstedt et al. 2006). Presence of P48, a membrane protein of *Mycoplasma bovis*, has also been utilized to develop specific, high-affinity aptamer sequences for an indirect diagnostic test of using competitive aptamer assay (Fu et al. 2014a, b).

2.1.4 *Clostridium perfringens*

C. perfringens, an enteric pathogen, releases several exotoxins affecting almost all domesticated animals. There are five major classes of toxins among a set of more than 15 and a few novel toxins, like NetB (Gibert et al. 1997; Petit et al. 1999; Keyburn et al. 2008). *C. perfringens* infections are identified by characteristic lesions associated with inflammation in early stages, leading to mucosal necrosis in advanced stages of infection (Timbermont et al. 2011). In the absence of antibiotics, *Clostridium* infection incidents increase dramatically which emphasizes on the need for improvement of diagnostic kits and continual surveillance of pathogen occurrence. The main challenge in development of diagnostic kits is that it should be able to differentiate between virulent and non-virulent strains to account for the endemic non-pathogenic strains of bird gut (Banerjee and Bhunia 2010). This led to the emergence of a cell-based detection biosensor (Yoo and Lee 2016) which uses mammalian cell line as the sensing element to monitor perturbations in

physiological activities of cells upon exposure to pathogens. Cell-based biosensors, besides pathogenicity detection, perform risk assessment by pathogen recognition. Biosensing devices for toxin sequence identification are based on oligoprobes immobilized microarray assay (Sergeev et al. 2004). After hybridization of oligoprobes to the target, fluorescently labeled amplicons can also be employed in the detection process (Al-Khaldi et al. 2004; Loy and Bodrossy 2006).

2.1.5 *Campylobacter*

Campylobacter is a thermophile and a microaerophilic pathogen of humans and animals. A few species of *Campylobacter*, such as *C. jejuni*, colonize intestine of poultry birds. Symptoms of *Campylobacter* infection range from asymptomatic infections to enteritis with vomiting, fever, and diarrhea; to abortion, infertility, lesions with edema of the mucosa of cecum and ileum, villous atrophy, and intraluminal accumulation of mucus. Contaminated or undercooked poultry meat is considered as the main source of infection transfer to humans. Established plate-based culture methods require 7 days for detection of its presence and strain identification. Strain differentiation between virulent and non-virulent pathogens using recommended cellular or biochemical procedures is difficult and transport-stress can make viable bacteria non-cultivable on selective medium (Wangroongsarb et al. 2011). PCR-based techniques are available which can rapidly detect viable organisms with improved sensitivity and reduced time frame, provided transport is carried out at the earliest, with minimal loss of sample.

For a limited number of samples, a melting point curve analysis (Barletta et al. 2013) based on PCR product of 16S rRNA amplification and fluorescence based RT-PCR methods are readily available for screening *C. jejuni.* In an attempt to increase specificity, robustness, and selectivity of the protocol, biosensor models are being developed using whole cells, nucleic acids, proteins, antibodies, and aptamers as the receptor. Among the proposed models, DNA-based biosensors are being considered as the most promising tool for cost-effective and rapid real-time detection of hybridized target nucleic acids. Most advanced propositions for development of these point-of-care devices include use of surface plasmon resonance (Manzano et al. 2016), electrochemical (Kubiăárová et al. 2000), acoustic (Jia et al. 2012), optical (Cecchini et al. 2012), and microwire based (Lu and Jun 2012) technologies.

Specific anti-*jejuni* antibodies are being explored as potential detectors in combination with magnetic particles (sensitivity up to 100 CFU/mL) or surface plasmon resonance with sensitivity up to 10^3 CFUs/mL (Wei et al. 2007). The major advancements in on-site detection of *Campylobacter* came with advent of lateral flow assays (Wadl et al. 2016) which although not extremely sensitive are highly portable and adaptable for multiplexing. Sensitivity of detection up to 5 nM has been attained by an optical biosensor developed by Gnanaprakasa et al. which uses hippuricase gene (hipO) as the target molecule.

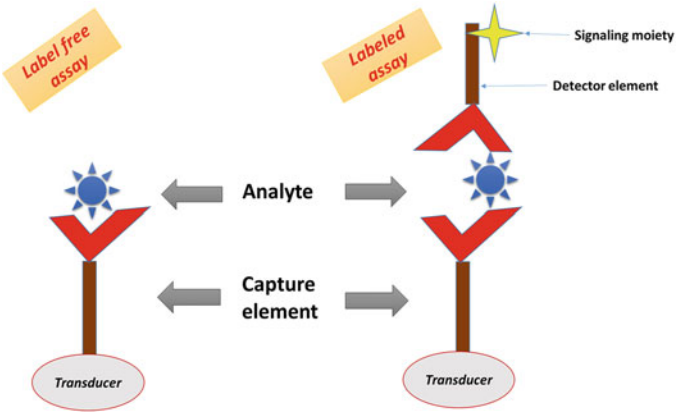

Fig. 4 Depiction of difference in detection via label-free and labeled assays: In a label-free assay, the analyte molecule can be directly detected, whereas the labeled assay utilizes an additional signaling moiety bound to the detector element to report the final result

2.2 Disease Detection

Some diseases are more common than others and affect alarming number of animals across the globe. Biosensor-based detection kits with label-free or labeled-assays (Fig. 4) have been developed for such diseases affecting the livestock health.

2.2.1 Foot and Mouth Disease (FMD)

FMD virus is the highly contagious pathogen affecting cloven-hoofed animals at a drastic pace. Although the mortality rate is relatively low, yet the morbidity and infection rate is tremendously high to cause a global economic impact of billion dollars every year (Knight-Jones and Rushton 2013). Common clinical symptoms include blister formation, tissue damage, and various other supplementary symptoms such as inhibited movement, excessive salivation, anorexia, and subsequent depression. The infection has been eradicated from most developed countries of the world but global outbreak due to a long-distance recurrence can cause massive economic losses. Despite being susceptible, most developing countries lack required resources for infection testing. Therefore, development and implementation of preventive, control, and coordination strategies are the critical task at hand. Development of laboratory based diagnostics has evolved from classical ELISA and RT-PCR tests to duplex one-step RT-PCR assays (Jamal and Belsham 2013) validated for simultaneous detection of the viral RNA and the host mRNA (Gorna et al. 2016). For on-field virus detection and subtype identification, a lateral flow assay (Fowler et al. 2014) and a chromatographic strip-based assay (Reid et al. 2001) have been developed. As a result of the success of lateral flow assay, the technique was further advanced by coupling it with reverse transcription loop-mediated isothermal amplification (RT-LAMP; Waters et al. 2014). This amplification then produced results on the lateral flow strip in the form of a test line visible by the naked eye. These methods

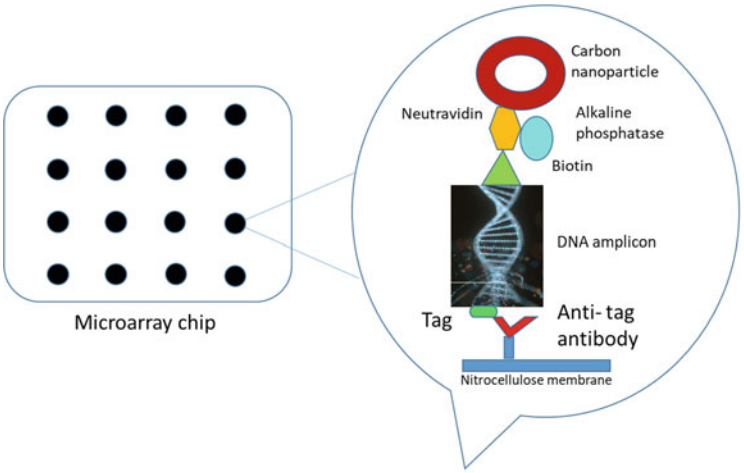

Fig. 5 Microarray based detection for bacteria causing mastitis: the figure represents the components embedded on every well of the microarray chip

emphasize on the possibility of development of more such optimized high-throughput assays which harbor in them a lot of potential for on-site virus screening.

2.2.2 Subclinical Mastitis

Mastitis is a multi-factorial disease manifested by the inflammation of milk producing udder parenchymal cells, leading to 10–20% decline in milk production. Clinical mastitis is characterized by evident changes in udder and milk, whereas subclinical mastitis stage seldom go undetected, making development of specific diagnostics critical. Subclinical mastitis can be caused by a range of bacterial strains including *Mycoplasma bovis*, *Staphylococcus aureus*, *Escherichia coli*, among many others. Thus, detection of such pathogens is the gold standard in mastitis detection. In prevalent scenario, tests developed for detection of subclinical mastitis include the modified white side test (MWT), the chloride estimation test, the bromothymol blue card test, California Mastitis Test (CMT), Udder Check Test®, determination of electrical conductivity, the modified Aulendorfer mastitis probe test (MAMP), infrared thermography, and monitoring of somatic cell count by PortaSCC® Test. Biosensor-based diagnostic kits developed for *M. bovis* are frequently used in preliminary indication of this diseased condition. To develop effective antimicrobial techniques and to control its epidemic tendencies, there is a need for rapid quantitation and characterization of the bacteria strains present in the samples. Despite occurrence of proteinases and multiple other related PCR inhibitors, many PCR- and RT-PCR-based kits are being extensively used with incorporated extraction process (Pisoni et al. 2010; Mujawar et al. 2013). A microarray-based biosensor for colorimetric detection and simultaneous identification of pathogens has been developed. The amplification is carried out with tags bound to specific PCR fragments which when captured between antibodies impregnated onto a nitrocellulose

membrane with carbon nanoparticles can be observed even by naked eye (Fig. 5). A few other nucleic acid, enzymatic, microfluidics, and biochip based techniques are being extensively explored for improved detection limit, "on-site" detection, and confirmation of the infection (Viguier et al. 2009).

2.2.3 Subclinical Ketosis

Subclinical ketosis is an underrated metabolic disorder prevalent in cattle characterized by increase of circulating ketone body concentration in the body fluids without appearance of any clinical symptoms. In its initial stages, it is characterized by loss of appetite, lethargy, and change in movement pattern. The negative energy balance of the animal during this phase, if unattended, causes substantial decrease in milk yield, reproductive performance, and risk of severe clinical ketosis resulting in adverse losses for animal health and economy (Ospina et al. 2010). The advancements in the field of nano-biosensors technology are being exploited to develop devices for real-time detection of beta-hydroxybutyrate in blood, milk, or urine; produced as a result of continual fatty acid breakdown in the body to meet the energy requirements. The biosensor could detect the beta-hydroxybutyrate to the extent of 0.05 mM (Weng et al. 2015a, b). Another study has reported a device for detection of β-HBA with quantum dots using nicotinamide adenine dinucleotide (NAD^+) as a cofactor on a microfluid platform with a detection limit at 35 μM (Weng et al. 2015a, b). Besides, an electrochemical biosensor has been developed by Veerapandian et al., for selective detection of β-HBA by immobilizing 3-beta-hydroxybutyrate dehydrogenase enzyme. Thus, development of handheld microfluidics based biosensor for on-site detection of subclinical ketosis ascertains massive potential for cost-effective, timely diagnosis (Veerapandian et al. 2016).

2.2.4 Bluetongue

Bluetongue is mainly a noncontagious infectious disease of ruminants and domestic species caused by the bluetongue virus of *Reoviridae* family. The infection is transmitted by the bite of a female *Ceratopogonidae* insect, and can even impact embryos via placenta, colostrum, or seminal fluid (Sperlova and Zendulkova 2011). Although the morbidity and mortality rate associated with bluetongue viral infection is rare, but it is important to note that the infection can go unnoticed for prolonged duration due to its asymptomatic manifestation and have grave economic impact on ovine and bovine rearing industries (Tabachnick 2002). These economic losses are not only due to death or decrease in production of animal products, but also due to indirect factors such as a widespread transfer of infection affecting large herds causing restriction in animal, serum, or semen export.

Epizootic Hemorrhagic Disease Virus Epizootic hemorrhagic virus is transmitted by the same vector (as for bluetongue virus) and exhibits similar symptoms but functions in contrast, leading to high risk of mortality and morbidity in cattle and sheep. Much alike the bluetongue virus, promising advances in epizootic diagnosis have been made through detection of hybridized viral DNA by fluorescent microsphere assays, which can further be extrapolated to multiplexing systems (Weis et al.

2015). Recently, a lateral flow assay for detection of epizootic hemorrhagic virus has also been established (Hanon et al. 2016).

2.2.5 Respiratory Disorders

Bovine respiratory syncytial virus (BRSV) is one of the most widespread and potent causative agent of respiratory disorders in bovine species. Ease of transmission of the infection from infected to healthy animals and associated secondary bacterial infections reinstate the crucial role of the virus in the subsequent welfare issues for the animals and the economic losses inflicted. Most commonly used method for curbing the infection and its symptoms is the use of antibiotics which raises a global concern of increase in the antibiotic resistant strains of pathogens. BRSV is a pneumovirus with a negative-sense RNA envelope, which has a 2–5 days incubation period before it manifests any clinical symptoms in the infected animal. Although antibody response can develop before expression of prominent clinical symptoms, when manifested these symptoms are localized to the upper respiratory tract and might last till 7–10 days post-infection. Due to difficulties in detection of negative-sense RNA or mRNA of BRSV, ELISA based diagnostic tests using F_0 and F_1 protein subunits were preferred over PCR of the mRNA sequences. For improvement of detection efficiency, electrochemical biosensors have been developed which use the BRSV-specific aptamers and antigens as receptor molecules. A molecular beacon model (Cai et al. 2013) was also created where streptavidin-HRP bound aptamer sequence was blocked at the hairpin loop structure of the beacon. Upon hybridization, the aptamer sequence gets exposed and becomes available for binding to the target nucleic acid sequence. This binding can then be quantified by tetramethylbenzidine (TMB), an HRP substrate (Rochelet et al. 2012). This method is highly specific and selective even in small volume, and with complex matrix sample. The most efficient method for BRSV detection utilizes the nanoparticle amplification immuno-PCR assay (Perez et al. 2011). The viral particle, for detection, is sandwiched between two antibodies where the first antibody is attached to a magnetic bead and the second is bound to gold nanoparticles. The first antibody enables the extraction of virus, and the second co-immobilizes the virus with the nanoparticle which is finally released in denaturation step of RT-PCR. Nanoparticle, besides improving the sampling sequence of the virus, increases PCR efficiency by 1000-fold by increasing *taq* DNA concentration and lowering the background signal thus bringing the detection limit to 4.1 PFU/mL (Perez et al. 2011). These advanced biotechnological techniques can significantly support the surveillance and diagnostic controls for enhancement of animal welfare.

2.3 General Health and Welfare

Advancements in the field of material, research, engineering, and decreasing costs of technology have led to a massive growth in the field of innovative diagnostic methods, with potential for a lot more. There is a need to develop non-invasive health monitoring tools for better diagnosis and treatment. Therefore, certain

biosensors have been designed and marketed that focus on analyzing the physiological parameters of the animals and form an informed analysis report of the animal health and its environment.

2.3.1 Metabolite Analysis

Metabolism monitoring of animals is an onerous task. Nonetheless, continual monitoring of metabolic activities and subsequently produced metabolites is crucial. Employing nanosensors for tracking the level of metabolites produced in all body fluids, including tears, is bringing a shift in trend of animal health monitoring and analysis (La Belle et al. 2014). Biosensors for detection of metabolites including glucose, ATP, lactate, ammonia, methane, acetone, etc. are being designed incorporating the electrochemical components with enabled radio communication. Disorders in animal metabolism can be indicated by concentration of specific biomarkers, such as non-esterified fatty acids. Under negative energy balance conditions of the animal, adipose fat is circulated in the form of these fatty acids and transported to liver for re-esterification. These circulating fatty acids are targeted for detection using redox active hybrid graphene oxide (GO) or modified ruthenium bipyridyl graphene oxide nanosheets by electrochemical analysis (Veerapandian et al. 2016). Other materials employed in novel fabrication of nano-biosensors include carbon electrodes, iron bound lipoxygenases, etc. Association of glucose metabolism in blood with several volatile organic compounds including ethanol, methanol, ketone bodies, and other exogenous compounds is the most useful source for immediate diagnosis of glucose level in the animal via a non-invasive approach. Analysis of volatile compounds can reflect a comprehensive composition of the organism's bloodstream and airways and subsequently indicate animal's health. Solid-phase and needle-trap micro-extraction procedures when combined with sophisticated analyzers such as mass spectrometry, laser spectroscopic techniques, quartz enhanced photoacoustic spectroscopy, etc., can provide extensive analysis of exhaled breath components to enable early diagnosis. Similarly, non-invasive detection of uric acid in saliva can be carried out using uricase enzyme as a biomarker in an electrochemical biosensor. This electrochemical sensor uses a microcontroller potentiostat integrated with a screen-printed electrode system. The real-time biosensor with a wearable monitor serves as a readily available method for general clinical analyses and therapy. Certain portable biosensors that are robust and resilient against extrinsic stressors have been adopted for metabolite monitoring from perspiration. A hand held form, belt form, RFID sensor patch (Rose et al. 2015), flexible tattoo, lateral flow assay strips are some of the advanced nanobiosensor prototypes being explored in sweat monitoring for health condition determination in animals. Biosensor applications via sweat analysis are being explored for detection of metabolites including lactate, sodium, glucose, potassium, certain metals, and body temperature. Though viable, these biosensors need further optimization for its feasibility and applicability for on-site detection.

2.3.2 Reproductive Health

Breeding is a crucial component of cattle farming. Heat detection is important for determination of a time frame most suitable for successful artificial insemination. Majority of the biosensors developed in the field of reproductive health aim at detection of ovulation phase and monitoring progesterone levels. Various scientific groups have successfully developed prototype models for the same. Zeidan et al. (2016) have developed an aptasensor for progesterone detection. The nanoenhancers are NIR-streptavidin-coated quantum dots, being used for ultrasensitive detection of progesterone molecules with the limit of detection up to 5 nM in phosphate buffer. Another such biosensor developed for progesterone is based on immobilization of anti-progesterone monoclonal antibodies onto a screen-printed carbon electrode (SPCE). The Herd Navigator® measures progesterone level in milk, predicts ideal insemination time, shortlists animals for pregnancy confirmation testing, indicates prolonged anoestrus, and predicts early abortion and probable cysts (Durkin and DeLaval 2010). The expensive nature of the device leads to a lack of on-field applicability necessitating improvement of steady detection conditions from low-cost sensors (Jónsson et al. 2011). A wireless intra-vaginal probe has been tested for the automation of oestrus detection. The probe senses movement, measures temperature and conductivity of the animal to provide reliable data. The developed system is power efficient and is capable of independent operation in small to intermediate farm systems, while large farms can be facilitated with battery operated relay repeaters (Andersson et al. 2015, 2016)

2.3.3 Stress Detection

Animal health is impacted by a wide range of intrinsic and extrinsic factors. All these factors including animal physiological condition, farm conditions, and change in general trend of environmental factors, have the potential to induce stress conditions in the animal. Currently, sound analysis and lactate level monitoring are the basic processes exploited for establishing biosensing devices to monitor stress levels in the animal. Sound is considered to be a reliable indicator of stress and is used in its analysis by a prototype using three binary-classifier support vector systems. They detect the emitted sound and then classify it for real-time evaluation from a surveillance system (Lee et al. 2015). Lactate detection based biosensors have been developed for several animal models, besides livestock, as they are an integral indicator of tissue homeostasis. Under stress conditions, animal metabolism produces excess pyruvate during glycolysis, which is stored in tissues as lactate and transported into blood. This increase in blood tissue level of lactate is the bioindicator of interest, and forms the basis of developing amperometric biosensors. Modified, non-invasive biosensors such as temporary tattoo-based sensors and RFID tag sensors are also available to monitor the levels of other important electrolytes and metabolites in the body (Neethirajan 2017). In the process of perfecting these detection devices, replacement of batteries with self-sustaining power source, optimization of packaging material, and incorporation of wireless transmission features is being worked upon. The potential of enzymatic biosensor for use in lactate monitoring is being explored which can be coupled with energy harvester and

micropotentiostat for its rapid detection by chronoamperometry. Such a device is based upon NAD^+ dependent lactate dehydrogenase enzymatic reaction and its suitability under laboratory conditions has already been verified (Garcia et al. 2016). Lactate variations can also be monitored by measuring its presence in saliva. Developing wearable "lab-on-a-chip" device consisting of carbon nanotubes or graphene is being explored for its potential in use of health-care technology.

2.3.4 Behavior and Movement

The most reliable method for early detection of any deviation from healthy condition of the farm animal is the monitoring of its behavior and movement. Any changes from the regular pattern recorded for every animal can relay important information about the activity level and general well-being of the animal. Motion sensor, audio and video recording, physiological testing, and global positioning system (GPS) are the technologies being extensively exploited in development of biosensors. These devices can be used to record body weight and size, feeding and rumination, oestrus and activity levels. One such scenario where movement monitoring is crucial is overseeing grazing efficiency to ensure proper feed conditions for optimal growth of animal (Neethirajan 2017). This grazing behavior of the cattle can be observed by analysis of location, posture, and movement of the jaw (Hertem et al. 2014). Multiple versions of biosensors have been developed for jaw movement analysis, which include pressure (mechanical) sensors, microphone (acoustic) sensors, acceleration, and electromyography sensors. Another such device has been developed to detect estrous and health parameters by the use of wireless sensors which measures the associated physiological conditions of the individual animal. A wide range of modified devices such as collars, microsensors, thermal sensors, motion-history image based technology, etc. have been developed which record normal behavioral patterns of the animal and then employ classification algorithms to analyze variation in behavioral pattern (Spink et al. 2013; Eastwood et al. 2016). This data when organized and analyzed can be indicative of general reproductive health trend of animals.

2.3.5 Farm Monitoring

Conventional methods of farm monitoring involve a lot of manual labor in data recording, updation, and maintenance, paving way to higher chances of human error. Lack of data-sharing capabilities by manual recording of data, cost-inefficiency of global positioning system, poor signal-to-noise ratio for voice entry systems are the basic drawbacks in recording mechanisms in on-site conditions. The most widely proposed system for monitoring activities is the use of Radio-Frequency Identification tags, i.e., RFID tags (Glennon et al. 2016). They can be helpful in tracking animal location, monitoring its behavior, or used in fattening or milking management (Fukatsu and Nanseki 2009). There are a few legal concerns associated with some advanced features added as tools for training or data acquisition. With a fixed and coordinated pressure sensor, these biosensors can integrate data acquisition and measurement systems to be used for health monitoring. Precision livestock farming has revolutionized strategies for animal monitoring and welfare. Treading upon the

principles of hazard analysis critical control point and quality management, these techniques enable judicial utilization of resources to maximize productivity (Banhazi et al. 2012). With precision farming and breeding, farmers can be benefitted manifold by improved status of marketing opportunities and economic development. These processes help farmers in making informed decisions about optimization of feed, early disease detection and treatment, supply chain management, and real-time monitoring of animals, manage health and fertility data; while the data analysis is carried out based on suitable algorithms (Mudziwepasi and Scott 2015; Wathes et al. 2008). One such device which is available for feed control and management in broiler chickens is known as the Flockman™ (Corkery et al. 2013). It monitors the feed consumption and allows the farmers to decide the kind of feeding practices to be followed at different time of growth. Besides the feed, Flockman can also analyze the impact of different environmental factors, such as heat and humidity on health and growth of chickens. Devices similar to those as aforementioned are being developed and improved for detection of various hormonal levels in the animal. The potential of integration of wireless sensors needs to be investigated which would enable better utilization of online health monitoring systems by networking and data-sharing capabilities.

3 Challenges

Despite the multidisciplinary applicability of these biosensors, their commercialization is still in its nascent stage. The most probable reasons for this challenge include the precision of detection, non-specificity, stability, and reproducibility of the fabricated sensing system.

1. Slow acceptability of these technologies in commercial farms is affected by its economic feasibility and frequent usage. Initiatives need to be taken to emphasize on the importance of biosensors as an emerging tool for efficient clinical management in remote, resource-deficit, and decentralized regions.
2. Another major limitation in the biosensor development arises because of the gap between the conception of an innovative thought and development of a promising concept at a laboratory scale followed by its real-time validation with clinical cases. The major limitation leading to such a discrepancy is thought to be the lack of raw samples in microfluidics and their lack thereof in experimentation on final device marketed. Besides, for successful implementation and utility of every biosensor, there lies an integral requirement of the adaptation of these devices to the real-time conditions in the field with minimal risk.
3. Additional challenge in clinical application of biosensors is to compensate for the time and resource requirement for sample preparation. In dense biological samples, such as body fluids, inadequate processing of sample often leads to practical challenges in on-field processing, such as the matrix effect. Developing chip-based sample preparation strategies for enriching the signal strength of the

target molecule of interest, neutralizing any accompanying inhibitors and substantial reduction in volume of sample used is crucial.
4. Post-optimization of all data collection strategies, the limitation that arises is the inefficient conversion of abundant data into useful information to take a decision in livestock management. Nevertheless, harmonization of methods across various platforms for the data collection needs to be carried out at the earliest for exploring complete potential of the developed technology.
5. Potential applicability of immuno-or apta-sensors depends on nature of the biomolecules and their ease and stability upon immobilization on to the sensor surface. There is an urgent requirement to undergo inter-laboratory testing for a wide scale validation and adaptability of the reliability of the data being generated by such biosensing devices.
6. The need to develop more robust and regenerative biosensors is still an area of concern. The performance of certain devices is questionable for minimal level of available analyte, thus posing serious limitations for developing affordable technologies for point-of-care diagnostics.

4 Conclusion

Immunosensing technologies and their constant evolution are emerging as the most popular domain in improving animal health. The rapid on-site diagnostic kits being developed provide the most suitable substitute for the traditional labor- and time-intensive protocols, utilizing specialized sophisticated techniques and instruments. Furtherance in fields such as electrochemistry, nanotechnology, and microfluidics, integrated with biological techniques, have led to an emergence in the field of point-of-care diagnostics. The advent of these on-site technologies can provide timely diagnosis and prophylaxis solutions in a cost-effective manner, thus revolutionizing livestock healthcare and management strategies as we know it. This multi-domain association has led to the advent of technologies such as smartphone-based microscopy, wearable sensors, 3D printing, and subsequent improvement in various label and label-free detection methods. The popularity of digital media is being incorporated in continual surveillance of animal health besides data collection and analysis for strategic management of the livestock farm. The steady commercialization of these biosensors is providing innovative approaches to address the global issue of animal health. Although it is currently viewed as a path towards an optimistic future, large scale adaptation and implementation of these methods still pose as the biggest challenge. Precision livestock farming techniques including bioacoustic monitoring, stress detection, farm monitoring, and uninterrupted observation of physiological characters of animal can help to devise a model system of animal breeding and maintenance.

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Methods for Enhancing Aptamer Affinity for Antigen Detection and Its Characterization

Komal Birader, L. Sai Keerthana, T. Yathirajarao, Jeannie Alice Barla, and Pankaj Suman

1 Evolution of Aptamers

Aptamers—the term derived by combining "Aptus" meaning to "fitting" in Latin and "meros" corresponding to "part" in Greek. So from where does the term aptamer got its significance? Or what actually corresponds to an aptamer? Are they naturally occurring or artificially produced? It was in the year 1990, when three different groups individually recited the advent of small nucleic acid molecules that have unique properties. Gold and Tuerk have described in their work about how specifically the stem loop structure formed by the naturally occurring RNA has high affinity towards bacteriophage T4 DNA polymerase (Tuerk 1990). Another group headed by Ellington used the word "aptamer" and described about the in vitro selection of aptamers for the RNA molecules isolated from a subpopulation of RNAs containing 10^{10} random sequences that undergoes folding forming a specific binding sites, that were specific for the organic dye used in the affinity chromatography (Szostak 1990). Joyce and Robertson in the same year reported the in vitro selection and amplification of catalytic RNA and affinity of the three-dimensional structure of the RNA enzyme (Joyce 1990).

Naturally occuring aptamer sequences like, the ribozymes or the riboswitches which allow the regulation of the gene expression by changing or attaining secondary or tertiary structure and binding to small metabolites (Tucker and Breaker 2005). Hence riboswitches are defined as the Nature's aptamers (Bunka and Stockley 2006). Many structural studies have been carried out, one of which confers the mechanism of ligand binding to the small nucleic acid molecules where the natural aptamers were synthesized by selecting them by in vitro selection method, by increasing the selection pressure or introducing mutations to investigate the changes

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in the action of the RNA stem loop structure upon these modifications (Hiraoa et al. 1999). The structural studies done by X-ray crystallography shows that modified or mutated aptamers bind at the same site of the ligand as the naturally occuring aptamer (Bunka and Stockley 2006; Cai et al. 2018). If we consider in vivo DNA sequences, then the operator region of a gene can be considered as an aptamer but the drawback was the binding of factors to these regions are reversible, hence a strong and long term affinity of the complex is not feasible in vivo conditions. Henceforth, for DNA aptamer selection in vitro selection is more preferable and reliable for achieving the isolation of high affinity sequences (Yi-yuan He 1996).

It can be assumed that due to difficulties in selection of the aptamers by in vivo method and selection of the naturally occurring nucleic acid sequences either RNA (ribozymes/riboswitches) or DNA was cumbersome taking several months for getting specific aptamers; it was then the Ellington group that first described and used the term SELEX—systematic evolution of ligands by exponential enrichment for the in vitro selection of aptamers (Tuerk 1990).

2 Selection of Aptamers

Since the prevalence of aptamers till date there have been a number of methods and techniques for the selection of aptamers—bead based SELEX (based on agarose beads for selection), Cell SELEX (selection of aptamers for the whole cell), HT SELEX (a high throughput method involving usage of protein bound beads) (Ogawa and Biggin 2012), CE SELEX (uses the capillary electrophoresis for selection), Microfluidic SELEX (a combination of traditional SELEX with microfluidics system), Graphene oxide based SELEX, Capture SELEX, etc., for various target molecules that can be macromolecules like proteins (Tuerk 1990; Tasset et al. 1997; Tahiri-Alaoui et al. 2002; Jayasena 1996), peptides (Susanne Leva et al. 2002; Ylera et al. 2002), amino acids (Geiger et al. 1996), oligosaccharides, few monosaccharaides (Yang et al. 1998), Nucleotides (Koizumi and Breaker 2000; Huizenga and Szostak 1995), Organic molecules (Jenison et al. 1994; Grate and Wilson 2001), hormones (Susanne Leva et al. 2002), single cells (Hu et al. 2017), metal ions, etc. (Ciesiolka et al. 1995; Hofmann et al. 1997; Patel 2000). But, the basic principle behind every method remains the same where the target molecule is incubated with the pool of nucleic acid sequences, removal of unbound sequences, amplification of the specific ones, and enrichment of the sequences by repeating the number of cycles (Fig. 1).

A number of aptamers have been selected after in vitro SELEX procedure and from this eclectic pool of aptamers, the one with highest affinity and specificity is preferred. The aptamers obtained after selection must be subjected for post-SELEX process wherein the fragments exposed to a lot of modifications to attain thermal stability, sustain nuclease activity, bioavailability, or sustained affinity (Gao et al. 2016a, b). The main property that is to be improved by post-SELEX process is to increase their affinity. There are numerous parameters that effect the aptamer affinity

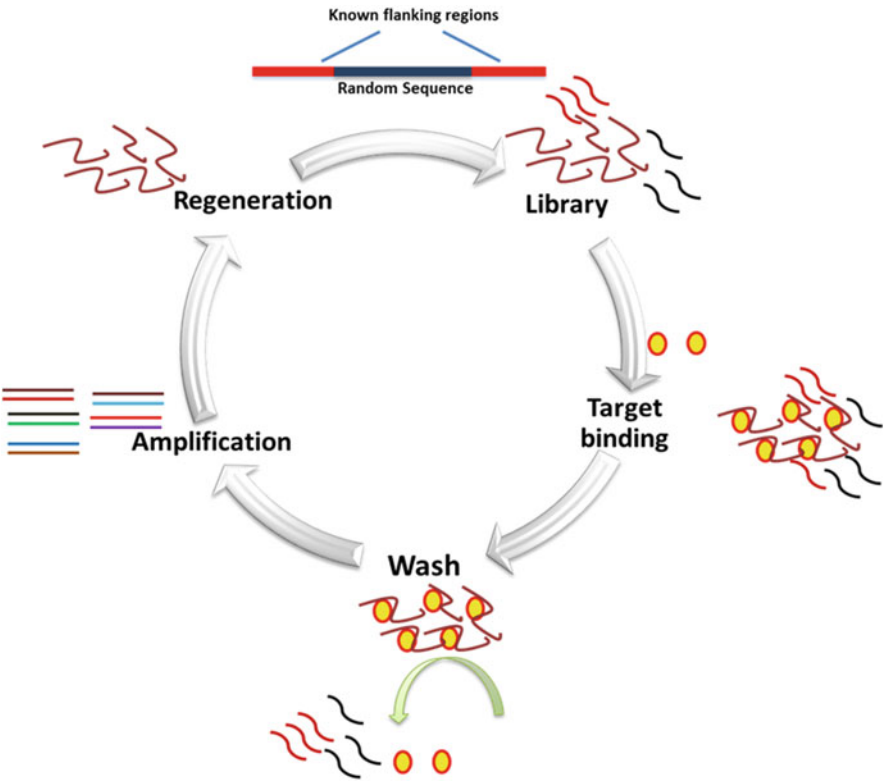

Fig. 1 Schematic representation of SELEX in a generalized manner

during selection as buffers, pH, temperature, and the length of the random region of the library (Hasegawa et al. 2016).

It is also noteworthy, to question what are the exact reasons for which there is reduction in the affinity of the aptamers. One could be the loss of high affinity aptamers during amplification. The secondary structure or the stem loop formed by the aptamers is the main part of the sequence that causes the binding to the target molecule. The stem loop structures formed are due to many interactions including Van der Waal's interactions, Hydrogen bonding, hydrophobic interactions, inter- and intramolecular interactions. The stable structure formed by some of the fragments becomes resistant for PCR amplification and is left unamplified causing loss of potential high affinity ligands. Although, this kind of limitation is overcome by non-biased PCR amplification method using emulsion PCR (Schutze et al. 2011; Shao et al. 2011). Another reason which is mentioned and discussed in lot of reviews is the limited molecular diversity, when compared with the protein sequence where it contains a combination of 20 different amino acids whereas in the traditional library the sequences ($\sim 10^{15}$) include a combination of only four naturally occurring nucleotides. In this context, the work done by Gold and his team on introduction

of unnatural nucleotides for increasing the affinity of the aptamers is noteworthy, where they worked on the selection procedure that includes the addition of amino acid like side chains for formation of molecules termed SOMAmers (slow off Rate modified aptamers) (Rohloff et al. 2014). In this review, we have discussed some of the methods to deal with these limitations to enhance affinity of potential aptamers. Here we have emphasized the post-SELEX process like truncation and modification of aptamers for enhancing its stability and affinity, and also asserting on the methods for determining the affinity of the aptamers.

3 Post-SELEX Process

The selection of aptamers for small molecules has always remained challenging when compared to large molecules like proteins and polysaccharides for example, few oligosaccharides when binds to its cognate aptamer, the length of the aptamer does not make any difference with the binding affinity which owes to the size of the target molecule (Gao et al. 2016a, b).On the contrary when the target is a small molecule like peptides, hormones, metal ions, nucleotides, amino acids, the length of the aptamer does play a significant role (Gao et al. 2016a, b). As it is a known fact that the stem loop structure formed by the nucleic acid sequences is the main reason for the binding to the target molecule, so it is considered that the shortening of the sequences may increase the affinity (Alhadrami et al. 2017). Another approach that is adopted for increasing the affinity that plausibly does not involve post-SELEX processing is the choice of appropriate library to be used for specific target. In this regard, many researchers have worked on trying different parameters for library construction, the library design which contains equal amounts of adenines, cytosines, thymines, guanines, or uracil leads to the library that selects that aptamers with good stability and affinity. The library designed by Choi et al., focused on more G or G+C content such that the chances of formation of G-quadruplex is high and provides stability to the structure of the aptamer (Choi et al. 2010) (Fig. 2).

4 Truncation of Aptamers

The random nucleic acid library that is used for selection of aptamers is usually as large as 80–100 nucleotide and as small as 20–30 nucleotides. By chemical synthesis, as long as 200 mer library has also been constructed which encompasses more than one target binding region (Aptatope), for targets that have many recognition sites like cell surfaces (Bruno 2015). Now while considering the length of the aptamer, one should also note that not all the nucleotides in the sequence are significant for the binding to the target molecule and the non-binders may also effect the affinity when small molecule targets are selected (Alhadrami et al. 2017). Truncation of aptamers is usually carried out after knowing the region in the sequences that has high probability to bind to the target. Through NMR and X-ray crystallographic studies, the number of binding sites and specific nucleotides that

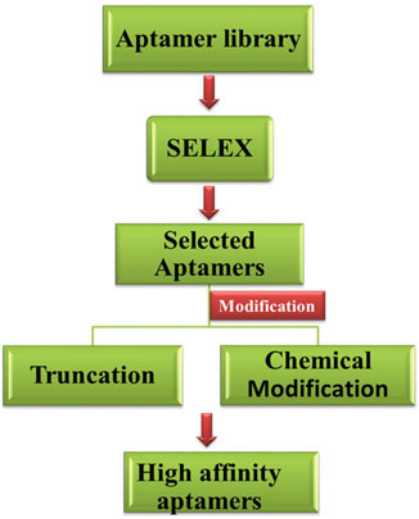

Fig. 2 Schematic pathway for generation of high affinity aptamers

bind to the target molecule can be predicted. By recognizing the binding sites of the target molecules in the aptamer sequence through the prediction of secondary structure aids in increasing the binding affinity. Tools like mfold for predicting the secondary or 3D structure are commonly used for this purpose (Zuker 2003). However, many other tools that can predict the secondary structure like Volfold, Sfold, AptaTrace are also available (Ding et al. 2004; Chan et al. 2005), AptaTrace (Dao et al. 2016).

Truncation of aptamers can be done by following the methods, which support the rational truncation by considering the secondary structures. Similar strategy was adopted by Alhadrami and Chinnappan et al., where they constructed a truncated aptamer for detection of Progesterone (P4) by splitting the aptamer in two parts, and determined the better affinity aptamer by performing fluorescent based assay using probes with a fluorescein and quencher. The aptamer fragment retaining the P4 binding domain showed better binding kinetics with high affinity than the original aptamer (Alhadrami et al. 2017).

Another small molecule target that has been studied in detail is cocaine, for the purpose to develop a sensor for its detection that has led to in-depth study of aptamers specific for cocaine. Stojanovic et al. have selected an aptamer for cocaine that folds to form a secondary structure formed on the basis of random and site directed mutagenesis and they could generate an aptamer with dissociation constant ~0.4–10 μM which forms a three way junction structure (Stojanovic et al. 2000a, b). Later, this work was continued for producing a split aptamer with relatively higher dissociation constant. But limitation to this was that it remained folded even in the absence of target molecule that gave a lot of noise when used in fluorimetric biosensor.

To overcome this, the wild type aptamer was split into 2–3 fragments which reduced the background noise but with slightly lower affinity (Stojanovic et al.

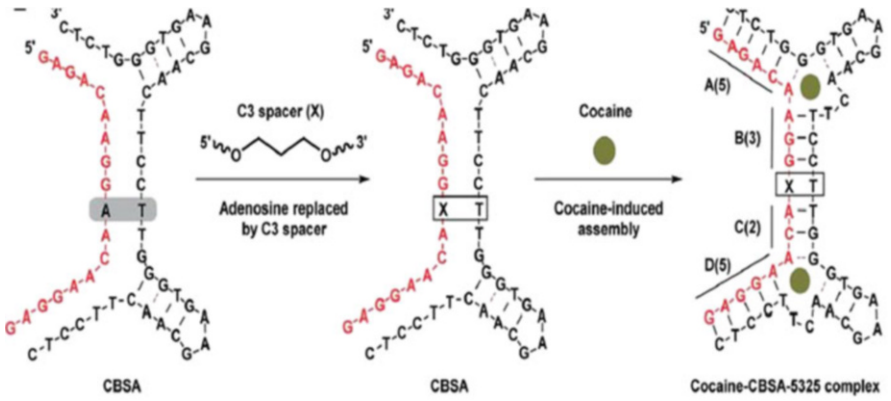

Fig. 3 Schematic representation showing pattern of aptamer splitting and substitution of certain nucleotides for increasing affinity and also the novelty in this type of splitting is the co-operative binding which enhances the affinity (Yu et al. 2017)

2000a, b; Landry 2002). With these truncated aptamers enzyme linked colorimetric assay was carried out which resulted in less background noise without enhancement of its affinity (Nie et al. 2013). To increase the affinity and stability of the 38-GC aptamer for cocaine which has 2.5-fold higher affinity than MNS 4.1 aptamer (Yu et al. 2017), further modifications were done on the basis of naturally occurring riboswitches which undergo co-operative binding to control gene expression depending on the concentration of the ligand (Maumita Mandal et al. 2006; Narasimhan Sudarsan et al. 2006). Yi Xiao et al. have done a novel work by introducing two tandem target binding sites in the split aptamer 38-GC which forms a three way junction secondary structure with three stem loops. Stem-3 having the binding site for cocaine and stem 1 and 2 aids in the stability of the three way junction structure upon target binding (Fig. 3).

It has been noted that many aptamers that are specific for small molecules undergo structure switching upon interaction with their cognate target molecules (Stojanovic et al. 2000a, b). Truncation of aptamers by enzymatic digestion using exonuclease III (Exo III) is another approach that has been used for cocaine aptamer. Upon binding of the target to the aptamer, the stem loop formed inhibits the enzymatic digestion a few nucleotides before the stem loop structure. The truncation by enzymes digestion was carried for small molecules like cocaine and its aptamer 38-GT (Roncancio et al. 2014) and ATP-33 for adenosine (Liu et al. 2012). The aptamer forms a TWJ structure which destabilizes upon Exo III digestion. In the absence of the target the enzyme digests the 35 nucleotide aptamer in 18 mer and 17 mer sequences, while in the presence of target there is accumulation of 35 mer sequence. By applying this kind of method for truncation of the aptamers used for constructing biosensors, the signal to noise ratio increases as well as sensitivity increases in the biosensing devices (Wang et al. 2018).

Truncation based on the secondary structure and the presence of conserved consensus sequence will lead to the aptamer with more stability and affinity. One

such example is the selection of aptamers against hemoglobin (Hb) and glycated hemoglobin (HbA1c) for designing a high throughput biosensor for detecting diabetes. Here, the aptamer G20 with three stem loop structure and G15 with three prominent stem loop structures for Hb and HbA1c, respectively, were generated. The G20 aptamer was having a 17 mer conserved sequence ACCCACCCACCAG CCCC and considering this truncation was done. First, G20T1 was designed with all three stem loops with removal of few terminal bases at 5′ end. The aptamer G20T2 (2nd truncated version of parent aptamer G20) was modified by removal of few nucleotides from the 5′ end and aptamer G20T3 (3rd trunctaed version of parent aptamer G20) was modified by excising nucleotides from either ends in such a way that it can form only one stem loop structure unlike the parent aptamer (Almusharraf et al. 2018). The aptamer G15 which binds to glycated hemoglobin, was modified in a similar fashion as aptamer G20 to form three truncated aptamers G15T1, G15T2 and G15T3. The Kd values of the wild type and the truncated aptamer are depicted in the table—showing that G15T1 had high affinity than the wild type aptamer whereas G15T2 showed no affinity to either Hb or HbA1c and G15T3 showed less affinity than the wild type. Similarly, among the truncated aptamers of G20, T1 has the highest affinity than the wild type and also other truncated versions (Fig. 4).

Yuan et al. have used the bioinformatics approach of aptamer docking for anticipating the binding motif between the last 15 nucleotides of Ap 1 which is originally 34 nucleotides in length when bound with the target molecule tobramycin using Autodock 4.0. By considering the binding site and the stem loop structure,

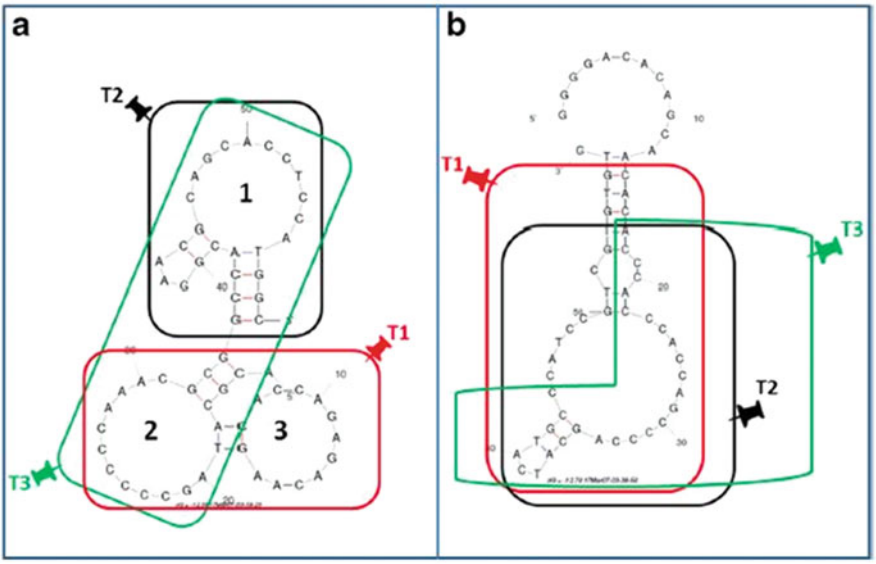

Fig. 4 Truncation pattern of the aptamer specific for hemoglobin and glycated hemoglobin G15 (**a**) and G20 (**b**). This truncation pattern included splitting of the aptamer based on the presence of number and part of stem loop structure

irrelevant nucleotides were removed to shorten the original aptamer into 26 nt, 20 nt, and 15 nt—Ap2, Ap3, and Ap4, respectively, and the affinities for these truncated aptamers were determined by fluorescent assays as 54.58 nM, 47.79 nM, and 42.12 nM, respectively, and the Kd of the original aptamer Ap1 was 52.37 nM. Considering the lowest Kd value of the aptamer Ap4, it was considered for the construction of an electrochemical biosensor for detection of tobramycin as it adds on the stability and low cost of production of the biosensor (Nie et al. 2018) (Table 1) (Fig. 5).

5 Modification of Aptamers

Modification of aptamers can be done either prior to the start of SELEX or as a post-SELEX protocol. For getting potential high affinity aptamers with high thermodynamic stability, modifications are required at every level that can be at the DNA/RNA aptamer backbone, complete base modification, the ring structure of the sugar modification, or modifying the linkage between the bases (Wang et al. 2011) (Fig. 6).

The sugar ring modification is generally carried out for stabilizing the nucleic acid structure, mostly the 2′ and 4′ regions of the sugar that are susceptible for nuclease degradation. Hence these are the sites to be modified for enhancing stability. The most common modification is the replacement of 2′ H with either 2′ fluorine (2′ F), 2′ amine ($2'NH_2$), or 2′ methoxy (2′ O-CH_3). The first aptamer drug approved by FDA—Macugen binds to VEGF165 with a sugar ring modification 2′ F in the pyrimidines and 2′O-CH3 in the purines (Ng et al. 2006). Locked nucleic acids (LNA) are unique set of nucleic acids where the ribose sugar has been modified through the formation of bridge between 2′ oxygen and 4′carbon. It induces the endo conformation and enhances the base stacking of the aptamers. The first chemical synthesis of LNAs was reported in 1997 (Satoshi Obika et al. 1997). Replacement or synthesis of aptamer library with the use of LNAs as the sugar moiety is preferential as it has less cytotoxicity and extreme thermal stability.

The LNA modified aptamers RNV66 against VEGF for breast cancer cell line has been successfully demonstrated (Edwards et al. 2015) to have the inhibitory effect of the LNA aptamer against VEGF. SPC 3649—Miravirsen and SPC 4955 are other successful LNA aptamers under clinical trials for chronic hepatitis C virus infection (Lanford et al. 2010).

Modification of the nucleic acid bases also imparts stability and high affinity to the aptamers. One such modification includes the addition of amino acid like side chain to the bases for higher affinity SOMAmers (decreased off-rate) (Rohloff et al. 2014). The nucleic acid bases are modified preferably at the 5th position, modification of deoxy uridine triphosphate (dUTP) to 5-(N-benzylcarboxamide)—5-BzdU in the aptamer AS1411 against a protein nucleolin produced in cancer cells, to develop an aptamer with nearly 2.5-fold higher specificity than the unmodified aptamer recognition of cancer cells (Lee et al. 2010).

Table 1 Comparative measures of aptamers of various molecules in their truncated and untruncated sequences and their affinities

S. No	Target	Aptamer	Sequence	Kd (nM)	Truncated aptamers	Sequence	Kd (nM)
1	Tobramycin	Ap 1	CGTCCACGGATCCATGGCACGTTATAGGTCGACG	52.37	Ap 2	TCGACATCCATGGCACGTTATGTCGA	54.58
					Ap 3	TGACTCCAGGCACTTAGTCA	47.79
					Ap 4	GACTAGGCACTAGTC	42.12
2	Progesterone	PG13	GCATCACACACCGATACTCACCCGCCA GATTAACCATTAGCCCACCGCCCACCCCCGCTGC	35	PG13T1	GCATCACACACCGATACTCACCCGCCTGAT	NA
					PG13T2	GATTAACATTAGCCCACCGCCCACC	2.1
3	Heamoglobin	G15	CAGAGACAAGCACACGCATAGCCCCCCAAACG CGGCCACGGAACGCAGCACCTCCATGGC	2.7	G15 T1	ACGCACACCAGAGACAAGTAG CCCCCCAAACGCG	1.8
					G15 T2	GCCACGGAACGCAGCACCTCCATGGC	NA
					G15 T3	ACGCATAGCCCCCCAAACGCGGCCA CGGAACGCAGCACCTCCATGGC	10.3
4	Glycated heamoglbin	G20	GGGGACACAGCAACACACCCACCCACCAGCC CCAGCATCATGCCCATCCGTCGTGTGTG	2.8	G20T1	ACACACCCACCCACCAGCCCCAG CATCATGCCCATCCGTCGTGTGT	0.2
					G20 T2	ACCCACCCACCAGCCCCAGCAT CATGCCCATCCGTCGT	14.4
					G20 T3	ACCCACCCACCAGCCCCAGCATCATGCC	31.5

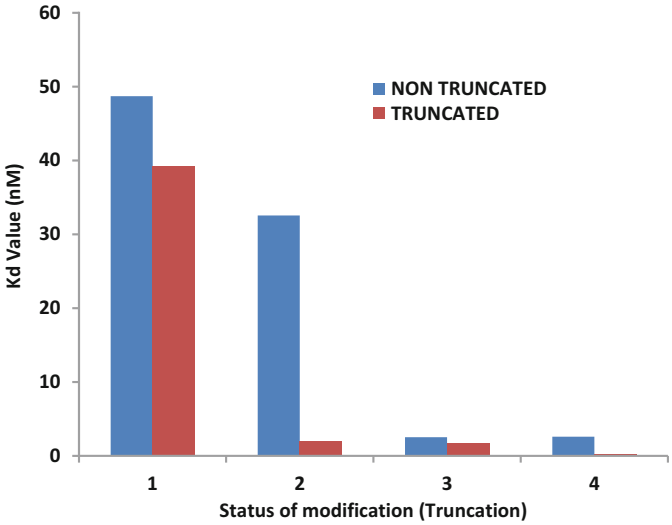

Fig. 5 Difference in the affinity values of the non-truncated and truncated aptamers

Modification of linkage groups involves the conversion of phosphodiester linkage with methylphosphonate, phosphorothioate, 3′ or 5′ capping with the phosphate moiety, addition of polyethylene glycol linkage, and biotin-streptavidin conjugates, etc. (Wu et al. 2010; Gao et al. 2016a, b). A combination of NMR and modification of aptamers led to development of highly sensitive and co-operative effect on aptamers selected for adenosine. A 27-nt long DNA aptamer Apt2a is specific for adenosine, where "2" denotes the number of binding sites of the aptamer. Adenosine interacts with the nucleotides of the aptamer Apt2a by reverse Hoogsteen mismatch, and these interactions were predicted based upon NMR analysis. The aptamer Apt2a (wild type) has a kd value of 16.4 μM to enhance the affinity and reduce the loss of heat, the structure of the aptamer was modified as Apt1a by insertion of a G5T mutant (i.e., G replaced by T at 5th position) and insertion of C to pair with G22. This modification led to reduction of the two binding sites to 1.1 ± 0.1 and Kd value changed to 12 μM. Similarly, to know the highest affinity that can be achieved by modification, the original aptamer Apt2a was again modified to get another truncated version Apt1b that has only one binding site with binding affinity of 14.1 μM (Zhang et al. 2017). The valency of the aptamers can also be modified to bivalent or multivalent forming either homologous—using same aptamer or heterologous—using different aptamers. By changing the valency, aptamers conjugated together to form a complex and bind to the same target but at different aptatopes. This strategy was applied for the aptamers specific for thrombin using a 15 mer dA linker, reducing the usage of the aptamer to ~30-fold and increasing affinity to approximately threefold. This was the first approach by Muller et al., for using of multivalent aptamers for enhancing its affinity (Muller et al. 2007). Thus, to show that valency of the aptamer has an effect on its affinity, lot of studies have been carried out where

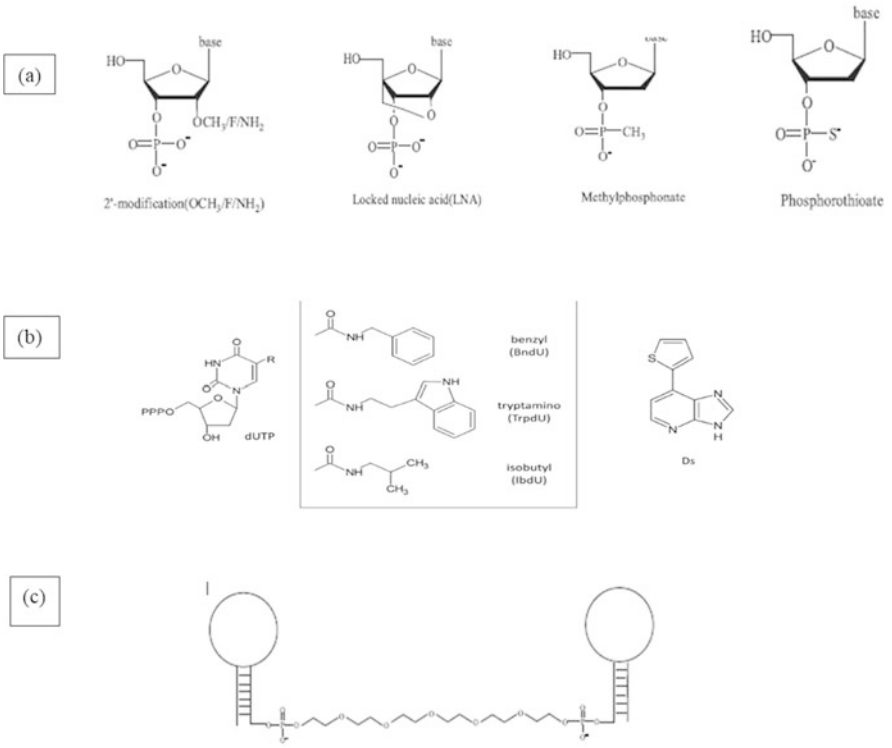

Fig. 6 Various modifications to increase affinity and stability of the aptamers (**a**) depict the modified sugar ring of the nucleic acids, (**b**) depict the modified bases of nucleotides that mostly favor the formation of G-quadruplex structures and (**c**) depict the formation of bivalent aptamer sequence by using the polylinker polyethylene glycol (PEG)

aptamers are bi/mltivalent having high affinity than their univalent counterparts, few of them are listed below (Table 2).

By the conventional method, selecting aptamers there is a significant chance of amplification bias during PCR amplification that occurs due to the inability to recognize the bound aptamers with the unbound ones. This issue was tackled by adopting the site directed mutagenesis, through which the starting material can be optimized to achieve specific aptamers in less number of SELEX cycles. Zheng et al. studied and worked on the aptamer related to saxitoxin, a paralytic shell fish poisoning toxin where they have selected an aptamer Apt stx ($K_d = 3.84$ μM) against saxitoxin. This aptamer was truncated and modified by site directed mutagenesis so that it can form a G-quadruplex structure resulting in increase in affinity that was determined by various methods (e.g. ELISA, cell bioassay, mouse bioassay) to be 133 nM (Zheng et al. 2015). The aptamer affinity increased by introducing nonhomologous random recombination (NRR) in a length controlled manner wherein the affinity was compared with the sequence after error prone PCR amplification

Table 2 Aptamers modified by increasing the number of binding sites, by various methods listed in column 4 that led to enhancement in the affinity of the aptamers towards its target molecule

Target	Valency	Affinity	Remark	References
Thrombin	Bivalent	k off ~ 1/50	16.6-fold higher than the monovalent aptamer	Youngmi Kim and Tan (2008)
VEGF	Bivalent	Kd = 30 pM	Constructed by using thymine linker	Yoshihiko Nonaka et al. (2013)
Cytotoxic T cell antigen-4 (CTLA-4)	Tetrameric	10 to 20-fold higher activity than the monomeric aptamer	–	Sandra Santulli-Marotto et al. (2003)
Membrane mediated human mIgM	Multivalent (tetravalent)	Kd = 17.1 nM	Constructed using PEG linker led to increased affinity from the monovalent aptamer Kd = 359 nM	Mallikaratchy et al. (2011)
Thrombin	Bivalent	Kd = 8.1 pM	with a 200-fold difference in affinity of monovalent aptamer	Ahmad et al. (2012)

(introduction of point mutation) and also with the parental strand and the resulted affinity was Kd = 131 nM showing nearly 15 to 20-fold lower affinity of PCR amplified sequence. Thus, this method of NRR can be used for diversifying the basic SELEX library that will greatly aid in increasing the affinity of the aptamers (Bittker et al. 2002). Nonaka et al., work is one that has been discussed many times in detail, they have used the in silico maturation method, where mutations were introduced in the VEGF specific sequence to form G-quadruplex and repeated this process for many cycles till high affinity sequences are obtained. By this process, they obtained sequences with affinity higher than parent aptamer VEap121—Kd 4.7 nM and the modified aptamer 3R02—Kd value 300 pM and a bivalent aptamer was constructed that has a Kd value of 30 pM (Nonaka et al. 2013).

Among various methods of post-SELEX process known till now, truncation of the aptamers is the process on which one can rely to improve the affinity from a range of micromolar to nanomolar or nanomolar to picomolar. The numerous methods of aptamer modifications, modification of the sugar ring structure, or the linkage modification or the protection of the ends of the aptamer (5′ and 3′ capping) aid in the stability of the aptamer and increase the resistance from nucleases. For increasing the affinity and specificity of the aptamer, the site directed mutagenesis, increasing the valency of the aptamer, and introduction of modified bases are promising techniques.

6 Characterization of Aptamer Affinity

The natural tendency of some molecules to bind to its target describes the affinity of that molecule. It can be determined by measuring either the dissociation constant (Kd) or association constant (Ka) that infers the binding strength between a ligand and its target. The lesser the Kd value of a molecule higher is its affinity. The affinity of the aptamer can be deduced from different methods and techniques like ELONA, Fluorescent based methods, equilibration methods, gel shift assays, Isothermal titration colorimetry, surface plasmon resonance spectrometry, fluorometry, etc.; some of them are described in this review.

6.1 Enzyme Linked Oligonucleotide Assay (ELONA)

ELONA is also described in few articles as Enzyme linked Aptamer Assay (ELAA) or Aptamer Linked Immunosorbent Assay (ALISA) that shares the same principle as ELONA. Gold and his team have contributed much in research related to aptamer selection and also its characterization. In 1995, they have submitted a patent describing a new method for determining the affinity of an aptamer (patent # US5789163A). ELONA is a technique in which the aptamers/oligonucleotides replace the antibodies from the traditional ELISA. Here the target is coated on the surface which binds with its specific biotin labeled aptamer and is detected by HRP bound to streptavidin, providing a colorimetric estimation of determining the Kd value. The VEGF binding aptamer was tested for its affinity by taking various serum samples and to compare the accuracy of both ELONA and ELISA (using commercially available hVEGF), they performed side by side with same samples. It was noted that the values obtained by ELONA were 15% higher when compared to ELISA (Drolet et al. 1996). The affinity of Protein A was also determined by ELONA and compared with the truncated version of the original aptamer that forms a G-quadruplex structure (Stoltenburg et al. 2016). Although there is quite reliability on this protocol but there is also a point of dispute over the labeling of biotin to the aptamers as this has been noted that the 5′ or 3′ biotin labeling is not constant and changes with the type of target and the binding site of the aptamer. Hence, one has to confirm the binding site before labeling the aptamer with biotin and then can perform ELONA as a secondary assay for confirming dissociation constant (Fig. 7).

6.2 Fluorescence Based Assay

Fluorescence based assay does provide a dependable procedure for affinity measurement; this assay is mostly adapted for constructing fluorescence biosensors apart from affinity conformation. The aptamer sequences are labeled with fluorophore at one end and quencher at the other end near the region which will be binding to the target molecule such that when the target binds, the aptamer will undergo a

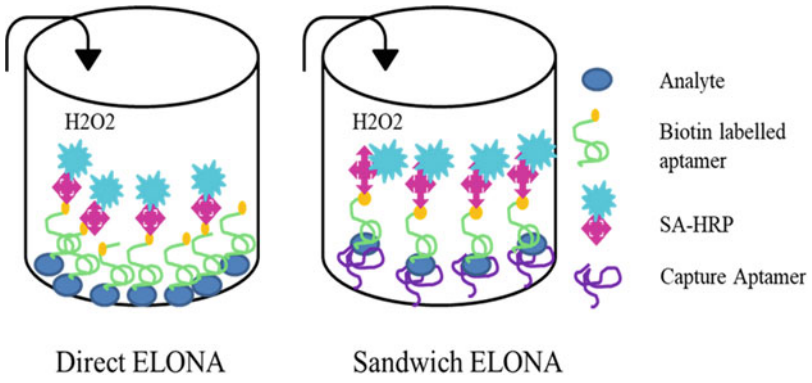

Fig. 7 Diagrammatic representation of direct and sandwich ELONA

conformation change causing change in intensity, that will mark for target binding and this property is used in fluorescence biosensors. By using different concentrations of aptamers and plotting a non-regression fitting graph dissociation constants can be drawn. Similar work has been done for determining affinity of progesterone specific aptamer PG13 and its truncated version PG13T1 where change in the Kd value of the original and truncated versions was observed (Alhadrami et al. 2017), for detection of cocaine (Yu et al. 2017) and kanamycin (Xing et al. 2015). This method has also been used as a displacement assay for the detection of ATP by taking ThT (Thioflavin T) as mediator molecules (as they are structurally similar), in replacement of split aptamers and introducing label free sensing technique (Ma et al. 2019). Penner et al. describe the use of polarization of the fluorescence molecule instead of relying on the fluorophore and quencher, as an advantage of the aforementioned method there will be no requirement of labeling of the sequence with many molecules and change in the conformation of the aptamer. It is useful for small target molecules like ochratoxin (OTA), relatively cost-effective and more specific compared to the fluorophore quencher system (Penner 2008) (Fig. 8).

6.3 Flow Cytometry

Flow cytometry is a technique that includes analysis and sorting of different types of cells in a population each labeled with a fluorescent molecule. The same principle applies for aptamer affinity too, wherein the aptamers selected for whole cell or cell specific marker are subjected to flow cytometry. This method of aptamer selection or affinity determination is mostly chosen for early and specific detection of various types of cancers (liver, breast, prostate, lung, brain and lymphomas) and affinity detection of pluripotent stem cells (Hou et al. 2015). For live *Pseudomonas aeruginosa* cells, the affinity of aptamer was determined by incubating bacterial cells with increasing conentrations of the aptamer (Soundy and Day 2017), for differentiating and selecting among mature and immature white adipocyte from a

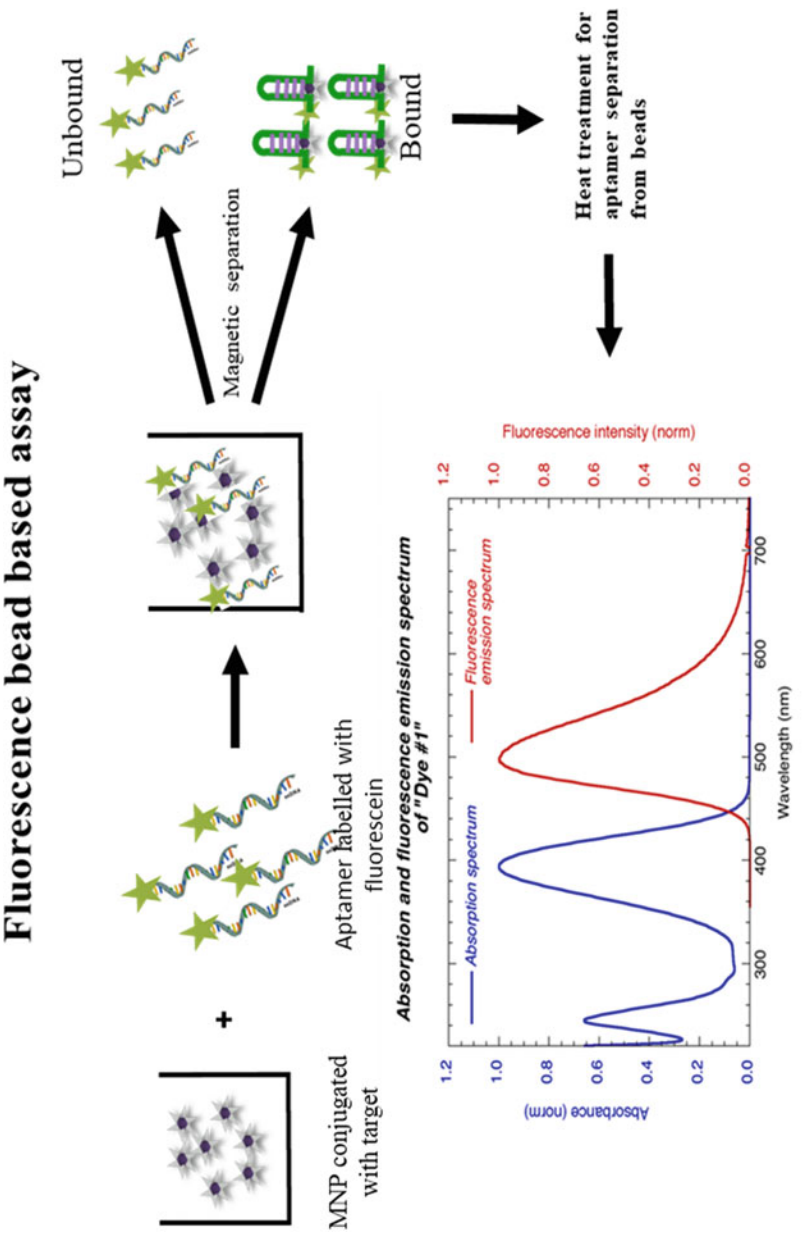

Fig. 8 Schematic representation of fluorescence-bead based assay for determining aptamer affinity

population of cells. The Kd was determined by using the formula $Y = B_{max}X/(Kd + X)$ from the sigma plot (Kim et al. 2014). There is a lot of work done in relation to determination of affinity and aptamer selection using flow cytometry and it is indeed a promising technique for live cell or whole cell specific aptamers used intensively in cancer research as aforementioned for the early prognosis of the disease.

6.4 Surface Plasmon Resonance

Surface plasmon resonance is a light dependent method, the target molecule in the mobile state interacts with its ligand immobilized on a sensor chip coated with dextran. This interaction causes the light to emit through a prism reflected off the inner sensor surface, the reflected light is measured by the detector. When there is no interaction the complete light is reflected off. When there is interaction, the light is reflected at an angle called as resonance angle, the resonance angle is proportional to the change in mass on the sensor chip. The changes thus are plotted on a sensogram and analyzed (Bakhtiar 2012) providing the association and dissociation constant of the aptamer towards its ligand. Usage of SPR provides a versatile and high throughput method for interaction studies of aptamer and its cognate target molecule. To showcase the versatility of SPR, intensive work has been done involving 12 molecules with varied frame of molecular weight some of them include citrulline, FMN, malachite green, ATP, TPP, glycine, etc. (Chang et al. 2014). Streptavidin coated chips are also used widely on which the biotin labeled aptamers are immobilized, the aptamer VEGF aptamer was dimerized (29 mer) and checked for its affinity using the streptavidin coated chip (Hijiri Hasegawa et al. 2008) and for daunomycin (Wochner et al. 2008) (Fig. 9).

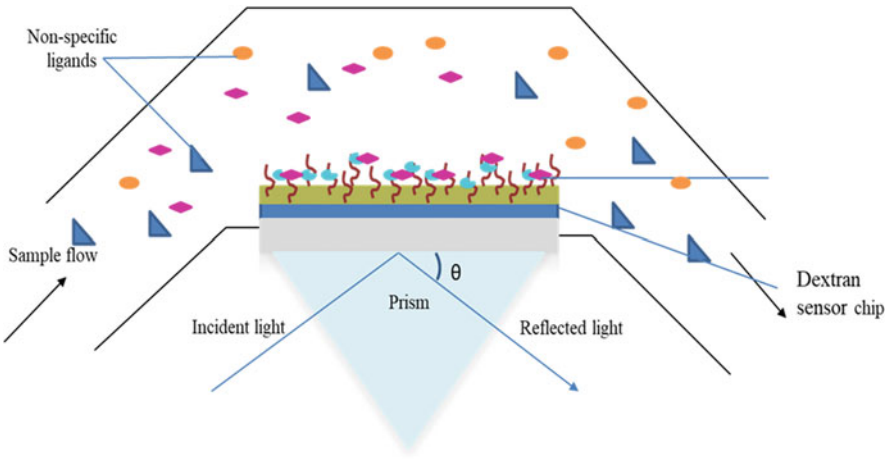

Fig. 9 Diagrammatic representation of working principle of surface plasmon resonance

6.5 Microscale Thermophoresis

Microscale thermophoresis uses fluorescent labeled molecules and measures its movement upon different temperature gradient caused due to infrared laser. Binding of these fluorescent labeled ligands with their targets leads to changes in the size, hydrophobicity, and movement of the molecules on exposure to infrared radiation. Thus, measures the sensitivity of the binding affinity. This technique has advantage over others in that it does not require to immobilize the molecules and can detect Pico molar range of targets present in the solution and also lags behind as it cannot infer the association and dissociation constant (Jerabek-Willemsen et al. 2011). MST have been used in number of studies to detect the affinity of molecules either through monitoring the intrinsic tryptophan in the proteins or the fluorophore bound to the molecules (Seidel et al. 2012), for adenosine affinity characterization (Jerabek-Willemsen et al. 2011).

6.6 Isothermal Titration Calorimetry

Isothermal tittration is a label free calorimetric assay where the heat absorbed or dissipated during titration is taken into consideration for determining the affinity. Titration is performed by taking ligand (aptamer) into the syringe and is titrated aganist the target molecule present in the calorimetic cell, the heat absorbed during this process is directly proportional to the amount of target bound to the ligand (Brown 2009). The advantage of this assay over others discussed earlier is that, it is a label free method, immobilization of the molecules on a chip is not required and calculates the affinity as well as thermostability of the molecules. While its requirement of high concentrations of both target and ligand stands as its major disadvantage. Chen et al. have studied the interaction mechanism of widely studied thrombin molecule; in this study they have evaluated the interaction by using SPR, CD spectroscopy, and also by ITC. The complete binding sites of the aptamer and the formation of G-quadruplex are discussed in detail in their work (Lin et al. 2011). The aptamer for whole cell *M. tuberculosis* H37Rv and BCG affinity towards its target was analyzed using ITC showing high Ka value providing a promising agent to be used as probe in the inhibitory assay against the virulent strains (Chen et al. 2007).

7 Conclusion

Aptamers have many applications in the field of research and are advantageous due to their property of low immunogenicity, require less cost of production and can be generated in no time (Nimjee et al. 2017). The aptamers for small molecules like hormones, peptides, small ions available are not reliable due to their low affinity. Although it has been proven many times that the aptamers have the ability to completely replace antibodies (Jayasena 1999), there are many factors that do restrain the affinity of these molecules when applied in drug therapy, as therapeutic

agent, signaling molecule, lateral flow detection assays, dipstick assays (Bunka and Stockley 2006; Sharma et al. 2017). The selection of aptamers for small target molecules is currently carried out mostly by the traditional SELEX process that consumes long time for getting high affinity aptamers. Though there are protocols already reported for achieving better affinity aptamer in short time period, but these methods do vary depending on the target molecule. Thus, there is a requirement of a method that is reliable. Another approach which can be followed for enhancing the aptamer affinity is by modifying the selected aptamer (post SELEX process) or modifying the aptamer library before selection which has been the main focus of this review. Through this review, we suggest that an aptamer if modified (truncated) based upon its structural studies can increase the aptamer affinity when compared with other modification techniques like sugar backbone modifications (LNAs or artificial nucleotides) that adds on cost of production of the aptamer. The characterization of the aptamer affinity requires an efficient and accurate method, though the calorimetric methods seem to be promising but it would be more appropriate to follow these methods as a validation to the affinity known by using the SPR, ITC, or MST technical methods.

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Application of Biosensors to Enhance Reproductive Efficiency and Production of Livestock and Poultry by Diverse Antigen Analysis

Pankaj Kumar, Shalini Chakraborty, Devika Nagar, Komal Birader, and Pankaj Suman

1 Introduction

Meticulous health monitoring of livestock is a beneficial aspect for getting higher economic value in farm industries. Livestock management with precision farming is expected to grow from US $3.20 billion in 2015 to US $7.87 billion by 2022 (markets and markets). Hence, the development of field applicable and cost-effective technology is the major requirement for the improved production in animals. Health threat of animal directly affects the food supply; ultimately the result is the effect on socioeconomic status of human. Infectious diseases are the leading cause of death for both human as well as animals. Animal is the source of more than 70% disease emergence in human (Vidic et al. 2017). Therefore, surveillance for zoonotic pathogens in animals is critical for managing these infections. Apart from these, reproductive abnormalities or reproductive failures are one of the most significant factors that limit the productivity of animals resulting in huge economic loss. Biosensors are rapid and efficient analytical tool for several infectious disease diagnoses. In the past three decades, several biosensors for the bacteria and virus detection have been developed. This chapter will review the progress made and the opportunities to develop biosensing platforms for improvement of animal production, and reproductive health. Several techniques being used for diagnosis of problems associated with livestock health, reproductive status and infections and their limitations have been summarized in Table 1.

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Table 1 Techniques used for diagnosis of problems associated with livestock health, reproductive status and infections

Biosensors	Principle/target	Limitation/difficulties	Reference
Health and behaviour monitoring			
Feeding behaviour analysis	a. Mechanical sensor b. Acoustic sensor c. Electromyography sensor	• Pressure sensor analyse the variable in number and duration of rumination, eating and resting phase	Benvenutti et al. (2017) Navon et al. (2013) Weng et al. (2015a)
Subclinical ketosis monitoring	a. Detection of β-hydroxybutyrate by immobilizing enzyme 3-hydroxybutyrate dehydrogenase b. Quantum dot based biosensor	• Serum based detection of BHB (detection limit 0.05Mm) • Serum based detection of BHB	Weng et al. (2015a, b) Neethirajan (2017)
Reproductive health monitoring			
Estrus detection	a. Monitor the vaginal conductivity and temperature b. Herd Navigator stepcount and leg tilt sensors	• There might be other factor for raising the body temperature of animals • Too much expensive	Andersson et al. (2015, 2016) Jónssona et al. (2011)
Pregnancy detection	a. Lateral flow test (LFT) b. Blood-based pregnancy tests (BioPRYN) and ELISA	• Test is optimized on the basis of progesterone concentration in milk (cost 275/test) • Based on PAGP and PSPB[a] concentration determination • Non invasive method • Required expertise	Waldmann and Raud (2016) Piechotta et al. (2011)
Disease detection			
Foot and mouth disease	a. Lateral flow immuno-chromatographic for the detection of antibodies against FMDV protein b. SpectroSens™ optical microchip sensors	• Antibody based, high cost per sample • Temperature sensor	Yang et al. (2015) Bhatta et al. (2012)
Bovine respiratory disease	a. To evaluate the exhaled N2O, CO and serum hepatoglobin concentration b. Field-effect transistor (FET) for direct potentiometric serological diagnosis of the BHV-1 viral protein via an	• Volatile organic compound (VOC) are not easily stable at room temperature • Not equivalent sensitivity as SPR and ELISA	Gray et al. (2016) Schaefer et al. (2012)

(continued)

Table 1 (continued)

Biosensors	Principle/target	Limitation/difficulties	Reference
	IgE-coated immunosensor		
Salmonella detection	a. culture of the suspected sample in strain specific medium followed by serological test b. Salmonella AD (lateral flow system) Lateral flow test device, named "Ingezim BTV CROM Anticuerpo"	• Need laboratory setup and expert to perform the test, time consuming • Require two step enrichment before the sample insertion on the strip • Detection is based on the BT virus specific antibody in animal serum	'International Standard Horizontal method for the detection, enumeration and serotyping of', salmonella 2017 Fang et al. (2014a, b) Hanon et al. (2016)
Paratuberculosis and brucellosis	a. Electronic nose developed to detect the VOC in serum sample	• Differentiate the sera from brucellosis and paratuberculosis infected animals and healthy animals at the population level	Knoblocha et al. (2009)

[a]PAGP pregnancy associated glycoprotein, *PSPB* pregnancy specific protein B, *VOC* volatile organic compound, *BHV* Bovine herpes virus

2 Biosensors to Enhance the Reproductive Efficiency of Farm Animals

One of the very important applications of biosensors in livestock is to successfully predict the early pregnancy and subsequently monitor the healthy pregnancy. A study in 2013 claimed to detect pregnancy in cows by monitoring the rise in temperature from the 5th to the 12th day after insemination (Nograles and Caluyo 2013). Earlier studies suggested that an increase in temperature maybe due to reaction of mother's immune system towards the newly implanted embryo in the uterus. This is a non-invasive method for the detection of temperature changes in the cow within a range of 40 m and most importantly without human intervention by using a personal computer connected to Xbee modules and MATLAB program and a sensor attached below the head of the Animal's tail (Nograles and Caluyo 2013). Another very recent study developed an Automatic Measurement System for Cattle's Surface Temperature (AMSCST) which can accurately detect surface and rectal temperatures of cows using a special sensor shell that fits onto the metatarsus of the hind leg, thereby presenting an automatic system that could provide better control over reproductive cycles and subsequent well-being as well (Kou et al. 2017). In relevance, a 2018 study by Barriuso et al., addressed a number of problems in farms by applying quite an arduous technological feat combining wireless sensor networks and multi-agent systems using the "Platform for Automatic coNstruction

of orGanizations of intElligent Agents" (PANGEA). Despite, it is being a technological coup; the system appears to be as user/farmer friendly as it gives alerts on mobile phones. This way one can detect the precise moment when the animal goes into labour making calving easier and under able supervision increasing chances of survival for both the new-born and the mother (Barriuso et al. 2018). Similarly, an automatic sensing technology has been put to use in poultry farms as well as where digital signal processors can detect sounds in an incubator to successfully predict the internal pepping stage of the chicken embryos (Exadaktylos et al. 2011; Costa-Silva et al. 2009). In real time not only acoustics, but a temperature sensing device on eggshells could predict the hatching time of individual embryos and provide definitive information on the biological age of the hatchlings for the better management of poultry industry (Romanini et al. 2013).

3 Biosensing Applications to Reduce Neonatal Mortality

Biosensors are also finding their applications in young animal survival which may address a very important problem of unforeseen premature mortality common in farms. Proper heating is a very important concern in the first three weeks of chick survival. Inadequate heating may lead to serious physiological problems as well as direct physical concerns of smothering when chicks huddle together. If it is not warm enough, it would have its own threats too. A study devised a noise analysis technique to make sure of the comfort of chicks during the heating phase (de Moura et al. 2008). A very potent reason for infant mortality of cows is respiratory diseases considering the lower capacity of the new-born immune system. A wearable temperature sensor node put on the base of the calf's tail can keep the farmer updated about the health and well-being of the small animals thereby ensuring economic propagation and production (Nogami et al. 2014). Piglet survival remains a very important issue considering unexpectedly high mortality in the early lactation phases (Alonso-Spilsbury et al. 2017). It has been suggested that observing various degrees of stress the foetus has to go through during birth, could help in identifying problems (Alonso-Spilsbury et al. 2017). There is no report of working biosensors in this field till date; hence it offers a good avenue for directed research ensuring lesser mortality.

4 Biosensors to Monitor Animal Behaviour

In animal biology, the behaviour is key. In farm management, behaviour of the animals can tell a lot about their well-being, probable sickness and readiness for reproduction and stressed conditions. Few studies that have made an impact on automatically sensing behavioural changes in farm animals will be discussed here. A recent study has developed automatic tracking systems for recording basic behavioural patterns in pigs ranging from standing postures to feed intake in response to stimuli. It makes the otherwise impossible single animal behaviour observations easier having a very positive impact on farm management and

commercial production (Matthews et al. 2016). A review has summarized different sensors and probable recordable behaviour triggers which when recorded could be put to use in piggeries allowing early disease diagnosis (Matthews et al. 2016). Automated surveillance of animal farm has been developed to monitor the changes in locomotion considering gait and daily activity (Song et al. 2008; Fernandez-Carrion et al. 2017), drinking pattern (Stephen et al. 2005), disease specific behaviour like coughing (Hemeryck et al. 2015). These sensors can automatically identify aggression problems in pigs. Thus, it is allowing a better and practical farm control. Such a sensor based on sensing kinetic depth can recognize behaviour in pigs (Lee et al. 2015). Similarly, real-time measurements for the detection of gait and probable lameness in cows have been put forward in a study (Van Nuffel et al. 2015). Radio frequency based systems can be used for successful monitoring in cattle farms to keep a record of personalized record of feed intake (Dahlke et al. 2008; Brown-Brandl and Eigenberg 2011). Another study suggest that oestrus in cattle may also be automatically detected using sounds (Chung et al. 2013). An automated method for oestrus detection and a suitable time for artificial insemination in cows can be achieved by "pedometry" (Nebel et al. 2000). It measures increased activity of cows in oestrus and a "pressure sensing radiotelemetric system" to look for any mounting behaviour and time of contact. Sometimes there may not be distinct behavioural changes during oestrus, so in such cases, measurement of body temperature maybe taken as an indication for ovulation (Kou et al. 2017). In poultries, the sound analysis maybe used to detect stress condition in laying hens (Lee et al. 2015). Feed intake by the birds can also be easily detected by a microphone analysing the sounds made by them (Aydin et al. 2014). Automatic tools have been developed to monitor and rescue the general activities like jumping and landing forces (Aydin et al. 2010; Banerjee et al. 2014). Computer visions maybe used for a variety of behavioural checks such as mounting, stretching, scratching, drinking, resting, spreading of wings, etc. (Pereira et al. 2013). A lot of such technologies are being sought and being made accessible for livestock monitoring.

5 Biosensors for Detection of Toxins in Animal Feed

Microfluidic devices have high capability to detect least number of antigens and toxins present in animal feed. Thus, it is finding potential applications in livestock farms for the animal feed monitoring. A microfluidic based sensor has been developed for detection of botulinum neurotoxin (Neethirajan 2017). It can detect the toxin to the extent of <30 pg/ml in spiked human serum (Babrak et al. 2016). L-glutamate monitoring in animal's body fluid is also important to know the cognitive function. Some implantable or wearable sensors can be developed for the continuous monitoring of L-glutamate level in the body of animal. As a major portion of ingested aflatoxins are excreted in the faeces, it is considered as an important source for aflatoxin analysis. Most of the methods for aflatoxin detection in food sample are based on high performance liquid chromatography (HPLC), tandem mass spectroscopy, and real-time mass spectrometry (Rahmani et al. 2010;

Iqbal and Asi 2013; Busman et al. 2015). All of the above-mentioned methods are time taking, need expertise and also required complete laboratory setup. Microplate based immunoassay, lateral flow immunoassay and different immunosensors are the commonly used methods for aflatoxin detection (Goryacheva et al. 2011; Anfossi et al. 2013).

6 Biosensor for Detection of Venoms in Envenomed Individuals

Venom is a salivary secretion containing toxin and generally used either for defence or predation in bees, ants, wasps, snakes, spiders, scorpions, etc. It is a viscous, clear, poisonous fluid, which is highly complex as these comprises many biologically active substances and broadly are neurotoxic; cytotoxic; hemotoxic; myotoxic in nature. Specific and rapid venom detection is difficult practice especially in case of snake venom. For the accurate administration of antivenom, it is important to know the species of snake and the type of venom produced by them. In clinical cases where species or type of venom has not been identified, the nonspecific polymeric antivenom is given which carry severe side effects and also the effectiveness of treatment is reduced (Teja et al. 2018). Therefore, venom detection within limited time frame is essential to encounter specific and early treatment; where detection by biosensor technology maybe helpful in specific and rapid determination of venom. There have been reports of few techniques employed for the detection of snake venom, such as avidin–biotin optical immunoassay (AB-OIA) based sensor for the detection of beta-bungarotoxin (β-BuTx), which is a neurotoxin from *Bungarus multicinctus* (Van Dong et al. 2002). An enzyme-linked immunoassay(ELISA) based detection in Bothrops and Lachesis (Heneine et al. 1990; Heneine and Catty 1993) and a highly specific, sensitive venom detection ELISA test (VDET) was reported for the detection of venom from Indian Cobra, Krait, Saw-scaled viper and Russell's viper with a single device in a short period of time (Shaikh et al. 2017). Other than these, there are reports of optical based immunoassay development which are rapid, sensitive and specific, thus these are considered as good biosensors in the field level (Lee et al. 2015). On the other hand, for the first time, an impedimetric biosensor was developed to detect phospholipase A2 from snake venom (Zehani et al. 2018). Another simple and rapid venom detection test, a gold nanoparticle based lateral flow assay (LFA) detects Indian Cobra venom and Russell's viper venom with high specificity and sensitivity is a potential field diagnostic test to detect envenomation (Pawade et al. 2016). Most recent crofer 22 APU steel-based biosensor has been developed that specifically detects the venom from *Bothrops* genus via electrochemical impedance spectroscopy (EIS) (Faria et al. 2018).

7 Biosensors for Detection of Metabolic Diseases in Farm Animals

Metabolic disorders are key problems in high yielding dairy cows specifically during pregnancy or during lactation period (Sundrum 2018). Clinical diseases mostly related to nutritional management are ketosis, milk fever, retained placenta, metritis, displaced abomasum and lameness. Subclinical ketosis is a metabolic disease associated with negative energy balance in dairy cattle resulting in decrease in milk yield. Early measurement of β-hydroxybutyrate (β-HBA) level is an ideal biomarker for diagnosis of subclinical ketosis, thus minimizing economic loss in dairy cattle. Recently, chip based, easy to use, affordable detection system for β-HBA has been developed by Weng and Colleage. In this method, the β-HBA has been detected within 1 min with the limit of 0.05 mM β-HBA in serum (Weng et al. 2015a, b). To enhance the β-HBA detection sensitivity, Nicotinamide adenine dinucleotide (NAD+) based biosensor has been developed that can detect β-HBA in blood and milk with the detection limit of 35 μM (Weng et al. 2015a, b; Neethirajan 2017). Increased concentrations of non-esterified fatty acids (NEFA) in biological fluids of cows are an efficient biomarker for metabolic diseases. Ruthenium bipyridyl complex-modified graphene oxide nanosheets ([Ru (bpy) 3]2þ-GO) based biosensor has been developed for early metabolic disease diagnosis in cattle. It is based on detection of circulating non-esterified fatty acid (NEFA) that is generally formed after the breakdown of adipose fat. Increased concentrations of NEFA in body cause severe problems like fatty liver, ketosis, displaced abomasum, and metritis. Thus, a higher expression of NEFA might be a good indicator for detection of metabolic diseases (Neethirajan 2017). Several techniques are currently available to evaluate the blood calcium levels through atomic absorption spectroscopy, ion-selective electrodes and chromophore-based spectrophotometric methods, but these techniques are very costly and require expertise in instrument handling. Recently, gold nanoparticle based colorimetric detection has been devised for a variety of targets including metal ions. The aggregation of ligand-functionalized gold nanoparticles upon binding to a target results in a colorimetric response that can easily be observed by naked eye.

8 Conclusion and Future Prospects

The current perspective of livestock farming is to create a complete management system to maintain healthy animals with high productivity. Apart from this, it is also important to continuously monitor the animal's health to avoid the economic loss. Most of the available diagnostic techniques are time taking, labour intensive, require sophisticated instrument and expertise to perform it accurately in well stabilized laboratory. To overcome these difficulties, it is important to develop field applicable, cheap, rapid and reliable method for early disease diagnosis in livestock. Although in developing countries, people have limited resources for their livelihood so with proper management, they may have profitable livestock farming with reduced

expenditure on health management and diseases. Development of portable and cost-effective biosensor in the veterinary science will be a big step towards accurate and early disease diagnosis.

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Clinical Implications of Cortisol and Bioanalytical Methods for Their Determination in Various Biological Matrices

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1 Introduction

Cortisol is one of the adrenal steroid hormones classified under the glucocorticoid class of hormones and primarily produced from cholesterol in both adrenal glands located at the top of kidney's. Cortisol acts as a biomarker for various ailments and plays an indispensable role in modulating numerous physiological processes such as maintaining blood pressure, renal system, immune function, glucose utilization, cardiovascular homeostasis, skeletal, carbohydrate metabolism, endocrine system, reproductive system, haematopoietic system and central nervous system (Gatti et al. 2009; Levine et al. 2007).

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Abnormal increase in cortisol production (i.e. hyperadrenocorticism or glucocorticoid hypersecretion), showing excessive level of cortisol in blood, will result into Cushing's syndrome. Cortisol secretion generally follows circadian rhythm. However, loss of circadian rhythm along with abnormal/loss of negative feedback mechanism of HPA axis will lead to the chronic level of cortisol inside circulating fluid ultimately resulting in clinical level of endogenous Cushing's syndrome which is manifested by muscle and bone weakness, hypertension, hypercholesterolemia, osteoporosis and obesity (McEwen 2009). Excessive cortisol secretion also results in the elevated level of fatty acids and amino acids in the blood as well as suppresses immune system and inhibits inflammatory responses. Cortisol when secreted in low amounts leads to Addison's disease with the symptoms of scars, hyperpigmentation on skin, hypoglycaemia, weight loss with decreased appetite, extreme fatigue, irritability and depression (Edwards et al. 1974).

2 Cortisol Structure and Properties

Cortisol is a hormone with low molecular weight (~362 Da), naturally occurring pregnane corticosteroid also known as 11ß,17α,21-Trihydroxypregen-4-ene-3,20-dione. Its molecular formula is $C_{21}H_{30}O_5$ with biological half-life of 6–8 h (Derendorf et al. 1991). It is lipid soluble hormone so its solubility in water is negligible (0.32 mg/mL), in DMSO it is 100 mM, while in ethanol it is around 25 mM (Fig. 1).

3 Cortisol Synthesis and Secretion

Cortisol was isolated as compound 'F' from adrenal gland as well as its structure was analysed by Edward Calvin Kendall and his colleagues in 1930. Adrenal glands are located on top of both the kidneys and possess two compartments i.e. externally

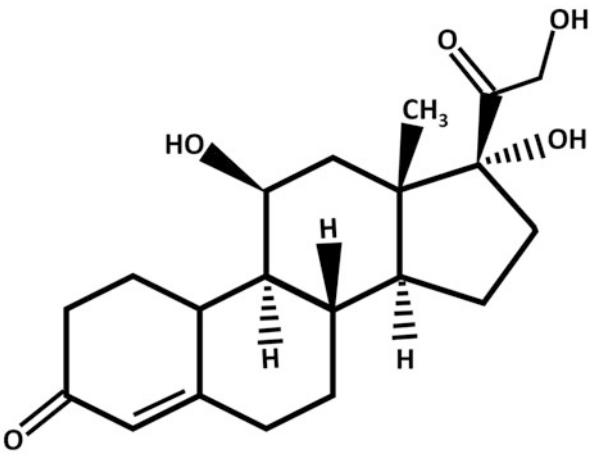

Fig. 1 Chemical structure of cortisol (F)

adrenal cortex and adrenal medulla at the core. Adrenal cortex is composed of three basic layers i.e. zona glomerulosa, zona fasciculata and zona reticularis. The middle layer i.e. zona fasciculata is known to be responsible for cortisol secretion. There are numerous factors which affect the cortisol secretion resulting in a dramatic variation in cortisol level in bloodstream. In particular, physiological or emotional stress influences significantly the cortisol secretion, which is why cortisol is also termed as a 'stress-hormone' (Holsboer and Ising 2009). The release of cortisol is regulated by hypothalamus–pituitary–adrenal (HPA) axis, a complex signalling system among the hypothalamus in brain, pituitary/hypophysis at the bottom of brain (connected to hypothalamus via pituitary stalk/ Infundibular stalk) and adrenals glands. HPA axis is the prime component responsible to control and maintain physiological process under varying environment. Figure 2 represents a schematic showing HPA axis control and cortisol secretion. External environmental factors initiate activation of

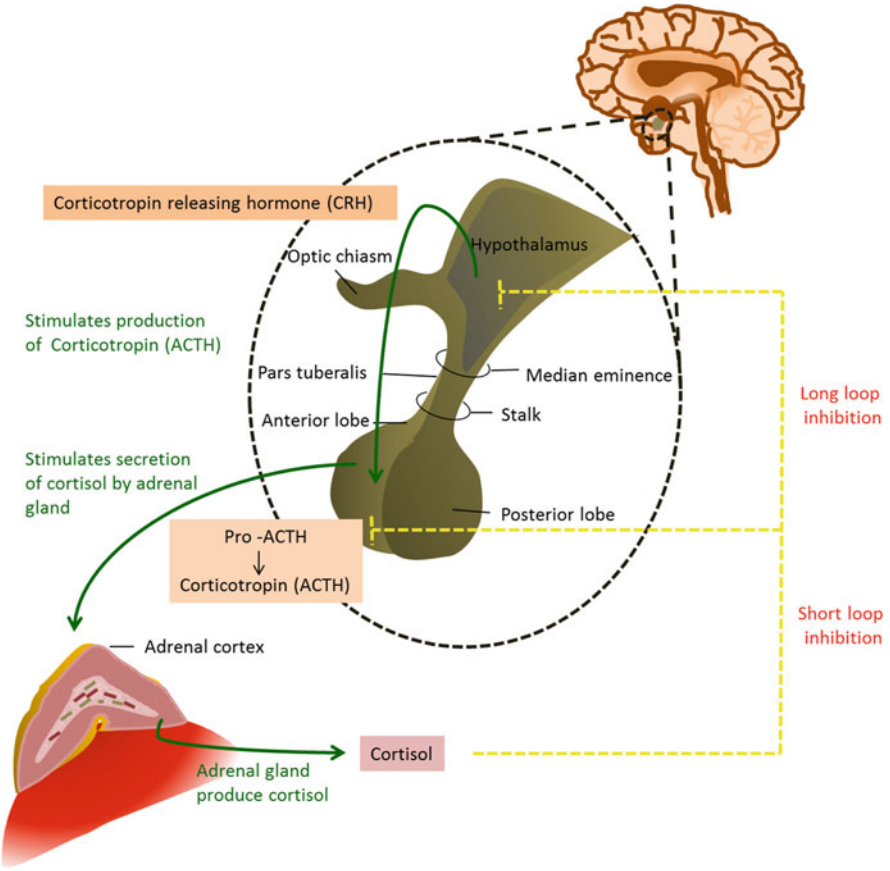

Fig. 2 Cortisol secretion controlled by HPA axis and feedback loop. External factors stimulate hypothalamus to release CRH, which leads to activation of the anterior pituitary to secrete ACTH. The stimulated ACTH, further triggers cortisol secretion from adrenal cortex

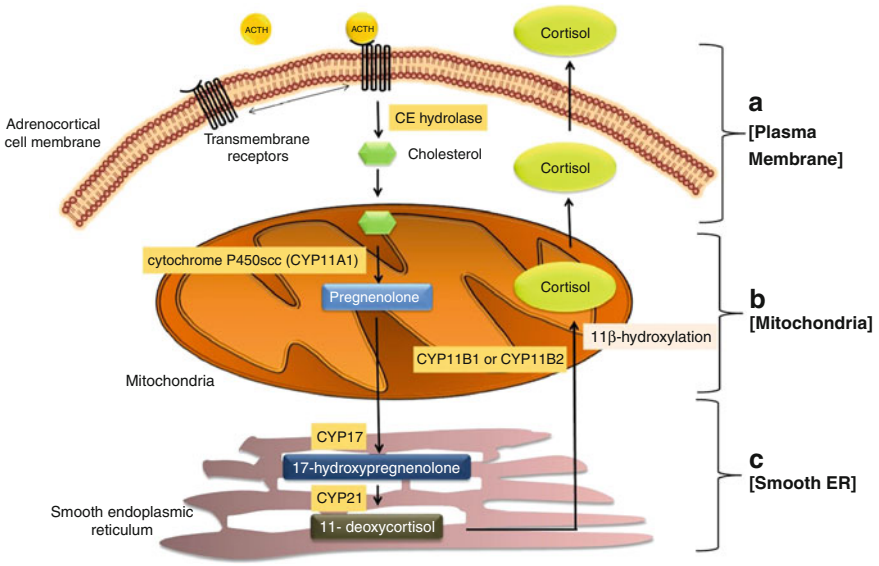

Fig. 3 Diagrammatic representation of cortisol synthesis. (**a**) The ACTH binds to cell membrane receptor and triggers the conversion of free cholesterol from cholesterol ester. (**b**) Free cholesterol is further transferred inside mitochondria and converted into pregnenolone using enzyme cytochrome P450. Moreover the pregnenolone further undergoes a set of reactions and is finally converted into 11-deoxycortisol under the influence of CYP17 and CYP21, inside smooth endoplasmic reticulum. 11-deoxycortisol is finally converted into cortisol inside mitochondria by 11ß-hydroxylase and is then released inside the bloodstream to influence their target sites

HPA axis by triggering the hypothalamus to release corticotrophin releasing hormone (CRH). Furthermore, CRH regulates the release of adrenocorticotrophic hormone (ACTH) from anterior region of pituitary gland that travels through blood stream to the adrenal cortex.

Adrenal cortex responds by cortisol biosynthesis as represented in Fig. 3 (Katsu and Iguchi 2016) that further governs the physiological processes. ACTH binds to specific receptors in the adrenocortical cell membrane that enables the conversion of cholesterol ester into free cholesterol with the help of cholesterol ester (CE) hydrolase. Free cholesterol is utilized by cytochrome P450 side-chain cleavage enzyme (CYP11A) and is converted from cholesterol to pregnenolone inside mitochondria. In further the pregnenolone is then converted to progesterone under the influence of 3-beta hydroxysteroid dehydrogenase and is further 17 hydroxylated to 17-alphahydroxypregnenolone using 17 alpha-hydroxylase (CYP17) inside smooth endoplasmic reticulum (SER). Finally, the hydroxylated progesterone is catalysed to cortisol by steroid 11beta-hydroxylase (CYP11B1) via 11-deoxycortisol intermediate. Cortisol regulates their secretion by negative feedback inhibition. Cortisol binds to the receptors on the cells of hypothalamus and pituitary gland, inhibiting the secretion of trophic hormones, ultimately resulting in

homeostatic alteration in HPA axis and cortisol concentration. The advantage of negative feedback inhibition is that it holds the level of hormone in an optimum physiological range. Extreme stress level results in abnormal level of cortisol in circulatory system altering the optimum physiological processes.

4 Health Disparities and Detection

Health can be defined as complete physical, social, mental well-being, and not just the absence of disease or ailment. Good health can be governed by various factors such as physical environment (immigrant status, geography), person's behaviour (gender or sex, ethnicity, race) and characteristics, social and economic environment (education, lifestyle and nutrition) (Djuric et al. 2008). Imbalance in such personal, social and economical attributes triggers illness. Various other factors such as psychological stress and nutrition also contribute to health disparities in population that are encountered with recurring stress of daily life. Multiple potential sources and reasons including, sexual orientation and globalization i.e. altered standard of living and competition result in an elevated level of psychological stress and are becoming a serious concern about life threatening diseases, as shown in Fig. 4 (Djuric et al. 2008). As an example of nutrition factor, under high stress conditions it will give rise to consumption of high dietary fats and food with high glycaemic index. Furthermore, it will result in low intake of micronutrients and vitamins that may ultimately give rise to elevated risk of heart disease, diabetes and obesity.

Health discrepancy explicate poor life quality, high healthcare charges, lower productivity and life expectancy. Several biomarkers such a *homeostasis* and *allostasis* have been shown to be influenced by psychological stress. Homeostasis defines the stability of physiologic system in a living organism and the ability of an organism to respond to such physical and psychological demands via activation of various physiologic process is referred as allostasis. Biomarkers can aid to characterize and compute the biological effect of psychological stress on aetiology of health discrepancy. Physiological stress detection and quantification at precise and

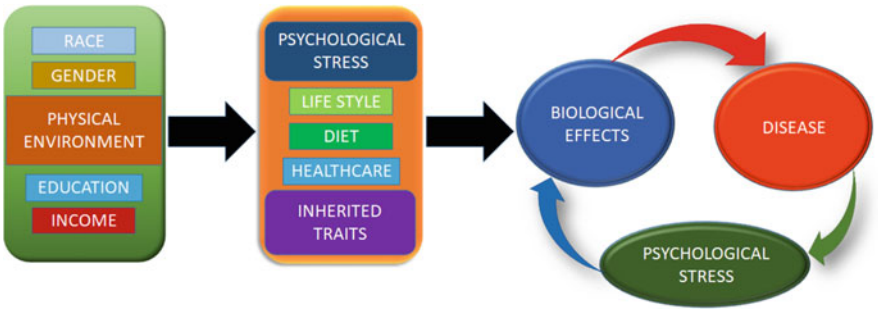

Fig. 4 Factors affecting health disparities that act independently and interactively, thus leading to differential health status in several population sets

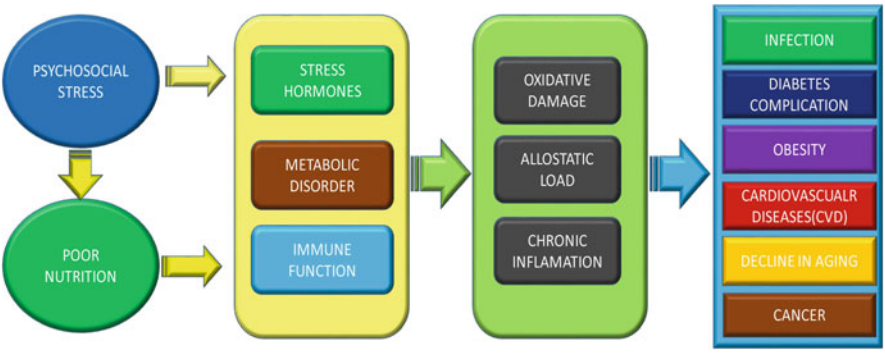

Fig. 5 Probable physiological intermediates for psychological stress

specific level are gaining attention for personalized health monitoring and diagnostics. Figure 5 depicts the schematic of the process and outcomes during psychological stress. Improved efforts are being made to establish analytical devices for stress monitoring and associated anomalies to achieve timely diagnostics and medication. Studies revealed a distinct association between stress and cortisol levels, hence cortisol has emerged as an important biomarker to ascertain psychological stress (Levine et al. 2007; Gatti et al. 2009). Cortisol variation could be a dominating precursor to define psychological and medical related stress such as sexual abuse stress, post-traumatic stress disorder (PTSD) and stress due to racism (Delahanty et al. 2000; Pervanidou et al. 2007). Since behaviour and environmental conditions trigger cortisol secretion, therefore quantification of cortisol has become an important diagnostic indicator for a point-of-care platform to map behavioural response pattern.

Total cortisol is accessible in two basic forms: 90% in bound state in serum and remaining 10% in free form. Out of total serum cortisol, 80% are bound to cortisol-bound globulin (CBG or Transcortin) and 10% with serum albumin (le Roux et al. 2003). CBG level shows abnormal variation during starvation, chronic nephrotic disorders, pregnancy, oestrogen therapy and while taking oral contraceptive pills; resulting in varying total cortisol level. Free cortisol is the sole biologically and physiologically active form that could reflect clinical status precisely and independently. Almost all the cortisol detection methods require significant levels of expertise, and sophisticated and expensive instruments as well as they lack real time data analysis. The static information is challenging for the doctors/technical personnel to predict accurate and quantitative diagnosis of the disease (Yaneva et al. 2009; Frasconi et al. 2009; Yang et al. 1994; Lewis and Elder 1985). Point-of-care devices are high in demand to pursuit best possible sensing platforms that could be portable, miniaturized, sterile, disposable, low power based, require minimum sample amount, consume least time while giving more precise and detailed information as well as the product must be economical enough to be easily accessible to poor and

unemployed population residing in remote locations (Ahn et al. 2004; Soper et al. 2006; Wang 2006; Chan et al. 2013; Loncaric et al. 2012).

5 Sources of Cortisol Detection

Detectable quantities of cortisol are secreted by the adrenal gland into the human circulatory system. Identification of these secretions can be performed using various bio-fluids such as saliva, blood, interstitial fluid (ISF), urine, sweat, tears and urine (Fig. 6) (Ruder et al. 1972; Shi et al. 2009; Yaneva et al. 2009). This section presents an assessment of some above-mentioned bio-fluids as detectors of cortisol quantities present inside the body.

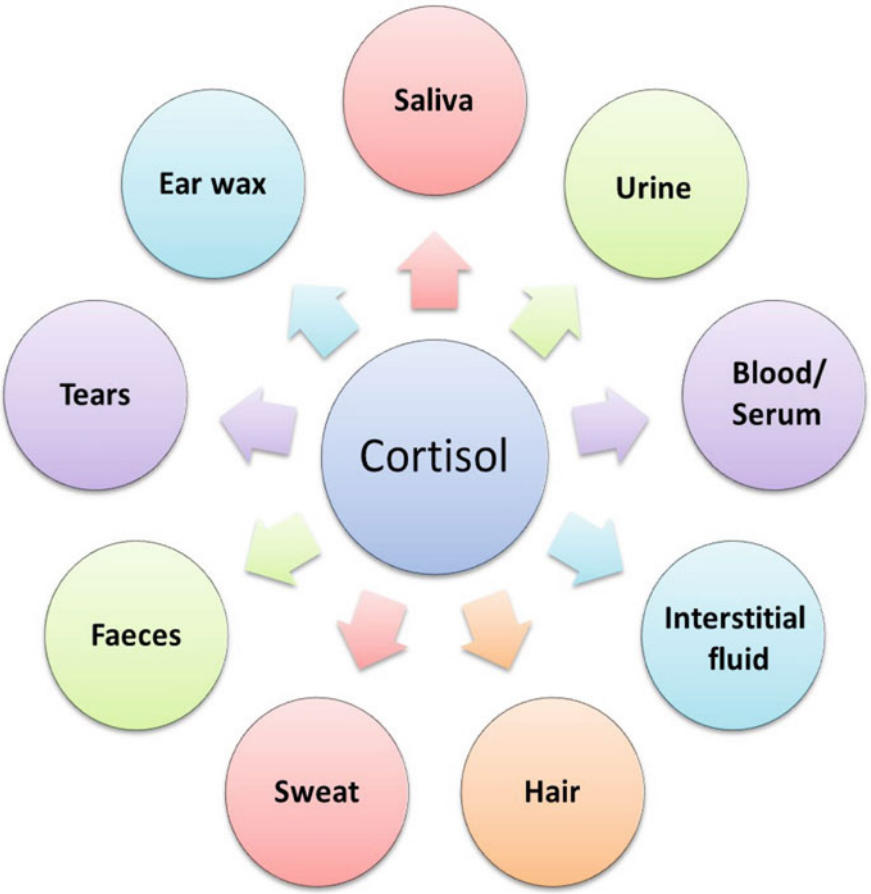

Fig. 6 Diverse sources for cortisol detection

5.1 Saliva

Cortisol detection and analysis using saliva as bio-fluid have recently gained the significant attention of the research community. It contains certain soluble biomarkers used for early disease detection. About 100–500 mL/day saliva is produced through human salivary glands, i.e. sublingual, submandibular, parotid and some minor salivary glands. The salivary constituents are synthesized and secreted by cells of salivary glands directly into saliva. Several serum components such as antibodies, cytokines, drugs and hormones are transported to saliva from capillary walls. Transportation of these molecules involves various mechanisms such as passive diffusion, active transcellular transport, pinocytosis and paracellular ultrafiltration (Fig. 7a). These mechanisms help in the transfer of molecule from the barrier of basement membrane and cells of the salivary gland to saliva. The speed of hormone transportation from blood to saliva is regulated by lipophilic layer of glandular epithelial cells and capillaries. In addition to being a non-invasive and painless collection of test sample (Van Caenegem et al. 2011), saliva based analysis has features which are absent in many other existing methods. First, there is a well-known correlation between the blood and salivary cortisol level. Second, unlike blood wherein 90% of the cortisol present is in a bound state, almost all the cortisol that exists in saliva is in an active or a free state. Third, unlike the 24 h urinary free cortisol (UFC) test, the method does not demand special arrangements for collection and handling of sample (VanBruggen et al. 2011). Because of all these comparative advantages, it has emerged as the most viable bio-fluid for detection and analysis of cortisol. In addition, with the standardization of collection procedures over the last few years, the results obtained using this technique are likely to be more robust to temporal variations as well as other external factors in comparison to other existing techniques.

However, there are two challenges that lie when using saliva as a bio-fluid for cortisol detection. The first challenge is obtaining sufficient concentration levels of

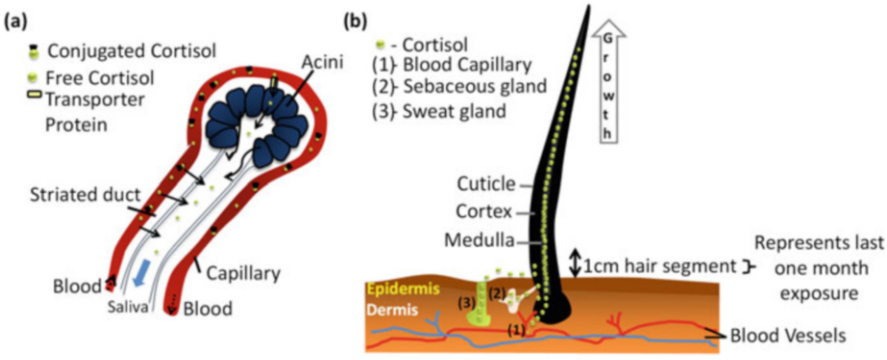

Fig. 7 Illustration of cortisol accumulation (**a**) in saliva and (**b**) in the medulla of hair follicle through active and passive diffusion process

cortisol for detection and analysis. Since saliva contains only the free cortisol or active cortisol, the concentration of obtained cortisol is generally less and lies in the range of 0.5 mg/dL to 0.05 mg/dL. This prerequisites the application of high sensitivity assays with high detection precision for efficient detection of cortisol concentration in saliva. Second, since the cortisol present in saliva is unstable at a room temperature, on-site storage and processing is another challenge.

In the literature, the authors have reported various applications of salivary cortisol assays. In particular, characterization of circadian rhythm using this technique has been reported by Price et al. (1983). Further, characterization of Cushing's syndrome and Addison's disease has been reported by Raff et al. and Lovas et al., respectively (Raff et al. 2011; Løvås et al. 2006). In addition, characterization of adrenal abnormalities and stress-related disorders has been reported by Granger et al. and Carpenter et al., respectively (Granger et al. 2012; Carpenter et al. 2011).

5.2 Blood

Among all the methods of bio-fluid sampling for cortisol detection, blood sampling remains the oldest one (Stevens et al. 2008; Gatti et al. 2009; Singh et al. 2014; El-Farhan et al. 2017). Measurement of cortisol levels in the blood is a two-step process. In the first step, the total amount of cortisol level in the blood is measured. Thereafter in the second step, the proportion of free cortisol level or the one in active state denoted by the Cortisol Free Index (CFI) is deduced using Coolen's equation (le Roux et al. 2003). The obtained values lie in the range of 25 mg/dL to 2 mg/dL. However, several other requirements such as sterility of equipment, specialized medical personnel and infrastructure for safe handling and storage, and no case of any infections put severe constraints on the reliability of the method. Further, since the method involves painful puncturing of veins, the stress produced while sampling often leads to a sudden rise in blood cortisol levels (Levine et al. 2007); thereby compromising the accuracy of the obtained results. As a consequence, blood remains the least desirable bio-fluid for sampling (Kaushik et al. 2014).

5.3 Interstitial Fluid (ISF)

Surrounding the cells in the human body, ISF is a major component of extracellular fluid. It provides a transfer medium for metabolites and proteins from blood to cells. It contains glucose, ethanol and cortisol molecules in proportions similar to blood. This removes the requirement of frequent re-calibration using blood sampling to acquire the concentration of the above-mentioned metabolites from ISF. However, since ISF is present beneath the skin, the process obtaining fluid samples for cortisol detection requires invasion through the skin. Various authors have reported different techniques to minimize this invasion and obtain ISF. A low-energy laser based ISF extracting system has been proposed by Venugopal et al. In this method, very small

micropores in the stratum corneum are created using a laser. Thereafter, a small amount of vacuum pressure is exerted and ISF is extracted from these micropores at a rate up to 10 μL/h (approx). Since, the insertion is just limited to stratum corneum, the method remains painless (Venugopal et al. 2008). In another work, they proposed a method for ISF detection by integrating the above laser extraction technique with an electrochemical detection system for cortisol detection. However, since in both the methods, the rate of extraction remains low at approximately 10 μL/h, the techniques remain inadequate for real time or point-of-care operations (Venugopal et al. 2011).

Taking a different approach from the techniques mentioned above, the authors El-Laboudi et al. and Prausnitz have proposed a method for ISF extraction using micromachined microneedles (El-Laboudi et al. 2012; Prausnitz 2004). ISF extraction from human skin using hollow microneedle array has also been reported (Mukerjee et al. 2004). A similar ISF extraction technique using microneedles made of glass for monitoring glucose has been proposed in 2006 (Wang 2006).

An interesting application of the above reported techniques would be in the design of wearable devices for slow but continuous sampling of body fluids such as ISF. However, to ensure sterility and protection from infections, the invasive component in these devices would have to be biocompatible and biodegradable.

5.4 Urine

Measurement of free cortisol, which is the active form of cortisol in the human body, can be obtained using 24 h urinary free cortisol (UFC) test. This test involves the collection and monitoring of urine samples over a 24 h period. Since excretion of hormones, salts and other waste chemicals takes place through urine, the concentration of these substances in urine can help in the diagnosis of any abnormality of organ generating the substance. For example, a drastic variation in cortisol levels in urine from the normal level which is 10–100 μg/24 h, is an indicator of adrenal abnormality (Brossaud et al. 2012).

Although, it is a non-invasive and painless method of obtaining body fluid for cortisol measurement, a long 24 h urine sample collection period severely limits the practical applicability of the 24 h UFC test. Since the collection and storage of these samples require special containers and a refrigeration unit, lab diagnosis not only becomes inconvenient, but also unreliable. In addition, other factors severely affecting the concentration levels of cortisol in urine such as pregnancy and diuretic medication not only limit the suitability of the method to non-real time detection, but also puts a question on the reliability of urine as a bio-fluid for cortisol detection itself.

5.5 Sweat

Sweat analysis using synthetic patches is a popular non-invasive method for diagnosis of certain bodily conditions such as the presence of excessive unwanted chemicals inside the body as well as genetic disorders such as cystic fibrosis (Prunty et al. 2004). Sweat as a bio-fluid for measurement of cortisol levels in the body has been proposed (Russell et al. 2012). In a more recent work, Xing et al. have reported a cloth based device patch for sweat collection integrated with an analytical device (Xing et al. 2013). However, there still lies a significant gap in the literature regarding cortisol detection in sweat.

In addition, since human perspiration is always interlinked with several external factors such as geographic location, physical activity and stress levels, ambient weather and individual genetic traits, reliability or accuracy of the method for measurement of cortisol levels is often a topic of debate.

5.6 Hair

Hair cortisol analysis can serve as a substitute to several other existing methods for monitoring the cortisol level in the body over a prolonged period of time ranging from a few days to several months (Koren et al. 2002). This is possible since hair sample collection and storage is not only easy, but can also be done by non-professional workers. In fact, the collected samples can be stored even at a room temperature and transported across a long distance. In addition, the sample collection procedure is entirely non-invasive and painless, reducing stress induced variations to the minimum. In toxological studies, forensic studies, doping and clinical assessment, the use of hair has attracted adequate or significant attention over the last few decades. In addition, because of the slow growth rate of human hair, this method can actually provide a picture of an individual's long-term cortisol secretion profile and subsequently individual's stress exposure level profile. As the hair grows, cortisol enters into the medulla of hair shaft from blood through passive diffusion (Fig. 7b). Thus, each preceding centimetre of hair corresponds to the gross serum free cortisol concentration accumulated in the month. Hair cortisol analysis is influenced by several factors. These factors include mental and physical health such as myocardial infraction and diabetes, age, sex, ethnicity and exposure of exogenous factors such as shampoo, hair dye and chemicals, etc.

In the literature, Koren et al. were the first to realize the possibility of cortisol detection using hair as the biological sample (Koren et al. 2002). In this work, authors have first collected the hair samples from wild hyraxes, thereafter employed a modified form of salivary ELISA protocol, to detect cortisol levels present in their body. The idea of using human hair as the biological sample for cortisol detection in the human body was first identified using salivary ELISA methodology (Sauvé et al. 2007). The obtained cortisol levels using human hair sampling were observed to lie in the range from 1.7 to 153.2 pg/mL. To further investigate for any correlation between this hair sampling method and other existing methods of cortisol detection,

the above obtained cortisol values were compared with that obtained using other biological samples such as 24 h urine, saliva and serum. Among the three, only 24 h urine reflected a positive correlation with the hair based sampling technique. Several other studies reported long-term documentation of hormones and drugs information that may be used for detection of stress or drug of abuse (Gao et al. 2010; Gow et al. 2010; Stalder et al. 2012; Manenschijn et al. 2011; Raul et al. 2004).

6 Cortisol Detection Techniques

There have been several approaches for the detection of cortisol from different sources of body fluids. Here is a short description of the techniques and their evolution in pursuit of better quantification of cortisol. Here, we present also in brief the shortcomings of the conventionally employed techniques and how these techniques can prove useful for integration into point-of-care applications. Table 1 highlights the various techniques used for cortisol detection along with their key features of detection and limitation ranges.

6.1 Chromatographic Techniques

The first in order or the oldest among all the techniques are chromatographic techniques for quantification of cortisol. This technique is mainly used for the separation of cortisol from different body fluids such as blood, urine, saliva, etc. by its selective adsorption on specific materials. Kabra et al. (1979) first described the use of liquid chromatography (LC) and detected the presence of cortisol in serum. Thereafter, Raul et al. (2004) developed a technique involving liquid chromatography (LC) and mass spectrometry (MS) to determine the cortisol and cortisone levels in hair samples of 44 subjects comprising both sexes. This method determined the levels of cortisol and cortisone affected by sex of subjects rather than properties of hair. This technology was further improved by Klopfenstein et al. (2011) for determining cortisol levels in plasma of blood. They studied cortisol production rate (CPR) by implementing steady-state infusion of deuterated cortisol. Moreover, MS helped in analysis of stable-isotope dilution and improving the selectivity by removing interfering components.

Another method which was introduced around LC was high-performance thin liquid chromatography (HPTLC) by Funk et al. (1981). This technique involved the use of thin layer of adsorbent material on substrates such as glass, plastic or aluminium foil. Also, this technique improved the detection of low concentration of cortisol conjugated with fluorescence molecule in plasma of human blood. In addition, HPTLC was used to detect more than one plasma samples in a single experiment. Thereafter, spectroscopic techniques such as ultraviolet, Raman spectroscopy, etc. were integrated with high-performance liquid chromatography techniques for rapid detection of cortisol. This coupling of techniques served better for detecting several samples at a single run but at the cost of expensive reagents and

Table 1 A Summary of the conventionally used techniques for cortisol detection highlighting their sensing platforms and sensing parameters

Cortisol detection techniques	Sensing platforms	Sensing parameters	Reference
Chromatography techniques	Liquid chromatography(LC) Liquid chromatography and mass spectroscopy (LC-MS) High-performance thin layer chromatography (HPTLC) HPLC-FLU (solid phase extraction)	LOD = ~2 ng/mL LOD = 1 pg/mL LOQ = 5 pg/mL LOD = 2.5pg/spot DR = 10–1000/spot LOD = 1 pg/mL DR = 0.22–7.45 mg/L	Kabra et al. (1979) Raul et al. (2004) Funk et al. (1981) Chen et al. (2010)
Immunoassay	Radioimmunoassay Electrochemiluminescence immunoassay (ECLIA) Chemiluminescence flow injection analysis (CL-FLA) Bioluminescent probe Competitive immunoassay based microchip integrated electrophoretic system Capillary electrophoresis (CE) and a laser-induced fluorescence based competitive immunoassay	LOD = 0.07/mL DR = 0.1–2.7 ng/mL DR = 0.36–36 ng/mL DR = 1–60 ng/dL LOD = 0.4 ng/mL DR = 1–60 μg/dL DR = 0.1 pg/mL to 100 pg/mL	van Aken et al. (2003) Shi et al. (2009) Kim et al. (2011) Koutny et al. (1996) Schmalzing et al. (1995)
Optical immunosensor	Quartz crystal microbalance (QCM) Microfluidic SURFACE plasmon resonance (SPR) immunoassay	LOD = 11 pg/mL DR = 5–100 pg/mL LOD = 0.27 ng/mL RT = 10 min	Ito et al. (2014) Mitchell et al. (2009)
Electrochemical immunosensor	Cyclic voltammetry and alkaline phosphatase (AP) enzyme Electrochemical impedance sensing (EIS) Single-walled carbon nanotubes (SWCNTs) based chemiresistive transducer Polyaniline(PANI)-Au nanocomposite label free electrochemical immunosensor Paper-based electrical biosensor Antibody-embedded on polymer coupled to field effect transistor (FET) Electro-reduced graphene oxide (e-RGO)	LOD = 0.36 pg/mL DR = 0.36 pg/mL to 0.36 ng/mL LOD = 14.9 ng/mL DR = 1 pg/mL to 10 ng/mL LOD = 0.36 pg/mL DR = 0.36 pg/mL to 0.36 ng/mL LOD = 0.64 ng/mL DR = 0.36 pg/mL to 3.6 μg/mL LOD = 3 pg/mL DR = 3 pg/mL to 10 μg/mL LOD = 1 ng/mL DR = 10 fg/mL to 10 ng/mL RT ≤1 min	Sun et al. (2008) Arya et al. (2010) Tlili et al. (2011) Arya et al. (2011) Khan et al. (2017) Jang et al. (2018) Tuteja et al. (2018)

analyte derivatization. Micellar liquid chromatography (MCL) was used by Hornilos et al. as an alternative to high-performance liquid chromatography (HPLC) since it offers more selectivity due to larger interactions of solutes with mobile and stationary phases (Izquierdo-Hornillos et al. 2005).

Further, Chen et al. made an attempt to improve the HPLC-fluorescence (FLU) method which was earlier developed by Zhang et al. to increase the detection performance by use of solid phase extraction (SPE) tip based electrospun nanofibres (Chen et al. 2010; Zhang et al. 2008). This technique determined the low concentration of free cortisol in saliva and solved the issues of the conventional system such as low extraction recovery, bad cleanup effect, etc. However, these techniques are still lacking the potential for specific determination at very low concentrations, portability and rapid detection time which undermines their application at point-of-care applications.

6.2 Immunoassay

The high specificity of antigen–antibody interaction is the most exploited strategy for determination of cortisol in specimens of body fluids. Due to this property, they are considered as gold standard for detection of specific marker and determination of analyte concentration. Radioimmunoassay was first reported for determination of cortisol in samples during the 1970s. However, due to the presence of radioisotopes involved in the process, there seemed a sharp decline from the 1980s for further usage in an immunoassay. Alternatively, fluorescence-labelled tags showed promising endeavour for use in the immunoassay. The measurement is done according to the fluorescence intensity detected which is proportional to the concentration of fluorescent tags. Different labels such as fluorescein isothiocyanate (FITC) (Kobayashi et al. 1979), horseradish peroxidase (HRP), biotin-streptavidin, a mixture of sulphuric acid and acetic acid have been used in cortisol immunoassay. The minimum cortisol level detected was 300 pM by using FITC-labelled cortisol immunoassay. During the years of labelled immunoassay, another technique caught attention for its potential namely electroluminescence immunoassay (ECLIA). This immunoassay is based upon optics of the chemiluminescent properties of intermediates. These intermediates undergo highly excitation state to produce an electrical excitation, thus producing light. ECLIA is therefore reported for several applications especially in diagnosis of cortisol detection for Cushing's syndrome, post-traumatic stress disorder (PTSD), obesity, athlete's performance. van Aken et al. integrated polyclonal sheep antibody in competitive ECLIA for measurement of cortisol in human saliva (van Aken et al. 2003). This added many advantages such as faster assay time, commercial availability of immunoassay and no pretreatment. The chemiluminescence (CL) property was improved by integrating flow injection assay (FLA) which further improved detection of cortisol in serum (Shi et al. 2009). More advancements were brought by Kim et al. (2011) through the fabrication of

bioluminescent probe for salivary cortisol detection. This was the first attempt at revealing the role of N-terminal extended ligand binding domain (HLBD) present in glucocorticoid response element in a reporter gene system and constructing two on–off switches for the molecular probe (Kim et al. 2011).

Competitive immunoassay integrated with electrophoresis for detection of free and bound labelled cortisol provided insights into another approach for immunoassay. Microchip was also integrated with the electrophoretic system (Koutny et al. 1996) such as capillary electrophoresis (CE) integrated with laser-induced fluorescence system (Schmalzing et al. 1995), CE integrated with amperometric detection using enzyme-substrate (HRP-3,3,5,5-tetramethylbenzidine dichloride (TMB)) (Jia et al. 2002). Among all the immunoassays that have been described so far, enzyme linked immunosorbent assay (ELISA) is the most sensitive labelled immunoassay and still widely used for protein concentration determination. The use of ELISA for cortisol detection has been used to validate the results obtained from modern techniques. Till date, many commercial available ELISA kits are directed towards the much lower limit of detection and a broader range of physiological cortisol levels. However, incorporating ELISA has its own demerits in view of point-of-care applications such as involving larger sample volumes and reagents, limited multiple assays, intricate assay steps and larger incubation periods.

6.3 Optical Immunosensor

The use of optics and label free immunosensing led to the development of another interest in the field of cortisol which offered lower detection limits, a broader range of detection and high selectivity. The method of cortisol detection techniques is based on surface plasmon resonance (SPR) and quartz crystal microbalance (QCM). Both techniques used are highly sensitive to binding of antigen–antibody interaction and offer much greater resolution in detection of cortisol in samples. In SPR, there occurs a shift in the resonating waves to higher wavelength upon adsorption of molecules on a conducting substrate. The principle of SPR works on the oscillation of valence electrons of the conducting substrate irradiated by light. This technique is highly essential in quantification of captured analytes with antibodies on substrate, which attracted its options for optimizing into point-of-care applications by reducing the optics and electronics involved. Whereas, in QCM the detection of cortisol is measured by resonating property of piezoelectric immunosensor (substrate) quantifying the antigen–antibody interaction. There occur micro-deformations at the piezoelectric crystal upon capture of analytes by the antibodies, which is further quantified as a change in the electrical signal. Mitchell et al. improved the performance of SPR with the development of cortisol-linker conjugate integrated in a microfluidic SPR immunoassay (Mitchell et al. 2009). On the other hand, Ito et al. developed a detection system comprising twin sensor chip on QCM that offered much lower detection limit of cortisol and broader range of detection (Ito et al.

2014). The cortisol detections are still limited to research laboratories which in future will find its place as commercially available POCDs.

6.4 Electrochemical Immunosensor

The introduction of label free immunosensing has opened up wide ranges of detection methods thus improving the speed, fabrication and analytical efficacy of the immunosensor for detecting a biomarker at point of care (Wan et al. 2013). Electrochemical immunosensor works on the principle of measuring electrical changes of a conductive substrate because of the adsorption of an analyte on the antibodies-functionalized surface. The microelectrodes which are integrated with the conductive substrate offer high sensitivity and lower detection limits. Sun et al. (2008) reported the use of microfabricated gold (Au) electrodes on which cortisol antibodies were immobilized. The further immunosensing was quantified by cyclic voltammetry (CV) based upon the reaction between alkaline phosphatase (AP) enzyme and antibodies in a p-nitrophenyl phosphate (pNPP) solution. Electric impedance spectroscopy was reported by Arya et al. (2010) for detection of cortisol where cortisol antibodies were immobilized on dithiobis (succinimydyl propionate) [DSP] modified interdigitated microelectrodes (IDμEs). It was further improved by the same group by developing ethanolamine(EA)/cortisol monoclonal antibody (C-Mab)/DTSP/Au based biosensor which improved the limit of detection and proposed its potential as a disposable cortisol detection in body fluids such as saliva, interstitial fluid (ISF), etc., catering to the needs of point-of-care application. Moreover, they introduced the use of electrophoretically fabricated polyaniline nanocomposite for immobilizing C-Mab/EDC-NHS to facilitate much higher order of detection and sensitivity compared to the previous immunosensors (Arya et al. 2011). For elaborating more on POC measurement, Tlili et al. (2011) developed a label free immunosensor where anti-C-Mab and cortisol analog (cortisol-3-CMO-NHS ester) were immobilized on single-walled carbon nanotubes based chemiresistive transducer (SWCNTs). Much advancements in microelectronics and circuitry design led to development of much smaller form factor immunosensors. A field effect transistor based cortisol sensor was developed by Jang et al. which addressed the issue of Debye screening length (λ_D) occurred from the sensing surface-electrolyte interaction (Jang et al. 2018); thus improving the detection limits of cortisol in artificial sweat in process. Thereafter, screen printed electrodes (SPE) came into the picture where miniaturization is necessary to meet the POC criteria. The use of graphene and its oxides in printing over substrate served as an attractive candidate for use in POCD. Khan et al. first attempted the use of graphene on paper-based electronic biosensor chip to detect salivary cortisol at POC. In this study, C-Mab is immobilized using DTSP and poly(styrene)-block-poly(acrylic acid) (PS_{67}-b-PAA_{27}) polymer and graphene nanoplatelets (GP) were used for biosensor design on filter paper (Khan et al. 2017). This added many

advantages over previously developed immunosensors such as excellent shelf life, faster detection time, no requirement of additional redox medium for electron exchange. Further, Tuteja et al. reported an electro-reduced graphene oxide (e-RGO) SPE which facilitated the first dual cortisol and lactate antibody-conjugated detection at the same time (Tuteja et al. 2018). It reported significant detection time such as less than 1 min that is quite revolutionary and beneficial at point of care. The discussed template offers a wide range of detection and possesses great potential in economic production of biosensor available at our hands reach.

7 Point-of-Care Diagnostics

Nowadays, the trending advanced standard of living and world globalization directly influence the health either at genetic or protein level and therefore they are considered as the major reason for various diseases. Since point-of-care (POC) devices may be involved in to treat anomalies specifically, these diagnostics are gaining high value in those research areas where specific and multiple replicability is required with high precision, financially economical and with less expertise required (Chan et al. 2013; Ahn et al. 2004; Yager et al. 2008; Loncaric et al. 2012; Wang 2006; Soper et al. 2006; Gracie et al. 2017; Price and Kricka 2007; Chandra 2016). For better diagnosis of the problem to completely eradicate or to permanently cure the disease, it is essential to monitor in real time. Point-of-care devices are in high demand for real time because of their miniaturized and compact sizes, minimized human error, easy to handle and economical as it required low volume of reagents and sample. For the detection of physiologically variable ailment at its initial stage for effective treatment within the physiological environment, wearable POC sensing devices are gaining attention nowadays, with promising results (Fotiadis et al. 2006; Hung et al. 2004; Chandra et al. 2017).

Traditional sensing devices and POC systems allow detection of cortisol precisely but have limitations of their availability at many places due to portability constrain, high cost, lengthy processing, long run time for analysis with requirement of trained personnel to operate. To overcome this situation, integration of these sensing platforms within microfluidic environment could enable an effective, and efficient product with certain outstanding features such as low cost, controlled environment mimicking physiological scenario in vitro, perfect fluidic control with high end repeatability of result.

Kumar et al. have fabricated an electrochemical immune-biosensor integrated with microfluidics involving micro-electro-mechanical system (MEMS) technology to reduce signal to noise ratio by efficiently regulating flow of liquid over gold (Au) nanowire. Functionalized Au nanowire is used to improve selectivity, sensitivity and to enhance the limit of detection of the sensor. Aligned gold nanowire acts as working electrode and covalent linkage chemistry has been used to couple cortisol antibodies. This biosensor response plot detected as linear in range of 10–80 μM. This setup was found to be simple and sensitive cortisol assay, relevant to biological fluids as well as serum cortisol detection (Kumar et al. 2007).

Yamaguchi et al. have proposed a miniaturized, portable, quick responsive, immune-chromatographic strip (5 × 1.5 × 50 mm^3) and reader (26.5 × 25.5 × 14.5 cm^3). For molecular determination of cortisol, a glucose oxidase-cortisol conjugate was synthesized on the strip (Yamaguchi et al. 2009). Yamaguchi et al. (2009) reported an improved version of POC that was non-invasive based salivary cortisol detection POC immunosensor with lateral and vertical fluid flow and control mechanism. They proposed an inverse correlation of cortisol concentration to glucose oxidase-cortisol conjugates detected electrochemically. The cortisol POC revealed salivary 0.1–10 ng/ml salivary cortisol computing 0.98 regression coefficient and 14% coefficient of variance (CV). The obtained regression value resembled very close to the value detected by commercially existing ELISA technique ($R^2 = 0.92$) (Masaki Yamaguchi et al. 2013).

A completely automatic low temperature co-fired ceramic based electrochemical immunosensing device for cortisol detection was established by Vasudev et al. (2013). They have proposed a computational fluid dynamics based a 3-dimensional microfluidic channel setup to confirm effective sample introduction following washing (Vasudev et al. 2013).

A miniaturized Potentiostat (LMP91000 chip) conjugated with Low Temperature Co-Fired Ceramics (LTCC) microfluidic manifold have been manifested by Kaushik et al. (2014) for the detection of cortisol at the level of picograms/millilitre with 5.8 μA (pg mL^{-1}) sensitivity. This device demonstrated cortisol detection in range of 0.01–500 ng/mL. The fabricated electrochemical cortisol immunosensor results were compared and validated using enzyme linked immunosorbent assay (ELISA) technique (Kaushik et al. 2014).

Complementary metal-oxide-semiconductor (CMOS) integrated in poly (dimethylsiloxane) (PDMS) based microfluidic immunosensor for quick and precise salivary cortisol estimation using optical detection method is devised by Pinto et al. (2017). They claim the device as quick responsive, non-invasive approach based on coating an immobilized antibody (Ab), a polyclonal anti-Immunoglobin G on a treated poly(dimethylsiloxane) surface. Cortisol in the sample is competed with (HRP)-labelled cortisol for Ab binding sites. HRP-labelled cortisol bound to capturing antibody conjugated with HRP enzyme reacts with chromogenic tetramethylbenzidine (TMB) to impart a blue coloured complex that is subsequently turned into yellow coloured solution upon adding an acid or any stop reagent. This yellow solution of HRP-labelled cortisol is quantified by measuring solution intensity through optical absorbance at 450 nm wavelength using CMOS photodetector (Pinto et al. 2017).

Onur Parlak et al. recently in July 2018 published an article proposing wearable biosensor platform for real time detection and analysis of cortisol from sweat. They have proposed organic electrochemical transistors (OECTs) integrated with biomimetic polymeric membrane. Organic electrochemical transistors (OECTs) are very eminent in the field of bioelectronics due to their extraordinary capacity to interface biology with electronics. Polymeric material has enough flexibility, chemical and physical stability in response to body temperature and certain deformations. Microchannels of the polymeric material are conductive and functionalized with a

cortisol sensitive membrane. Laser engraved microchannels have been created for quick and accurate sweat acquisition and exact delivery of sweat to the sensor interface resulting in non-invasive, user comfort and easily operated wearable point-of-care device (Parlak et al. 2018).

8 Available Products for Cortisol Detection

Irrespective of expanding usage of cortisol quantification during various psychological distresses and early diagnosis of diseases, its clinical efficacy is restricted due to absence of optimum technology platform for real time assessment and quantification of biological responses. Point-of-care platforms integrated with microfluidic tools could bring a breakthrough in terms of automated, rapid, with high precision, miniaturized wearable or easy to use devices to improve healthcare system. Various diagnostic analysers are available commercially; however, most of the tools are diagnostics for pets, wild animals, canines and felines as represented in Table 2. Only few POC platforms are available for human cortisol out of which four are listed in Table 3 along with their trade names, owner brand and the techniques used for development of these platforms. This shortage of appropriate tool for cortisol detections opens the opportunities to develop more precise and efficient cortisol sensing platform.

9 Summary/Conclusion

Cortisol is released from adrenal glands which are triggered by mechanism of HPA axis influenced by external environment. Cortisol secretion is essential in numerous body metabolism such as optimizing blood pressure, immune response, maintaining bone metabolism, glycogen catabolism for glucose synthesis. Correlation between physiological stress and cortisol secretion in patients suffering from certain ailments such as Addison's, Cushing's, insomnia and regular stressful routine is well established. Thus, real time cortisol level detection can contribute to early diagnosis of certain disorders. In this chapter, we have articulated various promising and established techniques as well as the methodologies implemented for early diagnosis of cortisol level and their sensitivity during physiological distress or imbalance in cortisol secretion from adrenal gland. Salivary fluid is found to be more efficient source for cortisol due to availability of unbound cortisol. Since free or unbound cortisol in saliva is only 10% of the total cortisol, it is therefore essential to establish techniques that must have the ability to detect even lowest level of cortisol efficiently and precisely. We have also illustrated several sources and sites of cortisol detection as well as the available commercial products for cortisol detection. Techniques based on non-invasive principles are gaining admiration as well as acceptance from large group of populations. Wearable biosensors miniaturized and integrated with microfluidics are revolutionary field of point-of-care diagnostics for cortisol detection.

Table 2 Cortisol detection devices available in market for animals and pets

Cortisol analysers	Manufacturing company	Test sample used	Highlighting features	Technique employed	Reference
VIDAS® cortisol S	Biomerieux	Serum, plasma or urine	Easy to perform, ready to use, have higher accuracy than gold standard radioimmunoassay (RIA)	ELFA technique (enzyme linked fluorescent assay)	Goyal et al. (2011), Proverbio et al. (2013)
SNAP® cortisol test	IDEXX Laboratories	Serum, plasma	Minimized nonspecific binding and enhanced results, ability to read weak positive results, multianalyte detection using single-SNAP device	SNAP ELISA technique	Gold et al. (2016), O'Connor (2015)
Element i immunodiagnostic analyzer	Heska	Serum, plasma	Immediate results, inclinic confirmation, completely automated Auto-dilution facility, rapid data management	Surface plasmon enhanced fluorescence (SPF) technology	InnovatioNews (2015)
AIA-360	Tosoh Bioscience	Serum, plasma	~36 test/h, first result in ~20 min, random access, simple touch screen operation, continuous processing	Competitive fluorescent enzyme immunoassay	Higgs et al. (2014)
Immulite 1000	Siemens Healthineers	Serum, plasma	Extensive menu, low cost, reliable Low volume detection	Random assay immunoassay	Higgs et al. (2014)

Table 3 Few point-of-care diagnostic products available for humans

Cortisol analysers	Manufacturing company	Test sample used	Highlighting features	Technique employed	Reference
VerOFy® and LIAM™ Cortisol	Goffin Molecular Technologies	Saliva and urine	Non-invasive specimens, rapid, easy to use, multiple disease detection from same sample, capable of working in non-traditional field settings	Immunochromatography	Miocevic et al. (2016)
i-calQ	i-calQ	Blood and saliva	Smartphone based solution, affordable, takes lesser time to detect, tests can be performed anytime and anywhere	Smartphone based lateral flow tests	You et al. (2013)
L'AuRa	LamdaGen Corporation	Saliva	Simple design for easy use, single or multiplexed target, rapid results, high sensitivity	ELISA	Dorn et al. (2007)
U-Check-It™	Bedell Innovations		Wearable device Monitoring of mood, cortisol, sleep, built in GPS tracker, compatible apps with android phones		Taj-Eldin et al. (2018)

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Receptors in Immunodiagnostics: Antibodies, Antibody Fragments, Single Domain Antibodies and Aptamers

Nachiket Shembekar

1 Introduction

Traditionally, assays for detecting proteins, carbohydrates and other organic molecules were carried out using specific antibodies generated against them in animals. Hence, such detection assays are called as immunodiagnostic or immunodetection assays. With the recent developments in the field of diagnostics, now it has also been possible to detect the analytes using nucleic acids such as aptamers and other similar molecules. Hence, collectively such detection assays have been termed here as immunodiagnostic assays. An ideal immunodiagnostic assay should be able to detect a target analyte from a complex sample such as blood, urine; it should be able to detect the lowest possible amount of the target in a sample, the assay should be easy to carry out and the assay has to be stable at room temperatures to be able to be carried out near the point of care. All such parameters rely heavily on one of the basic components of a diagnostic assay, a detection receptor. An immunodiagnostic method is basically an affinity based specific capture of the target molecules in a sample by the detection receptor. Conventionally, antibodies and other derivatives have been employed as detection receptors in the diagnostic assays. The detection of a desired analyte from a complex sample such as saliva, urine, tissues, etc. with a high affinity and specificity is only possible using biorecognition elements such as antibodies and other fragments, aptamers. This is due to the fundamental property possessed by the antibodies of interacting in a non-covalent way with the organic molecules like proteins, carbohydrates and other small molecules. In the context of antibody recognition, the organic molecule to which the antibody binds is commonly called as an antigen and the exact small regions where the antibody binds are called as epitopes. The unique biorecognition property of antibodies in turn can be attributed to their evolutionarily gained

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physiological function, that is, to specifically recognize foreign objects and pathogens entering the organism's body. Similarly, the nucleic acid aptamers that form tertiary structures can also bind to several organic molecules. This function of nucleic acids may also have been attained evolutionarily due to the physiological functions such as transcription and translation. The nature of an antigen–antibody interaction dictates the overall sensitivity and the specificity of the diagnostic assay. Hence, the desirable features of an ideal diagnostic assay such as lower limits of detection and high sensitivity can be achieved using high affinity biorecognition elements as the detection receptors. One of the greatest advantages of using antibodies in the immunodiagnostic assays is that the antibodies can be easily produced using animals as well as other recombinant technologies such as phage display, the knowledge for which is available for decades. These antibodies can be custom generated to recognize a specific epitope on a target protein or similar analyte molecules of choice. There are two key features of a typical immunodiagnostic assay: the formation of a complex between the receptor and the analyte and the detection of such complex formation. Immobilization of the detector molecules on the support matrices has transformed the way the immunodiagnostic assays are developed. The immobilization of the capture ligand helps in the generation of stable stationary antibody–antigen complexes which in turn facilitates their detection. To summarize, it is important to understand the structure, biochemical binding interactions, affinities and production process of biorecognition receptors for designing a diagnostic assay. In order to understand these issues, this chapter focuses on structural–functional aspects and methodologies to produce the detection receptors that are used in the immunodiagnostic assays such as whole mammalian antibodies (e.g. IgG), antibody derivatives (e.g. Fab and scFv fragments), single domain antibodies from camelids and sharks (e.g. V_HH and VNARs) and aptamers. The term 'whole antibody' has been used for an immunoglobulin molecule so as to differentiate between an antibody and the antibody fragments. Since the success of a diagnostic assay is based on the nature of its receptor.

2 Antibodies

2.1 Structure and Function of Antibodies

Antibodies or immunoglobulins (Ig) are globular proteins produced by the adaptive immune system of mammals, birds and certain other species that are used to specifically recognize and destroy a range of foreign objects such as pathogens, cancerous cells and allergens. The specific recognition of a target with high affinity and relative ease of production makes antibodies one of the widely used detecting agents in immunodiagnostics. The basic structure of an antibody consists of four polypeptides, two identical heavy chains and two identical light chains. Each heavy chain is bound to a light chain by a disulphide bond and other non-covalent interactions to form a heterodimer. Two such heterodimers are in turn bound together by disulphide bonds to form a monomeric structure of an antibody

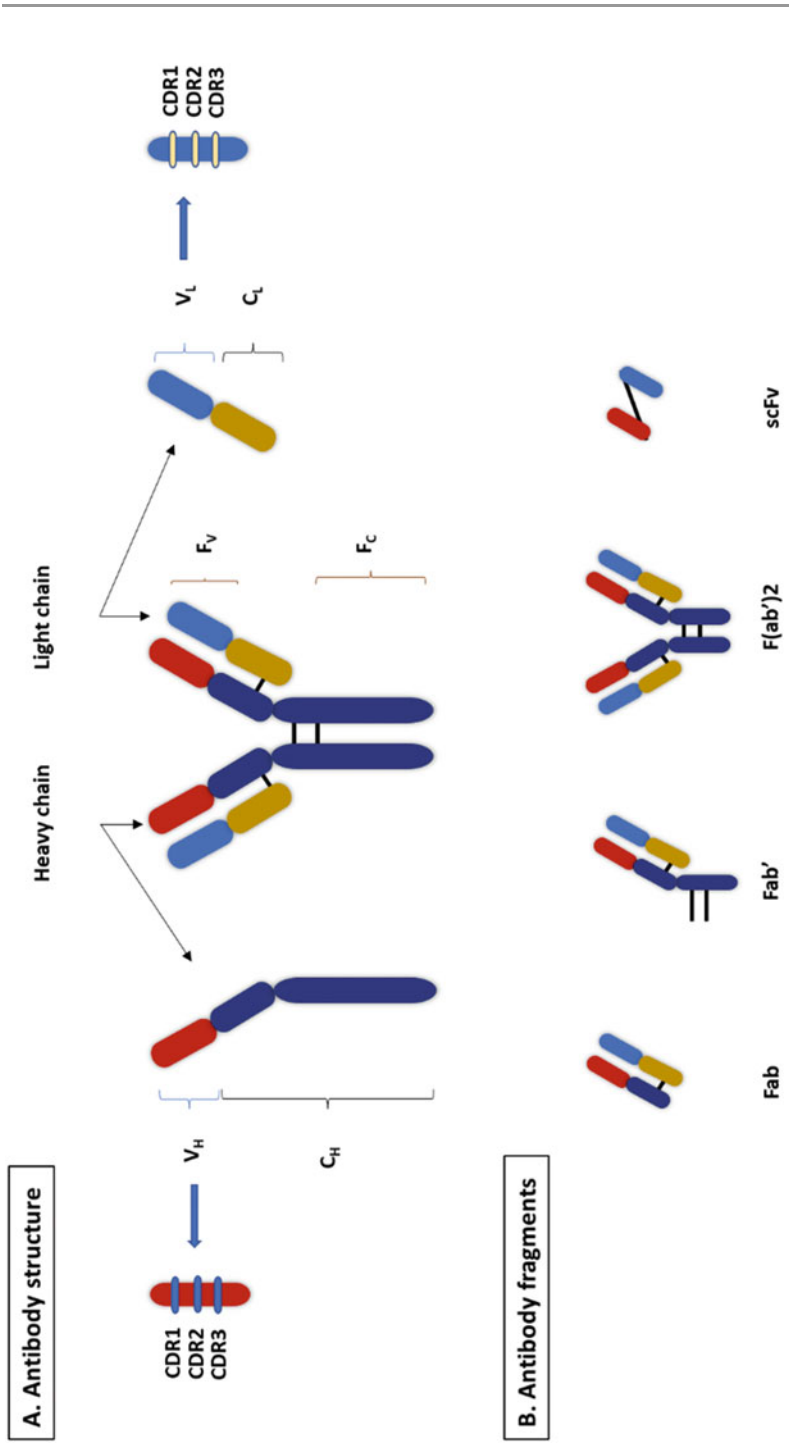

Fig. 1 The structure of an antibody molecule and various antibody fragments. (**a**) The whole antibody molecule is a dimer of heterodimers, made up of a heavy chain connected to a light chain via disulphide bonds (Black lines). Each heavy chain and a light chain have a variable portion (V_H, Red and V_L, Blue accent) and a constant region (C_H, Dark blue and C_L, gold). The V_H and V_L form the antigen binding fragment variable, F_V. The constant region of the heavy chain forms the

(Fig. 1a). In mammals, based on the molecular composition, the antibodies have been further classified into five main isotypes as IgA, IgD, IgE, IgG and IgM. The monomeric form of an IgG class antibody has an approximate molecular weight of 150 KDa. Each heavy chain and a light chain typically consist of an amino terminus variable region (V_H and V_L) and a C-terminal constant region (C_H and C_L) (Fig. 1a). Distinct stretches of 5–15 amino acids in the V_H and V_L regions show hypervariability in the sequence among different antibodies. These are called complementarity determining regions (CDRs) (Fig. 1a). The CDRs are involved in the interaction with the antigen or stabilizing the antigen–antibody interaction. Thus the V_H and V_L regions together form a fragment variable (F_v) that is uniquely specific for a particular antigen and hence is involved in specific antigen binding. The constant region (fragment crystallizable; F_c) confers additional cell-mediated functionality to the antibody such as opsonization and phagocytosis by other immune cells.

A typical antibody–antigen interaction is governed by non-covalent bonds such as electrostatic forces, hydrogen bonding, hydrophobic forces and Van der Waals forces. The complex formation between antigen–antibody is reversible and can dissociate depending on the strength of the interactions as well as the reaction conditions such as pH, temperature, etc. The overall strength of the interaction shown by antibody towards the antigen or vice versa is generally termed as affinity. The affinity of an antibody to a particular antigen is commonly expressed in terms of equilibrium dissociation constant (K_D). In simple terms, K_D is the concentration of the ligand required to saturate half of the available binding receptors. So, if the antibody is said to possess high affinity towards a particular antigen, it takes lower concentration of the antibody or the antigen to saturate the available binding sites. Thus higher affinity in turn means the value of the K_D will be lower. For a sensitive diagnostic assay, it is desirable to have high affinity antibodies that are capable of detecting lower concentrations of the antigens.

2.2 Production and Use of Antibodies in Immunodiagnostics

Antibodies are produced by the plasma cells of the immune system. Upon encountering a specific antigen, the B cells differentiate into either memory cells or plasma cells which then secrete antigen specific antibodies. Generating antigen specific polyclonal antibodies by immunizing animals has been one of the standard methods for obtaining antibodies for the immunodiagnostics. After the immunization,

Fig. 1 (continued) fragment crystallizable, F_C. The variable region contains three hyper-variable regions, that are responsible for antigen binding, called as complementarity determining regions (CDRs; CDR1, CDR2 and CDR3). (**b**) A Fab fragment is a monovalent antigen binding domain made up of $V_H - V_L$ and first constant region domains. A monovalent Fab fragment that also has some part of the Fc region and the functional thiol groups is called as Fab' fragment. A bivalent form of the Fab' fragment is called as F(ab')2. The V_H and V_L regions linked together via a short peptide linker are called as Single Chain Fragment Variable (scFv)

polyclonal antibodies can be obtained from the serum and other fluids of the animal. These antibodies can be further purified if required, using methods such as affinity chromatography. Polyclonal antibodies can be considered as a mixture of different antibodies secreted by various plasma cell clones that each recognizes a different epitope of the same antigen (Fig. 2a). Thus such polyclonal antibodies targeting multiple epitopes of the same antigen can make the immunodiagnostic assay more sensitive. However there are some disadvantages of using polyclonal antibodies. There can be significant variations in the immune response generated by the animals used for immunizations. This can be due to the differences in the genetic background of the animals or could simply be due to the immunological health of the particular animal. This can result in a lot-to-lot variation of the polyclonal antibody mixture. Secondly, the polyclonal antibodies can consist of a mixture of antibodies that are specific for the antigen as well as those that are cross-reactive with other antigens. This can make the diagnostic assay less specific. The later problem can be overcome to a certain extent by affinity purification of the polyclonal antibodies against the desired antigen or by prior cross-adsorption of the antibodies against the undesirable antigen.

The discovery of hybridoma technology by Kohler and Milstein in 1975 paved the way for generation of monoclonal antibodies (MAbs) specific for a particular antigen (Köhler and Milstein 1975). The hybridoma technology principally involves fusion of a primary B cell with a myeloma cell to create hybrid cells (hybridoma) that are immortal and can potentially secrete unlimited amounts of antibodies in the culture (Fig. 2b). Thousands of antibody secreting hybridoma cells can be generated by immunizing the animal with an antigen of choice and then using the B cells from the immunized animal to create hybridoma cells. Then by performing cloning or limiting dilution of single hybridoma cells a unique clonal population of cells (monoclonal) all of which secrete antibodies that recognize a single epitope can be created. In this way, MAbs specific for a desired antigen can be developed. Development of MAbs allowed for generation of uniform lots of antibodies without any variation. Also, the in vitro culture of hybridoma cells allowed for production of large quantities of antibodies without much dependence on the animals. The ability to produce custom designed MAbs for a specific antigen led to the development of specific and sensitive diagnostic assays that were not possible with the polyclonal antibodies. One of the disadvantages of using MAbs in the diagnostic assays is that if the antigen to be detected has undergone any mutations, it may no longer bind to the MAb and can thus escape the detection. To avoid such problems, a cocktail of MAbs can be used in the diagnostic assay to make it more specific and sensitive.

A modern more commonly used method of generation of antibodies is the phage display method (McCafferty et al. 1990; Winter et al. 1994). It is a method that combines recombinant DNA technology with the bacteriophage-bacterial expression system to discover novel, target specific ligands. Recombinant technology allows for generation of limitless diversity of the antibody ligands at the genetic level, whereas bacteriophage system allows for phenotypic expression/display of all such ligands (Fig. 3). Millions of filamentous bacteriophage that each display an antibody fragment on their outer coat protein are used to identify the antigen specific antibodies. In

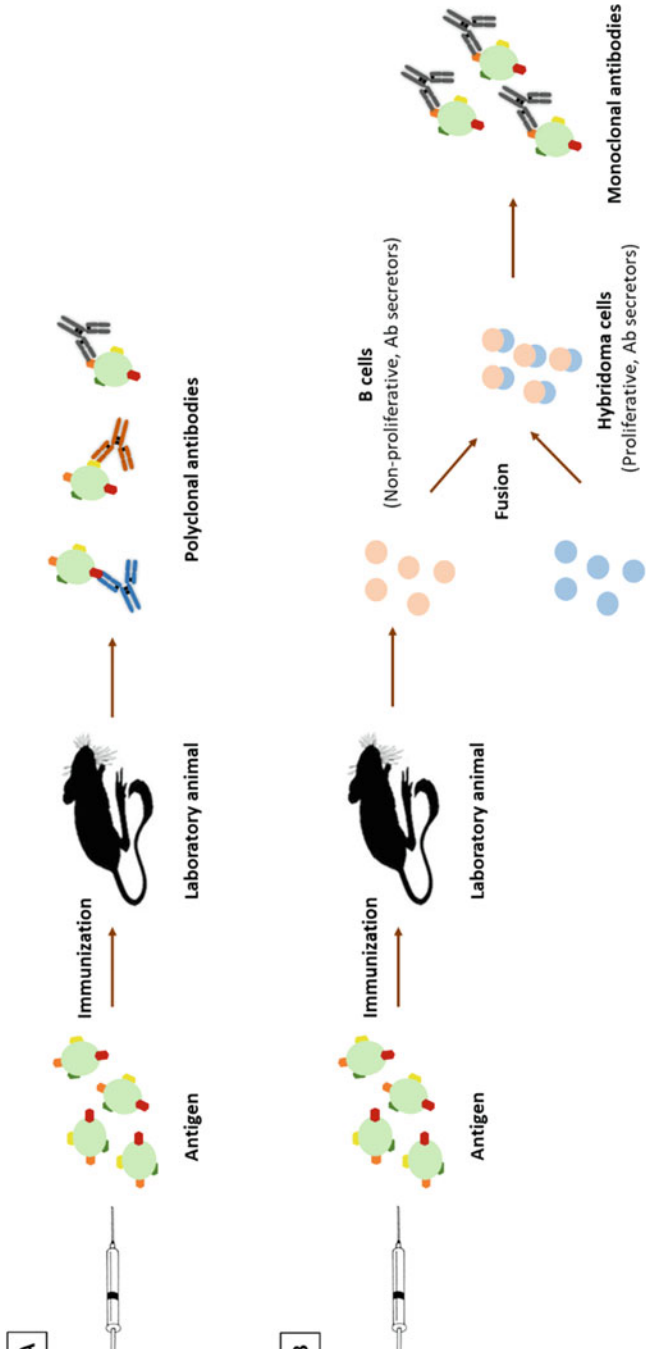

Fig. 2 Generation of antibodies from immunized animals. (**a**) Polyclonal antibodies can be obtained from the serum and other fluids of the animals immunized with the antigen. Polyclonal antibodies contain a mixture of antibodies that identify different epitopes of the same antigen. (**b**) Monoclonal antibodies that can recognize the same epitope of the antigen can also be generated from immunized animals such as mouse and rabbit. Fusion of antibody secreting cells with myeloma cells generates hybridoma cells that are immortal and can potentially secrete unlimited amounts of antibodies in an in vitro culture

the phage display screening methodology, an antibody encoding phagemid library is prepared by fusing the antibody V genes to the gene expressing one of the outer coat proteins (gIII or gVIII) of the bacteriophage. In this way, the antibody is displayed on the coat protein of the bacteriophage which in a way links the antigen binding phenotype of the antibody to its genotype. Depending upon the source of the antibody genes, the phage display library can be immune, naïve or a synthetic library. In the immune library, the sequences of the antibody ligands originate from the immune cells of the immunized animal or even from the human volunteers immunized or exposed to the antigen. In the synthetic phage display library, the antibody sequences are designed in silico which may or may not exist physiologically. A naïve library can have the antibody sequences derived from primary B cells that have not encountered the antigen. An antibody library that is a mixture of two or more of these types can also exist. However despite the origin of the genes, owing to the preparation of a bulk antibody library the heavy chain–light chain pairing of the parental antibody is most often lost. But this could also give rise to novel or better heavy chain–light chain ligands. The phage library displaying variety of ligands is then panned repetitively (3–8 cycles) against the desired antigen (Fig. 3). A panning is an assay similar to ELISA where the phages are allowed to bind to the antigen via the displayed specific antibody, while the non-specific phages that do not bind to the antigen get washed away (Fig. 3). After several rounds of such panning, specific antibody fragments get enriched which can then be sequenced to get the nucleotide sequence of the V region. After obtaining the sequences, the antibodies can be suitably expressed from prokaryotic or mammalian system. One of the advantages of using phage display method for generating antibodies is that it can be faster than the other traditional methods. Phage display method may also allow for discovering higher affinity ligands. This can simply be done by sequentially panning the phage display library with decreasing concentration of the antigen. Alternatively, the binding domains of the antibody ligands can be mutated at the genetic level during the generation of phagemid library. Although phage display technique offers several advantages over the other antibody generation methods, there are few disadvantages as well. Phage display method generally allows for generation of smaller antibody fragments such as Fab and scFv, which are not as stable as whole IgG. These smaller antibody fragments show low avidity of antigen binding as compared to the whole antibody. This may result in missing out on some of the best binders due to less avidity. Thus, ultimately the binders arising from phage display library may have to be expressed as a full length antibody or fusion proteins in vitro in mammalian cells, which can decrease their yield and increase the time.

The whole antibodies have been principal components of traditional diagnostic immunoassays which are based on principles of ELISA. In these assays, antibodies are immobilized on the solid support to capture the analytes in the reactions. Then a secondary antibody conjugated to an enzyme provides a colorimetric readout of the assay. There has also been progress with other detection methods where the secondary antibodies are conjugated to a radioisotope or a fluorophore to make the diagnostic assay more sensitive. The modern diagnostic assays are based on the lateral flow of the fluids that simultaneously allow for the capture and detection of

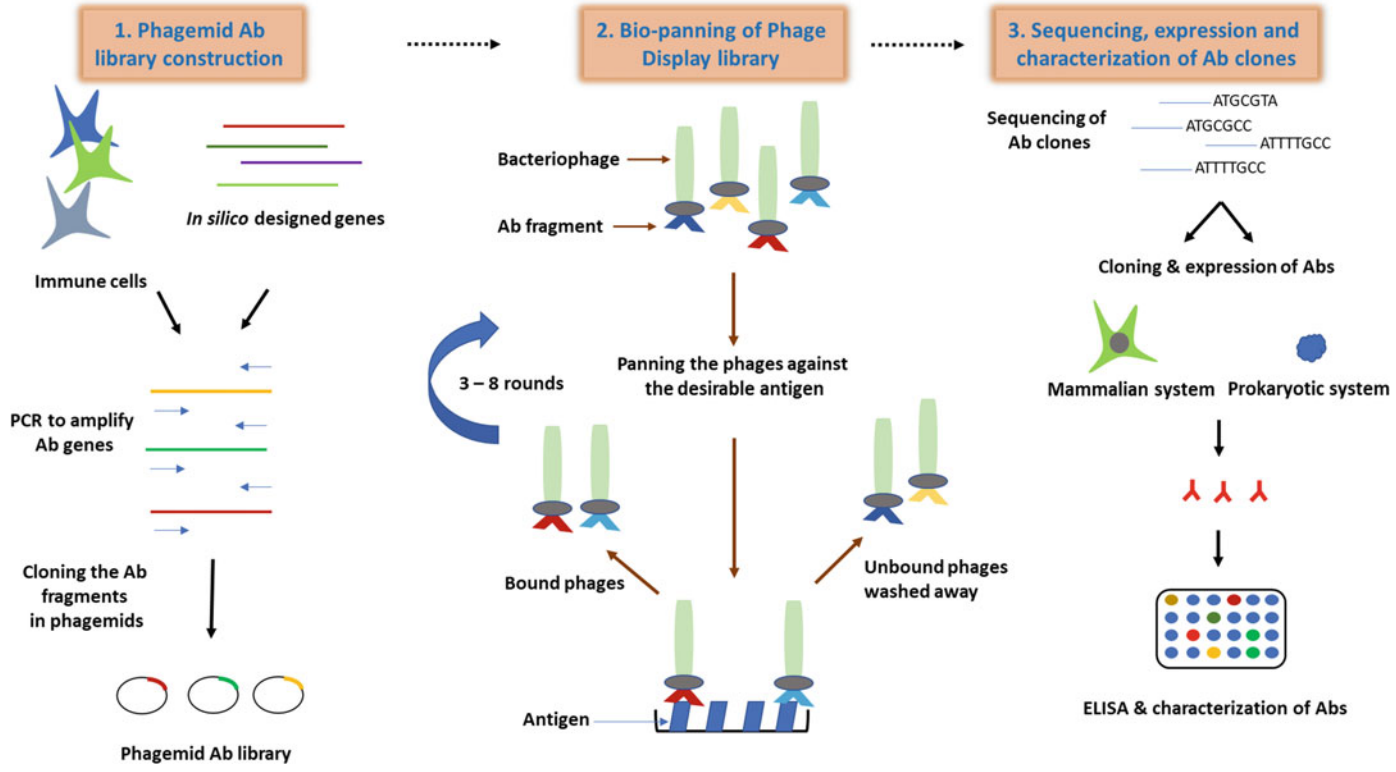

Fig. 3 Generation of antibodies from phage display library. The process of antibody generation using phage display library begins with a generation of a V domain library in phagemid. Introduction of such phagemids into a suitable prokaryotic host followed by infection of helper phages generates a bacteriophage library displaying antibody fragments on their surface. Such phages are then panned against a target antigen, wherein the unbound phages are washed away and the bound phages are amplified and rescreened. After finishing the panning rounds, the variable region antibody sequences can be sequenced from the selected phages. The selected antibodies can then be expressed as fragments or full length antibodies using suitable prokaryotic or mammalian expression system

the analyte in the sample. A well-known example of such kind of assay is a pregnancy detection kit which involves detection of human chorionic gonadotropin (hCG) hormone using specific immobilized antibodies. Another significant improvement in terms of the label free detection is the use of Surface Plasmon Resonance (SPR) technology. In this method, the antibodies are immobilized on the gold surface. When the antigen binds to the antibody, the change in the mass of the complex affects the light emission behaviour of the gold surface. To be able to be used as a detecting sensor in an immunoassay, the whole antibody needs to be immobilized on a solid support. In this immobilization process, the antibody activity is often lost due to the denaturation of the molecule or due to the improper orientation of the antigen binding site on the antibody after immobilization. One of the commonly used methods for immobilization of antibodies is by using protein G (from *Streptococcus* sp.) or protein A (from *Staphylococcus aureus*) (Shen et al. 2017). The protein G/A binds to the Fc portion of the antibody. So by pre-treating the solid surface with protein G/A, antibodies can be immobilized on the solid matrix of the diagnostic assay. Protein L (from *Peptostreptococcus magnus*) that binds to the light chain can also be used for immobilization. Another method for immobilization has been to make use of high affinity biotin–streptavidin interaction. The antibodies to be immobilized can be biotinylated and by pre-treating the solid surface with streptavidin the antibodies can be immobilized.

Antibodies have been used in the detection of various infectious diseases such as anthrax, leptospirosis; parasitic helminths; protozoans as well as novel emerging diseases. Antibodies have also been part of diagnostic assays in food and drug industry, environmental monitoring as well as in in vivo imaging. Recent developments such as antibody screening using droplet microfluidics technology can potentially allow for high-throughput antibody discovery in shorter time (Shembekar et al. 2016, 2018). This methodology neither requires immunization of the animal nor generation of a phage display library. The concept revolves around compartmentalization of a whole antibody binding assay in the aqueous droplets (~100 μm in diameter) that are surrounded by oil. Since the whole assay is compartmentalized, single B cells can be screened for antibody discovery on high-throughput scales. Because the antibody screening can be directly performed on single antibody secreting cells, primary cells can be employed in the screening process and the generation of hybridoma cells or the phages is not required. There has also been an attempt to use droplet microfluidics to preserve the heavy chain–light chain linkage by encapsulating single B cells in droplets (Rajan et al. 2018). Such microfluidics based technologies are being improved over the last few years and could provide a significant impetus to the field of antibody discovery. Each antibody discovery method has its own advantages and disadvantages. Thus a combination of traditional antibody generation methods along with the modern methods can offer a great novelty and diversity in generating the reagents for an immunodiagnostic assay.

3 Fab Fragments

3.1 Structure and Function of Fab Fragments

The whole antibody can be cleaved or synthesized into smaller antibody fragments that can still bind the cognate antigen. The smaller antibody fragments can allow for ease in production and their engineering. One of the most useful antibody fragments is called Fab (Fragment antigen binding) fragment. A Fab fragment is a monovalent antigen binding unit that consists of V_H and V_L domains, along with first constant region. Domain from heavy chain C_H1 and the constant region from the light chain C_L bound together with disulphide bonds (Fig. 1b). The molecular weight of a Fab fragment is approximately 50 kDa. A 55 kDa Fab monovalent fragment that also has some part of the Fc region and functional thiol groups is called as Fab' fragment. The functional sulphydryl group on the Fab' fragment can be used for alkylation or conjugation to other proteins and can also help in immobilization of the fragment on the solid supports used in the diagnostic assay. A bivalent form of the Fab fragment that also contains some part of the Fc region is commonly called as F(ab')2 fragment and is approximately 110 kDa.

3.2 Production and Use of Fab Fragments in Immunodiagnostics

The Fab fragments can be generated mainly via two ways: using enzymatic cleavage of the whole parent antibody or using recombinant DNA technology. A less commonly used method combines these two methods, where fragments are primarily generated using recombinant synthesis and then they are further broken down using proteolytic cleavage. In order to proteolytically produce Fab fragments from the whole antibody, a non-specific, thiol endopeptidase called Papain is used (Brezski and Jordan 2010; Vlasak and Ionescu 2014). Papain cleaves the peptide bond before the hinge region thus giving two Fab and the Fc region (Fig. 4a). If the intact Fc fragments are required, then this is the method of choice. Papain can also be used to generate F(ab')2. However, the production of F(ab')2 fragments is not very consistent due to the unavoidable over digestion of the parent molecule. Pepsin is another protease that cleaves the antibody molecule after the hinge region yielding an intact F(ab')2 fragment and degraded Fc region (Fig. 4b) (Vlasak and Ionescu 2014). The F(ab')2 fragment can then be mildly reduced to obtain monovalent Fab' fragments. The pure Fab fragments from both these methods can then be obtained by gel filtration or chromatographic methods. Another enzyme called Ficin is a preferred protease to generate F(ab')2 and Fab fragments due to its higher consistency of degradation of antibodies over the papain. In presence of varying concentrations of cysteines, the ficin can effectively either produce F(ab')2 fragment or 2 Fab fragments (Fig. 4c) (Mariant et al. 1991). The problem with the proteolytic cleavage method for generating antibody fragments is that some enzymes work only for particular antibody class or species and thus cleavage protocols have to be optimized for each and every antibody class. Another problem with the enzymatic or

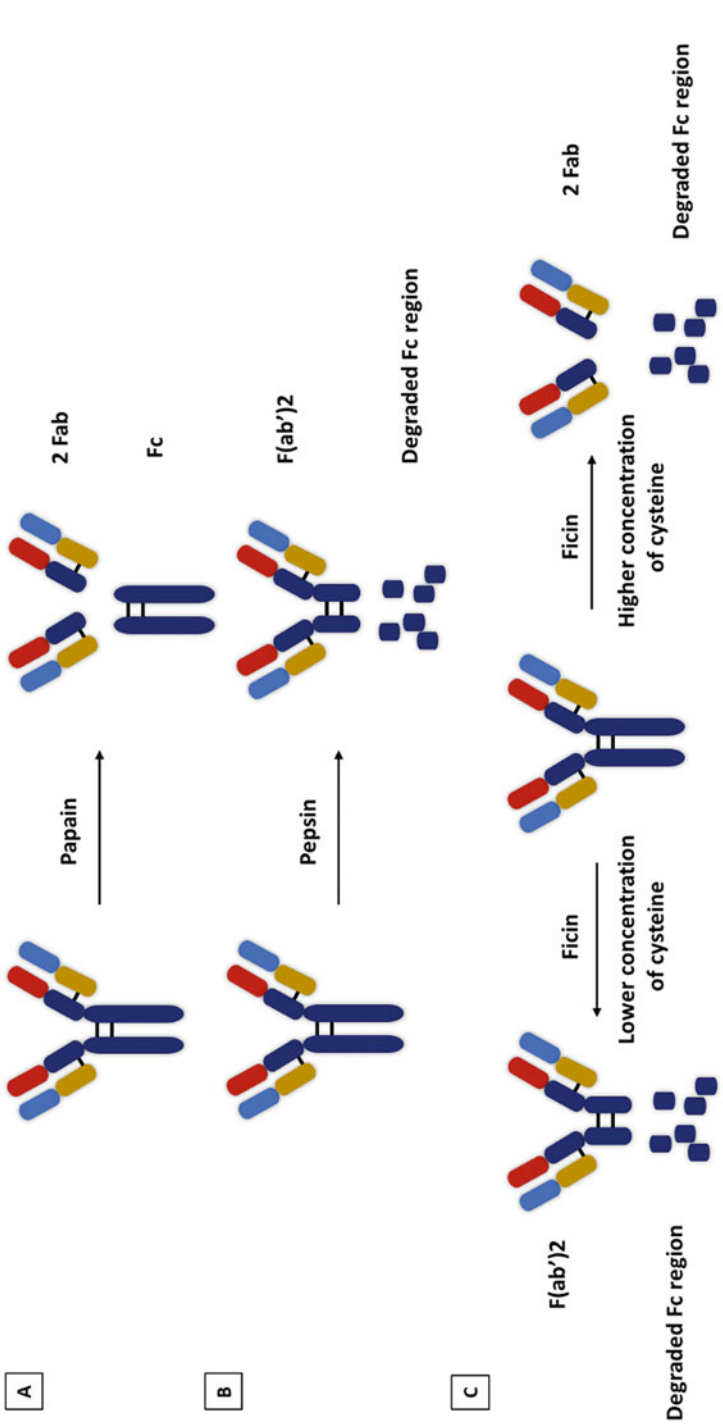

Fig. 4 Generation of Fab fragments from whole antibody by proteolytic cleavage. (**a**) A Fab fragment is generally cleaved off proteolytically from a whole antibody using a Papain enzyme. This process can also yield intact Fc fragment. (**b**) An enzyme called Pepsin is used to cleave F(ab')2 fragments from a whole antibody. Since the enzyme cleaves the whole antibody after the hinge region. (**c**) An enzyme called Ficin can cleave the whole antibody resulting into either F (ab')2 or Fab fragments, based on the concentration of cysteine residues

proteolytic cleavage method is that it can lead to the destruction of the antigen binding domains to some extent. In order to immobilize the Fab-related fragments on the solid surface, fusion with another protein is a preferred choice. Because it has been shown that the direct immobilization of the Fab-related fragments denatures their structure (Crivianu-Gaita and Thompson 2016). Several proteins such as albumin and protein G have been used to immobilize the fragments on the solid surfaces. Similar to the whole antibody molecule, these fragments can also be immobilized using biotin–streptavidin interactions. Due to their smaller size, they can be immobilized at higher densities as compared to the parental antibody molecule. Due to the presence of functional thiol groups, Fab and Fab' fragments could be immobilized on the maleimide or iodoacetyl activated surfaces (Welch et al. 2017). Out of all the Fab-related fragments, F(ab')2 fragments could potentially result in higher avidity antigen interaction, as compared to the Fab and Fab' fragments. This is simply because of the bivalent structure of the F(ab')2 fragment as compared to the monovalent Fab and Fab' fragments. The Fab fragments have been mainly used in immunoassays as compared to the Fab' fragments which have been used in the optical, electrochemical type of biosensors. Fab' fragments have also been used in the detection of insulin and TNF-α, which were fluorescence and luminescence based assay (Lee et al. 2005; Erikaku et al. 1991). In a competition based assay, the target analyte, phenytoin was already coated on the surface. Anti-phenytoin Fab' fragments were then allowed to bind to the target. When the sample flowed over the surface, presence of phenytoin displaced the Fab' fragment which resulted in the signal detection (Schiel et al. 2011).

4 Single Chain Variable Fragment (scFv)

4.1 Structure and Function of scFv

An antibody fragment smaller than the Fab fragment that contains only antigen binding V domains is commonly called as single chain variable fragment (scFv). The V domains of an antibody possess relatively weak interactions. Also due to the sequence variability in the Fv region, the fragment is unstable and its expression results in the dimerization of V_L domains. Hence, the V domains have been stabilized using different strategies such as disulphide linkers, peptide linkers and multimeric formations (Nelson 2010; Essen and Skerra 1993). A typical scFv contains a V_H and V_L domain connected from N to C-terminus of one another through a peptide linker. Thus the scFv retains the antigen binding function of an antibody but lacks the constant region. The most common linkers to produce monomeric scFvs are peptide linkers that are 15–30 amino acids long. The peptide linkers are commonly made up of Glycine and Serine residues such as $(Gly4\text{-}Ser)_3$ due to the flexibility that they impart to the antigen binding domains. However, charged residues such as glutamic acid and lysine have also been used by some groups to enhance the solubility of the scFv (Ahmad et al. 2012). The orientation of the V_H and V_L domains also plays a significant role in the functionality of the scFv. The comparative studies about the

scFv orientations using the same linkers have shown that the V_L—linker—V_H orientation provides higher antigen binding capacities than the V_H—linker—V_L. This is because the N-terminus of the V_L domain and the C-terminus of the V_H domain are involved in antigen binding (Desplancq et al. 1994). The linkers 0–10 amino acids tend to give rise to multimeric forms of scFvs. The multimeric forms can provide greater avidity for the antigen binding. These non-covalently linked dimers (diabodies) or trimers (tribodies) are difficult to generate in controlled conditions. Since the different multimeric formats are all in equilibrium. However, certain strategies such as PEG based fusion of two scFv fragments and inclusion of cysteine residues in the linker to allow disulphide bond formation could help in controlling the formation of multimeric forms.

4.2 Production and Use of scFv Fragments in Immunodiagnostics

Recombinant technology using phage display method is the most commonly used method for production of scFv fragments. A library of linked V_H and V_L regions is expressed in a bacterial system. As described above, with the help of a filamentous bacteriophage, this library of scFv fragments is then displayed on the phage particles. The phages are panned against the desired antigen to enrich the specific scFv fragments. The selected scFv fragments can then be expressed in soluble forms in suitable bacterial hosts. Using another approach, scFv fragments have been produced from hybridoma cells, splenocytes of immunized mice as well as from human peripheral B cells. In these cases, the V region of the antibody was cloned and expressed directly in a suitable prokaryotic host. To date, scFv fragments have been expressed using bacterial, mammalian, yeast, plant and insect cell systems. The bacterial system remains a preferred choice for scFv expression, since the engineering of the V region and the protein level modifications in the expressed scFv such as His- and other tags, fusion with another protein are easy to perform. There are different factors to be considered for the expression of the scFv fragments in the bacterial system. Expression of these fragments in the cytoplasm of the cells results in the production of the scFv protein as an insoluble aggregates in the inclusion bodies. The properly folded scFv fragments then have to be renatured from these inclusion bodies. Alternatively, the fragments can be directed using a signal peptide to be produced in the periplasmic space of the cells that lies between inner and outer membranes of gram-negative bacteria. This method of expression allows correctly folded scFv fragments to be produced in the oxidizing environment (Ahmad et al. 2012).

One of the major advantages of expressing scFv fragments in bacterial system is that they can be conveniently expressed as a fusion protein along with other target proteins. This can be useful for immobilization of these scFv fragments for an immunodiagnostic assay. For example, scFv fragments expressed with streptavidin peptide were immobilized on the biotin surfaces and scFv fragments fused with His-tags were immobilized on the solid surfaces using zinc- iminodiacetic acid (IDA) method or Ni-NTA coated gold surfaces (Crivianu-Gaita and Thompson

2016). Such methods non-covalently immobilize the scFv fragments on the solid surfaces. Similarly, using various other strategies scFv fragments have been covalently immobilized on the solid surfaces. Such strategies include modification of the C-terminal thiols and addition of reactive N-terminal Serine to the scFv fragments (Ros et al. 1998). Most of the strategies involving covalent immobilization of the scFv fragment on the solid surface give rise to a correct orientation of the scFv, with the antigen binding domain accessible to the antigen. One exception to this rule is with the covalent immobilization using free amines on the scFv for coupling on the solid surfaces, which may result in higher density of the immobilized fragments but does not ensure correct orientation (Howell et al. 1998).

As a diagnostic receptor, scFv fragments offer several advantages such as they are easier to produce in bulk, convenient to engineer, different immobilization strategies are well known and can be immobilized at higher densities on the solid surfaces as compared to the whole antibodies which means a sensitive diagnostic assay. scFv fragments have reportedly been successfully used as a diagnostic biosensors in the diagnosis of various infectious diseases and pathogens such as cholera, influenza virus, *Bacillus anthracis,* Hepatitis B virus and *Entamoeba histolytica*. scFvs from Chicken origin have also been demonstrated as useful biosensors for detecting *Mycobacterium bovis* (Ahmad et al. 2012).

5 Single Domain Antibodies

5.1 V_HH Antibodies: Structure, Function and Use in Immunodiagnostics

Certain mammals such as camels, llamas and dromedaries produce antibodies with only heavy chains and lacking the light chains. These antibodies are called as single domain antibodies or heavy chain only antibodies (HCAbs). Typically these antibodies contain two heavy chains joined via disulphide bonds. Such HCAbs usually have one variable domain (V_H) and two constant region domains (C_H) (Hamers-Casterman et al. 1993) (Fig. 5). The isolated antigen binding V_H of such HCAbs is commonly known as V_HH (variable domain of the heavy chain of HCAbs) or Nanobody® (Ablynx). The finger-like projection of N-terminal V_H domain is responsible for antigen binding. The molecular weight of a V_HH antibody is approximately 15 kDa, making it one of the smallest antibody fragments. One of the major advantages of V_HH antibody is that the smaller antigen binding area allows for binding to epitopes that are not accessible by other antibody formats such as catalytic sites of enzymes. From a therapeutic point of view, these antibodies are useful as they can penetrate into tissues. Due to the presence of hydrophilic amino acids in the framework of V_HH antibodies, they show higher solubility as compared to the classical V_H fragment (Muyldermans 2001). The V_HH antibodies have been mainly produced either from naïve or immunized phage display libraries. After immunizing the camelid with a desirable purified antigen or crude protein extracts or denatured antigens, V_HH fragments can be easily cloned into a phage display library. The

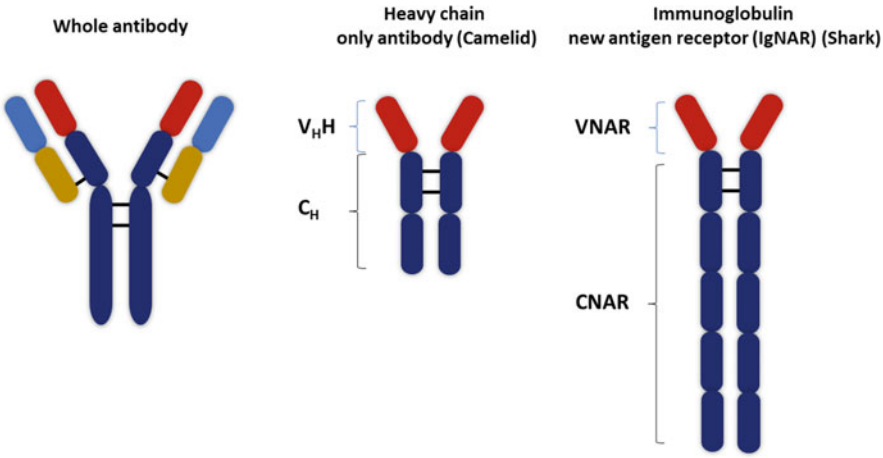

Fig. 5 Single domain antibodies from camelid and shark species. The human IgG antibody is a dimer of heterodimer made up from a heavy and a light chain. The antibodies found in camelid and shark animal species are made up of two heavy chains only. The camelid antibody consists of one variable and two constant regions, whereas shark antibody consists of one variable and five constant region domains in each heavy chain domain. The variable regions of these antibodies called as V_HH and VNAR are one of the smallest antibody fragments with a molecular size of about 15–12 kDa, respectively

antigen specific V_HH fragments can then be obtained by panning this phage display library against the target antigen. Due to their monomeric structure, small size and solubility they can be easily expressed using prokaryotic systems. Owing to the small size of about 450 base pair of their single coding exon, their genetic engineering is also easier (Muyldermans 2001). V_HH antibodies have also been generated using other display systems such as bacterial display system, ribosome as well as yeast surface display. V_HH antibodies are suitable biosensors due to their easier production and robust structure that can withstand harsh environmental conditions. They can be immobilized at higher densities as compared to the conventional antibodies, making the diagnostic assay more sensitive. Conversely, due to the smaller size of V_HH antibodies, they are likely to be inaccessible to the antigen after immobilization on the solid surface. Also, their intrinsic hydrophilicity can be problematic for their immobilization. Hence, hydrophobic C-terminal peptide tags such as his or myc or fusion to Fc portion has been used for their adsorption on the solid surfaces, with the Fc region providing the best results (Saerens et al. 2005). V_HHs have been used in diagnosis of infectious pathogens, parasites, cancer antigens as well as toxins. V_HH antibodies were isolated using an immune library against Trypanosome parasites which cause African sleeping sickness and Chagas disease. Some of the novel nanobody clones that were obtained were specific for the immunized trypanosomal antigen, whereas some clones showed genus specific broad reactivity. The broad reactivity of the clones was due to the penetration of the V_HH antibodies to access the conserved residues (Saerens et al. 2008). In certain

cases, V_HHs have shown more specificity as compared to conventional antibodies. For example, V_HHs have successfully been used to specifically distinguish between *Brucella* and *Yersinia* species in livestock (Abbady et al. 2012). Biotinylated V_HHs have also been employed as capture ligands on streptavidin beads to identify CEA biomarkers in cancer patient's sera (Even-Desrumeaux et al. 2010). A V_HH based agglutination reagent has also been developed for HIV diagnosis. A V_HH that is specific for red blood cell antigen is fused to the HIV p24 antigen. The presence of anti-p24 antibodies in patient sera then mediates red blood cell agglutination reaction (Habib et al. 2013). Such kind of diagnostic assays can be developed for various other pathogens. One of the advantages with V_HH antibodies is their thermostability. This has also been demonstrated by using anti-caffeine V_HHs which showed binding to caffeine even at 70 °C in the hot beverages (Ladenson et al. 2006).

5.2 Variable New Antigen Receptors (VNARs): Structure, Function and Use in Immunodiagnostics

Similar to the camelid animals, certain species of shark fishes also produce antibodies with single variable domains which are called as immunoglobulin new antigen receptors (IgNARs). These naturally occurring IgNARs are found in many different shark species such as nurse sharks, wobbegong sharks, smooth dogfish, banded hound sharks and some other cartilaginous fishes. IgNARs have been shown to function as immune response mediators similar to antibodies in these fishes. The intact IgNAR is made up of a disulphide linked homodimer of two chains each containing a variable new antigen receptor (VNAR) that is responsible for antigen recognition and five constant domain new antigen receptor (CNAR) (Kovaleva et al. 2014) (Fig. 5). The commercial research and development of VNARs has been under propriety control of some of the companies. One of the companies that has proprietary of producing VNARs by immunizing shark fishes, Elasmogen Ltd., has a trademark name of soloMERs™ for the VNARs. Another company that is involved in the research and development of shark VNARs is Ossianix Inc. The VNARs consist of two CDRs: CDR1 and CDR3, whereas CDR2 is almost absent. Usually CDR3s of VNARs are longer (15–17 amino acids) and structurally more diverse than the human IgGs. Interestingly, there are two other hyper-variable regions (HV2 and HV4) that are also known to participate in either antigen binding or in stabilizing the antigen-VNAR complexes, apart from CDRs (Barelle and Porter 2015). The VNARs are thought to be similar to the V_L of mammalian light chain or T cell receptor V regions. However, in terms of sequence homology to human V_H, VNARs are very distant with only 25% cross conserved amino acid residues (Roux et al. 1998). The VNARs are the smallest antibody fragments with a size of about 12 kDa. Due to such a small size they can efficiently recognize cryptic epitopes which cannot be accessed by other antibody fragments. VNARs contain many hydrophilic residues as compared to the conventional antibodies thus making them highly soluble and hence easy to express in bacterial system. VNARs are very stable molecules which can withstand temperatures of up to 100 °C and also extreme

conditions of pH such as 1.5, without much loss in activity (Steven et al. 2017). This is partly because of the presence of significantly higher numbers of cysteine residues in the VNAR structure. This stability also makes them highly suitable as diagnostic agents, where the diagnostic assay needs to be carried out near the point of care or the reagents need to be transported to distant locations. Similar to other antibody fragments, VNARs can also be commonly produced using prokaryotic system such as phage display system or soluble proteins. VNARs can be produced from naïve phage display library or using an immunized library. There are facilities available which can immunize shark fishes with the desired antigen. Thereafter the VNAR sequences can be amplified using PCR and cloned to produce immune phage display library. By panning such phage display library of VNARs, antigen specific single domain antibodies can be obtained. Some of this technology is under proprietary and patent control of some commercial companies. The shark VNAR domains have been reportedly used to detect markers from viral diseases and viruses such as Ebola virus haemorrhagic fever (EVHF), Sudan virus (SUDV), Tai Forest virus (TAFV) and Zaire Ebola virus with high sensitivity (Leow et al. 2017). VNARs have also been used to detect toxins such as staphylococcal enterotoxin B, ricin, botulinum toxin A and cholera toxin (Liu et al. 2007a, b). A VNAR isolated against AMA-1 protein of malarial parasite showed high binding affinity through its CDR3. The binding specificity of this VNAR was comparable with commercially available polyclonal and monoclonal antibodies as well as other smaller antibody fragments. In addition, the purified recombinant VNAR was also observed to show binding to the antigen at 45 °C as well as retained the refolding properties up to a temperature of 85 °C. Moreover, the VNAR also showed high stability under the proteolytic environment of murine stomach tissues (Griffiths et al. 2013; Henderson et al. 2007). Owing to the superior heat stability, negligible non-specificity and ability to detect cryptic epitopes, VNARs are being recognized as potent biosensors for diagnostic applications.

6 Aptamers: Structure, Function and Use in Immunodiagnostics

It is well known that nucleic acids can form tertiary structures that can bind specifically or non-specifically to proteins, nucleic acids or other organic molecules. Single stranded DNA or RNA chains which are selected by in vitro evolution to show binding to protein or other antigens are termed as aptamers. The aptamers show comparable affinities to the target antigen, similar to antibodies, in the range of nano- to pico-molar (Jayasena 1999). Aptamers are of the size of 1–2 nm, which allows for their immobilization at very high density on the solid surfaces resulting into superior sensitivity. The aptamers are developed using a selection process called as systematic evolution of ligands by exponential enrichment (SELEX) (Tuerk and Gold 1990) (Fig. 6). In this methodology, a large random library of single stranded nucleic acids (RNA or DNA) is screened against a target of interest. Those nucleic acids that bind to the antigen are then separated from the target using various

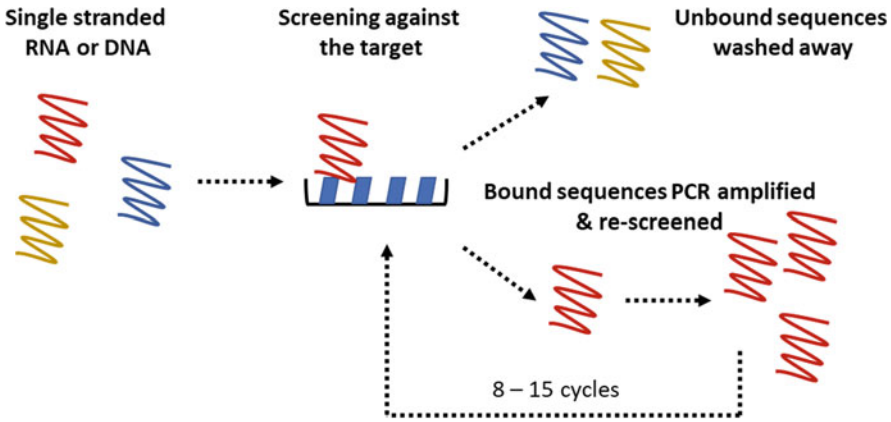

Fig. 6 Systematic evolution of ligands by exponential enrichment (SELEX) process. Single stranded RNA or DNA molecules called as aptamers that show binding to various organic molecules are generated using a process called as systematic evolution of ligands by exponential enrichment (SELEX). A random library of single stranded nucleic acid molecules is screened against the target antigen. The unbound aptamers are washed away, whereas the bound ones are eluted out. The selected aptamers are then amplified using PCR and then rescreened against the antigen. The screening cycle can be repeated for 8–15 rounds. The selected aptamers can then be chemically synthesized

methods such as affinity chromatography, size exclusion chromatography or electrophoresis. The selected nucleic acid molecules are then amplified and repeatedly screened against the target (up to 15 cycles) (Fig. 6). The generated aptamers using the SELEX process are generally 70–80 nucleotides long, but they can also be shortened by 20–30 nucleotides by removing regions which do not participate in the functional binding. In the end, once the desired aptamer sequence is obtained, the nucleotides can be synthesized chemically within a short period of time. The SELEX process has also been improved over the last few years that allows for selection of aptamers with very high specificity and affinity. For example, it is possible to increase the affinity of the aptamers by using only the specific short regions or domains of the target analyte in the subsequent screening rounds allowing for high affinity binding (Keefe et al. 2010). Some counter-SELEX processes also allow for selection of highly specific aptamers by removal of cross-reactive aptamers that can bind other structural analogs (Jayasena 1999). Aptamers are susceptible to nuclease degradation during the immobilization or the subsequent steps of the test assay. Hence, various strategies have been developed to prevent nuclease degradation as well as to increase the stability of the aptamers such as addition of amine or fluorine or PEG groups (Pieken et al. 1991). Aptamers have been generated against variety of bioorganic targets such as metal ions, amino acids, antibodies, antibiotics and others (Crivianu-Gaita and Thompson 2016). Aptamers have also been shown to differentiate between presence or absence of reactive groups such as hydroxyl and methyl (Crivianu-Gaita and Thompson 2016). Aptamers have been shown to be very sensitive in the detection of analytes, with capability of detection of as little as

250 pM of IgE antibody and 73 pM of thrombin molecules (Wang et al. 2015; Maehashi et al. 2007).

7 Comparative Analysis of Different Biosensors and Future Perspectives

The ease of production of the biosensors and their suitability in immobilization on the solid surfaces are some of the most important criteria in the selection of a biosensor for a diagnostic assay. In order to select the best biosensor for a diagnostic assay, various parameters such as specificity and affinity towards the analyte, stability and the ease of production are needed to be considered. Even though the individual biorecognition receptors can have better affinities or specificities on a case to case basis, all these parameters can be compared in a generalized way to select the best biosensors (Fig. 7).

In terms of ease of production and cost effectiveness, out of all the biosensors that have been discussed in this chapter, Fab fragments are overall easiest and cheapest to

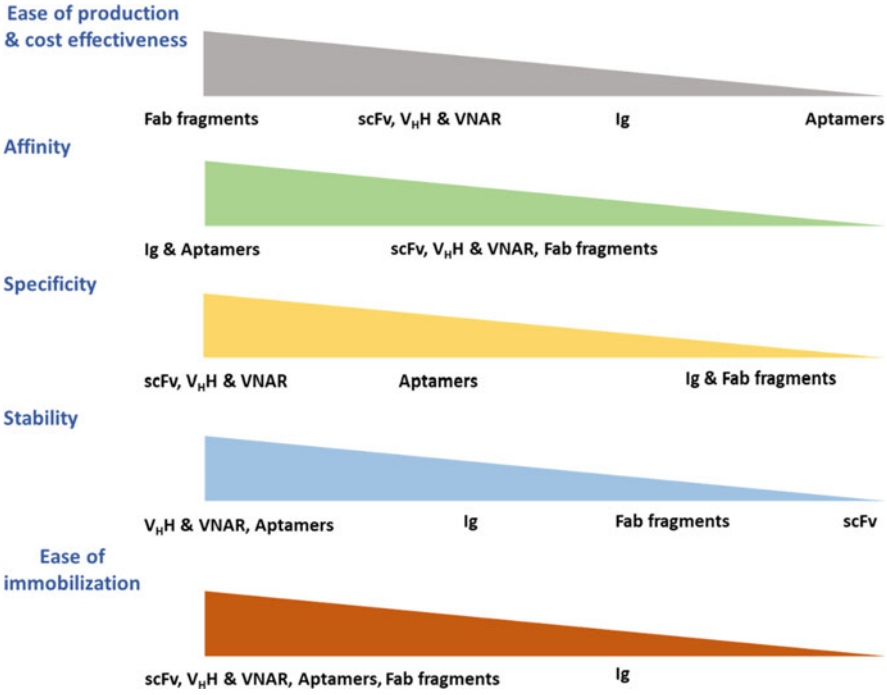

Fig. 7 Generalized comparative analysis of different receptors. The whole antibody (Ig), Fab fragments (Fab, Fab', F(ab')2), scFv, V_HH and VNARs and aptamers have been compared considering the parameters such as ease of production and cost effectiveness (Fab >> aptamers), affinity (Ig >> scFv & others), specificity (scFv >> Ig), stability (V_HH and VNARs >> scFv) and ease of immobilization (scFv >> Ig)

produce. Since these fragments are obtained by proteolytic or chemical cleavage, once the parent antibody is generated, the downstream cleavage methods can be applied in few days. The drawbacks with this methodology are the loss of functional activity of the fragments and the need to have large amounts of the parent antibody for this process. At the second number in terms of ease of production are the biosensors which are produced using phage display library, such as scFv and single domain antibodies that can be considered easiest to produce. Even though the generation of the phage display library is a laborious process and requires skilled manpower, once the library is generated, it can be used to generate antibody fragments against various different antigens. Also, in the downstream production, if the antibody fragments are expressed using prokaryotic system, then the overall manufacturing process becomes relatively easy. The whole antibodies that are produced either from animals or using phage display library are relatively difficult to produce. The animal immunizations and the subsequent antibody screening processes are laborious as well as costly. The whole antibody expression using the mammalian system makes the overall process even more laborious, even if the antibodies are screened using phage display library. The aptamers are costliest to produce due to the time consuming SELEX process. To identify aptamer sequences, nucleotide sequence library has to be prepared and the library needs to be screened repeatedly against the target, followed by PCR and purification of amplified products after each rescreening. However, once the desired aptamer sequence is obtained, its chemical synthesis can take few days.

In terms of affinity of the biosensing elements, aptamers and whole antibodies can be considered as the top choices. The whole antibodies isolated from immunized animals with repeated immunizations can attain affinities in the pico- to nano-molar range. Monoclonal antibodies isolated from rabbits have been consistently shown to have affinities in the pico-molar range (Feng et al. 2011). Due to the multiple rounds of selection, the aptamers can attain as much affinity as the antibodies have, which could be in the pico- to nano-molar range. Fab and scFv fragments isolated using phage display library generally tend to have lower affinities. Single domain antibodies generally have also been shown to have lesser affinity as compared to the whole antibodies. The affinities of all such antibody derivatives can be increased by adopting suitable screening procedures such as using decreasing concentrations of the antigens with the subsequent rounds of panning or dimerization of the molecules. In terms of specificity of the biosensors, the antibody fragments that are isolated from phage display library could possibly have higher specificity than the ones isolated from the animals. The Fab fragments cleaved from the polyclonal antibodies raised in animals could show non-specific binding to the closely related antigens. The aptamers also tend to have higher specificities due to a careful selection process. With regard to the stability of various antibody formats, the single domain antibodies show highest stability that can withstand high temperatures in the range of 80–90 °C as well as extreme pH. The aptamers are also considered to be highly stable biosensors that can retain binding to the target even after denaturation. The whole antibodies are on the second rank in terms of stability with the ability to withstand moderate room temperatures that can be suitable for the point of care

diagnostic assay. The Fab and scFv fragments are the least stable biosensors which can be stabilized with multimerization or by fusion with Fc fragments or other proteins. With regard to the ease of immobilization, scFv, aptamers and Fab fragments are relatively easier to immobilize as compared to the whole antibodies. Since there is a scope for addition of various functional groups to these fragments which confers suitability in their immobilization. Also due to their smaller size, it is possible to immobilize them at higher densities as compared to the whole antibodies, making the diagnostic assay more sensitive.

To conclude, the immunodiagnostics are being consistently improved over the last few years. The choice of biosensors largely determines the success of a diagnostic assay. Based on the target to be detected, type of diagnostic assay and method of detection, the biosensors have to be carefully selected. Newer methods of antibody screening procedures such as microfluidics which allow for rapid high-throughput screening can provide novel biosensors without the need for immortalization or library preparation.

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