Biosensors and Bioelectronics 47 (2013) 1–11

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Development of electrochemical immunosensors towards point of care diagnostics

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ARTICLE INFO

Article history:
Received 17 January 2013
Received in revised form
27 February 2013
Accepted 28 February 2013
Available online 14 March 2013

Keywords:
Electrochemical immunosensor (EI)
Point of care (POC)
Oriented immobilization
Nanotechnology
Multiplexing analysis
Integration

ABSTRACT

Electrochemical immunosensors (EI) has attracted numerous interests due to its inherent benefits over the other transduction schemes, such as a high sensitivity, ease of use, a possible automation and integration in compact analytical devices, mostly cheap and relatively simple technology of its production. Thus, EIs have great potential in point of care (POC) diagnostics for early detection of diseases. During last decades, numerous efforts have been put into EIs development. Firstly, different fabrication methods and amplification strategies have been employed to achieve high sensitivity. To be pointed, nanotechnology has been involved in the fabrication and signal amplification of EIs, which present great superiority. Secondly, EI arrays have been used for multiparametric analysis. Thirdly, several attempts have been made to construct integrated systems, which showed promising applications for POC test. Several of them are commercially available for POC use. Herein, we will review briefly the recent achievements and progress in developing EIs towards POC diagnostics.

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http://dx.doi.org/10.1016/j.bios.2013.02.045

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1. Introduction

Rapid, sensitive and selective detection of certain protein biomarkers in early diagnosis is critical for the cancer therapy. Recently, considerable attention has been paid to point of care (POC) diagnostic which can be performed at the bedside or in the clinic. The realization of POC requires not only fast, sensitive and selective detection, but also small, cheap and integrated device. Electrochemical immunosensor (EI), with the inherent advantages of high sensitivity, low cost, low power requirement and potential of automation, has been a promising approach for POC diagnosis (Centi et al., 2009; Tothill, 2009; Vestergaard et al., 2007; Von Lode, 2005; Warsinke, 2009; Warsinke et al., 2000).

To realize POC diagnostic, there are several demands to be satisfied such as high sensitivity, multitarget detection and integration. There are two key points for the improvement of sensitivity: (1) the way to immobilize the recognition layer onto electrodes; (2) the way to amplify the electrochemical signal of the binding event between the antibody and its antigen. Considerable efforts have also been devoted towards the following two key points: (3) multiplexing analysis; (4) integration of EI devices with greater capabilities for home-health care.

Although there has been substantial progress in the development of EIs, the way to POC diagnostic is still hard and long. This review tried to trace the development of EIs and summarize the challenges for them being applied to POC diagnostics.

2. The way to immobilize the recognition layer onto electrodes

The recognition layer immobilization is a potentially important prerequisite for the fabrication of EIs. For most of the current EIs, the recognition layers are proteins (antibodies or antigens), and the transducers are made from metals (e.g., gold, platinum), semiconductor materials (e.g., indium tin oxide, Iridium oxide) or carbon materials (e.g., carbon paste, glassy carbon, graphite). Based on the physical and chemical properties of both the electrode and the protein, a number of immobilization methods have been proposed. The key problem during the immobilization is how to fully maintain the protein's conformation and activity.

2.1. Physical immobilization

Physical immobilization is based on the adsorption of proteins onto electrode surfaces via noncovalent interactions, mainly electrostatic force, ionic bonds and hydrophobic interactions. However, physical immobilization often results in random orientation and weak attachment.

To enhance the attachment of proteins with electrode surface, several strategies were developed by entrapping proteins into a polymer matrix (Dai et al., 2003). As reported by Wilson and Rauh, antibodies were immobilized in electrochemically grown iridium oxide (IrO_x) thin film matrices (Wilson and Rauh, 2004). During the electrochemical growth of iridium oxide (IrO_x) films, antibodies were physically entrapped and available for immunological binding. Though physical entrapping of the proteins can enhance the attachment, the recognition efficiency is limit due to the burying of the active sites. Thus other non-physical immobilization strategies have been developed.

2.2. Covalent immobilization

Covalent bonds are mostly formed between side-chain-exposed functional groups of proteins with suitably modified electrodes, resulting in an irreversible binding and producing a high surface coverage.

The modification of carbon electrodes can be achieved by electrochemically activating the electrode surface, resulting in carboxyl groups for the binding with amines of proteins. We have

Table 1
Comparison of different approaches for covalent immobilization.

Electrode		Functional groups in capture probe	Cross linker	References
Metal	Gold	Amine	11-Mercaptoundecanoic acid	Ahmad and Moore (2012)
	Gold	Amine	22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid; 1,2-dithiolane-3-pentanoic acid	Nassef et al. (2008)
	Gold	Amine	Thiourea; thioctic acid; 3-mercaptopropionic acid	Limbut et al. (2006)
	Gold	Amine	Glutaraldehyde	Jiang et al. (2003)
	Gold	Amine	Carboxymethyldextran	Darain et al. (2005)
Semiconductor	Iridium oxide	Amine	(3-aminopropyl)triethoxysi-lane and glutaraldehyde	Salam and Tothill (2009)
	ITO	Amine	(3-glycidoxypropyl)-trimet-hoxysilane	Wei et al. (2009)
Carbon	Graphite		4-carboxymethylaniline	Wilson (2005)
	Graphite	Amine	Poly-terthiophene carboxylic acid	Corgier et al. (2005)
	Glassy carbon	Amine	Pyrrolepropylic acid	Dong et al. (2006)
	Boro-doped diamondn	Amine	o-Aminobenzoic acid	Preechaworapun et al. (2008)
	Carbon paste	Carboxylic	4-Carboxyphenyl diazonium	Hayat et al. (2011)

ITO: Indium–tin oxide.

reported the fabrication of an EI based on the electrochemical activating of carbon electrodes (Wan et al., 2011a).

Another important approach for modification of electrodes is using bi-functional crosslinking reagents. Table 1 lists some examples of bi-functional crosslinking reagents used in EIs, such as glutaraldehyde (Jiang et al., 2003), (3-aminopropyl)triethoxysilane and glutaraldehyde (Wilson, 2005), diazonium cation (Corgier et al., 2005; Hayat et al., 2011), (3-glycidoxypropyl)-trimethoxysilane (GPTMS) (Wei et al., 2009), thiol derivatives (Ahmad and Moore, 2012; Limbut et al., 2006; Nassef et al., 2008), and several polymers(Darain et al., 2005; Dong et al., 2006; Kim et al., 2010; Preechaworapun et al., 2008).

Salam and Tothill compared physical and covalent immobilization via amine coupling of carboxymethyldextran on the surface of the gold working electrode for the detection of *Salmonella typhimurium*. Around 250-fold increases in detection sensitivity was achieved by using covalent antibody immobilization (Salam and Tothill, 2009).

An potential problem with crosslinking reagents modified electrode is the slow electron transfer kinetics between the redox label and the electrode, which are separated by a poorly conducting layer. Wei et al. reported a controlled surface modification process, in which the antibody was immobilized on GPTMS-modified indium tin oxide (ITO) electrodes (Wei et al., 2009). They optimized the modification condition to achieve the best analytical performance based on a balance between the amount of surface-immobilized antibody and the electrochemical kinetics of the redox label. Under the optimized conditions, high sensitive detection for benzo[a] pyrene and pyrenebutyric acid were obtained with a detection limit of 2.4 and 10 ng/mL.

Covalent immobilization presents great advantages in the fabrication of EIs. However, there is always room for improvement. Two main approaches to improve the affinity efficiency of immobilized proteins are the oriented immobilization and the utilization of nanomaterial (NM).

2.3. Oriented immobilization

In some cases, immobilization may lead to partial or complete loss of protein activity, due to random orientation and structural deformation. Therefore, oriented immobilization is demanded for the improvement of the EI performance. Oriented immobilization means that the capture probes are immobilized in such a way that their recognition sites are uniformly arranged and exposed to the sample solution.

2.3.1. Oriented antigen immobilization

In the case of antigens, few researches have been reported about the oriented immobilization of antigen perhaps due to the structure diversity of antigens. Feng et al. reported an affinity ligand fused to the N/C-terminus of chimeric antigen for site-oriented immobilization, which resulted in significant enhancement of analytic signal (Feng et al., 2011). As reported by Rosales-Rivera et al., the oriented immobilization of the antigen on the surface has been achieved using a carboxylic-ended bipodal alkanethiol that is covalently linked with amino groups of the antigen (Rosales-Rivera et al., 2011).

2.3.2. Oriented antibody immobilization

Different from the oriented antigen immobilization, oriented immobilization of antibodies plays an indispensable role in the fabrication of EIs. Antibodies are constructed by four chains: two identical "heavy" and two identical "light" polypeptide chains, which together form a "Y-shaped" conformation (Fig. 1A). The arms of the Y-shaped molecule are antigen binding fragment (Fab), and the vertical portion is called the fragment-crystallizable (Fc). If the antibody molecules are attached on the surface randomly, there are four possible orientations (Fig. 1B): end-on (Fc attached to the support), head-on (Fabs attached to the support), sideways-on (one Fc and one Fab attached to the support), and flat-on (all three fragments attached to the support). In many cases, the actual orientation on a given surface may be a combination of all above if no specific approach was done to control the orientation, resulting in uncontrollable antigen binding efficiencies.

As the active sites of the antibody are on the Fab segment, the ideal orientation of the immobilized antibody is end-on, when the Fc adsorbs onto the adsorbent surface and the Fabs orient to the analyzing solution (end-on). Several approaches were developed to control the orientation of antibodies to improve the analytical performances of EIs.

2.3.2.1. Site-oriented antibody immobilization. One reported technique is site-oriented immobilization, in which Fabs of antibody are prepared and deposited onto gold surfaces via native thiol groups located at the hinge region. While the thiols were attached to the surface of sensor, the antigen binding site is away from the transducer surface (end-on orientation), resulting in a higher accessibility and activity of the immobilized receptor molecules. As shown in Fig. 1C, by using half-antibody and Fab, the antigen binding site was available to the analyte solution, resulting in high antigen affinity efficiency. Fabs can be obtained by using antibody engineering techniques (Romanazzo et al.,

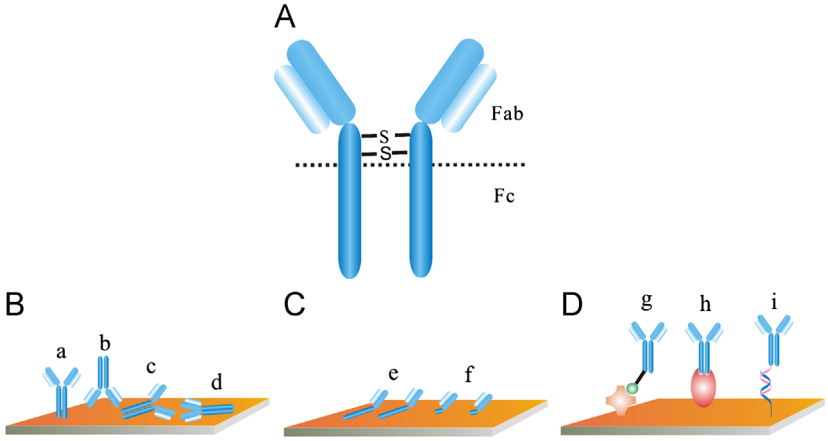

Fig. 1. Illustration of (A) mainly structure of antibody; (B) different orientation of randomly immobilized antibodies: (a) end-on, (b) head-on, (c) sideways-on, (d) flat-on; (C) oriented immobilization of antibody fragments: (e) half-antibody, and (f) Fab; (D) immobilization of antibodies through different bioaffinity systems: (g) avidin–biotin, (h) protein A, G or L, and (i) DNA-directed immobilization.

2010) or selectively cleaving IgG molecules by 2-mercaptoethylamine (Billah et al., 2010).

Several studies described the comparison of random and oriented immobilization of antibody fragments. Bonroy et al. compared the immunosensor responses of different immobilized antibody fragments, such as $F(ab)_2$ and Fab, with their parental IgG (Bonroy et al., 2006). It was demonstrated that the antigen binding signal produced by oriented immobilization of Fabs was 2-fold higher than that of randomly covalent immobilized full-length antibodies and $F(ab)_2$. Similarly, Vikholm-Lundin and Albers compared the performance of antibody, $F(ab)_2$ and Fab (Vikholm-Lundin and Albers, 2006). It seemed that the Fab was site-directly oriented and the response to target was fivefold compared to the randomly oriented antibodies and $F(ab)_2$ layer.

2.3.2.2. Bioaffinity immobilization. Another approach to achieve oriented antibody immobilization was developed on different bioaffinity interactions. A number of affinity systems have been reported in the fabrication of EIs, such as lectin-sugar (Ho et al., 2010b), protein A, G, or L system (Di Gao et al., 2006; Kerman et al., 2006; Liu et al., 2012), His-Tag system (Vallina-García et al., 2007), DNA-directed immobilization (Jung et al., 2007), affinity capture ligand system and so on (Fig. 1D).

Ho etl al. compared two approaches for oriented immobilization of antibodies: using the Fab fragment and affinity of boronic acid towards sugar moieties (Ho et al., 2010b). The sensitivity of the EI based on affinity of boronic acid was about 250 times higher than that using antibody fragment, which might be due to the less denatured protein and preserved immunobinding affinity.

Protein A, G, or L (ProA, ProG, or ProL) could selectively bind with the Fc of different types of IgG with high affinity, resulting in the oriented immobilization of antibodies with significantly improved antigen-binding capacity, sensitivity, and stability. Tran et al. compared the surface of silanized interdigitated electrodes by four methods: direct adsorption, covalent binding with a cross linker of glutaraldehyde, covalent binding with a cross linker of glutaraldehyde combined with anti-human IgG and covalent binding with a cross linker of glutaraldehyde combined with a bioaffinity of ProA (Tran et al., 2012). The results showed that the ProA method provided the highest signal for detection of analyte.

Recently, several attempts have been made to convert DNA arrays into protein arrays using DNA hybridization. A versatile biolinker for efficient antibody immobilization was prepared by site-specific coupling of ProG to DNA oligonucleotide (Jung et al., 2007). ProG tagged single-stranded DNA was sequence-specifically bound to complementary surface-bound DNA probes, resulting in the hybridization of ProG onto the DNA surface. Then antibodies could be attached to ProG in a controlled and efficient way. We have recently reported a reusable EI platform with novel 3D DNA nanostructure-decorated gold surfaces by taking advantage of DNA-directed antibody conjugation and high resistance to non-specific protein adsorption (Pei et al., 2011). This platform supports regeneration of the surface by using mismatched complementary DNA, which forms the basis of reusable immunosensors.

2.4. Nanotechnology utilized in the fabrication of EI

Due to the unique physical and chemical properties of NM, various biocompatible NMs have been successfully applied in bioanalyitical techniques (He et al., 2010a, 2010b; Hu et al., 2009; Li et al., 2008; Song et al., 2009, 2010; Yan et al., 2010; Zhang et al., 2009). While they are used in the fabrication of EI, NMs provide with high surface-to-volume ratio, more conformational freedom for protein immobilization and high surface reaction activity. Moreover, some NMs (usually metal nanoparticles or semiconductor nanoparticles) can effectively accelerate electron transfer between electrode and biomolecules, resulting in improved EI performance for target detection.

A wide variety of NMs have been applied in electrochemical biosensors and several comprehensive review articles have been reported in the field (Luo et al., 2006; Pumera et al., 2007). For example, Guo and Dong provided a comprehensive overview on the use of inorganic NPs for enhancing construction of diverse electrochemical biosensors (Guo and Dong, 2009). Joseph Wang reviewed a variety of new nanoparticle/biomolecule assemblies for advanced electrical detection of proteins (Wang, 2007). However, the NMs used in the fabrication of EIs are not systematically discussed. Thus we herein try to review the recent research on the NMs based EIs.

The illustration of the fabrication of NMs based EIs is shown in Fig. 2. In a typical procedure, NMs are firstly attached on the electrode, and capture probes are then immobilized on the NMs modified electrode. The orientation of capture probes immobilized on the surface of NMs is random. However, the surfaces of NMs provide a considerable large area for the immobilization of antibodies and more conformational freedom, resulting in a higher binding affinity to target.

The most commonly used NMs are gold nanoparticle (AuNP), carbon NMs, and other NMs, which will be discussed in the following sections respectively.

2.4.1. AuNP based EIs

AuNP is one of the most commonly used NM in this field of electrochemical biosensing (Pingarrón et al., 2008). In this review, we are going to discuss the universal procedure of fabricating AuNP-based EIs.

The first step is about how to modify electrodes with AuNPs. Several researchers reported the attachment of AuNPs onto gold electrode by using different bi-functional crosslinking reagents such as 4-thiophenol (Liu et al., 2011b), 1,6-hexanedithiol (Liu et al., 2009), and thiourea (Chullasat et al., 2011). Owino et al. reported the polymerization of thionine on the glassy carbon

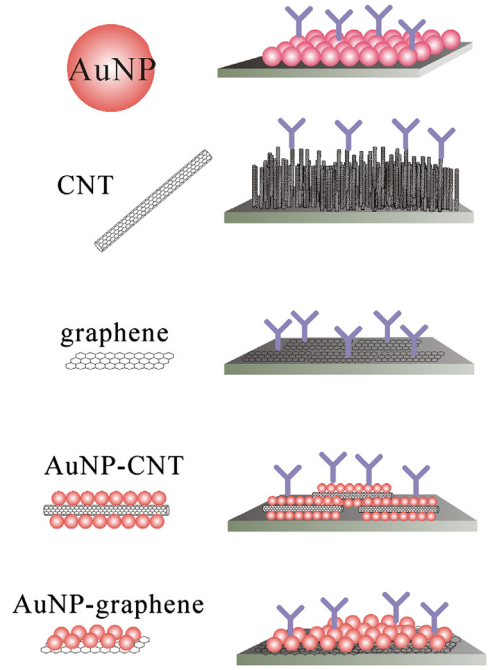

Fig. 2. Illustration of different types of NMs in the EIs fabrication. A typical process is the modification of the electrode with NMs followed by the attachment of capture probes.

electrode to form polythionine film followed by the AuNPs adsorption (Owino et al., 2008).

The second step is to attach the capture probe onto the AuNP surface. Several strategies have been reported including physical adsorption (Munge et al., 2009; Tang et al., 2006), MSA (Chullasat et al., 2011) or glutathione (GSH) as a spacer arm (Mani et al., 2009).

Recently, several other researchers proposed a different process by firstly preparing antibody conjugated AuNPs and then subjecting them to a gold electrode (Ahirwal and Mitra, 2010; Tang et al., 2007).

To improve the performance of AuNP based EIs, some scientists were devoted to arrange AuNPs on the electrode surface. Rusling's group reported a densely packed AuNP platform for the fabrication of EI (Mani et al., 2009). Chen et al. reported a kind of ordered 3D Au nano-prickle clusters by directly electrodeposition on glassy carbon electrode utilizing the spatial obstruction/direction of the polycarbonate membrane (Chen et al., 2010).

2.4.2. Carbon NMs based EIs

Carbon NMs are a class of carbon based NMs in different shapes which have been widely used in the fabrication of EIs.

Carbon nanotube (CNT) is a unique type of carbon NMs with a cylindrical nanostructure which has a length-to-diameter ratio up to 10,000,000. Rusling's group has reported a serial of EIs based on the immobilization of antibodies onto the single walled CNT forest (Malhotra et al., 2010; Munge et al., 2010, 2009; Yu et al., 2005, 2006). Single-wall CNTs were carboxyl functionalized and assembled on the electrode by using Nafion ionomer and Fe^{3+}-precipitated hydroxides, resulting in monolayers of vertically aligned single walled CNT forest. This layer features carboxylated nanotube ends, which provides a conductive, high area surface for covalent attachment of a large population of capture antibodies. As reported by several researchers, Multi-walled-CNT layer was also used to fabricate high sensitive EIs (Liu et al., 2011a; Viswanathan et al., 2009). Yun et al. have reported a label-free immunosensor based on CNT array electrodes (Yun et al., 2007). Anti-mouse IgG was then covalently immobilized on the nanotube array through –COOH groups on electrochemically activated nanotubes.

Ju's group has reported a series of EIs based on carbon NMs (Wu et al., 2007; Zhang et al., 2010). The acidic oxidation of carbon nanofiber could provide a porous carbon nanofiber membrane and a larger number of active sites for covalent binding of CA125, resulting in good precision, high sensitivity, acceptable stability and reproducibility. In another study, they proposed the colloidal carbon sphere (CSA) modified ITO electrode which was activated to generate carboxyl groups on the surface of the carbon spheres for the binding of antibodies.

Recently, graphene, a monolayer of carbon atoms tightly packed in a two-dimensional honeycomb lattice, have attracted numerous interests in designing EIs. Lin's group reported a EI based on graphene sheet (GS) platform, which could increase the surface area to capture a large amount of antibodies and thereby amplify the detection signal (Du et al., 2010). Wei et al. developed a series of EIs with the antibody immobilized onto TH and GS modified glassy carbon electrode surface (Cai et al., 2011; Wei et al., 2010a, 2010b). All of these immunosensors exhibited wide linear range, low LOD and good long-term stability. Reduced graphene sheets (RGSs) were also used for the fabrication of EIs (Wan et al., 2011b). As reported by Haque et al. they presented an electrochemically reduced graphene oxide (ERGO)-based electrochemical immunosensing platform, which was coated with a N-acryloxysuccinimide-activated amphiphilic polymer for antibody immobilization (Haque et al., 2012).

2.4.3. Other NMs based EIs

Several other nanoparticles were utilized in the fabrication of EIs. Lin's group developed zirconia nanoparticles (ZrO_2) platform for EIs, which were pre-coated on a SPE by electrodeposition and followed by antibody immobilization (Liu et al., 2008; Lu et al., 2011). Huang et al. developed a novel electrochemical impedimetric immunosensor based on O-carboxymethylchitosan surface modified Fe_3O_4 nanoparticles (Huang et al., 2010a). This EI was demonstrated to be reproducible by being treated with glycine–HCl buffer solution (pH 2.8). Li et al. proposed a novel EI for sensitive detection of PSA based on nitrodopamine functionalized iron oxide nanoparticles, which increased the loading capacity for biomolecules (Li et al., 2011). These NMs have contributed to the highly sensitive EIs by providing not just the reduced dimensions but the increased surface-to-volume ratio.

2.4.4. Nano-composites based EIs

Nano-composites were designed to improve the performance of pure NMs for antibody immobilization with higher specific surface and electron transfer rate. Huang et al. reported a disposable EI based on AuNPs/MWCNTs–chitosans composite film (Huang et al., 2010b). Cui and Zhu reported a AuNPs/colloidal carbon sphere hybrid material for the immobilization of protein in biosensing (Cui and Zhu, 2010). Graphene–AuNP nano-composites formed film with abundant adsorbing sites and large surface area can reserve the biocompatibility of the biomaterials which greatly increased the loading amounts, thus, significantly improves the performance of biosensing (Kong et al., 2011; Leng et al., 2011; Liu et al., 2011c; Tang et al., 2011a; Wang et al., 2010). As reported by Yuan et al. a new [Ag–Ag_2O]/SiO_2 nanocomposite material was employed as a connected material to absorb Ag–Ag_2O nanoparticles on SiO_2 surface for label-free determination of CEA (Yuan et al., 2010). All these formed hybrid architectures showed great potential in highly sensitive detection of proteins.

Though there are numerous strategies for the immobilization of recognition layers onto electrodes, the best one is still not achieved. To sum up, oriented immobilization can improve bioaffinity of the recognition layers and nanotechnology can improve the immobilization efficiency. A promising way to achieve the best immobilization strategy might be the combination of the two procedures.

3. The way to amplify electrochemical signal

Another way to achieve high sensitive EIs is to develop signal amplification methods.

3.1. Signal amplification by redox mediators

The horse-radish peroxidase (HRP) and alkaline phosphatase (ALP) conjugated antibodies are conventional enzymatic labels used in EIs, due to the fact that the enzymatic products are electroactive for electrochemical measurement (Das et al., 2007; Moreno-Guzmán et al., 2012; Serafín et al., 2011; Tan et al., 2009). However, conventional enzyme-based signal amplification approaches are not high enough for ultrasensitive detection. Redox mediators are used to facilitate the electron transfer between enzyme and electrode and thereby achieved an enhanced electrochemical response (Guo and Yang, 2005; Lai et al., 2009; Yang et al., 2010a, 2010b). As reported by Guo and Yang, ruthenium tris(2,2′-bipyridine) (Ru-bipy) was used as the electrochemical label and oxalate was used to amplify the oxidation current of Ru-bipy (Guo and Yang, 2005). Das et al. proposed a strategy for signal amplification by using p-aminophenol (AP) redox cycling by hydrazine (Das et al., 2007). The ALP-conjugated antimouse IgG was used as the detection label,

which generated AP by enzymatic reaction. This generated AP was electrooxidized at the ITO electrode modified with a partially ferrocenyl-tethered dendrimer. The oxidized product, p-quinone imine (QI), was reduced back to AP by hydrazine, which was then electrooxidized again to QI, resulting in a redox cycling. As a result, the electrochemical amplification for a very low detection limit (100 fg/mL) was realized by the redox cycling.

3.2. Signal amplification by multiple redox indicators

Different strategies for signal amplification have been developed by employing multilabels per detection probe such as multiple redox indicators, NM tracers, and multilabels on NM carriers. One approach to fabricate multiple redox indicators per detection probe is rolling circle amplification (RCA). RCA is an isothermal enzymatic amplification process, which produces a long DNA with repeat sequence for DNA detection probes binding. As reported by Lu et al. an immunosandwich was formed by capture antibody, target antigen and biotinylated secondary antibody (Lu et al., 2012). Biotinylated primer DNA was immobilized on the secondary antibody by biotin–streptavidin system. After the RCA of biotinylated primer DNA, a large number of affinity sites on the RCA products were provided to link DNA detection probes. Doxorubicin hydrochloride was utilized as an electroactive indicator to intercalate the CG–GC steps between the RCA products and the DNA detection probes. A considerable low detection limit of 0.06 pM was achieved by this system.

3.3. Signal amplification by NMs

In short, NMs based signal amplification strategies have been widely reported. In some cases, NMs can play the role of electrochemical labels due to their redox activities or enzyme activities. In other cases, NMs are used as nanovehicles on which antibodies and signal molecule are coimmobilized. Besides, Special nanoparticles from enzyme induced growth or deposition is used for the enhancement of sensitivity.

3.3.1. NMs as electrochemical labels

Inorganic NMs such as catalytic AuNPs or Quantum dots (QDs) have been demonstrated to catalyze electrochemical reactions, resulting in enhanced electrochemical signal. Ho et al. reported a sensitive EI using AuNPs as electrochemical signal probes (Ho et al., 2010a). The electrochemical signal from the bound AuNPs was obtained from the oxidization of AuNP and the reduction of $AuCl^{4-}$ by square wave voltammetry (SWV). As reported by Chen's group, AuNP-coated CNTs were used as nanocatalysts for EI (Tang et al., 2011b). The AuNPs immobilized on AuNP-CNTs could catalyze the reduction of p-nitrophenol and improve the reversibility, leading to a high sensitivity.

QDs were firstly used as electrochemical tracers by Wang's group for simultaneous detection of three target proteins (Liu et al., 2004). Four types of QDs (ZnS, CdS, PbS, and CuS) coated with different antibodies were used to provide distinct voltammetric peaks, whose position and size reflected the identity and concentration of the corresponding antigen. Viswanathan et al. developed three antibodies conjugated with specific nanocrystals (CdS, PbS and CuS) for electrochemical measurements (Viswanathan et al., 2012). The square wave anodic stripping voltammetry was employed to measure released metal ions from bound antibody nanocrystal conjugates, resulting in ultrahigh sensitivity and precision. QD–GS conjugates were used as electrochemical labels for signal amplified EI (Yang et al., 2011). The high sensitivity can be ascribed to the multi QDs loaded GS and the good conductivity of GS.

3.3.2. NMs as nanovehicles in EIs

As illustrated in Fig. 3, different NMs have been used as nanovehicles including AuNP, CNT, graphene, lipsome and their nanocomposites. Several samples are listed in Table 2. The nanovehicles can be based on irregular-shaped gold nanoparticles (ISNGs) (Tang et al., 2010b), CNTs (Yu et al., 2006) (Wan et al., 2011a), GS-TH (Yang et al., 2010a), GO (Du et al., 2011b), silica NP (Wang et al., 2006), SiO_2 (Zhong et al., 2009), and NDA–Fe_3O_4 (Li et al., 2011). Antibodies (or antigens) and electrochemical labels are coimmobilized on this nanovehicles to perform high sensitive detection of targets.

Lipsome is a unique nanocarrier due to their ability to carry different agents in the aqueous cavity. Ho's group described a series of EIs using ferrocene encapsulated and antibodies-functionalized liposomes as highly specific recognition labels for the amplified detection of several biomarkers (Liu et al., 2004; Viswanathan et al., 2009). The SWV was employed to analyze faradic redox responses of the released ferrocene carboxylic acid from the liposomes attached on the electrode surface. Yu's group reported ALP-encapsulated liposomes as primary single amplification strategy and biocatalytic metal deposition as secondary single amplification strategy for the detection of PSA (Qu et al., 2010). After the attachment of functionalized liposomes onto the electrode, surfactant was used to release the encapsulated ALP, which initiated the hydrolysis of ascorbic acid 2-phosphate to produce ascorbic acid. This dual signal amplification strategy was demonstrated several orders of magnitude higher sensitivity than the cut-off value of the PSA (LOD 7 pg/mL).

Nano-composites are also used for signal amplification. Comparing to pure NMs, nano-composites have larger specific area and higher efficiency of electron transfer. Zhu's group have developed a multi-enzyme functionalized label AuNPs which were uniformly assembled on the surface of poly (styrene-acrylic acid) nanospheres

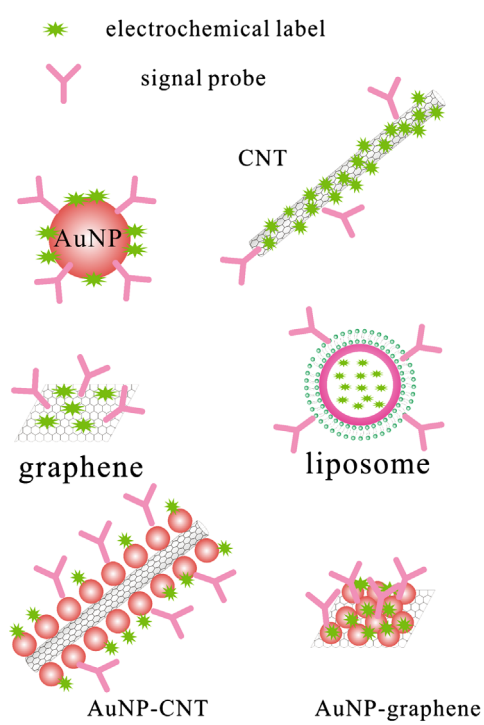

Fig. 3. Different types of NMs used as nanovehicles for signal amplification of EIs. Both the signal antibodies and electrochemical labels are coimmobilized onto the NMs.

Table 2
Examples of different NMs as nanovehicles for signal amplification.

NM	Electrochemical label	Target	Sensitivity	References
ISNG	HRP	AFP	10 pg mL^{-1}	Tang et al. (2010a, 2010b)
CNT	HRP	PSA	4 pg mL^{-1}	Yu et al. (2006)
GS	HRP	PSA	1 pg mL^{-1}	Yang et al. (2010a, 2010b)
GO	HRP	phosphorylated p53	0.01 nM	Du et al. (2011a, 2011b)
Silica NP	Guanine residues	Phosphorylated p53	2.0 pM	Wang et al. (2006)
SiO_2	HRP	Human IgG	0.1 nM	Zhong et al. (2009)
NDA- Fe_3O_4	HRP	PSA	4 pg mL^{-1}	Li et al. (2011)
Lipsome	Ferrocene carboxylic acid	CEA	1 pg mL^{-1}	Viswanathan et al. (2009)
Lipsome	ALP	PSA	7 pg mL^{-1}	Qu et al. (2010)
AuNP/PSAN	ALP	TNF-α	10 pg mL^{-1}	Yin et al. (2011)
Au/SiO_2	HRP	Human IgG	35 pg mL^{-1}	Wang et al. (2010)
Au/GS	HRP	CEA	10 pg mL^{-1}	Zhong et al. (2010)
Au/Fe_3O_4	HRP	CEA	1 pg mL^{-1}	Li et al. (2010)
MSN-AuNP	HRP	Norethisterone	3.58 pg mL^{-1}	Wei et al. (2010a, 2010b)

ISNG: irregular-shaped gold nanoparticle; CNT: carbon nanotube; GS: graphene sheet; GO: graphene oxide; NDA-Fe_3O_4: nitrodopamine functionalized iron oxide nanoparticles; AuNP: gold nanoparticle; PSAN: poly (styrene-acrylic acid) nanospheres; MSN: mesoporous silica nanoparticles; HRP: horseradish peroxidase; ALP: alkaline phosphatase; AFP: α-fetoprotein; PSA: prostate specific antigen; CEA: carcinoembryonic antigen; TNF-α: tumor necrosis factor α.

to conjugate ALP (Yin et al., 2011). Yao's group reported a novel sandwich-type EI for human IgG using Au/SiO_2 nanoparticles (NPs) with adsorbed HRP-anti-hIgG as the secondary antibody layer (He et al., 2010a). Lin's group designed another nanolabel with chitosan-protected graphene as core and multi-nanogold particles as shell (Zhong et al., 2010). Li et al. developed an EI using multilayered coating of AuNP on the magnetic Au/Fe_3O_4 composites (Li et al., 2010). As reported by Wei et al. amino-group functionalized mesoporous silica nanoparticles (MSN) immobilized with Au nanoparticles were utilized for the adsorption of HRP and the secondary antibody (Wei et al., 2010b). All these nano-composites based EIs featured ultrahigh sensitivity.

3.3.3. Nanoparticles growth or deposition in EIs

Enzyme or nanomaterials induced nanoparticle deposition has also been used for the sensitivity enhancement of EIs. AuNPs-induced silver deposition was a typically used sensitivity enhancement strategy. Zhang et al. reported a signal improvement strategy based on antibody labeled AuNPs and the addition of the silver enhancer solution (Zhang et al., 2008). The formation of silver precipitation on AuNPs was determined by electrochemical impedance spectroscopy. Qu et al. developed a signal amplification strategy based on graphene oxide (GO) initiated silver enhancement (Qu et al., 2011). The deposited metal silver onto GO produced redox current, resulting in a wide range of linear response, low detection limit, good reproducibility and stability. Yu's group proposed a series of EIs based on enzyme induced copper (Huang et al., 2008) and silver deposition (Tan et al., 2009). Zhang et al. reported an signal amplification strategy which employed glucose oxidase for the enzymatic deposition of AuNPs (Zhang et al., 2011). Electrochemical detection was conducted by monitoring the reduction of gold oxide, which was correlated to the amount of antigens in the solution.

In short, by using different signal amplification strategies, EIs feature ultrahigh sensitivity. To the best of our knowledge, the highest sensitivity was achieved by using CNT-AuNPs as nanolabels/nanocatalysts with a detection limit of 0.8 fg/mL (Tang et al., 2011b). However, there are still challenges faced by researchers due to the complicated assembly process of NMs. Thus there is a compromise between sensitivity and complexity.

4. Multiplexing detection and integration

When facing POC diagnosis of cancers, EI have to overcome several challenges. One of the major challenges is multiparametric analysis, as most diseases have more than one marker associated with their incidence. Another challenge is the system integration, which will eventually realize the POC diagnostic in a hand-held analyzer.

4.1. Multiplexing analysis

Simultaneous detection of panels of tumor markers can increase its diagnostic accuracy in early cancer detection. Among different analytical technologies, EIs present inherent advantages due to the fact that electrodes can be easily integrated. Multiplex channel electrode arrays are developed based on screen-printed technology that allow rapid production of large numbers of sensors relatively inexpensively (Renedo et al., 2007). The SPEs are fabricated by printing various inks such as graphite inks (Darain et al., 2005; Huang et al., 2012a, b; Moreno-Guzmán et al., 2012; Parker and Tothill, 2009; Rao et al., 2006; Wu et al., 2008; Yu et al., 2004) and gold inks (Escamilla-Gómez et al., 2009; Heurich et al., 2011; Radi et al., 2009; Serafín et al., 2012; Susmel et al., 2003) on different types of substrates.

The biggest challenges facing SPE arrays are the crosstalk and intervention. To overcome them, technologies like spaces separation, addressable signal readout and so forth were needed. As illustrated in Fig. 4A, we proposed an EI using a disposable 16 channel SPE array combined with a universal multilabel nanoprobe for the simultaneous detection of cancer biomarkers: PSA and Interleukin 8 (IL-8) (Wan et al., 2011a). The spatial separation of the electrodes enabled simultaneous detection of multiple proteins to be conducted in a single assay without amperometric crosstalk between the electrodes. Wilson and Nie reported a sensor array consisted of eight iridium oxide sensing electrodes, an iridium counter electrode, and a Ag/AgCl reference electrode patterned on a glass substrate (Wilson and Nie, 2006a). Additionally, they developed a chip-based sensors contained 12 eight-site biosensors and provided the analytical capability of a 96-well immunoassay plate (Wilson and Nie, 2006b). Several other SPE arrays were also developed, which enabled simultaneous immunoassay to be conducted without cross-talk between adjacent electrodes (Du et al., 2011a; Wu et al., 2008).

Besides the SPE arrays, microfabrication techniques have been adopted in the EI arrays. Kelley's group reported the use of a microelectronic chip platform to achieve multiplexed immunosensing of the ovarian cancer biomarker CA-125 (Fig. 4B) (Das and Kelley, 2011). This platform was based on a class of microelectronic chips that supported the growth of metal sensors with a variety of sizes and morphologies. The electrochemical signal was simply

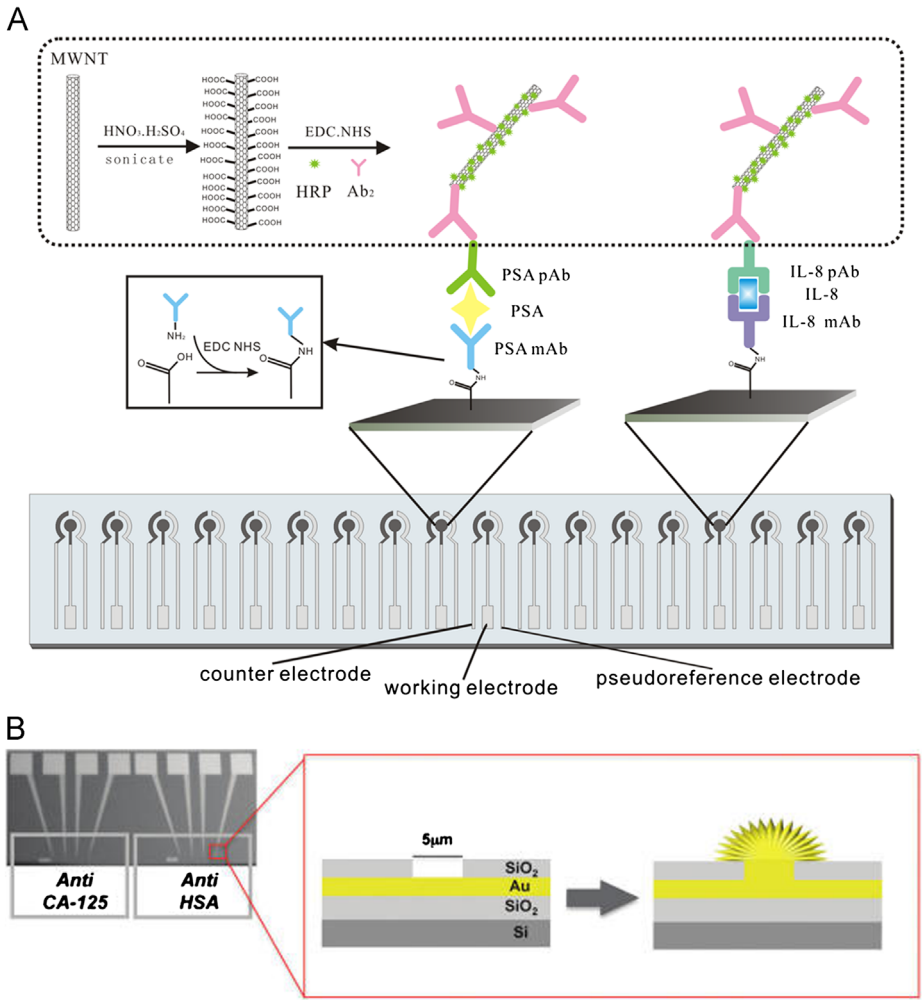

Fig. 4. Illustration of (A) SPE array for simultaneous detection of two different protein biomarkers. Copyright 2011 Elsevier. (B) Multiplexed microfabricated chip (left) featuring 5 μm openings for the electrochemical deposition of sensors (middle) and the generation of Au sensors by Au electrodeposition (right). Copyright 2011 American Chemical Society.

readout after the incubation of a protein-containing sample, without the cumbersome washing steps for the sandwich assay. And the LOD was remarkably down to 0.1 U/mL.

4.2. Integration

Another challenge facing POC system was the integration of EI into an analytical instrument. Some efforts have been made toward the integration of EI with flow-through technology to form automated systems. Chen's group reported an integrated automatic EI array has been designed for the simultaneous detection of 5-type hepatitis virus antigens (Tang et al., 2010a). The developed system integrated EI array with the flow-through system to realize automatic detection. Skldal et al. developed a portable detector ImmunoSMART, which was combined with the cyclone air sampler to detect the model bioagent E.coli DH5a in bioaerosols (Skládal et al., 2012). The level of 150 CFU/L in air was determined in 20 min. These developed systems showed promise for the automated detection.

However, these systems are disadvantageous to the further miniaturization. Thus the integration of EIs into a microfluidic format was developed, which provided new opportunities for high-throughput detection, automation and miniaturization.

Zhu's group developed a PDMS–AuNP composite microfluidic system that can reach attomoles LOD for protein detection (Zhou et al., 2010). CdTe and ZnSe QDs were used as the electrochemical labels and the square-wave anodic stripping voltammetry was

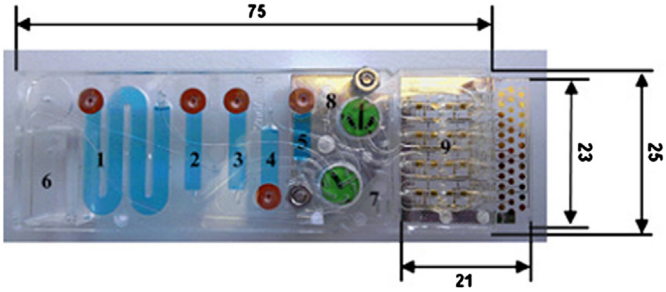

Fig. 5. Photograph of integrated microfluidic device. Copyright 2011 Wiley.

conducted in a microchannel via flow injection mode. Pereira et al. described a microfluidic device consists of a Plexiglas system with a central channel and a gold electrode, which was used for the detection of IgG antibodies specific to *Echinococcus granulosus* in human serum samples (Pereira et al., 2011). The proposed system presents reduced complexity and costs, which showed great promise for the future clinical application. Tan et al. reported a PDMS microfluidic immunosensor integrated with specific antibody immobilized alumina nanoporous membrane for rapid detection of foodborne pathogens Escherichia coli O157:H7 and *Staphylococcus aureus* with electrochemical impedance spectrum (Tan et al., 2011). The sensitivity was around 102 CFU/ml and the assay time was only 1–2 h.

Table 3
Examples of currently available POC biomarkers.

Type of disease	Biomarker
Cancer biomarker	AFP, CEA, PSA, PAP, p53, NSE, TNF-α
Pregnancy/ovulation	HCG, LH
Diabetes	HbA1c, urine albumin, creatinine
Cardiac diseases	CK-MB, troponins, BNP, Ddimers, cTnI/T, myoglobin
Infectious diseases	CRP, PCT
Viruses diseases	adenovirus, HIV, Hepatitis B, RSV, rotavirus, Influenza A and B

AFP: α-fetoprotein; CEA: carcinoembryonic antigen; PSA: prostate specific antigen; PAP: prostatic acid phosphatase; P53: tumor protein 53; NSE: Neuron specific enolase; TNF-α: tumor necrosis factor α; HCG: human chorionic gonadotropin; LH: luteinizing hormone; CK-MB: creatine kinase muscle and brain; BNP: B-type natriuretic peptide; CRP: C-reactive protein; PCT: procalcitonin; HIV: human immunodeficiency virus; RSV: respiratory syncytial virus.

As reported by Kellner, a fully automated microsystem housing an amperometric immunosensor was developed (Kellner et al., 2011). The microfluidic device integrated reagent reservoir, microvalves and detection zone with an electrode array (Fig. 5). The microsystem was computer controlled and can be used for the analysis of real patient serum samples. The analytical performance of the device for the amperometric detection of CEA and cancer antigen 15-3 (CA15-3) demonstrated excellent correlation with a commercial ELISA. This microsystem possessed great promising for POC testing.

5. POC applications of EI based systems

5.1. POC diagnostic biomarkers

POC diagnostics can be applied to a wide range of disease, such as cancer, diabetes, infectious diseases, and cardiac diseases. Several examples of the currently available POC biomarkers are presented in Table 3. As most diseases have more than one marker associated with their incidence, simultaneous detection of panels of disease markers can increase their diagnostic accuracy.

5.2. Commercially viable EI based system

As mentioned in the first part of this review, the eventual goal in the development of integrated EI systems is for POC diagnostics. Currently, several commercial EI-based systems are available for POC use, such as the i-STAT analyzer (http://www.poct.co.uk/index.cfm), Electro-Immunointerfaces and Surface Nanobiotechnology (ELISHA) system (http://www.immunosensors.com/), and the Asklepios system (http://genefluidics.com/products_asklepios.php#). Typically, a commercial EI-based system consists of two parts: electrochemical reader and disposable biochip. Analytes in a wide range of sample matrices can be detected by the recognition layers immobilized on biochips. Some of the designed biochips even realize the quantification of multiple proteins simultaneously.

As predicted by some researchers, the future POC system might become as common as personal computers or cellular phones, or might be integrated into small household items to monitor the daily health status of individuals. Thus there is still a long way to go. Additional research efforts are needed toward the development of POC systems with features of full integration, automation and miniaturization. Moreover, the cost should be lowered towards marketization.

6. Summary and conclusions

In conclusion, this review tried to trace the development of EIs based on the four key points: (1) the way to immobilize the recognition layer on electrodes; (2) the way to amplify electrochemical signal; (3) multiplexing analysis; and (4) integration of EI system with greater capabilities for POC diagnostics. On one hand, many researchers have taken advantage of the unique merits of NMs in the fabrication and signal amplification of EIs, resulting in ultrahigh sensitivity. Still there is much more room for progress, because the complicated fabrication processes of NMs limit their applications. On the other hand, several excellent studies have been done to perform multiplexing analysis and integration of EI-based POC systems. Some successful examples of integrated EI-based POC systems are commercially available, which indicates a developmental milestone of EI.

7. Future perspectives

Though there has been substantial progress in the development of EIs, additional research efforts are needed toward the development of POC systems with features of full integration, automation and miniaturization. Until now, most research efforts have focused on the methodology rather than the application of EIs. However, for the future commercialization, more and more researchers show concern for fabrication of EI-based POC systems. Thus experts in the field of microelectronics machine system are encouraged to participant. It can be expected that, with further development and resources, such POC systems are going to speed up the diagnosis of cancer and make analytical results available at patient bedside or physician office within only a few minutes.

Acknowledgments

The authors gratefully acknowledge financial support provided by the National Science Foundation (No. 21105048, 21205079), NUST Research Funding (No. 2011XQTR08). Shanghai Bureau of Quality and Technical Supervision (2012-04); the General Administration of Quality Supervision, Inspection and Quarantine of PRC (2012QK273).

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