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Review

Status of biomolecular recognition using electrochemical techniques

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ABSTRACT

The use of nanoscale materials (e.g., nanoparticles, nanowires, and nanorods) for electrochemical biosensing has seen explosive growth in recent years following the discovery of carbon nanotubes by Sumio Ijima in 1991. Although the resulting label-free sensors could potentially simplify the molecular recognition process, there are several important hurdles to be overcome. These include issues of validating the biosensor on statistically large population of real samples rather than the commonly reported relatively short synthetic oligonucleotides, pristine laboratory standards or bioreagents; multiplexing the sensors to accommodate high-throughput, multianalyte detection as well as application in complex clinical and environmental samples. This article reviews the status of biomolecular recognition using electrochemical detection by analyzing the trends, limitations, challenges and commercial devices in the field of electrochemical biosensors. It provides a survey of recent advances in electrochemical biosensors including integrated microelectrode arrays with microfluidic technologies, commercial multiplex electrochemical biosensors, aptamer-based sensors, and metal-enhanced electrochemical detection (MED), with limits of detection in the attomole range. Novel applications are also reviewed for cancer monitoring, detection of food pathogens, as well as recent advances in electrochemical glucose biosensors.

Contents

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1. Introduction

Molecular recognition plays a critical role in cell adhesion, surface wetting, tissue engineering, performance of biosensors and microarray technologies (Gellman, 1997; Sadik et al., 2002; Sampson et al., 2001; Twardowski and Nuzzo, 2003; Yan and Sadik, 2001). Until recently, these interactions are characterized at the molecular level using UV–vis spectroscopy, infrared spectroscopy, gel-electrophoretic mobility, nuclear magnetic resonance, circular dichroism and many other microarray techniques. These are, however, not easy for studying real-time biomolecular reactions either in solution or at interfaces (May and Russell, 2002; Sadik et al., 2002; Yan and Sadik, 2001).

The successful coupling of electrochemistry with naturally existing sophisticated biomolecules promises to generate novel, real time molecular recognition technologies that are sensitive and selective. Electroactive labels are commonly used in the detection of biochemical reactions at solid electrodes. Typically, one of the recognition partners is labeled or tagged with an electroactive species, such that the electrochemical property of this label is changed following the molecular recognition. Alternatively, a redox mediator is added to the solution being analyzed and the change in the electrochemistry of this species is used as a means of assessing the molecular recognition. Although an electroactive label allows the ultrasensitive detection of a single macromolecule, the need for such labeling may change the properties of a host macromolecule and the resulting change is not immediately known or could it be predicted beforehand. Moreover, the labeling step creates subtle variation in the binding affinities, conformational changes and associated kinetics of the biochemical reagents. Based on recent results with solid electrodes, it is clear that the function of many macromolecules changes from what is observed prior to the labeling step. In the worst case, molecules entirely loose their binding activity almost immediately after modification with the label. Consequently, the development of label-free, nanoparticle-modified electrochemical systems has been widely reported.

Recent reviews on electrochemical sensors have been reported for clinical analysis, assessing antioxidant capacity, ecotoxicity, environmental monitoring, food applications and sports medicine (Ahmed et al., 2008; Badihi-Mossberg et al., 2007; Gamella et al., 2006; Grieshaber et al., 2008; Hansen, 2008; Kumar and Chen, 2008a; Lambrianou et al., 2008; Nikolaus and Strehlitz, 2008; Polohova and Snejdarkova, 2008; Prieto-Simon et al., 2008; Rogers, 2006; Wang et al., 2008). This article, however, reviews the status of biomolecular recognition using electrochemical detection (ECD) in general. It discusses the challenges of assessing biomolecular recognition using electrochemical techniques, and focuses on how nanomaterials are being used to fabricate novel electrochemical biosensors and to enhance the analytical utility.

2. Label vs. label-free detection

Majority of the monitoring methods for biomolecular reactions require the application of a reporter or label (e.g., radio- or enzymatic- or fluorescent-labeling) to detect the molecular recognition between a ligand and its receptor. Among the most valuable labels are enzymes such as peroxidase, glucose oxidase (GOx), alkaline phosphatase, catalase or luciferase; electroactive compounds such as ferrocene or In^{2+} salts and a series of fluorescent labels including Cy5, ruthenium diamine complexes, phosphorescent porphyrin dyes and Alexa Fluor dyes (Cooper, 2003; Polohova and Snejdarkova, 2008). Most label-dependent platforms are based on the measurement of fluorescence (e.g., fluorescence resonance energy transfer or fluorescence polarization) or radioactivity (e.g., filter binding assays and scintillation proximity assays). These powerful technologies allow rapid determination of the affinities, and often the kinetics of a drug–receptor interaction with remarkable sensitivity (Luppa et al., 2001). However, the need for such labeling frequently changes the properties of host macromolecules and could sometimes result in total loss in bioactivity or stability (Table 1). Although techniques that employ labels are highly sensitive due to the analytical characteristics of the label applied, the concept of direct detection of biomolecular interactions offers potential simplicity (Fig. 1). Furthermore, the labeling steps involved in the indirect techniques impose additional time and cost constraints, and can in some cases interfere with the molecular interaction by blocking a binding site or leading to false negative responses. Many reporter compounds are also hydrophobic, and may create high background signals giving false positives (Andreescu et al., 2005, 2006, 2007; Cooper, 2003; Newman and Turner, 2005; Thevenot et al., 1999). In order to enhance the electrochemical sensitivity, particular attention must be paid to the inherent dependence of the electrochemical system on the trans-

Table 1
Comparing label vs. label-free electrochemical detection.

Labeled	Label-free
Utilize electroactive signal generating labels, e.g., enzymes, ferrocene, $Fe(CN)_6^{3-/4-}$; $Ru(bpy)_3^{3+/2+}$; $Os(bpy)_3^{3+/2+}$; methylene blue)	Detects a physical change in the system as a result of the biomolecular recognition
Labeled biosensors are more sensitive due to the amplification afforded by the enzymatic reaction or electroactive label	Less sensitive especially to molecular recognition involving small molecules, e.g., haptens, Ab–Ag
Requires additional steps (labeling and substrates enzymatic reaction)	Require fewer steps (no labeling step)
Additional steps increase the probability of error	More amenable to miniaturization
Electroactive label may require high redox potential, which may destroy the specificity of the biorecognition elements.	Suitable for both in-situ and ex-situ measurements (especially EIS)
	May facilitate the regeneration of the electrode surface using selected potential modulation (e.g., PAD).

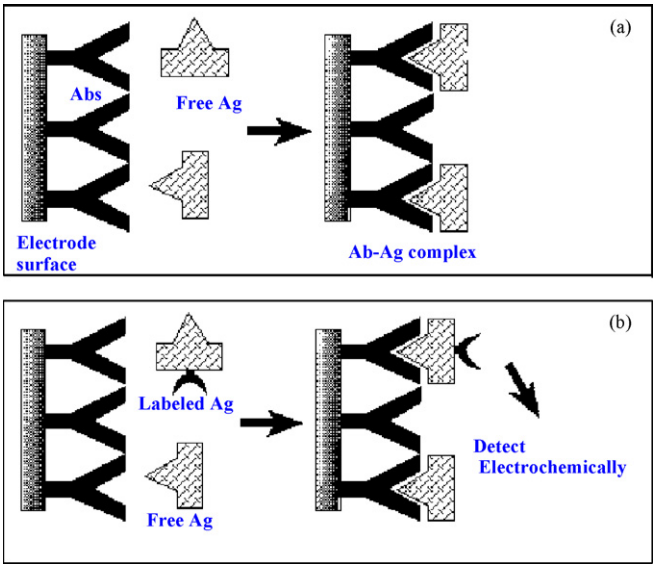

Fig. 1. Label vs. label-free electrochemical detection (see Table 1). The electrochemical detection is achieved by measuring the changes in the current, potential or charge of the labeled species or the resulting complex. Alternatively the electrochemical detection is possible via shifts in impedance, capacitance or admittance.

port rate of the redox couple to the electrode surface, i.e., electron transfer kinetics.

2.1. Electrochemical techniques for monitoring bioaffinity reactions

Electrochemical sensors for monitoring biomolecular reactions are mainly based on the detection of current or potential changes resulting from interactions occurring at the transducer/biomolecule interface. These sensors are designed by coupling the biorecognition elements (e.g., antibodies, DNA, receptors) to solid electrode surfaces (e.g., Pt, Au, Ag, graphite or carbon-based conductors) or electrode arrays, which respond to applied electrical impulses such as potential or current (Andreescu et al., 2005, 2006, 2007; Thevenot et al., 1999). Electrochemical techniques have played a major role in the move towards simplified testing, including home-use devices. Their rapid development is due to their relatively low cost, simplicity, sensitivity and ease of miniaturization, compared to optical, calorimetric or piezoelectric immunosensors. Indeed, easy-to-use self-testing glucose strips, based on screen-printed enzyme electrodes, coupled to pocket-size amperometric meters, have dominated the $5 billion/year diabetes monitoring market over the past two decades (Newman and Turner, 2005). Such disposable enzyme electrodes generate analytical information within 5–10 s in connection to 0.5–10 μL fingerstick blood samples. The utilization of antibodies in electrochemical sensing technologies has not been so successful. This could be attributed to the fact that there is no convenient, sensitive and selective electrical signal generation mechanism available. Consequently, many workers have developed several systems in which indirect electrochemical signal generation has been used (Abel and von Woedtke, 2002; Campbell et al., 2002; Stefan et al., 2002; Subrahmanyam et al., 2002; Zhang et al., 2003). These systems, at least defeat some of the very purposes of sensing technologies because they are multistep processes consuming lots of time and reagents. Therefore several challenges still face the pursuit of practical, usable electrochemical immunosensors. Amperometry is perhaps the most common transduction method used in biosensor development. Amperometric biosensors operate by applying a constant potential and monitoring the current associated with the reduction or oxidation of an electroactive species involved in the recognition process. The current generated is linearly related to the analyte concentration. They are more attractive because of their high sensitivity and wide linear range. Fig. 1 shows schematics for label vs. label-free electrochemical detection. New sensing concepts, coupled with numerous technological innovations, have opened the door to widespread clinical applications of amperometric devices (Cunningham, 1998; Heinemann and Halsall, 1985; Wang, 1999, 2006). The high sensitivity, specificity, simplicity, and inherent miniaturization of modern electrical bioassays permit them to rival the most advanced optical protocols. Amperometry is commonly utilized in enzymatic assays in which hydrogen peroxide is one of the products. H_2O_2 is oxidized at a constant potential to produce oxygen molecule. The electrons produced results in a measurable current that is directly proportional to the hydrogen peroxide concentration. There have been numerous reports showing that direct electron transfer from an electrode to redox proteins creates difficulty due to the location of the electroactive center being buried deep within an enzyme's structure or may result in electrode fouling (Bard and Faulkner, 2001; Cunningham, 1998; Heinemann and Halsall, 1985; Wang, 1999, 2006). The irreversible or slow electron transfer is mainly attributed to the fact that even though the biological reaction of interest may be thermodynamically favorable, the kinetically controlled heterogeneous electron transfer requires an inaccessible overpotential. Problems with the orientation issues of the macromolecule is also been widely recognized (Bard and Faulkner, 2001; Cunningham, 1998; Heinemann and Halsall, 1985; Wang, 1999, 2006). In that respect, mediators are needed to simultaneously achieve equilibrium with both the biocomponent and the electrode. This implies that the mediator serves as the electron acceptor in place of molecular oxygen. The use of mediators facilitates electron transfer at the mediator's potential without the requirement of a high overvoltage for the biological compound. Redox mediators are small sized compounds that enable the reversible exchange of electrons with the electrode. Electron transfer mediators (e.g., ferrocene, $K_3Fe[(CN)]_6^{3-/4-}$; $Ru(bpy)_3^{3+/2+}$; $Os(bpy)_3^{3+/2+}$; methylene blue) with fast rates of heterogeneous electron transfer have been shown to yield better sensitivity than oxygen/hydrogen peroxide mediator.

However, unlike enzymatic reactions that can be followed directly by product formation or substrate depletion using a coupled or indicator reactions, antibody-based recognition reactions are not themselves measurable. In electrochemical immunosensors, there exists no direct electron exchange between the immobilized antigen or antibody and the electrode. The binding interaction generates no products (unlike the enzymatic sensors) that can be readily measured electrochemically. Therefore indirect detection is still the norm. Much of the electrochemical immunoassay work was pioneered by Heineman's Group and was generally referred to as electrochemical immunoassay (ECIA) (Bard and Faulkner, 2001; Cunningham, 1998; Heinemann and Halsall, 1985; Wang, 1999, 2006). Basically, ECIA utilizes enzyme-labeled antibodies and could involve different assay formats including sandwich and competitive methods. Recently, other approaches have been reported to achieve good detection limits, address the issue of electrode stability and surface regeneration using electrochemical techniques.

2.2. Electrochemical bioaffinity sensors: benefits and challenges

The specificity, simplicity, and inherent miniaturization afforded by the advances in modern electronics enabled electrochemical sensors to rival the most advanced optical protocols. One major obstacle in implementing electrochemistry for the detection of biomolecular reaction lies in its inadequate sensitivity. Other obstacles in adapting electrochemistry to monitoring biomolecular recognition, especially using "label-free" techniques are (i) immobilization procedures usually lead to a partial loss of antibody binding capacity, and (ii) regeneration (i.e., release of the antibody–antigen (Ab–Ag) binding) that is most often incomplete or impossible due to the lability of the Ab. However, regeneration is necessary in order to be able to calibrate the sensor. It is worth noting that many of the remaining challenges pertaining to electrochemical detection, especially with respect to selectivity may be readily overcome by integration into other analytical systems such as online sampling, separation or by coupling with flow injection systems.

In order to enhance electrochemical sensitivity, particular attention must be paid to the inherent dependence of the electrochemical system on the transport rate of the redox couple to the electrode surface, i.e., electron transfer kinetics. The electrons come from redox reactions of either immobilized enzyme labels or electroactive labels on the biomolecules for the indirect sensors, redox active molecules such as potassium ferrocyanide $(K_3Fe[(CN)_6]^{3-/4-}$ in solution or immobilized redox probes for the direct sensors. Hence the sensitivity of electrochemical bioaffinity sensors still depends on the diffusivity of the electroactive species and fast electron transfer. Moreover, the rate of electron transfer at charged transducer surfaces is influenced by solvation of redox probes and other ionic species in solution (Bard and Faulkner, 2001). As stated

earlier, slow overall kinetics and sluggish electron transfer are the key factors for the low sensitivity in electrochemical sensors. Other factors that influence the performance of electrochemical sensors include (i) the type of immobilization chemistry (ii) the nature of the transducer surface and (iii) assessible redox potential, (iv) stability of the immobilized biomolecule, and (iv) as well as the tedious immobilization process. Some of the challenges are currently being addressed using numerous conceptual approaches including nanomaterials, microarray technologies, electrochemical impedance spectroscopy (EIS), MED, and nanoparticle-modified/incorporated electrodes.

2.3. Nanomaterials used in electrochemical biosensors

Nanomaterials, an emerging subdiscipline in chemistry has enabled the development of ultrasensitive electrochemical biosensors due to their high surface area, favorable electronic properties and electrocatalytic activity as well as good biocompatibility induced by nanometer size and specific physicochemical characteristics. Nanotechnology brings new possibilities for biosensor construction and for developing novel electrochemical bioassays. Nanoscale materials have been used to achieve direct wiring of enzymes to electrode surface, to promote electrochemical reaction, to impose barcode for biomaterials and to amplify signal of biorecognition event. The resulting electrochemical nanobiosensors have been applied in areas of cancer diagnostics and detection of infectious organisms.

The use of nanoscale materials including nanoparticles, nanowire, nanoneedle, nanosheet, nanotube, nanorod, nanobelt, etc. for electrochemical biosensing have seen explosive growth in the past 5 years, since the report for low-potential detection of NADH using carbon nanotube (CNT)-modified electrodes by Wang and co-workers (Musameh et al., 2002) and the first use of gold nanoparticles as labels for electrochemical immunosensors by Limoges and co-workers (Dequaire et al., 2000). Hundreds of research articles using nanomaterials for electrochemical bioassays have since been published and this continues to show an increasing tendency. There are dozens of reviews available which partly deal with use of nanomaterials for electrochemical nanobiosensors (Pandey et al., 2008; Pumera et al., 2007; Xiao and Li, 2008), more detailed reviews on carbon nanotube-based sensors (Agui et al., 2008; Gooding, 2005; He et al., 2006; Kumar and Chen, 2008b; Merkoci et al., 2005b; Rivas et al., 2007; Sirivisoot and Webster, 2008; Wang, 2005a,b; Wildgoose et al., 2006; Yogeswaran and Chen, 2008a) and nanoparticles-based biosensing (Katz et al., 2004; Luo et al., 2006; Merkoci et al., 2005a; Pingarron et al., 2008) can be found. Nanowire as sensing materials has also been reviewed (Yogeswaran and Chen, 2008b). Nanowires belong to a growing family of nano-objects, which also includes nanotubes, nanoparticles, nanorods and more. Like all wires, nanowires can serve as electrodes or interconnects between micro- and nanoelectronic devices. Additionally their dimensions are on the same scale as biomolecules, which unveils exciting possibilities for their interaction with biological species, such as cells, antibodies, DNA and other proteins.

Recently, Chen et al. (2008) reported positively charged Ni–Al layered double hydroxide nanosheets (Ni–Al LDHNS) for the first time as matrices for immobilization of horseradish peroxidase (HRP) in order to fabricate enzyme electrodes for the purpose of studying direct electron transfer between the redox centers of proteins and underlying electrodes. The immobilized HRP in Ni–Al LDHNS on the surface of a glassy carbon electrode (GCE) exhibited good direct electrochemical and electrocatalytic responses to the reduction of hydrogen peroxide and trichloroacetic acid (TCA). The linear detection results show that Ni–Al LDHNS provides a novel and efficient platform for the immobilization of enzymes and the fabrication of third-generation biosensors. ZnO nanosheet (Lu et al., 2008a) and other nanofibers and metal oxide as sensing materials in electrochemical biosensors were also reported (Tang et al., 2005; Vamvakaki et al., 2008; Zhou et al., 2008).

2.4. Electrochemical DNA microarray technologies

Today, an ideal biosensor platform is required to be not only miniaturized and cost-efficient but also capable of simultaneous detection of multiple analytes. The current trend toward creating point-of-care molecular diagnostic biosensors and massively parallel biorecognition arrays (microarrays) has introduced new technical challenges for the probes, transducers, and their detection apparatus. Microarrays can analyze large numbers of biological molecules using small amounts of material that are spatially segregated on a substrate (e.g., plastic, glass, semiconductor). The biological materials deposited or synthesized on the chip surface include nucleic acids, proteins, peptides, antibody and carbohydrates. The methods used for placing materials onto the chip surface rely on physical spotting, piezoelectric deposition, and in-situ synthesis (primarily for DNA). DNA synthesis methods include photolithography and electrochemical synthesis.

Development of DNA biosensors and DNA microarrays has increased tremendously over the past few years as demonstrated by the large number of scientific publications in this area (Audrey et al., 2008; Daniel et al., 2007). DNA microarrays (commonly called gene chips, DNA chips, or biochips) exploit the preferential binding of complementary single-stranded nucleic acid sequences. Unlike DNA biosensors, DNA microarrays are made from glass, plastic, or silicon supports consisting of tens to thousands of 10–100 μm reaction zones onto which individual oligonucleotide sequences have been immobilized (Hahn et al., 2005). The immobilization of a DNA probe in DNA biosensors is achieved directly onto a transducer surface. The exact number of DNA probes varies in accordance with the application. Contrary to DNA biosensors that allow single-shot measurements, DNA microarrays allow multiple parallel detection and analysis of the patterns of expression of thousands of genes in a single experiment. The most common method for analyzing hybridization events on DNA microarrays is fluorescence. The main technique that has been commercially successful is fluorescence. However, many different methods have been demonstrated including other optical techniques. Some techniques use an optical label that is attached to probes in the sample during sample preparation. Additional methods have also been developed, such as surface plasmon resonance, atomic force microscopy, and techniques that measure mass increases upon hybridization (e.g., quartz crystal microbalance, cantilevers).

Electrochemical detection offers several advantages over conventional fluorescence, such as portability, higher performance with lower background, less expensive components, and measurements could be made on turbid samples. Electrochemical transducers have often been used for detecting DNA hybridization due to their high sensitivity, small dimensions, low cost, and compatibility with micromanufacturing technology. There are numerous labeled electrochemical DNA biosensors where the tag can be an enzyme, ferrocene, an interactive electroactive substance (a groove binder, such as Hoechst 33258, or an intercalator), or nanomaterials and label-free electrochemical DNA biosensors have also been reported as reviewed in detail by Audrey et al. (2008), Daniel et al. (2007). Here we will only focus on the development of electrochemical DNA microarray biosensors. Four ECD methods for microarray applications that have been described in the literature (Kumar and Dill, 2005).

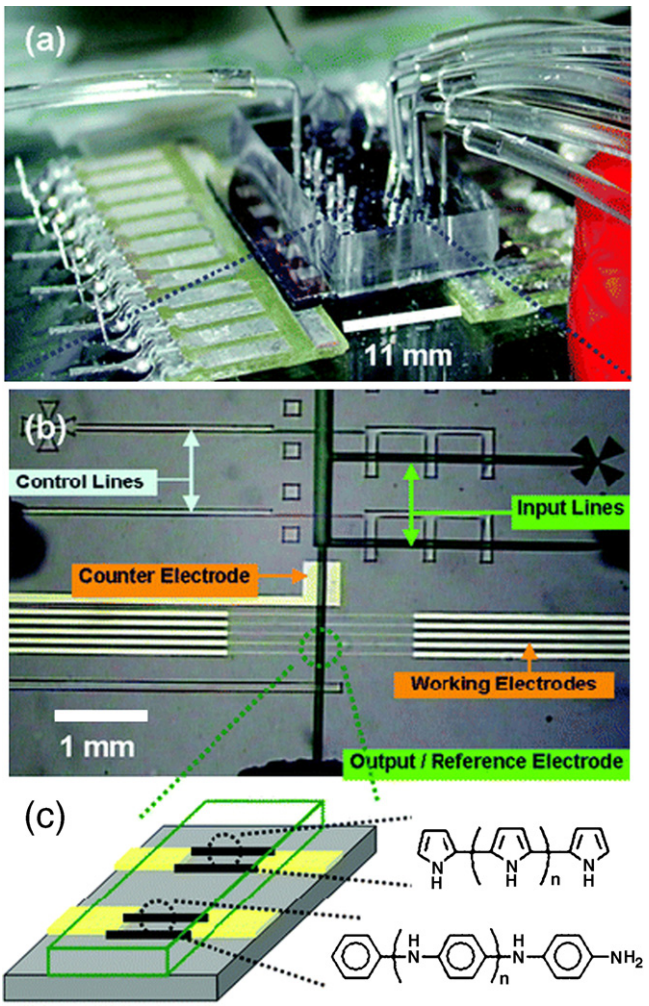

Fig. 2. (a) Actual view of the microfabricated and assembled integrated microfluidic device. (b) Optical micrograph of the integrated microfluidic device. Each microfluidic channel is 16 μm high and 100 μm wide. Each of the five pairs of electrode junctions is separated by a 2.0 μm-wide gap; the width and height of each electrode are 10 μm and 50 nm, respectively. (c) Schematic illustration of the electrochemical fabrication of CPNWs (in this case, polyaniline and polypyrrole) in the microfluidic channel. Reprinted with the permission from Wang et al. (2006). Copyright 2006 The Royal Society of Chemistry.

Microelectrode arrays are ensembles of microelectrodes, where single microelectrodes are wired in parallel with each one acting diffusionally independent, generating a signal which is thousands of times larger. The most common type is ordered disc microelectrode arrays (Davies et al., 2005; Hahn et al., 2005; Kumar and Dill, 2005). An example of microelectrode arrays are RAM™ electrodes where thousands of carbon wires are randomly sealed into epoxy resin producing random assemblies of microdisks (Davies et al., 2005). Microfluidic environments add value to biosensing tasks because they consume lower amounts of probe molecules and target analyte. Fig. 2 displays images of the microfabricated and assembled integrated microfluidic device containing conducting polymer nanowires (CPNWs) (Ordeig et al., 2006). The device comprises an array of Pt working microelectrodes (each pair separated by a 2 μm-wide gap) and a single platinum counter electrode, which are positioned within a microchannel of an overlaying two-layer PDMS microfluidic component. The reference Ag/AgCl electrode is placed just downstream of the working electrodes. The input channels can be used to deliver both the monomer precursor solutions for nanowire growth and the analyte solutions for nanowire sensing. The standard photolithography technique was used to fabricate the Pt microelectrodes on a silicon wafer possessing a thermally grown oxide layer. Initially, the integrated microfluidic device was completely filled with deionized water. By controlling the isolation valves, a solution of pyrrole or aniline monomer (driven by a back pressure of 2 psi) can be specifically introduced to the electrode junctions. After the monomer solution was delivered to the electrode junctions, one electrode on either side of the junction served as the working electrode for the electropolymerization of the corresponding CPNWs (Fig. 2c). The integration of electropolymerization and microfluidic technology provides several important advantages that allow a simple and rapid fabrication of high-quality CPNW sensors and their immediate utilization in situ in chemical and biological sensing.

A microelectrode array consisting of boron-doped diamond (BDD) microelectrode disks was also developed, a versatile electrode device with the advantages of both microelectrode arrays and of boron-doped diamond as an electrode material. To date, different BDD-microelectrode arrays have been reported. Basically, these devices are fabricated by creating a photoresist pattern on silicon onto which the BDD film is deposited. After this, the surface is spin coated with a polymer film (polyimide or Si_3N_4), which is then mechanically etched or via photolithography producing a protruding boron-doped diamond array consisting of 106 or 200 BDD disks with sizes between 5–30 μm diameter separated by a distance of 100–250 μm. The BDD-microelectrode arrays are excellent substrates for the deposition of a range of metals, copper, silver and gold allowing a single electrode array to act as a template for a microelectrode array of many different electrode materials for a variety of analytical tasks, all of which can be carried out with a single BDD-array after a suitable electrodeposition (Fletcher and Horne, 1999; Wang et al., 2006).

Microfluidic technology consisting of microfluidic mixer, valves, pumps, channels, chambers in a single chip device have been commercialized. The so-called CombiMatrix microelectrode array has been integrated to perform parallel immunoassays for detecting infectious particles (viruses and bacteria) in complex biological samples in a single, fully automated biochip device. The sensor is a miniaturized array of individually addressable microelectrodes controlled by active complementary metal-oxide-semiconductor (CMOS) circuitry. The devices with capabilities of on-chip sample processing and detection provide a cost-effective solution to direct sample-to-answer biological analysis for point-of-care genetic analysis, disease diagnosis, and in-field bio-threat detection (Liu, 2004; Simm et al., 2005). CombiMatrix's VLSI arrays of individually addressable electrodes using conventional CMOS integrated circuitry can be used in detecting various analytes via immunoassay protocols. These microarrays provide over 1000 electrodes per square centimeter. The chips are coated with a porous material on which specific affinity tags are synthesized proximate to selected electrode sites. The results for human α1 acid glycoprotein, ricin, M13 phage, *Bacillus globigii* spores, and fluorescein indicate that this method is one of the most sensitive available, with limits of detection in the attomole range. The detection range is 4–5 logs of analyte concentration, with an assay volume of 50 μL or less. CombiMatrix microarray platform provides for a host of multiplexed immunoassays because of the large number of electrodes available (Dill et al., 2004a,b; Liu, 2004).CombiMatrix developed an oligonucleotide microarray platform that contains 12,544 individual electrodes per square centimeter, and each working electrode is 44 μm in diameter (Ghindilis et al., 2007). First, a different ODN probe was synthesized at each microelectrode. After hybridization with the biotinylated target, the HRP-streptavidin conjugate allowed the electrochemical detection. The enzyme label catalyzed

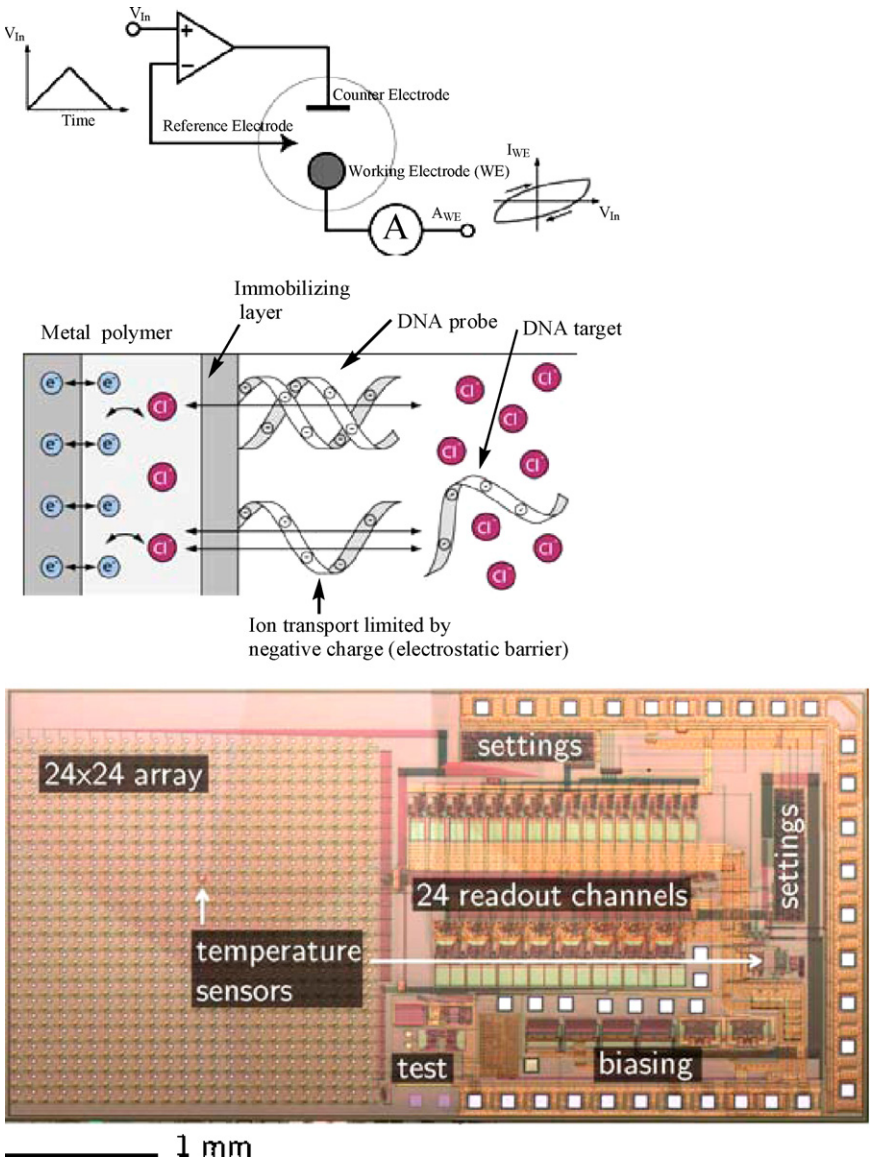

Fig. 3. Measurement principle chloride-counter ion travel kinetics are influenced by the extent of hybridization. A redox cycle is applied to the electrode and the electroactive polymer. CMOS Chip System Micrograph The chip contains electrodes, potentiostats, A/D converters and some digital circuitry units. Illustration of a cyclic voltammetry experiment and description of the label-free electrochemical DNA hybridization detection principle (TOP); The chip micrograph contains electrodes, potentiostats, A/D converters and some digital circuitry units (bottom) (reprinted with the permission from Heel, F. (2008). Copyright 2008 IEEE.

the oxidation of tetramethylbenzidine by H_2O_2. The detection limit was 7.5×10^{-13} M (http://www.Combimatrix.com).

CMOS multi-electrode DNA detection array for field and point-of-care usage (e.g., for the diagnosis of infectious diseases) was reported by developing a monolithic CMOS system carrying 576 electrodes and the necessary potentiostats on the same chip. The analog-to-digital conversion is also done on-chip. The measurement principle relies on cyclic voltammetry (CV) producing *I–V* curves, the shape of which depends on the electrochemistry occurring at the electrode surface (Fig. 3). The platinum electrodes are covered with an electroactive polymer (polypyrrole), which is cyclically oxidized and reduced. The kinetics of the chloride counterion exchange with the liquid phase upon this redox cycling is affected by the presence and concentration of negatively charged phosphate groups on the DNA strands. The number of these negative charges in close electrode vicinity increases upon hybridization. The kinetics of the ionic travel and, consequently, the shapes of the CV curves, are consequently altered through hybridization (Ghindilis et al., 2007). An integrated CMOS electrochemical sensor array capable of performing impedance spectroscopy, potentiometry, voltammetry, and ion-sensitive detection was reported by Hassibi and Lee (2006). The complete system is fabricated within a single chip and built in a standard digital 0.18-μm CMOS process with no post processing requirements.

Despite the enormous progress made in electrochemical nucleic acid biosensor research in the past several years (Choi and Park, 2004; Hassibi and Lee, 2006; Heer et al., 2008; Liang et al., 2004), to be one step closer to commercialization, this research must overcome several important hurdles. The first is validation of the biosensor results on a statistically large population of real samples rather than the commonly reported relatively short synthetic oligonucleotides. Another challenge is to multiplex the electrochemical biosensors into useful sensor arrays. Typically, arrays of 30–100 sensors are needed for diagnostic purposes. For example, breast cancer screening requires the testing of 20–30 cancer susceptibility genes plus positive and negative controls. Of the

many proposed electrochemical detection schemes, only a few attempts have been made to detect gene expression at the mRNA level (Priano et al., 2008; Xie et al., 2004).

2.5. EIS for monitoring bioaffinity reactions: new tricks for an old technique

Electrochemical impedance spectroscopy is capable of directly detecting the specific reaction between a receptor and its ligand. EIS is widely used to characterize variations in the electronic properties of bulk materials, and for investigating surface and interfacial processes on electrodes (Barsoukov and Macdonald, 2005; MacDonald, 1991). EIS principles have been of only minor significance for monitoring bioaffinity reactions. However, EIS represents one of the most powerful methods for directly probing the interfacial reaction mechanisms.

EIS provides a rapid approach for monitoring the dynamics of biomolecular interactions and it can be used to predict important aspects of bioaffinity sensors including surface reactivity, surface loading, binding constants and rates of reaction. It can contribute to the interpretation of fundamental biochemical processes via exact mathematical and physical models that are based on plausible linear and macroscopic phenomena. Numerous EIS-based bioaffinity sensors have been described for proteins, DNA–DNA, Ab–Ag or oligonucleotide–DNA interactions (Anderson, 1998; Jackson, 2000; Kolodner, 1996; Kolodner, 1995; Modrich, 1994; Schaeferling et al., 2002; Wetmur, 1991; Williams and Hochstrasser, 1997; Wilm and Mann, 1996; Xiao and Mansfeld, 1994). These sensors have been designed by immobilizing bioaffinity reagents (such as antigen, antibody, cells, DNA, enzyme layers, and semiconductors/thin silica layer) at the surface of a solid electrode. The binding of the molecular recognition partner is then verified through the detection of either a shift in impedance, or change in capacitance or admittance at the bulk of the electrode interface (Anderson, 1998; Patolsky et al., 1998, 1999; Wetmur, 1991). Hang and Guiseppi-Ellie (2004) utilized EIS for surface characterization of DNA immobilization and hybridization. Hamers an co-workers reported the direct electrical detection of hybridization at DNA-modified silicon surfaces and diamond thin films, based on field-induced effect (Cai et al., 2004; Yang et al., 2004a,b). Willner and co-workers used EIS to monitor the interfacial properties of layered electrodes upon formation of oligonucleotide complexes, and to precipitate enzymatic products (Patolsky et al., 1998, 1999). Our laboratory has extensively used EIS to study antibody–antigen interactions on conducting polymer-modified electrodes and other surfaces (K'Owino and Sadik, 2005; Sadik et al., 2002; Sargent et al., 1999; Sargent and Sadik, 1999, 2001; Sargent et al., 2001; Yan and Sadik, 2001). We have monitored the interfacial electron-transfer resistance of small organics, upon binding to the biotinylated dsDNA using the EIS (Sadik et al., 2002; Yan and Sadik, 2001). In a recent study, we described the underlying theory involved in the dynamics of antibody–antigen (Ab–Ag) interactions using linear, EIS technique (Sargent et al., 1999). The theory was used to test for antibody molecules sequestered within conducting polymer films (Sadik et al., 2002; Sargent et al., 1999). Recently, we reported the supramolecular docking and immobilization of biotinylated dsDNA onto a self-assembled monolayer of avidin using EIS and QCM techniques (Arora et al., 2001; K'Owino and Sadik, 2005; Katz and Willner, 2003; Patolsky et al., 2005; Sargent et al., 1999). Recent reviews further underscore the increasing usage of EIS for studying biological systems especially when no redox mediator is involved (K'Owino and Sadik, 2005; Katz and Willner, 2003). Recent reports showed unprecedented sensitivities for DNA detection using electronic amplification; it is possible to differentiate mutants at a concentration of 5×10^{-13} M from the normal gene at a concentration of 1×10^{-9} M (Ramanaviciene and Ramanavicius, 2004; Tansil and Gao, 2006). Direct detection methods are promising also using pulse amperometry (Ramanaviciene and Ramanavicius, 2004; Tansil and Gao, 2006). The amperometry method with ultra thin films of oligonucleotides-doped polypyrrole has been reported with LOD as low as 1.6 fmol in 0.1 mL (Tansil and Gao, 2006).

3. Recent advances in electrochemical bioaffinity sensors

3.1. Nanoparticles in biomolecular detection

Nanoparticles have been extensively used in bioaffinity sensors for nucleic acids and proteins (Tansil and Gao, 2006). These particles are unique because their nanometer size gives rise to high reactivity and other beneficial physical properties (electrical, electrochemical, optical and magnetic). Their applications can potentially translate into new assays that improve on current methods of biomolecular detection (Cai et al., 2002; Kerman et al., 2004). The redox properties of gold nanoparticles (coupled with silver enhancement) (Fig. 4) have led to their widespread application as electrochemical labels in biosensor development. These biosensors have remarkable sensitivity with detection limits in the pM range (Wang, 2003; Wang et al., 2003a).

In most cases, the metal nanoparticle can be oxidized to form metal ions that can be conveniently detected electrochemically. Ozsoz et al. (2003) reported the direct oxidation of gold nanoparticle tags following hybridization of the tagged target DNA and probe DNA that is covalently attached onto a graphite electrode. Wang et al. (2003a) used gold nanoparticles coated with ferrocenylhexathiol and streptavidin to monitor the DNA hybridization by using the ferrocene groups as the reporter molecule with a linear range for DNA between 7 and 150 pM. This approach requires no enzyme or enzyme substrate for amplification. Nanoparticles have also been coupled with magnetic particles to capture target DNA, which then hybridizes with a secondary probe DNA tagged to a metal nanoparticles. The nanoparticles were chemically oxidized and detected by anodic stripping voltammetry. The detection limit of 0.3 nM was reported using three different nanoparticle tags (ZnS, CdS, and PdS) (Wang et al., 2003b; Wang et al., 2002). A common problem with

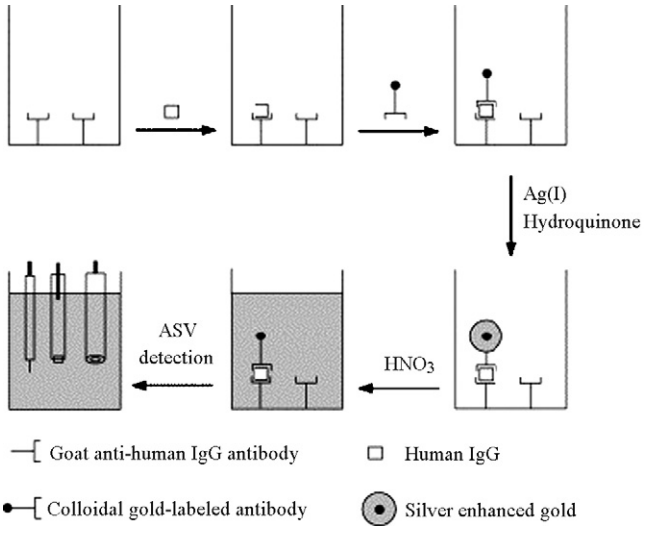

Fig. 4. Electrochemical detection of DNA hybridization using Au nanoparticles with silver enhancement (a) immobilization of target DNA (b) hybridization with Au nanoparticle labeled detection probe and Ag enhancement (c) voltammetric detection of Au redox signal (Copyright: Chu et al., Biosens. Bioelectron., 2005, 20, pp. 1805–1812; Wang et al., Anal. Chem., 2001, 73, pp. 5576–5581).

silver enhancement is a high background signal resulting from non-specific precipitation of silver onto the substrate electrode. Various electrode surface treatments and electrochemically or enzymatically controlled deposition methods of silver have been reported with the objective of reducing the silver related background signal and increasing sensitivity (Tansil and Gao, 2006).

Among various detection techniques for DNA hybridization, electrochemical detection is the most prevalent since it provides a simple, inexpensive, accurate and sensitive platform. Most of the electrochemical nanoparticle based DNA assays rely on anodic stripping (Park et al., 2001; Wang et al., 2001a,b) or conductance measurements (Wang et al., 2001b). The sensitivities of the anodic stripping techniques are improved by catalytic enlargement using silver enhancement. The assay of target DNA to the picomolar level has been achieved through these methods. However, the sensitivity of bioassays based on anodic stripping depends on the size of the metallic nanoparticle. This is limited to the preparation of 30 nm particles; otherwise the sensitivity of the assay is reduced by the high background signal caused by capture and deposition of silver. To overcome this restriction, Hwang et al. (2005) have developed a new system of electrochemical detection of DNA hybridization based on stripping voltammetry of enzymatically deposited silver. The target DNA and a biotinylated detection DNA probe hybridize to a capture DNA probe tethered onto a gold electrode. Neutravidin (NA) conjugated alkaline phosphatase binds to the biotin of the detection probe on the electrode surface converting the non-electroactive substrate to a reducing agent. The latter reduces the metal ions in solutions leading to the deposition of metal onto the electrode surface and DNA backbone. Hwang et al. have reported a remarkable detection of 100 aM (or 10 zmol) for DNA. Finally, additional steps may be required to remove the excess silver, after the silver enhancement step, which may otherwise affect the reliability of the stripping-based electrochemical detection. To overcome these drawbacks, we have developed a novel MED electrochemical scheme for probing the electronic properties of DNA binding with small molecules, toxins and Ab–Ag recognition.

3.2. Metal-enhanced electrochemical detection (MED)

The application of underpotential deposition (upd) of Ag monolayer as a means of enhancing the electrochemical sensitivity of biomolecular reaction was first reported by K'Owino et al., and successfully applied in the development of a new DNA platform via metal-enhanced electrochemical detection (MED sensors) (K'Owino et al., 2003). This is based on the discovery that immobilized metal layer, either as continuous film, particle, colloids or monolayer significantly amplifies the electrochemical signals, while reducing the reorganization energy following molecular recognition at DNA electrodes.

3.2.1. MED assay format

In MED, a solid-phase monolayer of silver is deposited onto gold surface using upd (Fig. 5). The surface is then modified with either a single-stranded, double-stranded nucleic acid, Ab or Ag recognition element. A significant change in the current is measured upon exposure to the binding partner (e.g., DNA, Ag or small organic molecules). These current changes are dependent on the concentrations of this binding partner. The variation in the redox current is proportional to the concentration of the DNA (or Ag) binding molecule. Experimental evidence is provided from cyclic voltammetry, differential pulsed voltammetry, scanning electron micrograph and energy-dispersive X-ray spectroscopy.

MED has been tested for the detection of parts-per-Trillion (ppT) levels of anticancer drug cisplatin and PCBs (K'Owino et al., 2003). A modified MED concept was also explored for detection of two base pair mismatches using *Microcystis* as model (Aluoch et al., 2005b; K'Owino et al., 2007). Results showed a linear response to target DNA sequence at pM concentrations. The approach offers an alternative route to ultrasensitive label-free assay, which has also been extended for antigen–antibody binding reactions. Fig. 5 demonstrates the principle and the format for MED. The typical electrochemical signals for the MED detection could be determined using cyclic voltammetry (as illustrated in Fig. 5), or differential

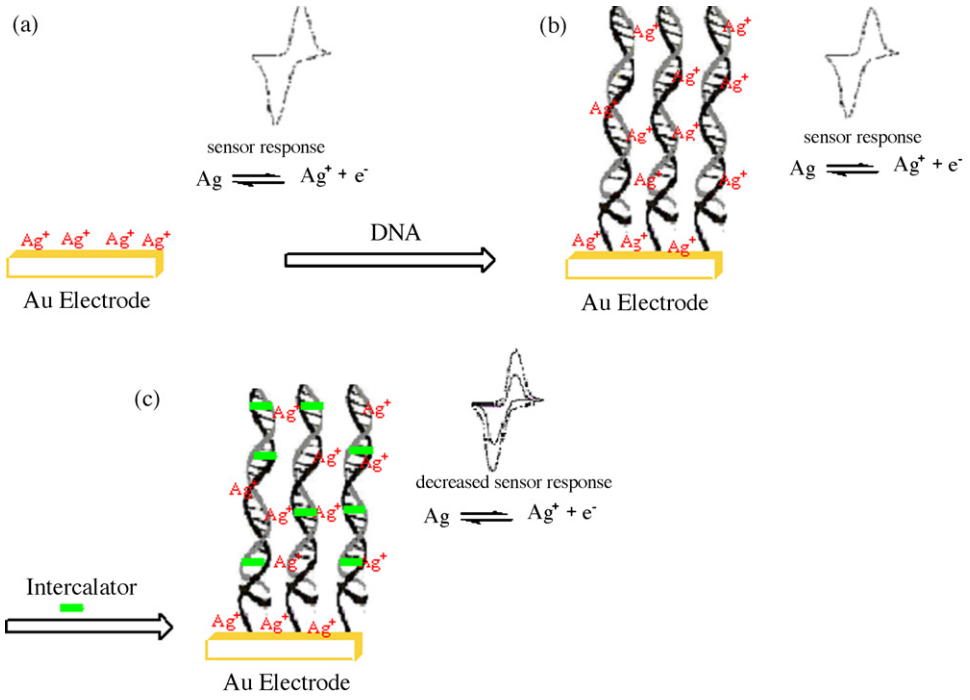

Fig. 5. Electrochemical detection of DNA-small molecule detection MED (a) preparation of silver monolayer on Ag using upd, (b) immobilization of double-stranded DNA and (c) intercalation of small molecules e.g toxins, drugs etc., which intercalates. MED concept has also been demonstrated for assessing Ab–Ag, DNA–DNA and DNA–protein interactions (~100–1000-fold enhancement in sensitivity recorded for bioaffinity monitoring).

pulse voltammetry (DPV). The resulting molecular recognition creates simultaneous insulation of the electrode surface by the immobilized biomolecules. Using the MED scheme, a detection limit of 10 pM was obtained for cisplatin–short sequence DNA interactions, which represents ~100-fold improvement compared with the previous (1 nM) detection limit (K'Owino and Sadik, 2005; Yan and Sadik, 2001). This remarkable sensitivity signifies the suitability of the new scheme for monitoring DNA–cisplatin interactions. The detection may also be used to monitor other biomolecular reactions including DNA hybridization (K'Owino et al., 2003), mismatch detection (Aluoch et al., 2005b; K'Owino et al., 2007), DNA–protein, antigen–antibody (Ngundi, 2003), and DNA–RNA reactions.

3.3. MED implementation for DNA–small molecule interactions

Biosensors have been developed for polychlorinated biphenyls (PCBs) including electrochemical immunosensors (Benda and Sadik, 1998), DNA biosensors by Mascini an co-workers (Marrazza et al., 1999). Work by Yan and co-workers (Sadik et al., 2002; Yan and Sadik, 2001) in which Faradaic impedance spectroscopy was used as a detection technique is another example. However, the detection limits recorded in these studies (ca. 1 nM) needed improvement. We thus examined the utility of MED DNA biosensor in the detection of different PCB congeners. Samples studied included Aroclors 1221, 1242, 1254 and 1260. We are not aware of previous works for the in-vitro measurements of PCB toxicity and the direct interactions with DNA using Ag^0/Ag^+ redox couple. To achieve our goal, we performed DPV of the DNA modified upd-Ag electrode after incubation with different concentrations of each PCB Aroclors. Electrochemical responses of all the PCB Aroclors were compared with those of cisplatin.

The DPV results for cisplatin are shown in Fig. 6. As seen, DPV of the Ag^0/Ag^+ couple exhibited symmetric peaks for cisplatin. Similar DPV responses were recorded for all the PCBs studied. In addition, the peak width at half peak height ($E_{1/2}$) of these voltammograms varied from one analyte to the other. For instance, the $E_{1/2}$ value for the cisplatin was 53 mV while that of 1×10^{-2} ppb of Aroclor 1221 was 50 mV. Similarly, Aroclors 1242 and 1254 exhibited $E_{1/2}$ values of 48 mV and 60 mV, respectively. Consequently, the respective order of $E_{1/2}$ for PCBs was determined as 1221 < 1242 < 1254. Interestingly, this order tallied with the percentage chlorine by weight in the Aroclors: 21% for Aroclor 1221, 42% for Aroclor 1242 and 54% for Aroclor 1254. We therefore attributed this observation to size-limited DNA–PCB interactions. Plot of $\log_{10}$[PCB] vs. peak current decreased linearly (with R^2 values > 0.900) for Aroclors 1221, 1242 and 1260, respectively. These current decreases were attributed to the insulation of the electrode surface by the dsDNA following the binding of PCB Aroclors.

3.4. MED implementation for Ab–Ag recognition

MED concept has also been tested for the electrochemical detection of Ab–Ag reactions. Since silver is monodispersed on the gold surface, the antigen via cystamine, strongly chemisorbs at the gold electrode. The control CV experiment for the upd-silver using the antigenic monolayer electrode and phosphate buffer solution showed anodic and cathodic peaks at 155 and −2 mV. The peak currents recorded remained essentially constant after several cycles. This indicated that the presence of the antibody–antigen interaction is necessary for the predicted insulation of the electrode surface to occur. The extent of electrode surface insulation was observed to be dependent on the concentration of the antibody with upd-silver as the redox couple on the antigenic monolayer electrode, as was observed when potassium ferrocyanide was used as the redox couple. A linear decrease in

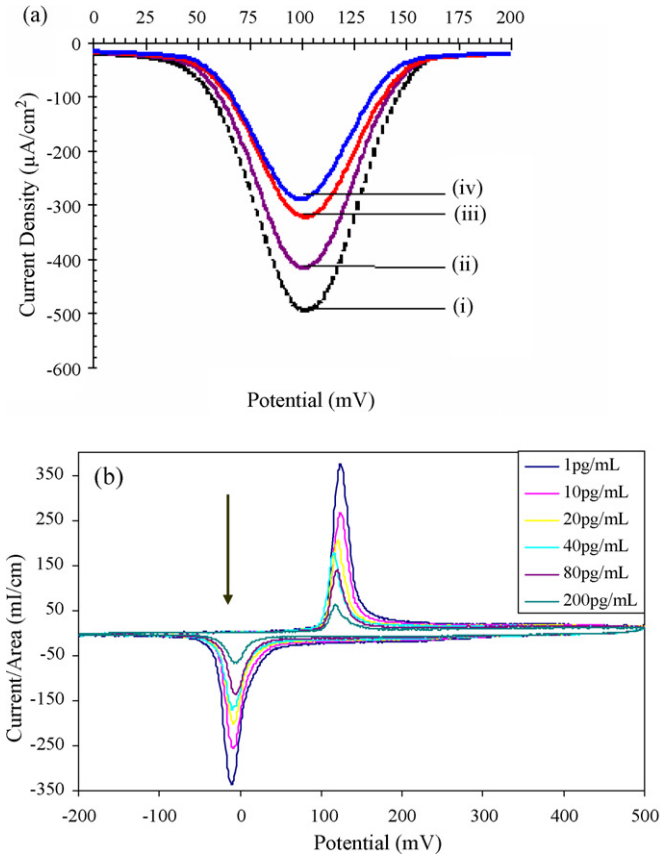

Fig. 6. Differential pulse voltammograms for MED technique using (a) different concentrations of cisplatin (i) of biotinylated ds DNA (ii) 1×10^{-5} nM cisplatin (iii) 1×10^{-4} nM cisplatin and (iv) 1×10^{-3} nM cisplatin. Conditions: PBS buffer (pH 7.3); and a scan rate 20 mV/s and (b) cyclic voltammograms showing the effect of increasing salivary amylase antigen concentration based on MED detection.

the peak current was observed as the concentration of the mouse anti-amylase was increased (Fig. 6). The calibration curve generated from plotting the peak current vs. the mouse anti-amylase concentration showed a linear range was between 3×10^{-3} and 1.6×10^{-2} ng/mL with a detection limit of 1.57 pg/mL (Aluoch et al., 2005a). For the analysis of Histatin, a linear detection range of 1–40 pg/mL and a detection limit of 0.626 pg/mL were recorded. This detection limit was 100-fold and 10-fold superior to the gold standard ELISA and the optical-based capillary fluorescence ELISA, respectively. The response of the biosensor to potential interferants was tested. The amylase-antibody-modified electrode had very minimal response for 1 μg/mL cystatin and histatin. Therefore cross-reactivity between, anti-cystatin and amylase–histatin was insignificant Superior analytical characteristics of the MED immunosensor were also realized compared to the solution based $Fe(CN)_6^{3-/4-}$ based system.

3.5. Aptamer-based electrochemical biosensors

Aptamers are artificial ligands consisting of single-stranded DNA and/or RNA sequences and are typically synthesized in vitro using systematic evolution of ligands by exponential enrichment (SELEX). These artificial nucleic acid ligands appear as attractive alternatives to antibodies owing to their relative ease of isolation and modification, tailored binding affinity, and resistance against denaturation that substantially enables them to be used as recognition elements for biosensing applications (Lee et al., 2008; Song et al., 2008; Tombelli et al., 2007; Willner and Zayats,

2007). To this end, extensive activities have been directed towards the application of aptamers as versatile materials for the design of biosensors. In particular, the combination of electrochemical methods (voltammetric, potentiometric and impedimetric detection) with the specific recognition properties of the aptamers has enabled the investigations of electrochemical aptasensors based on enzymes (Baldrich et al., 2004; Ikebukuro et al., 2005; Mir et al., 2006), nanoparticles and quantum dot (Hansen et al., 2006; He et al., 2007; Li et al., 2008; Numnuam et al., 2008) labels or on a binding-induced label-free detection by using intercalators (Baker et al., 2006; Cheng et al., 2007; Cho et al., 2008; Du et al., 2008; Le Floch et al., 2006; Li et al., 2007; Rodriguez et al., 2005). A variety of targets have been reported ranging from small molecules (Centi et al., 2007; Degefa and Kwak, 2008; Ikebukuro et al., 2005; Kim et al., 2007; Li et al., 2007; Radi et al., 2005; Strehlitz et al., 2008; Wu et al., 2007), 17-beta-estradiol (Kim et al., 2007), various proteins (He et al., 2007; Huang et al., 2008; Ikebukuro et al., 2005; Lai et al., 2007; Liao and Cui, 2007; Mir et al., 2006; Numnuam et al., 2008; Radi et al., 2006; Rodriguez et al., 2005; Shen et al., 2007; Zuo et al., 2007). These are both theoretically interesting and practically useful.

Different electrochemical aptasensors have been reviewed by Strehlitz and co-workers (Ikebukuro et al., 2005; Strehlitz et al., 2008) and mainly used for thrombin etc. protein detection (Ikebukuro et al., 2005; Mir et al., 2006). In one configuration, thrombin was detected using a sandwich format (Fig. 7). Sandwich assay formats are only applicable in the case of targets with multiple aptamer binding regions. Thrombin has two electropositive exosites, both of which are capable of binding a specific aptamer. The sandwich assay was performed by immobilizing a thiolated aptamer as a self-assembled monolayer on a gold electrode. The electrode was subsequently incubated with thrombin. In a second incubation step, a HRP-labeled aptamer was allowed to bind to the other thrombin exosite. The HRP-labeled aptamer was prepared by incubating a biotin-labeled aptamer with streptavidin-HRP, effectively forming a peroxidase-labeled aptamer. The HRP thus immobilized at the electrode surface, was measured electrochemically using hydrogen peroxide and osmium-based mediator ($[Os(bpy)^2(pyr\text{-}CH_2\text{-}NH_2)]Cl$). The current measured on the electrode was related to the quantity of thrombin by calibration. Aptamers have also been used as electrochemical beacons, which are oligonucleotide molecules able to undergo a measurable conformation change in response to the target analyte. A ferrocene-labeled aptamer beacon sensor has been used for thrombin detection, and an electrochemical aptamer-based beacon has been developed for cocaine detection (Li et al., 2007; Radi et al., 2006). These techniques described above are label-free, that is, neither the bioreceptor nor the target has to be covalently labeled with indicator molecules and this therefore omits a further step in the production process of the sensor.

So far, most electrochemical aptasensors have been based on the conformational change of the aptamers induced by the specific target binding, as schematically shown in Fig. 8A. In this context, the aptamers that were used as the probes for electrochemical target sensing were normally functionalized with a thiol group at one end so that they could be anchored onto the electrode (e.g., Au electrode) to make the as-prepared aptasensors essentially reagentless. The other end of the aptamer probes was labeled with redox moieties such as ferrocene (Li et al., 2007; Lu et al., 2008b; Radi et al., 2005) and methylene blue (Lai et al., 2007; Radi et al., 2006) for electrochemical sensing. This kind of electrochemical aptasensor with the aptamers as the probes has so far been employed for selective and sensitive electrochemical sensing of targets such as cocaine detection (Baker et al., 2006; Lu et al., 2008b), thrombin detection (Du et al., 2008; Lu et al., 2008b; White et al., 2008), ATP detection (Du et al., 2008; Shen et al., 2007; Zuo et al., 2007), platelet-derived growth factor (PDGF) protein detection (Degefa and Kwak, 2008; Ferapontova et al., 2008; Huang et al., 2008; Lai et al., 2007; Liao and Cui, 2007), and theophylline detection (Ferapontova et al., 2008). Contrary to what happens with DNA probes, the aptamers ability to bind their targets depends on folding and 3D structure. The specific binding of the targets to the surface-confined and redox-labeled aptamers essentially induces the conformational change of the aptamer probes and, as such, leads to the change in the distance between the labeled redox moieties and the electrode, which eventually results in the large variation in the voltammetric signal for electrochemical sensing of the targets. Moreover, the electrochemical aptasensors with the surface-confined aptamers as the probes may suffer from limitations from the complex procedures for sensor regeneration presumably due to the strong binding affinity of the aptamers toward the targets.

Recently, Lu et al. (2008b) reported a facile and general strategy for the development of aptamer-based electrochemical sensors, where the aptamer-complementary DNA oligonucleotides as the probes is based on the formation of a hairpin structure of cDNA probes through the hybridization of the tailor-made complementary sequences at their both ends caused by the target binding-induced dissociation of the aptamer (Fig. 8B). The prepared electrochemical aptasensors with the cDNA oligonucleotides as the probes and with the aptamers as the recognition elements substantially enable them to be used for the determination of targets in real samples. Similarly, Du reported a multifunctional label-free electrochemical biosensor based on an integrated aptamer for parallel detection of adenosine triphosphate (ATP) and alpha-thrombin, where a Au electrode as the sensing surface was modified with a part DNA duplex which contained a 5′-thiolated partly complementary strand (PCS) and a mixed aptamer (MBA) (Du et al., 2008).

Aptamer-based microarrays for the quantitation of multiple protein analytes have been developed (Cho et al., 2006; Evans et al., 2008; Kirby et al., 2004; Xu et al., 2005). A multiplex aptamer

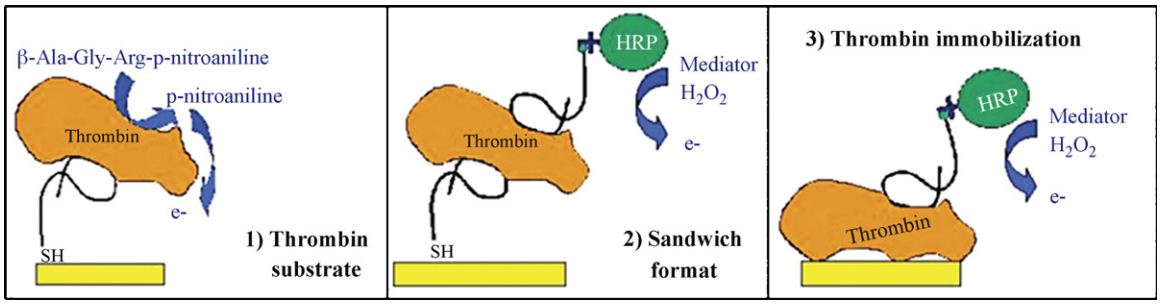

Fig. 7. Electrochemical aptamer-based sensor in a sandwich format. Reprinted with the permission from Mir et al. (2006). Copyright 2008 Elsevier.

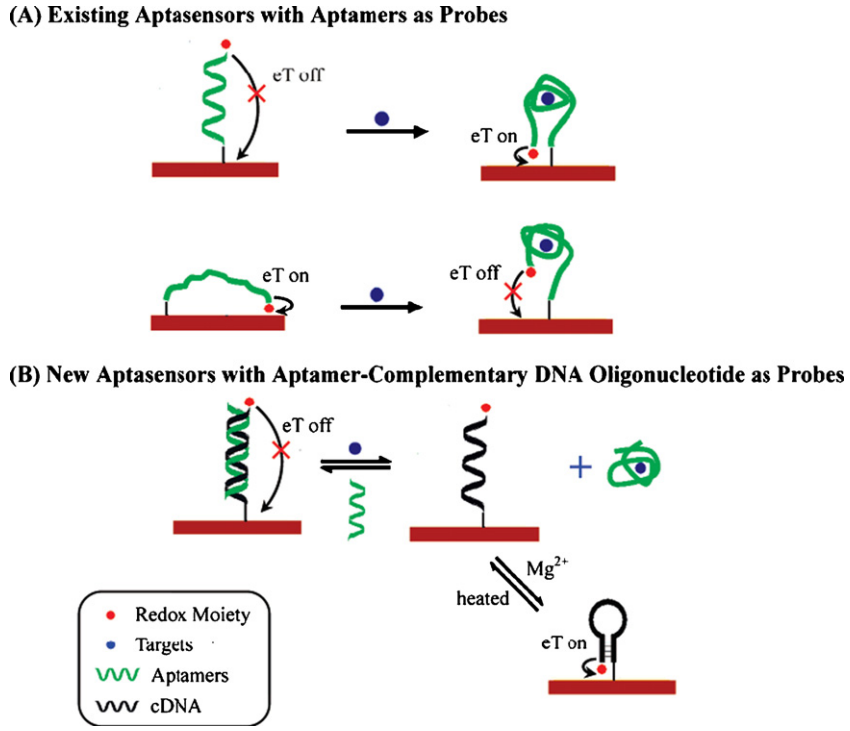

Fig. 8. (A) Aptasensors with aptamers as probes and (B) aptasensors with aptamer-complementary DNA oligonucleotide as probes. Reprinted with the permission from Lu et al. (2008b). Copyright 2008 American Chemical Society.

microarray was generated by printing two RNA aptamers (anti-lysozyme and anti-ricin) and two DNA aptamers (anti-IgE and anti-thrombin) on to either streptavidin (SA) or neutravidin-coated glass slides. Unlike most protein-based arrays, the aptamer chips could be stripped and reused multiple times. The aptamer chips proved to be useful for screening aptamers from in vitro selection experiments and for sensitively quantitating the biothreat agent ricin. Furthermore, aptamer arrays and biosensors will reveal the most effective tools for the detection of biomolecular interactions and the identification of protein targets, particularly with regard to those not detectable by known receptors like enzymes or antibodies.

4. Novel practical applications

4.1. Electrochemical biosensors for cancer monitoring

The possibility of using minimally invasive analytical instruments to monitor cancerous cells and their interactions with analytes provide great advances in cancer research and toxicology. Biosensors have emerged as a new technique for monitoring cancerous cells or their specific interaction with different analytes. The real success in the development of a reliable sensor for cell monitoring depends on the ability to design powerful instrumentation that will facilitate efficient signal transduction from the biological process that occurs in the cellular environment. The resulting sensor should not affect cell viability and must function as well as adapt the system to the specific conditions imposed by the cell culture.

Modern electrochemical bioaffinity sensors, such as DNA or immunosensors, have recently demonstrated great potential for monitoring cancer-related protein markers and DNA mutations. Although still at the basic research stage, it is envisioned that further development should transfer these electronic assays into small test cartridges. Representative examples are discussed in the following sections. Gao and co-workers (Tansil et al., 2005) recently reported on the ultrasensitive detection of cancer marker genes in mRNA extracted from human breast tissues (without a RT-PCR step), based on the catalytic oxidation of the guanine nucleobase by a redox threading intercalator. Willner and co-workers developed an amplified chronopotentiometric detection of telomerase activity extracted from 1000 HeLa cancer cells, in connection with hybridization of a biotin-labeled oligonucleotide to the telomere repeat units and subsequent binding of avidin-alkaline phosphatase (Pavlov et al., 2004).

Abnormal concentrations of certain proteins can indicate the presence of various cancers. For the past two decades Heineman's group has developed highly sensitive enzyme electrochemical immunoassays (Ronkainen-Matsuno et al., 2002). Such protocols rely on labeling of the antibody (or antigen) with an enzyme which acts on a substrate and generate an electroactive product that can be detected amperometrically. Enzyme immunosensors can employ competitive or sandwich modes of operation. In addition to enzyme labels, it is possible to use metal markers and redox tags for electronic transduction of antigen–antibody interactions.

The development of electrochemical immunosensors for measuring tumor markers was recently reviewed in this journal (Lin and Ju, 2005). It is well recognized now that simultaneous measurements of combination of tumor markers (i.e., distinct protein patterns) can play a major role in the diagnosis of cancer (Weisner, 2004). Protein signature patterns, reflecting different stages of the disease can be readily detected using immunosensor chips based on multiple antibody-functionalized transducers. Electrode arrays, having multiple individually addressable electrochemical transducers can be readily fabricated to meet the requirements of multi-analyte protein arrays. The use of a single enzyme label with such arrays requires proper spatial separation of the individual transducers to eliminate cross-talk problems due to diffusion of the enzymatically generated electroactive species from one electrode to the neighboring one. Wilson (2005) recently described a

dual electrode enzyme immunosensor for simultaneous amperometric measurements of the two tumor markers carcinoembryonic antigen (CEA) and α-fetoprotein (AFP). High sensitivity down to the 1 ng/mL level was reported, along with the absence of cross-talk effects. Nucleic acid ligands, known as aptamers, also offer great promise for electrochemical sensing of proteins. Label-free detection of aptamer–protein interactions has been documented recently in connection to electrochemical impedance spectroscopic transduction of the binding event (Rodriguez et al., 2005).

One-dimensional nanostructures, such as semiconductor- or conducting-polymer nanowires (NW), are extremely attractive for designing high-density protein arrays. Because of their high surface-to-volume ratio and novel electron transport properties, their electronic conductance is strongly influenced by minor surface perturbations (e.g., binding of biomolecules), hence indicating great promise for label-free real-time protein detection. Such 1D material thus offers the prospect of massive redundancy in nanosensor arrays and suggests great promise for assays of multiple disease markers in ultrasmall sample volumes. Several studies already indicated the potential of functionalized NW for highly sensitive real-time biodetection. For example, Lieber's laboratory demonstrated highly sensitive Protocols for monitoring DNA hybridization (Hahm and Lieber, 2004) or single viruses (Patolsky et al., 2004b) in connection to p-type silicon NW (SiNW) functionalized with PNA probes or antibodies for influenza, respectively. A very recent contribution from the same laboratory demonstrated the use of an antibody-functionalized silicon nanowire sensor array for the multiplexed label-free real-time monitoring of cancer markers in undiluted serum samples (Zheng et al., 2005). The biosensing utility of other 1D nanomaterials, such as conducting polymer NW or carbon nanotubes has also been illustrated (Katz and Willner, 2004; Ramanathan et al., 2005).

Recently, a simple and sensitive label-free electrochemical immunoassay electrode for detection of CEA has been developed. CEA antibody (CEAAb) was covalently attached on glutathione (GSH) monolayer-modified gold nanoparticle (AuNP) and the resulting CEAAb-AuNP bioconjugates were immobilized on Au electrode by electro-copolymerization with o-aminophenol (OAP). Electrochemical impedance spectroscopy and cyclic voltammetry studies demonstrate that the formation of CEA antibody–antigen complexes increases the electron transfer resistance of $Fe[(CN)]_6^{3-/4-}$ redox pair at the poly-OAP/CEAAb-AuNP/Au electrode (Tang et al., 2007). As more and more cancer antigens are being identified, this opens new possibilities for early cancer diagnoses based on enzymatic biosensing which is a key issue in cancer treatment.

An amplified electrochemical sandwich immunoassay based on a polyelectrolyte-coated ferrocene microcrystal was also reported recently (Mak et al., 2005). Capturing of the antibody-conjugated ferrocene microcrystal was followed by release of a large amount of the redox marker through the capsule wall (by a releasing agent), and led to a highly sensitive amperometric biodetection. The successful clinical realization of these ultrasensitive bioelectronic detection schemes requires proper attention to non-specific adsorption issues that commonly control the detectability of bioaffinity assays.

Inorganic nanocrystals offer an electrodiverse population of electrical tags as needed for multiplexed clinical testing. Different inorganic nanocrystal tracers (cadmium sulfide, zinc sulfide, copper sulfide, and lead sulfide) were used to differentiate the signals of four proteins or DNA targets in connection with sandwich immunoassay and hybridization assay, respectively, along with stripping voltammetry of the corresponding metals. Each binding event thus yielded a distinct voltammetric peak, whose size and position reflected the level and identity, respectively, of the corresponding antigen or DNA target (Liu et al., 2004; Zheng et al., 2008).

Recent advances in biosensor technology have been reported to increase the sample throughput in order to respond to current requirements for multianalysis. For example, breast cancer screening requires the testing of 20–30 cancer susceptibility genes plus positive and negative controls. Of the many proposed electrochemical detection schemes, only a few attempts have been made to detect gene expression at the mRNA level (Xie et al., 2004). Sadik and co-workers (Andreescu and Sadik, 2005; Andreescu et al., 2004) directed the research through a high-throughput, compact, portable, and easy to use sensor-electrochemical multisensor system, so-called the dissolved oxygen (DOX) system that enable measurement of cancerous cells activity as well as their interactions with chemical toxins based on the level of oxygen consumed by the cells. Cancer cells were monitored *in vitro* over a period of 70 h by inoculating the cell suspension directly on the multielectrode device. These experiments demonstrate the applicability of the impedance mapping technique in visualizing and quantifying physiological changes in the cell layer due to cellular processes as well as the effect of external chemical stimulus on cells (cell–drug interaction). Another system facilitates multidimensional (2D space and time) characterization of the cell cultures (Rahman and Bhansali, 2007; Rahman et al., 2008).

Cancer biomarkers identified from basic and clinical research, and from genomic and proteomic analyses must be validated. Ligands and probes for these markers can then be combined with detectors to produce biosensors for cancer-related clinical testing. Point-of-care cancer testing requires integration and automation of the technology as well as development of suitable sample preparation methods (Rasooly and Jacobson, 2006; Wang, 2005c, 2006). Biosensor-based diagnostics might simplify cancer screening and provide better approach towards early detection with improved prognosis. However, not many biosensors have been developed for cancer-related testing. One major challenge in harnessing the potential of biosensors is that cancer is a very complex set of diseases. Tumors vary widely in etiology and pathogenesis. In order to make precise diagnoses, it is essential to have highly sensitive detection methods to be able to measure very low concentrations at the early cancer stages. Currently, fluorescent imaging is still the main detection manner of cancer (Ju and Zhao, 2005; Kawde and Wang, 2004; Nie et al., 2007; Park et al., 2007; Rahman et al., 2006; Taitt et al., 2005). Miniaturization to bedside or even automated/continuous monitoring system for the desired level of sensitivity for cancer care purposes has not yet been achieved.

4.2. Electrochemical biosensors for food pathogens

Food borne pathogens pose a risk to food safety and are a threat to the global food supply chain. Correct detection and identification of food borne pathogens and other contaminants relying on conventional culturing techniques are very elaborate, time-consuming, and have to be completed in a microbiology laboratory. Automation in detection methods of food pathogens is highly desirable. Therefore, biosensor-based tools offer the most promising solutions and address some of the modern-day needs for fast and sensitive detection of pathogens in real time or near real time (Bhunia, 2008; Rasooly and Herold, 2006). The need for a more rapid, reliable, specific, and sensitive method of detecting a target analyte, at low cost, is the focus of a great deal of research. In particular, electrochemical biosensors for the detection of food pathogen have the advantage of being highly sensitive, rapid, inexpensive and highly amenable towards microfabrication and was reviewed by Ilaria and co-workers (Ahmed et al., 2008; Ilaria and Marco, 2008; K'Owino

and Sadik, 2005; Yang and Bashir, 2008), specifically amperometric, potentiometric, and impedimetric biosensors with special emphasis on new biorecognition elements, nanomaterials, and lab on a chip technology.

Recent advances in biosensor technology have been reported to increase the sample throughput in order to respond to current requirements for multianalysis. Generally, microelectrodes have great advantages over conventional electrodes for analytical measurements, such as low resistance, high signal-to-noise ratio, rapid attainment of steady state, and the use of small solution volumes etc. In particular microfabricated interdigitated array microelectrodes (IDAM) have received great attention, there are one or multiple electrode pairs in an IDA, and the distance between finger electrodes can be in micron to nanometer range in order to probe the volume close to the electrodes.

Impedance biosensors for bacteria detection are based on impedance analysis of the electrical properties of bacterial cells when they are attached to or associated with the electrodes (Yang et al., 2004a,b). The advances in microfabrication technologies have enabled the use of microfabricated microarray electrodes in impedance detection and the miniaturization of impedance microbiology into a chip format, which has shown great promise for rapid detection of bacterial growth. The integration of impedance technique with biosensor technology has led to the recent development of impedance biosensors that is expending rapidly for bacteria detection. Yang and Bashir (2008) reviewed the electrical/electrochemical impedance for rapid detection of food borne pathogenic bacteria including microchip micro fabricated microelectrodes-based (interdigitated array microelectrodes) and microfluidic-based Faradaic electrochemical impedance biosensors (microchips), non-Faradaic impedance biosensors, and the integration of impedance biosensors with other techniques such as dielectrophoresis and electro-permeability.

Magnetic particles are useful in separating target cells from a mixture of bacteria and food matrices and also help to concentrate separated cells into a very small volume with the help of a magnetic field and improve the sensitivity. Recently, a microfluidic flow cell with embedded gold interdigitated array microelectrode was developed and integrated with magnetic nanoparticle-antibody conjugates (MNAC) into an impedance biosensor to rapidly detect pathogenic bacteria in ground beef samples like *E. coli* O157:H7, which can produce toxins that damage the lining of the intestine, cause anemia, stomach cramps and bloody diarrhea etc. (Gomez-Sjoberg et al., 2005; K'Owino and Sadik, 2005; Radke and Alocilja, 2005; Varshney and Li, 2007; Varshney et al., 2007; Yang et al., 2004a,b).

Cretich et al. (2008) reported on a new, rapid and robust method for PDMS functionalization, based on chemisorption of copoly(DMA-NAS-MAPS) which provides an effective way to immobilize DNA fragments for the bacterial genotyping and food pathogen identification. Actually, PDMS surface bound biomolecules can be applied to DNA, protein and peptide microarrays, to biosensors and cell-culturing. Furthermore, PDMS, with its attractive physico-chemical properties and the ease of patterning is the material of choice for the development of lab-on-chip devices. Liao et al. (2006) reported the first species-specific detection of bacterial pathogens in human clinical fluid samples using a microfabricated electrochemical sensor array. Each of the 16 sensors in the array consisted of three single-layer gold electrodes—working, reference, and auxiliary.

Sadik and co-workers (Karasinski et al., 2005, 2007) describes the integration of a fully autonomous electrochemical biosensor (96-well-type electrodes array-DOX-dissolved oxygen sensor) with pattern recognition techniques for the detection and classification of bacteria kingdom at subspecies and strain levels. The operation principle of a high-throughput 96-electrode DOX sensor was by measuring the difference in the oxygen consumed by different bacteria classes and strains over time and the oxygen reduction current was monitored at a fixed potential (−700 mV vs. gold), where the oxygen reduction current is maximum to ensure the highest sensitivity of the system. Additional selectivity was provided using pattern recognition, which allowed a better differentiation and classification of bacteria.

4.3. Recent advances in electrochemical glucose biosensors

Since Clark and Lyons proposed in 1962 the initial concept of glucose enzyme electrodes (Clark and Lyons, 1962), we have witnessed tremendous effort directed toward the development of reliable devices for diabetes control. Different approaches have been explored in the operation of glucose enzyme electrodes. We also have witnessed tremendous progress in the development of electrochemical glucose biosensors, as reviewed by Wang (Wang, 2005a, 2008; Wang and Musameh, 2004). Owing to the similar dimensions of nanoparticles and redox proteins such nanomaterials can be used for effective electrical wiring of redox enzymes. Various nanomaterials, including gold nanoparticles or carbon nanotubes, have thus been used as electrical connectors between the electrode and the redox center of glucose oxidase.

Carbon nanotubes represent additional nanomaterials that can be coupled to enzymes to provide a favorable surface orientation and act as an electrical connector between their redox center and the electrode surface. Particularly useful for this task have been vertically aligned CNTs that act as molecular wires (nanoconnectors) between the underlying electrode and a redox enzyme (Liu et al., 2005; Patolsky et al., 2004a). Willner and co-workers (Patolsky et al., 2004a) demonstrated that the edge of single-wall carbon nanotubes (SWCNT) can be linked to an electrode surface. Such enzyme reconstitution on the end of CNT represents an extremely efficient approach for 'plugging' an electrode into glucose oxidase. Electrons were thus transported along distances higher than 150 nm with the length of the SWCNT controlling the rate of electron transport. An interfacial electron-transfer rate constant of $42\,s^{-1}$ was estimated for 50 nm long SWCNT. Efficient direct electrical connection to GOx was reported also by Gooding and co-workers in connection to aligned SWCNT arrays (Liu et al., 2005). At present, activation of the bioelectrocatalytic functions of GOx by nanoparticles or CNT requires electrical overpotentials (beyond the thermodynamic redox potential of the enzyme redox center). Improving the contact between the nanomaterial and the electrode might decrease this overpotential.

Subsequently, nanowires as sensing materials were also used for hydrogen peroxide and glucose sensors to overcome the overvoltage in the detection of H_2O_2 (Cusma et al., 2007; Lu et al., 2007; Qu et al., 2007). Gold nanowires were prepared by an electrodeposition strategy using nanopore polycarbonate (PC) membrane, with the average diameter of the nanowires about 250 nm and length of about 10 μm. The nanowires prepared were dispersed into chitosan (CHIT) solution and stably immobilized onto glassy carbon electrode surface. The modified electrode allows low potential detection of hydrogen peroxide with high sensitivity and fast response time. Glucose oxidase was adsorbed onto the nanowire surface to fabricate glucose biosensor as an application example. The detection of glucose was performed in phosphate buffer (pH 6.98) at −0.2 V (Lu et al., 2007; Qu et al., 2007). Similarly, platinum nanowires (PtNWs) prepared with the help of porous anodic aluminum oxide (AAO) templates have been solubilized in chitosan together with carbon nanotubes (CNTs) to form a PtNW-CNT-CHIT organic–inorganic system. The PtNW-CNT-CHIT film modified electrode offered a significant decrease in the

overvoltage for the hydrogen peroxide and was showed to be excellent amperometric sensors for hydrogen peroxide at −0.1 V over a wide range of concentrations with the sensitivity recorded being 260 $\mu A\,mM^{-1}\,cm^{-2}$. By linking glucose oxidase, an amplified biosensor toward glucose was prepared which exhibits a selective determination of glucose at −0.1 V (Cusma et al., 2007). The application of polypyrrole-coated glucose oxidase nanoparticles (GOx/Ppy) in electrochemical biosensor design was also described, and the increase of K_M by over 10 times was determined for polypyrrole-coated GOx, compared with native GOx (Ramanavicius et al., 2005). A conductivity-based glucose nanobiosensor based on conducting-polymer-based nanogap has been developed by Tao and co-workers. Such nano junction-based sensor was formed by using polyaniline/glucose oxidase for bridging a pair of nanoelectrodes separated with a small gap (ca. 20–60 nm) (Forzani et al., 2004).

Ultimately, one would like to eliminate the mediator and develop a reagentless glucose biosensor with a low operating potential, close to that of the redox potential of the enzyme. In this case, the electron is transferred directly from glucose to the electrode via the active site of the enzyme. The absence of mediators is the main advantage of such *third-generation biosensors*, leading to a very high selectivity (owing to the very low operating potential). Oxidized boron-doped diamond electrodes also indicated recently some promise for mediator-free glucose detection based on direct electron transfer (Jing and Yang, 2006).

The ideal sensor would be one that provides a reliable real-time continuous monitoring of all blood glucose variations throughout the day with high selectivity and speed over extended periods under harsh conditions. Continuous glucose monitoring thus addresses the deficiencies of test-strip-based meters and provides the opportunity of making fast and optimal therapeutic interventions (i.e., insulin delivery) (Wilson, 2005; Wilson and Gifford, 2005). Typically, a needle-type multi-electrode array for the hypodermic continuous glucose monitoring sensor was fabricated using MEMS technology. The developed multielectrode sensor has four electrodes of two working (Pt) electrodes, one counter (Pt) electrode, and one reference (Ag/AgCl) electrode. Two working electrodes are for the enzyme and non-enzyme electrodes which measure glucose concentration and the background current (Jung et al., 2004). This would minimize short-term crises and long-term complications of diabetes leading to improved quality and length of life for people with diabetes. Glucose biosensors are thus key components of closed-loop glycemic control systems for regulating a person's blood glucose. The concept of closed-loop (sense/release) systems is expected to have a major impact upon the treatment and management of other diseases and revolutionize patient monitoring (Heller, 2005).

The challenges for meeting these demands include rejection of the sensor by the body, miniaturization, long-term stability of the enzyme in-vivo calibration, short stabilization times, baseline drift, safety, and convenience etc. The sensor must be of a very

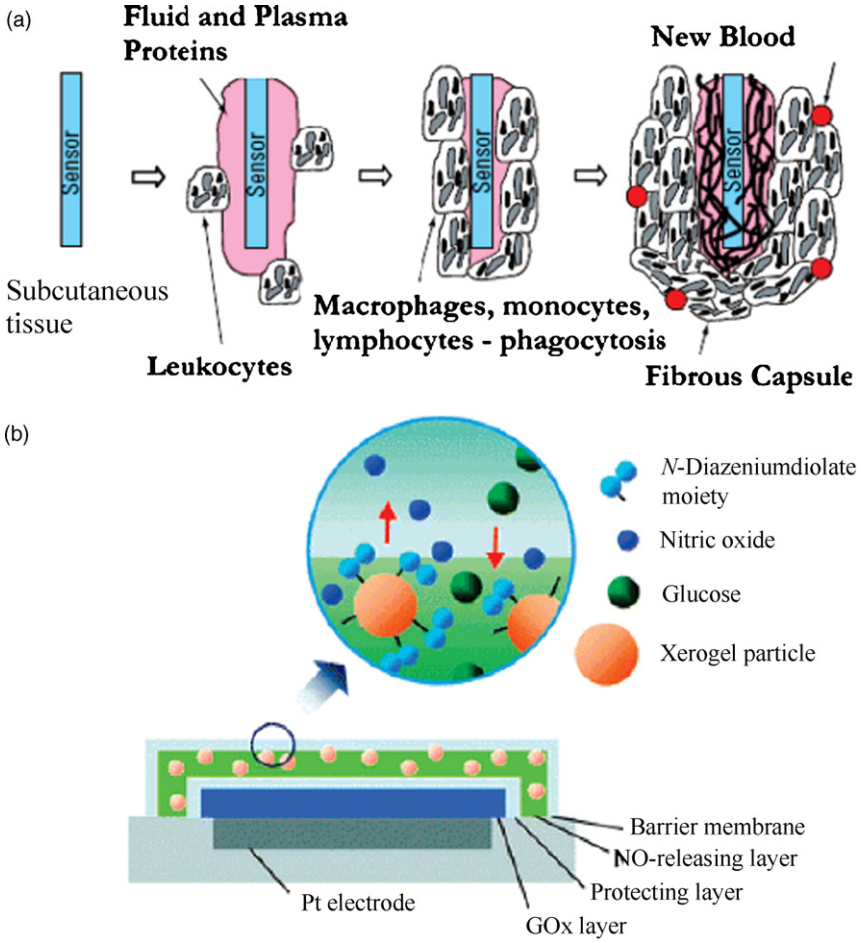

Fig. 9. (a) Inflammatory response of implantable sensor in the subcutaneous tissue. Sequence of events that leads to formation of fibrous capsules around chemical sensor. Reprinted with the permission from Frost, M., et al. (2006). Copyright 2006 American Chemical Society. (b) Nitric oxide releasing coating for improved biocompatibility of glucose biosensors. Schematic presentation of the hybrid xerogel/polyurethane glucose biosensor employing NO-donor-modified sol–gel particles supported in a polyurethane matrix. Reprinted with the permission from Shin et al. (2004). Copyright 2004 American Chemical Society.

tiny size and proper shape to allow for easy implantation resulting in minimal discomfort. Alternative sensing sites, particularly the subcutaneous tissue, have thus received growing attention. The subcutaneous tissue is minimally invasive, and its glucose level reflects the blood glucose concentration. However, such subcutaneous implantation generates a wound site that experiences an intense local inflammatory reaction. This inflammatory response associated with the wound formation is characterized with problems such as scar tissue formation accompanied by adhesion of bacteria and macrophage and distortion of the glucose concentration in the immediate vicinity of the sensor (Fig. 9a).

Recent approaches for designing more biocompatible in vivo glucose sensors focused on preparing interfaces that resist biofouling. These include a controlled release of nitric oxide (NO) (Frost and Meyerhoff, 2006; Gifford et al., 2005; Oh et al., 2005; Shin et al., 2004). NO is an effective inhibitor of platelet and bacterial adhesion. Such NO-release glucose sensors were prepared by doping the outer polymeric membrane coating of previously reported needle-type electrochemical sensors with suitable lipophilic diazeniumdiolate species (Gifford et al., 2005) or diazeniumdiolate-modified sol–gel particles (Fig. 9b). The use of nanomaterials for improved electrical contact between the redox center of GOx and electrode supports and enhanced "genetically engineered" GOx has attracted increased interest. Recently, genetically engineered periplasmic glucose receptors as biomolecular recognition elements on gold nanoparticles (AuNPs) have been developed as sensitive and reagentless electrochemical glucose biosensor. The receptors were immobilized on AuNPs by a direct sulfur-gold bond through a cysteine residue that was engineered in position 1 on the protein sequence (Andreescu and Luck, 2008). In addition, this will result in the advances in new "painless" in-vitro testing, artificial (biomimetic) receptors for glucose, advanced biocompatible membrane materials, the coupling of minimally invasive monitoring with compact insulin delivery system, new innovative approaches for non-invasive monitoring, and miniaturized long-term implants.

5. Conclusions and future trends

The current trends and challenges in electrochemical detection of biomolecular reactions have been reviewed. The successful coupling of nanomaterials with naturally existing sophisticated biomolecular systems has been explored for the development of ultrasensitive electrochemical sensors. Nanoscale materials have been used to achieve direct wiring of enzymes to electrode surface, to promote electrochemical reaction, to impose barcode for biomaterials and to amplify signal of biorecognition event. The resulting electrochemical nanobiosensors have been applied in areas of cancer diagnostics, detection of pathogenic organisms, food safety, environmental measurements and clinical applications. Electrochemical microfluidic technology consisting of microfluidic mixer, valves, pumps, channels, chambers in a single chip device have been commercialized. Aptamer-based microarrays for the quantitation of multiple protein analytes have been developed. The uniqueness of EIS for characterizing the electrochemical behavior of biomolecular system in which several processes may be coupled and occur at different rates has been explored. Metal-enhanced electrochemical detection concept has been demonstrated for the sensitive detection of Ab–Ag, DNA–DNA, DNA–drug and DNA–toxin interactions. The MED concept exhibits superior analytical characteristics over HPLC and ELISA for the detection of endocrine disrupting chemicals such as bisphenol A, nonyphenol and diethylstilbestrol (Ngundi, 2003). Similarly, the MED strategy has been extended to the detection of salivary amylase with picomolar detection limits (Aluoch et al., 2005a,b; K'Owino et al., 2007). The key issue to be addressed in the future is the increasing demand for higher sensitivity and selectivity that will allow molecules to be monitored in real-time at a minimal cost. Others should include issues of validating the biosensor on statistically large population of real samples rather than the commonly reported relatively short synthetic oligonucleotides, pristine laboratory standards or bioreagents; multiplexing the sensors to accommodate high-throughput, multianalyte detection as well as application in complex clinical and environmental samples.

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