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Review Article

Electrochemical Aptamer-Based Biosensors: Recent Advances and Perspectives

Abd-Elgawad Radi

Department of Chemistry, Faculty of Science, Mansoura University, Dumyat 34517, Egypt

Correspondence should be addressed to Abd-Elgawad Radi, abdradi@yahoo.com

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This paper reviews the advancements of a wide range of electrochemical aptamer-based biosensors, electrochemical aptasensors, for target analytes monitoring. Methods for immobilizing aptamers onto an electrode surface are discussed. Aptasensors are presented according to their detection strategies. Many of these are simply electrochemical, aptamer-based equivalents of traditional immunochemical approaches, sandwich and competition assays employing electroactive signaling moieties. Others, exploiting the unusual physical properties of aptamers, are signal-on (positive readout signal) and signal-off (negative readout signal) aptasensors based on target binding-induced conformational change of aptamers. Aptamer label-free devices are also discussed.

1. Introduction

Biosensors are devices detecting the presence of a target by using a particular recognition element and then monitoring the mass, optical, electronic, or magnetic signal changes, which are induced by the interaction of the recognition element and the analyte of interest. Molecular recognition is consequently the key for the sensor performance. The recognition elements were initially isolated naturally from living systems; now, they are available by synthesis in the lab, including receptors, enzymes, antibodies, nucleic acids, molecular imprints and lectins. The mostly used recognition components for clinic diagnostics and genomics/proteomics studies are antibodies and nucleic acids based on affinity assays owing to their special high sensitivity and selectivity in the affinity to target molecules. Antibodies are produced by immune system when it responds to antigens (i.e., toxins, chemicals, drugs, and virus particles, spores, bacterial toxins, and other foreign substrates). Antibodies were generated by animal immunization, and now, cell clone technology can produce poly/monoclonal antibodies in large quantities. The antibodies still encounter the challenges of pH and temperature sensitivity, short shelf life, easily degradation, and consequently, its repeatable usage is also a problem.

Aptamers, first reported in 1990, are attracting interest in the areas of therapeutics and diagnostics [1–3]. Aptamers are specific oligonucleic acid sequences (ca. 30 to 100 nucleotides), which recognize specific ligands and bind to various target molecules ranging from small ions to large proteins with high affinity and specificity. The term aptamer derives from aptus that means to fit. The RNA or DNA aptamers molecules are selected in vitro (selection evolution of ligands by exponential enrichment, SELEX process) from vast populations of random sequences. Aptamers are often called synthetic antibodies and can mimic antibodies in a number of applications. The selected aptamers bind their targets with affinities and specificities that can be comparable to those of antibodies. Aptamers present some advantages compared to antibodies, especially accurate and reproducible chemical production. Moreover, Aptamer offering chemical stability under a wide range of buffer conditions, resistant to harsh treatments without losing its bioactivity, and the thermal denaturation is reversible for aptamer. While it is important to remain proteins in a moisturized environment to maintain their bioactivity, and the physical or chemical denaturation is irreversible for antibodies. Aptamer has a wide range of molecular and therapeutic targets, including amino acids, any class of proteins (enzymes, membrane

proteins, viral proteins, cytokines and growth factors, and immunoglobulins), drugs, metal ions, other small bio-/organic/inorganic small molecules, and even whole cells. However, antibody is only employed for immunogenic compounds. Aptamers are small in size, cost effective, offering remarkable flexibility and convenience in designing their special structure. Moreover, combinatorial chemical synthesis offers a wide variety of methods for aptamer sequence modifications such as the terminal tagging chemical groups.

Biosensors based on aptamers as biorecognition elements have been coined aptasensors. The aptamers were initially used as therapeutic agents. For example, aptamer that selectively binds thrombin, a multifunctional serine protease that plays an important role in procoagulant and anticoagulant functions, was developed with the purpose of application as an anticoagulant [4]. Only recently, the aptamers have been used as recognition elements in biosensing. The first aptasensor was reported in 1996, with an optical biosensor based on fluorescently labeled aptamers [5]. To date, the best investigated aptamers are those for thrombin. The first electrochemical aptasensor with an amperometric sandwich-based biosensor based on glucose dehydrogenase-labeled signaling aptamers was described in 2004 [6]. The field of research is progressing so rapidly that new achievements have appeared, especially those focused on electrochemical methods of detection. Electrochemical devices have received considerable recent attention in connection to the transduction of aptamer interactions. Electrochemical transduction presents considerable advantages over optical, piezoelectric or thermal detection. The electrochemical detection offer high sensitivity and selectivity, compatibility with novel microfabrication technologies, inherent miniaturization, low cost, disposability, minimal simple-to-operate, robust, power requirements, and independence of sample turbidity. This paper examines electrochemical aptasensor discussing surface immobilization techniques and different detection schemes used to detect target analytes.

2. Immobilization of Aptamers

The crucial step in electrochemical aptasensors development is the immobilization of aptamers to an electrode surface, and it is important to develop strategies for reliable immobilization of aptamers so that they retain their biophysical characteristics and binding abilities, as well as for minimizing nonspecific binding/adsorption events. In principle these strategies are similar to those applied previously for the immobilization of single- or double-stranded DNA in genosensors or DNA biosensors for detection of DNA damage [7].

The methods of immobilization based on physical adsorption of DNA by means of electrostatic interactions are in general not suitable due to low stability caused by aptamers desorption from the surface. The common pathways for immobilizing a stable, flexible and repeatable aptamer layer surface are chemical covalent attachment, via avidin-to-biotin conjugation [8], and self-assembling the thiolated aptamer onto gold substrate using a thiol-alkane linked to the aptamer sequence [7]. streptavidin-polymer-coated indium-tin oxide electrode for immobilizing a DNA aptamer against lysozyme have been also designed.

Mixed two-component alkanethiol self assembly monolayers of recognition and shielding components, similar to those used in DNA hybridization sensors, are extremely attractive for achieving the desired balance between high loading, minimal nonspecific interactions, and preferred/accessible orientation. Such mixed coassembly monolayers have been widely used in DNA hybridization sensors and are being employed for the design of electrochemical aptasensors [9, 10]. Aptamers can be attached to the solid support at either the 5′-end or the 3′ end; both positions have been reported as being used for aptasensor development. However, there are very few studies looking at the effect of the two types of end attachment. Recent work suggests that it depends on the particular aptamer [11] although for biological targeting, it may be that the 3′ end is more suitable, since the 3′ end is the primary target for exonucleases, and thus, its coupling to the solid support would simultaneously confer resistance to nucleases.

It is also highly advantageous to explore the possibility of immobilization of aptamers onto novel materials, especially through the covalent linking approaches onto gold films/particles, silicates and silicon oxide surfaces, quantum dots, carbon fabricated nanotubes, and carbohydrates or dendrimers [12]. The advantage of dendrimers is their high stability and relatively large surface in comparison with flat electrode [13, 14]. An aptasensor based on a polyamidoamine dendrimer modified gold electrode was developed for the determination of thrombin. Amino-terminated polyamidoamine dendrimer was firstly covalently attached to the cysteine functionalized gold electrode through glutaraldehyde coupling. Subsequently, the dendrimer was activated with glutaraldehyde, and amino-modified thrombin aptamer probe was immobilized onto the activated dendrimer monolayer film. Poly(amidoamine) dendrimers were also used for aptamer immobilization [14], using glutaraldehyde for crosslinking of avidin to a dendrimer surface then immobilization of biotinylated aptamers.

Multiwalled carbon nanotubes (MWCNTs) were used as modifiers of screen-printed carbon electrotransducers (SPCEs) to immobilize 5′ amino linked aptamer sequence showed improved characteristics compared to the bare SPCEs [15].

The nanotubes were pretreated with carbodiimidazole-activated Tween 20 and 3-end of thrombin aptamer was modified by groups, which allowed covalent binding of thrombin aptamer [16] for detection of thrombin. single-walled carbon nanotubes SWCNTs allowed covalent attachment of the amino aptamers at the surface of field effect transistor [17] for effective detection of IgE. A Nafion-multiwalled carbon nanotubes coated electrode modified with electrochemical probe of methylene blue was designed [18], and gold-platinum alloy nanoparticles Au-PtNPs were electrodeposited onto the electrode surface for the immobilization of aptamer. For pathogen detection, an aptamer attached to an electrode coated with SWCNTs interacts selectively with bacteria [19] resulting highly accurate and

reproducible electrochemical response at ultralow bacteria concentrations.

The effect of aptamer structure and immobilization platform on the efficiency of thrombin characteristics was investigated with aptasensors based on glassy carbon electrodes covered with multiwalled carbon nanotubes (MWNTs) [20]. Aptamers with one or two binding sequences GGTTGGTGTGGTTGG specific for thrombin and poly(dA) and poly(dT) tags able to form dimeric products (aptabodies) were used to establish significance of steric and electrostatic factors in aptasensor performance. The electropolymerization of methylene blue onto MWNTs significantly improved electrochemical characteristics and sensitivity of thrombin detection against bare MWNTs. Amine-modified capture thrombin-binding aptamer probe (12-mer) was covalently conjugated to the MWCNTs-modified glassy carbon electrode (GCE) [21]. The target aptamer probe (21-mer) contains TBA (15-mer) labeled with ferrocene (Fc), which is designed to hybridize with capture probe and specifically recognize thrombin, is immobilized on the electrode surface by hybridization reaction.

Aptasensing layer-by-layer (LBL) strategy was developed for protein detection using self-assembled multilayers with ferrocene-appended poly(ethyleneimine) (Fc-PEI), carbon nanotubes (CNTs), and aptamer [22]. The Fc-PEI, CNTs, and DNA aptamer are LBL assembled on the electrode surface via electrostatic interaction. The single-walled carbon nanotube (SWNT) network-based biosensor using aptamers as a protein recognition site have been successfully demonstrated [23]. Aluminum was first patterned on the substrate with CVD-grown oxide. Then, gold was electrolessly plated on the Al electrodes and SWNTs were dip-coated on the substrate. Finally, aptamers were attached on the surface of SWNTs and were used as a recognition site for human serum albumin.

The biocatalytic growth of high-density gold agglomerates on a gold electrode surface to form a carrier for aptamer probe immobilization was described [24]. The approach provides a simple strategy to promote the seed-mediated deposition of Au from $AuCl_4$ onto surface-attached Au nanoparticles (AuNPs) in the presence of reductive coenzyme and surfactant. This nanostructured platform is effective and prospective toward the aptamer probe immobilization.

3. Electrochemical Aptasensors Detection Schemes

The electrochemical aptasensors can be divided into three broad classes depending on the assay format and the method of detection. These detection schemes will be reviewed. The first class of electrochemical aptasensors is sandwich- and competition-type assays. The electrochemical sandwich assays are reminiscent of the exceedingly well-established ELISA (enzyme-linked immunosorbent assay) approach, in which an electrode-bound aptamer is used to bring a complex composed of the target and some redox-active species to the electrode. Another commonly employed immunochemical approach is the competition or displacement assay in which unlabeled target molecules compete with exogenously added, redox-labeled target molecules for a limited number of binding sites on the sensing electrode. The second class of electrochemical aptasensors are based on detecting targets adsorbed to an aptamer-modified electrode surface using electrochemical impedance spectroscopy. The third class of electrochemical aptasensor involves the use of electrochemistry to monitor binding-specific conformational changes in an electrode-bound aptamer. This class of electrochemical aptasensors, for which no antibody-based analogue has been reported, appears to offer particular promise with regard to rapid, reagentless detection under realistically complex, real-time conditions. The approach is relatively insensitive to nonspecific binding of interferants and allows using them in complex sample matrices. Nevertheless, these systems present a limited application, whereas target binding-induced strand displacement appears as a more generalizable procedure. Sandwich structure strategy offers the advantages of high sensitivity and simple operation for biosensor fabrication when compared to the strategy by using only one recognition element to capture and label the target molecules. In a sandwich structured aptasensor, the target should have two or more recognition elements including aptamer, one is utilized as capturing element to be immobilized on electrode surface and catch target molecules, and the other one serves as probing element to marker the target with electroactive molecules or nanoparticles.

3.1. Sandwich or Competition (Displacement-) Type Electrochemical Detection. Sandwich or competition (displacement-) type electrochemical detection approaches have adapted by several groups. An electrochemical assay based on the aptamer and the signal of amplification of nanoparticles (NPs) was constructed for the determination of thrombin [25]. Aptamers immobilized on the electrode and AuNPs could be assembled with the target protein to form a sandwich structure. Differential pulse voltammetry was employed to detect the CdS NPs loaded on the surface of the Au NPs through the linker DNA, which was related to the concentration of the target protein. The assay took advantage of the amplification ability of Au nanoparticles carrying multiplex CdS NPs and the specific affinity of aptamers. Thrombin was detected in real samples with high sensitivity and good selectivity.

A sandwich structure detection model by using antibody as the capturing element, aptamer as the detecting element, and methylene blue as the electroactive marker intercalating into the aptamer bases was introduced [26]. An immobilization interface consisting of nanogold-chitosan composite film was used to improve the conductivity and performance characteristics of the electrode. The capturing antibody was linked to the glassy carbon electrodes modified with composite film via a linker of glutaraldehyde. Au nanoparticles as the electroactive labels tagged at probing aptamer was used to measure thrombin on a screen-printed carbon electrode using differential pulse voltammetry signal. The signal was further amplified by hybridizing the aptamer with its

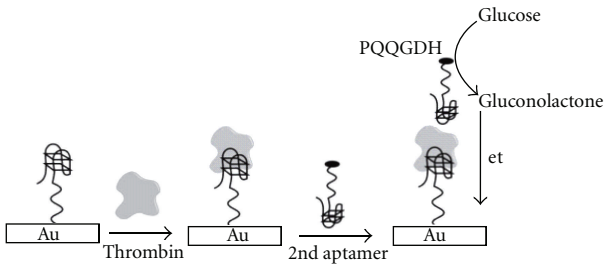

Figure 1: Sandwich assay: the immobilized aptamer captures thrombin and aptamer labeled with pyrroquinoline quinone glucose dehydrogenase ((PQQ)GDH) generates electrical current upon glucose addition.

complementary DNA, which was also labeled with gold nanoparticles [21]. Consequently, more gold nanoparticles were attached to each target protein, and thus, the signal was significantly enhanced.

A sandwich-type aptasensing system was constructed of two different aptamers which recognize different positions of thrombin (Figure 1) [6, 27]. One aptamer was immobilized onto the gold electrode for capturing thrombin onto the electrode and the other was used for detection. The aptamer for detection was labeled with pyrroquinoline quinone glucose dehydrogenase ((PQQ)GDH), and the electrical current, generated from glucose addition after the formation of the complex of thrombin, gold immobilized aptamer, and the (PQQ)GDH-labeled aptamer on the electrode, was measured. The increase of the electric current generated by (PQQ)GDH was observed in dependent manner of the concentration of thrombin.

A method utilized antibodies immobilized on the electrode surface to capture the protein target, the platelet-derived growth factor B-chain (PDGF-BB) as a model target, and the surface-captured protein was then sandwiched by an aptamer-primer complex was adapted for the detection of the amplified copies via enzymatic silver deposition then allowed enormous sensitivity enhancement in the assay of target protein [28].

A sensitive electrochemical aptasensor was successfully fabricated for the detection of adenosine triphosphate (ATP) by combining three-dimensionally ordered macroporous (3DOM) gold film and quantum dots (QDs) [29]. 5′-Thiolated ATP-binding aptamer (ABA) was first assembled onto the 3DOM gold film. Then, 5′-biotinated complementary strand (BCS) was immobilized via hybridization reaction to form the DNA/DNA duplex. The tertiary structure of the aptamer was stabilized in the presence of target ATP, the duplex can be denatured to liberate BCS. The reaction was monitored by electrochemical stripping analysis of dissolved QDs which were bound to the residual BCS through biotin-streptavidin system. The unique interconnected structure in 3DOM gold film along with the built-in preconcentration remarkably improved the sensitivity.

An ultrasensitive and highly specific electrochemical aptasensor for thrombin based on amplification of aptamer-gold nanoparticles-horseradish peroxidase (aptamer-AuNPs-HRP) conjugates was successfully developed [30]. In this electrochemical protocol, aptamer1 (Apt1) was immobilized on core/shell Fe_3O_4/Au magnetic nanoparticles (AuMNPs) and served as capture probe. Aptamer2 (Apt2) was dual labeled with AuNPs and HRP and used as detection probe. Remarkable signal amplification was realized by taking the advantage of AuNPs and catalytic reactions of HRP. The presence of proteins, such as human serum albumin, lysozyme, fibrinogen, and IgG did not show significant interference with the assay for thrombin.

An ultrasensitive aptasensor for the electronic monitoring of proteins through a dual amplified strategy was presented [31]. The target protein thrombin is sandwiched between an electrode surface confined aptamer and an aptamer-enzyme-carbon nanotube bioconjugate. The analytical signal amplification is achieved by coupling the signal amplification nature of multiple enzymes with the biocatalytic signal enhancement of redox recycling. This approach could be an attractive alternative to other common PCR-based signal amplification in ultralow level of protein detection.

A sandwich format of magnetic nanoparticle/thrombin/gold nanoparticle and thiocyanuric acid was presented for detection of thrombin [32]. An aptamer I was immobilized on the magnetic nanoparticles, aptamer II was labeled with gold nanoparticles. The magnetic nanoparticle was used for separation and collection, and gold nanoparticle offered excellent electrochemical signal transduction. The significant signal amplification was further implemented by forming network-like thiocyanuric acid/gold nanoparticles. The presence of other proteins such as BSA and lysozyme did not affect the detection of thrombin.

Electrochemical aptasensing of three configurations for thrombin detection was reported [33]. In the most straightforward configuration, the thrombin interaction with an aptamer selective for thrombin was detected electrochemically by the quantification of p-nitroaniline produced by the thrombin's enzymatic reaction [33]. Thrombin was also detected using an enzyme labeled sandwich format. Peroxidase-labeled thrombin was incubated with the aptamer and the interaction was measured electrochemically by detection of a diffusional mediator generated in a peroxidase catalyzed reaction (Figure 2). In a third strategy also employing an enzyme label, thrombin was immobilized on the sensor surface and incubated with a biotin labeled aptamer. The sensor was subsequently incubated with streptavidin-HRP (horseradish peroxidase), which bound to the biotin on the aptamer. The aptamer was again quantified by the electrochemical detection of a peroxidase catalyzed reaction. This strategy was applicable to competitive assays for detection of unlabeled thrombin.

Polsky and coworkers used platinum nanocrystals on the secondary aptamer as electrocatalysts to reduce hydrogen peroxide and transfer electrons directly from the electrode [34]. Using this approach, they report thrombin detection at 1 nM, which is more than 25-fold below the aptamer solution-phase dissociation constant.

A nanoporous gold- (NPG-) based electrochemical aptasensor for thrombin detection was developed [35]. The substrate electrode NPG was in situ fabricated by a facile

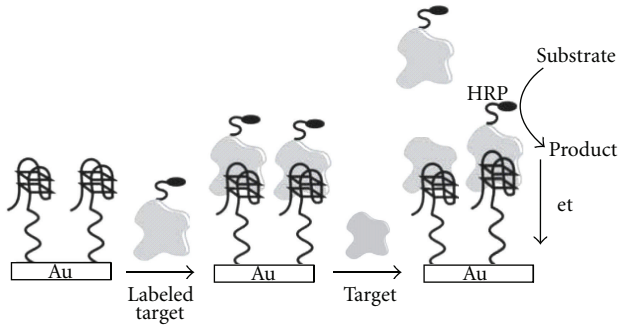

Figure 2: Competition (or displacement) assays: target molecules in a sample displace labeled-target molecules previously bound to the sensor surface.

one-step square wave potential pulse treatment. The electrochemical aptasensor was fabricated using a layer-by-layer assembling strategy. The sandwich structure was formed via thrombin connecting the aptamer-modified NPG and the aptamer-modified Au nanoparticles (AuNPs). The AuNPs was modified with two kinds of single-strand DNA (ssDNA). One was aptamer of thrombin, but the other was not, reducing the cross-reaction between thrombin and its aptamer on the same AuNP. The electrochemical signal produced by the $[Ru(NH_3)_6]^{+3}$ bound to ssDNA via electrostatic interaction was measured by chronocoulometry [36]. This NPG-based aptasensor also exhibited excellent sensitivity, due to the amplification effects of both NPG and AuNPs.

The advantages of aptamer, nanomaterial, and antibody to design an electrochemical sandwich immunoassay for the ultrasensitive detection of human immunoglobulin E (IgE) was combined by using methylene blue (MB) as electrochemical indicator [36]. The sandwich structure was fabricated by using goat antihuman IgE as capturing probe. Aptamer-Au nanoparticles (NPs) conjugates were used both as a sandwich amplification element as well as an accumulation reagent of MB. Once the aptamer-Au NPs conjugates specifically bind to electrode surface, MB molecules were accumulated on its surface by the specific interaction of MB with G base of aptamer-Au NPs conjugates. Therefore, with the increase of human IgE concentration, more aptamer-Au-NPs conjugates were bound, and thus, more MB molecules were accumulated. This sensing system showed excellent specificity for the detection of human IgE against other proteins: BSA, human IgA, and human IgM.

An electrochemical detection based on enzymatic silver deposition has been proposed to detect thrombin [37]. The target protein, thrombin, was first captured by thrombin-binding thiolated aptamer self-assembled monolayers (SAMs) on the gold electrode surface and then sandwiched with another biotinylated thrombin-binding aptamer for the association of alkaline phosphatase (Av-ALP). The attached Av-ALP enzymatically converted the nonelectroactive substrate p-aminophenyl phosphate (p-APP) to p-aminophenol (p-AP) which could reduce silver ions in solution leading to deposition of the metal onto the electrode surface. Linear sweep voltammetry was used to detect the amount of deposited silver which reflected the amount of the target protein captured into the sandwich configuration.

A multifunctional electrochemical strategy based on a dual-aptamer for the detection of adenosine and thrombin in one-pot was developed, based on biobarcode amplification assay [38, 39]. The capture DNA aptamer I was immobilized on the Au electrode. The functional Au nanoparticles (DNA-AuNPs) were loaded with barcode-binding DNA and aptamer II. Through the specific recognition for thrombin, a sandwich format of Au/aptamerI/thrombin/DNA-AuNPs was fabricated. After hybridization with the PbSNPs-labeled barcode DNA, the assembled sensor was obtained. The concentration of thrombin was monitored based on the concentration of lead ions dissolved through differential pulse anodic stripping voltammetry.

A disposable electrochemical assay involving magnetic particles and carbon-based screen-printed electrodes (SPCEs) was developed for the detection of C-reactive protein (CRP) [40]. The assay was based on a sandwich format in which a RNA aptamer was coupled to a monoclonal antibody and alkaline phosphatase (AP) was used as enzymatic label. After the sandwich assay, the modified magnetic beads were captured by a magnet on the surface of a graphite working electrode and the electrochemical detection was thus achieved through the addition of the AP substrate (α-naphthyl-phosphate) and α-naphthol produced during the enzymatic reaction was detected using differential pulse voltammetry. The assay was applied to the analysis of CRP free serum and serum samples.

A sensitively amplified electrochemical aptasensor was designed for adenosine triphosphate (ATP) detection [41]. In the sensing process, duplexes consisting of partly complementary strand (PCS1), ATP aptamer (ABA), and another partly complementary strand (PCS2) were immobilized onto Au electrode through the 5′-HS on the PCS1. Meanwhile, PCS2 was grafted with the Au nanoparticles (AuNPs) to amplify the detection signals. In the absence of ATP, probe methylene blue (MB) bound to the DNA duplexes and also bound to guanine bases specifically to produce a strong differential pulse voltammetry signal. In the presence of ATP, the ABA-PCS2 or ABA-PCS1 part duplexes might be destroyed, which decreased the amount of MB on the electrode and led to obviously decreased DPV signal. Therefore, such PCS1-ABA-PCS2/AuNPs sensing system could provide a promising signal-amplified model for aptamer-based small-molecules detection.

The self-assembly of labeled aptamer subunits in the presence of their substrates provides a method for the fluorescence or electrochemical detection of the substrate [42]. For electrochemical detection of cocaine, the thiolated aptamer subunit is assembled on an Au electrode. The methylene blue-labeled subunit binds to the surface-confined fragment in the presence of cocaine. The amperometric response of the system allows the detection of cocaine.

An aptamer-based sandwich assay with electrochemical detection for thrombin analysis was proposed using Au nanoparticles [43]. The primary aptamer was immobilized on the surface of a screen-printed carbon electrode (SPCE) and

the secondary aptamer was immobilized on Au nanoparticles. The electrochemical reduction current response of Au nanoparticles was monitored for the quantitative detection of thrombin. The effect of interfering proteins such as bovine serum albumin (BSA) was investigated. Control experiments also involved the use of an aptamer that has a binding affinity to immunoglobulin E (IgE).

An electrochemical method for the detection of thrombin based on a gold-nanoparticles sensing platform and usage of stripping voltammetry technique was developed [44]. The aptamer was immobilized on a screen-printed electrode modified with gold-nanoparticles by avidin-biotin technology. The oxidation of gold surface resulted in gold oxide formation upon polarization served as a basis for analytical response. The cathodic peak area was found proportional to thrombin quantity specifically adsorbed onto electrode surface. Binding of thrombin to an aptamer has also been detected using the ferricyanide/ferrocyanide redox couple as electrochemical indicator.

The Au nanoparticles-doped conducting polymer nanorods electrodes (AuNPs/CPNEs) were prepared by coating Au nanorods (AuNRs) with a conducting polymer layer [45]. The AuNRs were prepared through an electroless deposition method using the polycarbonate membrane as a template. The AuNPs/CPNEs combining catalytic activity of ferrocene to ascorbic acid were used for the fabrication of an ultrasensitive aptamer sensor for thrombin detection. Sandwiched immunoassay for r-human thrombin with NH_2-functionalized-thrombin-binding aptamer (Apt) immobilized on AuNPs/3D-CPNEs was studied through the electrocatalytic oxidation of ascorbic acid by the ferrocene moiety that was bound with an antithrombin antibody and attached with the Apt/3D-CPNEs probe through target binding. The selectivity and the stability of the proposed thrombin aptamer sensor were excellent, and it was tested in a real human serum sample for the detection of spiked concentrations of thrombin.

A simple electrochemical approach for the detection of thrombin, using aptamer-gold nanoparticles-modified electrodes was presented [46]. 1,6-Hexanedithiol was used as the medium to link Au nanoparticles to a bare gold electrode. Anti-thrombin aptamers were immobilized on the gold nanoparticles surfaces by self-assembly. The use of gold nanoparticles results in significant signal enhancement for subsequent detection. The total amount of aptamer probes immobilized on the gold nanoparticle surface is six-fold higher than that on the bare electrode, leading to increased sensitivity of the aptasensor.

The electrochemical thrombin detection system was developed using two different aptamers recognizing different parts of the protein in sandwich manner [47]. Aptamer 1-thrombin-ap2 glucose dehydrogenase complex was formed in the presence of thrombin, and a response current of the enzyme label was obtained.

An ultrasensitive label-free bioelectrochemical method for rapid determination of thrombin has been developed by directly detecting the redox activity of adenine (A) nucleobases of anti-thrombin aptamer using a pyrolytic graphite electrode [48]. The bioelectrochemical protocol involves a sandwich format Thrombin, captured by immobilized anti-thrombin antibody on microtiter plates, and was detected by anti-thrombin aptamer-Au nanoparticle biobarcodes. The adenine nucleobases were released by acid or nuclease from Au nanoparticles bound on microtiter plates. Differential pulse voltammetry was employed to investigate the electrochemical behaviors of the purine nucleobases based on the well-defined adenine signal. There was substantial amplification and thrombin can be detected at a very low level of detection as the nanoparticle carries a large number of aptamers per thrombin binding event. This method has been used to detect thrombin in complex matrix such as fetal calf serum with minimum background interference.

A method for the determination of platelet-derived growth factor BB (PDGF-BB) was developed using an electrochemical immunosensor with an aptamer-primed, long-strand circular detection probe [49]. Rabbit antihuman PDGF-B polyclonal antibody was immobilized on the electrode to serve as the capture antibody. The detection probe was synthesized via polymerase extension along a single-stranded circular plasmid DNA template with a primer headed by the anti-PDGF-B aptamer. In the presence of the analyte, the aptamer-primed circular probe was captured on the electrode via the formation of an antibody/PDGF-BB/aptamer sandwiched complex. The electroactivity indicator methylene blue was adsorbed on the electrode surface via the analyte-sandwiched complex with long-strand circular DNA, thus yielding a strong oxidation peak current of methylene blue in square wave voltammetric signal for the quantification of PDGF-BB. This strategy allowed electrochemical detection with enormous signal amplification arising from the long-strand localized circular probe.

Immense effort has been placed on the realization of immunoassays exploiting displacement of redox-labeled target molecules, due to the ease of use and applicability to immunochromatographic strips and immunosensors. Most of the efforts reported to date focus on the use of a redox-labeled target molecules target that is displaceable by the unlabeled target molecules toward which the antibody has higher affinity. Limited success has been achieved due to difficulty in obtaining redox-labeled target molecules targets to which the antibody has enough affinity to bind while at the same time having lower levels of affinity in comparison to the unlabeled target molecules to facilitate displacement. Aptamers, in contrast to antibodies, require the formation of a three-dimensional structure for target binding and can, thus, be anticipated to have a much higher affinity for binding its target rather than a modified form of the target (e.g., redox-labeled target). This phenomenon can be exploited for the development of a displacement assay, using enzyme-labeled target as a displaceable molecule.

In the first, Baldrich et al. measured thrombin at concentrations down to 5 nM via competition between horseradish peroxidase (HRP) modified thrombin and unlabeled target molecules in the sample [50].

The coupling of aptamers with the coding and amplification features of inorganic nanocrystals offer a highly sensitive and selective simultaneous bioelectronic detection of several

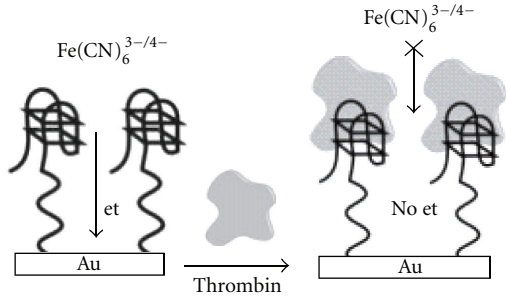

Figure 3: Impedimetric aptasensor: the binding of a target to the aptamer reduces the electron transfer (et) of a small redox mediator diffusing to the electrode surface and the increase the interfacial resistance, which provides a means of measuring the concentration of bound target via impedance spectroscopy.

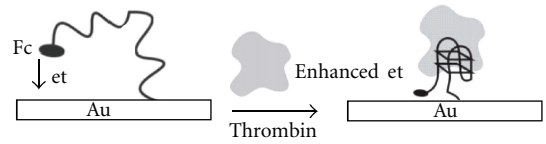

Figure 4: Electrochemical aptasensor based on the binding-induced folding of aptamers. In the absence of thrombin, the aptamer is largely unfolded, allowing for frequent collisions between the terminal redox moiety and the electrode. Upon target binding, the aptamer folds, enhancing electron transfer and producing a signal-on aptasensor.

protein targets [51]. This is accomplished in a single-step displacement assay in connection to a self-assembled monolayer of several thiolated aptamers conjugated to proteins carrying different inorganic nanocrystals. Electrochemical stripping detection of the nondisplaced nanocrystal tracers results in a remarkably low detection limit, that is, significantly lower than those of existing aptamer biosensors. The new device offers great promise for measuring a large panel of disease markers present at ultralow levels during early stages of the disease progress.

A modified RNA-aptasensor for the detection of small molecules in biological samples was presented [52]. A competitive displacement assay was applied to the detection of aminoglycoside neomycin B in whole milk using a fully 2′-O-methylated RNA aptamer with faradaic impedance spectroscopic detection. Neomycin B in solution displaces the aptamer from its complex with the SAM-immobilized neomycin B. The reusable aptasensor is capable of discriminating neomycin B from paromomycin, which differs from it in the substitution of a single amine group with a hydroxyl one. The modified endonuclease-resistant RNA aptamer maintains the exquisite selectivity of the natural aptamer and allows the examination of biological samples of high protein content.

3.2. Label-Free Impedance Spectroscopy Detection. The advantages and the limitations of using label-free detection strategies have been highlighted [53]. An impedimetric aptasensor using a mixed self-assembled monolayer composed of thiol-modified thrombin binding aptamer and 2-mercaptoethanol on a gold electrode is reported for thrombin detection [54]. The changes of interfacial features of the electrode were probed in the presence of the reversible redox couple, Fe(CN)$_6^{3-/4-}$, using impedance measurements. The electrode surface was partially blocked due to the self-assembly of aptamer or the formation of the aptamer-thrombin complex, resulting in an increase of the interfacial electron-transfer resistance detected by electrochemical impedance spectroscopy or cyclic voltammetry (Figure 3).

An electrochemical impedance spectroscopy method aptamer-based array consisting of single-stranded DNA containing a hairpin loop, was reported for detection of Human IgE. [55]. The binding of aptamers immobilized on gold electrodes leads to impedance changes associated with target protein binding events. A hybrid modified layer containing aptamers and cysteamine was fabricated on the photolithographic gold surface through molecular self-assembly, to increase the binding efficiency for proteins. Human IgE could be specifically captured by the aptamer and stand well above the self-assembled monolayer (SlAM) surface. The impedance spectroscopy detection provided higher sensitivity and better selectivity for aptamer-modified electrodes.

An aptamer-based biosensing assay for label-free thrombin detection and quantification by measuring the change in electrochemical impedance upon thrombin-aptamer complex formation was demonstrated [56]. A self-assembly of the DNA aptamer on a microfabricated thin film gold electrode, followed by the recognition of the protein binding event via monitoring the interfacial electron transfer resistance with electrochemical impedance spectroscopy.

A bifunctional derivative of the thrombin-binding aptamer with a redox-active ferrocene (Fc) moiety and a thiol group at the termini of the aptamer strand was synthesized [57]. The ferrocene-labeled aptamer thiol was self-assembled through S-Au bonding on a polycrystalline gold electrode surface. The aptamer-modified electrode was characterized electrochemically by cyclic voltammetry, differential pulse voltammetry, and electrochemical impedance spectroscopy. The modified electrode showed a voltammetric signal due to a one-step redox reaction of the surface-confined ferrocenyl moiety of the aptamer immobilized on the electrode surface. The impedance measurement, in agreement with the differential pulse voltammetry, showed decreased faradaic resistances in the same sequence. The "signal-on" upon thrombin association could be attributed to a change in conformation from random coil-like configuration on the probe-modified film to the quadruplex structure. The Fc oxidation signal increased in the thrombin concentration (Figure 4). The molecular beacon aptasensor was amenable to full regeneration and could be regenerated 25 times with no loss in electrochemical signal upon subsequent thrombin binding.

Different model systems, such as thrombin-antithrombin antibody, and Rev-peptide—anti-Rev aptamer were presented. In order to improve the signal-to-noise ratio, the use of reference sensors has been explored. The interaction of prostate specific antigen (PSA) to an anti-PSA antibody was shown to demonstrate the detection at concentrations as low as 10 nM.

An aptamer immobilization method based on electrically addressed fabrication has been developed for the preparation of aptamer-modified arrayed electrodes, by which the human IgE aptamer was oriented and immobilized on the gold electrode surface [58]. The optimization of the experimental conditions including the applied potential, time, and scan rate of potential was investigated. The method was successfully used to immobilize the aptamer onto the desired electrodes, pixel by pixel, based on the electrically addressed approach. Compared to the control electrodes, the resulting aptamer-modified electrodes showed their specific recognition for human IgE. The method owns several advantages such as rapid and simple immobilization as well as its automatic addressed capability by the electric approach.

A label-free electrochemical impedance aptasensing protocol utilizes the affinity interaction between the thrombin and the self-assembled DNA aptamer on gold electrode [59]. The specific interaction increases the electrode interfacial electronic transfer resistance. The resistance signal is then amplified by using guanidine hydrochloride to denature the captured thrombin for increasing the hydrated radius of the thrombin, consequently blocking the electron transfer from solution to electrode.

A label free, reagentless aptasensor for adenosine was developed on an ISFET device. The separation of an aptamer/nucleic acid duplex by adenosine led to the aptamer/adenosine complex that altered the gate potential of the ISFET [60]. The immobilization of the aptamer/nucleic acid duplex on an Au-electrode and the separation of the duplex by adenosine monophosphate (AMP) enabled the electrochemical detection of adenosine by faradaic impedance spectroscopy. The separation of the aptamer/nucleic acid duplex by adenosine and the formation of the aptamer/adenosine complex resulted in a decrease in the interfacial electron-transfer resistance in the presence of $[Fe(CN)_6]^{3-/4-}$ as redox active substrate.

An electrochemical biosensor utilizing pyrolyzed carbon as a working electrode for the aptamer-based thrombin detection was presented [61]. Electron transfer resistance changes due to thrombin binding onto the carbon surface modified with thrombin aptamer were measured using electrochemical impedance spectroscopy techniques.

A biosensor applying aptamer as probe and nonfaradic electrochemical impedance spectroscopy as the detection method for neuroinflammatory cytokines has been developed [62]. Platelet-derived growth factor BB (PDGF-BB), one of the important cytokines involved in neural inflammation has been selected as a detection target. Binding of PDGF to its aptamer immobilized on the silicon electrode surface leads to a decrease in capacitance measured by nonfaradic electrochemical impedance spectroscopy. The online measurement result exhibited negligible response for nonspecific adsorption but significant signal changes for the specific target. The biosensor design was promising for in vivo monitoring, as the nonfaradic strategy did not require any reagent to be loaded when performing the test, together with the ability of online measurements.

An impedance-sensor with two different geometries have been compared for the detection of Rev peptides with a molecular weight of 2.4 kDa [63]. Planar, two-dimensional interdigitated capacitor (IDC) sensors as well as three-dimensional nanogap sensors have been used. The specific interaction of the Rev peptide to an immobilized RNA anti-Rev aptamer (9.2 kDa) was detected for peptide concentrations in the range of $100\,nM^{-2}\,\mu M$. For the IDC sensor, only peptide concentrations above 500 nM gave detectable signals. For the nanogap sensor, the binding process was clearly visible for all concentrations applied. The higher sensitivity of the nanogap compared to the IDC is ascribed to the improved surface-to-volume ratio. [64]

A label-free aptasensor of mixed self-assembled monolayers (SAMs) composed of a thiol-modified PDGF binding aptamer and 6-mercaptohexanol (MCH) on a gold electrode [65] for platelet-derived growth factor (PDGF) protein was reported [65]. The SAMs were characterized by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), and differential pulse voltammetry (DPV) before and after binding of the protein using $[Fe(CN)_6]^{3-/4-}$, a redox marker ion as an indicator for the formation of a protein-aptamer complex. The electron transfer resistance). R_{et} was used to monitor the binding of the target protein. The results showed that without any modification to the aptamer, the target protein can be recognized effectively at the PDGF-binding aptamer SAMs at the electrode surface. Control experiments using nonbinding oligonucleotides assembled at the electrode surfaces showed that there was no formation of an aptamer-protein complex. The DPV signal at the aptamer functionalized electrode showed a linearly decreased marker ion peak current in presence of target protein. Thus, label-free detection of PDGF protein at an aptamer modified electrode has been demonstrated.

A bifunctional aptamer that includes two aptamer units for cocaine and adenosine 5′-monophosphate (AMP) was blocked by a nucleic acid to form a hybrid structure with two duplex regions [66]. The displacement of the aptamer by any of the substrates alters the interfacial electron transfer resistance at the electrode surface, thus providing an electronic signal for the sensing process.

Label-free electrical detection of a panel of peptide aptamers that recognise specific protein partners of the cyclin-dependent kinase (CDK) family interactions has been achieved by direct measurement of variations at open circuit potential (OCP) using an accurate differential voltage measurement [67]. Different peptide aptamers immobilized on gold electrodes were used for the detection of human CDK2 and CDK4. The interaction of the peptide aptamers with CDK proteins was successfully detected by direct OCP measurements. Variations in charge transfer resistance and in protein/double-layer capacitance were investigated by means of electrochemical impedance spectroscopy with charged redox markers in solution. The electrical detection of protein interactions could be achieved by direct measurement of OCP variations using suitable differential voltage instrumentation.

An electrochemical impedance biosensor utilizing pyrolyzed carbon film for aptamer-based thrombin detection was presented [68]. Thrombin aptamer was grafted onto the pyrolyzed carbon surface using carbodiimide-mediated

chemistry, followed by Triton-X 100 and BSA treatment to reduce nonspecific binding of thrombin. Electron-transfer resistance changes due to thrombin binding onto the carbon surface were measured, using electrochemical impedance spectroscopy. Pyrolyzed carbon can provide a new approach for miniaturization, integration, and low-cost fabrication in electrochemical biosensors.

GNPs electrodeposited on GCE used as a platform for the immobilization of the thiolated aptamer can improve the sensitivity of an EIS biosensor for the determination of thrombin [69]. In the measurement of thrombin, the change in interfacial electron transfer resistance of the biosensor using a redox couple of $[Fe(CN)_6]^{3-/4-}$ as the probe was monitored. The association and dissociation constants of three different immobilized aptamers binding with thrombin were measured and the difference of the dissociation constants obtained was discussed.

Electrochemical protein biosensors using aptamers probe doped in polypyrrole and subsequent electrochemical impedance spectroscopy have been successfully developed [70, 71]. Two targets, platelet-derived growth factor and immunoglobulin E, have been also tested [70]. A sensitive and real-time biosensor for inflammatory cytokine detection has been successfully measured in both offline ElS characterization and real-time impedance monitoring [71].

An aptamer-based sensor development, utilizing a model system of human alpha thrombin interacting with a thiolated DNA aptamer, immobilized on gold electrodes [72]. EIS measurements took place in the presence of iron ferrocyanides.

A simple and highly sensitive electrochemical impedance spectroscopy (EIS) biosensor based on nano-MnO_2 as a platform for the immobilization of the aptamer was developed for the determination of adenosine [73]. In the measurement of adenosine, the change in interfacial electron transfer resistance (R_{et}) of the biosensor using a redox couple of $[Fe(CN)_6]^{3/4}$ as the probe was monitored. The sensor was shown to exhibit high sensitivity, desirable selectivity and good stability.

An impedimetric electrochemical biosensor was developed for the label-free and selective detection of leukemia cells based on aptamer-modified gold electrode using electrochemical impedance spectroscopy (EIS) technique [74]. The thiol-terminated aptamer selected for acute leukemia cells was self-assembled onto the gold electrode surface as recognition probe, which was characterized by cyclic voltammetry (CV) and EIS using $Fe(CN)_6^{3-/4-}$ as a redox probe. The electron-transfer resistance R_{et} of $[Fe(CN)_6]^{3-/4-}$ on the sensor surface increased substantially upon incubation of aptamer-modified electrode in cell solution. The work provided a simple, convenient, low-cost, and label-free method for early leukemia diagnosis.

A label-free and sensitive faradic impedance spectroscopy (FIS) aptasensor based on target-induced aptamer displacement was developed for the determination of lysozyme [75]. The aptasensor was fabricated by self-assembling the partial complementary single-strand DNA (pcDNA)-lysozyme binding aptamer (LBA) duplex on the surface of a gold electrode. The introduction of target lysozyme induced the displacement of the LBA from the pcDNA-LBA duplex on the electrode into the solution, decreasing the electron transfer resistance of the aptasensor. The fabricated aptasensor shows a high sensitivity, good selectivity, and satisfactory regeneration. This work demonstrates that a high sensitivity of the fabricated aptasensor can be obtained using a relatively short pcDNA.

Faradaic impedance spectroscopy and ion-sensitive field-effect transistor (ISFET) were applied to sense aptamer-substrate complexes [76]. The methods utilized anticocaine aptamer fragments that self-assembled, in the presence of cocaine, to a supramolecular aptamer fragments/cocaine complex on the electrode surface or ISFET gate. One of the aptamer fragments is assembled on a Au electrode or the ISFET gate. The second thiolated aptamer fragment is used to modify Au NPs that are used as amplifying labels for the two detection schemes. The impedimetric and ISFET methods enabled the analysis of cocaine.

A protein assay method based on a DNA array was developed in which human immunoglobulin E (hIgE) and its DNA aptamer were used as an analytical model [77]. The target protein hIgE was captured by the aptamer in homogeneous solution and then the resulting hIgE-aptamer complex was hybridized onto probes self-assembled on the DNA array. The charge transfer resistance (R_{ct}) of electrodes before and after hybridization were measured by electrochemical impedance spectroscopy (EIS). To test the selectivity of the method, four different probes with one-to-three mismatched bases were immobilized on respective electrodes. The results showed that the complex could be hybridized and detected out on the electrodes modified with the fully complementary sequences. In addition, the DNA array could be employed to analyze multiple samples selectively with the matched aptamer.

A reusable label-free electrochemical nucleic acid aptasensor for the determination of cocaine by the immobilization of thiolated self-assembled DNA sequences on a gold nanoparticles-modified electrode was constructed [78]. When cocaine was complexed specifically to the aptamer, the configuration of the nucleic acid aptamer switched to a locked structure and the interface of the biosensor changed, resulting in a variation of the corresponding peak current of an electrochemical probe ($[Fe(CN)_6]^{3-/4}$ as monitored by cyclic voltammetry and electrochemical impedance spectroscopy (EIS).

A sensitive aptamer-based electrochemical biosensor to detect human immunoglobulin E (IgE) was designed [79]. 5′ Biotin labeled 45 mer DNA aptamer sequence was immobilized onto streptavidin coated graphite surfaces. Interaction between human IgE and DNA aptamer was monitored by electrochemical impedance spectrometry.

A multispecific electrochemical array with eight individually addressable gold working electrodes for rapid biosensing of 2.7 kb-long target Yersinia pestis DNA and for protein sensing of ricin toxin chain A (RTA) in the presence of redox agent were designed [80]. The array allowed to incorporate multiple negative controls in the course of a single binding experiment as well as to perform parallel identical

experiments to improve reliability of detection. Eight individual EIS measurements were completed in 15 min. The array is disposable, economical, and easy to use.

A dual RNA/peptide aptamer probe for simultaneous detection of PSMA (+) and PSMA (−) prostate cancer cells using electrochemical impedance spectroscopy was reported [81]. This approach can be applied as a general tool for early diagnosis of prostate cancer.

Aptamer-based capacitive label-free biosensors for monitoring aptamer-protein recognition events, based on charge distribution under the applied frequency by nonfaradaic impedance spectroscopy (NFIS) was reported [82, 83]. The biosensors based on gold interdigitated (GID) capacitor arrays functionalized with synthetic RNA aptamers. The RNA aptamers served as biorecognition elements for C-reactive protein (CRP). The signal is generated as a result of the change in relative capacitance occurring as a result of the formation of an RNA-CRP complex on GID capacitors. The RNA-protein complex on GID capacitors could be extended to the development of electrical biosensor systems for the early diagnosis.

Two modified aptamers, a partially (ATA) and a fully O-methylated aptamer (FATA), were proposed as recognition elements for the detection of tobramycin at therapeutic range in human serum [84]. A displacement assay was developed using faradaic electrochemical impedance spectroscopy (F-EIS) as a detection technique. The affinity constant, KDb, for both aptamers was estimated, and the selectivity towards other aminoglycosides was also tested.

Lysozyme has been detected selectively in a mixture containing a large excess of six proteins and amino acids (both electroactive and nonelectroactive) by combining aptamer-coated magnetic beads and chronopotentiometric stripping measurements of the captured protein (in connection to the intrinsic electroactivity of the protein) [85]. The protein measurement by adsorptive chronopotentiometric based on scanning the guanine bases of the guanine-rich secondary aptamer. When involving PCR reaction to amplify these guanine bases, fM level of detection limit has been obtained. The approach has also been employed for electrochemically investigating amino acid amides by using guanine-rich DNA aptamer as the electroactive marker.

The effect of aptamer structure and immobilization platform on the efficiency of thrombin binding and its detection using electrochemical impedance spectroscopy (EIS) characteristics was investigated with aptasensors based on glassy carbon electrodes covered with multiwalled carbon nanotubes (MWNTs) [86]. Aptamers with one or two binding sequences GGTTGGTGTGGTTGG specific for thrombin and poly(dA) and poly(dT) tags able to form dimeric products (aptabodies) were used to establish significance of steric and electrostatic factors in aptasensor performance. The electropolymerization of methylene blue onto MWNTs significantly improved electrochemical characteristics and sensitivity of thrombin detection against bare MWNTs.

The biosensors based on DNA aptamers immobilized by electrostatic adsorption onto electropolymerized methylene Green imprinted with DNA have been developed and examined for thrombin detection using electrochemical impedance spectroscopy and potentiometry [87]. The addition of DNA at the electropolymerization stage followed by acidic treatment of the coating significantly improved the efficiency of electrostatic adsorption of the DNA aptamer and provided sensitive detection of thrombin.

An amperometric aptasensor based on DNA aptamers immobilized by avidin-biotin method or by electrostatic adsorption onto multiwalled carbon nanotube layer contained methylene blue have been developed and examined for thrombin detection in buffer and in spiked blood serum [88]. The presence of MB increases the binding capacity of the surface layer and enhances the range of thrombin concentrations to be determined.

An artificial receptor formed by hybridization of two DNA aptamers for human thrombin (aptabody) was reported [89]. The aptasensor based on multiwalled carbon nanotubes allowed to detect thrombin with detection limit, 3 times better in comparison with conventional aptamer.

A potentiometric detection of DNA-protein interactions has been proposed [90]. The polymeric phenothiazine dyes, methylene blue and methylene green, were electrochemically deposited onto the glassy carbon electrode and covered with double stranded DNA (dsDNA) as a target for antibodies (DNA sensor) or DNA aptamer specific to human α-thrombin. The developed potentiometric biosensors can be used for preliminary diagnostics of autoimmune diseases and thrombin detection with sensitivity comparable to traditional methods.

Electrochemical indicator methylene blue and differential pulse voltammetry allowed to determine charge transfer from electrode surface to the thrombin bounded on a DNA aptamer with high selectivity in comparison with nonspecific binding caused by human IgG or human serum albumin [91]. The method of detection thrombin-aptamer interaction based on measurement the charge consumption from the electrode covered by DNA aptamers to an electrochemical indicator methylene blue (MB), which is bounded to a thrombin.

An electrochemical sensor to detect interferon (IFN)-γ, a selective marker for tuberculosis pleurisy, using its RNA or DNA 5′-thiol-modified aptamer probe immobilized on the gold electrode [92]. Interaction between IFN-γ and the aptamer was recorded using electrochemical impedance spectroscopy. IFN-γ was detected in fetal bovine serum, a mimicked biological system, which has similar components to pleural fluid.

A multifunctional reusable label-free electrochemical biosensor based on an integrated aptamer for parallel detection of adenosine triphosphate (ATP) and α-thrombin, by using electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV), was reported [93]. 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). The unimolecular MBA contained small-molecule ATP-binding aptamer (ABA) and also protein α-thrombin binding aptamer (TBA). Thus, the aptasensor could be used for detection of ATP and α-thrombin. The aptasensor held several advantages such

as label-free detection, high sensitivity, regeneration, multifunctional recognition, and sensing ability such as the simultaneous detection for multianalysis.

A sandwich system of aptamer/thrombin/aptamer-functionalized Au nanoparticles (Apt-AuNPs) was fabricated as the sensing platform [94]. The change of the interfacial feature of the electrode was characterized by electrochemical impedance analysis with the redox probe $[Fe(CN)_6]^{3-/4-}$. The three-level cascaded impedimetric signal amplification was developed: Apt-AuNPs as the first-level signal enhancer, the steric-hindrance between the enlarged Apt-AuNPs as the second-level signal amplification, the electrostatic-repulsion between sodium dodecylsulfate (SDS) stabilized Apt-AuNPs, and the redox probe $[Fe(CN)_6]^{3-/4-}$ as the third-level signal amplification. The aptasensor based on the enlargement of negatively charged Apt-AuNPs showed an increased response of the electron-transfer resistance to the increase of thrombin concentration.

A sandwich sensing platform was fabricated, in which the thiolated aptamers are firstly immobilized on a gold substrate to capture the thrombin molecules, and then, the aptamer functionalized Au nanoparticles (AuNPs) are used to amplify the impedimetric signals [95]. Such designed aptamer/thrombin/AuNPs sensing system could not only improve the detection sensitivity compared to the reported impedimetric aptasensors but also provide a promising signal amplified model for aptamer-based protein detection.

A label-free electrochemical aptasensor based on direct immobilization of the redox probes on an electrode surface was reported [96]. The gold electrode coated Nafion was firstly modified with redox probe-thionine (Thi) through ion exchange adsorption. Then, negatively charged nano-Au and positively charged Thi were layer-by-layer (LBL) self-assembled onto the modified electrode surface, which formed multilayer films for improving the amount of redox probes and immobilizing thiolated thrombin aptamers (TBA). In the presence of target thrombin (TB), the TBA on the multilayer film could catch the TB onto the electrode surface, which resulted in a barrier for electrontransfer, leading to decrease of the current. The method avoided the cubosome redox probe labeling process, increased the amount of redox probe, and reduced the distance between the redox probe and electrode surface.

A label-free electrochemical impedimetric aptasensor based on an anti-lysozyme-aptamer as a molecular recognition element, was developed for the detection of lysozyme [97]. Improvement in sensitivity was achieved by utilizing gold nanoparticles (AuNPs), which were electrodeposited onto the surface of a gold electrode, as a platform for immobilization of the aptamer. To quantify the amount of lysozyme, changes in the interfacial electron transfer resistance (R_{et}) of the aptasensor were monitored using the redox couple of an $[Fe(CN)_6]^{3-/4-}$ probe. The aptasensor also showed good selectivity for lysozyme without being affected by the presence of other proteins.

A reusable aptamer-based impedimetric biosensor using Amino-terminated IgE aptamers were covalently attached to carboxyl-modified a a nanocrystalline diamond (NCD) film using carbodiimide chemistry for detection of human immunoglobulin E (IgE) [98]. The formation of aptamer-IgE complexes caused a significant change in the capacitance of the double-layer, in good correspondence with the IgE concentration. The NCD-based aptasensor was demonstrated to be highly selective even in the presence of a large excess of IgG.

3.3. Aptasensors Exploiting Conformational Changes in Aptamers. A classic biosensor directly transduces ligand-target binding events into a measurable physical readout. More recently, researchers have proposed novel biosensing strategies that couple ligand-induced structural switching of biomolecules with advanced electronic transducers [99]. This approach has proven to be a highly general platform for the development of new biosensors. In this account, a series of electrochemical nucleic acid sensors that use target-responsive DNA structures, employing surface-confined DNA structures with appropriate redox labels, which can monitor target-induced structural switching of DNA or aptamer-specific small molecule probes by measuring electrochemical currents that are directly associated with the distance between the redox label and the electrode surface.

An electrochemical biosensor for single-step detection of a homodimer protein PDGF-BB based on proximity-dependent surface hybridization assay was built up [99]. The strategy relied on simultaneous recognition of a target molecule by a pair of affinity probes, which was a prerequisite for efficiently promoting the ferrocene-labeled tail sequences of the proximate affinity probe pair to hybridize together with surface-tethered oligonucleotide, thus triggering the redox current of ferrocene at the electrode. The strategy, as a universal methodology for developing high-performance biosensors, was demonstrated using an aptamer probe to a homodimer protein PDGF-BB, and the aptasensor showed intrinsic high sensitivity, excellent resistance to nonspecific interferences, and ready reusability.

An ultrasensitive, reagentless, target label-free electrochemical aptasensor for thrombin detection was constructed [100]. The aptasensor was based on a chronoamperometric beacon system for biomolecular recognition. The ferrocene-labeled aptamer adopts a 3D conformational change when interacted with thrombin. Thus, the ferrocene label was approached to the microperoxise-11 (MP-11) attached on the electrode surface. The thrombin-aptamer interaction was detected via a microperoxidase mediated electron transfer between the ferrocene and the surface.

An electrochemical DNA aptamer-based biosensor for detection of interferon (IFN)-γ was described [101]. A DNA hairpin containing IFN-γ-binding aptamer was thiolated, conjugated with methylene blue (MB) redox tag, and immobilized on a gold electrode by self-assembly. Binding of IFN-γ caused the aptamer hairpin to unfold, pushing MB redox molecules away from the electrode and decreasing electron-transfer efficiency. The change in redox current was quantified using square wave voltammetry (SWV) and was found to be highly sensitive to IFN-γ concentration. The

aptasensor was specific to IFN-γ in the presence of overabundant serum proteins. The aptasensor could be regenerated by disrupting aptamer-IFN-γ complex in urea buffer and reused multiple times.

A signal on electrochemical sensing strategy for the simultaneous detection of adenosine and thrombin is developed based on switching structures of aptamers [102]. An Au electrode as the sensing surface is modified with two kinds of thiolated capture probes complementary to the linker DNA that contains either an adenosine aptamer or thrombin aptamer. The capture probes hybridize with their corresponding linker DNA, which has prehybridized with the reporter DNA loaded onto the gold nanoparticles (AuNPs). The AuNP contained two kinds of biobarcode DNA: one is complementary to the linker DNA (reporter), whereas the other is not (signal) and is tagged with different metal sulfide nanoparticles. The aptamer parts bind with their targets and fold to form the complex structures. As a result, the biobarcoded AuNPs are released into solution. The metal sulfide nanoparticles are measured by anodic stripping voltammetry.

A signal-on, reagentless target-responsive electrochemical aptamer switch (TREAS) for the development of aptamer-based biosensors for adenosine triphosphate ATP detection was designed [103]. The aptamer oligonucleotide dually labeled with thiol and ferrocene groups is hybridized with its complementary strand, and the thiolated duplex is self-assembled on a gold electrode. This duplex is responsive to the target ATP, which liberates the complementary strand and forms the aptamer-target complex. The electroactive ferrocene moiety, which is distal to the electrode surface in the absence of ATP, is moved to the proximal position during the binding-induced structural transition. This binding turns on the electron transfer and leads to measurable electrochemical signals for quantification of ATP.

A sensitive, label-free electrochemical aptasensor for adenosine triphosphate (ATP) has been developed based on gold nanoparticles (AuNPs) amplification [104]. The aptasensor was fabricated as a tertiary hybrid DNA-AuNPs system, which involved the anchored DNA (ADNA) immobilized on gold electrode, reporter DNA (RDNA) tethered with AuNPs and target-responsive DNA (TRDNA) linking ADNA and RDNA. Electrochemical signal is derived from chronocoulometric interrogation of $[Ru(NH_3)_6]^{3+}$ (RuHex) that quantitatively binds to surface-confined DNA via electrostatic interaction. The introduction of ATP triggers the structure switching of the TRDNA to form aptamer-ATP complex, which results in the dissociation of the RDNA capped AuNPs (RDNA-AuNPs) and release of abundant RuHex molecules trapped by RDNA-AuNPs. The incorporation of AuNPs in this strategy significantly enhances the sensitivity of ATP assay.

An electrochemical sensing strategy for highly sensitive detection of small molecules was developed based on switching structures of aptamers from DNA/DNA duplex to DNA/target complex [105]. A gold electrode was first modified with gold nanoparticles (AuNPs), and thiolated capture probe was immobilized onto the electrode via sulfur-gold affinity. Then, a sandwich-type strategy was employed, which involved a linker DNA containing antiadenosine aptamer sequence and reporter DNA loaded on AuNPs. In the presence of adenosine, the aptamer part bound with adenosine and folded to the complex structure. As a result, the reporter probes together with AuNPs were released into solution and reduced a decrease in peak current. The sensor exhibited excellent selectivity against other nucleosides and could be used to detect adenosine from real human serum samples.

A label-free electrochemical aptasensor introducing a probe immobilization technique by the use of a layer-by-layer (LBL) self-assembled multilayer with ferrocene-appended poly(ethyleneimine) (Fc-PEI) on an indium tin oxide (ITO) array electrode for detection of cocaine was first constructed [106]. The Fc-PEI and gold nanoparticles (AuNPs) were LBL assembled on the electrode surface via electrostatic interaction. Then, cocaine aptamer fragments, SH-C2, were covalently labeled onto the outermost AuNP layer. When the target cocaine and cocaine aptamer C1 were present simultaneously, the SH-C2 layer hybridized partly with C1 to bind the cocaine, which led to a decreased differential pulse voltammetry (DPV) signal of Fc-PEI. The sensor was specific to cocaine in complex biologic fluids such as human plasma and human saliva.

A strategy based on the utilization of the aptamer-complementary DNA (cDNA) oligonucleotides as the probes for electrochemical sensing was described [107]. The sequences at both ends of the cDNA are tailor-made to be complementary, and both the ferrocene redox moiety and thiol group are labeled onto the cDNA. The labeled cDNA are hybridized with their respective aptamers (i.e., ATP- and thrombin-binding aptamers) to form double-stranded DNA (ds-DNA) and the electrochemical aptasensors are prepared by self-assembling the labeled ds-DNA onto Au electrodes. Upon target binding, the aptamers confined onto electrode surface dissociate from their respective cDNA oligonucleotides into the solution and the single-stranded cDNA could, thus, tend to form a hairpin structure through the hybridization of the complementary sequences at both its ends. Such a conformational change of the cDNA resulting from the target binding-induced dissociation of the aptamers essentially leads to the change in the voltammetric signal of the redox moiety labeled onto the cDNA and thus constitutes the mechanism for the electrochemical aptasensors for specific target sensing.

A reusable electrochemical aptasensor for highly sensitive detection of adenosine had been developed using sensing interface of self-assembling the part DNA duplex hybridized by 5′-thiolated part complementary strand (TPCS) and 3′-ferrocene(Fc)-labeled adenosine-binding aptamer strand (FABA) through S-Au bonding on a gold electrode surface [108]. When the modified electrode was incubated in the adenosine solutions, the aptamer made structure switching to bind adenosine. As a result, Fc-labeled adenosine-binding aptamer strand was taken off from the sensing interface, resulting in a decrease of the redox current. The aptasensor was characterized electrochemically by cyclic voltammetry and electrochemical impedance spectroscopy.

4. Conclusion

Aptasensors appear as promising devices based on aptamers that are very small in size compared to other biorecognition molecules like antibodies or enzymes. Nonspecific adsorption phenomena are usually less pronounced on nucleic acid-derivatized surfaces as compared to protein derivatized ones. Generally, regeneration of aptamer derivatized surfaces is quite easy to perform. Aptamers can undergo multiple denaturation/regeneration cycles, whereas antibodies suffer from permanent degradation. DNA aptamers are suitable for designing reusable aptasensors, whereas RNA aptamers allow single shot measurements. The major limitation is due to RNA aptamer degradation by ribonucleases. This can be overcome by chemical modification of RNA aptamers or use of enantiomeric aptamers known as spiegelmers.

Currently, the SELEX is a highly automated procedure and only few days are necessary for development of aptamers for certain ligands. This is much shorter in comparison with the selection of antibodies, where usually several months are required. Due to effectivity of the SELEX, the library of aptamers against various ligands has become wider. On the other hand, the primary procedure does not result in all cases in aptamers with desired affinity. Therefore, optimization of aptamer structure is required. This optimization is performed through biased library generation. As a result, it is possible to select aptamers with sensitivity to small modification of the ligand. Aptamers can even distinguish the chirality of molecules and their secondary structure. In principle, there is no restriction in the type of target for which the aptamer can be selected.

Several problems related to the practical application of aptamers are still under study, for example, how the immobilization of aptamers to the supported films and their microenvironment will affect the aptamer structure and aptamer-ligand interactions. The aptamers configuration is sensitive to the salt composition; therefore, liquid composition may affect the aptasensor properties [109]. Problems are connected with the application of aptamers in the complex biological systems. Several proteins may interact with DNA aptamers nonspecifically. They could bind to the sugar-phosphate backbone of DNA, and thus mask the specific binding of analyte. The presence of nucleic acid in the biological liquids may cause hybridization with aptamers, and thus affect the aptamers conformation and maintaining the proper binding site. It should be also mentioned that currently only approx. 250 aptamers are available, while the number of various antibodies is much larger. The continuous growth of immune test is also due to lack of aptamer-based kits at the market. Thus, despite the advantages of aptamers over antibodies, further effort is required for wide spreading aptamers-based technology in practical applications. Given the rapid pace of advances in this field, the development of miniaturized, easy-to-use electrochemical aptasensor diagnostic systems for large-scale clinical testing seems a realistic goal.

Future direction will probably see growth of electrochemical microchips and nanochips. The development of microarrays based on DNA aptamers used as receptors can be seen as a logical continuation of the DNA-chip technology development although the principle of target recognition is not based on hybridization but is analogous to the immunochemical assay. In the near future, aptamer microarrays are expected to play a dominant role in proteomics, thus extending the use of aptamer-based microarrays.

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