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Review

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Electrochemical Sensors and Biosensors Based on Nanomaterials and Nanostructures

Chengzhou Zhu,[†,§] Guohai Yang,[†,§] He Li,[†,§] Dan Du,[†] and Yuehe Lin*[,†,‡]

[†]School of Mechanical and Materials Engineering, Washington State University, Pullman, Washington 99164, United States

[‡]Pacific Northwest National Laboratory, Richland, Washington 99352, United States

CONTENTS

Taking advantage of exceptional attributes, such as being easy-to-operate, economical, sensitive, portable, and simple-to-construct, in recent decades, considerable attention has been devoted to the integration of recognition elements with electronic elements to develop electrochemical sensors and biosensors.Various electrochemical devices, such as amperometric sensors, electrochemical impedance sensors, and electrochemical luminescence sensors as well as photoelectrochemical sensors, provide wide applications in the detection of chemical and biological targets in terms of electrochemical change of electrode interfaces.

With remarkable achievements in nanotechnology and nanoscience, nanomaterial-based electrochemical signal amplifications have great potential of improving both sensitivity and selectivity for electrochemical sensors and biosensors. First of all, it is well-known that the electrode materials play a critical role in the construction of high-performance electrochemical sensing platforms for detecting target molecules through various analytical principles. Furthermore, in addition to electrode materials, functional nanomaterials can not only produce a synergic effect among catalytic activity, conductivity, and biocompatibility to accelerate the signal transduction but also amplify biorecognition events with specifically designed signal tags, leading to highly sensitive biosensing. Significantly, extensive research on the construction of functional electrode materials, coupled with numerous electrochemical methods, is advancing the wide application of electrochemical devices. For example, Walcarius et al. highlighted the recent advances of nano-objects and nanoengineered and/or nanostructured materials for the rational design of biofunctionalized electrodes and related (bio)sensing systems.[1] The attractiveness of such nanomaterials relies on their ability to act as effective immobilization matrices and their intrinsic and unique features as described above. These features combined with the functioning of biomolecules contribute to the improvement of bioelectrode performance in terms of sensitivity and specificity. Our group recently presented a general overview of nanomaterial-enhanced paper-based biosensors including lateral-flow test-strip and paper microfluidic devices.[2] With different kinds of nanoparticles (NPs), paper-based biosensor devices have shown a great potential in the enhancement of sensitivity and specificity of disease diagnosis in developing countries.

This Review focuses on recent advances in electrochemical sensors and biosensors based on nanomaterials and nanostructures during 2013 to 2014. The aim of this effort is to provide the reader with a clear and concise view of new advances in areas ranging from electrode engineering, strategies for electrochemical signal amplification, and novel electroanalytical techniques used in the miniaturization and integration of the sensors. Moreover, the authors have attempted to highlight

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areas of the latest and significant development of enhanced electrochemical nanosensors and nanobiosensors that inspire broader interests across various disciplines. Electrochemical sensors for small molecules, enzyme-based biosensors, genosensors, immunosensors, and cytosensors are reviewed herein (Figure 1). Such novel advances are important for the development of electrochemical sensors that open up new avenues and methods for future research. We recommend readers interested in the general principles of electrochemical sensors and electrochemical methods to refer to other excellent literature for a broad scope in this area.[3,4] However, due to the explosion of publications in this active field, we do not claim that this Review includes all of the published works in the past two years and we apologize to the authors of excellent work, which is unintentionally left out.

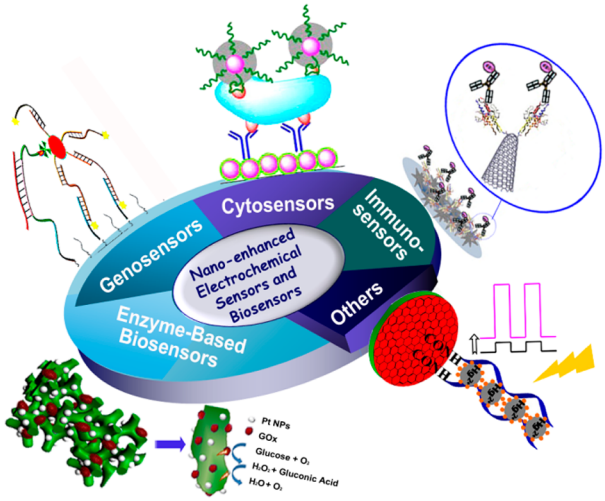

Figure 1. Schematic illustration of electrochemical sensors and biosensors based on nanomaterials and nanostructures, in which electrochemical sensors for small molecular, enzyme-based biosensors, genosensors, immunosensors, and cytosensors are demonstrated.

NONENZYMATIC SENSORS

The pursuit of electrochemical systems for bimolecular detection has received significant attention over the last two decades.[5] Electroanalysis toward small molecules is also of importance in a variety of areas. Enzymatic sensors possess high selectivity but suffer from limitations such as instability, complicated modification procedures, and critical microenvironmental factors. Such limitations stimulate the development of nonenzymatic electrochemical sensors with simple modification procedures and good stability. Enzyme-free electrochemical sensors have been widely used for determining the presence of hydrogen peroxide, glucose, and dopamine. The perspectives and current challenges of enzyme-free electrochemical sensors were discussed by Chen et al.[6] (142 references). Miao et al.[7] recently reviewed electrocatalysis and electroanalysis of nickel, itsoxides, hydroxides, and oxyhydroxides toward small molecules (85 references). Following are some examples of nonenzymatic sensors for the detection of small molecules.

Glucose. Glucose plays an important role in metabolism. Glucose biosensors have contributed significantly to clinical monitoring.[8,9] With regard to the electrode materials, metal, metal oxide nanostructures, and their hybrid nanocomposites are regarded to be the most promising materials currently used. Wang et al.[10] reviewed the progress made in recent years in the field of direct and nonenzymatic electrochemical sensing of glucose (221 references). Tian et al.[11] also reviewed the most recent advances in nonenzymatic glucose sensors based on various nanomaterials (125 references). Various nanomaterials with different shapes and compositions were synthesized to construct novel nonenzymatic electrochemical sensors for glucose detection.

Cao et al.[12] synthesized bimetallic PtCu nanochains through a water-based mild chemical route, compositions of which can be conveniently tuned at the mesoscopic scale by a facile dealloying process. Electrochemical measurements demonstrate that the sensors made by these PtCu nanomaterials are very sensitive and specific for glucose detection due to the wiring of dispersed crystals, porous nanostructure, clean surface, and synergetic electronic effects of the alloyed atoms. The resulted sensor performed very well in the detection of glucose in serum samples. Keerthy et al.[13] produced reduced graphene oxide (rGO) modified with platinum nanocubes and copper oxide nanoflowers. A low cost screen printing technology was used to fabricate such electrodes for point-of-care (POC) glucose monitoring. These sensors were highly specific to glucose in the presence of commonly interfering species like ascorbic acid (AA), dopamine (DA), uric acid (UA), and acetaminophen. It was discussed that copper oxide catalyzes glucose oxidation and Pt NPs act as a cocatalyst to enhance the electron transfer during the oxidation of glucose. Guo et al.[14] constructed a Ni/CdS bifunctional Ti@TiO_2 core−shell nanowire electrode through a hydrothermal and electrodeposition method. The resultant sensor based on the fabricated nanowire electrode exhibited great sensitivity in the electrochemical detection of glucose oxidation. The enhanced electrochemical performance on glucose sensing was attributed to the high dispersity of Ni NPs and the ability to discriminate the interfering species of CdS under the irradiation of visible light. The ability to combine the unique properties of individual nanomaterials provides a new and exciting frontier for the formation of novel electrodes.

Xu et al.[15] prepared α-Fe_2O_3 cubes in the presence of a hydrophobic iron-containing ionic liquid (IL) under hydrothermal conditions and tested the photoelectrochemical properties by means of the transient photocurrent responses of ITO electrodes which were modified with the as-prepared α-Fe_2O_3. The photoelectrochemical approach in the application of the glucose sensor was investigated. A glucose photoelectrochemical detection system based on a nonenzyme catalytic oxidation reaction has shown promising results with high sensitivity and fast response.

Hydrogen Peroxide. The rapid and accurate detection of hydrogen peroxide (H_2O_2) has practical importance in the field of bioanalysis as well as food safety and environmental protection.[16,17] Nagaiah et al.[18] reported a H_2O_2 sensor based on electrochemical deposition of Pd−Pt and Pd−Au NPs on spectrographic graphite. The sensitivity originates from the codeposition of Pd with either Pt or Au enhancing electrocatalytic activity for H_2O_2 reduction. Bai and Jiang[19] developed a H_2O_2 sensor based on copper sulfide NPs-decorated rGO. They investigated the application of this sensor in detecting H_2O_2 content in human serum and urine samples as well as H_2O_2 released from human cervical cancer cells. Their results have shown satisfactory recovery rates with good reproducibility, indicating potential applications in medical diagnosis. A

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powerful enzymatic mimetics employing graphene oxide (GO), carbon nanotubes (CNTs), and Pt nanocatalysts was fabricated by Wang's group.[20] The GO−CNTs−Pt nanocomposites exhibited peroxidase-like catalysis activity and electrocatalysis activities, that tested the colorimetric and electrochemical detection of H_2O_2. Moreover, sandwiched electrochemical immunoassays were demonstrated by using the GO−CNTs−Pt as catalytic tags. Such innovative nanostructure showed promise in the extensive catalysis applications in environmental, medical, industrial, and particularly aqueous biosensing fields.

Yang et al.[21] reported the development of a microwave-assisted strategy for the synthesis of nitrogen and boron codoped graphene with a hierarchical framework. The resultant sensor was able to selectively detect H_2O_2 in the presence of glucose and AA. Moreover, this electrode system could be easily functionalized with biomacromolecules to generate a cost-effective, highly sensitive, and biocompatible sensor for a variety of applications. Tian et al.[22] reported that ultrathin g-C_3N_4 nanosheets were fabricated by ultrasonication-assisted liquid exfoliation of bulk C_3N_4. They demonstrated the use of these nanosheets as an effective sensing platform for the detection of H_2O_2, which has been extended to the electrochemical detection of glucose in both buffer solution and human serum medium.

Wu et al.[23] developed a novel nonenzymatic electrochemical sensor based on a *p*-methoxy zinc porphyrin-C_{60} derivative (ZnPp-C_{60}), which was designed and synthesized by combining *p*-methoxy porphyrin and C_{60} moieties with a flexible methylene chain. Combined with theoretical information, the results showed that the ZnPp-C_{60} would be a novel material for construction of a nonenzymatic electrochemical sensor for H_2O_2 and nitrite analysis in a relatively wide potential range with high sensitivity and stability.

Cation. The sensitive and selective detection of toxic heavy metals coupled with a cost-effective and simple assaying procedure has paramount importance.[24] Recently, Cui et al.[25] developed a convenient and highly sensitive electrochemical detection platform for detecting copper. The sensor was fabricated on a glassy carbon electrode (GCE) through a layer-by-layer (LBL) assembling modification with multiwall carbon nanotubes (MWCNTs), poly(amidoamine) dendrimers, and dithiobis[succinimidyl-propionate] encapsulated Au NPs (DSP-Au NPs). The DSP modified sensor captures cysteamine (Cys) functionalized Ag NPs through the reaction between DSP and Cys. The presence of Cu^{2+} catalyzed cystocystamine regulated the assembly of Ag NPs on the sensor surface, resulting in the decrease of the electrochemical stripping signal of Ag NPs. With this strategy, the detection range of Cu^{2+} is 1.0−1000 nM with a detection limit of 0.48 nM. In addition, Sadhukhan and Barman[26] synthesized two-dimensional C_3N_4 under microwave irradiation and used it to modify GCE for the detection of Hg^{2+}. Due to its graphene-like structure, this sensor detected Hg^{2+} down to 0.09 nM. For a real application, the device fabricated by Rattanarat's group using screen-printing MWCNTs mixed carbon inks on polyester and then modified them with binanoparticles and ferricyanide which are supposed to enhance stripping signals and reduce Cu^{2+} interference.[27] The top layer of the device contains five wax-defined channels extending outward from an open sample reservoir. In order to achieve high selectivity and sensitivity, each channel contains an individual sample with pretreatment and detection zones. Under the optimized conditions, such a paper-based device can simultaneously detect Cd^{2+} and Pb^{2+} in the range of 5−150 $\mu g\ L^{-1}$.

Anion. Madhu et al.[28] prepared highly porous and heteroatom-enriched activated carbon (HAC) from banana stems. HAC was used to develop electrochemical sensors for the detection of nitrite in the application of monitoring environmental pollution. HAC exhibits noteworthy performance in the highly sensitive detection of nitrite. Their method was tested to determine nitrite in various water samples with acceptable results.

Conducting polymer-based modified electrodes have been extensively studied as sensing materials in the last decades.[29] Yang et al.[30] reported glassy carbon electrodes modified with polyaniline (PAni)-coated copper hexacyanoferrate for use as a cyclic voltammetry sensor. The application of the sensor was investigated in the detection of sulfite, which is used as a preservative in a variety of food and pharmaceutical products to inhibit enzymatic and nonenzymatic browning and also used in brewing industries as an antibacterial and antioxidizing agent.[31] They showed that the synergistic effect of these structures improved electrocatalytic activity toward detection.

Other Species. Most conducting polymer/graphene composites have excellent electrical conductivity. Gao et al.[32] developed electrochemically coated porous structure films of overoxidized polypyrrole/graphene (PPyox/GR) deposited onto GCE. This structure was successfully utilized as an efficient electrode material for the quantitative detection of adenine and guanine. With low background current, the permselective polymer coating improved the selectivity and sensitivity of microelectrodes for the electropositive purine bases.

Lin et al.[33] reported the hybridization of poly(luminol) (PLM) and poly(neutral red) (PNR) that is then enhanced by a conductive and steric hybrid nanotemplate using GO and MWCNTs. The PLM−PNR−MWCNT−GO mycelium-like nanocomposite is found to be electroactive, pH-dependent, and stable in the electrochemical system. This nanocomposite showed electrocatalytic activity toward NADH with a high current response and low overpotential. It also exhibited a high sensitivity of 288.9 $\mu A\ mM^{-1}\ cm^{-2}$ to NADH using amperometry.

Changes in glutathione concentration at the cellular level may be linked to diseases such as premature arteriosclerosis, leukemia, and diabetes. Lee et al.[34] described a modified GCE through electropolymerization of caffeic acid onto the electrode surface in the presence of either CNTs or nanocarbon, affording a nanocomposite with a high concentration of *o*-quinone moiety onto the electrode, that can be used for the detection of glutathione through an electrocatalytic process. Concentrations as low as 500 nM were detected.

Zhang et al.[35] prepared a phosphomolybdate functionalized graphene nanocomposite for the detection of AA, in which polyoxometalates can irreversibly adsorb onto carbon materials to form carbon nanocomposite structures. The amperometric signals are linearly proportional to the AA concentration in a wide concentration range from 1×10^{-6} to 8×10^{-3} M, with a detection limit of 0.5×10^{-6} M.

Recently, layered transition metal dichalcogenides have been extensively studied due to their structural similarities with graphene and their interesting physicochemical properties, along with their diverse exotic electronic properties.[36,37] Narayanan et al.[38] employed ultrathin MoS_2 sheet-based electrodes for electrochemical detection of dopamine (DA) as

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an important neurotransmitter in the presence of AA. The results showed that MoS_2 is expected to be a good electrode material for electrochemical sensing applications. Sun et al.[39] reported the Au NPs-decorated MoS_2 nanosheets synthesized by electrodeposition of Au NPs on MoS_2 nanosheets, which possess better properties than pure Au NPs and MoS_2. The composite film modified electrode showed excellent electrocatalytic activity toward the oxidation of AA, DA, and UA with three well-resolved oxidation peaks. The peak potential separations were large enough to individually or simultaneously detect AA, DA, and UA.

Nitric oxide (NO) plays an important role in physiological processes. It has been reported that some human diseases are related to their biological function.[40] Hunter et al.[41] utilized standard photolithographic techniques and a nitric oxide (NO) selective xerogel polymer to fabricate an amperometric NO microfluidic sensor. The sensor detected NO levels in small sample volumes (~250 μL) with low background noise. The detection sensitivity of 1.4 pA nM^{-1} was demonstrated along with the limit of detection (LOD) of 840 pM. This sensor exhibited excellent analytical performance in phosphate buffered saline. Moreover, the analytical performance of the device was investigated in simulated wound fluid and whole blood. The results showed that the sensor is able to measure NO in complicated biological samples. This proof of concept study demonstrated the feasibility of clinical application of this method. Metters et al.[42] reported screen printed single-walled CNTs (SWCNTs) sensors which were fabricated upon flexible polyester substrates. The screen printed SWCNTs sensors are benchmarked using potassium ferrocyanide (II), DA, hydrazine, and capsaicin. By using this sensor, the detection of capsaicin has been achieved at low micromolar levels. These electrodes hold potential in the development of disposable and highly reproducible sensors.

It is observed that sensors that exploit the unique properties of nanomaterials constitute the most rapidly expanding sensor research area. Moving forward, several areas of research will enhance the nanostructured sensing platform. For example, research in the storage and stability of the sensors will improve the shelf life of functional biosensors.

■ ELECTROCHEMICAL ENZYME-BASED BIOSENSORS

Electrochemical enzyme-based biosensors, a subclass of chemical sensors, combine the high specificity of the enzyme with the sensitivity of electrochemical transducers. Enzyme electrodes are electrochemical probes with a thin layer of immobilized enzyme on the surface of the working electrode. The enzyme is the most critical component of the enzyme electrode since it provides the selectivity for the sensor and catalyzes the formation of the electroactive product for detection. In the past two years, there were some review articles that focused on the development of various materials, techniques, and applications of electrochemical enzyme-based biosensors. Chen et al. described recent progress in electrochemical glucose biosensors and focused on some problems and bottlenecks in areas of enzymatic (glucose oxidase (GOx) based) amperometric glucose sensing (240 citations).[43] The focus of the review by de Oliveira's group is to present the current status and some trends in enzymatic nanoimmobilization.[44] The recent advance of lactate biosensors[45] and enzymatic uric acid[46] biosensors were also systematically discussed. In addition, Schneider and Clark presented different immobilization strategies that have been used to create Cytochrome P450s (CYPs) biosensors, with particular emphasis on mammalian drug-metabolizing CYPs and characterization of CYP electrodes.[47] Recent achievements in this area have focused on the development of a novel immobilization strategy and study of the direct electron transfer with the assistance of functional nanomaterials. Additionally, some other papers of interest will also be addressed.

Immobilization Strategies. The immobilization of enzymes is one of the key steps in developing high-performance biosensors, since it will affect the loading as well as the bioactivity of the enzymes. To date, different methods have been studied to achieve efficient enzyme immobilization, such as covalently binding enzymes onto the substrate surface or incorporating enzymes into different matrixes. The development of the synthesis of nanomaterials provides enormous opportunities for tailoring their properties, thus enhancing their functions and application in immobilization of enzymes.

A great number of nanostructured materials with different sizes, shapes, and compositions have been synthesized and utilized as novel electrode materials for immobilization of desired enzymes. Due to the homogeneous spherical shape, high conductivity, and large surface area for biomolecular conjugation, graphite NPs, consisting of several stacked graphene sheets, were reported to construct an enzyme biosensor to detect glucose in real samples.[48] After modification, GOx was linked with graphite NPs through an amide bond. This cost-effective approach will be applied to other electrochemical biosensors. Wågberg and co-workers reported on a novel sensing platform for H_2O_2 and glucose based on the immobilization of Pd helical carbon nanofiber (Pd-HCNF) hybrid nanostructures and GOx.[49] Small size and homogeneous distribution of the Pd NPs in combination with good conductivity and large surface area of the HCNFs significantly reduce the overpotential and enhance the electron transfer rate and therefore lead to a high performance glucose sensing platform. Malhotra and co-workers synthesized a series of nanomaterials, such as $NiFe_2O_4$,[50] Tm_2O_3,[51] and Cu_2O,[52] which were utilized as electrode materials for immobilizing bienzyme (cholesterol esterase (ChEt) and cholesterol oxidase (ChOx)). These fabricated bioelectrodes exhibit largely improved amperometric biosensing performance toward cholesterol.

Du and co-workers developed a series of robust organophosphorus pesticide (OPs) biosensors based on functional nanomaterials. As a typical example, a novel hydrolase biosensor, based on self-assembly of methyl parathion hydrolase (MPH) on the Fe_3O_4@Au nanocomposite, was developed for sensitive and selective detection of the OPs methyl parathion.[53] There were several advantages of this electrochemical biosensor. First, the Fe_3O_4nanocomposite provides an easy way to construct the enzyme biosensor and renew the electrode surface simply by an external magnetic field. The hydrolase is not poisoned by OPs and thus is reusable for continuous measurement. Moreover, Au NPs not only provide a large surface area, high loading efficiency, and fast electron transfer but also stabilize the enzyme through electrostatic interactions. The MPH biosensor shows a rapid response and high selectivity for detection of methyl parathion, with a linear range from 0.5 to 1000 ng mL^{-1} and a detection limit of 0.1 ng mL^{-1}. A nanohybrid of Au NPs, polypyrrole, and rGO (named as Au−PPy−rGO) was also prepared on the electrode, achieved by electrochemical deposition of rGO with pyrrole and the introduction of Au NPs.[54] Acetylcholinesterase (AChE) was

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further encapsulated in a silica matrix and immobilized on the Au−PPy−rGO nanocomposite by codeposition with $(NH_4)_2SiF_6$. The obtained nanohybrid of Au−PPy−rGO not only increased the surface area of the modified electrode but also showed excellent conductivity. AChE molecules were protected by a biocompatible 3D porous silica matrix to prevent them from leaking out and to retain their bioactivity. Furthermore, the fabricated AChE biosensor displayed high stability, excellent activity, and fast response to OPs. This assembly protocol is expected to be used for the immobilization of various enzymes and proteins, leading to robust biosensors.

Zhang and co-workers reported a facile electrochemical biosensing interface for sensitive glucose determination based on a Pt@BSA nanocomposite along with the covalent adsorption of GOx.[55] The electrocatalytic activity toward oxygen reduction was significantly enhanced due to the excellent bioactivity of anchored GOx and superior catalytic performance of interior Pt NPs. Upon the successive injection of glucose, the GOx-based biosensor catalyzed the oxidation of glucose to gluconolactone in the presence of oxygen, with the reduction peak current gradually decreased, making it suitable for glucose determination. In addition to the simple modification of enzymes on the surface of electrode materials, embedding them within a different matrix is widely reported. Cosnier's group synthesized polypyrrolic bipyridine bis-(phenantrolinequinone) Ru(II) complex/CNTs composites through electropolymerization, which were used for efficient enzyme entrapment.[56] Their ability to oxidize NADH while immobilizing enzymes during their electrodeposition represents a straightforward technique to design functional bioelectrodes. In presence of NAD^+, the resulting enzyme electrode exhibits high current densities for glucose oxidation with a detection limit of 1 μM glucose. Kale and co-workers succeeded in immobilizing GOD in a mixture containing silica sol−gel and poly(vinyl alcohol) composite film.[57] The sensitive nature of poly(vinyl alcohol) and improved stabilizing effect of prehydrolyzed tetraethyl orthosilicate created in the matrix was favorable for high sensitivity. At the same time, the presence of Au NPs in the immobilization matrix not only offered a biocompatible microenvironment but also efficiently improved electron transfer between the GOx/mediator and electrode surface.

LBL assembly has been selected as a reliable method to immobilize enzymes with preserved activity due to its simplicity and versatility. On the basis of the electrostatic interaction, poly(allylamine hydrochloride) (PAH) modified CNTs/Au composites were assembled with negatively charged enzyme, horseradish peroxidise (HRP) and ChOx, to fabricate a bienzyme biosensor for the detection of cholesterol.[58] The bienzyme biosensor showed more highly efficient electrochemical signal transduction. In addition, CNTs and Au NPs could enhance the electrochemical signal by catalyzing the response of H_2O_2 and effectively facilitate the electron transfer due to the good conductivity, further improving the detection sensitivity and stability. Considering the advantages in preserving enzyme activity of poly(ethylene imine) (PEI), Ferreira and coworkers fabricated β-galactosidase (β-Gal) immobilized in LBL films with PEI and poly(vinyl sufonate) on an ITO electrode modified with a layer of prussian blue (PB).[59] Lactose could be detected with an amperometric method with a sensitivity of 0.31 μA $mmol^{-1}$ cm^{-2} and a detection limit of 1.13 mmol L^{-1}, which is sufficient for detecting lactose in milk and for clinical exams. Compared to the immobilization of enzymes onto a substrate surface, incorporation of enzymes into a 3D matrix has the potential to increase the enzyme loading as well as to protect the enzyme from the surrounding environment. To increase the loading of GOx and simplify glucose biosensor fabrication, Yang and co-workers prepared a hydrogel from Fc modified amino acid phenylalanine, which was utilized for the incorporation of GOx.[60] The resultant hydrogel featured good biocompatibility and contained a significant number of ferrocene (Fc) moieties, which can be considered as an ideal matrix to immobilize enzymes for the preparation of mediator-based biosensors. Due to the improved enzyme loading and efficient electron transfer, the as-prepared glucose biosensor exhibited good performance for the electrochemical detection of glucose, such as high sensitivity, wide linear range, short response time, and good stability. Leopold and co-workers reported the xerogel biosensors containing composite films of (3-mercaptopropyl)-trimethoxysilane xerogel embedded with GOx, doped with Au NPs, monolayer protected clusters (MPCs), and coated with an outer polyurethane layer.[9] The MPC network within the sol−gel acts as a 3D extension of the working electrode area that allows for the biosensor's signal to have a decreased dependence on the diffusion of H_2O_2. It is found that the MPC-doped sol−gel glucose biosensors of this study are equal to or exceed comparable glucose biosensors reported previously. Similarly, 3D Pt NPs/PAni hydrogel heterostructures were also used as a novel matrix to load GOx and thus to construct highly sensitive glucose sensors (Figure 2).[61] On the

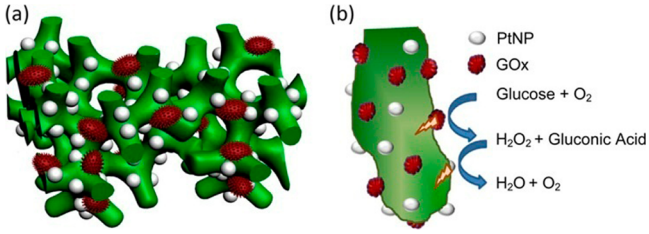

Figure 2. (a) Schematic representation of the 3D heterostructure of the Pt NP/PAni hydrogel, in which the PAni hydrogel acts as a matrix for the immobilization of the GOx enzyme and homogeneous loading of Pt NPs. (b) A 2D scheme showing the reaction mechanism of the glucose sensor based on the Pt NP/PAni hydrogel heterostructure. Reprinted from ref 61.Copyright 2013 American Chemical Society.

one hand, the Pt NPs/PAni hydrogel heterostructure-based glucose sensor synergizes the advantages of both the conducting hydrogel and the nanoparticle catalyst. On the other hand, the 3D porous structure of the PAni hydrogel favored the high density immobilization of the enzyme and the mass transfer of the glucose. The glucose enzyme sensor based on this heterostructure exhibited unprecedented sensitivity and low detection limit. Willner's group utilized enzyme-capped relay-functionalized mesoporous carbon NPs as effective bioelectrocatalytic 3D matrices to construct a glucose electrochemical biosensor.[62]

Direct Electron Transfer (DET). Enzyme-based electrochemical biosensors, especially the third-generation amperometric glucose biosensors, are fascinating because they function as the ideal biosensing model in the absence of mediators. The direct electrical communication of GOx can also contribute to the detection of glucose at low potentials which are slightly positive from the redox potential of GOx. Considerable attempts to overcome the long electron-tunneling distance

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were made to realize the direct electrochemistry of enzymes.[63] Liu and co-workers employed a whole-cell biocatalyst using a yeast surface displaying GOx and a constructed GOx-yeast/CNTs electrochemical glucose sensing platform.[64] Direct electrochemistry was achieved, suggesting that the host yeast cell did not have any adverse effect on the electrocatalytic property of the recombinant GOx. Ye and co-workers reported a DET glucose biosensor based on GOx self-assembled on electrochemically reduced carboxyl graphene.[65] It was noted that the conductivity of the graphene was improved because most of the oxygen-containing groups were eliminated after electrochemical reduction. Carboxylic acid groups remained and can effectively link with GOx. Well-defined and quasi-reversible redox peaks could be obtained. DET of GOx was also realized in bienzyme (glucoamylase and GOx) functionalized CNTs[66] and MnO_2 decorated rGO.[67] Although well-defined voltammetric peaks of direct electrochemistry of GOx have been achieved in previous reports, the detection of glucose based on the direct electron transfer of GOx has been rarely realized. However, it is worth noting that the determination of glucose based on electroreduction of enzyme-consuming O_2 at low potentials (close to the redox potential of GOx) should conceptually belong to the first-generation amperometric glucose biosensors, rather than the third-generation ones. To address this issue, Gorski and co-workers systematically investigated the signal transduction and enzyme activity in biosensors based on the GOx and CNTs embedded in a bioadhesive film of chitosan (CHIT).[68] This work focused on the on the role of DET in glucose sensing at a GOx/CNTs hybrid that was embedded in a CHIT on the electrode surface. Two main issues including the role of reactions relevant to the electrochemical glucose sensing the effect of CNT on the retention and enzymatic activity of GOx in CHIT films and in aqueous suspensions were studied. The well-defined voltammetric peaks of direct electrochemistry of GOx were observed regardless of CHIT. However, the DET was not the mechanistic basis for glucose sensing at a GOx/CNT-based biosensor, indicating that GOx molecules that were within the electron tunneling distance from CNT were not enzymatically active toward glucose. The biosensor was sensitive to glucose in air-equilibrated solutions based on the O_2-mediated enzymatic oxidation of glucose. The signal transduction relied on the net drop in a biosensor current that was caused by a decrease in a $4e^-$ O_2 reduction current and an increase in a $2e^-$ H_2O_2 reduction current. Moreover, they found that CNTs nearly doubled the retention of GOx in a biosensor, while CNTs significantly decreased the average enzymatic activity of GOx.

The third-generation biosensors based on enzyme DET were also reported in these two years. In a typical example, Cui and co-workers utilized functionalized planar boron-doped diamond (BDD) electrode as a biosensing platform for biomolecule immobilization with GOx as a test model.[69] In detail, BDD was treated with KOH and functionalized with 3-aminopropyltriethoxysilane (APTES). The free amino groups of GOx and APTES were cross-linked by glutaraldehyde (X), a bifunctional chemical to form a stable enzyme layer (GOx-XAPTES) on BDD. DET between the flavin adenine dinucleotide (FAD) center of GOx and the electrode was realized by using the APTES-glutaraldehyde conjugate as a molecular wire to form electron tunneling between the FAD center and BDD. Amperometric responses of the GOx electrode to glucose were illustrated in both aerated and deaerated buffer solution to address whether the signal response to glucose can be attributed to DET. Different from the other reports, the result confirmed the bioelectrocatalytic activity of the electrical contacted GOx. In the presence of glucose, glucose is oxidized by GOx in concurrence with the biochemical reduction of the GOx FAD to $FADH_2$. Niwa and co-workers investigated the effects of a bare ITO film electrode surface structure on human cytochrome (CYP3A4) by using polycrystalline ITO and amorphous ITO film.[70] They found DET from a human CYP layer or a CYP microsome adsorbed on ITO without any modification could be easily realized. Because of its larger surface area and negatively charged surface, the polycrystalline ITO film was a suitable electrode for the adsorption of CYP proteins while maintaining efficient DET and enzymatic activity. On this basis, the simple ITO interface was applied to drug metabolism and inhibitor evaluation. Wang and co-workers for the first time employed small molecular hydrogel as a surrounding matrix to stabilize Cytochrome c (Cyt c), further facilitating electron transfer between redox enzyme and electrode.[71] Significantly, the third-generation biosensors based on DET of Cyt c was successfully achieved to determine H_2O_2 at an optimized potential with high selectivity over other reactive oxygen species, oxygen, metal ions, AA, and so on, which provided a durable platform for real-time determination of H_2O_2 from live cells. In addition, Zhao et al. investigated the effect of three kinds of nanostructured silica−phytic acid (SiO_2−PA) materials with diverse morphologies as electrode materials including spherical SiO_2−PA (s-SiO_2−PA), rod-like SiO_2−PA (r-SiO_2−PA), and helical SiO_2−PA (h-SiO_2−PA) on the electrocatalytic activity toward DA detection based on the laccase biosensor.[72] Combining the direct bioelectrocatalyst, it was observed that that the laccase/h-SiO_2−PA-modified electrode showed the best electrochemical performances because helical SiO_2−PA could load more laccase and provide more spatial freedom in its orientation and thus facilitate DET of laccase.

Other Papers of Interest. This two year period witnessed some novel electrochemical detection strategies and methods in developing new enzyme-based biosensors. Vagin and co-workers reported a single-enzyme and membrane-free self-powered biosensor, in which both cathodic and anodic bioelectrocatalytic reactions are powered by cholesterol. Among them, ChOx was immobilized in a sol−gel matrix on both electrodes. Compared to either of the two individual electrodes, the self-powered sensor formed on the high surface-area carbon cloth electrodes, resulting in enhanced sensitivity.[73] To overcome the disadvantage of existing adenosine-5-triphosphate (ATP) biosensors, such as cascades of enzymatic reactions, Kucherenko's group developed a biosensor system consisting of two biosensors.[74] In detail, the first one was based on GOx and was designed to measure glucose concentration, and the other one was based on GOx and hexokinase and was sensitive toward both glucose and ATP. On this basis, simultaneous determination of glucose and ATP concentrations by two independent bioselective elements holds great promise in novel sensing devices. Reed and co-workers demonstrated electronic field-effect transistors (FETs) as sensitive devices. An Al_2O_3-passivated Si nanowire used to mimic transistor operation was created for measuring enzyme−substrate interactions via the monitoring of pH change.[75] The change in pH can be measured by the nanoribbon in solution in real time and is reflected in the change in drain current through the device. Urea in phosphate buffered saline (PBS) and penicillinase in PBS and urine can be effectively detected, at

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limits of detection of <200 μM and 0.02 units/mL, respectively. The enzyme kinetics can also be analyzed to accurately determine the kinetic constant. This direct, rapid, and label-free detection method can be readily generalized to many unrelated classes of substrates and enzymes. Schöning and co-workers reported a LBL nanofilm of polyamidoamine (PAMAM) dendrimer and CNTs on capacitive electrolyte-insulator-semiconductor (EIS) field-effect sensors for detecting urea.[76] Through the optimization of the arrangements of a LBL film and the enzyme urease, adequate film architecture urease sandwiched between the LBL film and another CNT layer [EIS-(PAMAM/CNT)-urease-CNT] exhibited a superior output signal performance and higher sensitivity of about 33 mV/decade by means of capacitance−voltage (C/V) and dynamic constant-capacitance measurements. It was determined that the presence of the additional CNT layer was needed to achieve a urea-based EIS sensor with enhanced properties.

Development of a novel electrochemical interface along with functional materials and enzyme immobilization plays a critical role for the rational design and construction of bioelectronic devices. Wang and co-workers designed a facile and effective electrochemical sensing platform for the detection of glucose and urea in one sample without separation; it was developed using CHIT-rGO/concanavalin A (Con A) as a sensing layer.[77] In this system, the CHIT-rGO with a large specific surface area was introduced to immobilize a large amount of Con A, exhibiting nice pH-switchable behavior to $Fe(CN)_6^{3-}$. The change of resistance to charge transfer or amperometric current in the presence of GOx or urease resulted from the change of glucose or urea concentration, thus realizing simultaneous detection of glucose and urea based on in situ pH-switchable enzyme-catalyzed reactions. Karra and Gorski studied the nafion-induced current amplification in dehydrogenase-based biosensors.[78] The fabricated biosensors were designed by sandwiching the enzyme−CHIT/CNTs film between an electrode and nafion film. The coating of such biosensors with nafion resulted in the current increase by up to 1000%, depending on the enzyme. The increase in the biosensor current was attributed to the pH-driven increase in the enzyme activity inside the two-film interface. The combination of the two-film interface with enzyme engineering to modify enzyme activity−pH profiles can lead to the enzyme-based biosensor devices with highly amplified current output. In addition, Liu and co-workers adopted an in-site immobilizing method to embed GOx in copolymer involving *N*,*N*-diethylacrylamide and methyl acrylic acid.[79] The effect of environmental stimuli, such as temperature, pH, the identity and concentration of anions, and the concentration of CO_2 in solution on the voltammetric response of Fc dicarboxylic acid ($Fc(COOH)_2$) at the film electrodes was investigated. This multiresponsive electrochemical behavior of the system could be further employed to maximize the electrochemical oxidation of glucose catalyzed by GOx entrapped in the films with $Fc(COOH)_2$ as the mediator in solution.

Future efforts were aimed at further miniaturization and integration of the electronic interface, further facilitating the development of advanced electroanalytical devices. For example, Wang and co-workers describe the first example of real-time noninvasive lactate sensing in human perspiration during exercise events using a flexible printed temporary-transfer tattoo electrochemical biosensor.[80] This flexible tattoo lactate sensor consists of a mediated lactate oxidase (LOx) working electrode, prepared by functionalizing the surface of the printed tattoo electrode with tetrathiafulvalene and CNTs, followed by tethering the LOx enzyme, and a biocompatible CHIT overlayer. The lactate biosensor was used for the electrochemical detection of sweat lactate, thereby substantiating its utility for the noninvasive assessment of lactate levels and degree of physical exertion. Mao and co-workers demonstrated a microfluidic chip-based online electrochemical system for in vivo continuous and simultaneous monitoring of glucose, lactate, and ascorbate in rat brain.[8] Taking advantage of single-walled CNTs in facilitating the electrochemical oxidation of ascorbate and dehydrogenases to selectively catalyze the oxidation of glucose and lactate, the microfluidic chip-based online electrochemical system allowed the integration of various detection units into a small device which is more suitable to establish a multicomponent analysis system with technical simplicity, near real-time nature, little crosstalk, and low cost.

GENOSENSORS

In electrochemical genosensors, single stranded DNA (ssDNA) fragments are immobilized onto the electrode surface as recognition probes for capturing the target DNA through hybridization. In the presence of hybrids, signals are generated via various mechanisms and then detected electrochemically. According to electrochemical detection principles, several important factors should be considered for the achievement of good sensitivity and selectivity in the biodetection. The past two years has witnessed substantial advances toward the development of a high performance electrochemical sensing platform for DNA detection. Recent review articles have focused on new methods and new signal amplification based on functional nanomaterials and enzymes in the DNA and RNA assays. Xu and co-workers recently evaluated the methods related to photoelectrochemical DNA biosensors.[81] This kind of photoelectrochemical DNA biosensor provides excellent sensitivity due to the separation and the different energy forms of the excitation source and the detection signal. In the another review article, the sandwich assay based on the biotechnologies and nanotechnologies for nucleic acids was also introduced.[82] Wu et al. summarized the latest developments in the application of nanomaterials as signal amplification elements in ultrasensitive electrochemical detection of DNA (136 citations).[83] From the point of their unique electrochemical properties, various nanomaterials with different signal amplification routes have been reviewed briefly. Meanwhile, some other new methods and related progress were also investigated in the development of electrochemical DNA sensors.[84−87] For example, a paper analytical device for quantitative detection of DNA was reported.[87] Here, we focus on the recent progresses in device fabrication, DNA probe design, enzyme-based amplification, nanomaterial-enhanced signal amplification, and other interesting methods.

Nucleic Acid Assay. *Design of DNA Probe.* The electrochemical DNA sensing platform consists of capture probes immobilized on the sensing surface for capturing targets and signal probes with electrochemical tags for signal generation. In the nucleic acid assays, good detection sensitivity can be achieved by optimizing hybridization conditions and improving hybridization efficiency. High detection specificity relies on the design of specific probes and the elimination of nonspecific binding on the sensing surface.[88,89] Under the circumstances, the design of a novel DNA probe was considered to be an efficient approach to enhance detection

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sensitivity. Due to the proper distance between the nucleobases, the rigid amido bonds, the high flexibility of the aminoethyl linkers, and intramolecular hydrogen bonding, the peptide nucleic acid (PNA) probe has great sequence specific affinity and stability and has received great interest in DNA sensors. On the basis of the advantage of the PNA−DNA hybridization, a rGO-based FET biosensor used for ultrasensitive label-free detection of DNA was reported.[90] A detection limit as low as 100 fM was achieved, which is 1 order of magnitude lower than that of the previously reported graphene FET DNA biosensor based on DNA−DNA hybridization. Fan and co-workers have demonstrated a new generation of electrochemical DNA sensors for sensitive and specific detection of microRNA (miRNA).[91] In their design, the use of a DNA tetrahedron ensures the stem-loop structure in a well controlled density with improved reactivity. The regulation of the thermodynamic stability of the stem-loop structure decreases the background signal and increases the specificity as well. The attached enzymes bring the electrocatalytic signal to amplify the detection. The combination of these effects improves the sensitivity of the sensor and can be applied to other miRNA detection methods. At the same time, DNA tetrahedral nanostructures containing a partially self-complementary region with a stem-loop hairpin structure were also innovatively designed.[92] An electrochemical redox label was attached to the reconfigurable tetrahedron edge in such a way that reconfiguration of this edge changed the distance between the electrode and Fc in the presence of target DNA. As mentioned above, the immobilization of the probe DNA on the surface of the electrode dictates the performance of the resulting sensor. However, it is very difficult to precisely control the DNA spatial orientation and position on solid surfaces via formation of the self-assembled monolayer using thiolated ssDNA molecules. A bovine serum albumin-monolayer-based probe carrier platform has been reported to improve the performance in comparison to a conventional thiolated ssDNA probe self-assembled monolayer-based electrochemical DNA hybridization biosensor.[93] When combined with an enzyme-amplification method, a detection limit of 0.5 fM was achieved with high specificity and was reproducible, which was primarily attributed to the enhanced spatial positioning range and accessibility of the probes on this novel platform.

Enzyme-Based Amplification. As is well understood, the application of redox labels is the simplest way to produce an electrochemical signal. However, a redox label can only transfer one or a few electrons to or from the electrode surface. The limitation of the number of electrons transferred directly affects the sensitivity of the DNA sandwich assay. High sensitivity is highly desired since the DNA levels are low in some real problems, such as in pathogen DNA detection and cancer or infectious disease DNA detection. Enzymes have been successfully used for detection of analytes providing both recognition and amplification of the binding event with a detectable readout. In this enzyme-based amplification system, DNA sensors based on enzymatic catalytic reactions, such as HRP[94,95] and alkaline phosphatase (ALP),[96,97] were used as a substitute for the redox label, thus providing high, steady, and reproducible signal amplification.

Besides, loading HRP onto various nanomaterials has become a promising way to further amplify the detection signal and achieve a lower detection limit for the analyte. Our group demonstrated an ultrasensitive electrochemical DAN sensor amplified by CNTs-based labels for the detection of human acute lymphocytic leukemia (ALL)-related p185 BCR-ABL fusion transcript.[98] Carboxylated CNTs were functionalized with HRP and target-specific detection probes to amplify the target hybridization signal. The activity of captured HRP was monitored by square-wave voltammetry measuring the electroactive enzymatic product in the presence of 2-aminophenol and hydrogen peroxide. The use of such labels greatly amplifies hybridization signals and enables the detection of full-length p185 BCR-ABL transcripts at subfemtomole levels, which corresponds to picograms of the target gene. The signal-amplified assay achieved a detection limit of 83 fM (5×10^{-18} mol in 60 μL) target oligonucleotides and has a 4-order-wide dynamic range of target concentration. The resulting assay allowed robust discrimination between the perfect match and a three-base mismatch sequence. Ju et al. reported that noncovalent $\pi-\pi$ interaction led to a stable monolayer stacking of ferric porphyrin on both sides of the GO and demonstrated a simple and convenient pathway to fabricate a universal peroxidase mimic by this GO-based nanocomposite.[99] When combined with the Au NPs−SWCNH modified electrode, the obtained trace label showed greatly enhanced peroxidase activity toward *o*-phenylenediamine (o-PD) oxidization in the presence of H_2O_2, which recognizes a biotinylated molecular beacon for specific electrochemical detection of DNA down to attomolar levels.

On the other hand, several novel electrochemical label-free methods using an enzyme-amplification strategy have been reported. For example, Gao's group developed a simple and ultrasensitive label-free miRNA biosensor, based on hybridized miRNA-templated deposition of an insulating polymer film and electrochemical impedance spectroscopic detection.[100] Upon hybridization, the neutral surface of the biosensor was converted to an anionic state by the hybridized miRNA strands. The deposition of the insulating polymer film, poly(3,3-dimethoxybenzidine), was then carried out by the HRP-catalyzed polymerization of 3,3-dimethoxybenzidine in the presence of H_2O_2. Such a tool may open a new paradigm in routine miRNA analysis with a detection limit of 2.0 fM.

Besides this postamplification strategy toward signal production by a hybridization event, there are target recycling and other strategies via nuclease. They have drawn more and more concerns owing to its striking improvement for the detection sensitivity toward target analytes.[101−103] Dual signal amplification can be readily realized due to the introduction of the functional nanomaterials. Ju and co-workers combined circular strand-displacement polymerization with silver enhancement to achieve a dual signal amplification.[104] After the molecular beacon hybridized with the target DNA and opened the loop part, the opened stem then hybridized with the primer assembled on Au NPs to initiate polymerization of the DNA strand, which led to the release of the target. The released target found another molecular beacon to trigger the polymerization cycle, resulting in the multiplication of the reporter Au NPs on the sensor surface. Sequentially, the Au NPs-promoted silver deposition afforded a signal trace for electrochemical stripping analysis of target DNA. This signal showed high selectivity and can be performed from 10^{-16} to 10^{-12} mol L^{-1} with a detection limit down to the subfemtomolar level.

Gao et al. described another amplification method for DNA detection by applying rolling circle amplification (RCA), which created a long ssDNA product and thus significantly enhanced the electronic responses of Si nanowire FET.[105] Because of the

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binding of an abundance of repeated sequences of RCA products, the fabricated nanosensor showed high sensitivity due to the enhanced signal-to-noise ratio (SNR). The biosensor has exhibited SNR > 20 for detection of 1 fM by employing the RCA amplification method, which exceeds the reported detection SNR by most previously reported DNA sensors.

Nanomaterial-Enhanced Signal Amplification. Given the limitation of the enzyme (i.e., poor stability and high cost), great attention has been paid to developing different kinds of functional nanomaterials, such as metal NPs, quantum dots (QDs), carbon-based nanomaterials, magnetic NPs, and polymers to design advanced genosensors. Because of their biological compatibility, high surface area, chemical stability, nontoxicity, excellent catalytic activity, and conductivity, the introduction of nanomaterials has greatly improved the analytical performance, amplified the detection signal, and stabilized the recognition probes or biosensing interface. It is well-known that the electrode materials as the key component are most widely used in electroanalytical investigations and play an important role in constructing high performance electrochemical sensing platforms to detect target molecules through different analytical principles. Jiao and co-workers synthesized the graphene/poly(xanthurenic acid) nanocomposite via a one-step synchronous electrochemical method.[106] Due to the synergistic effect, this graphene-based electrochemical platform showed an intrinsic advantage in highly sensitive impedimetric detection of DNA. They also synthesized sulfonated PAni-GO, which can be used as a novel electrode material to direct electrochemical detection of DNA.[107] A similar strategy was also reported to construct a label-free electrochemical DNA biosensor based on water-soluble electroactive dye azophloxine-functionalized graphene.[108] A sandwich-type DNA biosensor based on electrochemical coreduction synthesis of graphene-three-dimensional nanostructure gold nanocomposite films was developed with high sensitivity due to its high active surface area and high conductivity.[109] Other materials such as CHIT−ionic liquid[110] and mercury film/carbon nanotubes[111] as well as biocompatible nanostructured magnesium oxide-CHIT platform[112] were also used as advanced electrode materials to design high performance genosensors.

Various nanomaterials, especially Au NPs and carbon nanomaterials, have been used as excellent carriers for loading numerous signal elements such as enzymes, oligonucleotides, and redox labels. Yu and co-workers adopted a hairpin sequence as the capture probe with a restriction site introduced into its stem segment.[113] As shown in Figure 3, with high efficiency and high fidelity of *Eco*RI, the enzymatic cleavage reaction only occurs on those probes that retain their stem−loop structure without capturing the target, leading to a reduced background signal. In contrast, the capture probe is opened by the target hybridization, deforming the restriction site and forcing the biotin tag away from the electrode. Au NPs modified with a large number of Fc-signaling probes are captured on the basis of the biotin−streptavidin complexation. Furthermore, Fc tags can be dragged in close proximity to the electrode surface via hybridization between the signaling probes and the capture probe residues after *Eco*RI treatment, facilitating interfacial electron transfer and further enhancing the signal. This sensor achieves an ultralow detection limit to the zeptomole region and a wide dynamic range over 7 orders of magnitude. Zhang and co-workers modified Au NPs with two types of signaling reporter DNAs; one probe is complementary to the target DNA, while the other is not.

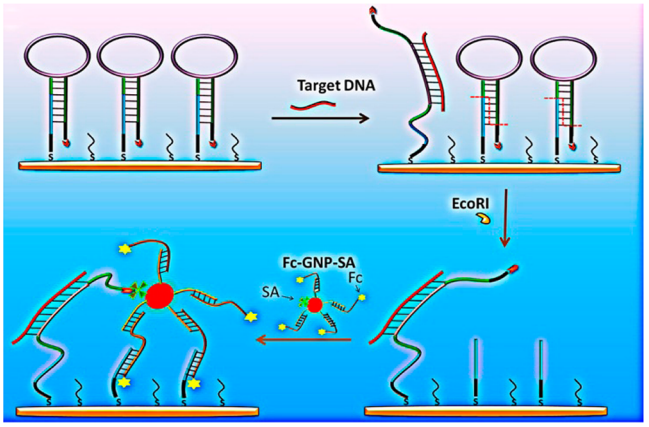

Figure 3. Design of the cooperative amplification-based electrochemical sensor for the zeptomole detection of DNA. Reprinted from ref 113. Copyright 2013 American Chemical Society.

The presence of nonmatched probe reduces the cross-reaction between target DNA and matched probe on the Au NPs, resulting in increased sensitivity of the sandwich-type DNA biosensor.[114]

The newly developed nanomaterials can act as electroactive tracers for signal amplification by numerous signal species directly from themselves. Combined with effective methods for determination of nanotracers, ultrasensitive electrochemical DNA-based assays have been easily developed. Liu and co-workers presented a novel strategy for simultaneous detection of multiple DNA targets based on the use of different encoding metal ions as tags. Metal ions bound to metallothionein molecules can be released after hybridization with DNA targets and then detected by stripping voltammetry.[115] Remarkable electrochemical properties of QD barcodes were also used as enhancing species to improve the signal. A novel dendritic QD nanocluster was constructed and used as versatile electrochemiluminescence (ECL) and electrochemical probes for the detection of DNA and cancer cells.[116] Dai and co-workers combined the high base-mismatch selectivity of the ligase chain reaction and the remarkable voltammetric properties of QD barcodes and provided the feasibility of sensitive multiplexed miRNA analysis detected by square wave voltammetry.[117]

Nanomaterials with enzyme-like characteristics were also used as a new method for signal amplification in genosensors. Huang and co-workers fabricated a sensitive gap-electrical biosensor based on self-catalytic growth of unmodified Au NPs as conductive bridges for amplifying DNA hybridization events.[118] In the presence of target DNA, the obtained dsDNA product cannot adsorb onto the surface of Au NPs due to the electrostatic interaction, which makes the unmodified Au NPs exhibit excellent GOx-like catalytic activity. Such catalytic activity can enlarge the diameters of Au NPs in the glucose and $HAuCl_4$ solution and result in a connection between most of the Au NPs and a conductive gold film formation with a dramatically increased conductance. On the contrary, the catalytic activity sites of Au NPs are fully blocked by ssDNA due to the noncovalent interaction between nucleotide bases and Au NPs. It is of great significance to explore the interaction between functional materials with electrochemical probes in a genosensor system, which provides wide opportunities to develop a novel sensing platform. Cui and co-workers found that the ECL of ruthenium(II) complex functionalized GO (Ru−GO) could be effectively quenched by

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Fc−ssDNA absorbed on the Ru−GO nanosheets. The Ru−GO has good discrimination ability over ssDNA and dsDNA. The mutant ssDNA target responsible for the drug resistant tuberculosis can hybridize with Fc−ssDNA and release Fc−ssDNA from the Ru−GO surface, leading to the recovery of ECL.[119] Zhang and co-workers demonstrated that the oxygen groups at the surface of CNTs together with the intrinsic electron properties of CNTs were the major reason for the suppression of ECL of $Ru(bpy)_3^{2+}$.[120] Utilizing this essential quenching mechanism, a new signal-on DNA hybridization assay has been proposed on the basis of the CNTs modified electrode, where the ssDNA labeled with $Ru(bpy)_3^{2+}$ derivatives probe (Ru-ssDNA) at the distal end was covalently attached onto the CNTs electrode. The quenched ECL signal returns in the case of the presence of complementary ssDNA. Xu and co-workers constructed a sensitive DNA biosensor based on ECL resonance energy transfer between RuSi@$Ru(bpy)_3^{2+}$ and Au@Ag_2S NPs.[121] According to the interaction between Au NPs and DNA immobilized on an electrode surface, Li and co-workers developed a novel DNA sensing method based on the ultrahigh charge-transfer efficiency of Au NPs.[122] Moreover, Bonanni and co-workers used MoS_2 nanoflakes as inherently electroactive labels to design a DNA sensor based on the differential affinity of MoS_2 nanoflakes toward ssDNA and dsDNA.[123]

Other Approaches for Signal Amplification. Zuo and co-workers demonstrated an ultrasensitive detection platform for miRNA by combining hybridization chain reaction (HCR) amplification and the tetrahedral DNA nanostructure probes.[124] Among them, 3D tetrahedral DNA nanostructure was the scaffold to immobilize DNA recognition probes to increase the reactivity and accessibility, while DNA nanowire tentacles are used for efficient signal amplification by capturing multiple catalytic enzymes in a highly ordered way. The synergetic effect of the DNA tetrahedron and nanowire tentacles has proven to greatly improve sensitivity for both DNA and miRNA detection. In fact, most of the reports on genosensors involve multiple magnification approaches mentioned above.Tang and co-workers combined HCR amplification with silver nanotags to electrochemically monitor nucleic acids with high sensitivity.[125] Due to the target-triggered long-range self-assembled DNA nanostructures and HCR, numerous silver nanotags were directly immobilized onto the long-range DNA nanostructures without the need of silver enhancement substrates and bioactive enzymes, each of which produces a strong electronic signal within the applied potentials. Under optimal conditions, the target-triggered long-range DNA nanostructures presented good electrochemical behavior for the detection of human immunodeficiency virus DNA at a concentration as low as 0.5 fM.

New Methods for DNA Detection. Nanopore analysis has emerged as the simplest single-molecule technique. Various ssDNA with similar lengths can slide through a-hemolysin (a-HL)-based protein nanopore at a bias voltage and lead to an indistinguishable signal for DNA detection. Kang et al. combined the DNA probe technique with nanopore detection and developed a new nanopore DNA biosensor.[126] The DNA sensor relies on the hybridization reaction between the short HBV target strand and deliberately designed DNA probes. It was demonstrated that the target HBV DNA could be detected with high sensitivity and selectivity. Chang and co-workers demonstrated an ion-exchange nanomembrane sensor for detection of DNA/RNA using the charge inversion phenomenon when negatively charged nucleic acids assemble on the surface of the positively charged membrane.[127] Changes in current−voltage characteristics were used to identify and quantify targets that hybridize with specific complementary probes covalently functionalized on the membrane surface. Furthermore, the fabricated sensor is specific and able to distinguish two base mismatches in the target sequence as well as capable of capturing and recording the target sequence from a heterogeneous mixture.

Electrochemical Detection of DNA Damage. DNA damage occurs frequently in organisms. Some endogenous and exogenous chemicals have been found to induce structural damages to nuclear DNA by base oxidation or modification. If unrepaired, these damaged DNA may lead to gene mutation and even tumor generation. Electrochemical genosensors are well qualified for the rapid screening of industrial and environmental chemicals for their potential geno-toxicity.[128−130] Specific types of sensors for the identification and quantification of DNA damage products such as methylated DNA bases were addressed in these two years.

DNA aberrant methylation represses gene transcription, deregulates gene expression, and causes various human diseases. Hence, detecting the DNA aberrant methylation level benefits the early diagnosis of some tumors and the epigenetic therapy for DNA methylation-related diseases. Ai's group developed a series of electrochemical methods for DNA methylation detection.[131,132] For example, they constructed a photoelectrochemical immunosensor to assay DNA methylation, where Bi_2S_3 nanorods were used as photoelectric conversion material. In this system, the methyl-CpG-binding domain (MBD) proteins were captured on the electrode surface through the specific interaction between MBD protein and symmetrical cytosine methylation in the CpG region of dsDNA. Then, an antihis tag antibody was captured to further inhibit the photocurrent and increase the detection sensitivity through the immunoreactions. This Bi_2S_3-based photoelectrochemical sensor possessed excellent photoelectron property and presented high detection specificity, even distinguishing single-base mismatched sequences.[132]

Xie and co-workers reported the highly sensitive detection of DNA methylation, methyltransferase activity, and inhibitor screening based on DNA-Au NPs signal amplification.[133] DNA hybrid methylated by DNA adenine methylation MTase can not be cleaved by *Mbo*I endounuclease and loaded more intercalated MB. It should be noted that MB was employed as an electrochemical indicator and DNA-modified Au NPs were used as a signal amplification unit because the DNA strands in this composite have strong adsorption ability for MB. On the basis of this principle, DNA methylation could be determined on the basis of the voltammetric signal change of MB.

■ IMMUNOSENSORS

Immunoassays are the detection platforms based on specific antigen−antibody recognition. They are well established standard biodetection methods used in clinical laboratories for disease diagnosis, in the food industry for food safety testing, and in monitoring environmental contamination.[134−138] Among immunosensors, electrochemical immunosensors are attractive and have received considerable attention because of their great features of being easily used and economical in mass production; having an excellent LOD with a small volume of samples; and being a paper-based simple analytical platform.

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Two recent review articles summarize the recent trends of electrochemical immunosensors toward POC diagnostics.[139,140]

Voltammetry and Amperometry-Based Immunoassay. Voltammetry and amperometry, such as linear sweep, differential pulse, square-wave, and stripping, are the most widely used electrochemical methods for immunoassay. Most of the strategies discussed below employ the sandwich immunoassay approach, in which the target antigen (Ag) is captured by its specific antibody (Ab_1) and detected by labeled secondary antibody (Ab_2).

Biomarkers and Bacteria Detection. The development of reliable, cost-effective, powerful detection, and monitoring strategies for cancer diagnosis is particularly important, due to the disease's prevalence, high rates of recurrence, and potential lethality.[141,142] Zhang et al.[143] designed a new anodic-stripping voltammetric immunoassay protocol for the detection of IgG, by using CdS QDs LBL assembled hollow microspheres as molecular tags. In a sandwich-type immunoassay format, subsequent anodic-stripping voltammetric detection of cadmium released under acidic conditions from the coupled QDs was conducted at an in situ prepared mercury film electrode. The new immunoassay is promising for enzyme-free and cost-effective analysis of low-abundance biomarkers. Singh et al.[144] presented a simple immunosensing scheme in which the incubation period was minimized without a large increase in the detection limit. This scheme was based on electrochemical-enzymatic redox cycling using GOx as an enzyme label, $Ru(NH_3)_6^{3+}$ as a redox mediator, and glucose as an enzyme substrate. Fast electron mediation of $Ru(NH_3)_6^{3+}$ between the electrode and the GOx label attached to the electrode allows high signal amplification. Benefiting from this, the detection limit for carbonhydrate antigen 125 (CA 125) was slightly higher than 0.1 U/mL.

Ren et al.[145] described an electrochemical biogate for a highly sensitive homogeneous electrochemical immunoassay by combining target-induced proximity hybridization with a mesoporous silica nanoprobe. The electroactive MB was sealed in the inner pores of MSN with single-stranded DNA. More importantly, the in situ recycling of the proximate complex could be achieved with nicking endonuclease Nt.BbvCI to open more DNA biogates for release of more MB, thus amplifying the electrochemical signal. The proposed assay showed a wide detection range from 0.002 to 100 ng mL^{-1} with a detection limit of 1.3 pg mL^{-1} for prostate-specific antigen.

Parshetti et al.[146] fabricated an ultrasensitive sandwich-type amperometric immunosensor for the detection of alpha-fetoprotein (AFP). Au/CHIT modified GCE and antibody-functionalized dumbbell-like Au−Fe_3O_4 heterostructures were used as the sensing platform and immuno-labels, respectively. The authors showed that the GCE modified with CHIT produced a high electrochemical response through the conjugation of more Au−Ab_1 and the dumbbell-like Au−Fe_3O_4 which served as a dual-probe to immobilize Ab_2 onto Au, as well as to reduce H_2O_2 by Fe_3O_4. That enhanced signal amplification.

Eletxigerra et al.[147] reported an electrochemical magneto-immunosensor for detecting a biomarker of tumor necrosis factor alpha (TNFα). The sensor was constructed by using magnetic microbeads and disposable screen-printed carbon electrodes, with the addition of hydroquinone as the electron transfer mediator and H_2O_2 as the enzyme substrate. After a thorough optimization of the assay, extremely low limits of detection were achieved: 2.0 pg mL^{-1} (36 fM) and 5.8 pg mL^{-1} (105 fM) for standard solutions and spiked human serum, respectively.

Bhimji et al.[148] developed the first electrochemical enzyme-linked immunosorbent assay to detect human immunodeficiency virus-1 (HIV-1) and HIV-2 in clinical samples. Excellent performance relative to a commercial gold standard test was obtained, which was based on the surface functionalization of SU-8 and oxidation current of *p*-aminophenol. Because of the heterogeneous nature of the assay, there is no interference by electroactive substances or electrode fouling.

The electrochemical immunoassay has also been used in other fields besides cancer biomarkers.[149] Grewal et al.[150] presented a method for label-free electrochemical detection of a protein from the enteric pathogen *Entamoeba histolytica* using cell-free yeast embedded antibody-like fragments (yeast-scFv) as novel affinity agents. The key architectural improvements were made, including: (i) avoiding use of secondary antibodies and (ii) utilizing yeast-scFv cell membrane fragments.

Tlili et al.[151] integrated two complementary detection strategies for the identification and quantification of *Escherichia coli* based on bacteriophage T4 as a natural bioreceptor for living bacteria cells. One involves screening and viability assays, employing bacteriophage as the recognition element in label-free electrochemical impedance spectroscopy. The other approach is a confirmation by loop-mediated isothermal amplification to amplify specifically the *E. coli* Tuf gene after lysis of the bound *E. coli* cells, followed by detection using linear sweep voltammetry. In another study,[152] biotinylated full antibody-based immunosensors have been optimized to enable the specific detection of pathogenic bacteria *S. pyogenes* in human saliva. Electrodeposited polytyramine was used as a base layer for the conjugation of biotinyl antibodies via a biotin-Neutr Avidin bridge. The impedance-based electrochemical immunosensor showed a linear response (100 cells/10 μL to 105 cells/10 μL) against *S. pyogenes* in cumulative incubation and 100 cells/10 μL to 104 cells/10 μL in single-shot incubation.

Our group[153] proposed a new approach for detecting/screening OPs poisons by simultaneously providing the results of dual biomarkers of both enzyme inhibition and enzyme adducts (Figure 4). Simultaneously, AChE enzyme activity of postexposure is also determined. The high detection sensitivity stems from enrichment of the electroactive product, thiocholine, on the surface of Fe_3O_4/Au nanocomposites followed by electrochemical oxidative desorption of thiocholine. In another work, we[154] presented the first report on the development of the Fe_3O_4 at TiO_2 magnetic NPs-based disposable electrochemical immunosensor with quantum dot-linked antibodies for sensitive and selective detection of the OP-butyrylcholinesterase adduct in human plasma. Fe_3O_4 at TiO_2 magnetic NPs not only selectively capture phosphorylated adduct by metal chelation but also directly separate it from biological matrices by simply exerting an external magnetic field.

Two new electrode functionalization strategies were developed by Prieto-Simón et al.[155] The first strategy relied on hydrazide-phenyl diazonium salts that were electrografted onto the gold electrode surface. The second strategy involved the use of mono- and dithiolated self-assembled monolayers carrying hydrazide functional groups. The immunosensors based on either a direct assay using electrochemical impedance spectroscopy or a sandwich-assay using differential pulse voltammetry for MS_2 phage detection were investigated. Their results showed that both immobilization protocols

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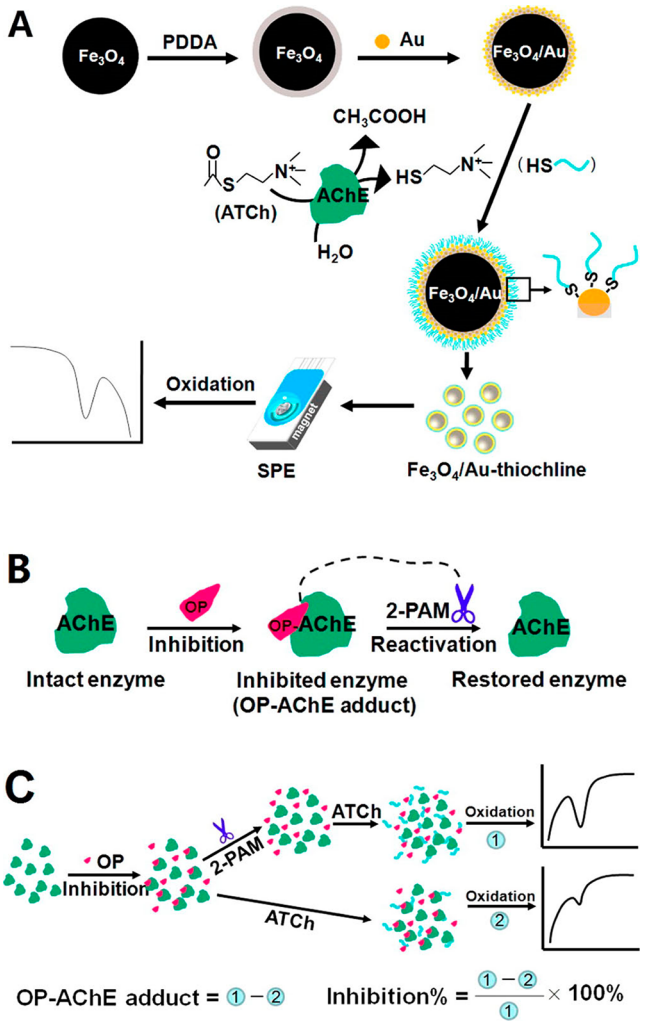

Figure 4. (A) Schematic illustration of the preparation of core−shell Fe_3O_4/Au magnetic nanocomposites and the electrochemical oxidative desorption process of thiocholine. (B) Schematic illustration of the measurement of AChE activity via the reactivation from an OP-exposed sample. (C) Parallel measurement of AChE activity in a postexposure sample with and without reactivation. Reprinted from ref 153. Copyright 2013 American Chemical Society.

efficiently controlled the orientation of antibodies and the permeability of the electroactive species in solution, resulting in strategies that can be easily tailored to prepare highly sensitive electrochemical immunosensors.

Amaya-González et al.[156] reported a highly sensitive approach for gluten analysis using aptamers as specific receptors, with the successful selection of aptamers for these water insoluble prolamins that was achieved choosing the immunodominant apolar peptide from α2-gliadin as a target for selection. The excellent features of the biosensors make the proposed method a valuable tool for gluten detection in foods.

Graphene. The combination of nanomaterials and immunosensors shows great potential for monitoring biomolecules and sensitive detection of target analytes. Nanomaterials can be used as carriers to load signal markers or directly as signal reporters for sensitively detecting biomarkers, and they can accelerate electron transfer when they are used as functional materials on electrode surfaces.[157,158] Nowadays, nanomaterials such as carbon materials,[159,160] colloidal nanocrystals (e.g., magnetic NPs, metal NPs, QDs), and other functional materials[161−164] are being developed to increase the sensitivity of the electrochemical detection of targets.

Graphene plays an important role in recent trends for immunosensors fabrication. Immobilization of the bioactive species is crucial for proper detection. Graphene offers an easy way to protect and stabilize these species. In addition to other nanomaterials, graphene-based immunosensors have exhibited good analytical characteristics and shown great promise for clinical applications. Recently, Wu et al.[165] introduced different approaches for the fabrication of graphene and the preparation of graphene-modified electrodes for electrochemical sensors (119 citations). Zhu et al.[166] provided an overview of electrochemical biosensing with graphene related materials and discussed the role of graphene in different sensing protocols (123 citations). For recent examples, Lin et al.[167] developed an electrochemical immunosensor for the detection of a cancer biomarker protein in serum at a low concentration with excellent signal-to-noise ratio. This reusable biosensor utilized a magnetic GO-modified gold electrode as the detection substrate. The fabrication method allows for reproducibility, ease of production and use, and storage stability enabling potential clinical use for rapid vascular endothelial growth factor detection. Gao et al.[168] constructed a sensitive sandwich-type immunosensor for detecting alpha-fetoprotein. Poly(diallyldimethylammonium chloride) (PDDA) protected PB/Au NPs/IL functionalized rGO (IL-rGO-Au-PDDA-PB) nanocomposite was fabricated and used as a signal amplification label to enhance the electrochemical response. Lu et al.[169] reported a novel, label-free, and inherent electroactive redox biosensor based on ultrathin Au−Pt nanowire-decorated thionine/rGO (AuPtNWs/THI/rGO). The AuPt NWs/THI/rGO composites not only favor the immobilization of antibody but also facilitate the electron transfer. It is found that the resultant AuPtNWs/THI/rGO composites can be designed to act as a sensitive label-free electrochemical immunosensor for the detection of carcinoembryonic-antigen.

Moreover, in recent years, the study of the graphene-based derivative, such as heteroatom-doped graphene,[170] GO,[171] and graphene nanoribbon,[172] has been popular and extensive, particularly with respect to electrochemical applications. Zhu's group[173] reported an approach to use the exceptional properties of the NG-based composite for the fabrication of an ultrasensitive electrochemical immunosensor based on a signal amplification strategy for the detection of matrix metalloproteinase-2. The design of the immunosensor also involved a polydopamine functionalized GO hybrid conjugated to horseradish peroxidase-secondary antibodies by covalent bonds as a multilabeled and biocompatible probe to increase the electrochemical response.

Other Nanomaterials. The biomolecule−nanomaterials hybrid system has excellent prospects for interfacing biological recognition events with electronic signal transduction so as to design a new generation of bioelectronic devices with high sensitivity.[161−164] Dendrimers have good conductivity and large potential windows, which are electrochemically important and make them novel solvent (electrolyte) systems, holding great promise in many studies of electrochemical biosensing.[174] Recently, a highly sensitive electrochemical immunosensor based on dendrimers/Au NPs as a sensor platform and MWCNT-supported multiple bienzymes (Ab_2/MWCNT/GOx/HRP) as labels was developed by Jeong's group for the detection of carcinoembryonic-antigen (CEA) The linear dynamic range of the proposed immunosensor covered a five-

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order wide concentration range in which CEA detection can be made without dilution. The LOD of the proposed CEA immunosensor was much lower than that of the conventional ELISA method.[175]

Li et al.[176] synthesized a novel ionic liquid, 4-amino-1-(3-mercapto-propyl)-pyridine hexafluorophosphate (AMPPH), which was used as a functional monomer to fabricate AMPPH-modified Au NPs (AMPPH-AuNPs) via a one-pot synthesis method. Rabbit antihuman IgG was immobilized onto the nanointerface based on AMPPH-Au NPs and used for human IgG immuosensing. The results indicate that AMPPH-Au NPs improved the immunosensing performance. Lou et al.[177] proposed a novel competitive electrochemical immunosensor by combining the electrochemical reduced GO-AuPd NPs platform with a Ag NPs functionalized polystyrene bionanolabel for the sensitive detection of human interleukin-6. In the meantime, they also introduced an electrically heated carbon electrode in the detection procedure of the immunosensor and further improved the sensitivity. An ultrasensitive immunoassay method based on the electrochemical measurement of PAni, which was catalytically produced by an HRP-functionalized Au NPs (HRP-Au NPs) probe at an immunosensor was developed by Lai et al. After performing a sandwich immunoreaction, the quantitatively captured HRP-Au NP nanoprobes could catalyze oxidation of aniline to produce electroactive PAni on the immunosensor surface. The electrochemical measurement of PAni enabled a novel detection strategy for the HRP-based immunoassay.[178]

Electrochemiluminescence. In the past two years, ECL has received much attention and become an important detection method.[179,180] Wang et al.[181] synthesized a novel functionalized material using surface-decorated fullerene to encapsulate hollow and porous palladium nanocages. The resultant functionalized material has a high specific surface area, good electrocatalytic ability, and efficient photocatalytic activity and has been used to construct an ECL immunosensor for the detection of *Streptococcus suis* Serotype 2. A wide linear detection range of 0.1 pg mL^{-1} to 100 ng mL^{-1} is acquired with a relatively low detection limit of 33.3 fg mL^{-1}. Qi et al.[182] reported an ultrasensitive ECL peptide-based method for the determination of cardiac troponin I, incorporating amplification of signal reagent-encapsulated liposome. The principle was based on the idea of encompassing heavy labels in larger carriers and on polyvalent binding motifs, employing the Ru1-encapsulated liposome peptides and the magnetic capture peptides. Ju's group[183] prepared a hemin functionalized graphene sheet via the noncovalent assembly of hemin on nitrogen-doped graphene that acts as an oxygen reduction catalyst to produce sensitive ECL quenching of quantum dots through the annihilation of dissolved oxygen, the ECL coreactant, by its electrocatalytic reduction. With the use of the catalyst with high loading of hemin as a signal tag of the secondary antibody, a novel ultrasensitive immunoassay for the carcinoembryonic antigen detection was demonstrated.

Photoelectrochemistry. Photoelectrochemistry is a newly developed analytical method and now attracts substantial research scrutiny in various fields.[81,184,185] Tian et al.[186] developed a photoelectrochemical immunosensor by incorporating graphene quantum dots (GQDs) and highly oriented silicon nanowires (SiNWs) for the determination of microcystin-LR in water samples. The GQDs/SiNWs heterostructure was employed for signal transduction and a biocompatible nanoscaffold for antibody immobilization. Zhang et al.[187] developed a new photoelectrochemical biosensor for ultrasensitive detection of monoclonal antibodies anti-Tn, which was used against the breast tumor-associated carbohydrate antigen Tn. The detection sensitivity of 1.0×10^{-13} g/mL was achieved. It should be noted that the CdSe QDs acted as both photosensitizer and an alternative multivalent form for carbohydrate antigen with high binding affinity. This design would facilitate testing for disease-related sugar markers as well as evaluate the immunogenic properties of carbohydrate vaccine candidates.

Other Papers of Interest. To achieve clinical or POC use, multiplexed electrochemical target detection has been investigated intensively in the last two years.[188−191] Rusling[192] reviewed multiplexed electrochemical protein detection and that of translation to personalized cancer diagnostics (60 citiations).

Wu et al.[193] reported the combination of a signal amplification strategy with a microfluidic paper-based analytical device for the quantitative analysis of four kinds of cancer biomarkers as model analytes, namely, alpha-fetoprotein, carcinoembryonic antigen, cancer antigen 125, and carbohydrate antigen 153. Signal amplification was achieved through graphene modification of the immunodevice surface to accelerate the electron transfer and also the use of silica NPs as a tracing tag to label the signal antibodies. Using the horseradish peroxidase-O-phenylenediamine-H_2O_2 electrochemical detection system, the potential clinical applicability of this biosensor was demonstrated in the detection of four candidate cancer biomarkers in serum samples from cancer patients.

Yang et al.[194] reported the design of a low-cost, portable intelligent microscale electrochemical device that can automatically deliver multiple reagents in a controlled manner. The successful adaption of a bubble-based cartridge to the screen printed electrode system leads to automatic and rapid sample delivery at the electrode surface in one step with minimal user intervention. They have performed sensitive and selective detections of several biological targets, including tumor biomarkers and H1N1 split influenza vaccine.

Inorganic−organic (poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate)) heterostructured light-emitting diodes (LEDs) based on ZnO nanorods were presented by Zhang et al. As a proof-of-concept, an advanced multiplexed photoelectrochemical immunosensor array was fabricated using the prepared LEDs as an excitation light source, and excellent performance for the detection of three different cancer biomarkers was achieved.[195]

Kong et al.[196] proposed a branched electrode platform for label-free, reagentless, and simultaneous tumor markers detection based on MB and PB, two different redox substrates. The branched sensing electrode fabricated via photolithography on an ITO electrode consists of two separate circular areas (2 mm in diameter, named as W1 and W2), which joined to a rectangular area for the electrical contact. By equipping each branched sensing pad with different redox active substrates and different antibodies, the sensor can simultaneously respond to multiple targets in the sample.

Liu et al.[197] reported on a protocol for a simultaneous competitive immunoassay for tetracycline (TC) and chloramphenicol (CAP) on the same sensing interface. Monoclonal anti-TC and anti-CAP antibodies were conjugated onto CdS and PbS nanoclusters, respectively. Cd (II) and Pb (II) ions were released from the surface of the corresponding nano-

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clusters by treatment with acid and detected by square wave anodic stripping voltammetry.

There has been substantial progress in the development of electrochemical immunosensors; however, a major challenge for electrochemical sensors is still simultaneous detection of multiple targets in complex biological samples with reliability, portability, low cost, rapid response, and excellent selectivity and sensitivity. Hence, the integration of electrochemical immunoassays into a disposable format will have great potential in the applications of clinical diagnostics, particularly for POC.

CYTOSENSORS

Considering the vital role of cells in life science and human health, cell-related bioassaying has become a hot research topic within the past decades.[198−203] Thanks to the development of nanotechnology, many kinds of novel nanomaterials have emerged to anchor recognition units such as antibody, aptamer, and receptors which can specifically and effectively capture cells, particularly cancer cells through binding the abnormal and overexpressed components such as proteins, glycans, and receptors on the surfaces of cancer cells based on "target-binding" technology. Developing highly sensitive cytosensors will have a great impact in health care. Notably, electrochemical cytosensing approaches play a more and more important role in the analysis and detection of target cells due to the inherent advantages such as miniaturization, easy operation, rapid response, satisfied sensitivity, high selectivity, affordability, and real-time and nondestructive analysis.[204−208] Recent advances on electrochemical cytosensors have been investigated in the detection of cell type and number, cellular physiological parameters, and crucial molecules on the cell surface or intracellular, pharmaceutical evaluation and screening. Different electrochemical methods have been used in the investigation, including common electrochemical methods, the ECL method, and the photoelectrochemical method in label-free or sandwich assays.[209−213]

Label-Free Cytosensing. For label-free electrochemical cytosensing, the key is to fabricate a biocompatible recognition interface onto the electrode, coupled with highly sensitive read-out electrical signal to report the cell-recognition events with the help of biofunctionalized nanomaterials. Jia's group reported an ultrasensitive electrochemical cytosensor for quantification of carcinoembryonic antigen (CEA)-positive BXPC-3 cells.[214] 3D architecture Au@BSA microspheres were prepared via a convenient and "green" synthesis route. These microspheres were employed to develop the sensing layer with the conjugation of monoclonal anti-CEA antibody (anti-CEA). With its excellent conductivity, stability, and biocompatibility, the 3D architectural Au@BSA microspheres were used to develop a label-free cytosensor. Highly specific detection of BXPC-3 cells with a broader detection range and a detection limit of 18 cells mL^{-1} has been achieved. Small molecules are also used for specific recognition of cells. An effective cytosensor using carboxymethyl CHIT-functionalized graphene (CMG-G) has been reported.[209] The electrochemical cytosensor was fabricated and functionalized with biocompatible CMC-G and a small molecule, folic acid. Electrochemical impedance spectroscopy (EIS) detection results have showed that the detection limit for HL-60 cells was achieved at 500 cells per mL. In addition, aptamers can be useful molecular probes for cell detection. Quantitative determination of human colon cancer DLD-1 cells were performed by an electrochemical aptasensor.[215] An effective biosensing interface between the MUC-1 aptamer anchored on biocompatible carbon nanospheres and Mucin 1 glycoprotein overexpressed on DLD-1 cells was investigated. Carbon nanospheres not only accelerated electron transfer but also supplied a highly stable matrix for the efficient immobilization of target MUC-1 aptamer, considerably amplifying the electrochemical signals and resulting in sensitive detection performance toward DLD-1 cells. In order to increase the selectivity and sensitivity of cytosensors, Peng's group employed a dual-aptamer recognition strategy for highly sensitive and specific detection of MEAR cells.[216] Two types of cell-specific aptamers, ss-TLC1c and ds-TLS11a, offered a unique nanobiointerface for cancer cell detection. The developed electrochemical cytosensor showed great reliable performance with satisfied sensitivity and specificity in detecting single MEAR cancer cells in 10^9 blood cells from a WBC sample. Real-time intracellular sensing is of great significance for advancing fundamental biological and clinical science. Competition strategy is another choice adopted for developing electrochemical cytosensors. It has been reported that ultrasensitive electrochemical detection of leukemia cells achieved a detection limit down to 10 cells.[217] The aptamer−Fe_3O_4 MNP/cDNA−Au NPs nanoprobes were employed to complete the competitive binding with leukemia cells. Due to a stronger binding affinity between the aptamer and targeting leukemia cells, the Fe_3O_4 MNP-bound aptamers preferred to form the aptamer−targeting cell complex and then dissociated cDNA−AuNPs nanoconjugates in the presence of CCRF-CEM cells. Thereafter, the residual Au NPs on the nanoprobes will perform Au NP-catalyzed silver deposition for amplified signal read-out. Pioneer work has been done by presenting a direct interface of vertically aligned single-walled carbon nanotubes (VASWCNTs) with eukaryotic cells (RAW 264.7 mouse macrophage cell line) for electrochemical study in an intracellular environment.[218] Combining the features of excellent high aspect ratios, efficient electron transport, and superior electrical conductivity, VASWCNTs were anchored onto an ITO substrate and then further wrapped by DNA, which will enter mouse macrophage cells through endocytosis. This allows real time monitoring intracellular events. Owing to the advantage of multiplexing ability, high through-output performance, and low reagent consumption, microfluidic devices were used to study the on-site real-time assay of the proliferation and apoptosis of HeLa cells.[219]

By virtue of its controllability and low background, ECL detection was widely used in cytosensing. A label-free ECL cytosensor was developed for specific determination of early apoptosis.[220] Assembled L-cysteine-capped CdS-QDs/PAni nanofibers (PAni-NF) were used for the stable and high loading immobilization of Annexin V, which recognize apoptotic cells through the interaction between Annexin V and PS exposed on the cell membrane during the cell apoptosis process. That resulted in a steric effect on the interaction between sensor and coreactants. More captured cells would lead to a lower ECL signal. A therapeutical effect could be evaluated by quantifying an early apoptotic HepG2 cell induced by resveratrol. The results show that the label-free ECL cytosensor holds a great potential for rapid detection of cell apoptosis and drug screening. The photoelectrochemical assay is a new and promising analytical approach for cytosensing. Low-toxic Ag_2S quantum dots were studied in the photo-electrochemical detecton of cancer cells.[213] As MCF-7 cells overexpress sialic acid (SA) on their membrane, boronic acid units were anchored onto a Ag_2S quantum dots-based

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photoelectric interface that captures MCF-7 cells by virtue of the interaction between boronic acid and the terminal SA moiety on the membranes of MCF-7 cells. The photocurrent decreased significantly after capturing MCF-7 cells. Because of the diffusion of the sacrificial electron donor to the surface of the electrode, the electron transfer after the immobilization of the cell on the electrode surface was blocked and resulted in the decrease of photocurrent.

Sandwich Cytosensing. The performance of sandwich cytosensing relies on the recognition probes specifically capturing the target cells and the signal probes specifically binding to captured target cells. Therefore, the key to fabricate high sensitive cytosensors is to develop high-performance recognition probes and signal probes, particularly, with the aid of emerging nanomaterials. Figure 5 illustrated a sandwich

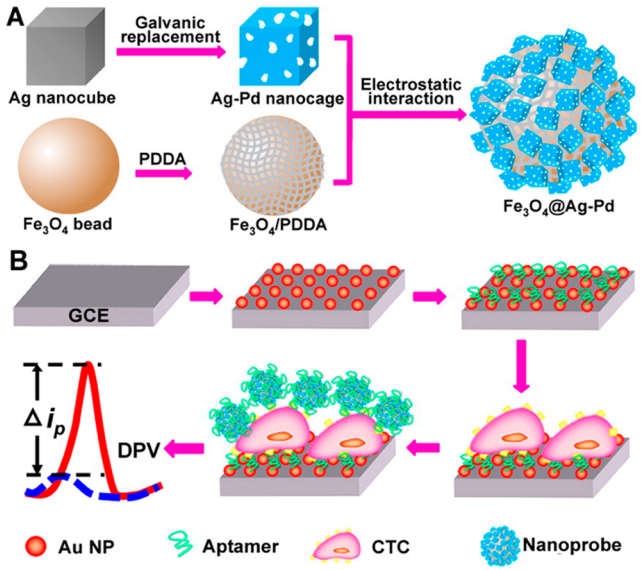

Figure 5. Schematic illustration of the fabrication of Fe_3O_4@Ag−Pd hybrid NPs (A) and the cytosensor assembly process (B). Reprinted from ref 205. Copyright 2014 American Chemical Society.

electrochemical cytosensor enhanced by robust nonenzymatic hybrid nanoelectrocatalysts.[205] A kind of Fe_3O_4@ Ag−Pd bimetallic nanocage core−satellite hybrid NP was found possessing significantly more robust electrocatalytic activities than the enzymatic peroxidase/H_2O_2 system for signal amplification in electrochemical cytosensing. The developed electrochemical cytosensor achieved detection limits of ∼4 MCF-7 and ∼5 T47D cells in a 1 mL sample. Cho's group reported an integrated multifunctional platform based on biotin-doped conducting polymer nanowires for cell electrochemical sensing.[221] Conductive disulfide-biotin doped polypyrrole nanowires with a well-ordered three-dimensional structure effectively immobilize streptavidin (SA) on the surface, and then, biotin labeled monoclonal antibodies were attached to SA. Circulating tumor cells (CTCs) were captured by antibodies on the sensing surface. HRP/antibody-conjugated NPs were employed as signal probes for quantification of CTCs. The detection range of CTCs is 10 to 1×10^4 cells, and a detection limit as low as 10 cells was reported. Zhu's group further reported a multiplex electrochemical cytosensing platform to simultaneously detect and classify both acute myeloid leukemia cells (AML) and acute lymphocytic leukemia cells (ALL).[222] In order to enhance the specificity of biointerection between recognition element and targeting cell, a multivalent recognition strategy has been proposed.[207] Poly(amidomine) dendrimer modified rGO offered a multivalent recognition interface for the immobilization of Concanavalin A (Con A), that significantly enhanced the cell capture efficiency and improved the sensitivity of the cytosensing for cell surface glycan. Two different aptamer-functionalized graphene-Au NPs were integrated as a recognition probe for capturing two different targeting cells. SBA-15 loaded with two kinds of redox-tags, HRP and cell-targeting aptamers, were used as probes for specifically capturing the targeting cells, amplifying the electrochemical signals, and retaining distinguishable signals for multiplex cytosensing. An ECL cytosensor was configured for dynamic evaluation of cell surface N-glycan expression.[223] Due to the specific recognition of Con A with mannose and the core trimannoside fragment of N-glycan, Con A as cell recognition element was immobilized onto carboxylic group functionalized MWCNTs. Con A was doped onto Au NP-modified Ru-$(bpy)_3^{2+}$-loaded silica nanoprobe serving as a signal probe for capturing mannose and N-glycan expressed on the K562 cell surface. There is an urgency to explore a portable and disposable device for cost-effective cytosensing. Yu's group made good contributions on developing lab-on-paper techniques for sensitive electrochemical or ECL detection of cells.[224,225] The constructed microfluidic paper-based portable analytical devices (μ-PADs or lab-on-paper) showed great promise in the detection of cells or monitoring cell-related activities, such as drug screening and other biological research.

CONCLUSIONS

Inherent sensitivity, simplicity, speed, and cost benefits continue to be strong driving forces for the development of electrochemical sensors and biosensors. In this Review, we have summarized remarkable advances in the development of novel ultrasensitive electrochemical assays based on nanomaterials and nanostructures.

There have been thousands of sensor papers published during the past two years, where electrochemical sensors represent the most rapidly growing class. Compared to other methods, such as spectroscopy and chromatography, the electrochemical measurements are much cheaper and simpler and easier to miniaturize, which makes them more suitable for POC detection, particularly for delivering benefits for resource-limited areas in both developed and developing countries. Besides that, a wide variety of strategies are used to improve the efficacy of sensing. Signal amplification for detection to utilize NPs as carriers or tracers, catalysts, and electronic conductors and produce a synergic effect among catalytic activity, conductivity, and biocompatibility has been achieved.

New developments in nanotechnology and material science as well as in custom engineering of biorecognition components have advanced the progress of useful and reliable electrochemical sensors and biosensors. The materials and biomaterials with rich nanostructures not only improve the electronic properties and increase the effective electrode surface for transferring electrochemical signal but also produce detectable signals for indirect detection of targets. Thus, the resulting methods possess high sensitivity and good specificity. The synergy of multifunctional materials, recognition elements, and electrochemical methods is improving the selectivity, stability, and reproducibility, thus promoting the development of sensors for assays and bioassays.

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Significant advances have been made in several areas related to the design and application of electrochemical sensors and biosensors. However, there is still much effort needed to implement these ultrasensitive sensors and biosensors for real-world applications. The integration of electrochemical sensors into (paper-based) microfluidic formats with the incorporation of unique materials for detection needs to be extensively explored in the future. The development of these systems would also lead to significant advantages compared to the current analytic systems, in terms of simplicity, speediness, cost, and automation. We envision that a sensing device that can simultaneously monitor the levels of cell, DNA, RNA, protein, and small molecule-related markers in a single miniaturized and user-friendly format will offer the promise of practical applications.

AUTHOR INFORMATION

Corresponding Author

*E-mail: yuehe.lin@wsu.edu. Tel: +1 509 335 8523.

Author Contributions

§C.Z., G.Y., and H.L. contributed equally.

Notes

The authors declare no competing financial interest.

Biographies

Chengzhou Zhu graduated with a BS degree in chemistry from Shandong Normal University (P. R. China) in 2007. He joined the group of Prof. Shaojun Dong at the Changchun Institute of Applied Chemistry and received his Ph.D. degree in January 2013. Since then, he has done postdoctoral work with Prof. Alexander Eychmüller supported by the Alexander von Humboldt Foundation in Dresden University of Technology. Currently, he is a postdoctoral research associate in the School of Mechanical and Materials Engineering at Washington State University under the supervision of Prof. Yuehe Lin. He has coauthored over 48 peer-reviewed publications. His scientific interests focus on carbon and metal nanomaterials for electrochemical applications.

Guohai Yang received his B.S. degree from Lanzhou University in China in 2008. He earned his Ph.D. degree in chemistry under the supervision of Prof. Junjie Zhu at Nanjing University in 2013. Currently, Dr. Yang is working as the postdoctoral fellow with Prof. Yuehe Lin in the School of Mechanical and Materials Engineering at Washington State University. His research interests mainly focus on the development of electrochemical sensor arrays based on functional materials.

He Li is a professor at School of Biological Science and Technology in University of Jinan. He received his Ph.D. degree from Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences in 2004. In 2004, Dr. Li joined University of Jinan in China. He worked as a research associate conducting research in biochemical analysis and nanomedicine in Professor Yuehe Lin's group at Washington State University in 2014. His research interests are mainly focused on developing various functional nanomaterials for cancer diagnosis and therapy.

Dan Du received her Ph.D. in Analytical Chemistry from Nanjing University in 2005. She joined Central China Normal University in 2005 and was promoted to Full Professor in 2011. Currently, she is a Research Professor at Washington State University. Her research interests include nanomaterials for biosensing and drug delivery, immunosensors for early detection of biomarkers, and functional nanomaterials for bioanalysis. Dr. Du has published more than 100 papers, with ~2800 total citations and an *h*-index of 33.

Yuehe Lin is a professor at Washington State University and a Laboratory Fellow at Pacific Northwest National Laboratory. He has been actively working in the nanotechnology area, particularly in the development of new nanobioelectronic devices and nanomaterials for biomedical diagnosis and drug delivery. His other research activities include synthesizing functional nanomaterials for energy and environmental applications. Dr. Lin has published ~300 papers, with ~18 000 total citations and an *h*-index of 72.

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