

Recent advances in electrochemical glucose biosensors: a review

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Glucose detection is of great significance in biomedical applications. Principles, methods and recent developments in electrochemical glucose sensors are reviewed here. Special attention is given to the discussion on some problems and bottlenecks in areas of nonenzymatic and enzymatic (glucose oxidase-based) amperometric glucose sensing.

1. Introduction

Glucose detection is of great importance in a variety of fields ranging from biomedical applications to ecological approaches.¹ In clinical medicine, diabetes mellitus is one of the leading causes of death and disability in the world. This metabolic disorder results from insulin deficiency and hyperglycemia is reflected by blood glucose concentrations higher than the normal range of about 3.9–6.2 (empty stomach) or 3.9–7.8 (2 h after food) mM. The quantitative monitoring of blood glucose is of great clinical importance, which can greatly reduce the risks of diabetes mellitus-induced heart disease, kidney failure, or blindness.^{2,3} About 9,000 peer-reviewed articles relevant to glucose sensors have been recorded in the ISI web of knowledge. As shown in Fig. 1, the number of glucose sensor-relevant articles has maintained an increasing trend over the last 10 years. Electrochemical and optical methods have been extensively developed to monitor glucose. Obviously, photons are measured in optical methods, while electrons are measured in electrochemistry. Hence, the wireless characteristic of optical methods makes them very convenient for bioimaging and *in vivo* biosensing. Many optical methods, such as absorptiometry (and reflectometry), fluorescence, and surface plasmon resonance (SPR), are highly effective for glucose sensing, the readers may read relevant papers and reviews for details.^{4–11}

Electrochemical methods, especially amperometric methods, have been widely utilized in glucose sensing. The nonenzymatic sensing and the enzymatic biosensing of glucose are two main categories of glucose sensing which have been widely investigated and utilized. Amperometric nonenzymatic glucose sensors based on the direct electrochemical oxidation of glucose have triggered great interest and

have been widely exploited. The greatest advantage of amperometric nonenzymatic glucose sensing over enzymatic biosensing is that the former has addressed the problem of insufficient long-term stability, which is the most common and serious problem for enzymatic glucose sensors originating from the intrinsic nature of enzymes. A lot of metal materials, such as noble metals (*e.g.* Au and Pt) and their composites, have been widely exploited as electrode materials in nonenzymatic glucose sensing owing to their high electrocatalytic ability, high sensitivity and good selectivity to the electro-oxidation of glucose.^{12–20} The history of nonenzymatic glucose sensing can be traced back to the direct electro-oxidation of glucose to gluconic acid in a sulfuric acid solution at a lead anode reported by Walther Loeb in 1909. Studies of direct electro-oxidation²¹ and electroreduction²² of glucose in alka-



Fig. 1 The numbers of glucose sensor-relevant articles published in the last 10 years (data collected from ISI web of knowledge).

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line ($\text{pH} > 11$) and acidic ($\text{pH} < 1$) solutions continue to date.¹ Nevertheless, the adsorption of oxidation intermediates of glucose (e.g. CO) or active species in the solution (e.g. Cl^-) may notably block the electrode activity for direct electro-oxidation of glucose, which is one of the most serious problems for nonenzymatic glucose sensors.^{23,24} In addition, the amperometric nonenzymatic glucose sensors usually offer analytical selectivity inferior to the amperometric enzymatic glucose sensors because it is not easy for these electrocatalytic materials to be as specific as enzymes to catalyze the glucose oxidation.

Glucose oxidase (GOx)-based amperometric enzyme electrodes play a key role in the move to simple, easy-to-use blood glucose monitoring, since GOx is relatively low in price as well as having high bioactivity and stability.²⁵ The entire field of enzyme electrodes can trace its origin back to the enzyme electrode fabricated by Clark and Lyons in 1962. Clark's original patent of amperometric enzyme electrode (Clark, L., Jr., U.S. Patent 33,539,455, 1970) covers the conversion of electroinactive substrates to electroactive products with the utilization of enzymes. Biosensors taking enzymes as their molecular recognition components are endowed with many advantages, such as high selectivity and sensitivity. With the rapid development of biology, chemistry, physics, medicine, and electronic technology over the years, enzyme-based biosensing is becoming one of the most active research areas in analytical chemistry due to its incomparable advantages over other techniques. Biosensors have been extensively studied since the work of Clark, and today glucose biosensors account for about 85% of the entire biosensor market in the world, due mainly to the notable biomedical significance of the rapid and convenient assay of blood glucose.²⁶ The huge market size of glucose biosensors makes diabetes a model disease for developing new biosensing concepts. Important reviews on electrochemical glucose biosensing have been published recently. For instance, Wang has reviewed the principles of operation, history, recent developments, and current status of electrochemical glucose biosensors.²⁶ Heller *et al.* have reviewed the electrochemical glucose sensors and their applications in diabetes management.¹

Glucose dehydrogenase (GDH) is another kind of enzyme used in glucose biosensing and is also utilized in fabricating commercial test strips for blood glucose.^{27–30} GDH-based amperometric biosensors are advantageous in being able to be operated at lower detection potentials than the first-generation GOx-based sensors, and their performance is not influenced by the oxygen level in the analyte solution.³¹ Pyrroloquinoline quinone (PQQ)-dependent GDH (PQQGDH) and β -nicotinamide adenine dinucleotide (NAD)-dependent GDH are the two main types of GDH that have been applied in biosensors. PQQGDH is no longer looked upon as a candidate for physiological glucose determination because of the requirement of suitable detergents for solubilization and purification for membrane-bound PQQGDH, as well as the low selectivity and poor thermal stability of water-soluble PQQGDH.³⁰ For NAD-dependent GDH-based amperometric biosensors, the need for the addition of the soluble NAD cofactor complicates

the analysis system to some extent.^{25,31} Moreover, the electrochemistry of both the oxidized form (NAD^+) and the reduced form (NADH) of NAD cofactor suffer from their irreversible characteristic, and the direct oxidation of NADH at unmodified electrodes requires a high overpotential owing to its sluggish electron-transfer kinetics. In addition, the common requirement of artificial electron acceptors for electrochemical measurements also greatly restricts the wide application of GDH.³² All of these drawbacks result in less popularity of GDH compared with GOx utilized in glucose biosensing.³³

Isoenzyme 2 of hexokinase functions in sugar sensing and glucose repression in *Saccharomyces cerevisiae*.³⁴ Hexokinase-based glucose biosensing has been recognized as a reference method for blood glucose determination because of its ultrahigh specificity. Hexokinase is highly recommended to be used in automated analyzer and emergency tests and the relevant kinetic mechanisms have been extensively examined by researchers.^{34–36} But hexokinase was not so widely used in the research of glucose biosensing as GOx, probably because of its relatively higher price, lower stability, and the need for ATP in its enzymatic reaction.

A number of studies have also been carried out to find a less invasive means to monitor blood glucose levels.^{37–39} Mid-infrared emission spectroscopy,⁴⁰ the metabolic heat conformation method,⁴¹ and a GlucoWatch design based on electroosmotic flow of subcutaneous fluid to the surface of the skin and detection of glucose with an enzyme electrode,⁴² have been exploited to monitor blood glucose non-invasively or minimally invasively. It has also been suggested that tear fluid can serve as a substitute for blood and non-invasive glucose monitoring has the potential to revolutionize diagnosis of diabetes.^{43,44} The development in non-invasive glucose monitoring has been reviewed in 2008 and it is expected to make greater progress in the future.³⁹

Many reviews have been published on glucose sensing, but some important aspects on this topic have not been fully understood or not attracted enough attention. The great scientific and clinical importance of glucose sensors and the fast development of core and peripheral technologies also require continuous updating in their research statuses. This review mainly discusses the principles of electrochemical glucose sensors, including nonenzymatic glucose determination and GOx-based enzymatic glucose biosensing, their recent developments and current status, the major strategies for enhancing their performance, as well as the key challenges and opportunities in their further development and applications. Given the long history and broad field of electrochemical glucose biosensors, the authors apologize for the inevitable oversights of some important contributions.

2. Nonenzymatic electrochemical glucose sensors

The nonenzymatic sensing of glucose based on the direct electrochemistry of glucose (oxidation or reduction) is a rapid

and cost-effective approach. Noble metals such as Pt^{15,17,45} and Au^{46,47} and their composites^{14,48,49} were usually chosen to develop nonenzymatic sensors in early research studies. Recently, Park *et al.* and Toghill *et al.* reviewed electrochemical nonenzymatic glucose sensing.^{50,51}

Three major problems exist in the direct oxidation of glucose on a conventional noble metal electrode: (1) the sensitivity of glucose sensing is restricted by the relatively sluggish kinetics of glucose electro-oxidation on conventional electrodes; (2) the activity of noble metal electrodes is often impaired by the irreversibly adsorbed oxidation intermediates of glucose and the adsorbed chloride ions (in our opinion, a permselective film against anionic Cl⁻ will help here); and (3) the selectivity of nonenzymatic glucose sensors is relatively poor, since some other sugars and some endogenous interfering species can also be oxidized in the potential range of glucose oxidation.^{18,48,52} A high real surface area (with high roughness factor) of an electrode is strongly expected to aid highly sensitive and selective electro-oxidation of glucose because the adsorption of glucose on the electrode surface is a prerequisite step, and the electro-oxidation of the interfering electroactive species of ascorbic acid, uric acid, and *p*-acetamidophenol is independent of the electrode roughness because it is diffusion-controlled. Nowadays, many attempts have been made to develop various nanomaterials with distinguished characteristics to provide new opportunities for fabricating novel nonenzymatic glucose sensors. But the adsorption of chloride is very significant on the rough electrode surface. Alkaline conditions were demonstrated to be effective in eliminating the effect of chloride because of the pre-occupation of the OH group on the electrode surface.⁵³

As is widely accepted, the catalytic process of nonenzymatic glucose oxidation includes the process of hemiacetalic hydrogen atom abstraction occurring simultaneously with the adsorption of the organic species, which is considered as the rate determining step in the catalytic process of glucose electro-oxidation. An “incipient hydrous oxide adatom mediator” (IHOAM) model was proposed by Burke and the hydroxide premonolayer formation was demonstrated to be one of the key points for the electrocatalytic process of glucose.⁵⁴ The importance of the “active” hydroxide anions in the vicinity of the electrode surface produced by the dissociation of water (shown in eqn (1)) to the electro-oxidation of glucose and many other organic molecules is well known.^{45,51} The oxidative adsorbed hydroxide radical expressed as MOH_{ads} is formed by the chemisorption of hydroxide anions to the reductive metal adsorption site (expressed as M as shown in eqn (2)) and is believed to be the catalytic component of electrocatalysts for glucose.



It is believed that the chemisorbed MOH_{ads} takes part in the slow step of glucose oxidation. As is seen in the above equations, the increase in the OH⁻ concentration definitely

promotes the formation of MOH_{ads}.⁴⁹ So nonenzymatic glucose sensing is a pH-dependent reaction and an alkaline environment is beneficial to it, which is the reason why a higher sensitivity of nonenzymatic glucose sensing is commonly observed in a higher pH environment.

Various nanomaterials, such as palladium nanoparticles supported on functional carbon nanotubes,⁵⁵ Ti/TiO₂ nanotube array/Ni composites,⁵⁶ boron-doped diamond nanorods,¹⁸ electrospun palladium (iv)-doped copper oxide composites nanofibers,⁵⁷ three-dimensionally ordered macroporous platinum templates,⁴⁹ polycrystalline Pt electrodes,⁴⁵ nanoporous Au,⁵⁸ Cu nanoclusters/multiwalled carbon nanotubes (MWCNTs) composites,⁵⁹ and highly dispersed Ni nanoparticles embedded in a graphite-like carbon film electrode,⁶⁰ were developed as excellent electrocatalysts for nonenzymatic glucose sensing in alkaline media. Porous tubular palladium nanostructures was also applied to nonenzymatic glucose sensing in 0.10 M pH 8.1 PBS.⁶¹ It was also reported that the nonenzymatic electro-oxidation of glucose is greatly enhanced at Ni and Cu compared with Pt and Au electrodes as a result of their electrocatalytic effect mediated by surface-bound Ni²⁺/Ni³⁺ and Cu²⁺/Cu³⁺ redox couples.^{62,63} All the above nanomaterials exhibit sensitive and selective responses to glucose electro-oxidation in alkaline solutions, in which the existence of abundant OH⁻ can largely promote the formation of MOH_{ads} and greatly reduce the adsorption of Cl⁻. As can be seen, various Pt, Au, Cu, Ni, Pd, Ti, TiO₂, and carbon-based nanomaterials can be utilized for nonenzymatic glucose sensing in alkaline media because of the stability and excellent catalysis of these materials or their oxides/hydroxides under high pH conditions.

However, the alkaline environment may cause surface degradation of the electrocatalysts and thus limit their lifetime. In addition, the efficiency of nonenzymatic glucose sensors is usually characterized in neutral physiological media but the physiological concentration of Cl⁻ can greatly suppress glucose adsorption in neutral media, and the physiological pH also significantly affects the electrocatalytic activity of the metal electrode to the oxidation of glucose. Various other nanomaterials have also been reported to be effective in detecting glucose in neutral media. For example, highly ordered Pt nanotube array electrodes were demonstrated to be sensitive, selective, and stable enough for nonenzymatic glucose sensing.⁶⁴ Park *et al.* reported enzyme-free glucose sensing using mesoporous Pt,¹⁷ and the mesoporous surface endowed the sensor with an excellent anti-poison ability, which retained sufficient sensitivity in the presence of excessive chloride ions (0.10 M KCl). The nanoporous PtPb networks¹⁴ synthesized by Wang *et al.* showed high sensitivity, high selectivity and excellent resistance towards poisoning by Cl⁻. It was also reported by Sun *et al.* that glucose can be selectively electro-oxidized on Pt-Pb alloy (Pt₂Pb) electrodes at remarkably more negative potentials compared with pure Pt surfaces, and more stable and larger responses can be obtained on Pt₂Pb because of its high surface roughness factor and particular nanostructure.²⁰ Gao *et al.*

developed a facile one-step ultrasonication-assisted electrochemical method to synthesize nanocomposites of graphene and PtNi alloy nanoparticles and demonstrated their use for highly selective and sensitive nonenzymatic glucose detection.⁴⁸ Platinum, copper sulfide, and tin oxide nanoparticles-carbon nanotubes (CNTs) hybrid nanostructures exhibited excellent sensitivity, selectivity, and stability in nonenzymatic glucose sensing.¹⁵ Palladium-single-walled carbon nanotube hybrid nanostructures showed good electrocatalytic activity toward the oxidation of glucose in neutral media even in the presence of a high concentration of chloride ions.¹⁶ Copper micropuzzles, which are high-quality Cu microplates that undergo *in situ* large-scale assembly into puzzle-like patterns, were synthesized with the assistance of glucose and applied as a nonenzymatic glucose sensor exhibiting a good sensitivity and a wide range of detection concentrations for glucose at -0.67 V in pH 7.40 phosphate buffer solution (PBS).⁶⁵ Xie *et al.* reported the preparation of Au-film electrodes in a glucose-containing Au-electroplating aqueous bath for a high-performance nonenzymatic glucose sensor and glucose/O₂ fuel cell.⁶⁶ Relatively active metals (*e.g.* Cu) can be electrochemically dissolved unless potentiostated at very low potentials, thus their applications in nonenzymatic glucose sensing at high potentials are limited in neutral media. Au nanomaterials are widely exploited as artificial enzymes because of their excellent stability and excellent catalytic effects. Gold nanoparticles (AuNPs) prepared *via* citrate reduction exhibit intrinsic GOx-like activity.⁶⁷ Small Au nanoclusters (3.6 nm) were found to have intrinsic GOx-like catalytic activity, which can catalyze the oxidation of glucose with the cosubstrate O₂ similar to that of the natural enzyme of GOx, with the production of gluconate and hydrogen peroxide.⁶⁸ Luo *et al.* also reported the self-catalyzed, self-limiting growth of GOx-mimicking AuNPs and found that the H₂O₂ generated from AuNPs-catalyzed glucose oxidation can induce the AuNPs' seeded growth in the presence of HAuCl₄.⁶⁹ The artificial enzymes here catalyze glucose oxidation in an oxygen-containing neutral solution and electricity is not required, and are quite different to the above nonenzymatic electrocatalysts for glucose electro-oxidation. The artificial enzymes here may lead to future amperometric glucose sensors integrating both enzyme-mimic catalysis and electrocatalysis for improved readouts.

3. Three generations of enzyme-based amperometric glucose biosensors

GOx is widely utilized in the majority of commercially available glucose sensors because of its low cost and high selectivity and sensitivity. Since Clark and Lyons proposed the initial concept of glucose enzyme electrodes in 1962,⁷⁰ tremendous efforts have been made towards the improvement of GOx-based amperometric biosensors for blood glucose determination.⁷¹ The first glucose enzyme electrode fabricated by Clark and Lyons took a thin layer of GOx entrapped over an oxygen

electrode *via* a semipermeable dialysis membrane to catalyze glucose oxidation in the presence of O₂ as shown in eqn (3), with the O₂ consumption detected by a Pt cathode according to eqn (4) and used as the signal for glucose determination.



The accuracy and precision of a GOx electrode based on O₂ consumption were greatly reduced by the variation in background oxygen in the samples. Updike and Hicks provided a smart solution to this problem by using two oxygen working electrodes (one covered with the enzyme) and measuring the current differences.⁷² The amperometric monitoring of the H₂O₂ product in GOx-based glucose determination was firstly proposed in 1973 by Guilbault and Lubrano⁷³ and various amperometric enzyme electrodes have since been described.

The biocatalytic reaction of GOx-based glucose biosensing involves the reduction of the flavin group in the enzyme (GOx(FAD)) by reacting with glucose to give the reduced form of the enzyme (GOx(FADH₂)) (as shown in eqn (5)), followed by the reoxidation of GOx(FADH₂) by the electron acceptor (Med_{ox}) to regenerate the oxidized form of the enzyme (GOx(FAD)), as shown in eqn (6). The regeneration of the original state of GOx(FAD) in the enzymatic cycle is vital, otherwise one enzyme molecule can take effect only once and the next enzymatic reaction cycle will cease.



According to the nature of Med_{ox}, amperometric glucose biosensors can be classified into the three following generations. The physiological mediator O₂, the artificial (synthetic) electron acceptor, and the electrode potentiostated at a potential positive of the formal potential of GOx, are used as the Med_{ox} to regenerate GOx(FAD) in the first-, second-, and third-generation amperometric glucose biosensors, respectively, as shown in Fig. 2.

3.1. First-generation amperometric glucose biosensors

The first-generation amperometric glucose biosensors take the physiological mediator O₂ as the Med_{ox} to regenerate GOx(FAD) and detect glucose based on monitoring the consumption of O₂ or the generation of H₂O₂ in the process of the enzymatic reaction. O₂ is the physiological electron acceptor of GOx and thus the electron communication must be very fast. While electrochemical reduction of O₂ is usually used to monitor the O₂ consumption for glucose quantification, both anodic oxidation and cathodic reduction of H₂O₂ can be employed to monitor the enzymatic generation of H₂O₂, and the anodic oxidation of H₂O₂ can favorably regenerate/replenish O₂ for improving the enzymatic cycling. The first-generation biosensing mode based on the measurement of O₂



Fig. 2 Summary of enzymatic glucose oxidation mechanisms, presented as the first, second and third generation sensors.⁵¹ Redrawn with permission from ref. 51, copyright 2010 by ESG (www.electrochemsci.org). Note that glucose assay at a first-generation amperometric enzyme electrode can be conducted in the three following amperometric signaling modes, *i.e.*, cathodic detection of O_2 and H_2O_2 as well as anodic detection of H_2O_2 , and the anodic detection of H_2O_2 to regenerate/replenish O_2 is obviously more favorable for enhancing the enzymatic reaction cycle (*versus* the other two modes).

consumption or H_2O_2 formation has the advantage of being simple, stable, and being able to be used in miniaturized devices.

The response of first-generation glucose sensors is directly related to the O_2 concentration in solution, thus oxygen tension plays an important role in the determination. The fact that normal O_2 concentrations are about 1 order of magnitude lower than the physiological level of glucose is known as “oxygen deficit”. The upper limit of linearity of first-generation amperometric glucose biosensors is greatly reduced by the “oxygen deficit” and their sensitivity to glucose is also restricted by the O_2 concentration in solution. Attempts have been made to conquer these drawbacks of O_2 -dependent amperometric glucose biosensors. The use of a mass transport-limiting film to increase the O_2 /glucose permeability ratio was proposed to address the “oxygen deficit”, and the two dimensional cylindrical electrode designed by Gough’s group is a successful example.^{74–76} Scientists also designed oxygen-rich carbon paste enzyme electrodes or constructed an air diffusion biocathode using O_2 directly from air to improve the O_2 supply to conquer the O_2 limitation.^{77–79} Other materials that can effectively enrich O_2 are also expected to be utilized in the first-generation amperometric glucose biosensors to conquer the O_2 -limit problem.

An anti-interferent ability is one of the prerequisites for an effective biosensor. A lot of coexisting oxidizable species in biological fluids, such as ascorbic acid, uric acids, and some drugs (*e.g.* acetaminophen), can be co-oxidized at the relatively high potential used for H_2O_2 electro-oxidation, so they all contribute to the current response and thus compromise the

selectivity and the accuracy of the glucose measurement. Considerable efforts have been devoted to improve the anti-interferent ability of first-generation amperometric glucose biosensors. As reported, there are mainly two effective protocols in minimizing interferences: (1) immobilizing the enzyme through a permselective film, which can diminish or inhibit the electroactivity of interferents but can allow the sufficiently high electroactivity of H_2O_2 or O_2 at the enzyme electrode;^{80,81} (2) decreasing the H_2O_2 -detecting potential by using catalysts immobilized at the enzyme electrode.⁸² Prussian blue (PB), which is called “artificial peroxidase” because of its high selectivity and catalytic activity for the reduction of H_2O_2 , has been widely utilized in the highly selective biosensing of glucose at low potentials. For instance, Zhao *et al.* prepared poly(diallyldimethylammonium chloride) (PDDA)-protected PB nanoparticles to catalyze the enzymatically generated H_2O_2 at low potentials and inhibit the responses of interferents.⁸³ Li *et al.* fabricated a highly sensitive molecularly imprinted electrochemical sensor based on the double amplification by an inorganic PB catalytic polymer and the enzymatic effect of GOx.⁸⁴ The incorporation of GOx into Langmuir–Blodgett films based on PB was also applied to an amperometric glucose biosensor operated at very low potential.⁸⁵ We realized the sensitive determination of glucose in both H_2O_2 -oxidation and H_2O_2 -reduction modes on a PB modified Au electrode with GOx immobilized.⁸⁶ Electropolymerized poly(toluidine blue O) film was also effectively used as the redox mediator that contributes to the low potential detection of glucose at a carbon nanotube modified glassy carbon electrode.⁸⁷ Various nanomaterials, such as CNTs,⁸⁸ platinum nanoparticles,^{89,90} metallized carbons,⁹¹ and composite nanomaterials,^{92,93} have also been demonstrated to be effective in improving the selectivity of resultant GOx-based amperometric biosensors by lowering the determination potential of H_2O_2 by virtue of the excellent catalytic effect of nanomaterials.²⁶ In addition, some biological enzymes for H_2O_2 (*e.g.* horseradish peroxidase (HRP)) have been co-immobilized to develop bienzymatic amperometric electrodes for cathodic detection of H_2O_2 . For instance, the co-immobilization of HRP to catalyze the reduction of GOx-generated H_2O_2 at low potentials is effective in improving the selectivity of glucose sensors.^{94,95} Furthermore, a higher degree of technical sophistication in the amperometric detection device can efficiently improve the selectivity for the glucose assay, *e.g.* introduction of an interferent-pretreatment unit to electrochemically remove the interfering species before they reach the biosensor surface.^{96–98}

3.2. Second-generation amperometric glucose biosensors

The 10-fold excess of glucose over oxygen in blood makes the “oxygen deficit” the most serious problem for the first-generation amperometric biosensors.⁹⁹ A highly successful approach to increase the electron-transfer rate of biosensors is the use of another artificial Med_{ox} to mediate the GOx cycling instead of oxygen.²⁶ The artificial Med_{ox} can be either a solution-state mediator that can diffuse into and out of the enzyme active site,⁹⁹ or an immobilized mediator by attaching it directly to the enzyme, entrapping it in the enzyme film, or using a redox-conducting polymer that can shuttle its

electrons to and from the enzymatic active site.^{100,101} Biosensors using artificial electron acceptors to shuttle electrons from the redox center of the enzyme to the surface of the electrode are called second-generation biosensors.^{102–104} The effective mediators for GOx include ferrocene derivatives, conducting organic salts (particularly tetrathiafulvalene-tetracyanoquinodimethane, TTF-TCNQ), ferricyanide, quinone compounds, transition-metal complexes, and phenothiazine and phenoxazine compounds.^{1,26,89,105–107} Interestingly, ferricyanide is not recommended as a highly efficient electron mediator for GOx in some reports, although the molecular mechanism remains unknown at present.^{106,108}

The catalytic process of second-generation amperometric glucose biosensors includes the following three steps: (a) the transfer of electrons (and protons) from glucose to the two FAD reaction centers of GOx, which are reduced to FADH₂, (b) the transfer of electrons from the FADH₂ centers to the artificial mediators, and thus the mediators transform from Med_{ox} to their reduced state Med_{red}, and (c) the transport of electrons through the artificial mediators to the electrode. The current signals produced by the oxidation of Med_{red} are used for glucose determination in the second-generation biosensing mode. So the effective interaction between mediators and enzymes, which is necessary for the realization of the effective shuttling of electrons between redox active centers of GOx and electrode, is essential in the second-generation biosensing mode. Diffusion mediators satisfy well this demand. However, the use of soluble mediating species cannot be used in implantable probes.^{109,110} Various strategies have been suggested for tailoring the mediators in the electrode-supported enzyme film. Chemically binding the mediators with the polymer backbone used for fabricating the biosensor has been widely utilized to stabilize artificial mediators.^{109,111} Hale *et al.* have investigated some systems where the mediating species were chemically bound to the polysiloxane to allow the close contact between the FAD/FADH₂ centers of the enzyme and the mediator, yet prevented the latter from diffusing away from the electrode surface.¹⁰⁹ Dong's group reported supramolecular organized multilayers constructed by MWCNTs modified with ferrocene derivatized poly(allylamine) redox polymer and GOx by electrostatic self-assembly and thus achieved the construction of a reagentless biosensor.¹¹¹ Hydrogels containing Os complexes were also used to fabricate second-generation amperometric biosensors with wide use in fundamental science and important practical applications.^{112,113} An electron-conducting crosslinked polyaniline (PANI)-based redox hydrogel, formed in one step at pH 7.2, was used to electrically wire the GOx and form an effective glucose electro-oxidation catalyst, and the electro-oxidation of glucose at 0.3 V vs. Ag/AgCl was thus realized.¹¹⁴ Willner's group fabricated the integrated electrically contacted CNTs/ferrocene/GOx electrodes and used them for the bioelectrocatalyzed detection of glucose.¹¹⁵ Hydroquinonesulfonate ions were co-immobilized in polypyrrole films with GOx to realize glucose sensing in the absence of mediator in solution.¹¹⁰ The attachment of redox mediators or relays directly to the enzymes is also widely reported. Schuhmann *et al.* reported the electron-transfer between GOx and electrodes *via* redox mediators bound with flexible chains to the enzyme surface.¹⁰¹ Scientists have also

tried to covalently link the ferrocene derivative to the enzyme molecule, but this was shown to be more complicated and less successful.¹⁰¹ In contrast, Sekretaryova *et al.* reported the successful stable immobilization of both the enzyme and the mediator, avoiding covalent linking of the latter, by exposing the enzymes to water-organic mixtures with a high content of organic solvent.⁹⁹ In our opinion, in contrast to a second-generation amperometric enzyme electrode involving a solution-state (diffusion-based) artificial mediator, the immobilized mediator case should provide a compromise between the three simultaneously-occurring but somewhat mutually contradictory events below, *i.e.*, the stable immobilization of the mediator (limited movement), the high electron/proton-exchange efficiency between the immobilized mediator and the immobilized enzyme, and the high electron-exchange efficiency between the immobilized mediator and the electrode surface. Hence, immobilization of the artificial mediator both near the enzyme's redox center and near the electrode surface is very important for the fast mass-/electron-transfer between the mediator, the enzyme, and the electrode, otherwise, the enzyme-mediating efficiency and/or the amperometric signaling efficiency will decrease or even fully die away.

3.3. Third-generation amperometric glucose biosensors

The third-generation amperometric glucose biosensors are fascinating because they function in 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 slightly positive of the redox potential of GOx (around -0.50 V vs. Ag/AgCl).^{26,116} Achieving the direct electron communication of enzymes depends significantly on the distance between the redox-active cofactor and the electrode surface.^{117,118} Various attempts to overcome the long electron-tunneling distance were made to realize the direct electrochemistry of enzymes. The reconstitution of apo-proteins on cofactor-modified electrodes and the reconstitution of apo-enzymes on cofactor functionalized Au nanoparticles were extensively employed as a versatile method to align redox enzymes on electrodes.^{29,119–124} Whereas this method is effective in electrically wiring redox enzymes with electrodes, the complicated procedures inhibit its wide use in practice. Yehezkeli *et al.* reported a method to electrically wire the enzyme and transform it from an oxidase to a hydrogenase by biocatalytically implanting Pt nanoclusters into GOx *via* thermodynamically reducing different metal salts to metallic nanoclusters with the reduced cofactor FADH₂.¹²⁵ Various nanomaterials have also been reported to achieve the direct electrochemistry of GOx.^{126,127} Shan *et al.* reported the direct electrochemistry of GOx and glucose biosensing based on polyvinylpyrrolidone-protected graphene.¹²⁶ Alwarappan *et al.* carried out a series of works to employ graphene-GOx for glucose detection, and the promotion of glucose biosensing and the direct electrochemistry of GOx have been realized with the aid of graphene.^{117,118,128} Wang *et al.* detected glucose based on the direct electron transfer reaction of GOx immobilized on highly ordered PANI nanotubes.⁹⁷ Amine-terminated ionic liquid functionalized CNTs-AuNPs were developed by Gao *et al.* to investigate the direct electron

transfer of GOx.¹²⁹ Holland *et al.* enabled the direct electrical communication between GOx and electrode through a simple site-specific modification of GOx to display a free thiol group near its active site and thus facilitated the site-specific attachment of a maleimide modified gold nanoparticle to the enzyme.¹³⁰

Although well-defined voltammetric peaks of direct electrochemistry of GOx have been achieved in a lot of studies, the detection of glucose based on the direct electron transfer of GOx has been rarely realized.⁹⁷ Most GOx exhibiting good direct electrochemical peaks of GOx still need mediators to catalyze the oxidation of glucose.^{131,132} In fact, some reports on GOx electrochemistry do not clearly demonstrate that the direct electrochemistry comes from the intact (or still sufficiently bioactive) enzyme, thus any claims on the third-generation amperometric biosensing of glucose should be made very carefully. In our opinion (as also reminded by one of the referees of this article),¹³³ the observed well-defined voltammetric peaks of direct electrochemistry of GOx probably come from the enzyme molecules whose activity has been greatly decreased because of the destruction of enzyme conformation or the release of flavin. With the use of the electrochemical quartz crystal microbalance (EQCM) technique, we measured the electroactivity of sodium dodecyl benzene sulfonate (SDBS)-treated GOx adsorbed on a MWCNTs/Au electrode as a function of the enzymatic specific activity (defined as the enzymatic activity per unit mass of enzyme) of the adsorbed GOx. We thus experimentally found for the first time that the electroactivity and the enzymatic specific activity of the immobilized GOx responded oppositely in the presence of MWCNTs and SDBS, and the portion of the adsorbed GOx showing electrochemical activity exhibited almost no enzymatic activity.¹³³ A similar conclusion has also been experimentally drawn by Wang and Yao.¹³⁴ Obviously, the significance of realizing such a direct electrochemistry of partially or fully denatured GOx for glucose detection is worthy of reconsideration. It must be emphasized that the determination of glucose based on electroreduction of enzyme-consuming O₂ 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. Instead, a distinguished decrease in the reduction current and an increase in the oxidation current of direct electrochemistry of GOx should be simultaneously observed in the presence of glucose (*versus* its absence) for a successful third-generation GOx-based glucose biosensor. Hence, a third-generation GOx-based glucose biosensor can efficiently work at potentials slightly positive of the redox potential of GOx around -0.50 V *vs.* Ag/AgCl^{26,116} (*e.g.*, at -0.30 V *vs.* Ag/AgCl). Generally, the direct electron communication between the deeply buried redox active center of the enzyme and the electrode surface requires the conformational change of the enzyme, which may result in an obvious loss of enzymatic activity. An appropriate balance between the enzymatic and electrochemical activities is vital for the third-generation amperometric glucose biosensors, and it seems that we still have a long way to go in this respect. In our opinion, the third-generation amperometric biosensing is optimistically expected to be globally realized through effectively connecting the redox

active center of the enzyme to the electrode by using nano-/subnanosized conducting wires of little interference to the enzyme conformation.¹²⁵

4. The construction of electrochemical cells for amperometric glucose biosensors

The fabrication of various electrochemical cells and their utilization in glucose sensing has been widely reported. A flow-through electrochemical detector for glucose based on a GOx-modified microelectrode incorporating redox and conducting polymer materials was developed by Rohde *et al.*¹³⁵ Ito *et al.* fabricated a microfluidic device for glucose detection using a microsized direct methanol fuel cell as an amperometric detection power source.¹³⁶ The improvement of electrochemical detector cells is highly desirable because of their extensive use in many areas. The effective immobilization of enzymes, the protection of the bioactivity of enzymes, and their integration and applications are the key factors for the fabrication of successful electrochemical cells.

4.1. The immobilization of enzymes on sensing electrodes

The immobilization of enzymes on solid interfaces is one of the crucial factors for the fabrication of enzyme-based devices, which has been realized by various strategies, such as physical adsorption, covalent attachment, and physical entrapment or encapsulation, *etc.*¹³⁷ Although immobilization can improve the long-term and operational stability of enzymes, this is often at the expense of significant loss in the catalytic activity of the immobilized enzymes. So the maintenance of enzymatic activity, the stability of immobilized enzymes, and the efficiency of the enzyme-based devices for application in biosensing are mainly considered in choosing an optimal immobilizing strategy.

Physical adsorption is a simple way to immobilize enzymes, which can effectively maintain enzymatic activity under mild experimental conditions. However, stability is one of the most serious problems for the wide applications of adsorbed enzymes, which is greatly affected by a combination of factors, *e.g.*, pH, ionic strength, temperature, surface tension, charges, and matrix. Scientists have conducted a lot of excellent research on enzyme adsorption and have greatly improved the stability of adsorbed enzymes. For example, He *et al.* realized the activity and thermal stability improvements of GOx upon adsorption on biocompatible core-shell poly(methyl methacrylate)-bovine serum albumin (PMMA-BSA) nanoparticles.¹³⁸ Raftlike lipid domains were demonstrated to be an ideal adsorption surface for enhancing the stability of adsorbed proteins.¹³⁹ The adsorption behavior and mechanism of enzymes on highly oriented pyrolytic graphite, CNTs, nanoporous materials, self-assembled monolayer (SAM) on gold, and dipalmitoylphosphatic acid monolayer have been extensively investigated recently.^{138–147}

Covalent attachment is considered as one of the most effective methods for the stable immobilization of enzymes and is widely employed to fabricate biosensors.^{148–150} For instance, Xu *et al.* reported the covalent immobilization of GOx

on well-defined poly(glycidyl methacrylate)-Si(111) hybrids from surface-initiated atom-transfer radical polymerization and the immobilized GOx exhibited a corresponding relative activity of about 60% and an improved stability during storage over that of the free enzyme.¹⁵¹ Wan *et al.* fabricated an amperometric glucose biosensor by covalent immobilization of GOx with the pendant hydroxyl groups of chitosan using 1,4-carbonyldiimidazole as the bifunctional linker and the spatially biocompatible microenvironment greatly enhanced the amount and biocatalytic activity of the immobilized enzyme.¹⁵² Pandey *et al.* realized the significant enhancement in the activity of GOx by covalently immobilizing GOx onto chemically synthesized thiolated gold nanoparticles and the covalently immobilized GOx thiolated nanoparticles exhibited a response time of 30 s, a shelf life of more than 6 months, and improved tolerance to both pH and temperature.¹⁵³ We suggested a simple and rather universal method for the highly efficient immobilization of enzymes by aqueous electro-deposition of enzyme-tethered chitosan for sensitive amperometric biosensing.¹⁵⁴ While many protocols for covalent immobilization of enzymes employ their amino acid residuals, the glycosyl-affinitive boronic acid polymer was suggested to covalently immobilize the enzyme at the glycosyl sites (so-called boronic acid–diol interaction), which should less affect enzymatic activity *versus* covalent immobilization of enzymes at their amino acid residuals.¹⁵⁵

Entrapment or encapsulation of an enzyme inside a solid matrix (*e.g.* polymers, redox gels, sol–gel-derived glasses, and carbon pastes) is preferred by most workers because of its advantages in enzyme immobilization, *e.g.*, the good maintenance of enzymatic activity and the relatively high stability of immobilized enzymes. The protocol of entrapping enzymes in electrodeposited polymers offers a film-thickness/site controllability higher than many other methods and is thus appropriate for preparing biosensing ultramicroelectrodes in miniaturized devices. Electropolymerized nonconducting films are widely used to prepare enzyme biosensors because of their high resistance against electrode fouling and high anti-interferent capability against some coexisting electroactive substances.^{156–159} However, a relatively low load of enzymes was observed in the electrosynthesized films because the entrapment of enzymes was mainly due to the presence of enzymes in the vicinity of the growing films.¹⁶⁰ Recently, we reported the immobilization of enzymes in dopamine and noradrenalin polymers, which can yield electrode-supported films much thicker than the traditional insulating polymer films owing to their special melanin-like porous structures. To our knowledge, the electropolymerization of dopamine and noradrenalin and the utilization of the resultant polymers to immobilize biomacromolecules were investigated in our group for the first time.¹⁶¹ The dopamine and noradrenalin polymers were demonstrated to be quite useful in entrapping biomacromolecules at high loading/activity, and the glucose-assay selectivity was also very high.^{86,89,106,161,162} For the first time, we have introduced a preoxidation (chemical, electrochemical, or enzymatic) step before the electropolymerization of monomers, which combines the respective advantages of chemical oxidation polymerization and electropolymerization and largely improves the biosensing performance (Fig. 3).^{86,89,160,162–164}



Fig. 3 Procedures for immobilization of GOx via the chemical preoxidation electropolymerization and conventional electropolymerization protocols. Reprinted with permission from ref. 160, copyright 2008, American Chemistry Society.

Chemical oxidation synthesis alone was also utilized by our group to prepare high-performance enzyme films.^{106,165} However, coating the working electrode with a polymeric film often leads to lower signals and longer response times because substrates have to diffuse into the matrix to interact with the entrapped enzyme, so the exploitation of excellent permselective films that can effectively reject interferences but well keep the biosensing performances is highly desirable.

In the last 20 years, the main development of electrochemical glucose biosensors can be attributed to the exploitation of various functional nanomaterials to improve the performance of the resultant biosensors. Conducting nanomaterials can greatly enhance the electron communication between enzymes and electrodes. Amperometric glucose biosensors based on various nanomaterials have been extensively studied in recent years, and some typical examples are listed in Table 1.^{26,28,93,115,125,166} Zhu *et al.* wrote a critical review of glucose biosensors based on the carbon nanomaterials of CNTs and graphene.¹⁶⁷ Rahman *et al.* reported a comprehensive review of glucose biosensors based on nanostructured metal oxides.¹⁶⁸ A review on glucose and H₂O₂ biosensors based on a silver nanoparticles-modified electrode was written by Rad *et al.*¹⁶⁹ Iwamoto *et al.* reported the activity enhancement of a screen-printed carbon electrode by modification with gold nanoparticles for glucose determination.¹⁷⁰ Qiu *et al.* fabricated aligned nanoporous PtNi (np-PtNi) nanorod-like structures and used them in electrocatalysis and biosensing.¹⁷¹ The np-PtNi nanorods exhibit remarkably improved electrocatalytic activity towards ethanol oxidation and H₂O₂ oxidation/reduction compared with the commercial Pt/C catalyst, and the GOx modified np-PtNi electrode can sensitively detect glucose over a wide linear range.¹⁷¹ Nanocomposites have also been used to fabricate high-performance amperometric glucose biosensors,^{172,173} *i.e.*, nanoleave-shaped copper oxide decorated MWCNTs composites,¹⁷⁴ Pt-MWCNTs-alumina-coated silica nanocomposite,¹⁷⁵

Table 1 Examples of nanomaterials widely used for the fabrication of GOx-based amperometric glucose sensors in recent years^a

Nanomaterial	Electrode composition	Sensitivity/ $\mu\text{A mM}^{-1} \text{cm}^{-2}$	Linear Range/mM	LOD/ μM	Year	Ref.
Pt	boron nitride nanotubes-PANI-PtNPs	19.0	0.01–5.5	0.18	2011	93
	PtNPs-MWCNTs-PANI	16.1	0.003–8.2	1.0	2011	177
	Pt-DENs/PANI/CNTs	42.0	0.001–12	0.50	2009	178
	Pt nanoclusters-MWCNTs	12.8	0.003–12	1.0	2012	179
	hollow Pt decorated MWCNTs	22.8	0.0012–8.4	0.40	2011	180
Au	AuNPs-MWCNT	19.3	0.02–10	2.3	2011	181
	AuNPs-hydrogel microstructures	100	0.1–10	0.37	2011	182
	AuNPs	47.2	0.001–5	1.0	2012	183
	crystalline AuNPs-MWCNTs	5.7	0.05–22	20	2009	184
Ag	AgNPs/CNT/chitosan	136	0.0005–0.05	0.10	2009	185
	PVP-Ag nanowires	22.4	2–20	N/A	2012	186
	AgNPs/PANINFs	N/A	1–12	0.25	2012	187
MWCNTs	MWCNTs-CS nanowire	5.03 $\mu\text{A mM}^{-1}$	1–10	N/A	2011	188
	PB/MWCNTs-GOx-CS-ICPTES	15.2	0.025–1.3	7.5	2011	189
	Pt/FeyOx-MWCNTs/CS	N/A	0.006–6.2	2.0	2010	190
	CS-PB-MWNTs-PtCo	23.4	0.0015–1.1	0.47	2011	191
	PtPd-MWCNTs	112	0.062–14	0.031	2012	192
	Fe ₃ O ₄ @SiO ₂ /MWNTs	58.9	0.001–30	0.80	2010	193
	polyelectrolyte-SWCNTs	157	0.5–5.0	5.0	2012	194
SWCNTs	SWCNTs-PVP-Os	56.0	N/A	N/A	2009	141
	Nafion-SWCNH	15.0	0–6.0	6.0	2008	132
Graphene	PdNPs/CS-graphene	31.2	0.001–1.0	0.20	2011	195
	TiO ₂ -graphene	6.20	N/A	1.0	2012	196
	Pt-Au/AuNPs-graphene	N/A	30	1.0	2010	197
Graphene oxide	graphene nanosheet	N/A	2–40	3.0 \pm 0.5	2010	117
	CS-Fc/GO nanocomposite	10.0	0.02–6.8	7.6	2011	198
	ERGO	22.8	0.005–12	1.0	2011	199
	RGO-PAMAM-Ag	75.7	0.032–1.9	4.5	2012	200
	ERGO-AuPdNPs	267	0.5–3.5	6.9	2011	201
Metal oxides	ZnO nanofiber	70.2	0.25–19	1.0	2010	202
		19.5	0.2–2.0	50	2010	173
	TiO ₂	9.90	up to 1.5	1.3	2011	116
	nanostructured metal-oxides	N/A	N/A	N/A	2010	168
	PEDOT-NiO HS	16.9	up to 1.5	N/A	2010	203
Others	graphene-chitosan-ZrO ₂	7.60	0.2–1.6	46	2012	204
	nanoporous PtNi nanorod	2.00	0.5–21	20	2012	171
	magnetic polymer	110	0.002–2.6	0.33	2010	165
	PDA-PtNPs PBNCs	99.0	0.0005–4.5	0.09	2009	89
	polyaniline nanotubes	97.2 \pm 4.6	0.01–5.5	0.3 \pm 0.1	2009	97
	exfoliated graphite nanoplatelets	14.2	up to 6.0	10	2007	205
	Si nanowire-AuNPs	N/A	0.1–0.8, 1–16	50	2010	206

^a LOD: limit of detection; PtNPs: Pt nanoparticles; Pt-DENs: dendrimer-encapsulated Pt nanoparticles; AgNPs: Ag nanoparticles; PANINFs: polyaniline nanofibers; CS: chitosan; ICPTES: 3-isocyanatopropyltriethoxysilane; PVP: poly(vinylpyridine); SWCNH: single-walled carbon nanohorns; PdNPs: Pd nanoparticles; Fc: ferrocene; GO: graphene oxide; ERGO: electrochemically reduced graphene oxide; RGO: reduced graphene oxide; PAMAM: polyamidoamine; AuPdNPs: gold-palladium (1 : 1) bimetallic nanoparticles; PEDOT: poly(3,4-ethylenedioxythiophene); NiO HS: NiO hollow spheres; PDA: polydopamine; PBNCs: polymeric bionanocomposites.

Pt-dispersed hierarchically porous electrode,¹⁷⁶ and boron nitride nanotubes-polyaniline-Pt hybrids.⁹³

4.2. The determination of enzymatic activity

The tremendous potential of enzymes as highly efficient catalysts is commonly recognized, but the poor stability of enzymes greatly limits their practical applications in many cases. As a kind of high-performance biocatalyst, enzymes express their unique catalysis on specific reactions based on their enzymatic activity, which is one of the key factors of enzymes. But the enzymatic activity tends to be influenced by the external environment. The protection of the activity of enzymes is of great importance in various enzyme-based devices. So the development of novel strategies which can effectively protect the activity of enzymes is important and relevant.

The analysis and determination of the enzymatic activity of immobilized enzymes is an important approach and provides critical evidence for investigating the enzyme reaction mechanism, evaluating the enzyme immobilization procedure, improving the enzyme immobilization material and constructing novel high-performance enzyme-based biosensors. Wang *et al.* reported new insights into the effects of thermal treatment on the catalytic activity and conformational structure of GOx studied by electrochemistry, IR spectroscopy, and theoretical calculations.²⁰⁷ Willner's group developed a novel protocol to follow the biocatalytic activities of GOx by electrochemically crosslinked enzyme-Pt nanoparticles composite electrodes.⁹⁰ Wu *et al.* investigated the effects of different types of ionic liquids on enzymatic catalysis of GOx and pointed out that the nature of ionic liquids is the main factor that affects the electrocatalytic activity of the GOx

towards the oxidation of glucose.¹⁸ Recently, Jensen *et al.* presented a novel method combining protein adsorption studies at nanostructured quartz crystal microbalance sensor surfaces (QCM-D) with optical (SPR) and electrochemical methods (cyclic voltammetry), allowing quantification of both bound protein amount and activity.²⁰⁸

In addition to the analysis of total enzymatic activity of immobilized enzymes by measuring the amount of products (*e.g.* H₂O₂) in the oxidation of β-D-glucose *via* a spectroscopic method, electrochemical method, or titration, the quantitative determination of enzymatic specific activity is also very important. Bourdillon *et al.* used a radioactive ¹²⁵I labeling method to quantify the immobilized enzyme and realized the detection of enzyme activity by analyzing the cyclic voltammetric responses recorded in the presence of glucose with ferrocene methanol as the mediator.²⁰⁹ The quantification of enzymatic specific activity includes the quantification of the amount of immobilized enzymes (*m*_{GOx}) and the detection of total enzymatic activity of immobilized enzymes. Our group conducted a series of biosensing studies based on evaluating the enzymatic specific activity of the immobilized enzyme for optimizing biomolecule-immobilization materials and methods.^{86,133,154,160,164} Enzymatic specific activity (ESA) is expressed as $ESA = n_{H_2O_2} / m_{GOx}$, where *n*_{H₂O₂} is the amount of enzymatically generated H₂O₂ in the first 60 s of enzymatic reaction on the electrode and *m*_{GOx} is the mass of GOx involved in enzymatic reactions. The quantification of immobilized enzymes was realized *via* the quartz crystal microbalance (QCM) technique, which can detect an electrode-mass change down to the nanogram level (generally equivalent to submonolayer modification). The immobilized enzyme (the *m*_{GOx} in this case) can be quantified from its resultant frequency decrease according to the Sauerbrey equation. The enzymatic specific activity of GOx immobilized on poly(*o*-phenylenediamine)/GOx-glutaraldehyde/PB/Au electrode was determined to be 2.4 kU g⁻¹.⁸² Later, the dopamine and noradrenalin polymers of special melanin-like porous structure were reported to be able to encapsulate GOx with an enzymatic specific activity as high as (16 ± 0.6) kU g⁻¹ by a conventional electropolymerization protocol and (34 ± 1) kU g⁻¹ by a chemical preoxidation-involved electropolymerization protocol.⁸⁶ The determination of enzymatic specific activity is expected to be effective in evaluating the performance of materials and protocols used in biomacromolecular immobilization.

The enzymatic activity can also be utilized in inhibitive analysis of environmental pollutants. Toxic Hg²⁺, Cu²⁺, Pb²⁺, Cd²⁺, and Ag⁺ were reported to be able to strongly interact with enzymes and inhibit the bioactivity of enzymes.^{210,211} Many immobilized enzymes have been used for inhibitive assays of heavy metal ions.^{212–216} GOx of low cost, good stability, and high specific activity has been widely used for the inhibitive assays of several heavy metal ions. Malitesta *et al.* reported heavy metal ion determination by GOx-based amperometric biosensors.²¹⁷ The inhibitive determination of heavy metal ions by an amperometric GOx biosensor was also investigated by Guascito *et al.*²¹⁸ Enzyme inhibition-based biosensors for food safety and environmental monitoring were extensively reviewed by Amine *et al.* in 2006.²¹⁹ Recently, we have developed a comprehensive experimental platform based on

QCM and electroanalysis techniques to quantitatively study heavy metal ions–enzyme interactions and amperometric inhibitive assays of heavy metal ions and realized the quantification of the number of the bound heavy metal ions per GOx molecule at various inhibition percentages. This platform is expected to find wide applications in enzymatic inhibitive assays and quantitative studies of the inhibition effects of heavy metal ions on many other redox-event-relevant enzymes.²²⁰

4.3. The integration of electrochemical cells for biosensors

Electrochemical glucose biosensors have played a key role in the move to simple, one-step blood glucose testing because they satisfy well the demands of personal (home) glucose testing. Various novel glucose biosensors are expected to be applied to fabricate blood glucose test strips, which are manufactured and marketed by numerous companies and have been popularly used by diabetic patients. Implantable glucose sensors^{221,222} are highly expected to serve well as point-of-care probes. So the integration of electrochemical cells is one of the key points for the application of amperometric glucose biosensors in many areas. In conventional laboratories, the electrochemical cells for biosensors are commonly integrated with three separated electrodes inserted in the corresponding electrolyte solution, and the corresponding enzymatic reactions take place on the enzyme-modified working electrode. The developed screen-printed three-electrodes realized the effective simplification and miniaturization of integrated electrochemical cells.²²³ The majority of personal blood glucose monitors rely on disposable screen-printed enzyme electrode test strips.²²⁴ Such electrode strips are commercially produced by the screen-printing micro-fabrication or vapor deposition process.²²⁵ The construction of strips is illustrated in Fig. 4 (left). Each strip contains the printed working and reference electrodes, and the working electrode is modified with enzyme, mediator, stabilizer, surfactant, linking, and binding agents. These reagents are commonly dispensed by ink-jet printing technology and deposited in the dry form.²⁶

The miniaturization of device and acceleration of analytical rate are pursued in the development of test strips. The test strips made in the 1990s utilized a carbon-working electrode and an Ag/AgCl reference electrode system. For these strips, a high operation voltage (400 mV), a large volume of blood (20 μL) and a long test time (around 20–25 s) were required. For the test strips made in late 1999, the operation voltage was lowered to 300 mV and the blood sample was automatically transferred into the reaction area at the edge of the strip by a capillary mechanism, with the sample volume reduced to 3 μL and the test time reduced to 10 s by utilizing a spacer and a hydrophilic membrane cover to restrict the reaction zone. The sample volume was further reduced to 0.5–2 μL and the test time was shortened to 5 s with the great improvement in quality of screen-printing for an example made in mid-2000. Three electrodes are adapted and printed on the support on the latest test strips (Fig. 4 (right)) to improve the test accuracy and minimize interference from the blood sample. Various membranes such as mesh, filter, and surfactants are often incorporated into the test strips to ensure a uniform coverage of sample and separate the blood cells. Cross contamination and drift are eliminated from these single-use devices and



Fig. 4 Cross section of a commercial strip for self-testing of blood glucose (left, reprinted with permission from ref. 26, copyright 2008, American Chemistry Society) and the test strips designed in 2008 (right, reprinted with permission from ref. 225, copyright 2009, Elsevier).

high clinical accuracy is ensured with a high degree of sophistication being essential.

The biofuel cell is another example of the integration of GOx-based amperometric glucose biosensors.²²⁶ An enzyme-based biofuel cell can work in the following way: the oxidase-modified electrode in the anodic compartment oxidizes the fuel substrate, while transferring electrons to the electrode; the enzyme electrode in the cathodic compartment reduces the oxidizer, while transferring electrons from the electrode to the oxidizer. Biofuel cells are considered as a novel kind of energy conversion technology which can be operated under mild conditions and serve as *in vivo* power sources for bioelectronics,²²⁷ which have become one of the most popular research areas and hold great promise in the many applications of biomolecules. Integrated, electrically contacted thin film-modified enzyme electrodes (the second and the third generation of enzyme electrodes) are widely utilized in fabricating biofuel cells.²²⁸ Our group has conducted a series of investigations on biofuel cells by utilizing an electrochemical noise device.^{66,105–107,162,229} Currently, GOx as biocatalyst at the anode is considered as the most promising type of implantable biofuel cell.²³⁰ Biofuel cells are also expected to be used in implanted cardiac pacemakers and self-powered biosensing devices, which have been studied and reported by some scientists and deserve continued efforts.^{231–236} A number of excellent reviews on biofuel cells have been reported,^{228,237–240} and the interested reader may read them for details.

5. Conclusions

Numerous publications on electrochemical glucose sensors and the undamped activity of this research field persuasively reflect the importance of glucose sensing. Major fundamental and technological advances have been utilized to enhance the capabilities and improve the reliability of glucose measuring devices. GOx-based amperometric glucose biosensors are expected to be ideal models for the fabrication of diverse

biosensing devices. However, there are still a lot of challenges ahead of us, the exploitation of excellent materials, the detailed investigation of the tricky problems related to the enzyme-based devices, the development of miniaturized implantable amperometric biosensors, and the investigation of the relationship of the catalytic function of enzymes with the conformation of the enzyme are still worthy of special research and are also of great significance for the real revolution and innovation of enzymatic biosensors. The exploitation of ideal sensors with reliable real-time continuous monitoring of all blood glucose variations with high selectivity and speed over extended periods is still a challenge worthy of further research efforts. The development of miniaturized implantable biosensors and biofuel cells are expected to find wide applications. Nonenzymatic electrocatalytic materials and artificial enzymes are also very important research topics at present, which may lead to high-performance catalysts for glucose sensing and relevant organic electrosynthesis.

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