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Reviews

Third-Generation Biosensors Based on the Direct Electron Transfer of Proteins

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Recent progress in third-generation electrochemical biosensors based on the direct electron transfer of proteins is reviewed. The development of three generations of electrochemical biosensors is also simply addressed. Special attention is paid to protein-film voltammetry, which is a powerful way to obtain the direct electron transfer of proteins. Research activities on various kinds of biosensors are discussed according to the proteins (enzymes) used in the specific work.

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1 Introduction

Biosensors, a concept that originated from the guidance of nature, is a subject of great interest in recent years. A biosensor is an analytical device comprising of a biological recognition element directly interfaced to a signal transducer, which together relates the concentration of an analyte, or group of analytes, to a measurable response. Electrochemical biosensors are analytical devices in which an electrochemical device serves as a transduction element. They are of particular interest because of practical advantages, such as operation simplicity, low expense of fabrication and suitability for real-time detection. Since the first proposal of the concept of an enzyme-based biosensor by Clark, Jr.,[1] significant progress in this field has been achieved with the inherited sensitivity and selectivity of enzymes for analytical purposes.

However, a major barricade in an electrochemical system lies in the electrical communication between the biological recognition element, such as redox proteins, and signal transducers, here the electrode materials. The electron-transfer rates between large rodox proteins and electrode surfaces are usually prohibitively slow because of the deep burying of the electroactive prosthetic groups within the protein structure, the adsorptive denaturation of proteins onto electrodes, and the unfavorable orientations at the electrode.[2] The way to achieve efficient electrical communication has been among the most challenging objects in the fields of bioelectrochemistry. In summary, two ways have been proposed. One is based on the so-called "electrochemical" mediators, both natural enzyme substrates or products (first-generation biosensors, mostly oxygen), and artificial redox mediators (second-generation biosensors, mostly dye molecules, conducted polymers, *etc*.).[3,4] The other approach is based on the direct electron transfer of proteins (third-generation biosensors). With its inherited simplicity in either theoretical calculations or practical applications, the latter has received far greater interest despite their limited applications at the present stage.[5]

Until now, the methods used to achieve the direct electron transfer of proteins, which are the basis of third-generation biosensors, are still very limited. Among them, protein-film voltammetry (PFV) is a very promising technique.[6-8] PFV examines a film of electroactive proteins that is adsorbed directly onto the electrode surface.[9-13] The protein is immobilized on an electrode surface as an absorbed electroactive film; by applying a potential, electrons are driven in and out of the active sites. Signals are obtained from extremely small sample quantities (monolayer coverage or less), and steady-state catalysis and redox-linked activation can be studied using slow scan rates or potential step techniques.

In this report, we will describe the general properties of the three generations of electrochemical biosensors and, especially, the recent progress in third-generation biosensors. We explain the concept of protein-film voltammetry and illustrate how it can be applied to third-generation biosensors in great detail.

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2 A Retrospect to the Development of Electrochemical Biosensors

After the pioneering work of Clark, Jr. on biosensors, his idea of a glucose oxidase (GOD) based-oxygen (O_2) electrode, despite its greatly reduced popularity in the present literature, still has a significant share in present commercial biosensors.[14] The ubiquitous oxygen acts as a mediator to communicate electrons between the enzyme and the electrode. Provided that the enzyme is immobilized at the electrode surface, this kind of biosensor can be directly used in test systems (whole blood, food, *etc*.). However, the first-generation biosensors have many drawbacks: the applied potential is too high, which adds chances for possible interference; the concentration of dissolved O_2 is fluctuant, which brings about systematic complexity; the tenuity of dissolved O_2 significantly decreases electrical currents, which influences the detection limit.[15-17]

The idea of artificial mediators was thus proposed to overcome the inherited drawbacks of a natural mediator. Some small redox-active molecules can diffuse in and react with the active site of the protein (enzyme), and diffuse out and react with the electrode surface, thus shuttling the electrons between the protein (enzyme) and the electrode. These mediators can efficiently decrease the applied potential of the biosensors,[18] and therefore decrease the interference from electrochemically oxidizable compounds present in real samples. Further progress in the development of second-generation biosensors was achieved by using flexible polymers onto which mediating functionalities were covalently bound.[15,19] However, the redox mediators used in conjunction with redox proteins are in no way selective, but rather general redox catalysts, facilitating not only the electron transfer between electrode and protein, but also various interfering reactions.

Since most "electrochemical" mediators lack selectivity, researchers have continued to look for better ways to accomplish electronic coupling between redox proteins (enzymes) and electrodes. Enzymes are famous for their superior selectivity and high affinity toward substrates. If a direct electron transfer of enzymes is available, they can work in a potential window close to the redox potential of the enzyme, itself, and thus be less susceptible to interfering reactions. The very first reports on direct electron transfer with a redox-active protein were published in 1977 when Hill[20] and Kuwana[21] independently showed that cytochrome *c* (cyt *c*) on gold and tin-doped indium oxide electrodes, respectively, exhibited virtually reversible electrochemistry, as revealed by cyclic voltammetry. Cyt *c* is a small redox protein that is active in biological electron-transfer chains, but has no enzyme properties. These first publications were soon followed in 1979 by reports that direct electron transfer was also possible for larger redox proteins with enzymatic activity (oxidoreductases or "redox enzymes"). It was shown that, in the presence of the enzyme substrate (molecular oxygen), laccase modified carbon[22] and peroxidase modified carbon electrodes[23] revealed direct electron transfer. These findings occurred some 10 years after the first paper on an enzyme-based amperometric biosensor was published.[24]

For biosensors based on direct electron transfer, *i.e*. third-generation biosensors,[25] the absence of mediators is the main advantage, providing them with superior selectivity, both because they should operate in a potential window closer to the redox potential of the enzyme and are therefore less prone to interfering reactions,[16] but also because of the lack of yet another reagent in the reaction sequence, which simplifies the reaction system. Another attractive feature of the system, based on direct electron transfer, is the possibility of modulating the desired properties of an analytical device using protein modification with genetic or chemical engineering techniques on one hand and novel interfacial technologies on the other hand.[5]

However, it is a pity that most enzymes cannot exhibit direct electron transfer at normal electrode surfaces. Great efforts have been taken in direct protein electrochemistry, but only a few proteins (enzymes) have been proved to exhibit direct electrochemistry, even with rapid development of surface technology and modern knowledge of chemically modified electrodes. To achieve direct electron transfer has been a "bottleneck" in developing third-generation biosensors. Up to now, extensive studies have been carried out toward finding novel surface functionalization, new electrode materials, and new proteins (enzymes) that have direct electron-transfer properties. Another way is to obtain a direct electrochemistry of proteins (enzymes) that are not natural electron-transfer proteins, or to exploit possible new enzyme activities for proteins (enzymes) that are known to exhibit direct electrochemistry at certain electrode surfaces by genetic or chemical-engineering techniques.[26,27] Nevertheless, a major disadvantage of this method is its high cost and technical difficulties (*e.g*., protein denaturation and renaturation, which are crucial for obtaining genetic-engineering products). Consequently, a new and promising approach, protein-film voltammetry, appears in view of its simplicity and effectiveness.

3 Protein-Film Voltammetry

PFV has proven to be a powerful way to obtain the direct electrochemistry of proteins as well as to investigate how electron-transfer coupling occurs at active sites, and how catalytic electron transfer through an enzyme is controlled.[7] In the protocol, the protein under investigation is adsorbed on a suitable electrode as a stable mono-/submonolayer film of molecules, each oriented for facile electron transfer. In this way, the problems of sluggish protein diffusion and the kinetics of interaction of the protein at the electrode are overcome, and it is also possible to exploit the unique abilities of electrochemical methods to detect and quantify the complex, redox-coupled chemical reactions that occur at the active sites.

PFV has several advantages over conventional voltammetry in which the protein molecules are free in solution, including the fine-tuned redox status of the entire sample, waveform definition, efficient screening for reactivities, sample economy, sensitivity and stoichiometry, and fast reactions. It has already led to many interesting and important discoveries in the field of protein chemistry linked directly to conformational change, ligand (substrate) binding, or ion (proton) transfer. Our studies have given an electrochemical approach to the investigation of ligand binding of myoglobin (Mb) in model membranes[28] and the allosteric effect of ATP on hemoglobin (Hb)[29] by PFV.

The electron-transfer reactivity of some proteins can be greatly enhanced when incorporated in certain membranes. It was observed that the heterogeneous electron-transfer rate constant for Mb was increased to about 1000-fold in liquid-crystal films of didodecyldimethyl ammonium bromide (DDAB).[30] Meanwhile, studies have shown that lipid membranes play a very active role concerning the functions of proteins.[31-33] For example, cyt *c* obtained *N*-demethylase activity by forming a supramolecule with a synthetic lipid bilayer membrane.[32] This approach can be viewed as "protein

engineering *via* noncovalent interactions". Specific microenvironments provided by lipid membranes might be responsible for the functional modulation or conversion of the proteins.[33] Therefore, the incorporation of proteins in membranes might not only enhance the electron-transfer reactivity in cases where proteins have sluggish electron transfer, but also lead to novel enzyme activity for a protein entrapped in the membranes.

Accordingly, PFV provides diversified choices for biosensor materials. Many excellent and innovative studies have recently been performed by a very diverse group of researchers. The choice of film materials has become more various, including water-insoluble surfactants, hydrogel polymers, lipids and nanoparticles. These films have been proved to be able to increase the direct electrochemical and catalytic properties of proteins. Besides the most studied heme-proteins, many other kinds of proteins have also been adopted to prepare third-generation biosensors, and thus more kinds of small substrates can be detected.

Here, we would like to summarize the recent work in third-generation biosensors based on the direct electron transfer of individual proteins by PFV in detail. A typical third-generation biosensor system based on PFV is shown in Fig. 1. The most widely used proteins in the third-generation biosensor field are cyt *c*, Mb, Hb and horseradish peroxidase (HRP).

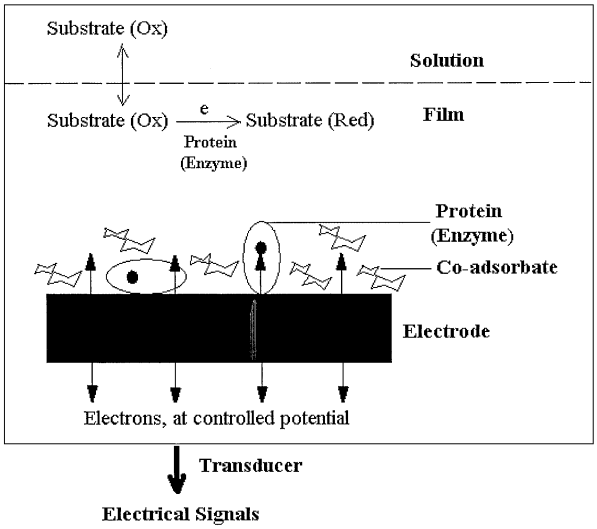

Fig. 1 A typical third-generation biosensor system based on PFV.

4 Proteins Employed in Third-Generation Biosensors

4·1 Cytochrome *c*

Cyt *c* is one of the most popular proteins for biochemical studies. It is a mono-heme protein, which participates in the electron-transfer chain both in mitochondria and chlorophyll. Generally, cyt *c* presents many advantages for use as a biosensor material: cyt *c* is easy to be obtained and is inexpensive; it is very stable and insensitive to the pH values, temperature, or organic environments. Until now, the direct electrochemistry of cyt *c* has been well examined in many membrane-bound states. Electrochemical and *in-situ* spectrophotometric studies of the interaction of cyt *c* with phospholipids have shown that the electrostatic interaction between cyt *c* and cardiolipin might lead to a rapid direct electron transfer.[34] The direct electrochemistry of cyt *c* embedded in films of anionic-type lipids was also obtained *via* ion-exchange.[35]

Although no catalytic activity of cyt *c* has been described in living systems, it was found to possess some peroxidase activity *in vitro*, especially when bound to lipid membrane.[36,37] The structure of the lipid is important to its peroxidase activity.[37] The possible reaction mechanism involves the generation of a high-valent oxioiron(IV) porphyrin radical cation from cyt *c* by the action of one molecule of hydrogen peroxide (H_2O_2). Other enzymatic activities, such as *N*-demethylase activity and cyt P450-like activity, have also been demonstrated upon incorporation in certain lipid membranes.[32,38]

Besides lipid membranes, cyt *c* has also been incorporated in other materials for the fabrication of biosensors. Many different self-assembled monolayers (SAMs) on gold electrodes, such as metallothioneins, thiolactic acid (TLA) and mixed-thiol (mercaptoundecanoic acid/mercaptoundecanol), have been reported.[39-41] A strong correlation between the electron-transfer rate constants and the hydrogen-bonding ability of the SAM was identified.[42] The direct electrochemistry and electrocatalysis of cyt *c* on deoxyribonucleic acid (DNA) film have also been well observed.[43,44] Cyt *c* can be deposited as a stable and electrochemically active film on a DNA-modified glassy carbon, gold, platinum, and transparent semiconducting, tin oxide electrodes. Moreover, two-layer modified electrodes containing cyt *c* and a DNA film were prepared by the deposition of cyt *c* on a DNA film-modified electrode, which was found to be electrocatalytically oxidation active for L-cysteine, ascorbic acid, NH_2OH, N_2H_4 and SO_3^{2-}.[43]

Recently, nanomaterials have aroused great interest in the study of electron transfer between proteins and electrodes due to their unique properties. The accumulation and reactivity of cyt *c* in mesoporous films of TiO_2 phytate and activated single-wall carbon nanotubes (SWNT) were documented.[45,46] Other studies have demonstrated that cyt *c* immobilized on colloidal gold modified carbon paste electrodes could also maintain its activity and electrocatalyze the reduction of H_2O_2.[47] The apparent Michaelis constant (K_m) value for this sensor was found to be 2.28 ± 0.17 mM, allowing measurements down to 0.01 mM H_2O_2. Our group has recently employed silver nanoparticles.[48]

4·2 Myoglobin

Mb (MW ~17000) is a water-soluble protein containing a single heme group. It plays an essential role in both oxygen storage and transportation in mammalian muscle. The electrochemistry of Mb at a bare indium oxide electrode has been reported. Nevertheless, its heterogeneous electron-transfer rate is slow and highly dependent on the purity of the protein and pretreatment of the electrode.[49-51] Cationic surfactant films, such as DDAB, were then successfully put into use.[52] It was later shown that the choice of the membrane was rather versatile. Mb exhibited nearly the same electron-transfer reactivity in many different membranes from natural lipids to artificial lipids.[30] Other studies have proposed that the surfactant molecules could interact with the electrode surface in a specific manner and anchor the protein molecules so as to align in a suitable orientation.[53] Besides, the direct electrochemistry of Mb was obtained on other interfaces, such as on a DL-homocysteine self-assembled gold electrode.[54] Furthermore, nanoparticles were used to improve the electron-transfer reactivity of the protein. The direct electron transfer for Mb assembled on nanocrystalline TiO_2 film has been reported; TiO_2 film could not only offer a friendly platform to assemble

protein molecules, but also enhance the electron-transfer process between protein molecules and the electrode.[55]

Many interesting phenomena in the sense of biosensors were observed in systems of Mb-membranes. Biosensors of trichloroacetic acid (TCA), ethylene dibromide (EDB), tetrachloroethylene (PCE), trichloroethylene (TCE), dichloroacetic acid (DCA), nitrite, O_2 and H_2O_2 were recently reported. These involved Mb depositing in various films,[56-61] including dihexadecylphosphate (DHP)-polycationic poly(diallyldimethylammonium) (PDDA) polyelectrolyte-surfactant complex films, polyacrylamide (PAM) hydrogel films, chitosan (CS) films, DDBA films and gluten films. As a good example, Mb-CS films were made by casting a solution of proteins and CS on pyrolytic graphite electrodes, which demonstrated both excellent direct electrochemistry and electrocatalysis to O_2, TCA, nitrite, and H_2O_2.

With the high stability and easy availability of Mb, it has also provided a powerful means for environmental monitoring and degradation of organohalide pollutants. It was found that Mb in a DDAB and polyionic complex, didodecyldimethylammonium polystyrenesulfonate film, effectively catalyzed the reduction of organohalide pollutants along with a significant lowering of the activation free energy, resembling the function of cyt P450 in microbial reductive dechlorinations in the environment.[62] Other enzyme activities of Mb in membranes were also noticed. Mb in both DDAB films and in layer-by-layer nanoassembled films with DNA showed nice enzyme activity toward styrene epoxidation.[63,64] The Mb-DNA membrane would be useful for monitoring pollutants, as well as in the electrochemical detection of DNA damage.[65] Moreover, poly(ethylene oxide)-modified Mb was prepared and adapted to applications in organic solutions[66] and at high temperature.[67]

4·3 Hemoglobin

Hb (MW ~64500) is a tetramer heme protein composed of two α and two β subunits. It functions as an oxygen vehicle in red blood cells. The electron-transfer reactivity of Hb is physiologically hampered, though it contains four hemes, which are known to act as electron-transfer centers in other proteins, such as cyt *c*.[68] On the other hand, it does undergo oxidation and reduction at the heme center in certain *in vivo* cases. For example, it takes part in the erythrocyte methemoglobin reduction pathway, which is a way to keep low levels of physiologically inactive methemoglobin in the circulating blood.[69]

Numerous efforts have been taken to obtain the electrochemical response of Hb at solid electrode surfaces. We found out that Hb, after being simply treated with dimethyl sulfoxide (DMSO), exhibits a direct electrochemical response at a pyrolytic graphite electrode.[70] Other materials, such as rhein,[71] and many kinds of biomimetic membranes, have been employed to obtain the direct electrochemistry of Hb. However, unlike some other small heme proteins, such as cytochromes, it is difficult for Hb to exhibit heterogeneous electron-transfer processes in most cases.[72] The reason for such low electron-transfer reactivity is clear, since Hb is not designed as an electron-transfer protein and the electroactive center of each subunit is deeply buried in the electrochemical insulted peptide backbone. Vitreoscilla sp. haemoglobin is a two-subunit hemoglobin, and it has demonstrated a better electron-transfer ability than four-subunit hemoglobins.[73]

Nanotechnology has provided a novel way to enhance the electron-transfer rates between hemoglobin and the electrode. As in the case of cyt *c* and Mb, nanocrystalline TiO_2 film has been proposed as a promising interface for the immobilization of Hb.[55] Not only has the direct electron-transfer process of Hb at a nanosized TiO_2 film electrode been achieved, but the inhibitive effect of nitric oxide (NO) on Hb has also been investigated.[74] Gold nanoparticle has been famous for its good biocompatibility. With the help of these gold nanoparticles, Hb can exhibit a direct electron-transfer reaction without being denatured.[75] To improve the stability of these particles, a kind of gold nanoparticle protected by lipid (DDAB) was invented.[76] The electron transfer of Hb at electrodes modified with colloidal clay nanoparticles has also been well studied due to its simplicity, effectiveness and low cost.[77]

Through non-covalent interactions with films, Hb not only displays enhanced electrochemical activity, but has also been functionally converted from an oxygen storage protein to a redox enzyme. Similar to Mb, Hb embedded in films of DDAB, polyion-surfactant DHP-PDDA, CS, dimyristoyl phosphatidylcholine (DMPC), polyacrylamide hydrogel, or poly(ester sulfonic acid) has displayed catalytic properties for the electroreduction of nitrite, O_2, TCA or organohalide.[58,60,78-81] Similar effects were often found in different films, and at the same time, different enzymatic activities were noticed in one film, which showed that the functional conversion of Hb was somewhat complex and independent of the structure of the membranes.

Hb has long been known to have some intrinsic peroxidase activities due to its close structural similarity with peroxidase. It is possible to improve its peroxidase activity by changing the structural orientation in the vicinity of its heme sites.[82] Considering the fact that a noncovalent interaction in a supramolecular assembly system significantly alters the heme orientation,[83] it might be a feasible way to functionally convert Hb to a peroxidase-like enzyme by introducing a membrane environment. SP sephadex is a chromatographic medium for protein purification. We proposed that Hb could exhibit a direct electron-transfer reaction after being entrapped in a Sephadex membrane; meanwhile, the peroxidase activity of the protein in the membrane was also greatly enhanced. Thus, an Hb/SP Sephadex membrane-based H_2O_2 biosensor was prepared.[84] Meanwhile, biosensors for H_2O_2 were also developed by depositing Hb in films of egg-phosphatidylcholine (PC), gluten, CS, kieselgubr clay and montmorillonite clay, *etc*.[60,61,85-89] It is noticeable that Hb encapsulated in films not only acquires many new properties, but can also be protected from environmental variations, such as the low pH of external solutions[90] and H_2O_2 inactivation.[91] Hence, it will be a very promising way to fabricate novel H_2O_2 biosensors. Besides, it was reported that Hb immobilized on an Au colloid-cysteamine-modified gold electrode also displayed the features of a peroxidase, and gave an excellent electrocatalytic response to the reduction of H_2O_2.[75]

We have also endeavored to construct direct protein-based biosensors for NO. NO has been recognized as a signal-transduction molecule, and the determination of NO has drawn increasing attention. NO has been shown to interact with the heme protein *in vivo*, and thus PFV provides a novel approach to study their interactions. We presented electrochemical evidence concerning the interaction between Hb and NO, making use of an Hb-DNA modified electrode.[92] A novel NO biosensor was accordingly proposed, which showed good sensitivity and nice stability.[93] Hb incorporated in other polyelectrolytes, such as polyethyleneimine (PEI) films, also demonstrated nice catalytic activities for NO detection.[94] PC is a component of a biological membrane, and provides a mimic environment for the functioning of proteins and enzymes. A NO biosensor based on Hb incorporated in a PC film and immobilized at a pyrolytic graphite electrode has also been

developed with the linear range of 0.1 – 300 μM and a detection limit of 0.1 μM.[95] Montmorillonite clay and gold colloid particles are also good materials for fabricating Hb-based biosensors.[96,97] Considering their good stability, nice selectivity, high sensitivity and easy construction, these biosensors have shown great promise for the rapid determination of traces of NO.

The concept of "fuzzy nanoassemblies"[98] provides a general and convenient way to fabricate multilayer protein films by consecutive electrostatic adsorption of alternative polyanions and polycations, which allows the design of a protein-film architecture to desirable specifications and thickness as well as good stability.[99] The electrochemistry of a layer-by-layer assembly of ultrathin films of hemoglobin and clay nanoparticles at a pyrolytic graphite electrode was investigated. $\{Clay/Hb\}_{(6)}$ film electrodes effectively catalyzed the electrochemical reduction of TCA, O_2, and H_2O_2.[100] We fabricated well-structured, multilayer Hb/DNA films *via* a layer-by-layer self-assembly technique. The electron-transfer rate of Hb was greatly facilitated, and this Hb-containing film demonstrated nice catalytic capability toward NO, which showed promise in biosensing fields.[101]

4·4 Horseradish peroxidase

HRP (MW ~44000) is of great biotechnological interest due to its wide use in biosensors.[102] It is a kind of peroxidase that catalyzes the one-electron oxidation of a variety of substrates at the expense of H_2O_2. Much research has been conducted on the catalytical behavior of HRP and HRP-based biosensors. Various amperometric enzyme electrodes for H_2O_2 determination[103-108] were prepared with HRP immobilized in sulfonated polyaniline-polycation, polyvinylferrocenium (PVF^+), poly {pyrrole-co-[4-(3-pyrrolyl)butanesulfonate]}, polypyrrole, TiO_2 sol-gel matrix on a electropolymerized phenazine methosulfate (PMS), or other organically modified sol-gel glasses. Several HRP based biosensors for the detection of small molecules other than H_2O_2 have also been developed in recent years, which include polyphenol, *t*-butyl hydroperoxide, 2-butanone peroxide, cumene hydroperoxide, *t*-butyl peracetate, *etc*.[109,110]

However, since HRP is not a physiologically electron-transfer protein, its *in-vitro* electron transfer is rather sluggish. Compared with direct electron transfer in the presence of a substrate, the "true" direct electrochemistry of HRP in the absence of a substrate was more difficult to obtain. Biomimetic films have first been employed to reach that goal. Quasi-reversible electron transfer was observed when HRP was incorporated in DDAB, DMPC, DHP, ionomer poly(ester sulfonic acid), Eastman AQ29, *etc*.[111-113] In all of these cases, HRP retained its peroxidase behavior and H_2O_2 biosensors were therefore developed.

An inorganic material, such as kieselguhr clay,[114] has also been shown to be useful for fabricating third-generation H_2O_2 biosensors. The nice sensitivity and good stability of this H_2O_2 biosensor has also shown the effectiveness of this protein-film voltammetry technique. In addition, gold nanoparticles and carbon nanotubes are other promising materials used to fabricate novel HRP-based H_2O_2 biosensors.[115-118]

4·5 Other proteins

GOD is a flavoprotein composed of two identical subunits, each having a flavin-adenine dinucleotide (FAD) co-subunit as its redox center. It catalyzes the formation of gluconic acid from glucose, while the enzyme itself is turned from GOD (FAD) to GOD ($FADH_2$). Numerous biosensors based on GOD are typically illustrated as first- or second-generation biosensors. GOD is reported to be electroactive only in limited cases, while in most of these cases the enzymes have lost their enzymatic activities.[119,120] Recently chemical-engineering techniques have been widely used, and excellent results have been obtained[27,121] with major disadvantages of high cost and technical difficulties. Nanomaterials have proved to be effective. The electrochemical behavior of GOD in carbon nanotubes showed that the prosthetic FAD group was still attached to the apoenzyme with no loss of enzyme activity.[122] A similar result was also obtained on a graphite paste electrode.[123]

Cellobiose dehydrogenase (CDH) is an extracellular fungal enzyme with two domains, one containing FAD and the one containing heme. Direct electrochemistry was achieved between the heme of CDH and a gold electrode modified with cystamine or 3-mercaptopropionic acid. The electrocatalytic behavior of CDH was demonstrated as well by the addition of an enzyme substrate, cellobiose.[124]

Catalase is another redox enzyme. It has been known that heme catalases are tetramers. Each of the four active sites consists of an iron-protoporphyrin IX prosthetic group with a tyrosinate axial ligand. This enzyme functions in two ways: "catalytically", decomposing H_2O_2 into water and O_2, and "peroxidatively", oxidizing alcohol, formate or nitrite with H_2O_2. Its direct electrochemistry has been studied by embedding the enzyme in a liquid-crystal film of DDAB at pyrolytic graphite electrodes.[125] The direct electrochemistry of catalase adsorbed from DMSO on glassy carbon electrodes was also studied, which revealed that catalase could act as a possible catalyst of the electrochemical reduction of oxygen.[126]

NarGHI is a kind of quinol-nitrate oxidoreductase. This membrane-anchored protein directs electrons from quinol oxidation at the membrane anchor, NarI, to the site of nitrate reduction in the membrane extrinsic [Fe–S] cluster and Mo-bis-MGD containing dimer, NarGH. Liberated from the membrane, NarGH retains its nitrate reductase activity and forms films on graphite or gold electrodes, within which a direct and facile exchange of electrons between the electrode and the enzyme occurs. PFV has been used to define the catalytic behavior of NarGH in the potential domain, and a complex pattern of reversible, nitrate concentration dependent modulation of activity has been resolved.[127]

Quinohemoprotein alcohol dehydrogenase from Gluconobacter sp. 33 is a newly isolated, purified, and characterized alcohol dehydrogenase. The enzyme was studied when simply immobilized onto carbonaceous surfaces in order to establish its characteristics and suitability for sensor development. The sensor design was based on a direct-electron transfer pathway.[128]

Sulfite dehydrogenase from Starkeya novella is an alphabeta heterodimer comprising a 40.6 kDa subunit (containing the Mo cofactor) and a smaller 8.8 kDa heme subunit. The enzyme catalyzes the oxidation of sulfite to sulfate with the natural electron acceptor being cyt *c* (550). Its catalytic mechanism is thought to resemble that found in eukaryotic sulfite oxiclases. Using PFV and redox potentiometry, both Mo- and heme-centered redox responses have been identified from the enzyme immobilized on a pyrolytic graphite working electrode. Upon the addition of sulfite to the electrochemical cell, a steady-state voltammogram was observed and a K_m of 26 μM was determined for the enzyme immobilized on the working electrode surface.[129]

Recently, bi-enzymatic biosensors based on direct electron transfer between electrodes and two enzymes, often oxidase and peroxidase, have attracted ever-more interest. When oxidizing

glucose, oxidase produces H_2O_2. Peroxidase can be coupled with the oxidase to catalyze the generated H_2O_2, and the resulting signal from catalytic reduction can be measured. DMPC films incorporating GOD and peroxidases have demonstrated good analytical responses to glucose.[130] That provides evidence for the feasibility of dual enzyme-lipid films for biosensor fabrication. Another bi-enzymatic biosensor was also constituted through a GOD-entrapping cellulose triacetate membrane, layered onto a polymeric membrane embedding HRP, which was previously blocked onto a pyrolitic graphite electrode.[131] This biosensor showed good inertness and stability toward potential electrical interferents.

5 Conclusion

PFV has provided a promising method to achieve the direct electron transfer of proteins (enzymes), and thus for the study and fabrication of third-generation biosensors. By replacing the kind of proteins (enzymes) used, trying new electrodes, biomimetic membranes and nanomaterials, and using various modification methods, researchers are now developing third-generation biosensors with higher selectivity and a lower detection limit for the detection of ever-more species. Further advances in this field will play an increasingly important role in the sensor community. Their importance, complexity, and diversity will only increase in the years ahead.

6 References

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