Protein Based Electrochemical Biosensors for H$_2$O$_2$ Detection Towards Clinical Diagnostics

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**Abstract**: Hydrogen peroxide (H$_2$O$_2$) has a diverse array of physiological and pathological effects within the living cells depending on the extent, timing, and location of its production. Detection of H$_2$O$_2$ is important in food industry, clinical diagnostics, and environmental monitoring at lowest levels. Electrochemical biosensors are efficient as they can analyze biological sample by direct conversion into an electrical signal. Electrochemical sensors based on direct electron transfer (DET) of proteins were developed to achieve fast electron transfer by avoiding free-diffusing redox species with improved sensitivity. We summarize the prerequisites for the DET of proteins for immobilization on the electrode surfaces with recent developments in development of H$_2$O$_2$ sensors and future prospects in this field.

**Keywords**: Proteins · Clinical diagnostics · Biosensors · Hydrogen peroxide

1 Introduction

Hydrogen peroxide (H$_2$O$_2$) is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress related state [1]. H$_2$O$_2$ mediated pathways have been linked to asthma, inflammatory arthritis, atherosclerosis, diabetic vasculopathy, osteoporosis, and a number of neurodegenerative diseases [2–4]. H$_2$O$_2$ is a by-product of several enzymatic reactions that can be used as diagnostic tools for detection of the onset of various biological conditions [5]. H$_2$O$_2$ is not only involved in phagocytosis but also acts as insulin that helps the transport of sugar through the body [6, 7]. Another important application of H$_2$O$_2$ is that it creates intracellular thermogenesis, a warming of cells which is absolutely essential to life’s processes [8]. Hence, it is considered to be an important analyte because of its importance in clinical diagnosis. H$_2$O$_2$ is produced by all cells of the body for many different physiological reasons, the granulocytes produce H$_2$O$_2$ as a first line of defense against bacteria, yeast, virus, parasites, macrophages, and most fungi [9, 10]. It is involved in metabolic pathways which utilize oxidases, peroxidases, cyclooxygenase, lipoxygenase, myeloperoxidase, catalase and many other enzymes. H$_2$O$_2$ is often used in therapy, as well as other treatment modalities, on a routine basis, usually given by intravenous injection [11, 12]. H$_2$O$_2$ would help arthritis because of its ability to supply oxygen to oxygen-hating organisms causing arthritis (Streptococcus viridans). However, it is usually treated as an intermediate or by-product of metabolism and considered of minor significance in metabolic pathways except as it relates to biochemical disruption, tissue or cellular damage [13, 14]. Wlassoff et al. [15] reported a new treatment of cancer based on the innate overproduction of H$_2$O$_2$ in cancer cells. It is described that, Hydrogen peroxide serves as a prodrug in the presence of transition metal ions, such as iron delivered by ferrocene. Under the effect of ferrocene, hydrogen peroxide is split into hydroxyl anions and highly reactive hydroxyl radicals. These radicals cause oxidative DNA damage which induces apoptosis leading to elimination of cancer cells. Maramag et al. [16] reported the influence of H$_2$O$_2$ on the treatment of prostate cancer. Many studies describe the protective role of vitamin C (ascorbic acid) against cancer development and in treatment of established cancer. Vitamin C inhibits cell division and growth through the production of hydrogen peroxide, which damages the cells probably through an as yet unidentified free radical(s) generation/mechanism. Further, the role of H$_2$O$_2$ and hydroxyl radical formation plays a major role in the killing of Ehrlich tumor cells [17]. Also, the production of potent oxidizing species, including the hydroxyl radical (OH), has been demonstrated during treatment of intact human MCF-7 breast cancer cells with doxorubicin [18]. H$_2$O$_2$ has wide application in surgery and dentistry, particularly in the debridement of wounds [19]. Its use in sensitive areas such as carotid artery and vein indicates its value in safety cleaning wounds as shown in Figure 1. Thus direct or indirect detection of hydrogen peroxide is one of the central themes in the design and fabrication of various biosensors.

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In electrochemistry, $\text{H}_2\text{O}_2$ can be either oxidized or reduced directly at ordinary solid electrodes. However, these processes in analytical applications are limited by slow electrode kinetics and high overpotential which will reduce the sensing performance and may suffer from large interferences from other existing electroactive species in real samples such as ascorbate, urate, and nitrates [20,21]. Hence, the research on $\text{H}_2\text{O}_2$ detection is mainly focused on electrode modifications in order to decrease the overpotential and increase the electron transfer kinetics. Towards this, several types of nano-biomaterials such as redox proteins [22], transition metals [23], metal oxides [24], metal porphyrin [25], redox polymers [26], and carbon nanotubes [27] have been employed to perform electrocatalytic $\text{H}_2\text{O}_2$ detection. Furthermore, the size and structure of biomaterials (proteins) can be tailored for designing a novel sensing platform and enhancing sensing performance [28]. Highly sensitive and selective determination of single chemical at extremely low concentrations is important for environmental monitoring, drug screening and for clinical diagnostics. Recently, a detection method which is based on the development of nanopore for the precise detection of single chemicals has been reported [29–31]. It consists of a hub of the bacteriophage phi29 DNA packaging motor as a connector consisting of 12 protein subunits encircled into a 3.6 nm channel for the passage of dsDNA to enter during packaging and to exit during infection.

The electron-transfer rate between a redox protein and electrode surface is usually slow, which is the major obstacles of the electrochemical system. Significant efforts have been made to understand the biological systems control which electron transfer processes are possible (i.e., reduction potentials) and how fast they will occur (i.e., rate constants) [32]. To achieve efficient electrical communication between redox protein and electrode there are many challenging objects has to overcome [33]. The factors that affect the properties of these important biological electron transfer sites are generally considered as either intrinsic (an inherent behavior or property of itself) or extrinsic (modulated external factors) to the active site [34]. Apart from electron mediators the inherited property of direct electron transfer property of protein has received much attention to understand the biochemical process and also towards development of novel bioelectronic devices [35,36].

Here in this review, we analyze different protein immobilization methods for better electron transfer between protein to surface and also study the properties of proteins based direct electron transfer towards the electrocatalytic reduction of $\text{H}_2\text{O}_2$. Also this study summarizes the recent advances in protein based direct electron transfer systems and gain an insight into the materials used in the electrocatalytic sensing of $\text{H}_2\text{O}_2$ towards clinical diagnostics.

2 Methods of Protein Immobilization

Understanding protein adsorption at solid surfaces is important for the development of many fields in nanotechnology, such as biomaterial production for medical devices, drug delivery systems, or in vitro diagnostics. Protein adsorption to solid surfaces in aqueous environments is crucial and very complex process which is driven by the protein-surface forces including van der Waals, hydrophobic and electrostatic forces. Proteins are usually adsorbed on the surface of solid surface in a disordered orientation and suffer from conformational changes that lead to the inaccessibility of the metal center to the underlying electrode surfaces for enzymatic reactions [37]. The conformational change of protein in the adsorbed state will differ with the type of protein, depending on the substrate including hydrophobicity/hydrophilicity, environmental conditions such as temperature, $\text{pH}$ and ionic strength of the solution. Hence, controlled immobilization methods are required to retain the native properties of the protein [38]. The basic requirements for a successful immobilization requires; 1) stability and specific affinity of the proteins for the surface 2) uniformity on the surfaces 3) pos-
sibility to control the immobilization density of the immo-
ibilized proteins 4) preserving the native biological func-
tions of proteins 5) orientation of the protein to achieve
maximum binding density.

Most immobilization methods developed so far in-
volve in the modification or coating of the surface with
proper linkers to change/enhance the surface property to
provide functional groups for binding the protein. Alter-
natively, immobilization of proteins on a bare surface
with no modification exploits specific interactions be-
tween the protein and the surface, such as immobilization
on the Au surface via thiol (sulfhydryl) groups and neces-
sitates using an affinity peptide that is specific to the par-
ticular surface.

2.1 Physisorption

This method of immobilization of protein is based on the
physical adsorption of protein on the solid surfaces and
hence it causes little or no conformational change of pro-
tein or loss of its properties. Physisorption is based on the
van der Waals interactions between the adsorbate and the
substrate and also between the adsorbed molecules [46].
Physical adsorption generally leads to dramatic changes
in the protein microenvironment, and normally involves
multipoint protein adsorption between a single protein
molecule and a number of binding sites on the immobili-
sation surface. Moreover, even if the surface has a uni-
form distribution of binding sites, physical adsorption
could lead to heterogeneously populated immobilized
proteins. This has been known for unfavorable lateral in-
teractions among bound protein molecules. In general,
this kind of adsorption is completely nonspecific, i.e.,
almost all gases can physisorb under the certain condi-
tions to almost all surfaces. Further, physorbed mol-
ceules can leave the surface after certain amount of time.
The session of physisorption is an exothermic process
occurs at lower magnitudes of temperature at an enthalpy
of the order ~20 kJ mol\(^{-1}\) due to weak van der Waals
forces of attraction. This method has advantages such as,
the procedure require no expertise or special equipment
also it does not require surface treatment but it suffers
from protein leaching from the immobilization support
[47].

2.2 Chemisorption

Chemisorption requires a specialized substrate that
allows for a chemical linkage between the biomolecule
and the surface and this method often requires the expert-
tise of a chemist. Here in this case, the enthalpy is around
an order of magnitude ~200 kJ mol\(^{-1}\) which is much
higher than physisorption. Bifunctional silane/thiol cou-
pling reagents are usually used to form a chemical bond
with the side functional group of the amino acid/ carbox-
ylic acid residue of the protein at one end and with the
glass/Au surface at the opposite end [48]. Basically there
are three different procedures in coupling protein to the
underlying surface. In the first method, the substrate is
activated with some chemical linker and then the protein
is allowed to bind the substrate through the chemical
bonding. In the second method, biomolecules such as pro-
teins or antibodies are activated to sulfur containing mol-
ceules and allowed to self-assemble on the substrate. Fi-
nally, the third method involves in the protein binding to
substrate by ligand-receptor mechanism such as lock and
key interaction. The chemical attachment involves more
drastic conditions for the immobilization reaction than
the attachment through adsorption [49]. The covalent and
coordinate bonds formed between the protein and the
support can lead to a change in the structural configura-
tion of the immobilization protein. Such a change in the
enzymatic structure may lead to reduced activity, unavail-
ability of the active site of an enzyme for the substrates, altered reaction pathways or a shift in optimum pH.

2.3 Site-Directed Immobilization

In both the physisorption and chemisorption techniques, proteins attach to surface in different orientations leads to considerable activity loss. Hence, good orientation techniques have been proposed to increase the efficiency of biotransformation and to enhance the sensitivity of biological reactions. To achieve well-oriented immobilization site-specific immobilization was proposed. The approach to site-specific immobilization requires functionalization of the target molecule, tailoring the surface, or both. The strategy is to develop recombinant proteins incorporating histidine residues in either amino or carboxyl end of the protein or cysteine residues on specific parts of the protein [50,51]. When protein has cysteine residues in the correct regions, protein could be immobilized and addressed on the Au surface with good orientation and coverage. Table 1 presents the various protein immobilization methods on the solid surfaces to achieve intrinsic properties of the protein for better performance towards novel electrochemical sensors.

3 Direct Electron Transfer of Proteins

Many of the fundamental processes in nature depend on upon the redox processes of constituent biomolecules. Cell respiration involves the stepwise oxidation of organic substrates through a chain of redox reactions. To understand these biological processes, electrochemical methods offers as a prominent technique for examining the electron transfer properties. The molecules of particular interest are redox-active proteins and enzymes. Now-a-days, studies of direct electrochemistry of redox proteins at the electrode-solution interface are of great interest. There are two main approaches that can be imagined in exploring suitable and effective coupling between a protein and an electrode: a) direct and unmediated electron transfer; b) indirect and mediated electron transfer [52]. The most important and challenging, is certainly, the first option as it is based on the aspect of protein electrochemistry that efforts have been concentrated till date. Electrode surfaces have been sought at which a freely diffusing redox protein can take part in rapid and direct electron transfer.

The studies on direct electron transfer are a simple and informative means for understanding the kinetics and thermodynamics of biological redox processes. Further, it provides a model for the study of the mechanism of electron transfer between enzymes in biological systems, and establishes a platform for developing new kinds of biosensors.

There are several factors that influence the direct electron transfer between the electrodes and proteins. Generally these factors include; a) electroactive prosthetic groups buried deep within the protein structure; b) adsorptive denaturation of proteins onto electrodes, c) unfavorable orientations of proteins at electrodes. Several strategies and works have been reported for the direct electron transfer of proteins from its first reports on the direct electrochemistry of redox protein in 1977 [53,54]. Since then achieving reversible, direct electron transfer between redox proteins and electrodes without using any mediators and promoters had made great accomplishments. Chemical modification of electrode surfaces is not the only route towards rapid and reversible electron transfer between an electrode and redox protein. A general requirement for such an interaction is that the surface of the electrode is electrostatically compatible with the surface of the protein, particularly that part implicated in the electron transfer process. In fact, direct electron transfer has now been achieved between a variety of electrode surfaces and a range of redox proteins.

3.1 Direct Electron Transfer of Cytochrome c

Mitochondrial cytochrome c (cyt c hereafter) is a protein ubiquitous to all eukaryotic organisms and is the most widely studied protein with regard to its electrochemical properties because of its high solubility in water compared with other redox-active proteins. Cyt c transfers an electron from complex III to complex IV, membrane-bound components of the mitochondrial electron-transfer chain. Cyt c takes part in electron transfer within the respiratory chain in mitochondria. Electrons are transferred
from NADH and FADH₂ to dioxygen by proteins located in the inner mitochondrial membrane. These systems are organized into multi-protein complexes, of which complexes I (NADH-ubiquinone reductase) and II (succinic-ubiquinone reductase) accept electrons from matrix substrates (NADH and FADH₂, respectively) and reduce ubiquinone to ubiquinol, complex III (ubiquinol-cytochrome c oxidoreductase) catalyzes the transfer of electrons from ubiquinol to cyt c, and complex IV (cytochrome c oxidase) transfers electrons from cyt c to dioxygen. This whole process is coupled in the synthesis of ATP [55]. It is known that the lysine residues surrounding the heme crevice of the protein plays a vital role in binding interactions and electron transfer with its redox partners. Cyt c is an excellent model for studying the electron transfer of typical enzymes from a structure point of view. Further, cyt c is known to have some intrinsic peroxidase activity due to its close similarity to peroxidase. In addition to these properties, cyt c has several advantages for use as a biocatalyst. (a) The heme prosthetic group is covalently bound to the protein. This property may be important for catalysis in the presence of organic solvents; cyt c does not lose its heme catalytic group in these systems, while peroxidases do; (b) cyt c is active over a wide range of pH [from pH 2–pH 11]. No other enzyme is active over such a pH range; (c) cyt c is able to perform biocatalytic reactions even at higher temperatures and after chemical modification its thermo stability will be highly increased; and (d) cyt c is inexpensive. Cost and stability are the two main factors for biocatalysis on a large scale. The direct electrochemical measurements indicated that the reduction potentials ($E^\text{red}$) at pH 7 and 25°C is in the range of 0.260–0.280 V [56].

### 3.2 Direct Electron Transfer of Myoglobin

The biological function of myoglobin (Mb) is to buffer the oxygen concentration in respiring tissues. The affinity of myoglobin for oxygen lies between that for hemoglobin and for the cytochromes that make use of molecular oxygen in oxidative respiration. It is a kind of heme protein containing a single polypeptide chain with an iron heme as its prosthetic group [57]. The physiological function ofMb is to store dioxygen and increase the diffusion rate of dioxygen in the cell. Although Mb does not function physiologically as an electron carrier, it undergoes the oxidation and reduction process in the respiratory system. Thus, its electron-transfer reactions play essential roles in biological processes. It is an ideal model molecule for the study of electron transfer reactions of heme proteins, biosensing, and electrocatalysis. Towards the usage of Mb in electrochemical detection systems, Mb is a water-soluble heme-containing protein present in most mammals and vertebrates, and has been used extensively for investigating electron transfer and electrocatalysis with the protein immobilized on an electrode surface. Its main function is to store oxygen and enhance diffusion in the muscle. This globular protein has a single 153 or 154 amino acid chain with a molecular weight of ~17 kDa [58]. It is a versatile protein with high tolerance for chemical and mechanical environments and can be easily expressed and purified in large quantities in E. coli. Another important feature is, it can accommodate various mutations without any adverse effects on its conformational and functional properties. Mb contains a single iron-porphyrin center that can accommodate ferrous, ferric or ferryl oxidation states within this heme active site moiety.

Even though a wide range of reports is available for immobilization of proteins for carrying out direct electron transfer and electrocatalysis with electrodes, a direct comparison involving active site orientation of monolayer and multilayer covalent attachment of proteins is absent. This is mainly due to the difficulty to achieve a site-directed covalent tagging of the native conformation of Mb on the surface with the full control over active site orientation. Furthermore, formation of both a monolayer as well as a multilayer on the electrode surface is a challenging task. However, several researchers are working on Mb immobilized electrodes to achieve the direct electron properties towards the development of novel electrochemical sensors.

### 3.3 Direct Electron Transfer of Hemoglobin

Hemoglobin (Hb) is a heme-containing protein that consists of “globin fold” and it reversibly binds to molecular oxygen. Hb are found in human erythrocytes (red blood cells) with a concentration approximately 30% (w/v) or 20 mM in heme. Erythrocytes take up approximately 40–45% of the blood volume (hematocrit) and hence 100 mL of human blood contains approximately 15 g of Hb. It has a molar mass of approximately 67,000 g/mol and comprises of two α- and two β-subunits, each containing a heme or Fe(II)-protoporphyrin IX. Since each heme can bind to oxygen molecules, four molecules of oxygen bind to Hb tetramer. Several studies were performed on the electrochemical behavior of heme proteins to understand the properties and their biological activity. Hb is ideally used for the study of electron transfer reactions of heme proteins because of its commercial availability and its cost [59,60]. Further, Hb possesses functional groups that can be readily oxidized or reduced by chemical redox agents; it does not easily undergo facile redox reactions at electrodes. Unlike other small heme proteins such as cyt c, it, however, is difficult for Hb to exhibit heterogeneous electron-transfer process in most cases, which means Hb exhibits such a slow electron transfer that no useful currents appear at conventional electrodes, even when rather large overvoltages are applied due to its extended three dimensional structure and resulting inaccessibility of the electroactive centers as well as its strong adsorption onto the electrode surface for subsequent passivation [61].
3.4 Direct Electron Transfer of Ferredoxin

Ferredoxins (Fdx) from are one electron carrier iron-sulfur proteins that function as electron transport chains and oxidoreduction reactions. The Fe-S center takes part in single electron transfer reaction in which one Fe atom undergoes oxidoreduction between Fe$^{2+}$ and Fe$^{3+}$. Fdx are small, soluble, generally very acidic proteins that are involved mainly as electron carriers of low oxidation-reduction potential in fundamental metabolic process. Iron-sulfur proteins (non-heme iron proteins, Fe-S) which may contain one, two, three or four Fe atoms linked to inorganic sulfur atoms and/or through cysteine-SH groups to the protein molecule. Rubredoxins (Rd) comprises of single iron-sulfur cluster without acid-labile sulfur that are characterized by having iron in typical thiolate coordination, i.e., an iron center surrounded by four cysteine residues or sulfur-containing ligands. Oxidized rubredoxin usually exhibits the characteristic EPR spectrum of high-spin Fe(III) ion in a rhombic ligand field, $g = 4.3$; the reduced form gives no discernible EPR signal. Only negative redox potentials at pH 7 have been noted for those rubredoxins presently characterized. Desulfoferredoxin is a variant rubredoxin with a higher symmetry and distinctive EPR spectrum with $g$-values of 7.7, 4.1 and 1.8. The *Spinach* Ferredoxin contains Fe atoms in [2Fe-2S] redox site which are bridged by two sulfides and the tetrahedral coordination of each iron completed by cysteine residues. Fdx contains clusters of two iron atoms and represented as [2Fe-2S]$^{2+}$ with 97 amino acid residues (AA) which have a molecular weight ranging from 11000 to 11900 [62]. One electron reduction of the [2Fe-2S]$^{2+}$ site occurs at one of the iron atoms to give the reduced [2Fe-2S]$^{+}$ site. In the oxidized state, both iron atoms are in a similar chemical state, which appears from the chemical shift and quadrupole splitting to be high-spin Fe$^{3+}$. In the reduced state the iron atoms are different and the molecule appears to contain one high-spin Fe$^{3+}$ and one high-spin...
Fe$^{3+}$ atom. In the oxidized state the two high-spin ferric iron atoms where only one of these iron atoms accepts an electron on reduction. The spins of the iron atoms are coupled in such a way that there is no net spin in the ground state ($S=0$). When the ferredoxin is reduced one electron is transferred to the specific iron atom, which then becomes high-spin ferrous; thus in the reduced state there is one high-spin ferrous and one high-spin ferric iron atom [63]. Figure 2 illustrates the model envisages for the iron-sulfur clusters in different ferredoxin protein molecules. The Desulfovibrio gigas ferredoxin, a sulfate reducing organism, has a major role in sulfate reducing bacteria (SRB) metabolism and contains a [3Fe-4S] redox site. The [3Fe-4S]$^{1+}$ cluster undergoes a typical redox processes, accepting a total of 3 electrons: [3Fe-4S]$^{1+}$->[3Fe-4S]$^{0}$->[3Fe-4S]$^{2−}$/. The electrochemical behavior of this protein is complex and several transitions are observed in the different redox regions (one at −130 mV and at −690 mV). Apart from Fe in the cluster the disulfide (S–S) bridge can accept two electrons [64].

### 3.5 Direct Electron Transfer of Horseradish Peroxidase

Horseradish peroxidase is an important heme-containing enzyme obtained from plant source. It has attracted the attention of many researchers from a variety of disciplines because of its practical and commercial applications. Advances in understanding the structure and catalytic mechanism of horseradish peroxidase have been made using protein engineering and other techniques. Horseradish peroxidase is not one enzyme, but a group of large family of isoenzymes which have different molecular forms of the same enzyme that catalyze the same biochemical reaction but have distinct physical, chemical and kinetic properties arising from small differences in their amino acid sequence. HRP-C is the most abundant isoenzyme isolated from horseradish root. Horseradish Peroxidase is a heme protein with 308 amino acid residues. The N-terminal residue is blocked by a pyrrolidenecarboxyl residue that appears to be buried inside the polypeptide chain. The C-terminal peptides were sequenced with and without a serine residue, indicating a rather labile Asn-Ser peptide bond [65]. HRP-C contains iron (III) protoporphyrin IX (ferri-protoporphyrin IX), i.e. a heme group, as a prosthetic group in its active site. In addition to the four coordination positions with the nitrogen atoms of the porphyrin pyrrole rings, the heme iron has two axial coordination sites (the fifth and sixth positions) where binding can also occur. Horseradish peroxidase is one of the most widely used enzymes in analytical applications. Due to its characteristics, HRP meets all the requirements for a successful use in analytical systems, apart from that the ability of HRP to catalyze the oxidation of numerous chromogenic substrates enables the use of spectrophotometric detection systems, including fluorescence and luminescence, opening way to a wide range of procedures.

### 4 Applications of H$_2$O$_2$ Biosensors Based on Direct Electron Transfer of Protein

Biosensors have shown great potential towards health care and environmental monitoring systems. The crucial elements in the performance of the biosensor mainly depends on the components, among which is the active matrix materials (the layer between the recognition layer of biomolecules and transducer) plays a vital role in establishing good stability, sensitivity and the endurance of a biosensor. Several studies have led to the rapid development of wide range of biosensors with improved detection limits [66]. Further, with the advancements in nanotechnology and in the field of engineering much improved with miniature biosensing systems have made to possible. The operation principle of a biosensor is shown in Figure 3.

The biosensors which are based on direct electron transfer of proteins, the redox state of the analyte or associated species is altered by either intermediating storing the redox equivalents in a redox protein-integrated prosthetic group or transfer of electrons between a suitable co-substrate within the active site. The advantage of biosensors which are based on direct electron transfer is mainly the absence of mediators which makes these biosensors having better selectivity and less prone to interference [67]. Further they have an advantage that there is a prospect to modulate the desired properties by protein modification by recombinant engineering towards novel biosensor devices.

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![Fig. 3. Operation principle of biosensing detection system.](image-url)
Electron transfer is a fundamental reaction in biological systems and it is very important to understand the biological function and to design synthetic energy transduction systems. Basically, electron transfer can be considered as a transition between two electronic states, donor (D) and acceptor (A). According to Marcus theory, the electron transfer rate is determined by the electronic coupling between D and A ($V_{DA}$), the reaction free energy ($\Delta G$) and the reorganization energy ($\lambda$) by the following Equation 1 [68]

$$k_{ET} = \frac{2\pi}{h} \frac{V_{DA}^2}{\sqrt{4\pi\lambda k_B T}} \exp \left( -\frac{(\lambda + \Delta G^2)}{4\lambda k_B T} \right)$$

(1)

Three key parameters, electronic coupling $V$, driving force $\Delta G$, and reorganization energy $\lambda$, determine the ET rate. $\Delta G$ is defined through the difference of the D and A redox potentials. Further, these quantities may significantly deviate from their values measured in aqueous solutions or in organic solvents. The electronic coupling $V_{DA}$ decay exponentially with the distance $R$ between D and A

$$V \sim V_0 \exp \left( -\frac{\beta}{2}(R - R_0) \right)$$

(2)

The parameter $\beta$ is determined by the superexchange interaction of D and A with their surrounding [69]. ET can also occur through incoherent hopping including transiently populated electronic states localized on a bridge along with direct and bridge-mediated super exchange between donor and acceptor. Further, if the prosthetic group is deeply buried inside the molecule then the direct electron transfer with high rate is unlikely because of the exponential decrease of the tunneling probability with the increase of the distance between the redox partners.

There are in principle two experimental approaches to establishing whether DET is occurring between redox enzymes and electrodes [70]:

a) Indirect evidence based on observing a catalytic response current in the presence of the enzyme substrate.

b) Direct evidence from observation of independent electrochemical activity of the redox cofactor comprising the active site in the absence of substrate.

The electrochemical reactions occur at bare working electrode often suffer from interferences or surface fouling byproducts arising in the follow-up reactions, linked with the main electrochemical process. Modified electrodes can be a solution towards this which avoids the drawbacks of bare electrodes such as adsorption of molecules, unpredictable surface reactivity and sluggish kinetics.

Electrochemical biosensors consisting of enzymes illustrating the three generations in the development of biosensor (a) first generation electrode utilizing the $\text{H}_2\text{O}_2$ produced by the reaction; (b) second generation electrode utilizing a mediator (ferrocene) to transfer the electrons, produced by the reaction, to the electrode; (c) third generation electrode directly utilizing the electrons produced by the reaction [71] are shown in Figure 4. The following reaction occurs at the enzyme in all three biosensors:

Substrate ($2\text{H}^+$) + FAD-oxidase $\rightarrow$ Product + FADH$_2$-oxidase

(3)

This is followed by the processes:

(a) Biocatalyst : FADH$_2$-oxidase + $\text{O}_2$ $\rightarrow$ FAD-oxidase + $\text{H}_2\text{O}_2$

(4)

Electrode : $\text{H}_2\text{O}_2$ $\rightarrow$ $\text{O}_2 + 2\text{H}^+ + 2e^-$

(5)

(b) Biocatalyst : FADH$_2$-oxidase + 2 Ferricinium$^+$ $\rightarrow$ FAD-oxidase + 2 Ferrocene + 2$\text{H}^+$

(6)

Electrode : 2 Ferrocene $\rightarrow$ 2 Ferricinium$^+$ + 2$e^-$

(7)

(c) Biocatalyst/Electrode : FADH$_2$-oxidase $\rightarrow$ FAD-oxidase + 2$\text{H}^+$ + 2$e^-$

(8)
4.1 Based on Direct Electron Transfer of Cytochrome C

Cytochrome c which contains iron centered porphyrin and can easily undergo oxidation and reduction over a wide range of potentials which are varied by the protein environment around heme groups [72]. Due to the redox capability of heme proteins, cyt c widely used to study the mechanism of the catalytic process between redox enzyme and substrate and used in enzyme-based biosensors, especially H$_2$O$_2$ biosensors. However, electron transfer between cyt c and solid electrodes is usually slow [73]. Thus, it is necessary to search for a way to develop a cyt c modified electrode that will enhance electron transfer to the solid surface, while still maintaining well-behaved electrochemistry and good stability. Recently cyt c immobilized on Au nanoparticles on ITO substrate (cyt c/AuNP/ITO) was applied for the detection of H$_2$O$_2$ as shown in Figure 5 [74].

Generally, the direct electron transfer of cyt c at normal Au bulk electrode is difficult; however with the incorporation of gold nanoparticle the electron transfer properties of the protein are enhanced. Experimental results showed that AuNP’s can act as tiny conduction centers, which can facilitate the rapid transfer of electrons which have a very high surface to volume ratio. The developed cyt c/AuNP/ITO electrode showed a couple of well-defined and quasi-reversible redox peaks because of the Fe$^{3+}$/Fe$^{2+}$ redox center, which was responsible for electron exchange. With increment with scan rates both the anodic and cathodic peaks were increased linearly shown in Figure 6.

The electrocatalytic response for the detection of H$_2$O$_2$ based on the cyt c/AuNP/ITO electrode can be expressed by the following equation

\[
\text{cyt c} - \text{Fe(III)} + e^- \rightarrow \text{cyt c} - \text{Fe(II)}
\]

\[
2\text{cyt c} - \text{Fe(II)} + 2\text{H}^+ + \text{H}_2\text{O}_2 \rightarrow 2\text{cyt c} - \text{Fe(III)} + 2\text{H}_2\text{O}
\]

$I-t$ curves were obtained for cyt c/AuNP/ITO for additions of 20 µL of 200 mM H$_2$O$_2$ at a potential of $-0.1$ V vs. Ag/AgCl. The response was also confirmed on the bare ITO electrode and cyt c/ITO electrode which shows very less response and slight increment in the current comparative to cyt c/AuNP/ITO towards H$_2$O$_2$. As shown in Figure 7 the developed electrode cyt c/AuNP/ITO showed linear response up to a concentration of 6 mM having a detection limit of 0.5 µM.

A comparison Table 2 is provided for cyt c immobilized on various modified electrode surfaces based on the direct electron transfer properties of the protein towards the detection of H$_2$O$_2$.

4.2 Based on Direct Electron Transfer of Myoglobin

Myoglobin (Mb) generally has a slow electron transfer ($\sim 10^{-3}$ cm$^2$ s$^{-1}$) rate hence efforts have been made to en-
The mechanism of the electrocatalytic reduction of H$_2$O$_2$ is expressed as:

$$\text{Mb} (\text{Fe}^{3+}) + e^- \rightarrow \text{Mb} (\text{Fe}^{2+})$$

(11)

$$\text{H}_2\text{O}_2 + 2 \text{Mb} (\text{Fe}^{2+}) \rightarrow 2 \text{Mb} (\text{Fe}^{3+}) + 2 \text{H}_2\text{O}$$

(12)

The catalytic current is linearly increased with the additions of 10 µL aliquots of H$_2$O$_2$ and a calibration plot is established for the accurate determination of H$_2$O$_2$. The amperometric response of the Mb/CeO$_2$/ITO electrode to H$_2$O$_2$ was recorded through successively adding H$_2$O$_2$ to a continuous stirring PBS solution. The amperometric response has linear relationship with the concentration of H$_2$O$_2$ shown in Figure 9.

The developed sensor based on Mb/CeO$_2$/ITO electrode has a sensitivity of 5.4 µA mM$^{-1}$ cm$^{-2}$ with a detection limit of 0.6 µM. The calculated apparent Michaelis–Menten constant ($K_m^{app}$), which indicate the catalytic activity of the protein to its substrate, can be obtained from the Lineweaver–Burk equation:

$$I_{ss}^{-1} = I_{max}^{-1} + K_m^{app} (I_{max} C)^{-1}$$

(13)

where $I_{ss}$ is the steady current obtained after adding substrate, which can be obtained from amperometric experiments. $C$ is the bulk concentration of the substrate, and $I_{max}$ is the maximum current measured under the saturated substrate condition. The value of the apparent Michaelis–Menten constant ($K_m^{app}$) was 3.15 mM, suggesting that the biosensor exhibited high affinity for H$_2$O$_2$. The selectivity of the developed sensor is examined with different interference compounds such as Ascorbic acid, uric acid, sodium nitrite and sodium bicarbonate at 0.2 mM which is evident that there was minimal influence of interfering species on the H$_2$O$_2$ response.

Zhang et al. [89] reported a modified electrode of Mb immobilized on Au nanoparticles on ITO surface. The electrodes shows pair of redox peaks at $-0.23$ and $-0.09$ V related to Myoglobin. This Mb/Au/ITO electrode has good electrocatalytic response towards H$_2$O$_2$ with a linear range of 2.5–500 µM with the detection limit of 0.48 µM. The value of the apparent Michaelis–Menten constant ($K_m^{app}$) was $1.3 \times 10^{-4}$ M shows a high biological affinity to H$_2$O$_2$.

### 4.3 Based on Direct Electron Transfer of Hemoglobin

Many H$_2$O$_2$ sensors have been proposed during several years based on Hemoglobin (Hb) because of its peroxidase activity and commercial availability. Direct electrochemistry of proteins on an electrode surface has been studied to sensitively detect H$_2$O$_2$ without the additional
Fig. 7. (a) Current-time curve obtained for ITO/AuNP/cyt c electrode upon successive addition of 20 µL aliquots of 200 mM H$_2$O$_2$ to 5 mL stirred 10 mM HEPES buffer at pH 7 with an applied potential of –0.1 V under nitrogen atmosphere; chronoamperometric curve obtained for (b) cyt c/ITO and (c) AuNP/ITO obtained by the addition of 20 µL aliquots of 200 mM H$_2$O$_2$ in 5 mL stirred solution of 10 mM HEPES buffer at the potential of –0.1 V under nitrogen atmosphere. Figure reproduced with permission from: Reference [74], © 2012 Elsevier.

Table 2. Summarizes the values of selected quantities measured from cyt c immobilized electrode towards H$_2$O$_2$ sensing that were reported in the literature.

<table>
<thead>
<tr>
<th>Electrode matrix</th>
<th>Detection limit (µM)</th>
<th>Linear range (mM)</th>
<th>$k_s$ (s$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt c/AuNP/ITO</td>
<td>0.5</td>
<td>–</td>
<td>0.69</td>
<td>[74]</td>
</tr>
<tr>
<td>cyt c/RTIL-PDDA-AuNPs/MUA-MCH/Au</td>
<td>5.0</td>
<td>0.04–3.45</td>
<td>2.65</td>
<td>[75]</td>
</tr>
<tr>
<td>cyt c/PFS-DNA/Au</td>
<td>0.72</td>
<td>0.003–1.83</td>
<td>0.78</td>
<td>[76]</td>
</tr>
<tr>
<td>cyt c/MWNTs/GCE</td>
<td>350</td>
<td>0.002–0.42</td>
<td>4.0</td>
<td>[77]</td>
</tr>
<tr>
<td>cyt c/Nanoporous Au</td>
<td>6.3</td>
<td>0.01–12</td>
<td>–</td>
<td>[78]</td>
</tr>
<tr>
<td>cyt c/Au/Chit</td>
<td>9.8</td>
<td>0.85–13</td>
<td>–</td>
<td>[79]</td>
</tr>
<tr>
<td>cyt c/MPCE</td>
<td>0.146</td>
<td>0.02–24</td>
<td>17.6</td>
<td>[80]</td>
</tr>
<tr>
<td>cyt c/PAN-PDA/GCE</td>
<td>7.3</td>
<td>0.002–0.38</td>
<td>–</td>
<td>[81]</td>
</tr>
<tr>
<td>cyt c/GNPs/RTIL/MWNTs/GCE</td>
<td>3.0</td>
<td>0.05–11.5</td>
<td>0.78</td>
<td>[82]</td>
</tr>
<tr>
<td>cyt c/MPA/Au</td>
<td>1.0</td>
<td>0–0.25</td>
<td>1600</td>
<td>[83]</td>
</tr>
</tbody>
</table>

Table 3. Summarizes the values of selected quantities measured from Mb immobilized electrode towards H$_2$O$_2$ sensing that reported in literature.

<table>
<thead>
<tr>
<th>Electrode matrix</th>
<th>Detection limit (µM)</th>
<th>Linear Range (µM)</th>
<th>Reusability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mb/CeO$_2$/ITO</td>
<td>0.6</td>
<td>3.0–3000</td>
<td>2 weeks</td>
<td>[85]</td>
</tr>
<tr>
<td>Mb/HMS/GCE</td>
<td>0.062</td>
<td>4.0–124</td>
<td>3 months</td>
<td>[86]</td>
</tr>
<tr>
<td>Nafion/Mb/IL/GCE</td>
<td>0.14</td>
<td>1.0–180</td>
<td>–</td>
<td>[87]</td>
</tr>
<tr>
<td>Nafion/Mb/CGNs/GCE</td>
<td>0.5</td>
<td>1.5–90</td>
<td>–</td>
<td>[88]</td>
</tr>
<tr>
<td>Mb/Au/ITO</td>
<td>0.48</td>
<td>2.5–500</td>
<td>–</td>
<td>[89]</td>
</tr>
<tr>
<td>Mb/HSG/SN-CNTs/GCE</td>
<td>0.36</td>
<td>2.0–1200</td>
<td>–</td>
<td>[90]</td>
</tr>
<tr>
<td>GNRs@SiO$_2$-Mb/RTIL-sol-gel/GCE</td>
<td>0.12</td>
<td>0.2–180</td>
<td>3 weeks</td>
<td>[91]</td>
</tr>
<tr>
<td>Mb/EMIM-BF$_3$/HA/GC</td>
<td>0.6</td>
<td>2.0–270</td>
<td>–</td>
<td>[92]</td>
</tr>
<tr>
<td>Mb/clay-IL/GCE</td>
<td>0.73</td>
<td>3.9–259</td>
<td>–</td>
<td>[93]</td>
</tr>
<tr>
<td>Mb/SDS-GNPs-GR/BPG</td>
<td>0.012</td>
<td>0.5–7.5</td>
<td>4 weeks</td>
<td>[94]</td>
</tr>
</tbody>
</table>
electron transfer mediator. Several studies were performed by immobilizing Hb on different electrode surfaces, such as glassy carbon electrode [95], metal oxides [96], nanoparticles [97], carbon nanotubes [98] and graphene [99,100]. However, there is a great need to further enhance the direct electron transfer rate of the mediatorless H₂O₂ sensor for not only further increasing the sensitivity, but also improving the response time, since the sensor often suffers from slow response time due to its limited direct electron transfer rate between redox proteins and electrode.

The mechanism for electrocatalytic reaction of Hb towards H₂O₂ can be expressed as follows [101]:

\[
\text{Hb(Fe}^{3+}) + \text{H}_2\text{O}_2 \rightarrow \text{Compound I (Fe}^{4+}=\text{O}) + \text{H}_2\text{O} \quad (14)
\]

\[
\text{Compound I (Fe}^{4+}=\text{O}) + e^- + \text{H}^+ \rightarrow \text{Compound II} \quad (15)
\]

\[
\text{Compound II} + e^- + \text{H}^+ \rightarrow \text{Hb (Fe}^{3+}) + \text{H}_2\text{O} \quad (16)
\]

Chen et al. [102] proposed a strategy for modified electrode (Hb/Au/Hb/MWNT/GC) by the preparation nanohybrid film composed of multiwall carbon nanotubes (MWNT) and gold colloidal nanoparticles (GNPs) by using proteins as linker materials towards the detection of H₂O₂. The strategy is negatively charged MWNT was first modified on the surface of glassy carbon (GC) electrode, then, positively charged Hb was adsorbed onto MWNT films by electrostatic interaction. The [Hb/GNPs], multi-layer films were finally assembled onto Hb/MWNT film through layer-by-layer assembly technique. This Hb/Au/Hb/MWNT/GC electrode give a pair of well-defined redox peaks at −0.26 and −0.37 V at scan rate of 50 mV/s, characteristic of heme Fe(III)/Fe(II) redox couples of Hb, suggesting that direct electron transfer has been achieved between Hb and underlying electrode are shown in Figure 10.

As compared to those H₂O₂ biosensors only based on carbon nanotubes, the proposed biosensor modified with MWNT and GNPs displays a broader linear range and a lower detection limit for H₂O₂ determination. The linear range is from 2.1×10⁻⁷ to 3.0×10⁻³ M with a detection limit of 8.0×10⁻⁸ M at 3σ. The Michaelis–Menten constant \(K_M^{\text{app}}\) value is estimated to be 0.26 mM. Moreover, this biosensor displays rapid response to H₂O₂ and possesses good stability and reproducibility as shown in Figure 11. Table 4 summarizes the Hb immobilized on various modified electrode surfaces towards the electrocatalytic reduction of H₂O₂.
4.4 Based on Direct Electron Transfer of Ferredoxin

Many biosensors have been reported based on the direct electrochemistry of protein towards the electrochemical detection of $\text{H}_2\text{O}_2$. Until now, many proteins such as cyt c, Mb, Hb has been extensively studied for the detection. These heme proteins have some disadvantages for example, Hemoglobin, which is a redox active protein consisting of four electroactive-iron (III) hemes as prosthetic groups enables reversible conversion of Hb-Fe(III) to Hb-Fe(II) but the rate of electron transfer from the protein to the surface of the electrodes modified directly by hemoglobin is slow; moreover a large, three dimensional structure of hemoglobin leads to inaccessibility of the redox centers that are located inside the protein. Therefore, direct electron transfer between the hemoglobin and electrode is difficult. Towards the development of new kind of protein based sensor, spinach ferredoxin has been successfully utilized for the electrocatalytic detection of $\text{H}_2\text{O}_2$ [112].

The Fdx/MUA/Au modified electrode shows good electrocatalytic activity towards the reduction of $\text{H}_2\text{O}_2$. The electrocatalytic response of the Fdx/MUA/Au towards $\text{H}_2\text{O}_2$ was investigated and the principle of catalyzing $\text{H}_2\text{O}_2$ was presented by the following equation.

\[
\text{Fdx}^{[2\text{Fe}-2\text{S}]}^+ + \text{e}^- \rightarrow \text{Fdx}^{[2\text{Fe}-2\text{S}]}^+ + \text{H}_2\text{O}_2
\]  \hspace{1cm} (17)

\[
\text{Fdx}^{[2\text{Fe}-2\text{S}]}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Fdx}^{[2\text{Fe}-2\text{S}]}^+ + \text{2OH}^-
\]  \hspace{1cm} (18)

From the above equations, in the bio-catalytic cycle of Fdx, at first it forms an enzymatic reducing agent as Fdx$^{[2\text{Fe}-2\text{S}]}^+$. This is catalytically active and can form an
adduct with H₂O₂, Fdx[Fe-2S]⁺⁻⁻H₂O₂⁻Fdx[Fe-2S]⁺⁺⁻⁻ subsequently it forms OH⁻ radical and then back to the resting state of the native enzyme, Fdx[Fe-2S]²⁺.

The Fdx/MUA/Au immobilized electrode showed a couple of well-defined and quasi-reversible redox peaks for the reduction of the Fe⁺³ ion in the [2Fe-2S]²⁺⁺ cluster, with the formation of [2Fe-2S]⁺⁺⁻⁻. The anodic peak potential (Epa) and cathodic peak potential (Epc) are located at 0.12 and 0.17 V (vs. Ag/AgCl), respectively. The formal potential (E⁰) is ca. 0.15 V and peak-to-peak separation (ΔEₚ) of 60 mV was observed. The developed sensor showed good amperometric response for the selective determination of H₂O₂ without any interference effects as shown in Figure 12.

The stability of the sensor have been examined by CV sweeps over a potential range of 0.4 to 0.2 V at 0.05 Vs⁻¹ for 50 cycles which retains its original value and the signal decreased 3.2% after storing at 4°C for 10 days.

4.5 Direct Electron Transfer of Horseradish Peroxidase Towards H₂O₂

HRP is an enzyme that catalyzes the oxidation of a wide variety of organic and inorganic substances, with H₂O₂ as electron accepter. In peroxidase based biosensors, H₂O₂ is reduced at low over potential due to the direct electron transfer between the electrode and HRP redox center. Thus, it is widely used enzyme for H₂O₂ detection biosensors. But, the redox center of HRP is surrounded in the protein matrix, which reduces the electrical conductivity of the enzyme and thus leads to poor electron transfer rates. Meanwhile the redox center is hidden inside the protein matrix of the enzyme; it can avoid interference of many molecules with large size. The catalytic center of the enzyme is deeply embedded in an insulating protein shell which results in sluggish electron-transfer kinetics, further it loses its bioactivity when it adsorbed directly onto the electrode surface. Therefore, appropriate promoters should be employed to facilitate the electron transfer and retain the biological activity of the immobilized enzyme. With the fast growth of nanotechnology, various nanomaterials such as metal nanoparticles [113], metal oxide materials [114], nanofibers [115], nanotubes [116], nanowires [117] have been widely used to modify electrodes, which are conductive to realize the direct electron transfer of the enzyme.

The general catalytic cycle proceeds through a sequence of reactions which can be described by the following equations

\[
\text{HRP (Fe}^{3+}\text{)} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I }[(\text{Fe}^{4+} = \text{O})] + \text{H}_2\text{O}
\]  
(19)

\[
\text{Compound I }[(\text{Fe}^{4+} = \text{O})] + e^- + \text{H}^+ \rightarrow \text{Compound II}
\]  
(20)

\[
\text{Compound II} + e^- + \text{H}^+ \rightarrow \text{HRP (Fe}^{3+}\text{)} + \text{H}_2\text{O}
\]  
(21)
In the first step of this cycle the HRP resting state react with H$_2$O$_2$ and is get oxidized to a high oxidation state intermediate known as Compound I, in which one electron removed from the ferric iron to the Fe$^{4+}$ oxyferryl center and an electron is removed for the porphyrin to give Porphyrin $\pi$-cation radical. In the second step involves a single electron oxidation of the substrate by compound I and the $\pi$-cation radical is reduced to give a second high oxidation state as compound II. Finally the compound II react with another substrate molecule from which an electron is extracted by the Fe$^{4+}$ oxyferryl to regenerate the resting state of the enzyme.

Zhang et al. [118] proposed a strategy for modified electrode (HRP/GO/Nafion/GCE) by immobilizing HRP on graphene oxide-Nafion nanocomposite film towards the application of H$_2$O$_2$ sensor. GO-based polymer composites provide a favorable microenvironment for HRP to realize its direct electron transfer, which allows it to be used for H$_2$O$_2$ sensing with great sensitivity. The cyclic voltammogram of HRP/GO/Nafion/GCE electrode display a couple of well-defined, stable and reversible redox peaks, which were attributed to the redox reaction of HRP with a formal potential ($E^\circ$) of $-369$ mV shown in Figure 13.

The electrocatalytic reduction of H$_2$O$_2$ at HRP/GO/Nafion/GCE was studied by cyclic voltammetry in the potential range of 0.30 to $-0.70$ V. When H$_2$O$_2$ was added reduction peak increases and decrease or no change in oxidation peak current is observed. These results demonstrate the typical electrocatalytic reduction processes of H$_2$O$_2$. The HRP/GO/Nafion/GCE electrode showed two reduction peaks at $-0.23$ V and $-0.39$ V corresponds to the reductions of O$_2$ and HRP.

It is clearly shown from the Inset of Figure 14 that HRP/GO/Nafion/GCE, the response current increased linearly with the concentration of H$_2$O$_2$ from 1.0 mmolL$^{-1}$ to 1.0 mmolL$^{-1}$ with the linear regression equation $I = 0.1182 + 12.82 C$ (I, $\mu$A; C, mmolL$^{-1}$) ($R = 0.9986$). The detection limit for H$_2$O$_2$ at a signal-to-noise ratio of 3 was found to be $4.0 \times 10^{-7}$ molL$^{-1}$. At HRP/Nafion/GCE, the linear range for the response current to the concentration of H$_2$O$_2$ was limited from 1.0 mmolL$^{-1}$ to 0.1 mmolL$^{-1}$. Michaelis–Menten model yields the apparent Michaelis–Menten constant $K_M^{app} = 0.684$ mmolL$^{-1}$ for HRP/GO/Nafion/GCE to the reduction of H$_2$O$_2$ (I$_{cat}$= $-0.099 + 68.40C$, I$_{cat}$, $\mu$A; C, mmolL$^{-1}$; $R = 0.9999$). The stability of the electrode was tested, and it was found that HRP/GO/Nafion/GCE could be repeatedly scanned without significant decrease of peak currents. The cathodic peak current of HRP/GO/Nafion/GCE was decreased by less than 5% after 4 weeks of storage. Table 5 summarizes the HRP immobilized on various modified electrode surfaces towards the electrocatalytic reduction of H$_2$O$_2$. 

![Cyclic voltammograms of (a) HRP/GO/nafion/GCE, (b) HRP/Nafion/GCE and (c) Go/Nafion/GCE in 0.1 molL$^{-1}$ pH 7.0 PBS at a scan rate of 100 mVs$^{-1}$. Figure reproduced with permission from: Reference [118], © 2007 Elsevier.](image1.png)

![Cyclic voltammograms of HRP/GO/nafion/GCE in 0.1 molL$^{-1}$ pH 7.0 PBS containing H$_2$O$_2$ upon increasing concentrations (B) Amperometric response of (a) GO/Nafion/GCE, (b) HRP/Nafion/GCE and (c) HRP/GO/Nafion/GCE in 0.1 molL$^{-1}$ pH 7.0 PBS at $-0.57$ V on successive additions of H$_2$O$_2$. Inset: Calibration curve of the electrocatalytic current versus H$_2$O$_2$ concentration. Figure reproduced with permission from: Reference [118], © 2007 Elsevier.](image2.png)
Review

were achieved. Many research groups are continuously working to develop protein based biosensors while very few proteins will display the direct electron transfer while most cases mediator is used for better electron transfer functions. Further, there is not any standard protocol to immobilize protein on electrode surface to achieve direct electron transfer reactions. Though several studies on conventional electrodes have achieved considerable results for the detection of H2O2, but with help of nanotechnology much better sensing methodologies towards H2O2 can be developed. Also, artificial enzymes that mimic natural enzymes could be another alternative for efficient way to develop biosensors. Natural proteins/enzymes are very sensitive to the environment and susceptible to denature relatively on unmodified electrodes also with increment in temperature or pH of the solution. The poor stability of natural enzymes becomes a barrier towards the fabrication process of biosensors and long-time usage in real world applications. Apart from these, as the cost of proteins are high which further limits the mass production of biosensors. Hence, artificial enzymes can be an alternative as they are more robust and can be easily tailor-made to the desired properties for constructing and better biosensing applications. Novel nanobiomaterials are expected to come with better understanding of the biological process and will continue the developments of H2O2 sensors towards clinical diagnostics, food safety and environmental monitoring.

5 Conclusions and Future Prospects

In recent years numerous reviews have addressed electrochemical H2O2 biosensors based on the catalytic properties of redox proteins and enzymes. This review is mainly concentrated on recent advances based on the direct electrochemistry of protein for electrocatalytic H2O2 determination. Several methods with variety of electrode surface materials are used to immobilize the proteins in order to enhance the direct electron transfer rate and provide proteins with suitable microenvironment. With the development of novel electrode surfaces mediator-free biosensors with greater enhancements in sensitivity and selectivity were achieved. Many research groups are continuously working to develop protein based biosensors while very few proteins will display the direct electron transfer while most cases mediator is used for better electron transfer functions. Further, there is not any standard protocol to immobilize protein on electrode surface to achieve direct electron transfer reactions. Though several studies on conventional electrodes have achieved considerable results for the detection of H2O2, but with help of nanotechnology much better sensing methodologies towards H2O2 can be developed. Also, artificial enzymes that mimic natural enzymes could be another alternative for efficient way to develop biosensors. Natural proteins/enzymes are very sensitive to the environment and susceptible to denature relatively on unmodified electrodes also with increment in temperature or pH of the solution. The poor stability of natural enzymes becomes a barrier towards the fabrication process of biosensors and long-time usage in real world applications. Apart from these, as the cost of proteins are high which further limits the mass production of biosensors. Hence, artificial enzymes can be an alternative as they are more robust and can be easily tailor-made to the desired properties for constructing and better biosensing applications. Novel nanobiomaterials are expected to come with better understanding of the biological process and will continue the developments of H2O2 sensors towards clinical diagnostics, food safety and environmental monitoring.

Acknowledgements

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Table 5. Summarizes the values of selected quantities measured from HRP immobilized electrode towards H2O2 sensing that reported in literature.

<table>
<thead>
<tr>
<th>Electrode matrix</th>
<th>Detection limit (µM)</th>
<th>Linear range (µM)</th>
<th>Sensitivity (µA·mM⁻¹·cm⁻²)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP/NanoCeO2/ITO</td>
<td>0.5</td>
<td>1.0–170</td>
<td>8.44</td>
<td>[119]</td>
</tr>
<tr>
<td>HRP/Au NP/MPA/Au</td>
<td>0.16</td>
<td>0.48–1200</td>
<td>311.72</td>
<td>[120]</td>
</tr>
<tr>
<td>HRP/Composite-3</td>
<td>0.009</td>
<td>0.01–0.22</td>
<td>7.8</td>
<td>[121]</td>
</tr>
<tr>
<td>HRP/laponite/chitosan</td>
<td>5</td>
<td>29–1400</td>
<td>19.7</td>
<td>[122]</td>
</tr>
<tr>
<td>HRP-flower ZnO-AuNP-Nafion/GCE</td>
<td>9.0</td>
<td>15–1100</td>
<td>–</td>
<td>[123]</td>
</tr>
<tr>
<td>HRP/HIL/TNT-GNP</td>
<td>2.2</td>
<td>15–750</td>
<td>–</td>
<td>[124]</td>
</tr>
<tr>
<td>Nafion/HRP-GNS-TiO2/GCE</td>
<td>5.9</td>
<td>41–630</td>
<td>–</td>
<td>[125]</td>
</tr>
<tr>
<td>HRP/PTMSPA@GNR</td>
<td>0.06</td>
<td>10–1000</td>
<td>0.021</td>
<td>[126]</td>
</tr>
<tr>
<td>Clay-HRP-Clay/AuCS-GCE</td>
<td>9.0</td>
<td>39–310</td>
<td>–</td>
<td>[127]</td>
</tr>
<tr>
<td>HRP/Au NAE</td>
<td>0.42</td>
<td>0.74–15000</td>
<td>45.86</td>
<td>[128]</td>
</tr>
</tbody>
</table>

Similarly, Catalase (Cat) is also a heme enzyme, which is present in all aerobic organisms having a molecular weight of ∼240000 composed of four identical subunits with each containing a single heme prosthetic group. The heme group consists of a protoporphyrin ring and a central Fe atom, where iron is usually in the ferric oxidation state as its stable resting state. As a catalyst, the enzyme functions either in the catabolism of H2O2 or in the peroxidative oxidation of small molecule substrates by H2O2. Under normal physiological conditions, enzyme controls the H2O2 concentration so that it does not reach toxic levels that could bring about oxidative damage in cells. The mechanism of disproportionation of H2O2 catalyzed by cat can expressed as

\[ \text{H}_2\text{O}_2 + \text{Cat(Fe}^{\text{III}}\text{)} \rightarrow \text{Compound I} + \text{H}_2\text{O} \] (22)

\[ \text{H}_2\text{O}_2 + \text{Compound I} \rightarrow \text{Cat(Fe}^{\text{III}}\text{)} + \text{O}_2 + \text{H}_2\text{O} \] (23)

where Cat(FeIII) is the resting state of the enzyme, Compound I is a two-equivalent oxidized form of Cat(FeIII) containing an oxyferryl heme (FeIV=O) and a porphyrin π-cation radical. H2O2 first oxidizes Cat(FeIII) to form Compound I and H2O, and then reduces Compound I to Cat(FeIII) and produces O2. This enzyme can act either as a reductant or as an oxidant in the reactions, and returns to its resting state after one catalytic cycle, while H2O2 undergoes dismutation to produce H2O and O2.

References
