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Recent advances on developing 3rd generation enzyme electrode for biosensor applications

Priyanki Das [a], Madhuri Das [a], Somasekhar R. Chinnadayyala [b], Irom Manoj Singha [a], Pranab Goswami [b,*]

[a] Centre For Energy, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India
[b] Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India

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ABSTRACT

The electrochemical biosensor with enzyme as biorecognition element is traditionally pursued as an attractive research topic owing to their high commercial perspective in healthcare and environmental sectors. The research interest on the subject is sharply increased since the beginning of 21st century primarily, due to the concomitant increase in knowledge in the field of material science. The remarkable effects of many advance materials such as, conductive polymers and nanomaterials, were acknowledged in the developing efficient 3rd generation enzyme bioelectrodes which offer superior selectivity, sensitivity, reagent less detection, and label free fabrication of biosensors. The present review article compiles the major knowledge surfaced on the subject since its inception incorporating the key review and experimental papers published during the last decade which extensively cover the development on the redox enzyme based 3rd generation electrochemical biosensors. The tenet involved in the function of these direct electrochemistry based enzyme electrodes, their characterizations and various strategies reported so far for their development such as, nanofabrication, polymer based and reconstitution approaches are elucidated. In addition, the possible challenges and the future prospects in the development of efficient biosensors following this direct electrochemistry based principle are discussed. A comparative account on the design strategies and critical performance factors involved in the 3rd generation biosensors among some selected prominent works published on the subject during last decade have also been included in a tabular form for ready reference to the readers.

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* Corresponding author. Tel.: +91 361 2582202; fax: +91 361 2582249.
E-mail address: pgoswami@iitg.ernet.in (P. Goswami).

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1. Introduction

Fast, specific and sensitive transduction of biochemical signals for quantitative or semi-quantitative detection of analytes of interest are the vital functional factors for a biosensor to be used for practical applications. The amperometric transducer-based biosensors are widely acclaimed not only for their inherent potential to exhibit these functional properties but also for bearing the scope of scaling down their size with tailored low production cost, easy fabrication, and simple operation with low or no sample loss (Wilson, 2005). However, the operational principle and the design approach being utilized to develop amperometric biosensors may largely influence the aforesaid functional entities and annexed characteristics. Facile electron transfer between the biocatalytic reaction and electrode is necessary to improve the functional property of the bioelectronic device. The amperometric transducer based biosensors function by the production of a current when a potential is applied on the working electrode in an electrochemical setup in response to the analyte of interest.

Enzyme-based amperometric biosensor comprises immobilized/confined enzyme(s) (mostly oxidoreductase) as the chemically selective layer over a highly conductive support material/matrix acting as electrode to transduce biochemical signal to electrical one under the influence of a suitable applied potential (Hirst and Stevens, 1985; Willner et al., 2006). If the signal/response accrued based on the electro-activity, primarily of co-substrate, product or co-product of the enzyme catalyzed reaction the category of the biosensor is termed as 1st generation, due to its primitive in nature. The first of its kind is the Clark oxygen electrode based glucose biosensor (Clark and Lyons, 1962). There are many drawbacks of the 1st generation biosensors such as, technical difficulty of maintaining air-tight sample chamber (if oxygen is used as redox indicator), and need of high redox potential for the redox indicator (e.g. $\sim +600$ mV versus SCE at Pt electrodes to oxidize H_2O_2) sometimes affects the specificity of the constructed biosensor. Coupling of electrons between the redox active centers of the enzyme and the electrode via some specialized small electroactive molecules to generate the response constitutes the 2nd generation biosensors. These specialized molecules are referred to as 'electron transfer mediators' (ETM), which shuttles electrons between the redox center of the enzyme and the electrode, at comparatively low over potential. The ETM also surpasses the role of molecular oxygen to take-up the electron from the reactive center of many redox enzymes catalyzing the aerobic oxidations of substrates (Gilardi et al., 1994). However, the leaching susceptibility of the soluble mediator to the sample solution, diffusion barrier of the mediator between enzyme–electrode interface, are some of the drawbacks affecting stability and reproducibility of the 2nd generation biosensors that prompted to explore 3rd generation biosensors. The biosensors of the 3rd generation category involve direct electrical communication between the redox centre of the enzyme and the electrode to generate the response (Ghindilis et al., 1997). These biosensors are characterized by high-selectivity and sensitivity, as they can operate in a potential window closer to the redox potential of the enzyme and the electron exchange between the redox centre of the enzyme and the electrode takes place without any diffusion barrier due to proximity of these two terminals (Gorton et al., 1999). This principle of direct electrochemistry has been known for over 30 years (Berezin et al., 1978; Tarasevich, 1979) and is also useful in identifying various distinctive properties of enzymes. One prominent application is the determination of redox potentials especially, where these relate to thermodynamically inaccessible or kinetically reactive species for which potentiometric methods are not suitable (Armstrong et al., 1988). The attractive feature of DET based biosensors is the possibility to regulate the desired properties through protein modification or interfacial engineering, which are pioneered and developed by Willner's group (Willner et al., 1996, 2006; Willner and Katz, 2000; Zayats et al., 2005) etc, Dong's group (Chen et al., 2007; Chi et al., 1994; Dong and Guo, 1995; Dong and Chi, 1992; Jiang et al., 2006; Jin et al., 2003) and Gooding's group (Ciampi and Gooding, 2010; Gooding et al., 2003; Liu et al., 2006a; Liu and Gooding, 2006) etc. Examples of different enzymes involved in DET include cytochrome c, glucose oxidase (GOD), azurin, multicopper oxidases (e.g. laccase, ascorbate oxidase, ceruloplasmin, bilirubin oxidase (BOD)) and several peroxidases (microperoxidase, horseradish peroxidase (HRP) etc). The conventional configurations of three generations of the biosensor are illustrated in Fig. 1.

The present review article summarizes the principle, characterization, and recent advancement in the fabrication strategies of the 3rd generation enzyme electrode for amperometric biosensors application. Effort has been made to incorporate major review papers that largely cover the development on the subject since its inception. The challenges and future perspectives for developing 3rd generation biosensors are also highlighted here.

2. Principles on 3rd generation biosensor

The direct electron transfer (DET) between the redox centre of enzyme and the electrode is the central requirement to the 3rd generation biosensors. Hence, the contact of the redox enzyme with the conductive electrode is essential to facilitate electron exchange. The feasibility of electron exchange between the redox centers of proteins and the electrodes may be explained by the electron-transfer (ET) theory of Marcus (Marcus and Sutin, 1985). The ET rate constant (K_{ET}) between a donor and acceptor pair is given by Eq. (1), where, d and d° are the distance separating the electron and donor, and the van der Waals distance, respectively, β is the electron-coupling constant and ΔG° and λ are the free energy change and the reorganization energy accompanying the electron-transfer process, respectively.

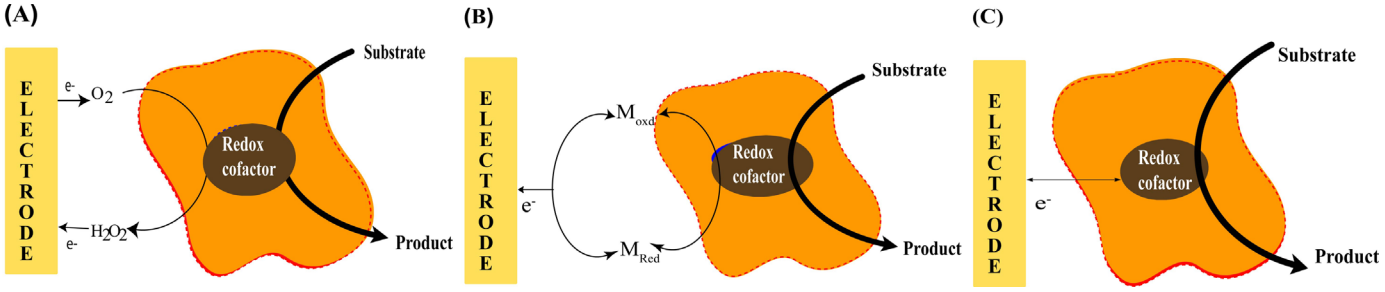

Fig. 1. Response mechanisms of different generations of amperometric enzyme biosensors, A: 1st generation biosensors where primarily, co-substrate/co-product is used as redox indicator, B: 2nd generation biosensors where artificial redox mediator is used to relay the electrons, and C: 3rd generation biosensors where direct electron transfer between enzyme and the electrode is established to generate the response.

$$K_{ET}\alpha \exp\left[-\beta(d-d^{\circ})\right].\exp\left[-(\Delta G^{\circ}+\lambda)^2/(4RT\lambda)\right] \quad (1)$$

It can be inferred from Eq. (1) that DET between two redox sites depends on three major factors: the reorganization energy qualitatively displaying the conformational rigidity of the redox compound in its oxidized and reduced form, the potential difference between the involved redox centers, and separation between the redox sites (Carter et al., 1995). In enzymes bearing insulated prosthetic group and deeply buried active site, the electron transfer rate may be impeded due to the exponential decrease of the tunneling probability with the distance of the involved redox centers (Schmidt and Schuhmann, 1996). In biological system, some redox proteins experience high electron transfer rates supported by the amino residues surrounding the redox centers, which adjust to a comparative position of redox centers and make their distance as close as possible. Thus, the tropism of redox protein on the electrode surface is an important factor to realize its DET (Wu and Hu, 2007). For studying DET majority of the methods utilize the concept of protein film voltammetry (PFV) where the redox protein is adsorbed as a stable mono-/submonolayer film of molecules on an electrode surface and probed by a variety of electrochemical techniques (Armstrong et al., 1997; Rusling et al., 2008; Zhang and Li, 2004). The thin film preserves the chemistry of the active site of the redox proteins and facilitates fast electron transfer due to proximal distance with the electrode (Léger et al., 2003). The protein molecules in PFV are arranged as a perfect monolayer, each behaving independently.

In the protein film, the active sites of the enzymes are easily accessible to species in solution such as, protons, metal ions, ligands, catalytic substrates, thus allowing the study of coupled reactions (Hirst, 2006). PFV overcomes the problems of slow protein diffusion and the kinetics of interaction of the protein at the electrode surface. It has several advantages over conventional voltammetry in which the protein molecules are free in solution, including efficient screening for reactivities, sharp redox status of the entire sample, wave-form definition, requirement of a minuscule quantity of sample, high-sensitivity, stoichiometry, and fast reactions (Zhang and Li, 2004). Further, in PFV the electrode can be swapped between solutions of different pH and composition, permitting measurement under conditions in which the protein has short life span (Hirst, 2006). The protein in the thin film may have good orientation on the electrode surface and its relative position could be modified by the amino acid residue surrounding the active site. It shortens the distance between the active center and the electrode which increases the electron tunneling rate efficiently (Zhang et al., 2009). However, to realize the desired results from PFV the formation of a proper interface for adsorption and electrical contact of the protein with the electrode in an innate state, and correct interpretation of voltammetric data are considered important (Hirst, 2006).

Electron transfer process is largely dependent on the specific properties of the enzyme, such as distance of the redox center from the protein surface, accessibility of the active site, the nature of the redox cofactor, the inherent protein stability and the mode of its immobilization on the electrode surface (Schmidt and Schuhmann, 1996). There are various types of weak or strong protein–electrode interactions that can be customized to initiate the DET by modifying either the protein or the electrode structure (Cooney et al., 2008). The redox enzymes may be divided into three groups. First, those with their active site cofactors weakly (or loosely) bound to the protein e.g. NADH, NAD^+ based enzymes. The active site cofactors can diffuse out of the enzymes during electron transfer processes. Second, the active center is located at the periphery of the enzyme (e.g. peroxidases) allowing easy exchange of electrons with electrode because of short electron tunneling distances. Third, those with their active sites deeply embedded in the protein matrix which hampers DET from the redox center to the electrode, e.g. flavin adenine dinucleotide (FAD) based enzymes (Heller, 1992).

3. Characterization of the 3rd generation biosensor

Electrochemical techniques, primarily cyclic voltammetry (CV) has been widely used to characterize various parameters of the biosensors (Rusling et al., 2008). The data from the anodic (I_{pa}) and cathodic (I_{pc}) peak currents versus scan rate plots of the immobilized enzymes electrode are discerned to characterize the redox processes on the electrode surface such as, surface controlled or diffusion controlled process, reversible or quasi-reversible, surface coverage area (Γ), electron transfer rate constant (k_s), electron transfer co-efficient (α) and number of electrons transferred in the reaction (n) (Murray, 1984). Γ of the enzyme on the electrode can be calculated using the relation, $\Gamma = Q/n\ FA$, where, A is the real surface area of the electrode, Q is the charge obtained by integrating the peak current area and F is the Faraday's constant. The value of α and k_s are estimated by measuring the anodic (E_{pa}) and cathodic (E_{pc}) peak potentials at various CV scan rates (υ) using Eqs. (2)–(4) (Laviron, 1979):

$$E_{pa} = E^{\circ\prime} + 2.3RT/(1-\alpha)nF \log \upsilon \quad (2)$$

$$E_{pa} = E^{\circ\prime} - 2.3RT/(1-\alpha)nF \log \upsilon \quad (3)$$

$$\log(k_s) = \alpha \log(\alpha) + (\alpha)\log \alpha - \log(RT/nF\upsilon) - \alpha(\alpha) n\, F\Delta E_p/2.3RT \quad (4)$$

where $E^{\circ\prime}$ is the formal potential, and R, T, and F have their usual significance.

The magnitude of k_s indicates the efficacy of the DET between the immobilized enzymes and electronic unit. The increasing height of the redox peak in CV with increasing concentration of substrate implies the involvement of DET principle in sensing the substrate of interest by the constructed enzyme electrode (Cooney et al., 2008; Vatsyayan et al., 2010). A couple of extensions of the voltammetry techniques were also developed to increase the sensitivity of the analyses using DET principle. Pulse voltammetry such as, differential pulse voltammetry (DPV), square wave voltammetry (SWV) have sensitivity several orders of magnitude superior than CV. Different 3rd generation biosensors are electrochemically characterized by DPV (Chinnadayyala et al., 2014; Ghosh et al., 2015), and SWV (Brondani et al., 2013; Janegitz et al., 2012; Moccelini et al., 2011). The electrochemical impedance spectroscopy (EIS), which offers to understand the charge transfer behaviors of the thin film layers deposited on the electrode surface is also employed to discern the performance of enzyme electrodes (Mani et al., 2013; Song et al., 2014; Wang et al., 2014).

For morphological characterization of the bioelectrodes various advanced techniques such as, Scanning electron microscopy (SEM) (Wang et al., 2014), Transmission electron microscopy (TEM) (Song et al., 2014), and atomic force microscopy (AFM) (Saxena et al., 2011) are frequently used. Again for chemical characterization of the material being used in the fabrication of the biosensor, techniques like UV–vis absorption spectroscopy (Li et al., 2014), Energy Dispersive X-ray spectroscopy (EDX) (Ghosh et al., 2015), Fourier transform infrared spectroscopy (FT-IR) (Mani et al., 2013), Raman spectroscopy (Wang et al., 2014), X-ray photoelectron spectroscopy (XPS) (Liang et al., 2013), X-ray diffraction (XRD) (Unnikrishnan et al., 2013) etc are commonly employed.

4. Fabrication strategies and applications of the 3rd generation biosensor

The DET of redox protein (or enzyme) may not be plainly achievable in a simple thin film assembly due to several reasons such as, low electronic conductivity of the surrounding amino acid chains of the protein (Polsky et al., 2007), unfavorable orientation or denaturation of protein molecules on the electrode surface (Wang and Wang, 2004) and the distance-dependent heterogeneous long-range ET. The approaches commonly being used to immobilize redox protein on the electrode surface, such as non-specific adsorption (Gole et al., 2001; Jia et al., 2002) and covalent attachment (Keating et al., 1998) are difficult to control and may yield randomly bound proteins with poor orientation or inappropriate alignment of the redox center with the electrode, which lead to inefficient ET on the electrode surfaces. Therefore, it is important to modify the surface in such a way that an active site of an enzyme can be in close proximity of the electrode surface without altering its essential enzymatic activity (Shleev et al., 2005). Different strategies have been explored in this regards (Prévoteau et al., 2010), which include nanofabrication of electrode, immobilization of the biocatalysts in redox units tied to the polymers (Rajagopalan et al., 1996), positioning of the enzymes on relay cofactor tethered by the process of reconstitution (Blonder et al., 1997) etc.

4.1. Nanofabrication of electrode

Since the beginning of the last decade, there has been a paradigm shift of biosensors research towards the application of various nanomaterials. The highly conductive nature of many nanomaterials and their dimensional similarity with the redox proteins have made DET possible between the nanomaterial conjugated redox proteins and electrode (Katz and Willner, 2004; Niemeyer, 2003; Willner et al., 2006). The effectiveness of nanomaterials on establishing DET is not only limited to simple redox proteins like cytochrome c (Zhang, 2008), peroxidase (Huang and Tsai, 2009), GOD (Wu et al., 2012), cholesterol oxidase (ChOx) (Saxena et al., 2011) etc but also has been realized in massive multimeric redox proteins, like, large catalase (CAT) (tetramer Mr∼90 kDa) (Vatsyayan et al., 2010), alcohol oxidase (AOx) (octamer Mr∼675 kDa) (Das and Goswami, 2013) etc. as pioneered in the authors lab not only for biosensor applications but also for generating current in biofuel cells (Das et al., 2014). As an example, CAT was physically immobilized on the electrode surface along with MWCNT, anionic and cationic polymer by exploiting the electrostatic principle as the stabilizing force that favored electron hopping between the redox centre of the enzyme and the electrode (Fig. 2). The value of k_s and Γ of the immobilized CAT were $1.05 \pm 0.2\ s^{-1}$ and 2.1×10^{-10} mol cm^{-2}, respectively. The biosensor exhibited a linear faradic current response against H_2O_2 in the concentration range of 10–5 mM (Vatsyayan et al., 2010), indicating it's potential H_2O_2 biosensor application. In addition to carbon nanotubes (CNT) s, varieties of other nanomaterials have been used to promote DET, such as, colloidal AuNPs (Zhao et al., 2006), silver nanoparticle (AgNP) (Murata et al., 2009), biomimetically synthesized silica nanoparticle (Zamora et al., 2009), porous nanosheet-based ZnO microsphere (Lu et al., 2008a) for 3rd generation glucose, D-fructose, H_2O_2, and H_2O_2 and $NaNO_2$ biosensors applications, respectively. Nanomaterials also provide a surface for increase enzyme loading on the electrode surface and in many cases the surface is endowed with a favorable microenvironment for maintaining the activity of the enzymes on the electrodes. The enzyme loading may also depend on the pH value, ionic strength, electrode supports, pore size and shape as well as hydrophobicity of the immobilizing matrix.

Electrodes are also modified on the molecular scale with CNTs to promote DET because of their unique structure and electronic properties (Vashist et al., 2011). The high aspect ratios allow the tubes to be fitted into proteins enabling DET with enzymes, having normally inaccessible redox centers. The internal cavities and external sides of the CNT wall furnish a platform for the accommodation of various biomolecules. This feature has inspired in coupling CNTs with GOD (Wu et al., 2012), CAT (Vatsyayan et al., 2010), cyt P450 (Vatsyayan et al., 2011), AOx (Chinnadayyala et al., 2014; Das and Goswami, 2013) etc. Silicon dioxide coated Fe_3O_4 magnetic NP-decorated MWCNT were used to construct a new type of amperometric glucose biosensor (Baby and Ramaprabhu,

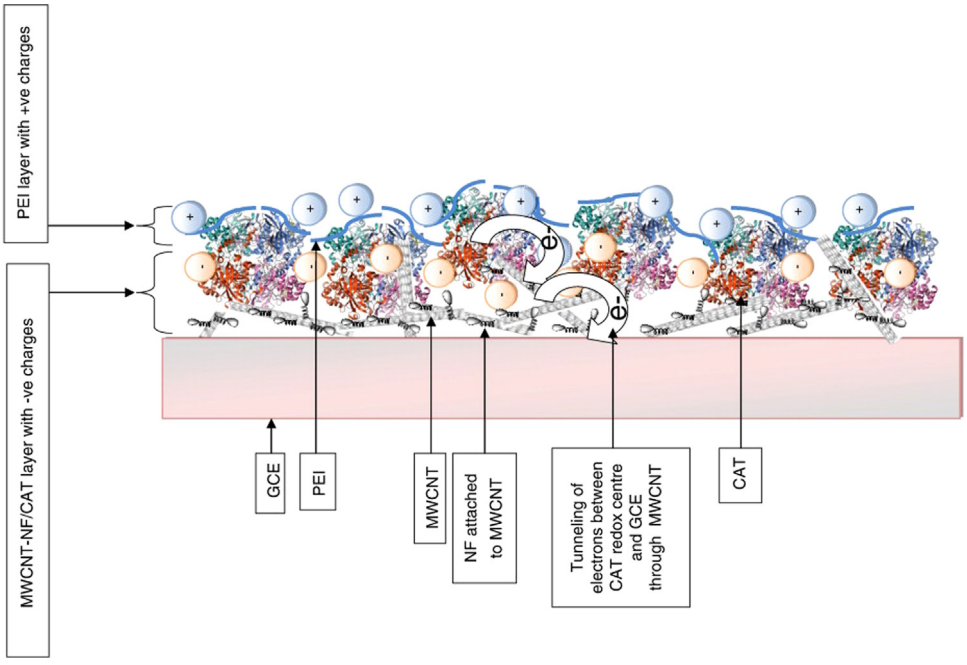

Fig. 2. Electrostatic interactions between the negatively charged underlying layer of MWCNT-Nf/CAT and the positively charged PEI layer utilized to develop bioelectrode (Vatsyayan et al., 2010), Copyright 2010, reprinted with permission from Elsevier.

2010). This biosensor exhibits a linear response from 1 μM to 30 mM with an excellent detection limit of 800 nM indicating its potential applications as 3rd generation glucose biosensor. Bamboo-shaped CN_2 nanotubes, synthesized by nitrogen atoms doping into CNTs, have been used for the immobilization of GOD (Jia et al., 2005). A pair of well-defined voltammograms for HRP has been obtained at the modified electrodes with a CNT/thionine/gold composite (Wang et al., 2008), a sol–gel-derived ceramic–CNT nanocomposite (Chen and Dong, 2007), CNT powder (Zhao et al., 2002), and N-CNT (Lyon and Stevenson, 2008). There are many other reports where CNT-modified electrodes have been used to study the DET for GOD also (Li et al., 2013; Mani et al., 2013; Wang et al., 2011).

Gooding and his coworkers presented a strategy for studying the electron transfer properties of microperoxidase 11 attached to the end of aligned SWCNTs. A cysteamine–derivatized gold electrode was placed in a dispersion of shortened SWCNTs in dimethylformamide (DMF) containing dicyclohexylcarbodiimide to convert the carboxyl group located at the end of shortened SWCNTs into active carbodiimide esters. Finally, microperoxidase was attached to the free ends of the tubes by incubation in a micro-peroxidase solution at 4 °C overnight (Fig. 3). The study proved that shortened SWCNT can be placed in close proximity to an electrode by self-assembly and act as a molecular wire to achieve efficient electrical communication between the underlying electrode and redox proteins (Gooding et al., 2003). With typical diameters of ~80 nm and lengths of the order of micrometers, Carbon nanofiber (CNF)s are also immensely attractive in bioanalytical applications owing to the combination of properties like, large surface area, non-toxicity, satisfactory biocompatibility, ease of fabrication, chemical as well as electrochemical stability, good electrical conductivity (Lu et al., 2008b).

Recently, a novel amperometric 3rd generation alcohol biosensor was developed using peroxidase coupled ferrocene activated AOx as biorecognition system. The sensor was fabricated by immobilizing ferrocene entrapped AOx and sol–gel chitosan film coated HRP on a MWCNT modified GCE through layer-by-layer technique. The activator ferrocene was entrapped in the protein matrix through a simple microwave based partial protein unfolding technique. The sensitivity of the biosensor with ferrocene entrapped AOx (150 $\mu A\ mM^{-1}\ cm^{-2}$) was significantly improved than the biosensor (90 $\mu A\ mM^{-1}\ cm^{-2}$) devoiding of ferrocene (Chinnadayyala et al., 2014).

Graphene (GR) was also reported to promote DET of enzymes on the electrodes thus offering as suitable material for biosensors applications (Sehat et al., 2014). It is a flat monolayer of carbon atoms strongly packed into a two-dimensional honeycomb lattice. Owing to large surface area and high intrinsic conductivity, GR was utilized to initiate direct electrochemistry of GOD for developing 3rd generation glucose biosensor (Unnikrishnan et al., 2013; Zhou et al., 2012). A GOD based biosensor using Nafion (Nf) and GR-gold disk electrode (GE) offering a wide linear range (2 and 14 mM) and a high-sensitivity (21.9 $\mu A\ mM^{-1}\ cm^{-2}$) for glucose was also fabricated (Hui et al., 2013).

Sizes of metallic NPs may be as small as channels and grooves in proteins, and hence can be used as nanoelectrodes by inserting it inside the enzyme, that electrically contact the redox center of the enzyme with electrodes (Xiao et al., 2003). Xiao group have used a 1.4 nm AuNP as electrical nanoconnector for wiring of the FAD-dependent GOD with an electrode (Xiao et al., 2003) for glucose biosensor application. An electrode surface modified with NPs attain a microenvironment similar to that of the redox proteins in native conformation in which, protein molecules find more space for orientation. Again, through the conducting tunnels of inorganic NPs, the shielding effect of the protein shell for DET is also decreased (Guo and Dong, 2009). Luo et al. (2004) showed that immobilization of GOD on the AuNP provides a higher stability to the biosensor, since AuNPs were able to adsorb the enzyme strongly thus preventing the enzyme leakage. The AuNP acts as an electrical nanoplug for the positioning of the enzyme on the

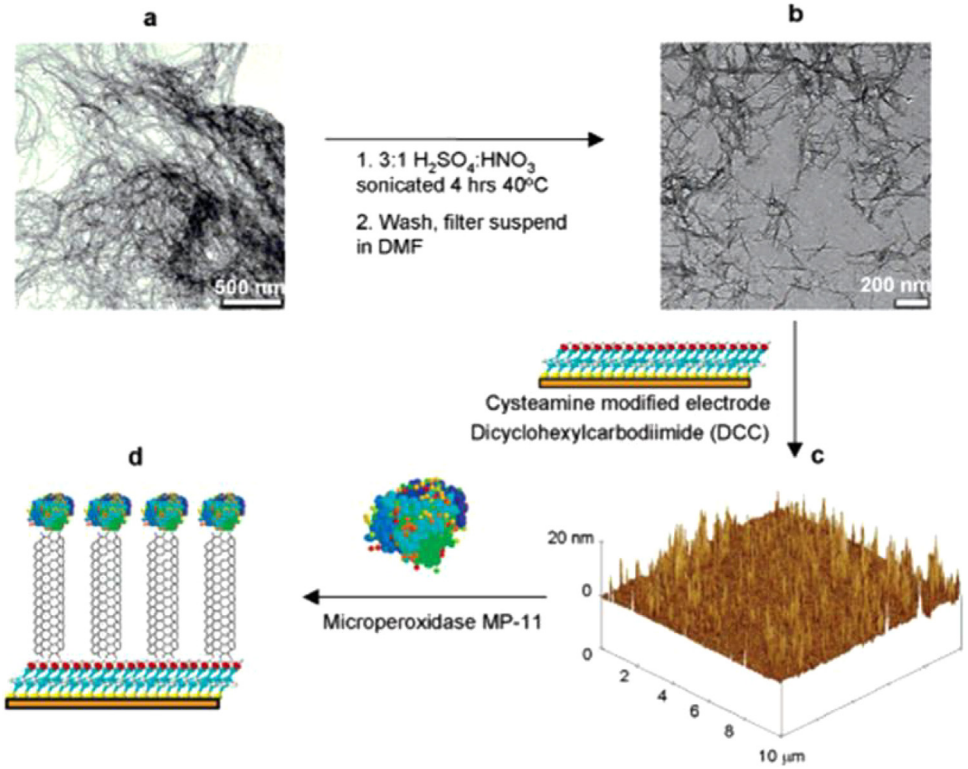

Fig. 3. The steps involved in the fabrication of aligned shortened SWNT arrays for direct electron transfer with enzymes such as microperoxidase MP-11 (Gooding et al., 2003), Copyright 2003, reprinted with permission from American Chemical Society.

electrode platform as well as for the electrical wiring of its redox center. This concept was extended to PQQ-dependent enzymes by the reconstitution of apo-glucose dehydrogenase on PQQ-functionalized AuNP assembled on a gold electrode surface (Zayats et al., 2005). Liu and his coworkers used Au colloid adsorbed cysteine-Nf modified platinum disk electrode to explore the direct electrochemistry of HRP and applied as 3rd generation H_2O_2 biosensor (Liu et al., 2006b). Again, the chemical modification of AuNP surface furnishes an efficient platform to improve the immobilization of biomolecules. For example, AuNP modified with cystamine and glutaraldehyde have been used for the immobilization of tyrosinase (Janegitz et al., 2012) and AuNP synthesized using PEI in ionic liquid (IL) have been applied in the immobilization of human sulfite oxidase (hSO), assisting DET between the enzyme and the modified electrode surface (Frasca et al., 2012) for phenol and sulfite biosensing applications, respectively. The effect of AuNPs sizes (13–21 nm) on the electrocatalytic activities of ChOx in AuNPs–ChOx hybrid systems was evaluated for cholesterol biosensors applications. The results infer that the electrocatalytic efficiency was improved with a decrease in the size of nanoparticles (Saxena and Goswami, 2012).

TiO_2 nanoparticles were used to modify a pyrolytic graphite electrode to accomplish the direct electrochemistry and electrocatalysis of HRP (Zhang et al., 2004a). HRP–titanate nano-sheets (TNS) electrode was fabricated by intercalation of HRP into layered titanate, for H_2O_2 biosensors application. The HRP–TNS electrode exhibited fast DET with high sensitivity, low detection limit and wide linear range towards H_2O_2. The excellent electrochemical performance of the electrode was attributed to the biocompatibility of the titanate sheets, providing better mass transport, more HRP loading ability and retention of activity of HRP by the porous structure of the HRP–TNS film to a large extent (Zhang et al., 2007). Jin group also developed a uric acid biosensor based on uricase immobilized on ZnO nanorods. The fabricated electrode exhibited DET of uricase, showing excellent thermal stability up to 85 °C and anti-interference ability. The sensor offers a linear dependence on the uric acid concentration in the range of 5 μM to 1 mM with a detection limit of 2 μM (Zhang et al., 2004b).

4.2. Polymer based approach

Polymeric materials are one of the most widely used supports in biosensor research. Conducting polymers have charge transfer properties due to the presence of a π-conjugated system in the polymer chain. It makes them compatible for integration with redox enzymes, permitting electron transfer to the electrode surface (Singh et al., 2009). Nf, chitosan (Chi), polypyrrole, polyaniline (PANI), polyphenol, polythiophene, poly-1,3-phenylenediamine, polyvinyl pyridine, polyvinyl alcohol, polycarbonate, osmium redox polymers, nylon are some of the examples of polymer (Sarma et al., 2009). Wang et al. (2009) developed a DET based glucose biosensor by entrapping GOD into the inner wall of highly ordered polyaniline nanotubes synthesized by using anodic aluminum oxide (AAO) as a template. This biosensor exhibits advantages of simplicity of construction, high-sensitivity (97.18 ± 4.62 μA mM^{-1} cm^{-2}), fast response time (3 s), efficient conservation of enzyme activity, and effective differentiation to common interfering species. Zhao et al. (2009) investigated PANI nanofibers as an electrode material for DET of GOD. After immobilization on this nanofibre matrix, GOD remains in its native conformation and undergoes effective DET reaction with a couple of well-defined, quasi-reversible redox peaks. The electrode displays good features in electrocatalytic oxidation of glucose with good reproducibility and stability.

The DET of CAT has been studied by using an amine functionalized-MWCNT, a room temperature ionic-liquid (RTIL) (1-butyl-3-methyl imidazolium tetrafluoro borate) [Bmim][BF4] for 3rd generation H_2O_2 biosensor application (Rahimi et al., 2010). Again, Li group fabricated a glucose biosensor based on [Bmim][PF6], mesoporous carbon (MC) and GOD (Sun et al., 2009). The DET of GOD could be achieved at the GOD/MC/[Bmim][PF6]-modified electrode. The cathodic and anodic peak currents for GOD at the GOD/MC/[Bmim][PF6]-modified electrode are a function of scan rate, indicating a surface-controlled thin-layer electrochemical behavior. The surface concentration of GOD was 7.05×10^{-8} mol cm^{-2}, which was much higher than the reported monolayer coverage. The values of k_s and α for the electron-transfer between GOD and the electrode were found to be 0.135 s^{-1} and 0.468, respectively. RTILs have also shown good compatibility with biomolecules and surfaces, so these can be used in electrochemical biosensors both as binder and conductor. Common advantages of RTILs include higher conductivity, good biocatalytic ability (Park and Kazlauskas, 2003), long-term stability (including stability at high-temperature), superior sensitivity, improved linearity, better selectivity, and the ability to fabricate biosensors with DET between protein and electrode. Du et al. (2007) prepared RTIL–SWCNT composite through grinding, and found high-stability of the composite. Direct electrochemistry was observed when heme-containing enzymes in combination with RTIL–SWCNT were immobilized on a GCE. A novel approach to construct an HRP-hydrophilic IL-AuNP-dotted, titanate nanotubes (TNT) biosensor for amperometric sensing of H_2O_2 was reported (Liu et al., 2012). HRP aqueous solution was used as a gelating agent for immobilization of the mixture of RTIL and AuNPs–TNTs nanocomposite onto the electrode surface. The biosensor responded to H_2O_2 in the linear range from 5×10^{-6} to 1×10^{-3} M with a detection limit of 2.1×10^{-6} M (based on the S/N=3). This study showed a promise for developing a non-poisonous, eco-friendly sensing platform for the construction of a 3rd generation biosensor for in vivo determination of environmental pollutants.

A bienzymatic 3rd generation glucose biosensor with GOD and HRP was reported (Zhu et al., 2007). The biosensor was prepared by dropping SWCNT onto an Au electrode followed by electro polymerization of GOD and HRP in polypyrrole (PPy) forming Au/SWCNT/GOD–HRP/PPy films facilitating direct electronic communication between the enzyme and the conducting substrate through SWCNT. The film reduces biocatalytically formed H_2O_2 at −0.1 V in 8 s, eliminating the interference of ascorbate, urate and acetaminophen thereby enhancing the selectivity of the biosensor (Zhu et al., 2007).

4.3. Self-assembled approach

Self-assembled monolayers (SAMs) are formed by chemisorptions of organothiols on a suitable support such as, gold or silver. Metal–sulfur bonds hold the ordered SAM onto the surface. The end R group facing the solution and the length of the hydrocarbon chains can be varied as desired. Except for extreme potential and extreme pH values, SAMs are quite stable. Bowden (1997) and Niki and Gregory (2003) have pioneered the SAMs of organothiols on gold electrodes to study direct protein electrochemistry. The structures of those SAMs require an electrode surface binding functional group (usually a molecular structure with a nitrogen, sulfur or phosphorus molecule) that promotes electron transfer by alternating π-electrons and a second functional head group for oriented attachment of the redox protein. Studies by Armstrong et al. (Armstrong et al., 1988; Armstrong and Wilson, 2000) and Bowden and co workers (Collinson et al., 1992) suggested that the orientation of the protein's (i.e. cytochrome c's) prosthetic group towards the electrode surface increased the rate of DET. According to the requirement, the electron transfer distance can be modified by changing the SAM alkyl chain length (Cooney et al., 2008). SAMs can provide ideal microenvironment for retaining the native conformation of proteins and enzymes, which enable us to systematically study the influence of experimental parameters on the

electron transfer kinetics of redox protein due to the well-defined structure of SAMs and simplified protein–SAMs system (Behera and Raj, 2007; Yue et al., 2006). SAM have also other advantages that make them especially suitable for biosensors, namely their ability to form ultrathin, ordered and stable monolayers, and the wide variety of available head groups leading to the ability to tailor the surface (Davis and Higson, 2005). Moreover, SAMs are very suitable systems for immobilizing biomolecules at a surface due to the strong, selective interaction of sulfur groups for gold (Davis and Higson, 2005). The 2D immobilization method (Wu and Hu, 2007), such as SAM (Chaki and Vijayamohanan, 2002), allows the enzyme to fix in right conformation on the electrode surface, which reduces the separation between the active site and electrode surface resulting in effective DET (Freire et al., 2003). Liang et al. (2013) reported DET of glucose biosensor, which was based on self-assembly of GOD on electrochemically reduced carboxyl graphene. This biosensor exhibited a linear response to glucose concentrations ranging from 2 to 18 mM with a detection limit of 0.02 mM. Peroxidase biosensors based on SAM-modified electrodes have also been reported. This is exemplified with the study of DET of immobilized HRP over Au colloids onto a cysteamine monolayer modified gold electrode surface. A pair of redox peaks ascribed to the direct redox reaction of HRP was observed. The biosensor exhibited an excellent electrocatalytic response to the reduction of H_2O_2 without the support of an electron mediator (Yi et al., 2000), confirming its potential application as 3rd generation H_2O_2 biosensor. Xu and Han (2004) fabricated a 3rd generation H_2O_2 biosensor by self-assembling AuNP on thiol-functionalized poly (styrene-co-acrylic acid) nanospheres. This biosensor showed high-sensitivity and good stability. Again, Xu group fabricated a biosensor for H_2O_2 using AuNP, hollow porous thiol-functionalized poly-divinylbenzene-co-acrylic acid (DVB-co-AA) nanospheres and enzyme HRP. Hollow porous thiol-functionalized poly-DVB-co-AA nanospheres were self-assembled onto a gold electrode to form a noncompact monolayer. The AuNP absorbed covalently onto the thiol groups of the hollow porous thiol-functionalized poly-DVB-co-AA nanospheres providing an active matrix for the immobilization of HRP, which greatly amplified the surface coverage of the functional monolayer. Experimental results indicated that the proposed fabrication method is of great interest for the development of highly sensitive, selective and stable 3rd generation H_2O_2 biosensor (Xu et al., 2007). A novel scheme for the fabrication of AuNP modified ChOx based enzyme electrode for quantitative determination of cholesterol has been reported (Fig. 4). Deposition of AuNP on the 1,6-hexanedithiol modified gold electrode, functionalization of the surface of deposited AuNP with carboxyl groups using 11-mercaptoundecanoic acid (MUA) and then covalent immobilization of ChOx on the surface of AuNP film using the N-ethyl-N′-(3-dimethylaminopropyl carbodiimide) (EDC) and N-hydroxy succinimide (NHS) ligand chemistry, are the steps associated with the process of fabricating the electrode. The fabricated biosensor was successfully used for the selective determination of cholesterol in human serum samples (Saxena et al., 2011).

The combination of sol–gel materials with nanomaterials is another area of increasing interest, since it may lead to the improvement of sol–gel film conductivity and of electron-transfer between electroactive biomolecules and electrodes. A self-assembled 3rd generation HRP biosensor was developed, by using a 3D sol–gel network, formed by hydrolyzation of a silicate monomeric precursor, to provide attaching sites for AuNP (Jia et al., 2002). Since AuNP self-assembled to silica-gel provided the necessary electron transfer pathway and, also, could be assembled into multilayers to increase the enzyme loading capacity, the resulting biosensor exhibited enhanced sensitivity and stability.

4.4. Reconstitution based approach

The redox center in enzymes is embedded in protein matrices exhibiting diameters in the range of 70–150 Å, so the spatial and steric separation of the centers from the electrode surface by the protein shell introduces the barrier for electron-transfer (ET) communication (Heller, 1990, 1992; Willner and Katz, 2000). A lot of research efforts were directed in the past two decades to develop methods for establishing electrical contact between redox enzymes and electrodes, and to overcome the steric hindrance for ET introduced by the protein matrices (Zayats et al., 2008). The reconstitution approach is a novel way of establishing electrical contact between the redox centers of enzymes and the electrode surfaces (Katz et al., 1999). In this approach, the cofactor is separated from the enzyme to yield the apo-enzyme structure, and the apo-enzyme is then reconstituted on the relay–cofactor-modified surface to yield the desired configuration (Fig. 5). Using this method, different redox enzymes have been electrically contacted with electrodes, using redox-active molecular units as charge transfer mediators. The flavoenzyme, GOD was electrically

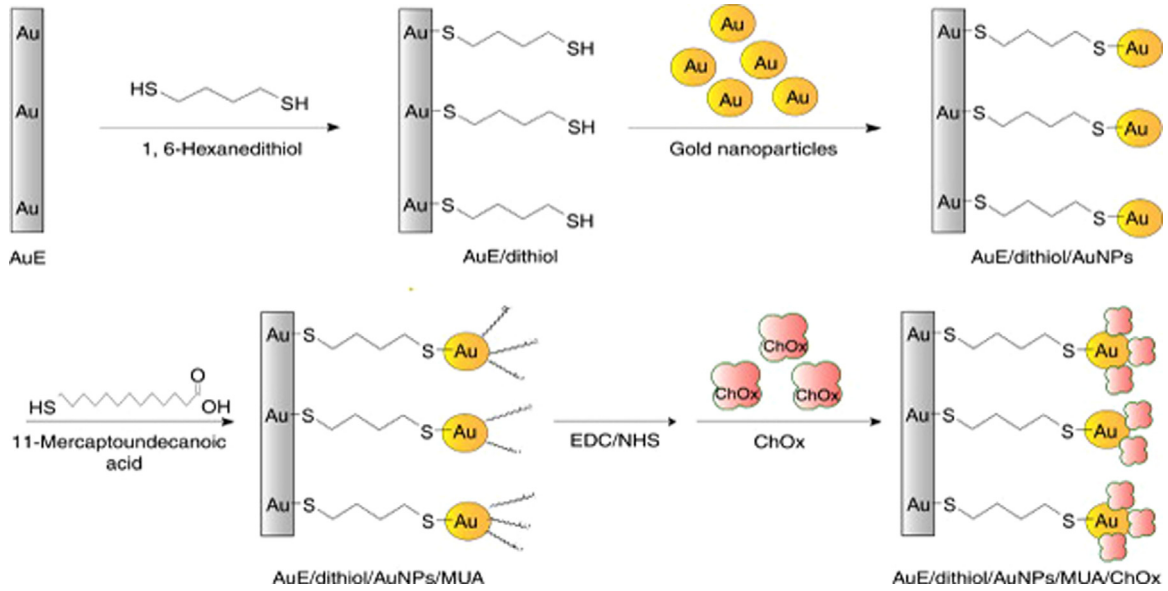

Fig. 4. The steps involved in the fabrication of AuNP modified cholesterol oxidase based enzyme electrode. (Saxena et al., 2011), Copyright 2011, reprinted with permission from Elsevier.

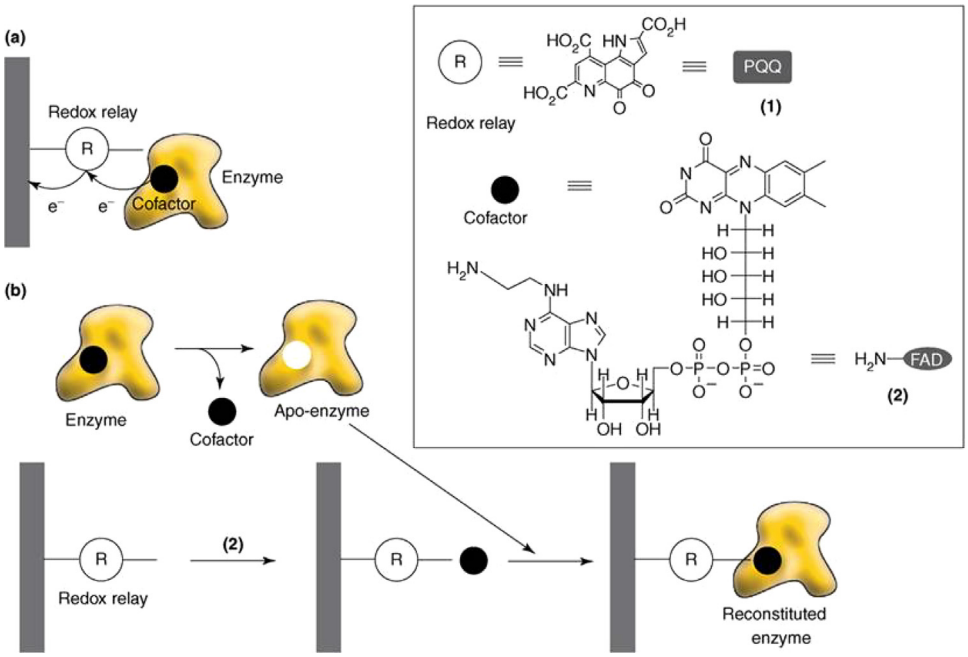

Fig. 5. Electrical wiring of redox enzymes (a) optimal configuration for the electrical contacting of a redox enzyme with the electrode (b) reconstitution of an apo-enzyme on a relay–cofactor monolayer for the alignment and electrical wiring of a redox enzyme. The structures of the redox relay molecule PQQ and cofactor amino-FAD are shown in the inset. (Willner et al., 2006), Copyright 2006, reprinted with permission from Elsevier.

contacted with an electrode by the reconstitution of apo–GOD on a PQQ (1) amino-derivatized FAD (amino-FAD) and (2) monolayer (Willner et al., 1996) (inset Fig. 5). In the case of flavoenzyme, the specific interactions between the flavin and the protein, as well as the conformation of the FAD within the active site of the protein, define the catalytic activity of the enzyme (Fruk et al., 2009).

Two general strategies are reported for assembling the reconstituted enzymes on electrodes. In one method, a relay cofactor dyad is assembled on the electrode, and the respective apoprotein is reconstituted on the surface to yield an aligned protein that is linked to the conductive surface by the relay component; whereas the second method involves the synthesis of the relay cofactor unit and the reconstitution of the apoprotein in solution. In both the configurations, the redox enzymes are electrically contacted with the electrode by means of the relay, a conductive molecular or polymer wire, a conductive nanoparticle, or even conductive carbon or doped silicon nanotubes. Reconstitution is also performed using CNT and conducting polymer wires. SWCNTs and MWCNT provide new graphite-like materials or cylindrical nano-dimensions. In order to achieve DET to the redox center of the enzyme molecules, FAD is first covalently attached to the SWCNT ends, and then GOD is reconstituted at the immobilized FAD (Patolsky et al., 2004).

There are numerous reports on the development of DET based bioelectrodes for various biosensors applications primarily, in clinical and environmental fields. A comparative account on some selected 3rd generation biosensors reported during the last decade has been made by focusing on some critical parameters frequently being used to express the efficiency of the biosensors as shown in Table 1.

5. Problems and challenges for developing 3rd generation biosensors

The denaturation of enzyme and the slow heterogeneous electron transfer in the enzyme–electrode interface are considered as major impediments in developing DET based efficient enzymatic biosensors. The DET based sensors exhibit relatively poor lifetime and stability. According to Marcus' theory (Marcus and Sutin, 1985) as described previously, the kinetics of electron transfer between the redox species and the electrode is determined by the distance between them. However, the gap between the prosthetic group of redox enzyme and the electrode surface is often large and the redox center in most cases is shielded by the protein shell; therefore the electron transfer via tunneling mechanism is rarely encountered (Gorton et al., 1999). Although different fabrication strategies have been utilized for direct transfer of electrons, the electrical contact efficiency (turnover rate of electrons) is usually significantly lower than the electron exchange rate between the redox center of the enzymes and their native electron acceptor or donors. The following twin reasons are ascribed to the inefficiency in some cases (Tel-Vered and Willner, 2010): (i) the tethering of the artificial redox-active electron relay units to the protein when performed on the chemically functionalized amino acid residues. Naturally, these modification sites are not sterically ideal for effective electrical wiring of the enzymes. Moreover, due to distortion of the protein back-bone by the chemical tethering of relay units, the enzyme is partially deactivated. For effective electron transfer, the number of relay units should be high as to maintain shorter electron transfer distances, which however results in an enhanced deactivation of the enzyme. (ii) The enzyme units linked to the electrode surface by covalent bridges or by physical entrapment in polymer matrices cause a random distribution of enzyme configurations with respect to the electrode. While some configurations of the enzyme exhibit effective electrical communication with the electrode, others are not sufficiently wired. As a result, the average efficiency of all enzyme orientations typically becomes low (Tel-Vered and Willner, 2010).

For DET the molecular bridge approach that provides a direct electron tunneling pathway between the redox active center and the electrode needs to fulfill a number of criteria. Firstly, because FAD (in case FAD based enzymes with specific example of GOD) is at least 13 Å from the surface of the glycoprotein, the bridge must be at least 13 Å long, for a connection between the redox active center of the enzyme and an electrode. Secondly, the bridge should be satisfactorily rigid so that it can project above a surface akin to a molecular post to provide room for the apo-enzyme to reconstitute around the surface-immobilized FAD without significant steric hindrance from the surface. Thirdly, each bridge must be placed apart on the electrode surface, so

Table 1
A comparative account on different parameters reported from various 3rd generation biosensors.

Fabrication strategy and Biosensor configuration	Enzyme (Redox active group)	Sensitivity (Analyte)	Detection limit	Response time	Linear Range	Stability	References
Nanofabrication							
Nf-MGF-GOD	GOD (FAD)	2.87 μA mM^{-1} cm^{-2} (Glucose)	0.25 mM	NA	1.0–12 mM	Biosensor was stored in PBS at 4 °C for 1 week; the reduction currents in air-saturated PBS containing 5 mM glucose retained 96% stability, while no obvious decrease was observed in N_2-saturated PBS.	(Wang et al., 2014)
GOD/cage-like PbS nanostructure	GOD (FAD)	11.02 mA mM^{-1} cm^{-2} (Glucose)	0.01 mM	7 s	5.0×10^{-5}–1.45 $\times 10^{-3}$M	Storage for 2 weeks at 4 °C shows a 5% loss of the bioactivity.	(Li et al., 2014)
Chit/HRP/KNs/Au	HRP (Heme)	750 μA mM^{-1} cm^{-2} (H_2O_2)	12 μM	1-2 s	0.04–6 mM	Biosensor retained 90% of its initial response current to H_2O_2.	(Cai et al., 2014)
ERGO–MWCNT/GOD/Nf	GOD (FAD)	7.95 μA$mM^{-1}cm^{-2}$ (glucose)	4.7 μM	5 s	0.01–6.5 mM	The modified film retained its 90% peak current even after 1 month.	(Mani et al., 2013)
MWCNT/Nf-AOx-PEI	AOx (FAD)	3 ± 0.08 μAmM^{-1} (ethanol)	5 μM	55 s	8–42 μM	No loss after 16 measurements carried out in 4hr, it retains about 90% of the original response after 4 weeks when stored in KPBS.	(Das and Goswami, 2013)
PsCDH NH_2-PD/SWCNT-GCE	CDH (FAD)	476.8 nAμM^{-1} cm^{-2} (lactose)	0.5 μM	4 s	1–150 μM	When stored at 4 °C, the shelf life of the sensor is longer than 6 months.	(Tasca et al., 2013)
HRP/nTOF/Ti-electrodes	HRP (Heme)	NA (H_2O_2)	1.5 μ M	NA	1–1000 μM	It retained 93% of its initial current response after18 days.	(Lee et al., 2013)
GOD/ MWCNTs–SnS_2/ Nf/GCE	GOD (FAD)	21.65 mAM^{-1} cm^{-2} (glucose)	4 μM	NA	0.02–1.95 mM	After 20 successive detections, the response retained 96% of the initial value. After storing for 3 weeks in 0.1 M pH 7.0 PBS at 4 °C, the biosensor showed a 6% loss of the bioactivity.	(Li et al., 2013)
GCE-PEI-AuNP-Laccase	Laccase (Copper)	(Catechol) 4.41 ± 0.007 μ A μM^{-1}	0.03 μM		0.36–11.00 μM	A relative response of over 80% was obtained at 90 days of evaluation.	(Brondani et al., 2013)
		(Guaiacol) 2.23 ± 0.003 μAμM^{-1}	0.03 μM		0.79–17.42 μM		
		(Pyrogallol) 1.22 ± 0.006 μAμM^{-1}	0.14 μM		1.74–19.60 μM		
		(Hydroquinone) 1.12 ± 0.006 μAμM^{-1}	0.21 μM		2.90–22.00 μM		
AR controlled ZnO NRs on Si/Ag electrodes	GOD Type VII from *Aspergillus niger*	(Glucose from urine)				Holds ~82, 86, and 94% of the initial performance even after 45 days for AR = 5, 30 and 60, respectively in 0.01 M PBS (pH 7.4)	(Ahmad et al., 2012)
ZnO NRs (AR = 5)	(FAD)	41.12 μA/mM cm^2	1 μM	< 6 s	0.0–12.0 mM		
ZnO NRs (AR = 30)		77.02 μA/mM cm^2	0.3 μM	< 5 s	0.0–14.0 mM		
ZnO NRs (AR = 60)		106.6 μA/mM cm^2	0.1 μM	< 2 s	0.01–17.0 mM		
Nf/Laccase-BP2000/GCE	Laccase (Cu)	99.84 μA mM^{-1} cm^{-2} (pyrocatechol)	0.003 mM,	15 s	0.003–5.555 mM	When the electrode was stored for 20 days at 4 °C, under the same experimental conditions (pH 7.0, room temperature, working potential 170 mV), the peak current of the oxidation of pyrocatechol was only decreased by 3.90%.	(Wang et al., 2012)
Ti/Au/BP/HRP-MB electrode	HRP (Heme)	54 μAμM^{-1} (H_2O_2)	0.075 μM	NA	10–500 μM	After 30 days, the residual response current of the biosensor was about 96%.	(Chatterjee and Chen, 2012)
GCE/HPt-CNTs-Chit/ L-cys/PDDA-Au/GOD	GOD (FAD)	20.1 m$AM^{-1}cm^{-2}$ (glucose)	0.4 μM	< 5 s	1.2–8.4 mM	The biosensor lost only 5.3% of the initial response after one weeks and the catalytic current response maintained more than 83.7% of its initial value after storage for 3 weeks.	(Wang et al., 2011)
AuNPs–GOD–MWCNTs–PVA/GCE	GOD (FAD)	16.6 μA mM^{-1} cm^{-2} (glucose)	0.2 mM	10 s	0.5–8 mM	Upto 1 month, when stored at 4 °C peak current only decreased slightly.	(Zhang et al., 2011)
GCE/MWCNT-NF/CAT/PEI	CAT (Heme)	30 μAmM^{-2} (H_2O_2)	~ 1 μM	~ 2 s	10–5 mM	75% of the CAT activity was retained even after 48 h of study.	(Vatsyayan et al., 2010)
TiO_2/CNT/Pt/GOD	GOD (FAD)	0.24 μAmM^{-1} cm^{-2} (glucose)	5.7 μM	< 3 s	0.006–1.5 mM	The sensor response to 0.01 mM glucose was decreased by 18% after one month mostly due to the decrease in the enzyme activity.	(Pang et al., 2009)
Polymer Based							
MP/ChOx/AuNPs-GSH/PDATT/GCE.	MP ChOx (Heme) (FAD)	0.054 ± 0.005 μAμM^{-1} (Cholesterol)	0.3 ± 0.04 μM.	0.5 ± 0.1 s μM^{-1}	10–130 μM	Examined over a period of one month during which the biosensor retained about 95% of its initial response to the	(Abdelwahab et al., 2010)

						cholesterol.	
CAT/RTIL/NH_2-MWCNTs/GCE	CAT (Heme)	4.9 nAnM^{-1} (H_2O_2)	3.7 nM	6 s	8.6–140 nM	The electrode was stored in PBS at 4 °C for 2 weeks and there was no observable change in response toward H_2O_2, showing high storage stability.	(Rahimi et al., 2010)
GCE/GCE	HRP (Heme)	1.44 mA cm^{-2}M^{-1} (H_2O_2)	0.2 μM	< 10 s	1–121 μM	In 50 mM pH 7.0 PBS retain 96% of its initial response current after 3-week storage.	(Lu et al., 2006)
Self assembled approach							
GOD self-assembled on ERCGR over GCE	GOD(FAD)	7 μAmM^{-1}cm^{-2} (glucose)	0.02 mM	NA	2–18 mM	Current of the modified electrode was reduced by 4.6% of its initial response after 2 weeks of storage at 4 ° C.	(Liang et al., 2013)
AuE/dithiol/AuNPs/MUA/ChOx	ChOx (FAD)	45.96 mAmM^{-1}cm^{-2} (Cholesterol)	34.6 μM	NA	0.04–0.22 mM	The bioelectrode retained ~95% of its initial response after 1 month, when stored at 4 °C in PBS.	(Saxena et al., 2011)
HRP-SAM-$AuNAE_{5h}$	HRP(Heme)	45.86 μA mM^{-1} cm^{-2} (H_2O_2)	0.42 μM	< 4 s	0.74–15 mM	When stored in pH 7 phosphate buffer at 4 °C the biosensor retained 96% of its initial activity for H_2O_2 after 4 weeks.	(Xu et al., 2010)
HRP/AuNP / poly (DVB-co-AA) nanospheres/AuE	HRP (Heme)	NA (H_2O_2)	0.5 μM	NA	1.0–8.0 mM	The biosensor was stored in the refrigerator at 4 °C, it retained 97.4% of its initial current response after intermittent (once a week) use over 60 days period	(Xu et al., 2007)
Reconstitution approach							
PTBA/FAD/GOD	GOD (FAD)	2.14 μA mM^{-1} cm^{-2} (glucose)	2.1 μM	~5 s	0.5–18 mM	The biosensor retained about 70% of its original response after 20 days by amperometric measurements in the presence of glucose solution (pH 7.5; temperature 25 ° C)	(Şenel et al., 2013a)
GOD/pol (aniline boronic acid/ GCE	GOD (FAD)	5.94 μA mM^{-1} (glucose)	0.24 mM	~12 s	1–17 mM	The biosensor retained about 72% of its original response after 50 days of storage at 4 ° C in PBS.	(Şenel et al., 2013b)

Table abbreviation, Antimony oxide bromide (AOB), Aspect-ratio (AR), Black Pearl 2000 (BP2000), Buckypaper (BP), divinylbenzene-co-acrylic acid (DVB-co-AA), Electrochemically reduced graphene oxide (ERGO), electrochemically reduced carboxyl graphene (ERCGR), Gold nanoparticles (AuNP), Gold electrode (AuE), Glutathione (GSH), Hollow nanostructured Pt (HPt), Horse radish peroxidase (HRP), $KNbO_3$ nanoneedles (KNs), 11-mercaptoundecanoic acid (MUA), mesocellular graphene foam (MGF), methylene blue (MB), Nanowire array electrodes (NAEs), Not available (NA), p-phenylenediamine (NH_2-PD), poly(diallydimethylammonium) chloride (PDDA), Poly-3,4'-diamine-2,2',5,2"-terthiophene (PDATT),Polyvinyl alcohol (PVA), Phanerochaete sordida (PsCDH), Poly-(pyrrolepropylic acid) (PPA), polythiophene boronic acid (PTBA), Titanium oxide film (TOF), nanowire array electrodes (NAE_{5h}) (5 h deposition time), zinc oxide nanorods (ZnO NRs).

as to provide room for apo-enzyme for reconstitution. Finally, transfer of electrons through the bridge should be rapid (Liu et al., 2006a). Tailoring enzymes to befit the immobilization for efficient electron transfer is also another promising aspect for DET. Holland et al. (2011) reported a simple genetically modified GOD enzyme to present a free thiol group near its active site that facilitated the site-specific attachment of a maleimide-modified AuNP to the enzyme, which enables direct electrochemical communication between the conjugated enzyme and the electrode (Holland et al., 2011).

6. Conclusion and future perspectives for developing 3rd generation biosensors

The major driving forces for developing 3rd generation biosensors are interference free (as they operate in a potential window closer to the redox potential of the enzyme), reagent less and label free detection of analyte. Since the appearance of first reports on DET between redox protein and electrodes (Eddowes and Hill, 1977; Yeh and Kuwana, 1977) the volume of research work performed worldwide is astounding as witnessed from the number of publications on the subject. However, the enormity of the work steeply increased since the beginning of 21st century with the concomitant increase in knowledge in the field of material science and polymer chemistry due to obvious reason of stunning effects of the advance materials emerged from these field for supporting DET of redox proteins on electrode surface. The major focus of this specialized branch of bioelectrochemistry is though growingly laid on developing 3rd generation enzyme electrode for biosensor application, an in-depth investigation on the subject is also largely prompted by the quest of understanding in vivo redox systems in cells, primarily, the oxidative phosphorylation and photosynthetic phenomena (Rasmussen and Minteer, 2014). The interesting information gathered so far (e.g. on many heme and copper based redox proteins) are supporting as underneath knowledge for conceptualizing 3rd generation biosensors. An efficient electron hopping between the redox center of the protein/enzyme and electrode implies favorable interfacial dynamics and chemical integrity of the protein at the electrode surface (Armstrong et al., 1988). Since thin protein film on the electrode surface has been identified as favorable approach to develop 3rd generation biosensors, the technique PFV has been evolved as most widely used technique to study the DET. The deconvolution of different PFV data enabled to assign redox mechanism, kinetics and thermodynamics relevant to the electron transfer and eventual chemical reaction on the electrode surface. However, for precise prediction of electron exchange route in DET based biosensors a deep understanding on starting from the native enzyme-substrate electron-exchange reaction, 3D structure of the protein including the distribution of electro-active amino acid moieties in the protein matrix, and the prospective route for electro-active interaction conduits of the redox protein with the surrounding electro-active environment (conductive nanomaterials, redox polymers etc.) is warranted.

An integrated approach utilizing the tools and techniques involved in molecular biology, spectroscopy, bioinformatics and computational chemistry and crystallography likely to offer comprehensive concept for tailor made construction of functional 3rd generation biosensors with different redox enzymes as bioelectrocatalysts. The DET based biosensors though, has been reported for a large number of targets with different fabrication approaches and concepts, the movement of these developments to acceptable products in market is not yet widely known. The primary reasons for this slow transition are presumed to be low-stability, reproducibility and high-cost of the constructs. Various biocompatible materials and approaches for fabrication of enzyme electrode though, have been proposed to increase the stability there are only limited reports on the studies on balanced performance factors for practical utility of the biosensors. To alleviate the cost, application of less expensive materials such as, paper, as sensor platform, which also comply the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment free, Delivered) mandate of WHO (Peeling et al., 2006) may be urged. Eventually, the reproduction of the fabrication concept of the DET based enzyme electrode on a low-cost biocompatible sensor platform following the emerging micro/nano-fabrication techniques and tools (Ledru et al., 2006; Shumyantseva et al., 2005; Xu et al., 2015; Zuo et al., 2008) will facilitate developing next generation biosensors in a lab-on-chip device for practical utility and commercial applications.

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