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Recent approaches to ameliorate selectivity and sensitivity of enzyme based cholesterol biosensors: a review

Anjum Gahlaut, Vinita Hooda, Vikas Dhull and Vikas Hooda

Centre for Biotechnology, Maharshi Dayanand University, Rohtak, India

ABSTRACT

The healthcare area is often reluctant to execute new technology unless they are proven to be safe, constructive and secure. Eventually, an aspiration stands for providing point-of-care testing service to allow a better estimation of the biochemical levels of a patient that entails an insistent remedial action. With increasing mortality rate due to cardiovascular diseases (CVDs) in present scenario, it has become the need of hour to develop more advance methods for their diagnosis, so that it can be determined at sensitive levels and can be prevented from being fatal. Elevated level of cholesterol in blood stream is one of the utmost risk factors which lead to CVDs. Discernible from the vast research in this field, worth of cholesterol biosensors is already recognized and flourished in the clinical analysis of brain and cardiac vascular diseases. It necessitates unremitting progress in the development of biosensing technology towards fabrication, miniaturization and multiplexing ability of cholesterol quantification devices so that they can endow with lab-on-chip-analysis systems to the medical field. Different strategies have been meticulously explored for the engineering of cholesterol biosensors utilizing nanocomposites, conducting polymers, nanotubes and nanoparticles. Foremost, this article reviews the contemporary evolution in cholesterol biosensors, which encompass various strategies for immobilization of enzymes and roles of various matrices and artificial mediators used for the biosensor fabrication. Still there remains an enormous challenge to congregate the demands of performance and yield in a cost effective manner for its application in successful treatments of CVDs.

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Introduction

Cardiovascular disease (CVD) is the prime cause of death in present world. According to statistical data analysis of CVD cases in US, American Heart Association has stated that 33% of US adults suffer from hypertension and every one out three deaths is due to CVD. Situation is relatively much worse in Europe where out of 4 million deaths every year 47% are due to CVD (As per data provided by British heart foundation). Cholesterol is popular as risk factor because the increase in blood cholesterol along with other factors, such as high blood pressure or diabetes, this risk of CVD increases even more. Elevated level of cholesterol in blood is one of the factors responsible for coronary artery disease, hypertension, nephritic syndrome or cirrhosis, atherosclerosis, heart attack and stroke. When high level of cholesterol circulates in the blood, it slowly builds up layer inside the inner walls of the arteries that feed the heart and brain. Together with other substances, it can form plaque, a thick, hard deposit that can narrow the arteries and making it harder for the blood to cross through. This crucial condition of stiffened and narrowed coronary arteries is known as atherosclerosis. The consequences may result into heart attack, stroke or even death [1]. In US total cost of CVD and stroke for 2009 has been estimated to be $312.6 billion (Cost includes diagnosis, medication and home health care).

Cholesterol ($C_{27}H_{46}O$) is found in almost all tissues of the body. It is a waxy substance which is amphoteric in nature with polar head and nonpolar hydrophobic tail. It exists in body in free as well as esterified form. About 70% of total cholesterol in blood is present in esterified form and its decreased level is usually associated with liver malfunctioning. Cholesterol is required by the body for performing various metabolic processes and cellular functions like intracellular transport, cell signalling and nerve conduction. It also acts as precursor in synthesis of vitamins, hormones and bile acids. Some cholesterol is synthesized in the body by liver and rest is taken in form of food. Eggs yolks, dairy products, meat and poultry are rich sources of cholesterol. Organ meats (liver, kidney, sweetbread and brain) contain very high level of cholesterol. Sometimes fat content is confused with cholesterol but it is not right because sometimes substance rich in fat may have low level of cholesterol and substance enriched with cholesterol may have low fat content. Liver has low fat content, but it has very high level of cholesterol in it [2]. Structure of cholesterol has been shown in Figure 1. The concentration of cholesterol less than 200 mg/dL is enviable and lowers the risk for coronary heart disease. The level of cholesterol above 200 mg/dL raises the risk, 200–239 mg/dL is borderline high, 240 mg/dL and above blood cholesterol faces twice the risk of coronary heart disease as someone whose

CONTACT Vikas Hooda advance.biotech@gmail.com Centre for Biotechnology, M. D. University, Rohtak 124001, Haryana, India

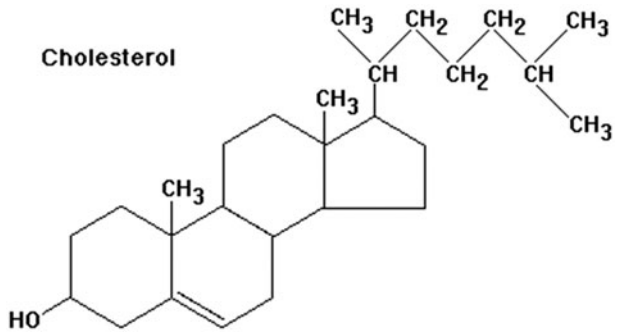

Figure 1. Structure of cholesterol.

cholesterol is below 200 mg/dL. Minimum level of cholesterol in the body which is considered ideal has decreased from 200 to 180 mg/dL with changing lifestyle of the people [3].

With increasing incidents of cardiac arrest and stroke around us and after diagnosing the cause behind them, more accurate and faster determination of cholesterol in the serum has gained public concern. Quantification of cholesterol is being done since years by different methods like colorimetric method [4], fluorimetric method [5], spectrophotometric [6], enzymatic [7] methods. Method using free enzymes with electrochemical methods has also been used [8]. Enzymes like peroxidase [9,10] which causes production of quinoneimine dye in the sample whose absorbance is proportional to the concentration of cholesterol in the sample. But these methods have several limitations like they require pre-treatment of sample, time consuming, tedious and require trained man power. Use of free enzymes in cholesterol determination is over-priced too as the free enzymes are discarded after use, or separation of free enzymes from solution is again time consuming. Immobilisation of enzyme enables its recycling. Biosensors having immobilized sensing material are getting considerable interest due to their ability to give rapid and sensitive response, compact size and reproducible nature. Due to these advantages they have found variety of applications which can aid us in our day-to-day life from health care to environment monitoring and food analysis to medical diagnostics [11].

Concept of biosensor

Biosensor is an analytical device which detects the biochemical change produced due to the presence of specific analyte. A signal is produced which is proportional to the concentration of the target analyte present in its proximity. Basic assembly of biosensor consists of recognition layer, transducer and processor. Recognition layer is the main sensing element of the biosensor; it is made up of biological component that can be an enzyme, a cell, antibody, receptor molecule or DNA. Recognition layer is immobilized either directly on transducer or on some other support present in its intimate contact. Transducer acts like a translator that recognizes the physico-chemical change (i.e. pH change, heat transfer, uptake or release of gases or specific ions, electron transfer and mass changes) and converts it into electronic signal and the processor converts it into readable digital output. Schematic representation of biosensor has been depicted in Figure 2. The concept of biosensor was pioneered by Clark for determination of glucose, thereafter concept has been explored widely for determination of various other molecules like cholesterol, lactate, uric acid, hCG, etc. Differentiating on the basis of type of transducer used, biosensors can be categorized into following:

Calorimetric biosensor

Enzyme catalysed reactions are usually exothermic. This phenomenon is used for analysing the biochemical change occurring on recognition layer. These biosensor measures the heat generated using thermistors. Cholesterol oxidase produce 53 kJ/mol, Glucose oxidase produces 80 kJ/mol [12].

Optical biosensor

Surface plasmon resonance biosensor is a kind of optical biosensor. Surface plasmon resonance transducers measure changes in refractive index of surface of the sensing element. When plane polarized incident light passes through the prism and strikes the metal at an adequate angle, it induces a resonant charge wave at the metal/dielectric interface that propagates a few microns. The total reflection is measured with a photo detector, as a function of the incident angle. For example, when an antigen binds to an antibody which is immobilized on recognition layer it is found to have increased reflectivity. This increase in reflectivity is due to the presence of antigen on the surface and directly proportional to its concentration [13].

Electrochemical biosensor

In electrochemical biosensor charge transfers from working electrode to reference electrode. It mainly works on three principles: i) Amperometric which detects change in current at constant potential, ii) Potentiometric which detects change in potential difference between working electrode and reference electrode at constant current [14] iii) Conductometric which detects change in electrolytic conductivity of the cell. Conductivity depends on ion concentration so; change in ion concentration due to biochemical change is interpreted using conductometric biosensor [15].

Piezoelectric biosensor

Piezoelectric crystals like quartz crystal vibrate under electric field and each crystal possesses a specific resonant frequency under a fixed strength of electric field. Change in resonant frequency occurs due to the absorption of particles on the surface of crystal. Change in frequency of crystal is proportionate with total mass deposited [16].

Enzymes involved in fabrication of cholesterol biosensor

Cholesterol biosensors contain cholesterol oxidase (ChO) and cholesterol esterase (ChE) as recognition element.

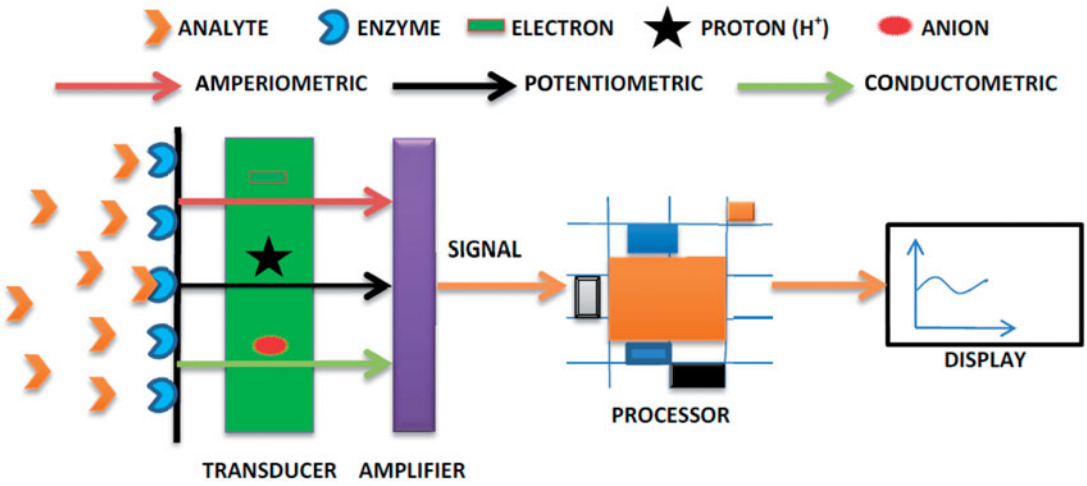

Figure 2. Basic principle of biosensor.

Cholesterol oxidase is a flavoenzyme which uses flavin adenine dinucleotide (FAD) as cofactor and catalyses the oxidation and isomerisation of steroids containing a hydroxyl group at 3′ position. ChO exists as monomer with molecular mass 55 kDa. ChO is commonly obtained from *Streptomyces hygroscopicus* and *Brevibacterium sterolicum*. ChO obtained from *Streptomyces hygroscopicus* is more sensitive and show linearity up to 20.7 mmol/L. It's another benefit is that it does not show interference due to the presence of hemoglobin [17]. Cholesterol oxidase belongs to flavin-specific oxidoreductases family. Its prosthetic group FAD is covalently attached to the core protein in *Streptomyces* and is non-covalently bound to the core enzyme in *Brevibacterium* [18]. Cholesterol esterase catalyses the hydrolysis of sterol esters into sterols and fatty acids. ChE is found in pancreas in bulk quantity, but it has been detected in other tissues also. It exists in polymeric form [19]. While determining cholesterol concentration in a given sample cholesterol esterase first hydrolyses esterified cholesterol and then cholesterol oxidase oxidizes cholesterol to produces cholest-4-en-3-one and hydrogen peroxide (H_2O_2). In amperometric biosensors electric potential is applied on H_2O_2 which get oxidized to produce electrons and the flow of electrons produces current. The current produced is directly proportional to the concentration of cholesterol present in the sample. Concentration of released protons is measured by potentiometric biosensor.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{ChE}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChO}} \text{Cholest} - 4 - \text{en} - 3 - \text{one} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Electric Potential}} 2H^+ + O_2 + \text{electrons}$$

A new approach to reduce interference is the use of Horse radish peroxidase (HRP). HRP has redox centre associated with ferroheme/ferriheme pair. Conversion of reduced state to oxidized state and vice versa is done through direct electron transfer. In its 3D configuration HRP has heme group present in outer region and facilitate direct electron transfer between redox centre and conduction sites present on transducer [20]. Regeneration of HRP is done via direct electron transfer.

$$H_2O_2 + 2H^+ + HRP_{Red.} \rightarrow 2H_2O + HRP_{Oxd.}$$
$$HRP_{Oxd.} + 2e- \leftrightarrow HRP_{Red.}$$

Enzyme immobilization

Immobilized enzymes are the enzymes physically confined or localized in a certain defined region of space without altering their catalytic activities, so that they can be used repeatedly and continuously. Immobilized enzymes are used in organic synthesis to fully exploit the technical and economical benefits of biocatalysts based on isolated enzymes. It will increase the enzyme specificity (k_{cat}/K_m) and reduce product inhibition [21]. Operational stability of enzyme also increases on immobilization. The concept of stabilization has been an important driving force for immobilizing enzymes [22]. Immobilization of enzyme on the surface of electrode provides reusability as well as cut the cost of enzyme preparation. Mode of enzyme immobilization is most critical step which decides the level of sensitivity and stability of biosensor [23]. Enzyme can be immobilized on different supports in different ways.

Physical adsorption

Adsorption is the simplest method of reversible immobilization based on weak forces like hydrophobic interactions, van der Waals forces, ionic and hydrogen bonds are involved in physical adsorption. Strength of interaction depends upon pH, salt concentration and temperature so that the interaction can be reversed by changing the pH, ionic strength, temperature or polarity of the solvent. Due to weak interaction enzyme fall off is a common problem with physical adsorption [24].

Covalent bonding

Immobilization of enzymes by formation of covalent bonds is the most stable mode of immobilizing. Covalent coupling is done either by activation of the matrix by addition of a

reactive functional group on a polymer or by modification of the polymer backbone to produce an activated group. Advantage of covalent bonding is the stable nature of the bonds formed between enzyme and matrix, fast response and reduced enzyme leakage but chemical modifications may lead to the denaturation of enzyme due to the alteration of their 3D conformation [25].

Cross-linking or co-polymerization

Cross-linking is the only method of enzyme immobilization that does not require a support. Cross-linking is covalent bonding between the molecules of an enzyme via polyfunctional reagents like glutaraldehyde, diazonium salt, hexamethylene diisocyanate, and N–N' ethylene bismaleimide. Though cross linking method of immobilization is cheap but indeed it requires purer form of enzyme. As it uses very little immobilized enzyme which need to have very high activity. Another demerit of using polyfunctional reagents is that they denature the enzyme and lower the activity [25,26].

Entrapment

Entrapment of enzymes within gels or fibres is the most suitable method when low-molecular weight substrates and products are involved in the process. Entrapment methods involve the occlusion of an enzyme inside a polymeric network that allocate the substrate and products to pass through but preserve the enzyme. But the entrapment of enzyme requires derivatization of enzyme free residues for gel formation, which may affect the enzyme activity [25].

Characterization of biosensors on the basis of support used

Varieties of natural and synthetic supports are being used for immobilization of enzymes for cholesterol determination. Cholesterol oxidase (ChO) and cholesterol esterase (ChE) are immobilized on different supports to determine the native as well as esterified cholesterol.

Organic and inorganic matrices based biosensors

Polymer matrices based biosensor

Polymers have gained great deal of scientific attention to be used as support for enzyme immobilization. More specifically the conducting polymers have gathered considerable interest due to their high-electrical conductivity, optical properties and mechanical strength. Conducting polymers like polypyrrole (PPy), polyaniline (PANI), polythiophene (PT) can be synthesized chemically by electrochemical polymerization [27]. These properties of polymers are exploited by using polymer matrix in transducer for enhancing the signal response and stability of biosensor. Cholesterol hydrolysing enzymes ChO and ChE has been immobilized on conducting polypyrrole films [28], PB/Polypyrrole (PPy) composite film [29], electropolymerized PPy films [30], poly(3-thiopheneacetic acid film/Pt electrode [31] and PANI–pTSA-Ag/ITO [32]. Chitosan (CS) is a green polymer due to its biodegradability and biocompatibility. It also shows high affinity for proteins and availability of reactive functional groups for chemical modifications which make it a perfect enzyme immobilization support [33]. Transducers have been fabricated using nano ZnO film on chitosan [34], Pt-Pd NPs-CS-GS nanocomposite [35], CNT-CS/GCE [36]. Along with conducting polymers non conducting matrices are suitable for enzyme immobilization. PVC is chemically inert insulating matrix. It has also been used to immobilize cholesterol hydrolysing enzymes. ChO and ChE were covalently immobilized on surface of PVC beaker which act as reaction cell and HRP was incorporated in carbon electrode [37].

Sol–gel matrices based biosensor

Sol–gel matrices hold remarkable physical rigidity, negligible swelling in aqueous solution, chemical inertness, high thermal and photochemical stability as well as tuneable porosity. Sol–gel matrices offer incredible possibilities for the promising encapsulation of heat-sensitive and fragile biomolecules like enzyme and protein due to their inherent low temperature process and biocompatibility [38]. These attractive features provide scope for their utility in optical and electrochemical biosensors. Cholesterol oxidase has been immobilized on silicic sol–gel matrix [39]. ChO has been co-immobilized with ChE covalently immobilized on tetraethylorthosilicate (TEOS) sol–gel films [40] and Sol gel/Pt NPs/CNT [41].

Membrane based biosensor

Membranes are also considered as good supports for enzyme immobilization. Besides being flexible and mechanically durable they are also cheap in cost. Also membranes are biocompatible in nature. Usually chemical modifications are required to make them suitable for enzyme immobilization. The cellulose acetate (CA) membrane gives the more active conjugate support-enzyme after chemical modification [38]. CA membrane has large pore size which facilitates the diffusion of substrate through it; this property of CA membrane is exploited while using CA membrane for immobilizing enzymes. ChO and ChE were co-immobilized on CA membrane and then the membrane was wrapped on carbon electrode incorporated with HRP which resulted in increased signal response [42]. ChO, ChE and HRP have also been co-immobilized on CA membrane mounted on Pt electrode [43]. Ultrathin dialysis membrane has also been used for immobilizing cholesterol hydrolysing enzymes [44]. Various other types of supports are also used for enzyme immobilization. Disposable screen printed electrodes are also used for immobilization of ChO. Screen printed electrodes are made of paste of conductive and dielectric inks which defines the working area and electric contacts [45]. Molecular wire type structure of 4-aminothiphenol has proved very efficient in electron transfer between enzyme and electrode [46]. Graphene modified graphite electrode [47], and oxygen electrodes have also been used in enzyme immobilization [48]. Properties of different types of electrochemical biosensors have been shown in Table 1.

Table 1. A brief account of various electrochemical biosensors used for the determination of cholesterol.

Sr.no.	Enzyme immobilized	Technique involved	Mode of immobilization	Transducer	Detection limit	Linearity range	Ref.
1.	ChO, ChE	Amperometry	Entrapment	Polypyrrole films	N.D.	1–8 mM	[28]
2.	ChO	Amperometry	Covalent binding	Polythiophene acetic acid/Pt electrode	N.D.	0–8 mM	[31]
3.	ChO, ChE	Amperometry	Covalent binding	PANI–pTSA-Ag /ITO	N.D.	20–400 mg/dL	[32]
4.	ChO	Amperometry		Pt-Pd-CS-GS nanocomposite	0.75 μM	2.2×10^{-6} –5.2×10^{-4} M	[35]
5.	ChO, ALP[a]	Amperometry	Co-immobilisation	CNT-Chitosan/GCE	10 μM.	0.05–2.0 mM	[36]
6.	ChO, ChE	Amperometry	Covalent binding	PVC and Carbon electrode	2.5 mg/dL	–	[37]
7.	ChO	Amperometry	Entrapment	Silicic sol gel matrix	1.2×10^{-7} mol/L	N.D.	[39]
8.	ChO, ChE	Amperometry	Covalent binding	Tetraethyl orthosilicate sol gel films	12 mg/dL	Upto 780 mg/dL	[40]
9.	ChO	Amperometry	Entrapment	Sol gel/Pt NPs/CNT	N.D.	4.0×10^{-6} –1.0×10^{-4} mol/L	[41]
10.	ChO, ChE, HRP	Amperometry	Adsorption	Cellulose acetate/ carbon electrode	2.5 mg/dL	2.5–6 mg/dL	[42]
11.	ChO, ChE, HRP	Amperometry	Covalent binding	CA membrane over Pt electrode	2.5 mg/dL	2.5–50 mg/dL	[43]
12.	ChO, ChE	Amperometry	Cross linking	Ultra thin dialysis membrane/ Oxygen electrode	–	15–80 mg/dL	[44]
13.	ChO, ChE	Amperometry	Cross-linking	Self-assembled monolayer of 4-aminothiophenol	–	25–400 mg/dL	[46]
14.	ChO, ChE	Amperometry	Covalent binding	Graphene modified Graphite electrode	15 μM	50–300 μM	[47]
15.	ChO, ChE	Amperometry	Cross linking	Oxygen electrode	N.D.	2–50 mg/dL.	[48]
16.	ChO	Amperometry	Covalent binding	Carbon nanotubes	10 mg/dL	50–400 mg/dL	[51]
17.	ChO	FFTCCV	Adsorption	MWNT/MnO_2 NPs.	0.3 nM	N.D.	[52]
18.	ChO	Cyclic voltammetry and amperometry	Covalent binding	MWCNTs	4.68×10^{-5} M	4.68×10^{-5}–2.79×10^{-4} M	[53]
19.	ChO, ChE, HRP	Cyclic voltammetry	Adsorption	Au NPs/ Ti electrode	N.D.	Upto 300mg/dL	[55]
20.	ChO	FFTCCV*	Covalent binding	CeO_2 NPs / MWCNT (SH) /AuNPs	0.2 nM	1–100 nM	[58]
21.	ChO	Amperometry	Covalent binding	Au NPs / Au electrode	34.6 μM	0.04–0.22 mM	[59]
22.	ChO	Amperometry	Cross linking	Nano structured polyaniline-SDS on ITO plates	N.D.	N.D.	[60]
23.	–	Amperometry	Adsorption	Fe_3O_4-SiO_2/MWCNT	N.D.	10 μM–4 mM	[61]
24.	ChO, ChE	Amperometry	Adsorption	Graphene/Pt NPs	0.2 μM	up to 12 mM	[62]
25.	ChO	Amperometry	Adsorption	ZnO NP/Au electrode	0.37×10^{-3} μM	$1–500 \times 10^{-3}$ mM	[63]
26.	ChO	Amperometry	Adsorption	Pt/Au ZnO Nano rods	0.03 μM	0.1–759.3 μM	[65]
27.	ChO	Potentiometry	Adsorption	Bilayer lipid membrane on ZnO nano walls	4×10^{-7}	1×10^{-6} –1×10^{-3} M	[66]
28.	ChO	Cyclic voltammetry and Chronoamperometry	Adsorption	Au-MWCNT-Chitosan	–	0.5–5 mM	[67]
29.	ChO, ChE, peroxidize	Amperometry	Adsorption	MWCNTs	N.D.	100–400 mg/dL	[68]
30.	ChO, HRP	Amperometry	Cross linking	NSPANI-AuNP-GR/ITO	25 mg/dL	Upto 500 mg/dL	[69]
31.	ChO, HRP	Amperometry	Adsorption	Graphite-teflon matrix	–	–	[70]
32.	ChO	Amperometry	Covalent binding	Platinum–Palladium–Chitosan–Graphene hybrid nanocomposites (PtPd–CS–GS) functionalized glassy carbon electrode (GCE)	0.75 μM	2.2×10^{-6}–5.2×10^{-4} M	[71]
33.	ChO	Amperometry	Covalent binding	2-Heptyl-4,7-di(thiophen-2-yl)-1H-benzo[d]imidazole) (BImTh) and 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)acetic acid (Fmoc-Gly-OH) on a graphite electrode	0.17 μM	–	[72]
34.	Apo-ChO	Amperometry	Covalent binding	Thiophene-3-Boronic acid and 3-amino-phenyl boronic acid on pencil graphite electrode (PGE)			[73]
35.	ChO	Amperometry	Covalent binding	(Z)-4-(4-(9H-carbazol-9-yl) benzylidene)-2-(4-nitrophenyl) oxazol-5(4H)-one (CBNP) on graphite electrode	0.4063 μM	–	[74]

[a]ALP: alkaline phosphatase; FFTCCV: Fourier Transformation Continuous Cyclic Voltammetry

Nano structures based biosensors

With evolution of nano scale materials, they have found vast scope in the field of biosensors and have revolutionized this field. Nanotubes, nanowires as well as nanoparticles are exploited for modifying the surface of electrode and recuperating the effectiveness of biosensors. CNTs were first used by Britto and his co-workers for developing an electrochemical sensor who demonstrated the electro catalytic properties of SWCNTs [49]. SWCNT is basically a sheet of carbon atoms folded to form cylindrical shape tube while MWCNTs are concentric rings of such cylindrical shapes. After that CNTs are being exploited for their properties of good conductivity and high surface to volume ratio [50]. Multi walled as well as single walled carbon nanotubes have been used as supports for immobilization of ChO and ChE as CNTs provides instant electron transfer. Positively charged ChO has been immobilized on negatively charged CNTs by in-channel flow technique [51]. MWCNTs composite films and MWCNTs with nanoparticles have been used as supports for immobilization of cholesterol oxidase, which has significantly improved the sensitivity of biosensor [52,53]. Various metal based nanoparticles like Gold, Silver, Platinum, ZnO, CeO_2 and MnO_2 are getting popularity these days. Nano particles increase surface area for immobilization of enzyme. NPs also enhance the rate of electron transfer, lower the working potential and improve the sensitivity of biosensors [54]. Some nano particles also take part in catalysis of chemical reaction involved. Use of nanostructures in biosensors has lowered the minimum detection limit up to nano molar concentrations. Nanoporous metal networks are also being used for enzyme immobilization [55]. Cholesterol oxidase had been immobilized on nanoparticles of CeO_2, MnO_2, ZnO, gold and platinum either by physical adsorption or covalent binding [41,52,56–58]. A biosensor based on Au NPs has shown that it not only provide larger surface area for enzyme loading but also forms nanostructured interface on the surface of electrode to improve luminol electrogenerated chemiluminescence (ECL) which aids in more sensitive detection of cholesterol molecules [59]. Polyaniline (PANI) films have property of high solubility and electrical conductivity. These properties are exploited along with increased interfacial area in nanostructured polyaniline films (NSPANI) [60]. SiO_2 coated on magnetic Fe_3O_4 dispersed on multi-walled carbon nanotubes forms paramagnetic nanocomposite layer (Fe_3O_4-SiO_2/MWCNT) which exploits electron transfer properties of nano Fe_3O_4 and MWCNTs, along with biocompatibility of nano sized silica [61] and graphene/Pt NPs [62]. Zinc oxide is also a preferable transducer. Along with being bio-safe it has remarkable electrical and thermal stability. That's why it is used in variety of ways to improve the sensitivity of biosensor viz. well crystallized flower shaped ZnO NPs [63], ZnO NPs [64], NanoZnO attached to CS [34], ZnO nanorods [65], ZnO nano walls [66]. Bilayer lipid membranes (BLM) in combination with ZnO nano walls have proved to increase the sensitivity in cholesterol determination. BLM exhibits lipophilic character thus attracts cholesterol molecules towards itself, and then the increased concentration of cholesterol molecules on membrane increases the sensitivity of biosensor [67]. ChO, ChE and HRP have been co-immobilized on MWCNTs [68], NSPANI-AuNP-GR/ITO [69] and graphite-teflon matrix [70] to overcome interferential problems arising due to electroactive species. Latest approach that has been implemented to improve the sensitivity is use of bimetallic nanoparticles. Electrodeposition of Pt-Pb NPs on CS and grapheme not only speed up the rate of electron transfer but also intensify the amount of ChO immobilized [35]. Various nanostructures based cholesterol biosensors have been characterized in Table 1 [71–74].

Another approach in making cholesterol biosensor is using alkaline phosphatase (ALP) with ChO on CNT-Chitosan on glassy carbon electrode (GCE). Phenol is produced by the action of ALP. Then oxidation of phenol is interpreted for cholesterol determination [36].

Role of mediators in cholesterol biosensors

High electric potential is required to oxidize H_2O_2, at such high electric potential various serum metabolites, ascorbic acid, uric acid, bilirubin, lactate, EDTA, metal ions and other electroactive species also get oxidized and interferes with the current produced by oxidation of H_2O_2 and show extra addition to the current actually produced by the hydrogen peroxide [75]. Mediators are electron transferring agents which participate in redox reaction and aid in rapid transfer of electrons. They get reduced at low potential and prevent other electro active species from oxidation. The redox potential of a mediator provides an appropriate potential gradient for electron transfer between enzyme's active site and electrode [76]. They oxidizes hydrogen peroxide on electrode surface and act as electron shuttle between enzyme and electrode mediators are immobilized on transducer by physical adsorption, covalent binding or polymer coating to provide instantaneous electron transfer. Electron mediators like cobalt phthalocyanine [77], ferrocene derivatives [78], phenothiazine derivatives [79], thionine [80] and titanium oxides have been used in cholesterol sensing to overcome interferential problems. Mode of action of mediators has been shown in Figure 3.

Prussian blue modified poly pyrrole composite films have also been used for immobilizing cholesterol oxidase for determining cholesterol [30]. Catalysis rate of PB is comparable to that of peroxidase that is why some researchers also refer it as "Artificial peroxidase" [81]. Ferrocene monocarboxylic acid has been co-entrapped with ChO on electropolymerized polypyrrole films [29]. In another research work reconstituted ChO was immobilized covalently on Au electrode along with pyrroloquinoline quinone (PQQ) and fabricated a sensor selective towards interferents with greater accuracy [82]. Table 2 [29,30,77–83] illustrates the characteristics of mediators based

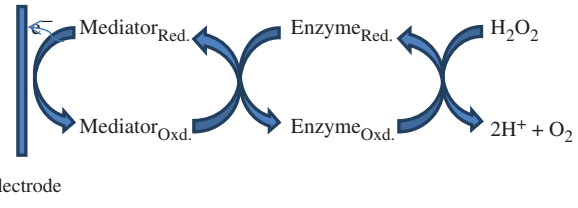

Figure 3. Role of mediator in electron transfer.

Table 2. Characteristics of mediator based electrochemical cholesterol biosensor reported in literature.

Sr. no.	Enzyme immobilized	Technique involved	Transducer	Mediator	Linearity range	Ref.
1.	ChO	Amperometry	Electropolymerized PPy film	Ferrocene monocarboxylic acid	12.4 μM	[29]
2.	ChO	Amperometry	Polypyrrole (PPy) composite film	Prussian blue (PB)	$10^{-5} \sim 10^{-4}$ mol/L	[30]
3.	ChO	Amperometry	Cobalt phthalocyanine screen-printed carbon electrode	Phthalocyanine	6×10^{-5}–5×10^{-3} mol dm^{-}	[77]
4.	HRP	Cyclic voltammetry and Chronoamperometry	Hydroxymethyl ferrocene modified carbon paste electrode	Ferrocene derivative	1×10^{-6}–1.5×10^{-4} M	[78]
5.	ChO	Amperometry	–	Phenothiazine	–	[79]
6.	ChO	Amperometry and Cyclic voltammetry	Gold electrode	Thionin	–	[80]
7.	ChO	Amperometry	SAM of cystamine	Pyrroquinoline quinone (PQQ)	–	[82]
8.	ChO, HRP	Amperometry	Poly(thionine)-modified glassy carbon electrode (GCE/PTH)	Hydroquinone (HQ)	25–125 μM	[83]

Table 3. Most commonly used methods adopted for the fabrication of cholesterol biosensors.

Sr. no.	Enzymes immobilized	Technique involved	Mode of immobilisation	Transducing surface	Detection limit	Linearity range	Ref.
1.	ChO	Surface Plasmon resonance	Covalent binding	AUT/Au electrode	N.D.	50–500 mg/dL	[84]
2.	ChO	Surface Plasmon Resonance	Physical adsorption	10-carboxy-1-decanethiol(CDT)/Au electrode	4.64 mg/dL	50–500 mg/dL	[85]
3.	ChO, ChE	Optical and gravimetric	Adsorption	Polystyrene sulfonate/polyethylene imine	–	–	[86]
4.	ChO	Fibre optics	Entrapment	Graphite powder on Silicon film	–	0.15–3 mM	[87]
5.	ChO	Fluorimetry	Covalent binding	Pt-octaethylporphine/Algilica beads	N.D.	1.25–10 mM	[88]
6.	ChO	Electrochemiluminescence	Covalent binding	Hemin-functionalized graphene (hemin-GR)	1.0 nM	3.3–1500 nM	[89]
7.	ChO	Electrochemical–Photoelectrochemical (PEC) dual-mode	Covalent binding	Graphene (G) sheets interconnected-graphene embedded titanium nanowires (TiO_2(G)-NWs) 3D nanostacks (designated as G/Ti(G) 3DNS)	6 μM	–	[90]

cholesterol biosensors. Artificial mediators have been employed in various sensors to eradicate the inferential problems due to other electro active species in the sample. Various techniques other than electrochemical method like optical and flurometric methods are also involved in cholesterol determination. ChO has been covalently immobilized onto 11-amino-1-undecanethiol hydrochloride (AUT) monolayer fabricated on gold substrates [84], 10-carboxy-1-decanethiol (CDT)/Au electrode [85] for cholesterol determination through surface plasmon resonance. Other optical biosensors based on fibre optics have also been fabricated by immobilizing cholesterol oxidizing enzymes on polystyrene sulfonate/polyethylene imine and graphite powder on silicon film [86,87]. Cholesterol determination has been done flurometrically by immobilizing ChO on oxygen sensing Pt-(II)-octaethylporphine modified algilica beads [88]. Table 3 [84–90] shows the properties of cholesterol biosensors based on different techniques.

Future prospects

Manual and wireless controlled biosensors are being designed and commercialized in healthcare industry for monitoring and tracking a CVD in patients with ease. Biosensors go beyond just diagnostics as they are endowed with real-time data logging. They prepare both doctors and alert patients for probable health issue in the near future. The potential of cholesterol biosensors is apparent to physicians for the monitoring of elevated level of cholesterol in the blood stream which leads to CVDs. Successful biosensors must be versatile to sustain enhanced comprehension of the bioreagent immobilization and miniaturization must be viable to allocate automation for parallel sensing with ease of operation in a cost-effective manner. The deployment of microfluidics, nanomaterials, software and biomarker patterns will make these devices highly potential and more pertinent for point-of-care early diagnosis of CVDs. With a concerted multidisciplinary approach, these advancements in the sensor technologies will successfully formulate them from the research and development laboratory to improve patient compliance. This extensive research has provided variety of methods for electrochemical analysis of blood cholesterol but to harness their full potential miniaturization of these biosensors is to be cogitated so before these biosensors are available to common man a long way is still to go. The deciding factors for the popularity of cholesterol biosensors in the near future will be the level of their sophistication, cost-effectiveness, reliability, portability, availability and commercialization. Although there is huge market potential but still except for few commercial successes, many of the prototypes

of biosensors have continued to lag behind to bag a significant place in market. In Future innovations, wearable and wireless smart mobile tools will transmit biomedical data to the designated healthcare professionals to diagnose the health status through a non-invasive method in a better way and thereafter tailor treatment according to the individual patient's requirements. Disposable and simple user-friendly biosensing devices will be preferred for the mass production. Undoubtedly, biosensors have a very bright future. Enthusiastically, nanosize-based sensors should be coalesce with biochips along with modern electronic elements so that they can strongly draw attention to utility when ensuring the smallest measuring systems, mobile, inexpensive and wide expediency in diagnostics.

Disclosure statement

No potential conflict of interest was reported by the authors.

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