Chapter 1
Introduction and Overview

Health being a major concern for humankind, healthcare industry is expanding at a rapid pace worldwide. The key to sound health lies in the regular assessment of health status, disease onset and progression, and continuous monitoring of treatments outcome. Biosensor technologies offer the potential to fulfill these criteria through an interdisciplinary combination of approaches from nanotechnology, chemistry and medical science. Biosensors find their application in medicine, pharmacy, food and process control, environmental monitoring, defense and security, but most of the research is driven by medical diagnostics. Bionanotechnology offers unprecedented opportunity to unlock the secrets of biology on an entirely new level and open a new era in healthcare. Currently available detection methods are based on table-top laboratory equipments which are expensive and require trained personnel for operation. The urgent demand to move clinical analysis from centralized laboratories to clinics, camps and patients self-testing at home has put tremendous pressure to develop compact, disposable and mobile devices that offer quick detection, are user-friendly, cost-efficient and suitable for mass production.

Early and accurate diagnosis plays an imperative role in determining predisposition or prognosis of the disease. The treatment of the diseases and the outcomes of the treatment are affected by the delay in the early diagnosis. Even simple routine biochemical tests are time-consuming, require well-trained technicians and expensive laboratory infrastructure with continuous supply of chemicals resulting in high costs.
Such diagnostic challenges have necessitated the development of new technologies nearer to the patient sites. In this context, biosensors are emerging as compact, portable, hand-held, faster and cost-effective devices. These devices can be used for bedside, near-patient, satellite or remote, and decentralized testing. The entire clinical community is witnessing a paradigm shift from the conventional diagnostic devices to automated point-of-care devices that can fulfill the increasing demand of medical sector including direct unprocessed specimen utilization and the minimum need of electronic or mechanical maintenance. Biosensors provide accurate and immediate analysis of samples (blood, serum, urine, saliva etc) for certain chemicals, metabolites etc to the clinical care team thereby facilitating immediate clinical decision and treatment for the patients. Numerous tests including blood glucose, blood gas concentration, coagulation, expression of cardiac markers, drugs of abuse, pregnancy, hemoglobin, urine analysis and infected diseases etc. are feasible with biosensors. A number of clinical biochemical studies such as blood glucose/lactate/cholesterol, nucleic acid sequence analysis, proteins/peptides, combinatorial synthesis, toxicity monitoring, immunoassays, and forensic analysis are the focused areas for developing the total analysis systems. The enduring dream of providing inexpensive and real-time monitoring of clinically relevant analytes can be envisioned through the use of nanotechnology.

Nanotechnology entails the creation and manipulation of functional materials, devices and systems at length scales between 1-100 nm where unique phenomena enable novel applications. At this length scale, the properties of materials differ significantly from the atoms, molecules or bulk materials of the same composition. Nature has been engaged in its unfathomable and uncanny nanotechnology project since the dawn of life.
It is only recently that humans have begun to understand nature through the control of matter at the nanoscale. The possibility of maneuvering matter at the nano-level was first proposed by Richard Feynman in 1959 during his lecture entitled ‘Room at the Bottom’ where the assembly of atomic blocks at the molecular level was discussed. In the nanoscale regime, materials (especially metal and metal oxides) can be thought of as neither atomic species which can be represented by well defined molecular orbitals, nor as standard bulk materials which are represented by electronic band structures, but rather by broadened discrete energy states. Quantization of energy levels drastically alters the properties of nanoparticulate systems and the size-dependent properties of materials at the nanoscale stem from the weird world of quantum physics. For example, crystals in the nanometer scale have a low melting point and reduced lattice constants, since the number of surface atoms becomes a significant fraction of the total number of atoms and the surface energy plays a significant role in the thermal stability. Bulk semiconductors become insulators when the dimension is sufficiently reduced. Although bulk gold does not exhibit catalytic properties, Au nanocrystal demonstrates to be an excellent low temperature catalyst.

Tunability and tunneling are the prominent quantum effects at nanometer scale which give birth to several unique properties like tailorable surface functionality, improved electrocatalytic activity, photoemission etc, which have proven to be of high utility in different areas of biomedical sciences such as therapeutics, drug delivery, clinical diagnostics etc. The molecular biology shows synergy with nanotechnology as biological entities are made up of ordered elements well within the 100 nm scale. With the advent of nanotechnology, the biological research has been revolutionized and man is
now attempting to unfold secrets of life and mimic nature by building analogous structures. Among all the aspects of nanotechnology applications, researchers are exhaustively working to improve the health standards through the development of bioanalytical devices.

A biosensor is a compact analytical device incorporating a biological or biologically derived sensing element integrated with a physicochemical transducer that translates a biological response into an electrical signal. The aim of a biosensor is to produce response in proportion to the concentration of an analyte or a group of analytes. From determining the presence of various analytes, compounds and living microbes to signaling when a cellular event takes place, biosensors can provide researchers and medical personnel with critical information. Among the various biochemical analytes, estimation of cholesterol in blood has recently become clinically important and demands utmost attention since the elevated level of cholesterol is associated with complicated cardiac problems such as hypertension, cerebral thrombosis, arteriosclerosis and heart attack. Cholesterol exists in two forms in the blood: (i) free cholesterol (30%) and (ii) esterified cholesterol (70%). Cholesterol is carried in human plasma by a series of proteins containing micelles known as lipoproteins. Low density lipoprotein (LDL), known as bad cholesterol in laymen terminology, is cholesterol-rich lipoprotein that transports body cholesterol to the liver. Accumulation of cholesterol may lead to plaque formation which may block the arteries and blood streams and may result in heart related disorders. Thus, early detection of free cholesterol, total cholesterol and LDL are essential to prevent cardiac disorders.
For the fabrication of an efficient biosensor, immobilization of biomolecules on a matrix plays a crucial role. The matrix should ensure high loading of biomolecules while retaining their biological activity and should provide easy access of the active sites towards the target analytes. Nanomaterials owing to their high surface area to volume ratio, tunable surface reactivity, stability in physiological conditions, electron transfer capabilities etc prove advantageous for biomolecule immobilization and signal transduction over the conventional bulk materials. Among various nanomaterials, nanostructured thin films of metals and metal oxides due to the ease of surface functionalization, biocompatibility, high stability, electrocatalytic activity and unique electron transfer capability hold the capability of markedly enhancing the sensitivity and selectivity of a biosensor. In particular, these nanostructured matrices act as self-mediated electrodes and facilitate the transfer of electrons from active site of proteins to the electrode interface during electrochemical reaction. Incorporation of the electroactive molecules further enhances the electrochemical current and results in direct electron transfer phenomenon, leading to higher sensitivity, faster response time and lower detection limits. These exclusive properties of nanostructured metals and metal oxides offer excellent prospects for interfacing biological recognition events with electronic signal transduction for designing next generation bioelectronic devices.

In the present thesis, the nanostructured matrices of iron oxide and gold have been prepared and their catalytic properties have been enhanced through the incorporation of electroactive molecules like prussian blue and triphenylphosphine. The nanostructured films have been utilized for the immobilization of enzyme molecules for the electrochemical detection of free cholesterol and total cholesterol. This chapter gives a
detailed review of literature on the reported cholesterol biosensors, which have been classified into three generations depending on their reaction mechanisms. Finally, the main objectives and a brief outline of the investigations carried out in this thesis are presented.

1.1. Biosensor

Biological molecules exhibit certain properties that are essential for the fabrication of nanoscale electronic devices. These biological materials are involved in catalytic reactions[1], electron transfer and ion transport[2], signaling and signal transduction and also display conformational switching[3]. This has led to the emergence of biomolecular electronics, an interdisciplinary field that exploits the signal detection and processing capabilities of biological materials[4]. The biological molecules exhibits properties like recognition specificity, color change on application of current or light, electron and ion transport, transfer of molecules from one location to another and signaling cascades that can be used for amplification of an optical or electronic signal[5]. These properties can be utilized to develop electronic switches, gates, storage devices, biosensors and biological transistors[6]. One of the fascinating applications of biomolecular electronics is the development of sensing devices, which come up from the integration of biomaterials with a transducer[7]. Biosensors are developed by immobilization of desired sensing biomolecules to a support and coupling with a physicochemical transducer. The construction and working principle of a typical biosensor is shown in Figure 1.1.
1.2. **Elements of Biosensor**

A biosensor is a self-contained analytical device that incorporates a biologically active material in intimate contact with an appropriate transduction element for the purpose of detecting (reversibly and selectively) the concentration or activity of chemical species in a sample[8]. The biological elements include enzymes, antibodies, nucleic acids, aptamers, micro-organisms, biological tissues, organelles etc[9]. An important ancillary part of a biosensor is the matrix that holds the biological sensing elements, provides stability to the biomolecules and facilitates the signal transduction mechanism[10]. The method of transduction depends on the type of physicochemical change resulting from the sensing event [Figure 1.2]. The signal can result from the change in proton concentration, release or uptake of gases, such as ammonia or oxygen, light emission, absorption or reflectance, heat emission, electron generation etc. The transducer converts this signal into a measurable response such as current, potential, temperature change, or absorption of light through electrochemical, thermal, or optical means[11].
1.3. Classification of Biosensors Based on Recognition Element

1.3.1. Biocatalytic Sensors

Biocatalytic sensors incorporate biological components such as enzymes, whole cells or tissue slices that recognize the target analyte and produce electroactive species or some other detectable outcome[12]. Enzymes, globular proteins composed mainly of the 20 naturally occurring amino acids that catalyze biochemical reactions, are the oldest and still most commonly used recognition element in biosensors[13]. Enzymes can increase the rate of a reaction significantly relative to an uncatalyzed reaction. The enzyme–substrate interactions can be probed by kinetic studies. Because of their complex molecular structures, enzymes often have exquisite specificity for their substrate molecules and can detect individual substances in a complex mixture, such as urine or blood, very selectively[14]. This eliminates the need for time-consuming, labor-intensive, and interference-prone sample pretreatment and separation steps used in composite methods. The arrangement of amino acids at the active site of the enzyme, often found at
the centroid of the protein, bind with the specific substrate making the enzyme selective for one type of substrate molecule. Many enzymes also incorporate small non-protein chemical groups, such as cofactors or prosthetic groups, into the structures of their active site that help determine substrate specificity[15]. Most of the enzymes used in biosensor fabrication are oxidases that consume dissolved oxygen in order to reoxidize and produce hydrogen peroxide as the by-product[16]. The inherent selectivity of enzymes often circumvents the signals produced by interfering species that are sometimes found in complex samples. However, enzyme activity is often further modulated by other components such as activators and inhibitors[17]. Personal blood glucose monitoring devices are the most successful commercial application of biocatalytic sensors.

![Figure 1.3: Enzyme-substrates reaction: An example of biocatalytic sensors.](http://faculty.ccbcmd.edu/~gkaiser/biotutorials/proteins/fg7.html)

1.3.2. Bioaffinity Sensors

Affinity sensors rely on the selective and strong binding of biomolecules such as antibodies, membrane receptors or oligonucleotides with a target analyte to produce a measurable signal[18]. The molecular recognition in affinity sensors is mainly determined by the complementary size and shape of the binding site to the analyte of interest. The high affinity and specificity of the biomolecule for its ligand make these
sensors very sensitive and selective[19]. The binding process such as DNA hybridization or antibody–antigen complexation is governed by thermodynamic considerations[20]. Immunosensors are antibody-based affinity biosensors where the detection of an analyte, an antigen or hapten, is brought about by its binding to a region of an antibody[21]. Immunoassays and immuno sensors have been developed for both quantitative and qualitative applications. Immunosensors can be used to detect trace levels (ppb, ppt) of bacteria, viruses, drugs, hormones, pesticides, and numerous other chemicals[22].

![Figure 1.4: Antigen-antibody interaction: An example of bioaffinity sensors.](image)

Nucleic acids have been less commonly used as the biorecognition element in affinity sensors compared to antibodies. Biorecognition using DNA or RNA nucleic acid fragments relies on either complementary base-pairing between the sensor’s nucleic acid sequence designed for some particular analyte of interest, or generating nucleic acid structures, known as aptamers, that recognize and bind to three-dimensional surfaces, such as those of proteins[23, 24]. DNA affinity probes are typically used in medical diagnostics to detect cancers, viral infections, and genetic diseases[25].

1.4. Classification of Biosensors Based on Transducer

1.4.1. Electrochemical

Electrochemical biosensors combine the sensitivity of electroanalytical methods with the inherent bioselectivity of the biological component. The biological component in the
sensor recognizes its analyte resulting in a catalytic or binding event that ultimately produces an electrical signal monitored by a transducer that is proportional to analyte concentration[26]. Most biosensors use electrochemical detection for the transducer because of the low cost, ease of use, portability, and simplicity of construction[27]. The reaction being monitored electrochemically typically generates a measurable current (amperometry), a measurable charge accumulation or potential (potentiometry) or alters the conductive properties of the medium between electrodes (conductometry)[28]. Use of electrochemical impedance spectroscopy by monitoring both resistance and reactance in the biosensor has also become common[29]. Electrochemical sensing usually requires a reference electrode, a counter or auxiliary electrode and a working electrode, also known as the sensing or redox electrode. The reference electrode, commonly made from Ag/AgCl, is kept at a distance from the reaction site in order to maintain a known and stable potential. The working electrode serves as the transduction element in the biochemical reaction, while the counter electrode establishes a connection to the electrolytic solution so that a current can be applied to the working electrode. These electrodes should be both conductive and chemically stable[30].

1.4.2. Optical

An optical biosensor is a compact analytical device, having biological sensing element, integrated or connected to, an optical transducer system which may detect changes in the absorbance, luminescence, polarization, or refractive index[31]. The optical biosensors may involve direct detection of the analyte of interest or indirect detection through optically labeled probes[32]. Biosensors employ the fiber optic probes on the tip of which enzymes and dyes (often fluorescent) have been co-immobilized. These probes consist of
at least two fibers. One is connected to a light source of a given wavelength range that produces the excitation wave. The other, connected to a photodiode, detects the change in optical density at the appropriate wavelength[25]. Surface plasmon resonance (SPR) transducers measure minute changes in refractive index at and near the surface of the sensing element. For example, when an antigen binds to an antibody that is immobilized on the exposed surface of the metal the measured reflectivity increases. This increase in reflectivity can then be correlated to the concentration of antigen[33].

1.4.3. Acoustic

Electroacoustic devices are based on the changes in the properties like mass density, elasticity, viscoelasticity, electrical or dielectric of a membrane made of chemically interactive materials in contact with a piezoelectric material[34]. Bulk acoustic wave and surface acoustic wave propagation transducers are commonly used. In the first, a crystal resonator (usually quartz) is connected to an amplifier to form an oscillator whose resonant frequency is a function of the properties of two membranes attached to it. The latter is based on the propagation of surface acoustic waves along a layer of a substrate covered by the membrane whose properties affect the propagation loss and phase velocity of the wave[35].

1.4.4. Calorimetric

Calorimetric transducers measure the heat evolved or absorbed during some biochemical reaction. These devices can be classified according to the way heat is transferred. Isothermal calorimeters maintain the reaction cell at constant temperature using Joule heating or Peltier cooling and the amount of energy required is measured. Heat conduction calorimeters measure the temperature difference between the reaction vessel
and an isothermal heat sink surrounding it. Finally, the most commonly used is the isoperibol calorimeter that also measures the temperature difference between the reaction cell and an isothermal jacket surrounding it. However, in this case the reaction cell is thermally insulated (adiabatic). This calorimeter has the advantage of being easily coupled to flow injection analysis systems[36].

1.5. Importance of Cholesterol

In animal tissues, cholesterol (cholest-5-en-3β-ol) is the most abundant member of a family of polycyclic compounds known as sterols. In essence, it consists of a tetracyclic cyclopenta[al]phenanthrene structure with an iso-octyl side chain at carbon 17. The four rings (A, B, C, D) have trans ring junctions, and the side chain and two methyl groups (C-18 and C-19) are at an angle to the rings above the plane with β stereochemistry (as for the hydroxyl group on C-3 also). There is a double bond between carbons 5 and 6. Thus, the molecule has a rigid planar four-ring nucleus with a flexible tail. Some of the cholesterol present in humans is esterified; that is, the hydroxyl group that projects from C₃ is attached to a fatty acid residue with an ester linkage[37].

![Figure 1.5: Structure of free and esterified cholesterol.](http://lipidlibrary.aocs.org/Lipids/cholest/index.htm)

Cholesterol is a ubiquitous component of all animal tissues (and of some fungi) and occurs in the free form, esterified to long-chain fatty acids (cholesterol esters) and in
other covalent and non-covalent linkages in animal tissues, including the plasma lipoproteins[38]. Cholesterol has vital structural roles in membranes and in lipid metabolism. It is a biosynthetic precursor of bile acids, vitamin D and steroid hormones (glucocorticoids, oestrogens, progesterones, androgens and aldosterone)[39]. In addition, it contributes to the development and working of the central nervous system, and it has major functions in signal transduction and sperm development[40]. It is found in covalent linkage to specific membrane proteins or proteolipids (‘hedgehog’ proteins), which have vital functions in embryonic development[41].

**Figure 1.6: Structural components of low-density lipoproteins.**

Most cholesterol is not dietary in origin; it is synthesized internally. The liver is the major site of cholesterol synthesis in body. Other tissues like the intestine, the skin, or the nervous tissues also produce it[42]. An adult can synthesize approximately 800 mg of cholesterol every day. In addition, an individual’s diet also provides substantial amount of cholesterol. Because there are two “independent” sources for cholesterol, the internal levels of the sterol must be controlled in order to avoid reaching levels giving rise to health issues. Continuous conversion of cholesterol into bile acids in the liver prevents the body from becoming overloaded with cholesterol, as its excessive accumulation in the
tissues is harmful. In normal humans, excess cholesterol is excreted in the feces either in the form of neutral sterols or after conversion to bile acids[43]. Cholesterol is minimally soluble in water; it cannot dissolve and travel in the water-based bloodstream. Instead, it is transported in the bloodstream by lipoproteins - protein "molecular-suitcases" that are water-soluble and carry cholesterol and triglycerides internally.

The apolipoproteins forming the surface of the given lipoprotein particle determine from what cells cholesterol will be removed and to where it will be supplied. The large number of small dense LDL particles are strongly associated with promoting atheromatous disease within the arteries[44]. For this reason, LDL is referred to as "bad cholesterol". High-density lipoprotein (HDL) particles transport cholesterol back to the liver for excretion. Having large numbers of large HDL particles correlates with better health outcomes, and hence it is commonly called "good cholesterol"[45].

![Lipid Concentration](mgdl⁻¹) Classification Risks
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Concentration (mgdl⁻¹)</th>
<th>Classification</th>
<th>Risks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>&lt;200</td>
<td>Desirable</td>
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<tr>
<td></td>
<td>200-239</td>
<td>Borderline</td>
<td></td>
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<tr>
<td></td>
<td>≥240</td>
<td>High risk</td>
<td></td>
</tr>
<tr>
<td>Low Density Lipoprotein</td>
<td>&lt;100</td>
<td>Optimal</td>
<td>Disordered levels of the cholesterol and its sub fractions may lead to heart attack, brain stroke, atherosclerosis, coronary heart disease and other cardiovascular diseases.</td>
</tr>
<tr>
<td></td>
<td>100-129</td>
<td>Near optimal</td>
<td></td>
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<tr>
<td></td>
<td>130-159</td>
<td>Borderline</td>
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<tr>
<td></td>
<td>160-189</td>
<td>High risk</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥190</td>
<td>Very high risk</td>
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<tr>
<td>High Density Lipoprotein</td>
<td>&lt;35</td>
<td>Very high risk</td>
<td></td>
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<tr>
<td></td>
<td>35-59</td>
<td>High risk</td>
<td></td>
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<tr>
<td></td>
<td>&gt;60</td>
<td>Desirable</td>
<td></td>
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<tr>
<td>Triglycerides</td>
<td>&lt;100</td>
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<td></td>
<td>100-199</td>
<td>Borderline risk</td>
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<td></td>
<td>200-399</td>
<td>High risk</td>
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<td></td>
<td>≥400</td>
<td>Very high risk</td>
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*Table 1.1: Serum cholesterol and triglycerides levels (mgdl⁻¹) and risk of heart diseases.*
Though cholesterol plays a central role in many biochemical processes, it is primarily known for the association of cardiovascular disease with various lipoprotein cholesterol transport patterns and high levels of cholesterol in the blood. In conditions with elevated concentrations of cholesterol (hypercholesterolemia), cholesterol promotes atheroma formation in the walls of arteries, a condition known as atherosclerosis, which is the principal cause of coronary heart disease and other forms of cardiovascular disease[45]. Increased concentrations of HDL correlate with lower rates of atheroma progressions and even regression. Abnormally low levels of cholesterol are termed hypocholesterolemia and some studies suggest that low levels of cholesterol are linked with depression, cancer and cerebral hemorrhage[46]. The level of esterified or total cholesterol in the blood should be less than 200 mgdL\(^{-1}\). The cholesterol level above 240 mgdL\(^{-1}\) can pose serious threat to the heart and brain related diseases. The LDL cholesterol level should be less than 160 mgdL\(^{-1}\) (or 100 mgdL\(^{-1}\)for someone already having heart disease). The HDL removes excess cholesterol from plaques and thus slows the growth of plaques, helping to reduce the risk of heart attack or stroke. So a high HDL level is a good sign of a healthy body; but a low HDL cholesterol level (less than 40 mgdL\(^{-1}\)for men and less than 50 mgdL\(^{-1}\)for women) also indicates a greater risk[47].

1.6. Conventional Detection Techniques for Cholesterol

The breakthrough in determination of cholesterol dates back to the late nineteenth century with the development of a practical color reaction described by Salkowski[48]. It was modified by the Nobel Prize winner Windaus, who also discovered that digitonin quantitatively precipitates cholesterol and does so relatively easily[49]. The gravimetric method developed by Windaus was the first reference procedure for the determination of
cholesterol. The methods of cholesterol detection have been classified into two main
groups: direct reactions and extraction methods, which are subdivided into partial
purification of cholesterol with organic solvents and complete isolation of cholesterol. In
direct reaction methods, reagents are added to the samples without any pretreatment so
that there is no phase separation[50]. On the other hand, extraction methods, as the name
implies, involves extraction of the cholesterol from the samples into a suitable solvent
followed by a color reaction after the purification[51]. Some of the different methods
used to analyze cholesterol are grouped and reviewed according to the analytical
techniques used.

1.6.1. Spectrophotometric Methods

Spectrophotometry refers to the measurement of absorption and emission of light by
materials. Liebermann and Burchard were the pioneers in the colorimetric methods of
cholesterol determination, which they established in 1885. The basic procedures later
used by others were more or less modified versions of their methodology. Three
compounds, acetic anhydride, glacial acetic acid, and sulfuric acid, are required for the
preparation of Liebermann-Burchard (L-B) reagent. In 1969, Edward Kim and Morris
Goldberg prepared and used a single stable L-B reagent for serum cholesterol assay[52].
The absorbance studies were then carried out at 625 nm. The above study gave a standard
deviation value of ± 5.7, which was deemed acceptable.

In 1934, Rudolf Schoenheimer and Warren M. Sperry reported the free and total
cholesterol determination in blood and other biological materials[53]. Amounts of
cholesterol ranging between 0.02 and 0.15 mg could be determined. The procedure
involved precipitation of cholesterol with digitonin, and then a color reaction was applied
to the precipitate. A modified L-B reaction was employed, which allowed direct comparison of the color given by digitonin with that of cholesterol in the range 610-620 nm using a sensitive photometer. In terms of accuracy, this method gave values very close to those found in the Windaus procedure.

Enzymatic methods have also been extensively investigated. Charles C. Allain and co-workers, proposed in 1974 a method preferable to older ones because of its specificity, dynamic range, and simplicity[16]. No prior treatment of the sample was required. Cholesterol esters are hydrolyzed to free cholesterol by cholesterol ester hydrolase. The free cholesterol produced is oxidized to cholest-4-en-3-one by cholesterol oxidase with the production of hydrogen peroxide. The hydrogen peroxide thus generated is measured by the oxidative coupling of 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm. The method yielded coefficient of variation of 0.5% for cholesterol concentration of 384.4 mgdl\(^{-1}\) and coefficient of variation of 1.0% for a cholesterol concentration of 46.9 mgdl\(^{-1}\).

Extraction methods generally present the disadvantage of being time-consuming, and require considerable manipulative skills and care in the process. Also, the L-B color is known to fade on exposure to light. On the other hand, direct methods suffer from the presence of interfering chromogens, hemoglobin, and bilirubin. Another shortcoming is the dilution of the final color produced due to the presence of water in the serum in cases where standards in organic solvents are used.

1.6.2. **Mass Spectrometric Method**

An isotope dilution/ mass spectrometric (ID/MS) method for total serum cholesterol was developed independently at the Karolinska Institute (KI) and the National Bureau of
Standards (NBS), now National Institute of Standards and Technology (NIST). The compatibility of both studies was then assessed in a study published in 1982[54]. In the KI procedure, cholesterol-\(d_4\) and purified unlabeled cholesterol served as labeled internal standard and primary standard respectively. Calibration curves were made from the average of two independent molecular-ion ratio measurements for the unlabeled and the labeled cholesterols separately and on four such standard mixtures. After saponification using alcoholic potassium hydroxide and extraction, ratios of the molecular-ions at m/z 390 and 386 from the two forms of cholesterol were independently measured twice by gas chromatography-mass spectrometry (GC-MS)[55]. On the other hand, the NBS method involved the use of cholesterol-\(d_7\). After saponification, the extracted cholesterol was converted into the trimethylsilyl ether derivative by overnight reaction with 0.5 mL pyridine/hexamethyldisilazane/trimethylchlorosilane (6:4:3). Combined GC-MS with selected ion monitoring at m/z 465 and 458 was used. Each observed ratio for a sample was converted into a cholesterol-\(d_7\) to -\(d_0\) weight ratio by a linear interpolation between the ratios observed for two standard mixtures[56]. GC-MS showed that lathosterol interfered in the method at the KI. The NBS results were found to be more precise; but the mean values for the second series of analysis by the KI method lied within ± 1.3% of the NBS values. Still, the ID/MS analyses required rigorous sampling, were time-consuming and required expensive instrumentation[54].

1.6.3. Electrophoresis Method

The electrophoresis allows fractions of cholesterol carrying entities, namely HDL, LDL, VLDL, and also lipoprotein(a) [Lp(a)], to be quantified[57, 58]. Lp(a) was described in 1963 as a genetic variant of LDL and has been established as an independent predictor of
coronary artery disease, stroke, and retinal occlusion. The study by Nauck and co-workers described the rather difficult separation of Lp(a) from the other cholesterol fractions, overcoming the requirement for preparative ultracentrifugation by using agarose gels in which Lp(a) and VLDL are resolved electrophoretically[59]. Cholesterol and triglycerides were determined enzymatically; VLDL, LDL, and HDL were isolated by sequential ultracentrifugation at less than 1.006 kg/L for VLDL, between 1.019 and 1.063 kg/L for LDL, and between 1.063 and 1.21 kg/L for HDL[60]. Lp(a) was then prepared from the regenerate fluid of a dextran sulfate-based LDL aphaeresis system by sequential centrifugation and gel filtration. Lipoprotein separation followed according to the Lipid Research Clinics Program’s protocol. Agarose gel lipoprotein electrophoresis and staining was then performed with a rapid electrophoresis system (REP). Electrophoresis was carried out at 250 volts for 40 minutes, and cholesterol was enzymatically stained by cholesterol dehydrogenase and nitroblue tetrazolium chloride as dye. The gels were scanned densitometrically at 570 nm with the densitometer of the REP system. Lp(a) was determined with a polyclonal antibody on a Behring nephelometer. This method had excellent agreement with most published immunological methods; the total coefficient of variations for LDL and HDL were < 9.5% throughout, the coefficient of variations for VLDL and Lp(a) were between 7.8% and 23.3% and between 6.8% and 17.7% respectively[61].

1.6.4. Chromatography

Chromatography allows the separation, identification and quantification of different forms of cholesterol with high accuracy. In 1998, D. J. Fletouris and co-workers presented a rapid method for the determination of cholesterol in dairy products by direct
saponification and using a capillary column gas chromatography (GC) system equipped with a flame ionization detector (FID). The linearity was acceptable, with an estimate of 98.6% overall recovery. The overall precision was found to be 1.4%[62].

Liquid chromatographic methods have been researched as well, with the use of high performance liquid chromatography (HPLC) either with a normal phase or a reverse phase[63]. The article by Michael D. Greenspan and co-workers mentioned a combination of a normal phase chromatographic system with spectral detection using a diode array detector in the separation and identification of cholesteryl esters, triglycerides, ubiquinone, α-tocopherol, dolichol, cholesterol, 7-dehydrocholesterol, and retinol[64]. This technique is advantageous in that it allows integration of two close chromatographic fractions as various wavelengths can be scanned.

In 1990, Indyk et al. reported the simultaneous liquid chromatography determination of cholesterol, phytosterols, and tocopherols in foods[65]. It describes a quality-control sample preparation scheme to overcome the disadvantages of conventional saponification strategies. The HPLC system is coupled in series to a variable-wavelength detector, a fluorescent detector, and a dual-pen chart recorder. Serial detection was done by UV (210-214 nm) and fluorescence measurements ($\lambda_{ex} = 295$ nm; $\lambda_{em} = 330$ nm). Though this method gave estimated values that agreed with literatures values and had the advantages of being rapid and simple, as well as selective and sensitive towards tocopherols, the better efficiency of the gas chromatographic systems compared to this method was acknowledged.
1.7. Electrochemical Detection of Cholesterol

Determination of cholesterol in human and animal blood, foodstuffs and other complex mixtures using conventional techniques is often difficult and beset with time consuming and expensive sample preparation prior to the actual assay. In the clinical laboratories, spectrophotometry is extensively used; however, it requires expensive instrumentation and is relatively complex to use as numerous steps like daily calibration with manufacturer supplied standard, prior sample preparation etc are involved. The usual turnaround time in the clinical lab is at least 24 hours per analysis and the cost is relatively high. The medical practitioner and consequently the patient must often wait before the result of such an analysis is known, thus delay the implementation of any needed corrective therapy. For the foregoing reasons, there is a need for the rapid and reliable device for the quantitative assay of cholesterol. The electrochemical biosensors offer several advantages over conventional detection techniques i.e. they are simple, sensitive, accurate and reproducible, require little sample preparation, deliver the assay rapidly, can be easily miniaturized and are inexpensive to produce and to use[66].

The electrochemical detection of cholesterol employs the enzyme molecules immobilized onto the suitable substrates. The enzyme molecules catalyze the formation of the electroactive product which can be monitored directly using amperometry, in which the produced current is measured in response to an applied, constant voltage[67]. Alternatively, the disappearance of the redox active reactant in an enzyme-catalyzed reaction can also be monitored by the electrode. The rapid enzymatic catalysis can also sometimes provide significant signal amplification in a biosensor allowing the detection to a very low level. The use of enzyme molecules makes the detection of cholesterol
highly selective and the electrochemical technique allows detection to a very low level of cholesterol. Thus, combining the selectivity of the enzymes towards cholesterol and the sensitive detection of electrons generated during biochemical reaction using electrochemical techniques can overcome the limitations of the conventional techniques and lead to low-level determination of cholesterol[68]. Extensive research is being carried out to develop high performance electrochemical biosensors by utilizing the smart nanomaterials with high electrochemical properties.

1.7.1. Sensing Elements for Cholesterol

For electrochemical detection of cholesterol, mainly two sensing elements have been used i.e. cholesterol oxidase (ChOx) and cholesterol esterase (ChEt). Besides these, Shumyantseva et al. have shown that flavocytochrome P450scc (RfP450scc) can be utilized for amperometric determination of cholesterol with the sensitivity of 13.8 nAµM$^{-1}$[69]. Antonini et al. have utilized the engineerized cytochrome P450sccK201E for the fabrication of cholesterol biosensor which resulted in high activity and linearity upto 200 µM[70]. Paternolli et al. have utilized the Langmuir–Blodgett films of cytochrome P450 for cholesterol measurement and have achieved linearity upto 750 µM[71].

**Cholesterol Oxidase**

ChOx, a monomeric bi-functional flavin adenine dinucleotide (FAD) containing enzyme (flavoenzyme), belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with oxygen as acceptor. ChOx catalyzes the oxidation of 3$\beta$-hydroxysteroids and then isomerization of the intermediate, $\Delta$5-6-ene-3$\beta$-ketosteroid (cholest-5-en-3-one) yields $\Delta$3-4-ene-3$\beta$-ketosteroid (cholest-4-en-3-one) [72]. ChOx possess potent insecticidal activity, besides its use to track cell cholesterol. ChOx is also
implicated in the manifestation of some diseases of bacterial (tuberculosis), viral (HIV) and non-viral (Alzheimer’s) origin because of its ability to alter physical structure of the cell membrane due to the conversion of cholesterol into cholest-4-en-3-one. In biotechnology, it is used for the production of a number of steroids. Being highly selective and sensitive, it finds widespread use in the determination of serum cholesterol[73].

1.7.1.2. Cholesterol Esterase

ChEt is a glycoprotein that belongs to the alpha/beta-hydrolase fold family [74] and aggregates to a hexamer in the presence of salts [75]. ChEt catalyzes the hydrolysis of sterol esters into their component sterols and fatty acids. Bile salts, such as cholate and its conjugates, are required to stabilize the enzyme in its native polymeric form and to protect it from proteolytic hydrolysis in the intestine[76]. It finds clinical applications in the determination of cholesterol in serum and plasma, with cholesterol oxidase or peroxidase [16]. It hydrolyzes a wide range of ester substrates including cholesteryl esters, acylglycerides, phospholipids [77], retinyl esters [78], vitamin esters, and phenyl esters [79]. The enzyme has also been found to have amidase activity [80]. Also, it is useful as a biocatalyst because of its ability to catalyze transacylation reactions in a water-limited environment [81].

1.8. History of Electrochemical Biosensors

The history of electrochemical enzyme biosensors began with the development of the first device based on glucose enzyme electrodes by Clark and Lyons of the Cincinnati Children’s Hospital in 1962 [82]. Their electrode relied on a thin layer of glucose oxidase (GOx) entrapped over an oxygen electrode via a semipermeable dialysis membrane.
Measurements were based on the monitoring of the oxygen consumed by the enzyme-catalyzed reaction.

\[
\text{Glucose} + \text{Oxygen} \xrightarrow{\text{Glucose oxidase}} \text{Gluconic Acid} + \text{H}_2\text{O}_2 \quad \cdots (1.2)
\]

A negative potential was applied to the platinum cathode for a reductive detection of the oxygen consumption.

\[
\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O} \quad \cdots (1.3)
\]

The entire field of biosensors can trace its origin to this original glucose enzyme electrode. Clark’s original patent [83] covers the use of one or more enzymes for converting electroinactive substrates to electroactive products. The effect of interference was corrected by using two electrodes, one of which was covered with the enzyme, and measuring the differential current. Clark’s technology was subsequently transferred to Yellow Spring Instrument (YSI) Company, which launched in 1975 the first dedicated glucose analyzer (Model 23 YSI analyzer) for direct measurement of glucose in 25 µL whole blood samples. Updike and Hicks further developed this principle by using two oxygen working electrodes and measuring the differential current for correcting oxygen background variation in samples [84]. In 1973, Guilbault and Lubrano described an enzyme electrode for the measurement of blood glucose based on amperometric monitoring of the hydrogen peroxide (H₂O₂) product [85].

\[
\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \quad \cdots (1.4)
\]

The resulting biosensor offered good accuracy and precision for 100 µL blood samples. Use of electron acceptors for replacing oxygen in GOx-based blood glucose measurements was demonstrated in 1974. Continuous ex-vivo monitoring of blood glucose was also proposed in 1974, while in-vivo glucose monitoring was demonstrated
by Shichiri et al. in 1982. During 1980s, considerable research focused on the development of mediator-based ‘second-generation’ glucose biosensors[86, 87], introduction of commercial screen-printed strips for self-monitoring of blood glucose[88, 89] and use of modified electrodes and tailored membranes/coatings for enhancing sensor performance[90]. In the 1990s, extensive activities were directed toward the establishment of electrical communication between the redox center of GOx and the electrode surface and the development of minimally invasive subcutaneously implantable devices[91-93]. Enzyme-based biosensors can be historically divided into three generations depending on their reaction mechanisms. First-generation biosensors used molecular oxygen as the electron acceptor whereas second-generation biosensors replaced oxygen with an artificial redox mediator. Third-generation biosensors utilized directly coupled enzyme electrodes.

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1916</td>
<td>Nelson and Griffin proposed the immobilization of proteins: adsorption of invertase on activated charcoal.</td>
<td>[94]</td>
</tr>
<tr>
<td>1956</td>
<td>Leland C. Clark Jr. invented the oxygen electrode.</td>
<td>[95]</td>
</tr>
<tr>
<td>1959</td>
<td>Rosalyn Sussman Yalow and Solomon Aaron Berson developed the radioimmunoassay which allows sensitive determination of hormones such as insulin based on antigen–antibody reaction.</td>
<td>[96]</td>
</tr>
<tr>
<td>1962</td>
<td>Clark and Lyons developed the first amperometric enzyme electrode for glucose.</td>
<td>[82]</td>
</tr>
<tr>
<td>1963/69</td>
<td>Garry A. Rechnitz and S. Katz introduced first potentiometric biosensor for urea determination which was based on urease immobilized on an ammonia electrode. They coined the word ‘biosensor’, earlier such devices were called enzyme electrodes or biocatalytic membrane electrodes.</td>
<td>[97]</td>
</tr>
<tr>
<td>1970</td>
<td>Bergveld introduced the Ion-Selective Field-Effect Transistor.</td>
<td>[98]</td>
</tr>
<tr>
<td>1972</td>
<td>Betso et al. showed for the first time that direct electron transfer</td>
<td>[99]</td>
</tr>
</tbody>
</table>
of cytochrome c could be realized at mercury electrodes.

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1975</td>
<td>First commercial biosensor (Yellow Springs Instruments glucose biosensor, pen-shaped single use electrode) was launched.</td>
</tr>
<tr>
<td>1982</td>
<td>First fibre-optic based biosensor for glucose was developed.</td>
</tr>
<tr>
<td>1983</td>
<td>First surface plasmon resonance (SPR) based immunosensor was proposed.</td>
</tr>
<tr>
<td>1984</td>
<td>Cass <em>et al.</em> introduced the first ferrocene-mediated biosensor for amperometric determination of glucose.</td>
</tr>
<tr>
<td>1987</td>
<td>The MediSenseExacTech™ blood glucose biosensor (strips/pen model and disposable) was launched.</td>
</tr>
<tr>
<td>1988</td>
<td>Adam Heller and Yinon Degani introduced the electrical ‘wiring’ of redox centers of enzymes to electrodes through electron-conducting redox hydrogels.</td>
</tr>
<tr>
<td>1991</td>
<td>Tatsuma and Kajiya proposed first mediator-based electrochemical sensor for free and total cholesterol detection respectively.</td>
</tr>
<tr>
<td>1992</td>
<td>i-STAT launches hand-held blood glucose analyser.</td>
</tr>
<tr>
<td>1993</td>
<td>Crumbliss <em>et al.</em> developed first electrochemical cholesterol biosensor based on nanoparticles (colloidal gold).</td>
</tr>
<tr>
<td>1996</td>
<td>Glucocard was launched.</td>
</tr>
<tr>
<td>1997</td>
<td>IUPAC introduced the definition for biosensors in analogy to the definition of chemosensors.</td>
</tr>
<tr>
<td>1998</td>
<td>The LifeScan FastTake blood glucose biosensor was launched.</td>
</tr>
<tr>
<td>2003</td>
<td>Schuhmann <em>et al.</em> introduced the use of electrodeposited paints as immobilization matrices for biosensors.</td>
</tr>
<tr>
<td>2012</td>
<td>Maus <em>et al.</em> patented a health monitoring and diagnostic device (LIFESTREAM cholesterol meter).</td>
</tr>
</tbody>
</table>

Table 1.2: Milestones achieved in the field of biosensors.

### 1.9. Advantages in Electrochemical Cholesterol Biosensor

The first report on the electrochemical determination of cholesterol was published in 1991 by Tatsuma *et al.* who assayed the cholesterol with a bioelectrode containing
horseradish peroxidase and cholesterol oxidase immobilized as a bilayer onto the tin (IV) oxide plate[103]. Ferrocene monocarboxylic acid in a 0.1M citrate buffer (pH 5.9) was used as the redox mediator. The linear detection range of the sensor was 1-7 mM with a maximum response of 20 nA cm$^{-2}$. Kajiya et al. fabricated a sensing electrode with cholesterol oxidase and cholesterol esterase in polypyrrole film and ferrocenecarboxylate was used as the electron mediator[104]. This cholesterol sensor yielded a maximum response current of 400 nA cm$^{-2}$ with a linear detection range of only 0-0.5 mM. In 1993, Crumbliss et al. utilized immobilized horseradish peroxidase on colloidal gold deposited on glassy carbon along with ChOx-ChEt entrapped in carrageenan hydrogel as the sensing electrode. The redox mediator was either ferrocene or ferrocenecarboxylic acid deposited on the glassy carbon surface. The linear detection range was 0-9 mgdl$^{-1}$ and the response current was 6.6 μA cm$^{-2}$[105].

All of the above single redox mediator systems suffered from poor sensitivity and a narrow range of linear response restricted to low cholesterol concentrations. In 1997, Guo et al. patented a sensor for the amperometric assay of cholesterol where sensing electrode comprised of polyester film coated with conducting graphite containing dimethylferrocene as the first redox mediator and the second redox mediator comprises 3,3′,5,5′-tetramethylbenzidine. The presence of the second redox mediator greatly amplifies the current flow produced by the presence of cholesterol and produces linear correlation of current flow with concentration over an extended range (40-500 mgdl$^{-1}$)[108]. In 2003, Hesagawa et al. patented the cholesterol biosensor produced by forming an electrode system comprising three electrodes on an insulating base plate by screen printing and subsequently forming an enzyme reaction layer comprising an oxidase and
an electron mediator on the electrode system. The proposed biosensor exhibited linearity in the cholesterol range of 0-116 mgdl$^{-1}$ in acidic conditions (pH 4-5)[109]. Maus et al. in 2003 patented a health monitoring and diagnostic device (LIFESTREAM cholesterol meter)[110]. Thus, the advent of electrochemical cholesterol biosensor began in 1991 and the first cholesterol device was patented in 2003.

The development of the biosensing device involved numerous efforts for the optimization of immobilization matrices. The matrices should be reproducible and resistant to a wide range of physiological pHs, temperature, ionic strength and chemical composition. Conducting polymers[111-113], carbon nanotubes (CNTs)[114-116], nanoparticles (NPs)[117-119], sol-gel/hydro-gels[120-122] and self-assembled monolayer (SAM)[123-125] modified metals and carbon surfaces are commonly used to prepare solid electrode systems and supporting substrates. For electrochemical determination, electrodes such as platinum (Pt), gold (Au), indium tin oxide (ITO) and carbon-based materials such as graphite, carbon black and carbon fiber are preferred due to their excellent electrical and mechanical properties. These materials have a high chemical inertness and provide a wide range of anode working potentials with low electrical resistivity. Based on the mechanism of the enzymatic reaction, cholesterol biosensors can be classified into three generations.

1.9.1. **First-Generation Biosensors**

First-generation enzyme biosensors rely on the use of the natural oxygen cosubstrate and generation and detection of hydrogen peroxide [Eq. 1-2]. The biocatalytic reaction involves reduction of the flavin group (FAD) in the enzyme by reaction with substrate to
give the reduced form of the enzyme (FADH$_2$) followed by reoxidation of the flavin by molecular oxygen to regenerate the oxidized form of the enzyme (FAD).

\[
\text{Substrate} + \text{FAD} - \text{Enzyme} \rightarrow \text{FADH}_2 - \text{Enzyme} + \text{Product} \quad \text{... (1.5)}
\]

\[
\text{FADH}_2 - \text{Enzyme} + \text{O}_2 \rightarrow \text{FAD} - \text{Enzyme} + \text{H}_2\text{O}_2 \quad \text{... (1.6)}
\]

\[
\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \quad \text{... (1.7)}
\]

The first-generation cholesterol biosensors relied on the use of conducting polymers, though some nanomaterials have also been used. Polypyrrole (PPy) is the most studied polymer[104, 126], while polyaniline (PANI), dianimonaphthalene and poly(vinylferrocenium) have also been used for fabrication of cholesterol biosensors[127-130]. In 1999, Vidal et al. reported the entrapment of ChOx within a Ppy film electropolymerized in a flow system[131]. The PPy film was overoxidized to provide it with anion-exclusion properties in order to minimize the interference of electroactive species such as ascorbic acid and uric acid. In 2001, Vidal reported the preparation of a bilayer film consisting of a PPy inner layer with entrapped ChOx and an outer layer of poly(o-phenylenediamine) (oPPD) on Pt electrode[111]. The combined exclusion properties of PPy and oPPD enabled the biosensor configuration to increase the selectivity of the biosensor and the interfering electroactive species, especially uric acid, showed a negligible permeation factor. These biosensors were satisfactorily applied to the determination of their respective substrates in control serum samples. In 2001, Brahim et al. developed cholesterol biosensors by entrapment of ChOx within composite polymeric film consisting of poly(2-hydroxyethyl methacrylate) (p(HEMA)) hydrogel intimately combined with electroactive Ppy[132]. The optimized cholesterol biosensor exhibited a linear range of 0.5-15 mM and detection limit of 120 μM.
Table 1.3: Characteristics of the first-generation cholesterol biosensors reported in literature.

Wang et al. utilized PANI as the matrix and studied the effect of Triton X-100. The response current of PANI/ChOx electrode increased with potential from 0.35 to 0.6V versus saturated calomel electrode and became independent of the concentration of Triton
X-100 at 0.6V [149]. Later, Singh et al. fabricated the total cholesterol biosensor by covalent immobilization of ChEt and ChOx onto PANI films. The biosensor exhibited response time of 40 s, linearity from 50 to 500 mgdl\(^{-1}\), sensitivity as 7.5\(\times10^{-4}\) nAmg\(^{-1}\)dl and a lifetime of about 6 weeks[133]. Solanki et al. used poly(aniline-co-pyrrole) film and the sensitivity of 93.35 AmM\(^{-1}\) was achieved[145]. Thus, the conjugated organic polymers owing to their conducting nature, compatibility with biomolecules and ease of synthesis and handling, have proven to be suitable candidates for the stable immobilization of enzymes and the amperometric detection of cholesterol.

There are a few reports on the use of nanomaterials for fabrication of first-generation cholesterol biosensors. Ramaprabhu et al. utilized gold nanoparticles (AuNPs) decorated graphene nanoplatelets and achieved sensitivity of 314 nA\(\mu\)M\(^{-1}\)cm\(^{-2}\) and linear response up to 135 \(\mu\)M[118]. Hahn et al. fabricated highly sensitive and wide linear-range detecting cholesterol biosensors based on zinc oxide nanorods (ZnO NRS). They have shown that the loading of biomolecules and electron transport properties can be tuned by controlling the dimension of nanorods. The biosensor with ZnO NRs of aspect ratio~60 exhibited the reproducible sensitivity of 74.10 \(\mu\)AmM\(^{-1}\)cm\(^{-2}\), wide linear range up to 16.0 mM, and fast response time of \(<2\) s[139]. Thus, the use of nanomaterials improved the detection range and the sensitivity of the biosensors as they provide high surface area for enzyme loading and a compatible microenvironment helping enzyme to retain its bioactivity.

**Role of Matrices:**

In first-generation biosensors, the role of conducting polymers and nanomaterials based matrices is limited to the entrapment of enzyme molecules which provides stability to the
enzymes. The enzymatic reaction proceeds through the utilization of dissolved O$_2$ in order to reoxidize the enzyme molecules and the liberation of H$_2$O$_2$ as the product of the reaction[150]. The amperometric measurement of H$_2$O$_2$ requires application of a potential at which endogenous reducing species, such as ascorbic and uric acids and some drugs (e.g. acetaminophen) are also electroactive[150]. The anodic contributions of the oxidizable constituents of biological fluids can compromise the selectivity and hence the overall accuracy. Also, since oxidase-based devices rely on the use of oxygen as the physiological electron acceptor, they are subject to errors accrued from fluctuations in the oxygen tension and the stoichiometric limitation of oxygen[151]. These include fluctuations in the sensor response and a reduced upper limit of linearity. The use of polymers in some cases reduce the electrochemical reduction potential of H$_2$O$_2$, however, the sensitivity and selectivity of the biosensors remain limited.

1.9.2. Second-Generation Biosensors

Second-generation enzyme biosensors rely on the use of non-physiological electron acceptors which are able to shuttle electrons from the redox center of enzymes to the surface of electrode. Enzymes do not directly transfer electrons to conventional electrodes because a thick protein layer surrounds its flavin redox center, which introduces a spatial separation between electron donor-acceptor pair. This restricts direct electron transfer, in accordance with distance dependence of electron transfer rate[150]:

$$K_{et} = 10^{13}e^{-0.91(d-3)e^{-[(\Delta G+\lambda)/4RT\lambda]}}$$

(1.8)

where, $\Delta G$ and $\lambda$ correspond to the free and reorganization energies accompanying the electron transfer, respectively, and $d$ the actual electron transfer distance. The minimization of the electron transfer distance (between the immobilized enzyme and the
electrode surface) is thus crucial for ensuring optimal performance. Accordingly, different innovative strategies have been suggested for establishing and tailoring the electrical contact between the redox center of enzyme and electrode surfaces.

Particularly useful have been the artificial mediators that shuttle electrons between the FAD center and the surface by the following scheme:

Substrate + FAD – Enzyme $\rightarrow$ FADH$_2$ – Enzyme + Product ... (1.9)

FADH$_2$ – Enzyme + 2Med(ox)$\rightarrow$ FAD – Enzyme + 2Med(red) ... (1.10)

2Med(red)$\rightarrow$ 2Med(ox) + 2e$^-$ ... (1.11)

where, Med(ox) and Med(red) are the oxidized and reduced forms of the mediator. Such a mediation cycle produces a current dependent on the analyte concentration. Mediators are re-oxidized at relatively low potentials and generate a current when they come in contact with the working electrode. Diffusional electron mediators such as ferrocene derivatives, ferricyanide, hydroquinone and p-phenylene diamine have been widely used to electrically contact enzyme molecules. As a result of using these electron carrying mediators, measurements become largely independent of oxygen partial pressure and can be carried out at lower potentials that do not provoke interfering reactions from coexisting electroactive species[150]. In order to function effectively, the mediator should react rapidly with the reduced enzyme (to minimize competition with oxygen), possess good electrochemical properties (such as a low redox potential), must be nontoxic and chemically stable (in both reduced and oxidized forms). By carefully selecting a mediator and a suitable redox potential, the transduction event at the second-generation biosensor could be measured in a potential range where other possible sample
components such as ascorbic acid, urea etc are not oxidized or reduced thereby minimizing interferences[151].

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Mediator</th>
<th>Sensitivity</th>
<th>Det. Limit (mM)</th>
<th>Linear Range (mM)</th>
<th>K_m^app (mM)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChOx/Nano-ZnO/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>2.27×10^-5 AM^-1</td>
<td>0.013</td>
<td>0.12-10.36</td>
<td>0.025</td>
<td>[152]</td>
</tr>
<tr>
<td>ChOx/NiO-CHIT/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>3.12×10^-2 AM^-1</td>
<td>1.12</td>
<td>0.25-10.36</td>
<td>0.67</td>
<td>[153]</td>
</tr>
<tr>
<td>ChOx/ZnO-CHIT/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>5.44 AM^-1</td>
<td>0.13</td>
<td>0.12-7.77</td>
<td>0.223</td>
<td>[154]</td>
</tr>
<tr>
<td>ChOx/CeO_2-CHIT/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>1.81 AM^-1 cm^-2</td>
<td>0.13</td>
<td>0.25-12.95</td>
<td>0.0906</td>
<td>[155]</td>
</tr>
<tr>
<td>ChOx/AuNPs/PDDA/MWCNTs/GCE</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>2.23×10^-1 AM^-1</td>
<td>0.004</td>
<td>0.02–1.20</td>
<td>0.89</td>
<td>[156]</td>
</tr>
<tr>
<td>ChOx/HRP/AuNPs/PDDA/MWCNTs/GCE</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>1.86×10^-1 AM^-1</td>
<td>0.002</td>
<td>0.01–1.05</td>
<td>2.18</td>
<td>[156]</td>
</tr>
<tr>
<td>ChOx/Au</td>
<td>Hydroxymethyl ferrocene</td>
<td>0.13×10^-9 AM^-1</td>
<td>0.006</td>
<td>Up to 2.1</td>
<td>2.94</td>
<td>[157]</td>
</tr>
<tr>
<td>ChEt–ChOx/ MWCNT/SiO_2–CHIT/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>3.8×10^-4 AM^-1</td>
<td>0.016</td>
<td>0.25-12.95</td>
<td>0.05</td>
<td>[158]</td>
</tr>
<tr>
<td>ChOx/CHIT NF–AuNPs/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>1.02 AM^-1</td>
<td>0.0005</td>
<td>0.001-0.04</td>
<td>-</td>
<td>[159]</td>
</tr>
<tr>
<td>(PAH-MCNT–AuNPs/HRP-ChOx)/Si</td>
<td>p-phenylene diamine</td>
<td>0.38×10^-3 AM^-1</td>
<td>0.02</td>
<td>0.18-11.00</td>
<td>-</td>
<td>[160]</td>
</tr>
<tr>
<td>GCE/PTH/ChOx/HRP</td>
<td>Hydroquinone</td>
<td>0.18 AM^-1 cm^-2</td>
<td>0.006</td>
<td>0.02-0.12</td>
<td>-</td>
<td>[161]</td>
</tr>
<tr>
<td>ChOx-CHIT/P9/ MWCNT/SPCE</td>
<td>K_d[Fe(CN)_6]</td>
<td>1.23×10^-2 AM^-1</td>
<td>-</td>
<td>2.59-12.95</td>
<td>-</td>
<td>[162]</td>
</tr>
<tr>
<td>MWN(Ti(SH)–Au/CHIT–IL/ChOx</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>0.20×10^-2 AM^-1</td>
<td>-</td>
<td>0.5–5.0</td>
<td>-</td>
<td>[116]</td>
</tr>
<tr>
<td>ChOx/G-Au/GCE</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>3.14 AM^-1 cm^-2</td>
<td>0.00005</td>
<td>0.05-0.35</td>
<td>1.22</td>
<td>[163]</td>
</tr>
<tr>
<td>ChOx/G-PVP-PANI</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>3.47×10^-2 AM^-1</td>
<td>0.001</td>
<td>0.005-0.01</td>
<td>-</td>
<td>[129]</td>
</tr>
<tr>
<td>ChOx/ NiFe_2O_3/CuO/FeO-CHIT/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>1.66×10^-4 AM^-1 cm^-2</td>
<td>0.81</td>
<td>0.12-12.95</td>
<td>0.21</td>
<td>[164]</td>
</tr>
<tr>
<td>ChOx/NS–CeO_2/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>80.31 AM^-1 cm^-2</td>
<td>-</td>
<td>-</td>
<td>2.06</td>
<td>[120]</td>
</tr>
<tr>
<td>ChEt-ChOx-CHIT/HRP/SPGE</td>
<td>K_d[Fe(CN)_6]</td>
<td>2.55×10^-12 AM^-1</td>
<td>-</td>
<td>2.81-13.00</td>
<td>-</td>
<td>[165]</td>
</tr>
<tr>
<td>ChOx/CHIT-SnO_2/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>1.34 AM^-1 cm^-2</td>
<td>0.13</td>
<td>0.26-10.36</td>
<td>3.8</td>
<td>[117]</td>
</tr>
<tr>
<td>ChOx/AuNPs-ODA/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>1.085×10^-3 AM^-1</td>
<td>0.61</td>
<td>0.65-12.95</td>
<td>-</td>
<td>[166]</td>
</tr>
<tr>
<td>ChOx/CHIT-SiO_2–MWNT/ITO</td>
<td>K_r/K_d[Fe(CN)_6]</td>
<td>1.31×10^-1 AM^-1</td>
<td>-</td>
<td>1.29-16.83</td>
<td>-</td>
<td>[167]</td>
</tr>
</tbody>
</table>

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Table 1.4: Characteristics of the second-generation cholesterol biosensors reported in literature.

Second-generation cholesterol biosensors have been fabricated using nanomaterials, nanomaterial-polymer composites or the self-assembled monolayer (SAM) of thiols, silanes etc. The potassium ferro/ferricyanide (K₃/K₄[Fe(CN)₆]) is the most widely used redox couple, while hydroxymethyl ferrocene, hydroquinone, p-phenylene diamine have also been used. Parra et al. fabricated cholesterol biosensor by direct adsorption of ChOx on Au electrodes[157]. The artificial mediators like hydroxymethylferrocene, thionin, nile blue and azure A were studied as electron acceptors for reduced ChOx. Cholesterol was amperometrically determined at +0.5V (vs SCE) with a detection limit of 60 µM and a sensitivity of 0.13 µAmM⁻¹. Later, they developed a rapid, simple and reproducible two-step method for constructing cholesterol biosensor by covalently bonding ChOx to a 3,3’-dithiodipropionic acid di(N-succinimidyl ester) (DTSP)-modified Au electrode[169]. The optimized bioelectrode in presence of...
hydroxymethylferrocene exhibited the sensitivity of 54 nAmM$^{-1}$ and the detection limit of 22 μM. Tiwari et al. assembled ChOx and ChEt on the surface of graphene by intermolecular attractive forces while Au NPs were incorporated into the hetero-assembly to enhance the electro-biocatalytic activity. The hybrid structures improved the sensitivity of cholesterol (0.13 μAmM$^{-1}$) and demonstrated wide linear range (0.05-0.35 mM) and low detection limit (0.05 mM)[167]. Chailapakul et al. reported the fabrication of paper-based cholesterol biosensor using graphene-polyvinylpyrrole-polyaniline (G-PVPy-PANI) nanocomposite. This sensing system was successfully applied for the determination of cholesterol in human serum[129]. Arya et al. developed total cholesterol biosensor based on dithiobissuccinimidyl propionate (DTSP) SAM onto Au surface[172]. The response was studied in the presence of $K_3/K_4[Fe(CN)_6]$ and the linear range of 50-400 mgdl$^{-1}$, $K_m^{app}$ value of 0.95 mM and the shelf-life of more than 50 days was obtained. Later, they utilized the SAM of N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane (AEAPTS) onto the ITO substrate for covalent immobilization of ChOx[177]. Kumar et al. then covalently immobilized ChOx and ChEt onto a biocompatible SAM of (3-glycidoxypropyl)trimethoxysilane (GPTMS) onto ITO electrode[124]. The total cholesterol could be estimated in the linear range of 1.5-6.1 mM. The bioelectrode exhibited high sensitivity of 0.351 mAmg$^{-1}$dl, $K_m^{app}$ value as 0.43 mM and could be used more than 10 times with a shelf life of up to 10 weeks. Thus, the ordered arrangement of thiols and silanes resulted in the regular assemblies of enzyme molecules which improved the performance of the biosensors.

Nanomaterials, possessing unique optical and electrical properties due to electron and phonon confinement and biocompatibility have been used as the alternative matrices
for enzyme immobilization and to improve stability and sensitivity of biosensors[178]. Nanostructured matrices may result in facile electron transportation across the electrode-electrolyte interface. Yang et al. used nickel hexacyanoferrate NPs on histidine modified CNTs based matrix and interference free determination of cholesterol was studied at −0.2V (versus SCE)[179]. The bioelectrode exhibited response time of < 20 s and linear range of 0.005–3mM. They have shown that nanostructured composite exhibits efficient electron transfer ability and thus, larger electrochemical response. Wisitosoraat et al. demonstrated the fabrication of highly sensitive cholesterol sensor utilizing a vertically aligned CNT electrode with electopolymerized enzyme immobilization. The biosensor exhibited sensitivity of 0.22 μA mg⁻¹dl and the detection range of 100-300 mgdl⁻¹[176]. Sharma et al. utilized different phases of nanostructured iron oxide for the sensitive determination of cholesterol using K₃/K₄[Fe(CN)₆] as the artificial mediator. The electrochemical properties were tuned by encapsulation with different organic and inorganic molecules. Thus, tailoring the properties of the nanostructured materials may further enhance the performance of the biosensors in terms of sensitivity, prolonged activity of biomolecules, stability etc[180].

Role of Matrices:
Different nanomaterials, polymers, composites, organic thiols, silanes etc have been used for the preparation of matrices. Their role is to allow ordered and covalent immobilization of enzyme molecules, to increase the loading of enzyme molecules and to enhance the enzymatic activity. Also, the matrices may facilitate the electron conduction across the electrode-electrolyte interface and thus may result in enhanced electrochemical response. However, the second-generation biosensors require the use of mediators to
lower the working potential of the biosensors. This may perhaps result in the chemical interference from the mediator, thereby making the biosensor mechanism complex.

1.9.3. Third-Generation Biosensors

Third-generation biosensors rely on the electrical coupling of the enzyme molecules with the electrode resulting in direct electron transfer phenomenon. This can be achieved by direct electrical contact between the enzyme and the electrode, immobilizing the enzyme and mediator in a conducting polymer, or ‘wiring’ the enzyme to the electrode by immobilizing it in a redox polymer. The matrix facilitates the electro-oxidation of the enzyme molecules according to the following scheme:

Substrate + FAD – Enzyme → FADH₂ – Enzyme + Product  ... (1.12)
FADH₂ – Enzyme → FAD – Enzyme + 2e⁻  ... (1.13)

The operating potential of the bioelectrode is close to that of the redox potential of the enzyme which enroutes direct electron transfer between the redox sites of enzyme and the electrode. The co-immobilized mediators or the flexible redox polymer help to transport electrons between the enzyme’s active site and the working electrode surface in an array of rapid electron relays and hence generate high current densities. The enzymes immobilized in flexible redox polymers that are covalently attached to the electrode have been called ‘wired enzymes’. The critical challenges must be overcome for the successful realization of this direct electron-transfer route owing to the spatial separation of the donor-acceptor pair. The 3rd-generation sensors are ideal for repeated measurements since neither mediator nor enzyme need to be added. This self-contained nature also lowers the cost per measurement and opens up possibilities for continuously monitoring the analytes.
Matharu et al. have used the SAM of 4-aminothiophenol (4-ATP) for the development of biosensors for detection of free and total cholesterol. The molecular wire-type behavior of short 4-ATP molecules promoted electron transfer between enzyme and the electrode surface due to its conjugated structure and thus eliminated the need for any artificial mediator[184]. Interference free estimation of free and total cholesterol was realized at low operating potential of 0.33 V with sensitivities of 542.3 nAmM\(^{-1}\) and 886.6 nAmM\(^{-1}\), respectively. Later, Zhu et al. developed a 3\(^{rd}\)-generation cholesterol biosensor based on the direct electron transfer of ChOx immobilized on Au NPs decorated MWCNTs[181]. Direct electrochemistry of ChOx on the electrode surface was obtained and under optimal conditions, the biosensor displayed a linear response in the concentration range from 0.01-5.00 mM with detection limit of 4.3 \(\mu\)M. The detection range of free cholesterol in human serum sample was in good agreement with that determined by the well-established spectrophotometric method. In 2013, Cao et al. utilized platinum-palladium-chitosan-graphene (Pt-Pd-CHIT-G) hybrid nanocomposites functionalized glassy carbon electrode[119]. The nanocomposite accelerated direct electron transfer from the redox enzyme to the electrode surface and under optimal conditions, the biosensor exhibited linearity range as 0.0022-0.52 mM, the limit of
detection as 0.75 μM, $K_m^{\text{app}}$ value as 0.11 mM, response time <7 s, and complete elimination of interference from the potential analytes. The 3rd-generation biosensors have resulted in the reduced response time owing to the direct electron transfer phenomenon and the reduced interference from the potential analytes.

**Role of Matrices:**

The 3rd-generation cholesterol biosensors have been fabricated using nanomaterials that possess electrocatalytic properties or the electroactive molecules having delocalized electronic structure. These interesting matrices provide high surface area for enzyme loading and their biocompatible nature provides conducive microenvironment to help biomolecules retain their activity. Besides these, such matrices facilitate electron transportation across the electrode-electrolyte interface and result in direct electron transfer from the active sites of the biomolecules to the electrode surface. The absence of the artificial mediators reduces the response time of the biosensors and completely eliminates the interference of the potential analytes that may result in false signals.

**1.10. Motivation of the work**

Because of its unique structure as a lipid and a steroid, cholesterol plays an essential role in the normal functioning of many animals including humans, serves in the build-up of the structure of many membranes and as a precursor of steroid hormones and bile acids. However, high levels of cholesterol in the blood and the occurrence of cardiovascular or coronary heart diseases have strong correlation. The presence of excessive cholesterol in the blood brings about conditions like hypertension, coronary artery disease, or stroke, when atherosclerotic plaques form in arteries and occlude blood vessels. High blood cholesterol does not present any symptoms, so there is no other way to be aware of the
potential risk of developing heart disease than to take a blood test and have a cholesterol profile. So there is an obvious need for developing methods which allow fast, precise, and reliable measurements of the amount of cholesterol present in the blood and in foodstuffs.

This research work is an effort to explore the possibility of developing low cost, simple, efficient and highly reliable strategy for detection of cholesterol for its routine quantification. It is aimed at improving the state of art biosensing devices by structural innovation brought in through the recent developments in nanotechnology. Incorporation of nanostructured metal oxides and metals on electrode surface would improve the biosensor efficacy such as detection limit, sensitivity, selectivity, detection time and long term functional stability of the devices. Though this research is applicable to a wide variety of biomolecules, free and total cholesterol have been chosen as the targeted candidates for detection, due to its high importance in clinical health care and point-of-care applications. With this motivation, the main objectives in this thesis are described in the next section.

1.11. Overview of the Thesis

The objective of the thesis is to ascertain protocols for using latest advancements in nanoscience for quick and reliable detection of cholesterol and to develop novel electrochemical biosensors. Attempts have been made to prepare, characterize, optimize and tailor the nanostructured films of metals and metal oxides for the strategic development of electrochemical biosensors for precise and reliable quantification of free cholesterol and total cholesterol. It is anticipated that the incorporation of electroactive
molecules along with nanostructures may prove advantageous to improve the efficacy in terms of sensitivity, selectivity, detection limit etc.

The thesis has been divided into seven chapters.

**Chapter 1** highlights the importance of biosensors in the healthcare industry and the distinctive properties exhibited by certain biomolecules that can be exploited for the fabrication of biosensors. The urgent need to develop the rapid, inexpensive and reliable biosensing device for cholesterol has been emphasized and the merits and demerits of the available detection techniques have been described. A detailed literature review on the advances in the electrochemical detection of cholesterol has been given and the role of the matrices in the performance characteristics of the fabricated biosensors has been emphasized.

**Chapter 2** details the protocols for the preparation of nanostructured matrices and the fabrication of the bioelectrodes. The formulae that have been used for the evaluation of the kinetic and sensing parameters of the bioelectrodes have been mentioned. Electrochemical techniques such as cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) that are used to study the response characteristics of the fabricated bioelectrodes have been discussed. The various experimental techniques such as Atomic force microscopy, UV-Visible spectroscopy, FTIR spectroscopy, X-ray Diffraction, X-ray photoelectron spectroscopy, Transmission electron microscopy, Scanning electron microscopy etc., that have been used to characterize the nanostructured materials, matrices and the bioelectrodes have been described.

**Chapter 3** details the studies relating to the preparation of Fe$_3$O$_4$ NPs and the phase transformation of these uncapped NPs to $\alpha$-Fe$_2$O$_3$ NPs during electrophoretic deposition.
onto ITO substrates. The *in-situ* oxidation of NPs during electrophoretic deposition is circumvented using surface passivation of the Fe$_3$O$_4$ NPs with organic shell (carbon) as well as inorganic shell (silica) while retaining biocompatibility of Fe$_3$O$_4$ NPs. XRD and XPS studies reveal the transformation of Fe$_3$O$_4$ NPs to α-Fe$_2$O$_3$ NPs on electrophoretic deposition and retention of phase of Fe$_3$O$_4$ NPs on encapsulation with carbon and silica, respectively. The fabricated cholesterol biosensors employing Fe$_3$O$_4$@C and α-Fe$_2$O$_3$ nanocrystalline films exhibit sensitivities of 193 and 218 nAmg$^{-1}$dlcm$^{-2}$, respectively, from CV studies and sensitivities of 0.42 and 0.90 Ωmg$^{-1}$dlcm$^{-2}$, respectively, from EIS studies. The comparable sensitivities for biosensors obtained using Fe$_3$O$_4$@C and α-Fe$_2$O$_3$ NPs suggest that encapsulation of Fe$_3$O$_4$ NPs with carbon do not significantly affect electrocatalytic activity of Fe$_3$O$_4$ NPs while adds to the stability of the NPs.

**Chapter 4** describes the studies relating to the fabrication of mediator-free biosensor based on prussian blue encapsulated Fe$_3$O$_4$ NPs for detection of free cholesterol. The iron oxide nanoparticles of size ~10 nm have been encapsulated into four nanometer thick shell of prussian blue and then electrophoretically deposited onto ITO substrate. The immobilization of cholesterol oxidase has been done onto the nanostructured film to investigate the kinetic parameters and the biosensing characteristics. The fabricated bioelectrode exhibits electron transfer coefficient and charge transfer rate constant of 0.45 and 45.15 s$^{-1}$, respectively. Direct electron transfer properties of the nanostructured film result in 3$^{rd}$-generation cholesterol biosensor. The bioelectrode exhibits high sensitivity (2.15 mAM$^{-1}$cm$^{-2}$), low K$_m$ value (0.07 mM), good stability and high selectivity towards cholesterol.
Chapter 5 reports the fabrication of bienzyme functionalized nanostructured Au electrode for the mediator-free determination of total cholesterol. The one-step electrochemical route for the synthesis, functionalization and deposition of Au nanostructures via electroreduction of gold chloride onto the ITO coated glass plates has been proposed. The covalent biofunctionalization of the optimized Au electrode has been done with ChEt and ChOx to investigate the kinetic parameters and the sensing characteristics. The bioelectrode shows surface-controlled electrode reaction with electron transfer coefficient and charge transfer rate constant of 0.68 and 7.09 s\(^{-1}\), respectively. Under the optimal conditions, the bioelectrode undergoes direct electron transfer reaction and exhibits high sensitivity of 0.53 AM\(^{-1}\)cm\(^{-2}\) and low detection limit of 1.57 µM for cholesterol ester without use of any redox mediator.

Chapter 6 demonstrates a novel one-step electrochemical method for controlled synthesis of electroactive gold nanoparticles (Au NPs) in an organic medium using an organometallic precursor Au(PPh\(_3\))Cl [Ph- phenyl]. The hierarchical assembly of Au nanostructures has been tuned on ITO surface during electrochemical reduction of Au(PPh\(_3\))Cl using cysteamine. The presence of triphenyl phosphine in Au film enhances the electrocatalytic activity resulting in higher charge transfer kinetics. The ChOx as a model enzyme has been immobilized on various fabricated nanostructured Au films. Direct electron transfer properties of nanostructured Au films result in 3\(^{rd}\)-generation cholesterol biosensor. The biosensing performance of electrodes with different Au nanostructures towards cholesterol has been investigated using CV without use of any artificial redox mediator. The optimized bioelectrode exhibits high sensitivity of 4.22 AM\(^{-1}\)cm\(^{-2}\) and low detection limit of 5.49 µM. This biosensor shows excellent selectivity,
stability and reproducibility and thus can be utilized for health care diagnostics application.

**Chapter 7** summarizes the research work reported in this thesis, concludes the thesis and also presents the future scope of the work.

1.12. Conclusions

An introduction to the biosensors has been given. The importance of the cholesterol and the risk associated with the disordered cholesterol levels has been described. The need for the regular monitoring of the cholesterol level in the blood has been emphasized and the detection techniques available have been discussed. The advances in the electrochemical cholesterol biosensors have been described and literature survey has been done. Finally, the overview of the present thesis has been given.