CHAPTER 9

Advances in Electrochemical Cholesterol Nanosensors

Mitali Saha

Department of Chemistry, National Institute of Technology, Agartala-799046, Tripura, India

mitalisah@gmail.com

Abstract

Electrochemical biosensors have benefited extensively from advances in nanomaterials (e.g., metal nanoparticles, carbon nanotubes and graphene). Among the various biomolecules, cholesterol has aroused considerable interest in recent years on account of its being an important marker in clinical diagnosis. Recent research has shown that cholesterol plays an important role in the brain synapses and also in the immune system including protection against cancer. As biosensors have the ability to detect and measure constantly the concentration of specific organic or inorganic substances in desired specimens, the thrust to monitor the presence of cholesterol in blood is driving the need for the development of efficient cholesterol biosensors. This chapter highlights the recent advances in the field of enzymatic and non enzymatic cholesterol sensors with the use of nanotechnology.

Keywords

Nanomaterials, Electrochemical, Cholesterol, Biosensor

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1. Introduction

Nanotechnology is one of the most exciting forefront fields and impacts several areas of material science. It is also useful in solving challenging bioanalytical problems. The combined efforts of chemists, biochemists, physicists and engineers towards coupling of biological molecules with electronics have been the key to recent successful developments in the area of biosensors. The last decades have shown unprecedented interest in the development of analytical devices for the detection, quantification, and monitoring of different biological and chemical compounds.

Cholesterol and its fatty acid esters are the main constituents of mammalian cell membrane and are precursors of other biological materials, such as bile acid and steroid hormones. Accurate measurement of cholesterol level in blood has long been recognized as an important clinical test for diagnosing cardiovascular diseases. Currently, many patients are using disposable cholesterol strips for self monitoring of their blood cholesterol levels. Recent improvement in the biosensor technology has been made by using various types of materials for their fabrication such as conductive electroactive polymers, composite materials, composites of transition metal-based complexes and organometallic compounds, sol–gel and hydro–gel materials, nanomaterials, nano-metal composites, nano-metal oxides, etc. This chapter highlights the recent advances in the developments of nanomaterials based enzymatic and non enzymatic cholesterol biosensors.

2. Overview of biosensors

A biosensor is a self-contained device consisting of two functional parts. A bioselective element is in direct contact with a physical transducer, which transforms the information

from biorecognition domain into an electrical or optical signal. The amplitude of such signal depends on the concentration of the analyzed compound (analyte) in the sample. For a biosensor to be pragmatic, efficient and capable it must inherently possess the following features and the biocatalyst must be highly specific for the analyte under investigation:

- It must not be labile when subjected to normal storage conditions
- It should exhibit good and uniform stability over a significant number of assays
- The reaction should be independent of various physical parameters
- The cofactors/coenzymes must be co-immobilized with the biocatalyst
- Accurate, precise and linear results must be obtained without the need to resort to dilution/concentration
- While using the biosensor for invasive monitoring, care must be taken to ensure that the probe is tiny and biocompatible and should eschew toxic or antigenic effects.

3. Electrochemical biosensors

Electrochemical biosensors provide an attractive solution for analyzing the content of a biological sample due to the direct conversion of a biological event to an electronic signal. However, converting biological information to an easily processed electronic signal is challenging due to the complexity of connecting an electronic device directly to a biological environment. An electrochemical sensor is a device that transforms electrochemical information into an analytically useful signal. Electrochemical sensors are usually composed of two basic components, a chemical (molecular) recognition system which is the most important part of a sensor and a physicochemical transducer which is a device that converts the chemical response into a signal that can be detected by modern electrical instrumentations. These two parts form a working (or sensing) electrode. A reference electrode and sometimes a counter electrode are also used in electrical measurements.

3.1 Potentiometric biosensors

Potentiometric biosensors are based on ion-selective electrodes (ISE) and ion-sensitive field effect transistors (ISFET). The primary outputting signal is possibly due to ions accumulated at the ion-selective membrane interface. Current flowing through the electrode is equal to or near zero. The electrode follows the presence of the monitored ion resulting from the enzyme reaction [1]. There are three types of ion-selective electrodes which are of use in biosensors: (a) Glass electrodes for cations (e.g. normal pH electrodes) in which the sensing element is a very thin hydrated glass membrane which generates a transverse electrical potential due to the concentration-dependent competition between the cations for specific binding sites. The selectivity of this membrane depends upon the composition of the glass. The sensitivity to H^+ is greater than that achievable for NH_4^+ ; (b) Glass pH electrodes coated with a gas-permeable membrane selective for CO_2 , NH_3 or H_2S . The diffusion of the gas through this membrane causes a change in pH of a sensing solution between the membrane and the electrode which is then determined and (c) Solid-state electrodes where the glass membrane is replaced by a thin membrane of a specific ion conductor made from a mixture of silver sulphide and a silver halide.

3.2 Amperometric biosensors

Amperometric biosensors are quite sensitive and more suited for mass production than the potentiometric ones [2]. The working electrode of the amperometric biosensor is usually either a noble metal or a screen-printed layer covered by the biorecognition component [3]. Amperometric biosensors can work in two- or three-electrode configurations. The former case consists of reference and working (containing immobilized biorecognition component) electrodes. The main disadvantage of the two-electrode configuration is limited control of the potential on the working electrode surface with higher currents, and because of this, the linear range could be shortened. To solve this problem, a third auxiliary electrode is employed so that the voltage is applied between the reference and the working electrodes, and current flows between the working and the auxiliary electrodes. The amperometric biosensors are often used on a large scale for analytes such as glucose, lactate and sialic acid [4, 5]. Biological agents such as model *Bacillus cereus* and *Mycobacterium smegmatis*, the serological diagnosis of *Francisella tularensis*, a pharmacology study and the detection of pesticides and nerve agents have also been described [6-9].

4. Nanomaterials based cholesterol sensor

Importance of cholesterol biosensors has already been recognized in the clinical diagnosis of cardiac and brain vascular diseases. Nevertheless, the practical application of

a majority of the fabricated cholesterol biosensors is ordinarily limited by their inadequate performance in terms of one or more analytical parameters including stability, sensitivity and detection limit. In recent years, with the development of nanotechnology, novel nanomaterials are being fabricated and their useful properties and applications in biosensors are being examined. For example, nanomaterials-based biosensors, represent integration of material science, molecular engineering, chemistry and biotechnology which can improve the sensitivity and specificity of biomolecule detection, hold the capability of detecting or manipulating atoms and molecules, biomolecular recognition, pathogenic diagnosis and environment monitoring. Nanoscale materials offer distinctive size tunable electronic, catalytic and optical properties which opened new opportunities for designing highly efficient biosensor devices. Incorporation of nanomaterials in biosensing devices has been found to improve the electroactive surface, electronic conductivity and biocompatibility of the electrode surfaces. All these parameter, ultimately result in enhancing the analytical performance of the biosensors.

4.1 Working principles of cholesterol oxidase and cholesterol esterase

The measurement of cholesterol level is usually performed using cholesterol esterase (CE), cholesterol oxidase (CO), and peroxidase (POD). In 1974, Roeschlau and Allain [10,11] described the first fully enzymatic method. Cholesterol esters were cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids (scheme-1). Cholesterol oxidase then catalyzes the oxidation of cholesterol to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide so formed can be used to oxidize an indicator in the presence of peroxidase. In this test, 4-aminoantipyrine and phenol are converted into quinoneimine dye in the presence of POD by the oxidizing action of H_2O_2 . The color intensity of the resultant dye is directly proportional to the cholesterol concentration and thus the level of cholesterol is determined by measuring the increase in absorbance at 512 nm.

Scheme 1. Mechanism of cholesterol oxidase and cholesterol esterase

The enzymatic method is the principal method used for measuring the level of cholesterol in plasma, which consists of solubilizing the cholesterol in an aqueous solution through its incorporation in lipoproteins. In order to measure accurately the cholesterol content of cells or tissue by this method, identification of a suitable solvent should be done to allow

good recovery of free and esterified cholesterol in solution and that is compatible with the substrates and enzymes. Previously, for cells and tissues, the use of the enzymatic cholesterol assay has been limited due to the inadequate quantitative recovery of free cholesterol and cholesterol esters in enzyme compatible solvents. In fact, it has been proposed that the enzymatic method cannot be used for this purpose due to poor extraction and solubilization, which results in a wrong estimation of the cholesterol mass compared with an analytical method [12].

4.2 Enzyme based cholesterol biosensor

Electrochemical biosensors based on immobilized enzymes have found wide application for detection of several biological substrates. In general, enzyme-immobilization methods can be divided into four groups; adsorption, entrapment, cross-linking and covalent attachment [13]. For the adsorption method, the enzyme is attached on the matrix either by hydrogen bonds or through various charge attractions. For example, a multilayer of poly(2,6-pyridinedicarboxylic acid) with negative charge in the first layer and poly(allylamine hydrochloride) with positive charge in the second layer immobilizes negatively charged horseradish peroxidase by adsorption [14]. The one step entrapment process by electro-polymerization, using conducting polymers, such as polypyrrole [15,16] and polyaniline [17], is a fast, convenient and popular method. During the entrapment process, the enzyme is entrapped inside the polymer and the thickness can be arbitrarily controlled by changing the polymerization time. In the cross-linking method, the use of a cross-linking agent such as glutaraldehyde [18] is necessary to link the enzyme and the substrate. However, in a covalent attachment, chemical bonds are formed between the enzyme and the matrix through functional groups, such as amino or carboxylic groups. For instance, urease can be covalently immobilized on a copolymer film of poly(N-3-aminopropyl pyrrole) and polypyrrole, to fabricate an urea biosensor [19]; cholesterol oxidase (ChO) can be bound on a polyaniline-carbon nanotube composite film for a cholesterol biosensor [20]. The most recent approaches do not make use of mediators, but monitor directly the redox behavior of the enzyme at the electrode surface. A redox reaction between an enzyme and an analyte can be transduced to an electrical signal via subsequent direct electron transfer between the enzyme and the electrode. For the majority of enzymes, the protein matrix of the enzyme insulates the active site and thus prevents the direct electron transfer. In such cases, chemical mediators are commonly employed as transducers to convert the enzyme activity into an electrical signal. Carbon nanotubes (CNT) have been recognized as one of the most promising electrode materials since the first electrode application in the oxidation of dopamine in 1996 [21]. With the discovery of carbon nanotubes by Iijima, the combination of CNT with polyaniline (the polymer with strong biomolecular interactions,

excellent stability and very good electronic property) was found to improve significantly the electrical, mechanical properties and reversible control of conductivity both by charge-transfer doping and protonation.

Since then, the research has been focused on their electrocatalytic behaviors toward the oxidation of biomolecules and their performance has been found to be much superior to those of other carbon electrodes in terms of reaction rate, reversibility and detection limit. Cholesterol biosensor based on enzymatic action at carbon nanotube modified electrode has been studied by various groups [22-25]. Mechanism of enzymatic detection of cholesterol at CNT modified electrode proposed by Solanki et al. [24] and Yan et al. [25] is shown in scheme 2.

Scheme 2. Proposed biochemical reaction at ChEt–ChOx/MWCNT/SiO₂–CHIT/ITO bioelectrode.

Metallic nanowires, in particular Au nanowires are very promising as smart building blocks in emerging electronic and sensing applications [26-29]. As a bio-sensing material, metallic nanowires can be covalently modified with functional groups like –SH, –COOH and –NH₂ [30, 31], which can be linked further to a specific enzymes, such as cholesterol oxidase and cholesterol esterase. Sandro Carrara et al. [32] has shown that modification of screen-printed rhodium–graphite electrodes with gold nanoparticles integrated with cytochrome P450sc yields a bioelectrocatalytic system for cholesterol measurements. B.D. Malhotra et al. [33] prepared a cholesterol biosensor by immobilizing cholesterol oxidase onto electrophoretically deposited conducting polymer film on indium-tin-oxide glass plate derived from nano-structured polyaniline (PANI)

colloidal suspension using N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide chemistry. These bio-electrodes exhibited linearity within the concentration range of 25 to 400 mg/dL of cholesterol, detection limit as 25mg/dL and sensitivity as 7.76×10^{-5} Abs (mg/dL)⁻¹. In the past few years, chitosan, which is a transformed polysaccharide, has been widely used as a biomaterial for immobilizing enzyme. It exhibits excellent membrane-forming ability, biocompatibility, nontoxicity, high mechanical strength, and hydrophilicity [34]. It has also been used as a medium for dispersion of CNTs in aqueous solution [35, 36].

Nanostructured oxides of metals such as tin, zirconium, titanium, zinc, iron, cerium and magnesium have been found to exhibit interesting non-toxic and catalytic properties, functional biocompatibility and nanomorphological surface activity. These materials also show enhanced electron-transfer kinetics and strong adsorption capability, providing suitable environments for the immobilization of biomolecules and resulting in enhanced electron transfer and improved biosensing characteristics. Scientists are now inclined to use biocompatible nanomaterials as suitable matrixes for the enzyme immobilizations for the effective detection of cholesterol [37–41]. Among nanomaterials, ZnO has attracted much attention due to its wide range of applications. ZnO as a wide band gap (3.37 eV) semiconductor plays an important role in sensors, actuators optics and optoelectronics, due to its semiconducting, pyroelectric and piezoelectric properties.

The discovery of graphene in 2004, added a new dimension to electrochemical biosensor research [42]. The unique thin structure of graphene nanosheets with an excellent capability for the rapid signal communication endorses their potential for such practical applications. Researchers have fabricated a sensitive sensor that can detect minute traces of cholesterol in human blood serum. They made the sensor by modifying multilayered graphene sheets with a compound derived from sugar molecules. This sensor is potentially useful for measuring the levels of serum cholesterol. The researchers found that the sensor was able to detect cholesterol even in the presence of interfering biochemicals such as salts, amino acids and lipids which are usually present in human blood serum. The sensor could detect cholesterol at micromolar concentrations, retaining stability and sensitivity even after 100 cycles of electrochemical studies [43]. The use of graphene can avoid the problems associated with metal alloy NP and CNT. The unique properties of graphene (fast electrontransportation, high thermal conductivity, excellent mechanical flexibility and good biocompatibility) justify its potential applicability as electrochemical biosensor [44-47].

4.3 Non- enzymatic cholesterol biosensor

The reproducibility of enzyme-based sensors is not very good and the activity of the enzyme is liable to be affected by temperature, pH, and toxic chemicals. Although, the enzyme-based sensors show high selectivity and excellent sensitivity, the activity of enzyme decreases with the use of the sensor and the enzyme is easily denatured during its immobilization due to the intrinsic nature of enzyme [48]. In order to overcome the disadvantages of enzymatic cholesterol sensors, nonenzymatic cholesterol sensors have been designed and fabricated, which have several attractive advantages, such as simple fabrication, high stability, reproducibility, low cost and freedom from oxygen limitations. Dai et al. synthesized porous tubular Ag nanoparticles by electrodeposition of Ag into a CdS modified porous anodic alumina template and subsequent removal of CdS [49], the modified electrode has a wide linear range but the sensitivity is relatively low.

It is the first example of a nonenzymatic cholesterol biosensor based on Ag nanoparticles. This biosensor possessed an acceptable reproducibility, good stability and low interferences. Park and co-workers fabricated a Pt nanoparticle modified macroporous gold electrode [50], while Lee used nanoporous Pt electrode for the nonenzymatic determination of cholesterol [51]. These two Pt-based sensors have good sensitivity but the detection limit was not enough. These sensors are highly effective at detecting low levels of cholesterol. Lee et al. [52] prepared a layer-by-layer assembled CNT network as a matrix for loading platinum nanoparticles in order to accomplish the non-enzymatic determination of cholesterol. The sensor has good anti-interferent properties in the presence of glucose, ascorbic acid, and dopamine. Hai Binh Nguyen et al. [53] reported a novel cholesterol biosensor based on electrochemical microelectrode with graphene films coated on PANi/Fe₃O₄ films which showed high sensitivity ($74\mu\text{A}\text{mM}^{-1}\text{cm}^{-2}$), fast response time ($<5\text{ s}$) and correlation coefficient square (R^2) of 0.9986. According to these authors, by taking the layer-by-layer fabricated electrode and advantage of graphene like pattern, excellent analytical quantification of cholesterol sensor like fast response time, high selectivity and reliability, high sensitivity can be obtained. Singh et al. [54] reported a new non enzymatic sensor using functionalized nanographite as a working electrode, Ag/AgCl as a reference and platinum as a counter electrode. Under optimum detection conditions, the constructed sensor had a linear range of 50 to 500 mg/dl for cholesterol with a correlation co-efficient of 0.99784. Sensitivity of the developed cholesterol sensor was $1.0587\text{ }\mu\text{A/mg}$. This biosensor showed good reproducibility, stability and low interferences.

5. Conclusion

During the last 40 years, various biosensors have been developed encompassing a wide range of applications but the number of commercially available biosensors is limited. Different strategies have been explored for the construction of cholesterol biosensors that employed nanomaterials along with other materials as sensing elements. Yet there is much scope for further research. Among the various detection techniques of cholesterol, voltammogramic biosensing method has been recently developed as an extremely significant technique. Unfortunately, the biosensor market has not expanded as predicted. But, the combination of several new techniques, derived from chemistry, biochemistry, materials science and electronics will lead to the development of accurate and low cost commercial cholesterol biosensor which will surely boost the market of biosensors for health care. In fact, miniaturization will play an important role in the trend of biosensor development in the near future.

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