

Development of Nanoprobe for the Determination of Blood Cholesterol

Jeyashanthi-Navamani*, Ravikumar-Palanisamy, Gurusamy R, Murugasami-Ramasamy and Arumugam S Velalar college of Engineering and Technology, Erode, Tamilnadu, India

Abstract

Measurements of serum cholesterol levels are important in the diagnosis and classification of hyper lipoproteinemias. Elevated cholesterol levels may occur with hypothyroidism, nephrotic syndrome, diabetes, and various liver diseases. The present investigation was carried out for the development of rapid, highly sensitive and economic cholesterol biosensor for the determination of blood cholesterol. The PVP encapsulated ZnS nanoparticals have been synthesized and characterized by Fourier Transform InfraRed (FTIR) spectroscopy, UV-visible spectroscopy, and Atomic force microscopy techniques in the present investigation. The size of the nanoparticles is found to be ranging between 21-22 nm.

The surface morphology of the sensing area of functionalized MWCNT was examined using Scanning Electron Microscopy (SEM). The length of the MWCNTs is found to be approximately 2 microns. In the IR spectrum of functionalized MWCNTs, the presence of bands at 1636 cm⁻¹ and 3434 cm⁻¹ confirmed the presence of carbonyl and hydroxyl moieties of carboxylic acid group.

Cholesterol Oxidase was immobilized on to the ZnS nanoparticles and Multiwall Carbon Nanotubes (MWCNTs) which they were placed on a glassy carbon electrode surface using Nafion by LBL assembly technique. Cyclic voltammetric study reveals that the fast electron transfer between electrodes was achieved through the incorporation of ChOx into the Nafion-ZnS-MWCNTs film. ZnS and MWCNTs played an important role in facilitating the electron transfer between the ChOx and the electrode surface.

The cholesterol bioelectrode shows detection range of 10-450 mg/dl and good linearity is obtained in 50-450 mg/dl (1.3 -11.6 mM) range with linear regression coefficient 'R2' as 0.986. The Michaelis-Menten constant K_m value obtained as 0.84 mM using Lineweaver-Burke plot by plotting 1/V vs 1/Conc. The low K_m value 0.84 mM indicates high affinity of immobilized ChOx to cholesterol. It exhibits optimum pH 7.0 and optimum temperature 35°C. Therefore, the bioelectrode fabricated in this study is promising for cholesterol detection in human blood.

Keywords: Multi walled carbon nanotubes; Cholesterol oxidase; Nafion; Michaelis-Menten constant; Zinc sulphate nanoparticles

Introduction

The estimation of blood cholesterol concentration is one of the most widely performed assays in clinical laboratory. Accurate measurement of cholesterol level in blood has long been recognized as an important clinical test for diagnosing the cardiovascular diseases and many patients are using the disposable cholesterol strips for self monitoring their blood cholesterol levels. The limiting step for commercialization of disposable cholesterol strip is immobilization procedures of the enzyme. The basic concept of enzyme based cholesterol sensor is refer to the glucose sensor was first described by Clark and Lyon [1]. Since the pioneer work, reasonable research work has been done to the development of cholesterol sensors. The majorities of sensors are based on electrochemical principles and use enzymes as biological component for analyte recognition. The most widely used active electrode materials are Carbon [2], conducting polymers [3] and transition metal oxides [4].

A number of electrochemical cholesterol biosensor have been developed employing cholesterol esterase, Cholesterol Oxidase and peroxidase immobilized onto octyl agarose gel, pyrole membrane, nylon mesh, screen printed strip, Carbon paste electrode, polypyrrole film, graphite-teflon matrix, dialysis membrane, conducting polypyrrole films, polyanniline films and nano sized materials. It is based on the following chemical reactions [5].

 $Cholesterolacetate + H_2O \xrightarrow{Cholesterolesterate} Cholesterol + Acidicacid$ $Cholesterol + O_2 \xrightarrow{Cholesteroloxidase} Cholestern - 3 - one + H_2O_2$

 $2H_2O_2 + Phenol + 4 - a minoantipyrine \xrightarrow{Peroxidase} Quinoe minedye(\lambda_{max} = 520nm)$

Conventional electrochemical biosensors are based on either glassy Carbon electrodes (GCE) or metal electrodes (Au, Pt or Cu for example) for amperometric or voltammetric analyte detection. Such electrodes have a series of disadvantages, including poor sensitivity and stability, low reproducibility, large response times, slow electron transfer reaction and a high potential for electron transfer reactions [6]. CNTs can overcome most of these disadvantages owing to their ability to undergo fast electron transfer and the resistance of CNT-modified electrodes to surface fouling.

Carbon nanotubes are among the most extensively researched materials today. This is the most versatile material, with the properties ranging from optical absorption and emission on one hand to the mechanical properties of bulk materials such as young's modulus, on the other [7]. The electrochemical reactivity of important biomolecules is enhanced by CNT, promote the electron-transfer reactions of enzymes containing redox center and enhance electrochemical reactivity, CNTmodified electrodes have been shown used for accumulating important biomolecules such as enzymes and for alleviating surface fouling effects such as in the NADH oxidation process. The sensitive CNT conductivity

*Corresponding author: Jeyashanthi-Navamani, Velalar college of Engineering and Technology, Erode, Tamilnadu, India, E-mail: njeyashanthi@gmail.com

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to the surface adsorbates permits the use of CNT as nanoscale sensors. CNTs are used in wide range of electrochemical biosensors ranging from amperometric enzyme electrodes to DNA chip.

Multiwall Carbon Nanotubes (MWCNTs) can be used as suitable intermediates between electrodes and enzymes, because of their high surface area, high surface to volume ratio, good electrical conductivity and significant mechanical strength [8]. MWCNT enhance electrocatalytic activity due to presence of edge-plane-like sites located at both ends and in the defect region [9]. Besides this, MWCNT produce changes in energy bands close to Fermi level [10]. MWCNT have recently been utilized for fabrication of electrochemical biosensor [11].

The nanostructured metal particles have profound applications in optics, optoelectronics, sensors, and actuators due to their semiconducting, piezoelectric, and pyroelectric properties [12]. Among these semiconductor nanocrystals or quantum dots have gained considerable interest during the past decade. These materials have unique technological applications for various photoelectronic devices or even for advanced biotechnology due to their size dependant physical and optical properties [13]. Nanomaterials prepared from metals, shaped into Nano particles have been widely used for their ability as electrode modification materials to enhance the efficiencies of electrochemical biosensors [14]. Metal nano particles can increase efficiency of photochemical reactions and greatly improve the catalytic activity of enzymes to generate novel photo electrochemical systems.

Metal nano particles possess high surface area, chemical stability and shows fast electron communication features [15]. The high electron communication features, high surface to volume ratio and electro-catalytic activity of the nanosized materials make them ideal as immobilization matrices, as transduction platform and/or mediators. To improve the stability, sensitivity, selectivity and other analytical characteristics of biosensors various conventional materials matrices have been used. Among them metal nanoparticles retain the bioactivity of the immobilized enzyme and enhance the sensing characteristics such as sensitivity, selectivity and low detection limit of the biosensors.

Among the several metal nano materials that have been tested for biosensing applications, ZnS represents an excellent material with a high isoelectric point that allows easy immobilization of an enzyme (with a low isoelectric point) through electrostatic interaction [16]. Furthermore, its high chemical stability and non-toxic nature, as well as its biocompatibility and high electron transfer capability make ZnS a promising material for building an enzymatic sensor by immobilizing the appropriate biomolecules without electron mediators. Such type of ZnS-based biosensors can also be used to further develop implantable biosensing systems.

Conducting electroactive polymers (CPs) have aroused considerable interest due to their electronic conducting properties and unique chemical and biochemical properties. Conducting polymers are multifunctional materials that can be employed as receptors as well as transducers or immobilization matrices in electrochemical biosensing. They are characterized by an extended π - conjugation along the polymer backbone, which promotes an intrinsic conductivity, ranging between 1E–14 and 1E2 S/cm [17]. Their electrical conductivity results from the formation of charge carriers ("doping") upon oxidizing (p - doping) or reducing (n - doping) their conjugated backbone [18]. They have the characteristics of organic polymers, such as light weight, resistance to corrosion, flexibility, and ease of fabrication [19]. When formed as nanostructures, conductive polymers acquire appealing properties: ease of preparation by chemical or electrochemical methods, sensitivity

towards a wide range of analytes, considerable signal amplification due to their electrical conductivity, and fast electron transfer rate [20].

CPs are easily synthesized and deposited onto the conductive surfaces by electrochemical polymerization. Coating electrodes with CPs open up numerous possibilities for the immobilization of biomolecules on the electrode surface. They improve the bioaffinity, electrocatalytic properties, electron transfer rate and direct communication to produce analytical signals. Co-immobilization of other molecules (enzymatic co-factors or charge-transfer mediators) by entrapment within CP films or by covalent binding on these films permits straight forward fabrication of reagentless biosensors [21].

Nafion, a sulfonated tetrafluorethylene copolymer, consists of a hydrophobic fluoroCarbon backbone (-CF2-CF2-) to which hydrophilic sulfonate groups (SO3-) are attached (Mauritz and Moore, 2004). The presence of negatively charged sulfonate groups in the membrane explains the high level of proton conductivity of Nafion. That has attracted much interest owing to its interesting properties such as biocompatibility, non-toxicity, low-cost, good film forming ability, high mechanical strength, high hydrophilicity, chemical and electrochemical stability, extremely low permeability to reactant species, selective and high ionic conductivity, and ability to provide electronic insulation [22].

In the present work, a novel nanoprobe for blood cholesterol determination has been constructed by LBL Self assembly technique. GC electrode was modified with ZnS, Nafion and excellent conductive MWCNTs. Cholesterol Oxidase simply entrapped into the ZnS/ MWCNTs composite and Nafion was used as to give stable and homogeneous cast thin films. Meanwhile, this new electrochemical system can utilize synergy effect between MWCNTs and ZnS to facilitate electron-transfer processes and retain the good bioactivity of Cholesterol Oxidase. This biosensing probe based on ZnS/MWCNTs/ Nafion/ChOx/GC electrode responded sensitively to cholesterol.

Experimental Procedure

Reagents

Cholesterol Oxidase (ChOx) from Nocardia sp, (E.C. 1.1.3.6), Nafion (5 wt% in lower aliphatic alcohols) and cholesterol were purchased from Sigma. Aldrich. 10 mg ml⁻¹ ChOx solution was obtained by dissolving ChOx in 0.05M PBS (pH 6.0) and stored at 4°C in a refrigerator. Different concentrations of cholesterol stock standard solutions were prepared in 0.05M PBS (pH 6.0). Cadmium nitrate (\geq 99.0%), sodium sulfide (\geq 99.0%), soluble starch, zinc acetate tetrahydrate (G.R, Glaxo laboratories India Ltd.), thiourea (G.R., S.D.Fine Chem.Ltd.), poly-N-vinyl-2-pyrrolidone (PVP, molecular weight 58,000, ACROS, USA) and N,N-dimethyl formamide (DMF, G.R., Merck, India). All other reagents were of analytical grade.

Preparation of cholesterol solution

Cholesterol solution was prepared by dissolving 400 mg of standard cholesterol followed by addition of nonaethylene glycol monododecyl by stirring till the solution was clear and colorless at 65°C. The hot phosphate buffer saline (PBS) pH 7.0 was added and volume made up to 100 ml. The solution was allowed to reach its normal temperature before being used and was stored at 4°C.

Apparatus

All electrochemical experiments were carried out on a cyclic voltameter CHI 660C (CHI Instruments Inc., Austin, USA). A

conventional three electrode system was used in this work. The asprepared electrode was used as the working electrode (1mm diameter). A platinum wire was used as the counter electrode and an Ag/ AgCl (saturated KCl) electrode was used as the reference electrode. Phosphate Buffer System (PBS) 0.05 M (pH 6.0) was always employed as supporting electrolyte.

Synthesis of PVP-encapsulated ZnS nanoparticles

The synthesis reaction was carried in the N,N-Dimethyl formamide (DMF) medium. Calculated quantity of zinc acetate, thiourea and PVP was dissolved in DMF under stirring of 150 rpm in a tightly closed glass container so as to obtain homogeneous solutions. The concentration of Zn²+, S²- and PVP used in the DMF were 7.5×10-² M, 15×10-² M and 5×10^{-4} M, respectively. The container with the reaction solution was thereafter placed in an oil bath, preheated at a temperature of 136 \pm 1°C, for 10 min under constant stirring, removed from the bath and cooled to ambient temperature. The pH of the transparent solution was about 6. The dispersed sulphide nanoparticles were collected from the DMF solution by adding a known volume of acetone (G.R., Merck, India). Immediate flocculation of nanoparticles occurred. Particles were collected using centrifugation at 6000 rpm. To remove the last traces of adhered impurities, the particles were washed thrice using acetone, each time collecting the particles using centrifugation as described above. The washed particles were dried at 60°C in air. For the measurement of the UV-Vis absorbance spectra, the dried particles were dispersed in high conductivity water (conductivity of water: $1.4 \times$ 10⁻⁵ mho/cm) [23].

Functionalization of MWCNT [24]

The MWCNTs were pre-cleaned in concentrated HCl by sonication for 30 mins followed by continuous stirring. The reaction mixture was centrifuged, filtered and rinsed thoroughly with distilled water. Then the black solid was heated at 225°C for 20 h. This was continued at 300°C for 1 h followed by sonication in HCl. The pre-cleaning performed by concentrated HCl is to purify the MWCNTs especially to eliminate the residual metal catalyst particles from the nanotubes. The pre-cleaned MWCNTs were then oxidized in hot concentrated HNO₃ solution for 48 h under refluxing conditions to remove impurities and generate surface functional groups. Purification of MWCNT surfaces prevents self-poisoning by foreign impurities while functional group generation enhances the electro catalyst formation.

Preparation of the chemically modified Nafion-MWCNTS-ZnS ChOx electrodes [25]

The procedure for the preparation of Nafion-MWCNTs-ZnS-ChOx modified electrodes was as follows: GC (Glassy Carbon) electrode with the diameter of 3mm was polished with 1.0 and 0.05 μm alumina slurry sequentially and then sonicated in distilled water, ethanol and distilled water for a few minutes, respectively. 0.1 mg MWCNTs were added into 1mL of 0.5 wt% Nafion solution, and this solution was ultrasonicated for several minutes to form a stable black suspension (1mg/mL). Then a homogeneous solution (solution A), which finally contained 2 mg/mL ChOx, 0.5 ml ZnS and 0.1 mg/mL MWCNTs, was formed by thoroughly mixing the MWCNTs-Nafion suspension with ChOx solution (10 mg/ml) and ZnS solution. Four microliters of the solution A was cast onto the surface of a GC electrode by using a 10 µL pipette to obtain Nafion-MWCNTs-ZnS- ChOx film modified electrode. A beaker was covered over the electrode so that water can evaporate slowly in air and a uniform film can be formed. This enzyme modified electrode was stored at 4°C in refrigerator when not in use.

For comparison, Nafion-ChOx/GC, Nafion-ZnS/GC, Nafion-ZnS-ChOx/GC and Nafion-MWCNTs-ChOx/GC electrodes were prepared using a similar procedure. Solution B containing Nafion and 2 mg/mL ChOx was used to prepare the Nafion-ChOx/GC electrode and solution C containing Nafion and ZnS was used to prepare the Nafion-ZnS/GC electrode. Similarly, solution D containing Nafion, 2 mg/mL ChOx and ZnS was used to prepare the Nafion-ZnS-ChOx/GC electrode while another solution E containing Nafion, 2 mg/mL ChOx and 0.2 mg/mL MWCNTs was used to prepare the Nafion-MWCNTs-ChOx/GC electrode. All the modified electrodes were stored at 4°C in refrigerator when not in use.

Assay of Cholesterol Oxidase (EC 1.1.3.6)

The reagent A (0.5% (w/v) Cholesterol Substrate Solution) reagent B (500 mM Sodium Acetate Buffer, pH 5.0) were pipetted out in Test and Blank Tubes. The tubes were mixed by inversion and equilibrated to 37°C. The reagent C (A solution 0.005 unit/ml of Cholesterol Oxidase in Reagent B) was added only to the Test tube. Immediately mixed by inversion and incubated at 37°C for exactly 30 minutes. Then reagent D (Ethyl Alcohol) was added to Blank and Test tubes. The reagent C was added to the Blank tube. The tubes were mixed by inversion, transferred to suitable cuvettes and recorded the absorbance at 243 nanometer (A_{243nm}) in a spectrophotometer.

Units/ml enzyme = (A $_{243nm}$ Test - A $_{243nm}$ Blank) × V $_1\!\times$ df / 30 × 18 × V $_2$

 $V_1 = Volume$ (in milliliters) of the stopped reaction

df = Dilution factor

30 = Time (in minutes) of the assay as per the Unit Definition

18 = Millimolar extinction coefficient of 4-cholesten-3-one

 $V_2 =$ Volume (in milliliter) of enzyme used.

Unit Definition: One unit will convert 1.0 $\mu mole$ of cholesterol to 4-cholesten-3-one per minute at pH 5.0 at 37°C [26].

In this work, ZnS nanoparticles are synthesized, characterized and used for the development cholesterol nanoprobe. The cholesterol nanoprobe is based on MWCNTs and ZnS nanoparticles with Cholesterol Oxidase (ChOx) immobilization in Nafian using Layer By Layer assembly technique. The electrochemical behavior of Nafion-ZnS-MWCNTs-ChOx modified electrode and the kinetics of the immobilized enzyme ChOx are studied.

Characterization of ZnS nanoparticles

Nanoparticle characterization is necessary to establish understanding and control of nanoparticle synthesis and applications. Characterization is done by using a variety of different techniques. Common techniques are electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), x-ray photoelectron spectroscopy (XPS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), ultraviolet-visible spectroscopy, dual polarization interferometry and nuclear magnetic resonance (NMR). ZnS nanoparticles synthesized in this work are characterized by ultraviolet-visible spectroscopy, Fourier transform infrared spectroscopy and atomic force microscopy.

UV absorbance spectrum of ZnS quantum dots

The absorption spectra of PVP encapsulated ZnS nanoparticles are

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shown in Figure 1. The onset of absorption is located at 290 nm (4.28 eV). The blue shift of absorption edge compared to bulk ZnS (onset is at 340 nm) clearly explains the quantum confinement effect of ZnS nanoparticles. The absorption edges of the nanocrystallites are also sharp, indicating that the synthesized particles have relatively narrow size distributions.

IR spectrum of ZnS nanoparticles

Infra red spectroscopy exploits the fact that molecules have specific frequencies at which they rotate or vibrate corresponding to discrete energy levels (vibrational modes). These resonant frequencies are determined by shape of the molecular potential energy surfaces, the masses of atoms and by the associating vibronic coupling. The IR spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infra red spectrum [27].

FTIR spectra of the PVP-capped ZnS are presented in the range 4000–500 cm⁻¹ is shown in Figure 2. It is observed that absorption peaks in the range 1558 and 1418 cm⁻¹ are ascribed to the C-H bonding. This is due to the formation of co-ordinate bond between the nitrogen atom of the PVP and the Zn²⁺ ions. Therefore, the FTIR studies strongly support the formation of PVP-encapsulated ZnS nanoparticles.

AFM image of ZnS nanoparticles

The atomic force microscopy or scanning force microscopy is one of the important tools for imaging, measuring and manipulating



materials at nanoscale (http://en.wikipedia.org/wiki/Atomic_force_microscope).

Figure 3 shows an atomic force microscopy image for Zinc sulphide nanoparticles, with scan size of 2 μ m × 2 μ m. Height profile was taken across a quantum dot which shows the height of the quantum dot. The height and width were found to be approximately 8 nm and 86 nm respectively. An approximate histogram plot across the area was taken which shows increased distribution of particles with size ranging between 21-22 nm.

Characterization of functionalized MWCNTs

The surface morphology of the sensing area of functionalized MWCNT was examined using scanning electron microscopy (SEM). The chemical property of the MWCNT was characterized by Fourier Transform Infrared Spectroscopy (FTIR).

IR spectrum of functionalized-MWCNT

The result on generation of surface functional groups obtained from FT-IR spectroscopy is shown in Figure 4. It is clear that several types of functional groups, particularly Carbonyl and hydroxyl groups have been generated on acid-oxidized Carbon nanotube surfaces as expected. Examination on surfaces of acid oxidized Carbon nanotubes was carried out using a Fourier transform infrared (FT-IR) spectrophotometer to ensure formation of desired surface functional







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groups (Figure 3 and 4). Also shows IR spectrum of unfunctionalized and functionalized MWCNTs, respectively. As seen from the spectrum of functionalized MWCNTs, the presence of bands at 1636 cm⁻¹ and 3434 cm⁻¹ confirmed the presence of Carbonyl and hydroxyl moieties of carboxylic acid group. This confirms that the Carbon nanotubes has been well functionalized with -COOH, which was absent in unfunctionalized MWCNTs. The presence of -COOH groups on the surface of the MWNTs is advantageous for a better deposition of semiconducting nanoparticles on the surface of the MWNTs.

SEM image of functionalized MWCNT

The Figure 5 shows the SEM image of functionalized MWCNTs. After functionalization of MWCNTs some amorphous Carbons are in the picture. The length of the MWCNTs is approximately 2 microns.

Electrochemical study of chemically modified electrode

Electrochemical analysis involves the measurement of potential (potentiometry) or current response (voltametry). This study focused on current measurement (voltametry). Cyclic voltametry, Square wave voltametry, Linear sweep voltametry, Rotating disk electrode, Chronoamperometry, Galvanostatic charge discharge technique and electrocatalysis using voltametry are the various types of voltametry [28].

Cyclic voltametry is the most widely used electrochemical technique. In cyclic voltammetric experiment the potential of an electrode is cycled voltametry from a starting potential Ei to a final potential Ef and then back to Ei. The potential at which the peak current occurs is known as peak potential Ep. At this potential the redox species has been depleted at the electrode surface and diffusion is limited by the current. The magnitude of the Faradaic current Ipa (anodic peak current) or Ipc (cathodic peak current) is an indication of the rate at which electrons are being transferred between the redox species and the electrode [29].

The electrochemical behaviors of the Nafion-ZnS-MWCNTS-ChOx modified electrode fabricated by layer by layer assembly technique in this work were investigated by cyclic voltammetry.

Electrochemical behavior of Nafion-ZnS-MWCNTS-ChOx modified electrode in 0.05 M PBS (pH 7.0)

In Figure 6, curve d is the cyclic voltammogram of a Nafion-ZnS-MWCNTs-ChOx/GC electrode in 0.05 M PBS (pH 6.0) at a scan rate of 50 mV/s, while curve c, curve b and curve a are the cyclic voltammograms of a Nafion-ZnS-ChOx/GC electrode, Nafion-ChOx/GC electrode and a bare electrode, respectively. From the cyclic voltammogram, no redox peaks were observed at the bare electrode (curve a), Nafion-ChOx/GC electrode (curve b), Nafion-ZnS- ChOx/GC electrode (curve c) and Nafion-ZnS-MWCNTs-ChOx/GC electrode (curve d).

Electrochemical behavior of Nafion-ZnS-MWCNTS-ChOx modified electrode in PBS solution with 30 mM cholesterol

In this work, the direct electrochemistry of ChOx on the Nafion-ZnS-MWCNTs modified electrode was investigated. In Figure 7, curve d is the cyclic voltammogram of a Nafion-ZnS-MWCNTs-ChOx/GC electrode in 0.05 M PBS (pH 6.0) with 30 mM cholesterol at a scan rate of 50 mV/s, while curve c, curve b and curve a are the cyclic voltammograms of a Nafion-ZnS-ChOx/GC electrode, Nafion-ChOx/GC electrode and a bare electrode, respectively. From the cyclic voltammogram, no redox peaks were observed at the bare electrode (curve a) and Nafion-ChOx/GC electrode (curve b), and the redox



Figure 5: SEM image of functionalized multi wall Carbon nanotubes.



peaks at the Nafion-ZnS-ChOx/GC electrode (curve c) were very small. In contrast, there was a pair of well-defined and quasi-reversible redox peaks at the Nafion-ZnS-MWCNTs-ChOx/GC electrode (curve d). The formal potential E° , the average of the anodic and cathodic peak potentials, was estimated to be -0.461 V.

The cathodic and anodic peak currents were of similar magnitude, with a ratio about unity. The anodic peak potential and the cathodic peak potential of curve d in Figure 4 and 7 were respectively -0.234 V and -0.688 V with a small peak potential separation ($\Delta Ep = 454 \text{ mV}$), revealing a fast electron transfer process.

All of these results indicate that the direct electron transfer of ChOx was achieved through the incorporation of ChOx into the Nafion-ZnS-MWCNTs film. ZnS QDs and MWCNTs played an important role in facilitating the electron transfer between the proteins and the electrode surface, which might be due to a higher charge detaching efficiency of quantum dots resulting from its quantum size effect and the favorable orientation of ChOx molecules in the composite film, etc.

Electrochemical behavior of Nafion-ZnS-MWCNTS-ChOx modified electrode in 30 mM cholesterol at various scan rates

The Figure 3 and 8 is the cyclic voltammograms of the Nafion-ZnS-MWCNTs-ChOx/GC electrode at various scan rates. The redox peak currents were scanning rate-dependent. With the scan rate increasing, the cathodic and anodic peak currents increased simultaneously. A linear relationship between them with scan rate from 0.05 to 0.2 V/s revealed that the electron transfer between ChOx and GC electrode could be easily performed at the Nafion-ZnS-MWCNTs-ChOx composite film.

Study of immobilised ChOx kinetics

Enzyme kinetics is the study of the chemical reactions that are catalyzed by enzymes. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction studied. Studying the enzyme's kinetics in this way can reveal the catalytic mechanism of the enzyme.

Effect of cholesterol concentration on the activity of immobilised ChOx

Michaelis–Menten kinetics of an enzyme describes the rate of enzymatic reactions, by relating reaction rate v to the concentration of a substrate [S]. Its formula is given by

$$\upsilon = \frac{V_{\max}[S]}{K_m + [S]} [30]$$



Figure 7: Cyclic voltammograms of (a) bare electrode, (b) Nafion-ChOx/GC, (c) Nafion-ZnS-ChOx/GC and (d) Nafion-ZnS-MWCNTs-ChOx/GC in 0.05M PBS (pH 6.0) with 30mM Cholesterol.



Figure 8: Cyclic voltammograms of the Nafion-ZnS-MWCNTs-ChOx/GC electrode in 0.05 M PBS (pH 6.0) with 30mM Cholesterol at various scan rates. Scan rate (from a-d) is 50, 100, 150, 200mVs⁻¹, respectively.

The Michaelis-Menten constant K_m is the substrate concentration at which the reaction rate is at half-maximum, and is a measure of the substrate's affinity for the enzyme. A small K_m indicates high affinity, meaning that the rate will approach V_{max} more quickly.

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The enzyme response to cholesterol concentration has been carried out. Figure 9 shows the enzyme activity to different concentrations of cholesterol. There are ten different concentrations of cholesterol standard solution (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/dl) have been applied. The enzyme activity was increasing according to the rise of cholesterol concentration. The Michaelis– Menten and Lineweaver–Burke plots were drawn to study the kinetics of immobilized Cholesterol Oxidase.

The immobilized ChOx exhibits detection range of 10-450 mg/dl, and good linearity is obtained in 50–450 mg/dl (1.3 -11.6 mM) range with linear regression coefficient 'R2' as 0.997.

The Michaelis-Menten constant (K_m) is a parametric statistic for describing the affinity between the enzyme and substrate in enzymatic reactions. In order to examine the activity of enzymes in biosensors, the apparent Michaelis-Menten constant (K_m) of ChOx was determined.







A Lineweaver-Burk double reciprocal plot based on the experimental data is shown in Figure 3 and 10. This is drawn by taking the reciprocal of both sides of the Michaelis–Menten equation. This is a linear form of the Michaelis–Menten equation and produces a straight line with the equation y = mx + c with a *y*-intercept equivalent to $1/V_{max}$ and an x-intercept of the graph representing $-1/K_{M}$ [31].

$$\frac{1}{\upsilon} = \frac{K_M}{V_{\max}[S]} + \frac{1}{V_{\max}}$$

The value of K_m (0.84 mM) is calculated from the intercept and slope of the Lineweaver-Burk plot (Figure 10). The low K_m value 0.84 mM indicates high affinity of immobilized ChOx to cholesterol. The smaller K_m value also indicates that the diffusion of substrate and product into and out of the membrane is easy in the present Nafion-ZnS-MWCNTs-ChOx/GC electrode composite matrix.

Effect of pH on immobilized ChOx activity

The pH of the solutions containing substrates can affect the overall enzymatic activity since, like all natural proteins, enzymes have a native tertiary structure that is sensitive to pH; denaturation of enzymes can occur at extreme pHs. It is well known that the enzyme activity is highly pH dependent and the optimum pH for an enzymatic assay must be determined empirically. It is best to choose a plateau region for enzyme assay so that the pH should not have any effect on enzyme activity. Stoytcheva [32] has studied the activity of the immobilized acetyl cholinesterase between pH 2 and pH 9. She has reported that the activity was decreased of approximately 70% at pH 2 compared to of that at pH 7.

The effect of different pH on the activity of immobilized Cholesterol Oxidase enzyme was examined and shown in Figure 11. The optimum enzyme activity was obtained at pH 7.0. The optimum pH value for the enzyme activity in homogeneous solution has been reported to be 7.0 [33]. Therefore, enzyme immobilization procedure did not cause a change in optimum pH of enzyme activity.

Effect of temperature on immobilized ChOx activity

The rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Centigrade rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results. In the case of enzymatic reactions, the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. Most enzymes rapidly become denatured at temperatures above 40°C [34].

Temperature exerts a marked influence on the Cholesterol Oxidase activity. The effect of temperature on the enzyme activity was examined in the range of $10-60^{\circ}$ C. The optimum temperature was found in the range of 35° C (Figure 12).

Conclusion

In this study, a novel nanoprobe (Nafion-ZnS-MWCNTs-ChOx/GC) was developed for cholesterol determination in blood by using LBL assembly technique. This study has shown that the ZnS nanoparticles and MWCNTs act as an effective mediator between the enzyme Cholesterol Oxidase and a glassy carbon electrode. Nafion was incorporated into a glassy carbon electrode upon which Cholesterol Oxidase has been immobilized. It provides all the features required for the analysis of cholesterol in clinical samples. Its performance rests on a number of features: the rapid rate of electron transfer between reduced





enzyme and the ZnS nanoparticles; the good electrochemical properties of MWCNTs and Nafion resulting in the effective confinement of the mediator to the electrode surface.

The optimum pH and temperature of the sensor was found to be 7.0 and 35°C respectively. The developed sensor was calibrated with 50-600 mg/dl of cholesterol and found to have linearity of 50-450 mg/dl. The calibration curve was drawn with initial velocity of the enzyme catalysed reaction Vi (One unit will convert 1.0 µmole of cholesterol to 4-cholesten-3-one per minute at pH 5.0 at 37°C) vs. different concentrations of standard cholesterol solution (mg/dl). The low K_m value 0.84 mM indicates high affinity of immobilized ChOx to cholesterol. The smaller K_m value also indicates that the diffusion of substrate and product into and out of the membrane is easy in the present Nafion-ZnS-MWCNTs-ChOx/GC electrode composite matrix.

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