# Cholesterol Oxidase nanoparticle preparation, characterization, and use in the improvement of a potentiometric cholesterol biosensor

**NEETU,** Research Scholar, Department of Chemistry, BABA MASTNATH UNIVERSITY ASTHAL BOHAR, ROHTAK- 124001, Haryana, INDIA
**SEEMA KUMARI,** Assistant Professor, Department of Chemistry, BABA MASTNATH UNIVERSITY ASTHAL BOHAR, ROHTAK- 124001, Haryana, INDIA
**RAVI KUMAR RANA ,** Professor, Department of Chemistry, BABA MASTNATH UNIVERSITY ASTHAL BOHAR, ROHTAK- 124001, Haryana, INDIA

## ABSTRACT

The commercial cholesterol oxidase nanoparticles (NPs) aggregates were created by the desolvation, cross linking by Glutaraldehyde, and Cysteamine dihydrochloride functionalization. FESEM, U.V, and Fourier transform infrared spectroscopy were used to analyse these enzyme nanoparticles (ENPs). The Cholesterol Oxidase NPs' FESEM pictures revealed that they ranged in size from 9 to 100nm, with an average of 22 nm. In comparison to natural enzyme molecules, the Enzyme NPs were extra steady, and had a longer shelf existence. By using glutaraldehyde coupling, the Enzyme NPs were immobilized on chitosan (CHIT) activated cellulose acetate (CA) membrane with 28.56% starting action of free cholesterol oxidase nanoparticles and a conjugation yield of 1.52mg/cm$^2$. To create a potentiometric cholesterol biosensor, an O-ring-mounted CA membrane was attached to the bottom end of a Fluoride ion -selective electrode (FLISE). Electrode was then coupled to a digital pH metre. At pH- 6.5 and 40°C, the biosensor responded at its best within 10 s. The biosensor was used to test the potentiometric determination of cholesterol in sera from people who seemed to be in good health and those who had cardiac issues. With a wide operating range of 0.003- 0.090 mM/Land a sensitivity of -58 mV/decade, biosensor had a LOD of 0.032µM/L. The added cholesterol in serum had a 106.33% analytical recovery. The current biosensor's coefficients of variations (CVs) were 1.92% for within batches and 2.32% for between batches. The serum cholesterol readings obtained using the reference technique enzymicmethod and the current biosensor had a strong correlation (r = 0.9999). L-alanine and pyruvate caused very little interference to the biosensor, although L-glutathione and glutamine did so somewhat. This interference was reduced by the use of a particular ion selective electrode. Cholesterol oxidase kept in 0.5M sodium phosphate buffer PBS at pH 7.0 at 4 °C, the Enzyme NPs-bound CA membrane was utilised up to 7-8 times daily for a total of 160 days.

***Keywords:*** Cholesterol Oxidase nanoparticles Cellulose acetate membrane, Potentiometric Cholesterol Biosensor, Fluoride ion-selective electrode FLISE pH or ion meter

**DOI Number: 10.48047/Nq.2022.20.17.Nq880290 Neuroquantology 2022; 20(17):2248-2265**

## 1. Introduction

All human body cells contain cholesterol, which is a lipid molecule (Baynes et al.,2005) . Around 5.17 mM of cholesterol are present physiologically in human serum (Machlan et al., 2000). High cholesterol levels can cause fatal illnesses like diabetes, cancer, and heart disease, cardiovascular, cerebral, etc. Determining the level of cholesterol in the human body is therefore crucial (Nauck et al., 2000, Staels et al., 2002, Ikonen et al., 2008). Calorimetric, spectroscopic, and electrochemical techniques have all been employed for the determination of cholesterol levels (Ibupoto et al., 2014). Potentiometric sensors, meantime, have been widely used as electro-analytical instruments for the past 50 years. With the use of a lipophilic ionosphere, their flexible chemical selectivity may be easily adjusted. With the publication of the first potentiometric enzyme sensor in 1969, rapid advancements in the field of potentiometric sensing have been shown, for example since they do not need an external power source and no current passes during detection. A strong foundation for the creation of new sensors that might measure an accurate concentration of cholesterol has been made possible by the development of novel devices with greater selectivity, rapid reaction time, and sensitivity. About 80% and 75% of deaths in men and women, respectively, are brought on by coronaryartery disease and heart attacks. Most elderly people with cardiovascular diseases are between the ages of 70 and 80. In the US, 85% of those over the age of 80, 71% of those between the ages of 60 and 80, 37% of those between the ages of 40 and 60, and 11% of those between the ages of 20 and 40 have CVD. The average age of death in developing nations is above 80, but it is approximately 68 in poorer ones due to coronary artery disease. Men are more prone to these diseases than women are, and they frequently manifest seven to ten years sooner in men. Cardiovascular disease is becoming more likely. Nearly all body tissues contain cholesterol ($C_{27}H_{46}O$). It is an amphoteric waxy substance, Cholesterol is necessary for several metabolic and cellular processes in the body, including intracellular -transport, cell communication, and nerve conduction. Additionally, it acts as a precedent for the production of Hormones, Vitamins, and Bile acids. Some cholesterol is produced by the liver; the rest is obtained from diet. There is a lot of cholesterol in poultry, pork, dairy products, and eggs. The highest cholesterol foods include egg yolks and organ meats including liver, kidney, sweetbreads, and brain. Bio sensing approaches are quantitative or semi-quantitative in nature, with biologically derived sensing components either incorporated into or in close proximity to the transducer (Turner et al., 1987; Thakur and Karanth., 2003). Guilbault and Montalvo presented the first potentiometric enzyme sensor for the detection of cholesterol in 1969. (Bhalla et al., 2016). For the purpose of determining cholesterol levels, numerous techniques have been developed. Three broad groups can be made from those techniques:

1) Traditional chemical techniques using the Abell-Kendall protocol,

2) fluorometric and colorimetric enzymatic assays, which are frequently used for assay kits and automated plate readers,

3) analytical instrumental approaches such gas and liquid chromatography or mass spectrometry.

Each of those approaches has particular benefits. Traditional chemical treatments are very cheap and easy to carry out, but multiple steps are necessary. Despite having expensive enzymes required, limits of detection (LOD) in enzymatic assays are typically low. The most precise and sensitive techniques are chromatographic and mass

spectrometric, but they also call for expensive equipment and considerable sample preparation(Hua li et al., 2019). A biosensor is described as analytical devices that combine biological detecting components such sensor system and a transducer. Existing and widely used traditional techniques for determining cholesterol include colorimetry, fluorimetry, gas chromatography, enzymiccolorimetry/spectrophotometry, and high performance liquid chromatography (HPLC) (Bisht et al., 2005). However, each of these approaches has a drawback of some kind, such as the need for expensive equipment, trained operators, or lengthy research times (Tiwari et al., 2009). Despite the fact that there are several approaches, the two that are usually employed for detecting cholesterol levels are potentiometric and amperometric biosensing (Bhalinge et al., 2016). Potentiometric approaches stand out among these two as extremely selective and unquestionably popular procedures. Potentiometric biosensors are viewed as apreferable alternative to conventional methods of detecting cholesterol because of their simplicity, speed, affordability, and high sensitivity. The conc. of cholesterol is then determined by surveillance the production of $NH_4^+$ or $CHO_3^-$ ions using their ISE. Guilbault and Montalvo created the first potentiometric cholesterol oxidase electrode to measure cholesterol using hydrolysis mediated by cholesterol oxidase (Lakard et al., 2004, Jiang et al., 2004). Enzymatic strategies for potentiometric transducers are more precise and selective. A biosensor with an enzyme layer directly on its surface has been created to measure the quantity of cholesterol (Chou et al., 2008). Potentiometric cholesterol biosensors established on ion-sensitive field effect transistors, are the best among the many types of modern biosensors because of their increased sensitivity and quick electronic turnout (output) (Bergveld, 2003). The cholesterol oxidase has been immobilised onto several supports for use in cholesterol biosensors. However, when enzymes are directly immobilised on a support, their denaturation results in the loss of their stability and activity. To solve this issue, the enzyme molecules were aggregated, then glutaraldehyde was used to crosslink them into nanoparticles (NPs), which were then immobilised. Due to their unique electronic, optical, mechanical, electrical, thermal, and catalytic properties as well as their increased surface area, enzyme nanoparticles have demonstrated enormous potential for enhancing enzyme electrodes (Kundu et al., 2013; Pundir, 2015; Pundir and Aggarwal, 2017; Narwal and Pundir, 2017). Therefore, using cholesterol oxidase NPs rather than natural enzyme molecules FlISE may improve the analytical capabilities of cholesterol biosensors. In order to build a potentiometric cholesterol biosensor employing a fluoride ions selective electrode, the current paper addresses the characterisation, and immobilisation of cholesterol oxidase nanoparticles onto cellulose acetate (CA) membrane (FLISE).

## 2. Experimental

### 2.1 Major Chemicals used

In all of the experiments, cholesterol oxidase from microorganisms (15U/mg from microorganism), Cholesterol and glutaraldehyde (25%), urea, chitosan(CHIT),ethanol, and methanol from SISCO Research Lab, Mumbai, Nessler Reagent from Hi-Media Lab. Pvt. Ltd. Mumbai, CA membrane from Whatman, GmbH-Dassel, Germany through Genetrix, New Delhi and cysteaminedihydro chloride from Fluka, Mumbai were used. All other chemicals were of analytical reagent (AR) grade. Double distilled water (DW) was used in all experiments. Reference filling solution (30 ml supplied with electrode), standard Ionic Strength Adjuster (ISA)

solution for preparing standards and samples were used.

### *2.2 Major instruments used*

In the current investigation, the following equipment and supplies were employed in the lab: The development of the potentiometric cholesterol oxidase biosensor employed a digital Ion/pH metre (model number LAMION 40) and Flouride Ion Selective Electrode (FLISE) from Van London Phoenix Co., USA through Satguru Enterprise, Hisar -125001 (Haryana). Weighing scale, UV-visible spectrophotometer (double beam manufacture lasany/ Japan) GJU Hisar, and FTIR spectrophotometer (digital model PGB 200, wensar) from GJU Hisar. Field emission scanning electron microscopy (FESEM) JSM-7610F Plus, JEOL, USA at GJU Hisar for taking FESEM image of cholesterol oxidase NPs.

### 2.3 Cholesterol oxidase nanoparticle preparation (ChOx-NPs)

In order to create cholestrol oxidase nano particle, ethanol added to 3ml of enzyme solution), at a dropping rate of 0.1 – 0.2ml/minute (Kundu et al., 2012) under 500 rpm continuous churning. Then, 1.8 ml of 2.5% glutaraldehyde will be added to the suspension of these nanoparticles, which will be continuously stirred at 500rpm for 24 hour sat 4°C to ensure, the cross linked of enzyme nanoparticles. Studies using UV, FESEM, and FTIR spectroscopy will be used to describe cholesterol oxidase-NPs. When not in use, cholesteroloxidase NPs will be kept at 4°C (Nidhi Kundu et.al., 2012). Aggregates of cholesteroloxidase nanoparticles were created as a result of interactions between cholesterol oxidase enzyme molecules caused by van der wall, hydrophobic, and electrostatic forces. These enzyme aggregates were made always insoluble while retaining their pre-organized structure and by the reaction of ammonia group on the enzyme catalyst surface, with a bifunctional crosslinking agent like Glutaraldehyde **(**$(CH2)_3(CHO)_2$ (Chawla et al., 2013). cholesterol oxidase enzyme NPs shape and size characterized as determined by FESEM. These NPs' comprehensive morphological examination and spherical form were seen in the FESEM photos.

### 2.4 Characterization of cholesteroloxidase NPs

Cholesterol oxidase NPs were examined under a field emission scanning electron microscope FESEM, their Ultra visible spectra were captured using a Ultra Violet spectrophotometer, and their FTIR spectra were captured using an FTIR mass spectrophotometer.

### 2.5 Immobilization of Cholesterol Oxidase Nanoparticles on modified Cellulose acetate membrane

Nanoparticles of cholesterol oxidase will be covalently fixed to the membrane. The membrane will first be cleaned with distilled water to get rid of any dirt before being air-dried for 30 minutes. To add -$NH_2$ groups to the membrane's surface, it will be exposed to 5% cysteamine for 24 hours at room temperature. Cleaning with 10% of methanol followed dried by air for 30 minutes will be used to remove unbound $NH_2$ groups. For two hours, the membrane will be submerged in 2.5% of glutaraldehyde (in 0.5 M PB, pH 7.0).at ambient temperature Glutaraldehyde will thoroughly cleanse the membrane. In order to covalently immobilise the cholestrol oxidase-NP aggregate suspension, it will be evenly distributed over the activated CA membrane andlefttthereovernightat4°C for immobilization. In order to examine the enzyme activity of the cholesterol oxidase -NP aggregates that are discarded, the cholesterol oxidase aggregates bound membrane will be washed. High resolution Field emmission scanning electron

microscopy (FE-SEM) of the immobilised membrane with the immobilised cholesterol oxidase aggregation will be used to verify the immobilisation (NidhiKundu et al., 2012)

**2.6 Cholester ol Oxidase NPs/CA membrane/FIIS electrode construction**

A flourideions specific-electrode (FLISE), functions as a transducer or sensor that transforms the activity of a few chosen ions dissolved, in a Lipid Solution into an electrical potential that can be monitored by a digital ionmeter. Flouride specific membrane and the reference-electrode are commonly used to construct the electrode's lower sensing portion. The concealing the filling hole with a rubber insert of the reference room of the electrode was also removed, along with the little black cap from the end of the Fl- ion selective electrode. The Orion™ ISE filling solution that came with the ISE was now added to the electrode. To get rid of any air bubbles that could have become trapped above the CA membrane, a light shakedown was applied to the ISE. Before usage, the ISE was submerged in DW for 30 minutes.

**2.7 Response measurement of potentiometric Cholesterol biosensor**

By serial dilution of the 0.1M, 1000 ppm, or 100 ppm fluoride standard, prepare two fluoride standards whose concentration is near the expected sample concentration. Add 50 ml of TISAB 1 or TISAB 3, to each 50 ml of standard. When calibrating, assume that the added TISAB has no effect on the standard concentration.

Place the more dilute solution on the magnetic stirrer and begin stirring at a constant rate. Assure that the meter is in the concentration mode. Lower the electrode tip into the solution. Adjust the meter to the concentration of the fluoride standard and fix the value in the memory according to the meter manufacturer's instructions after stabilization of the reading. Rinse the electrode with distilled water and blot dry. Place the more concentrated solution on the magnetic stirrer and begin stirring at a constant rate. Lower the electrode tip into the solution. Adjust the meter to the concentration of the fluoride standard and fix the value in the memory according to the manufacturer's instructions after stabilization of the reading. For low level measurements, place the rinsed, dried electrode into a solution containing equal volumes of distilled water and TISAB 1 or TISAB 3 (or add 10 ml of TISAB-2 to each 100 ml of distilled water). After stabilization, fix the blank value in the meter according to the meter manufacturer's instructions. After rinsing the electrode and blotting dry, place the electrode tip into the sample diluted with an equal volume of TISAB 1 or TISAB 3. After stabilization, read the concentration directly from the meter display. The calibration should be checked every 1-2 hours. Assuming no change in ambient temperature, place the electrode tip in the first fluoride standard. After the reading has stabilized, compare it to the original reading. A reading differing by more than 0.5 mV or a change in ambient temperature will necessitate repetition of Steps 2-8 (2-9) above. The meter should be re-calibrated daily, by lowering the concentration of a 1000ppm standard solution, two standards of fluoride (Fl-) of 1 ppm and 1000 ppm were created. The Electrode was attached to a ion digital or pH metre, and the cholesterol oxidase – nanoparticles with the help of an O-ring, bonded CA membrane was put over the lower/sensitive end of the ISE. It was then placed in a 200 ml beaker containing 100 ml of standard solution at 1ppm and 10ml of Ion strength regulator, and the mixture was well agitated. After one minute, the memory value was set to -57mV in accordance with the calibration guidelines provided by the meter's manufacturer. The FLISE/electrode

tip was then cleaned with tissue paper after being washed with DW. The electrode was put into a 200 ml beaker with 10 ml of ISA and 1000ppm of standard solution, and the mixture was well agitated, value in mV was recorded when a steady reading was shown, and it was -60mV. Electrode was then cleaned with DW, dried with tissue paper, and placed in a solution of 100ml buffer solution (0.5M, pH -7.0) Containing 0.1ml cholesterol. The measurement in mV was immediately captured from the metre display after 1minute and was -55mV. Every 1-2 hours, the electrode was re-calibrated by repeating the aforementioned procedures.

**2.8 Optimization of improved potentiometric Cholesterol biosensor**

Different dynamic features of the immobilised compound on the CA layer-covered FLISE composite anode were taken into consideration, such as how pH, hatching temperature, brooding duration, and the focus of the substrate (cholesterol) affected the protein cathode's optimal operating states. A terminal via sophisticated particle metre operating at a constant temperature assisted in the estimations.

**2.9 Application of Cholesterol biosensor**

The Pt. BDS Postgraduate Institute of Medical Sciences hospital provided sera samples from apparently healthy and ill patients with cardiac disorders. The cholesterol content of these sera samples was measured by the present-biosensor as characterised for its ability to assess reaction under its optimal conditions, with the exception that the cholesterol was replaced by serum (0.1ml), which was added into 10 ml of reaction buffer (0.5M, pH-7.0). Cholesterol concentration was extrapolated from an optimally generated cholesterol concentration and biosensor response standard curve.

**2.10 Evaluation of a Biosensor for Cholesterol**

In order to assess the cholesterol biosensor, the analytical performance was calculated in terms of percentage analytical recovery, limit of detection (LOD), accuracy, and correlation.

**2.11 Cholesterol Oxidase enzyme stability after mounted on CA membrane**

By monitoring its reaction weekly, the storage integrity of the Cholesterol Oxidase Nanoparticles -CA membrane was investigated under 60 days. After being cleaned in DW, the membrane was kept in 0.5M sodium phosphate buffer, pH 7.0, at 4 °C while not in use.

## 3. Results and discussion

**3.1 Cholesterol Oxidase NPs and their Aggregates Preparation**

Aggregates of cholesterol oxidase NPs were prepare dutilising the ethanol by desolvation procedure. By adding ethanol drop wise to the natural cholesterol oxidase solution, while holding temperature constant at 4°C, the cholesterol oxidase-NPs aggregates were created. Because the hydration layer around the native cholesterol oxidase molecules shrank, cholesterol oxidase nanoparticles (NPs) were created. Cholesterol Oxidase-NP aggregates were created as a result of interactions between the cholesterol oxidase molecules, including Vander Waals, hydrophobic, and electrostatic forces. These enzyme aggregates were made completely insoluble while retaining their preorganizeds structure and, consequently, their activity by reacting -$NH_2$ groups that Cysteamine Dihydrochloride placed on an enzyme surface with a functional cross linking agent such as glutaraldehyde $(CH2)_3(CHO)_2$. Because Cholesterol Oxidase NPs aggregates may position themselves, a considerable increase in their activity as

compared to the original Cholesterol Oxidase molecules. The activation may be brought on by conformation changes that the aggregating enzymes molecules undergo.

### 3.2 Characterization of Cholesterol Oxidase NPs

As determined by FESEM, the form and size of the aggregated of cholesterol oxidase nanoparticles are shown in Fig.2 Cholesterol Oxidase nanoparticles had a diameter that ranged from 50 to 100 nm. This demonstrated that 4 - 9 native cholesterol oxidase molecules joined together to create a single, spherical NP of cholesterol oxidase. By using UV-visible absorption spectroscopy, these Cholesterol Oxidase NPs aggregates were further studied. Native Cholesterol Oxidase's UV-visible absorption spectra revealed a peak at 280nm, which was caused by the absorption of peptide bonds and aromatic amino acids. Additionally, following the production of NPs, the peak of the cholesterol oxidase's aromatic bond moved from 240 to 280 nm (Cholesterol Oxidase NPs). The creation of Cholesterol Oxidase NPs aggregates was confirmed bya rise in this absorbance peak, which showed that the cholesterol oxidase molecules concentrated without anyalteration in the fundamental structure. The absorbance of the Cholesterol Oxidase NPs gradually increased in comparison to natural Cholesterol Oxidase, according to a study of the UV spectra of the enzymes.



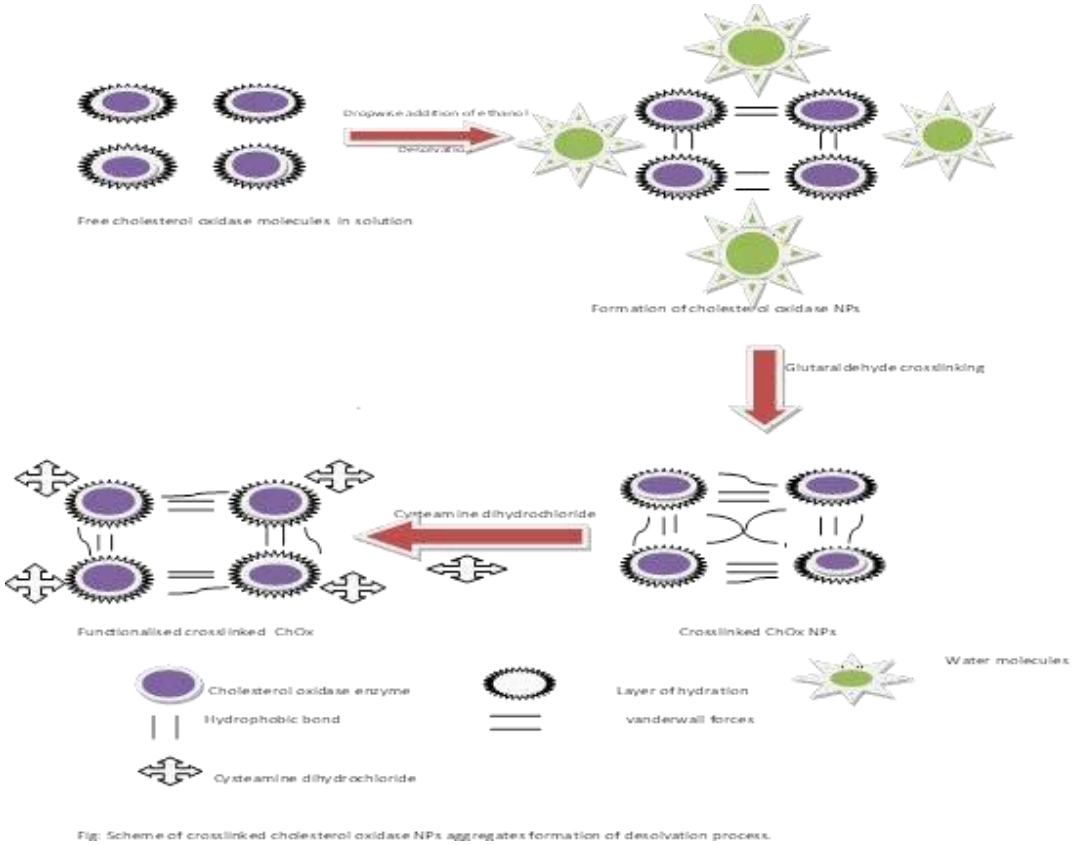
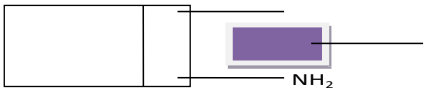



Figure 1 shows a schematic of the desolvation process used to create aggregates of cross-linked cholesterol oxidase nanoparticles.

**3.3 Aggregates of cholesteroloxidase-NP are immobilized on the CA membrane.**

Through glutaraldehyde coupling, cholesterol oxidase nanoparticles were covalently bound onto CHIT activated CA membrane. First, chitosan introduced free amino groups onto the CA membrane, activating it. The aldehyde groups of the functional glutaraldehyde interacted with these free ammonium groups on the CA membrane. The reaction between the additional free aldehyde groups and the amino groups, on the surface of the cholesterol oxidase nanoparticles aggregates now formed the Schiff –base in between the activated CA membrane and the aggregates, guaranteeing their immobilization on the CA membrane.




**3.4 Characterization of the CA membrane bound cholesterol oxidase NPs**

**3.4.1**

**By**

**FESE**

**M**

Fig. 2 displays the FE-SEM images of the naked CA membrane. These FESEM photos made it easy to examine how the membrane was modified step by step. The naked CA membrane's hollow beaded shape was seen in the FESEM photos. However, cholesterol oxidase-NP aggregates immobilized CA membrane demonstrated clusters of beads in spreader, filled, and globular beads aggregation of enzymes in the form of beaded structures throughout the surface of the CA membrane, which supports the immobilization of cholesterol oxidase-NP aggregates on CA membrane. Following covalent immobilization of cholesterol oxidase-nanoparticles onto CA membrane through glutaraldehyde coupling between amino groups of cysteaminedi hydrochloride, the immobilized enzyme retained with 182.56% retaining activity as a free enzyme with a conjugation yield of 1.52 mg/cm2, demonstrating an an increase in the free enzymes' activity. By FTIR spectra

**3.4.2 By FTIR Spectra**

FTIR spectra for free cholesterol oxidase enzyme are shown in curve 1 of Fig. 11 from 4000-500cm-1 wavenumbers, while curve 2 depicts the development of cholesterol oxidase NPs aggregates that have been verified by FTIR spectroscopy. FTIR Spectra of cholesterol oxidase and cholesterol oxidase-NPs revealed the stretching- vibration bands at transmittance (3437.30- 3436.58cm-1) are are due to asymmetric and symmetric stretching vibrations of $CH_2$ and $CH_3$ groups, (2076.24- 2063.17cm-1) are due to $CH_2$ symmetric stretching vibrations, (1638.04- 1636.61cm-1) due to C=C stretching due to presence of glutaraldehyde. In comparison to free cholesterol oxidase's activity of 15.8 moles/min/ml, the NPs of cholesterol oxidase shown greater activity (18.4 moles/min/ml).

**3.5 Establishment of a potentiometric cholesterol biosensor**

By securing Cholesterol Oxidase NPs aggregates bounds on CA membrane over the bottom end of the Fluoride ion selective electrode (FLISE) with an aid of O-ring and attaching it to a pH metre, a potentiometric cholesterol biosensor was created. Potentiometric response was based on FLISE, which has benefits

including a straightforward process, a quick reaction time, non destructive analysis, a broad moderate selectivity and range, and widespread application for the measurement of several ions. When the CA membrane closes, a potential is created as a result in the time-dependent behaviour of the rxn buffer.


**Fig: 2 An potentiometric cholesterol biosensor based on cholesterol oxidase NPs aggregates immobilized on CA membrane over the lower sensitive and of FIISE**



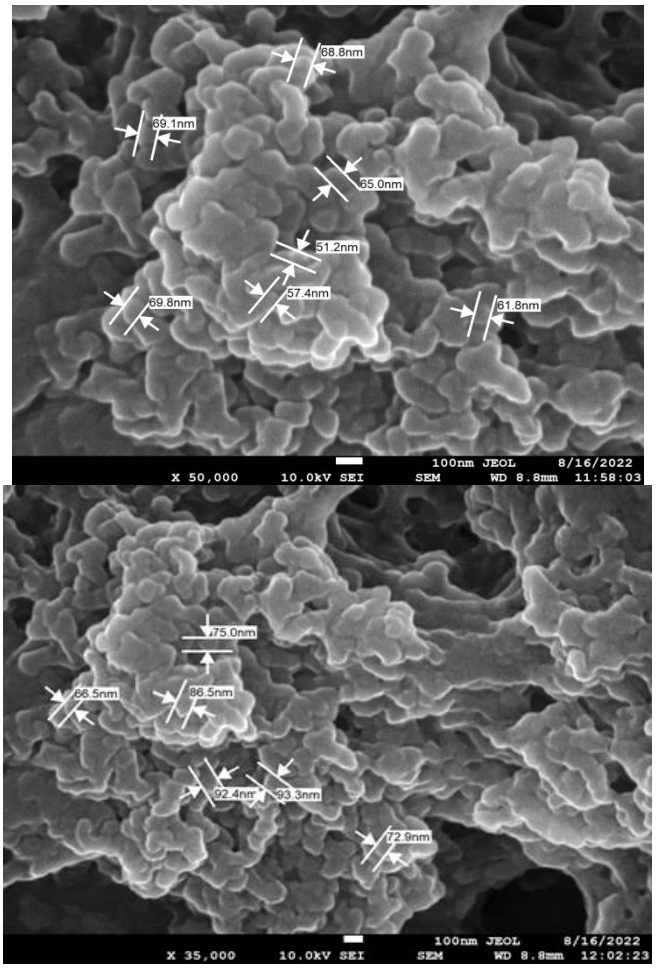
**3.6 Measurement of the cholesterol biosensor's response**

Biosensor's reaction Time was examined across the range of 5 - 120 seconds, with 10 second intervals. The biosensor reacted quickly to the substrate when cholesterol was introduced to the buffer (sodium phosphate buffer 7.0), achieving 98% of constant current in (fig.). The response time was previously reported potentiometric cholesterol biosensors based on ChOx/Znonanorods/Agcl (10s), ChOx/ nanowires/CO3O4 (10s), ChOx/Graphene/Agcl/PBS (5s), ChOx/Lipid film/Zno/Agcl (5s), and ChOx/ Znonanowall/Al (10s) (5s).

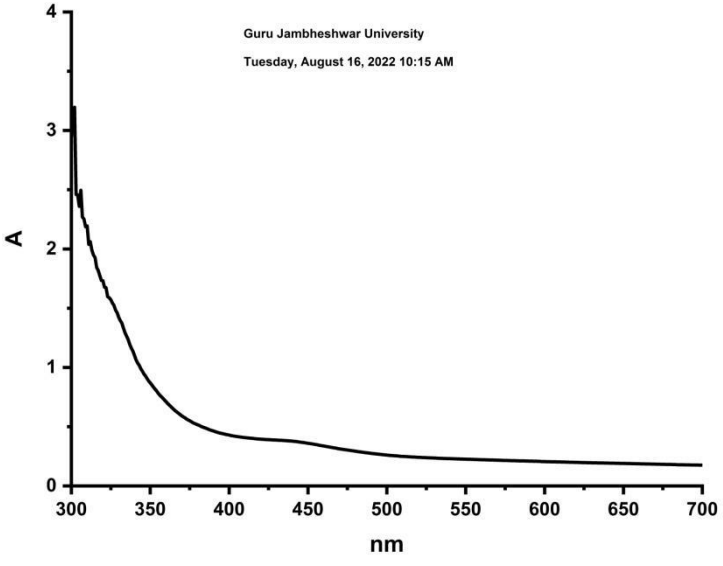
| Sr. no | Types of electrode | Linear range | Detection limit | Potential | Sensitivity | Time | pH/Precision | Storage/stability | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 1. | ChoX/Znonanorods/AgCl | 1 * $10^{-6}$ to 1 * $10^{-6}$ | - | | 35.2Mv | 10 sec | - | _ | Israr et al.,2010 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2. | ChoX/Graphene/AgCl/PBS | - | $10^{-7}$ M | 64mV | 5 sec | 7.0 | _ | Nikoleli et al., 2013 |
| 3. | ChoX/nanowires $CO_3O_4$ | 1x10$^{-7}$M to1 x10$^{-3}$M | 5 x10$^{-8}$M | 94.031mV | 10 sec | 7.3 | _ | Ibupoto et al., 2014 |
| 4. | ChoX/Lipid Film/Zno/AgCl | 1x $10^{-6}$ M to 1 x $10^{-3}$ M | 4 x10$^{-7}$M | 57mV | 5 sec | - | _ | Psychoyios et al.,2012 |
| 5. | ChoX/ CA-CNT | $10^{-3}$ to 10$^{-8}$M | $10^{-8}$ M | - | - | | _ | |
| 6. | ChoX/ Znonanowall/ Al | 1 x10$^{-6}$ to 1 x $10^{-3}$ M | - | 53mV | 5 sec | | _ | Israr et al., 2011 |

Table 1- A comparison properties of various potentiometric cholesterol biosensors


88.8nm
39.1nm
65.0nm
51.2nm
57.4nm
69.8nm
61.8nm
X 50,000 10.0kV SEI SEM WD 8.8mm 11:58:03
100nm JEOL 8/16/2022
75.0nm
66.5nm
86.5nm
92.4nm
93.3nm
72.9nm
X 35,000 10.0kV SEI SEM WD 8.8mm 12:02:23
100nm JEOL 8/16/2022




Guru Jambheshwar University
Tuesday, August 16, 2022 10:15 AM
A
nm
0
1
2
3
4
300
350
400
450
500
550
600
650
700

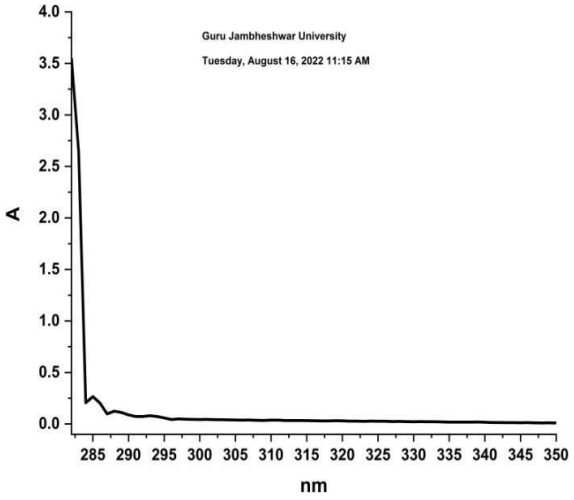



**Fig: 3** Cholesterol Oxidase NPs FESEM images in (A). (B) The natural cholesterol oxidase and cholesterol- oxidase nanoparticle UV spectra.

**3.7 Optimization of improved holesterol biosensor**

At pH 6.5, the immobilized cholesterol oxidase-NPs aggregates based biosensor shown its best performance (fig). The loss of $NH_2$ groups may be the cause of this drop in ideal pH. The optimal pH was similar to previously reported potentiometric cholesterol biosensors based on ChOx/Graphene/Agcl/PBS (pH-7.0) and ChOx/nanowires/$CO_3O_4$ (pH-7.3).

The maximal reaction of the immobilized cholesterol oxidase-NPs aggregates was seen at 40°C, which is greater than that of the free cholesterol oxide seen zyme (37°C). This improvement in thermo-stability of ENP aggregates, which form as a result of the aggregation and crosslinking of enzyme molecules, may explain the rise in the optimal temperature of cholesterol oxidase. There was a hyper bolicr relationship between biosensor response and cholesterol in the range 2 -300µM. The response was constant after 44µM. The current cholesterol biosensor's operational range was from 0.003 to 0.090mM, which is lower than potentiometric cholesterol biosensor based on (1 x $10^6$ - 1 x $10^{-6}$M), nanowires $CO_3O_4$ (1x $10^{-3}$M), lipid films (1x $10^{-6}$ - 1 x $10^{-3}$M), CA- CNT Oils ($10^{-3}$ -$10^{-8}$M), Znonanowalls (1x$10^{-6}$-1x$10^{-3}$M).

**3.8 Evaluation of a Biosensor for Cholesterol**

**3.8.1 Lower detection limit**

The current biosensor's lower detection limit was 0.032µmol/L, which is greater than potentiometric cholesterol biosensors that use grapheme/lipid film(0.1µm/L), Znonanowalls/Lipid film(0.4µm/L),ChOx/Ezetimibe/wire(0.25µm/L), ChOx/CO3O4(0.0035µm/L).

**3.8.2 Sensitivity**

The enhanced cholesterol biosensor used in this study has a sensitivity of -58 mV, therefore it is superior than previously published Potentiometric cholesterol biosensors, based on Chox/Znonanoroads/Agcl (35.2 mV), Chox/Graphene/Agcl(64mV), Chox/Lipid film/Zno(57mV), Chox/Znonanowall/Al(53mVChOx/nanowires/$CO_3O_4$( -94mV).

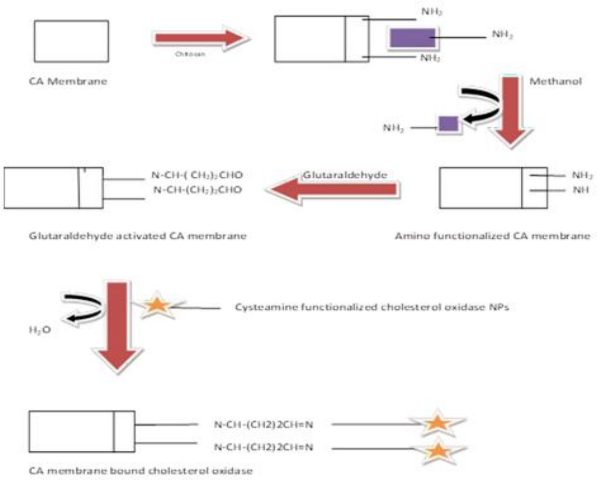
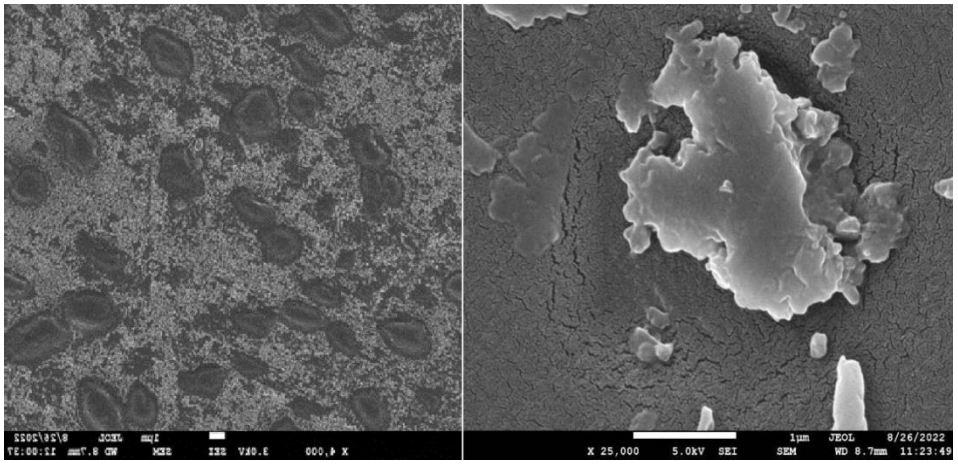



**Fig: 4** schematic representation of steps /chemical reaction involved in the fabrication of cholesterol oxidase NPs/CA membrane

A B





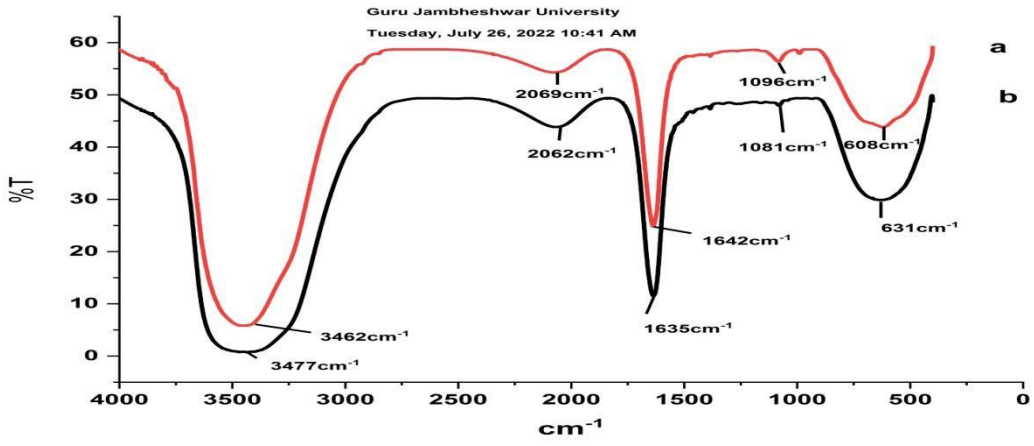



**Fig: 5 FSEM images of pure membrane and bounded membrane and FTIR spectra of cholesterol oxidase and cholesterol oxidase NPs**

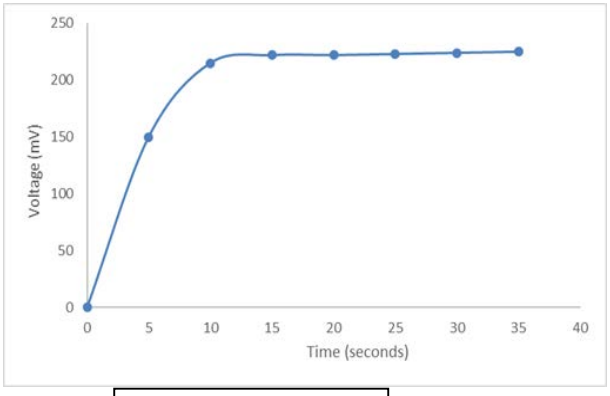
### 3.8.3 Analytical recovery

To check the accuracy of the present cholesterol biosensor method, the analytical recovery of added cholesterol in serum was determined. The average analytical reoveries of cholesterol ladded to blood serum at levels of 5.0Mm, 10mM, 15mM, 20mM were varied from 97.82%, 99.61%, 98.5% and 98.37% respectively(Table) demonstrating the high reliability of the present improved cholesterol biosensor.

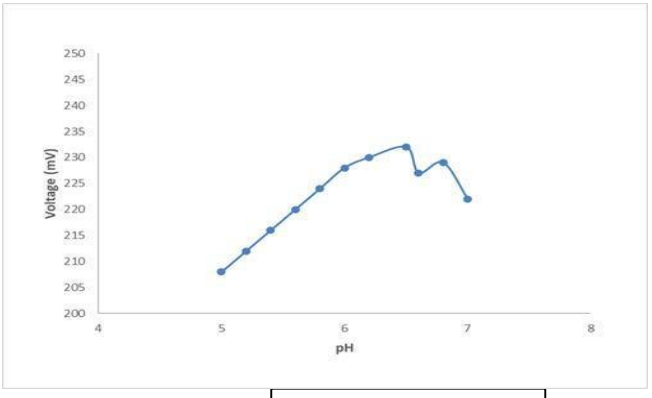


| Cholesterol added to sera (mM) | Cholestrol found in sera(mM) | Recovery(%) |
|---|---|---|
| 0 | 80 | 100 |
| 5 | 84.89 | 97.82 |
| 10 | 89.96 | 99.61 |
| 15 | 94.77 | 98.5 |
| 20 | 99.67 | 98.37 |

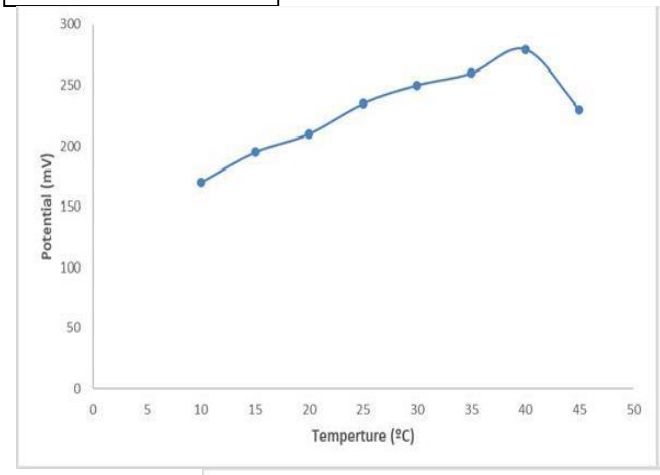
**Table: 2** Determination of analytical recovery of added Cholesterol in the serum samples spiked with known amount of Cholesterol using improved potentiometric biosensor as measured by CA/Cholesterol oxidase NPs/flourideISE.

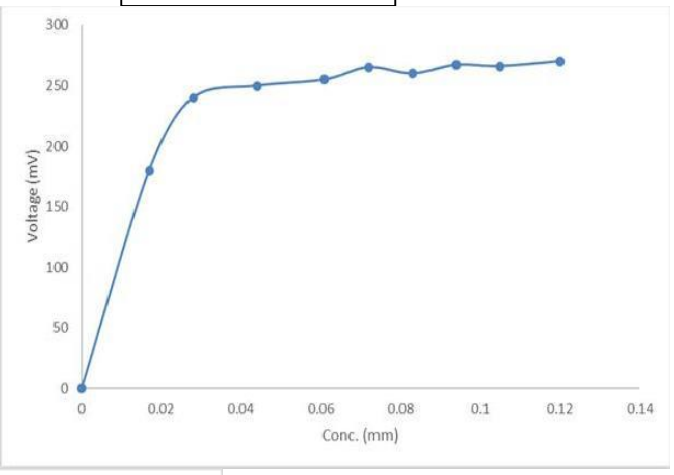


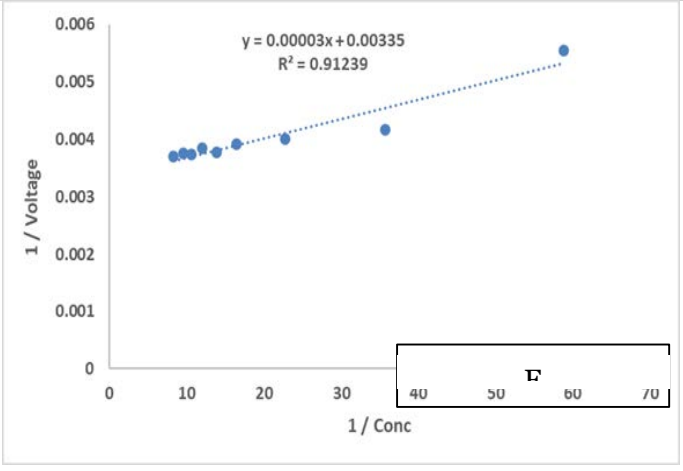

A




B




E



**Fig: 6** Based on the cholesterol oxidase NPs/CA membrane, the cholesterol biosensor's potential response is shown in Fig. 5(A). (B) Cholesterol Oxidase NPs/CA Membrane Response as a Cholesterol biosensor based on pH Effect. (C) Cholesterol Oxidase ChOx NPs/CA membrane-based cholesterol biosensor response to incubation temperature. (D) Cholesterol Oxidase NPs/CA membrane-based cholesterol biosensor response to cholesterol concentration. (E) LB plot showing the impact of cholesterol conc. on the response of the cholesterol biosensor, based on the

response of the Cholesterol oxidase NPs/CA membrane (D).

### 3.8.4 Precision

The measurement of cholesterol in the blood sera on the same day after and after 7 day of storage at 4°C resulted in within and between sample coefficients of variations (CVs) of 1.92% and 2.32%, respectively. These precise findings show that the current biosensor approach has high repeatability and consistency.

| N | Mean | CV% | SD |
|---|---|---|---|
| Cholesterol(mg/dL) | Within assay(S) | | |
| 173.60 | 170.86 | 1.93% | 3.3 |
| 170.98 | | | |
| 169.44 | | | |
| 166.00 | | | |
| 174.28 | | | |
| Between assay (S) | | | |
| 166.40 | 168.99 | 2.32% | 3.82 |
| 161.23 | | | |
| 169.56 | | | |
| 162.00 | | | |
| 160.80 | | | |

**Table: 3** within and between-assay coefficients of variations for determination of Cholesterol in serum as measured by CA/Cholesterol oxidase NPs/fluoride ISE.



### 3.8.5 Correlation

The cholesterol levels in the 20 blood serum samples were calculated using the current biosensor technique compared to those obtained using the Enzymic Colorimetric kit method, which is utilised in Hospitals, to ascertain the correctness of the biosensor. Regression analysis was used to link the results acquired using the two different methodologies. Correlation coefficient (CV) was determined. CV showed aa strong connection (R = O.9999) between the values for cholesterol calculated by the reference method and the current biosensor. Current biosensing approach for determining cholesterol in serum is more straightforward, sensitive, precise, and quick when compared to reference methods.

### 3.8.6 Storage stability of CA/Cholesterol Oxidase NPs/$Fl^-$electrode

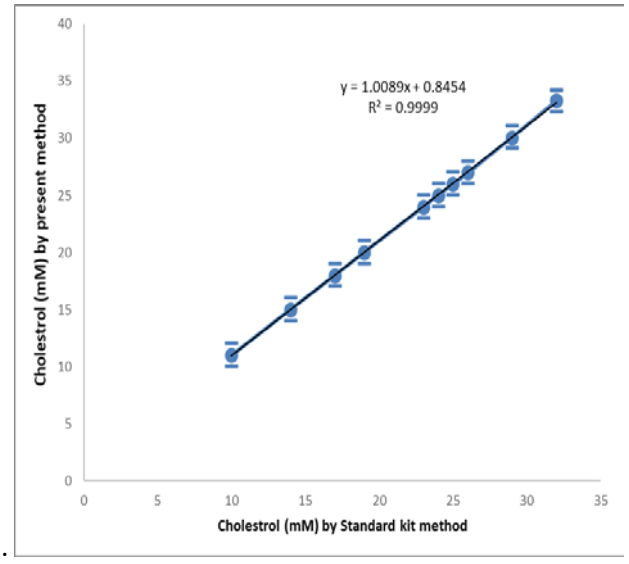
Upto 8–9 reuses, the enhanced cholesterol biosensor response measurement was 96% repeatable; however, after that, the membrane became saturated and had to be washed in DW before being reused 4-5 hours later. When kept in 0.5 M SPB at pH 7.0 at 4 °C, the ENPs-bound CA membrane was utilised for 160 days (Fig. 6B). When kept in a dry place (DW) at normal temperature, the FLISE electrode survived for 7 months. As a result, the shelf life of immobilised Cholesterol Oxidase was extended by the synthesis of cross-linked Cholesterol Oxidase NP aggregates.

### 3.8.7 Interference study

Some common cations and related metabolites were chosen to explore their effects on the response of the current biosensor at their physiological concentration. All of these interference investigations employed reaction buffer containing cholesterol at a final-concentration of 1.0mM. L-Glutathione and glutamine exhibited minimal interference with the biosensor, however L-Alanine and pyruvate caused some interference. This interference was reduced by using a particular ISE.

### 3.8.8 Potentiometric measurement of serum cholesterol

Serum cholesterol levels, as determined by the current biosensor, ranged from 12.5-90mg/dl in individuals who appeared to be in good condition and from 227 to 270 mg/dl in those who appeared to be suffering from heart disease. These numbers are similar to those previously reported for cholesterol biosensors.



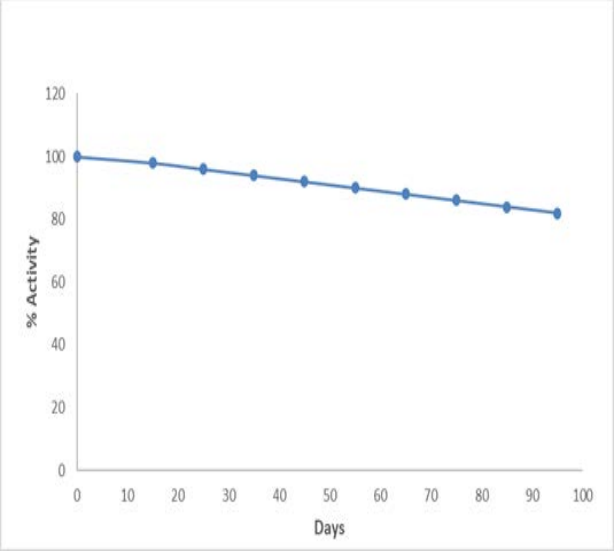



**Fig: 7** (A) shows the correlation between sera cholesterol readings measured using the traditional enzymatic kit approach and the current biosensor based on the cholesterol oxidase nanoparticles/CA membrane. Cholesterol Oxidase NPs/CA membrane storage stability at 4 °C.

## 4. Conclusion

Cholesterol Oxidase Nanoparticles aggregates were produced, analysed, and covalently immobilised onto CA membrane before being used to build a potentiometric Cholesterol biosensor with a Fluoride ion selective electrode FLISE. Biosensor demonstrated enhanced analytic capabilities in term of the sensitivity (-58 mV), excellent selectivity, expanded working range (0.003- 0.09mM), low detection limit(0.032µM), strong repeatability, great storage stability (160 days). It has been determined that using ENPs in the design Enzyme increases the analytical performance of sensors while also making them simpler to construct.

**References**

1. V.Rajesh,W.Bisht,W.Takashima,K.Kaneto,Biomaterials26(17),3683(2005)
2. G.G.Guilbault,J.G.Montalvo,J.AmChem.Soc1969,91,2164
3. Jiang Jingying, Ruikang K Wang. Comparing the synergistic effects of oleic acid and dimethyl sulfoxide as vehicles for optional clearing of skin tissue in vitro in Medicine and Biology 49(23), 5283, 2004
4. Nidhi Kundu, Sandeep Yadav and C.S. Pundir. Preparation and Characterization of glucose oxidase nanoparticles and their applications in dissolve oxygen metric determination of serum glucose J. Nanosci. Nanotechnology., 2012, 13, 1710-16
5. S. chawla, R. Rawal, C.S.Pundir. An electrochemical sulfite biosensor based on gold coated magnetic nanoparticles modified gold electrode Biosensors and Bioelectronics 31 (1), 144-150., 2013
6. S . Chawla, R Rawal, C.S. Pundir. An electrochemical sulfite biosensor based on gold coated magnetic nanoparticles modified gold electrode, Biosensors and Bioelectronics 31 (1), 144-150, 2015.
7. C.S. Pundir, R. Deswal, V. Narwal, J. Narang, Quantitative analysis of metformin with special emphasis on sensors: a review, Curr. Anal.

Chem. 13 (2017), https:// doi.org/10.2174/157341101366617 0907150509.
8. C.S. Pundir, Purification and properties of an oxalate oxidase from leaves of grain sorghum hybrid, Biochim. Biophys.Acta 1161 (1993) 1–5.
9. C. Pundir, V .Narwal, biosensing methods for determination of triglycerides : a review , biosens. Bioelectron.100 (2018) 214-227, https://doi.org/10.1016/j.bios.2017 .09.008.
10. V. Narwal, R. Deswal, B. Batra, V. Kalra, R. Hooda, M. Sharma, J.S. Rana, Cholesterol Biosens.: A review 143 (2019) 6–17
11. O. Lowery, N. Rosebrough, A. Fare, R. Randall, Int. J . Tissue React.(1951)
12. NikhilBhalla,PawanJollyandPedroustr ela.Thefirstpotentiometricenzymese nosorreported.,2016
13. M.S Thakur and Naikankatte G Karanth.Karanth.Adual enzyme amperometric biosensor for monitoring Organophosphorous pesticides in Biotechnology Teechniques 11(9): 653-655.,2013
14. Turner J,C et al. Rediscovering the social Group : A Self- Categorization Theory. Oxford and New YorkBlackwell., 1987
15. R. Bias, N. Potezny, J.B. Edward, A.M. Rofe, R. Conyer, R, oxalate determination by immobilized oxalate oxidase in a continuous flow system, Anal. Chem. 52 (1980) 508–511.
16. J. W. Baynes, M. H. Dominiczak, Medical Biochemistry, 2nd ed., Elsevier Mosby, Philadelphia, USA, 2005.
17. J. MacLachlan, A. T. L. Wotherspoon, R. O. Ansell, C. J. W. Brooks, Journal of Steroid Biochemistry and Molecular Biology 2000, 72,169.
18. M. Nauck, W. Marz, H. Wieland, Clinical Chemistry 2000, 46, 436.
19. B.Staels, Nature London 2002, 417, 699.
20. E. Ikonen, Nat. Rev. Mol. Cell Biol. 2008, 9, 125.
21. W. M. Sperry and F. C. Brand. J. Biol. Chem. 1943, 150, 315-324
22. T. Nakaminami, S. Kuwabata, H. Yoneyama, Anal. Chem. 1997, 69, 2367.
23. J. Bobacka, A. Ivaska, and A. Lewenstam. Chem. Rev. 2008, 108, 329-351.
24. Author links open overlay panelLi-Hua Li [a 1], Ewelina P. Dutkiewicz [b c 1], Ying-Chen Huang [b], Hsin-Bai Zhou [b], Cheng-Chih Hsu [b]https://doi.org/10.1016/j .jfda.2018.09.001