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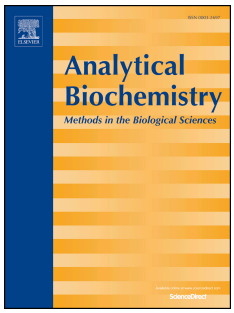

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Amperometric determination of serum total cholesterol with nanoparticles of cholesterol esterase and cholesterol oxidase

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Abstract

We describe the preparation of glutaraldehyde cross linked and functionalized cholesterol esterase nanoparticles(ChENPs) cholesterol oxidase nanoparticles(ChOxNPs) aggregates and their co-immobilization onto Au electrodefor improved amperometricdetermination of serum total cholesterol. The Transmission electron microscopic (TEM) images of ChENPs and ChOxNPs showed their spherical shape and average size of 35.4nm and 56.97nm respectively.Scanning electron microscopic(SEM) studies of Au electrode confirmed the co-immobilization of enzyme nanoparticles(ENPs). The biosensor exhibited optimum response at pH 5.5 and 40^0C within 5s, when polarized at +0.25 V versus Ag/AgCl. The working/ linear range of the biosensor was 10-700 mg/dl for cholesterol. The sensor showed high sensitivity and measured total cholesterol as low as 0.1 mg/dl. The biosensor was evaluated and employed for total cholesterol determination in sera of apparently healthy and diseased persons. The analytical recovery of added cholesterol was 90% while within and between batch of coefficient of variation (CV) were <2% &<3%. There was a good correlation(r=0.99) between serum cholesterol values as measured by standard enzymic colorimetric method and present method. The initial activity of ENPs/working electrode was dissipated by 50% during its regular use for 200 times over a period of 60 days, when stored dry at 4^0C.

KeywordsCholesterol, Bionanosensor, Cholesterol esterase nanoparticles,Cholesterol oxidase nanoparticles, Co-immobilization, Serum, Total cholesterol.

Introduction

Enzyme nanoparticles(ENPs) are the aggregated form of enzyme molecules in nanometer scale within the dimensions, 10-100nm. These ENPs exhibits unique optical, electrical, electronic, thermal, chemical and catalytic properties besides increased surface area.Direct immobilization of enzymes onto nanomaterials may cause its denaturation leading to loss of its activity. This problem was solved by aggregating the enzyme moleculesto form their nanoparticles (NPs) and cross linked within their self in controlled manner. This has resulted into promising strategies for development of improved biosensor in terms of limit of detection as well as current response [1].ENPs aggregates of few enzymes such as horse reddish peroxidase (HRP)[2], glucose oxidase (GOD) [3,4], uricase[5] and cholesterol oxidase (ChOx) [6] have been immobilized onto the surface of metal electrodes during the past one decade to construct improved biosensors for H_2O_2, glucose, uric acid and cholesterol, respectively.

Measurement of total cholesterol level in serum is very important, due to its presumed relationship to atherosclerosis&cardiovascular diseases, nephritis, myxedema, obstructive jaundice, diabetes mellitus and cerebral thrombosis [7].However, the recently reported cholesterol oxidase nanoparticles (ChOxNPs) based cholesterol biosensor, required the pretreatment of serum sample with 0.1ml cholesterylesterase(5mg/ml) at 37^0C for 10 min prior analysis, which complicates the procedure and renders it expensive and time consuming. This problem was overcome in the present work by co-electrodepositingcholesterol esterase nanoparticles (ChENPs) with ChOxNPs onto Au electrode.To the best of our knowledge, there has been no report on preparation of ChENPs and their co-immobilization/electrodeposition with ChOxNPsonto an electrode, so far. The co-electrodepositedenzymes form a single step procedure; hence activity is dependent on this step, as compared to multiple steps in case of individual enzymes. Further,co-electrodepositionprovides close proximity of enzymes, which lead to easy diffusion of intermediates between different enzymes in the reaction medium [8]. Thus, co-electrodeposition ofChENPs and ChOxNPsonto Au electrode areexpected to provide better ENPs electrode for total cholesterol determination.

Materials and methods

Reagents

4-Aminophenazone, HRP from Sigma Aldrich,phenol, cholesterol, ChOxfrom microorganism sp.;130 U/mg, cholesterol esterase (ChE) from porcine pancreas; 15 U/mg, triton X-100,glutaraldehyde, alumina slurry, chitosan, isopropanol and cholesteryl acetate from SRL Mumbai andethanolfrom ChangshuYangyuan Chemical, China were used. Gold wire (1.5 x0.05 cm) (23 carat) was purchased from the local market. All other chemicals were of analytical reagent (AR) grade. Double-distilled water (DW) was used throughout the experiments.

Instruments

Spectronic-20 D(Thermo Scientific, USA), All electrochemical experiments were performed on an Autolab PGSTAT 30 electrochemical workstation (Eco Chmie BV, The Netherlands) with the GPES 4.9 software,transmission electron microscope(TEM, JEOL 2100 F,Japan), scanning electron microscope (SEM, JEOL Japan, Model: JSM-6510) at Jawaharlal Nehru University, New Delhi).A modified Au electrode, a KCl-saturated Ag/AgCl electrode and a Pt electrode served as the working, reference, and counter electrodes respectively, in all electrochemical experiments. All currentresponseswere measured at room temperature against Ag/AgCl reference electrode. Double distilled water(DW) was used throughout the experiment. TEM and SEM images of ENPs and ENPs modified electrode were taken at Advanced Instrument Research Facility(AIRF) of Jawaharlal Nehru University New Delhi.

Dissolution of enzymes

Both ChE and ChOx were dissolved individually in 0.02 M sodium phosphate buffer (PB) (pH 7.0) at a concentration of 1.0 mg/ml and stored at 4 ^{0}C until use.

Assay of ChE

The assay of ChEwas carried out according to [9] with some modifications. The reaction mixture containing 1.7ml PB (0.05M, pH 7.0) containing 50 mmol/L tauro-deoxycholate, 0.1 ml ChEsolution and 0.1 ml ChOx solution was pre-incubated at 37 ^{0}C for 2 min. The reaction was started by adding 0.1 ml cholesteryl acetate solution (500 mg/dl). 1.0 ml color reagent was added and the reaction mixture was kept in dark at 37 ^{0}C for 10 min to develop the color.A_{520} of the color was read against control in Spectronic -20 and the content of H_2O_2 generated in the reaction was calculated from standard curve between H_2O_2concenteration vs. A_{520}.One unit of ChE is defined as the amount of enzyme protein required to generate 1.0

µmole of H_2O_2/min/ml. This $H_2O_2$was generated from oxidation of cholesterol by ChOx, which in turn produced from hydrolysis of cholesteryl acetate by ChE.

Preparation of cholesteryl acetate solution:

Fifty mg of cholesteryl acetate was dissolved in 1.0 ml of Triton X-100 by slowly heating and stirring, until the solution was clear. PB(0.05 M, pH 7.0) was added to get a final concentration of 50mg/dl and stored at 4^0C until use[10].

Preparation of color reagent:

The color reagentwas prepared by dissolving 50 mg 4-aminophenazone, 100 mg phenol, and 1.0 mg HRPin 100 ml 0.4 M PB (pH 7.0) and stored inan amber colored bottle at 4^0C and prepared fresh every week.

Assay of ChOx

Assay of ChOx was carried out in 15 ml test tube wrapped with black paper according to [9]with modification. The reaction mixture, consisting of 1.8 ml PB (0.05 M, pH 7.0) containing 0.4% triton X-100, 0.1 ml of cholesterol solution, and 0.1 ml of ChOx solution incubated for 10 min at 37 ^{0}C. Color reagent (1.0 ml) was added and kept at 37 ^{0}C for 15 min to develop the color. A_{520} was read and the content of H_2O_2 was interpolated from standard curve between H_2O_2 concentration and A_{520}.

One unit of ChOx is defined as the amount of enzyme protein required to generate 1µmole of H_2O_2 from cholesterol/min/ml.

Preparation and characterization of ChENPs:

Nanoparticles of ChEwere prepared separately by desolvation with ethanol and subsequent cross-linking with glutaraldehyde[4]. Firstly, 2 ml enzyme solution (2mg/ml) was transformed into ENPs by pipetting continuously 4 ml of desolvating agent, i.e. ethanol into 2 ml enzyme solution under continuous stirring at 500 rpm andat a dropping rate of 0.1ml/min. Afterdesolvation process, 1.8 ml of 2.5 % glutaraldehyde was added to these ENPs, and the suspension was stirred for 24 h, to ensure the cross linking of ENPs and thus forming ENPs. The resulting ENPs were purified by three cycles of differential centrifugation (14000xg. 10 min) at 4 ^{0}C and pellet was redispersed to the original volume in DW. Each redispersion step was performed in an ultrasonication bath over 5 min. Amino ($–NH_2$) groups were introduced

on ENPs by adding 0.12 g of cysteamine di-hydrochloride with constant stirring for 5-6 h. At last, functionalized ENPs were separated from free enzyme solution by centrifugation at 1200 rpm at 4 ^{0}C for 10 min, followed by its dispersion in 0.1M PB (pH 7.0)and stored at 4^{0}C.The shape and size of ChENPs was studied by taking their images in Transmission Electron Microscopeat Advanced Instrumentation Facility, Jawaharlal Nehru University, New Delhi on commercial basis.

Preparation and characterization of ChOxNPs

NPs of ChOx were also prepared and characterized as described above for ChENPs

Co-electrodeposition of ChENPs+ChOxNPs on AuElectrode: Construction of enzyme electrode

Prior to the surface modification, Au wire was cleaned with piranha solution (H_2SO_4:H_2O in 3:1 ratio) for 20 min. and then rinsed thoroughly with DW. The cleaned Au electrode was then polished with silica slurry. The mixture of ChENPs + ChOxNPs suspensionin1:1ratio, was electrochemically deposited onto polished Au electrode by cyclic voltammetry (CV) by immersing it into a mixture of 23 ml 2.5 m$MK_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1) and 2 ml of ChENPs + ChOxNPs mixture and then applying potential between -0.2 to +0.6 V (vs. Ag/AgCl) for 20 cycles at a scan rate of 20mVs^{-1}.ChENPs + ChOxNPs/Au electrode was rinsed with DW and then dried at room temperature.

Construction and response measurement of cholesterol biosensor:

An amperometric biosensor for total cholesterol wasconstructed by connecting ChENPs+ChOxNPs/Au electrode,as working electrode with an Ag/AgCl (saturated 3 M KCl) electrode as the reference electrode and Pt wire as auxillary electrode through potentiostat. All electrochemical characterization and measurements werecarried out using this three-electrode system. Cyclic voltammetric measurements were carried out in a three electrode cell containing 25 ml PB (0.1 M, pH 7.0) and 0.1 ml of cholesterol (50mg/dl). Current measurements were performed applying CV in the potential range, +0.1 and +1.0 V. To record cyclic voltammograms, the following instrumental parameters were used:Step potential +1 mV and scan rate20mVs^{-1}. All electrochemical measurements were carried out at room temperature.

Optimization of biosensor

Various kinetic properties of ENPs/working electrode were studied such as optimum pH, incubation temperature,response time and effect of cholesterol concentrations. To study optimum pH,the pH of reaction buffer (0.1M) was varied from pH 4.0 to 8.0 at an interval of 0.5 pH using sodium acetatein thepH range 4.0-5.5 and PBin the pH range 6.0-8.0. Similarly,for optimumincubation temperature, the reaction mixturewas incubated at different temperature ranging from 20-50^{0}C at an interval of 5.0 ^{0}C. The effect of cholesterol concentration by using cholesterol solution of different concentration in the range:Lower range: 0.1 to 10.0 mg/dl and higher range:10-700 mg/dl.

Determination of total cholesterol in serum

Intravenous blood samples (1 ml each) from apparently healthy and diseased individuals suffering from various hypercholesterolemia such as atherosclerosis, nephritis, diabetes mellitus, myxedema and obstructive jaundice individuals, both male and female, of different age groups were collected from local hospital of Pt. BDS University of Health Sciences and centrifuged at 5000 rpm for 5 min, and the supernatant(serum) was collected. The present biosensor was employed for determination of total cholesterol in the serum samples. The determination of cholesterol were carried out in the same manner as described for testing of cholesterol biosensor under optimal working conditions except that the cholesterol solution was replaced by serum. The concentrations of total cholesterol in sera were extrapolated from standard curve between cholesterol concentration(100-700 mg/dl) and current in µA, prepared under optimal assay conditions of ChOxNPs+ChENPs modified Au electrode.

Results and discussion

Characterization of ChENPs and ChOxNPs by TEM

TEM images of ChENPs and ChOxNPs showed their spherical shape, with diameter in the range of 10-100 nm (average 35.4nm and average 56.97nm respectively), confirming the formation of NPs(Fig. 1 &2). The size of ChOxNPs was similar to our earlier report[6].

Characterization of ChENPs and ChOxNPs by SEM

Fig.3(a)and 3(b) showed surface morphology of bare Au electrode and ChENPs+ChOxNPs/Au electrode respectively, as recorded by SEM. The SEM of bare gold electrode showed smooth surface while Fig. 3(b) showed globular surface morphology confirming the co-immobilizationof enzyme electrode.

Optimization of the biosensor

The optimum working conditions of the biosensor were studied in terms of effect of pH, incubation temperature, response time and substrate(cholesterol) concentration. The optimum pH was obtained at pH 5.5(Fig.4), which is lower than that of earlier reported cholesterol biosensor(Table1). The optimum temperature was at 40^0C(Fig.5), which is higher than earlier reported cholesterol biosensor, but lower thangraphene nanoparticles based biosensor (Table1). Hence, the subsequent experiments were carried out at pH 5.5 and 40^0C.The biosensor response in terms of maximum current was recorded within 5s, which is lower than earlier biosensor(Table1). There was a linear relationship between biosensor response and cholesterol concentration in the lower concentration range0.1-10mg/dl and higher concentration range, 10-700 mg/dl(Fig. 6).

Evaluation of biosensor

The following criteria was studied to evaluate the performance of present biosensor e.g. linearity, analytical recovery, detection limit, precision and correlation with standard method.

Detection limit

The detection limit (S/N=3) of the present biosensor was 0.1mg/dl , which is better/lower than earlier reported detection limits for enzymes immobilized onto ZnO (0.37 mg/dl) [11], ChOxNPs modified Au electrode (1.56 mg/dl)[6] carbon electrode (2.14 mg/dl)[15], alkylamine glass beads (5 mg/dl)[13], graphene nanoparticles (12.1 mg/dl)[17] , but equal to [Poly(3-TPAA)](0.1 mg/dl)[12] and higher than alkylamine glass beads(0.02 mg/dl)[14], MWCNTs (0.01 mg/dl)[16].

Linearity

There was a linear relationship between current (μA) and cholesterol concentration ranging from 0.1 to 10.0mg/dl and 10.0-700 mg/dl for the present biosensor, which is better than earlier biosensors based on ChOx immobilized onto ZnO(0.3-19.3 mg/dl)[11], [Poly(3-TPAA)] (0-0.3 mg/dl) [12], alkylamine glass beads (5-50 mg/dl)[13], alkylamine glass beads (0.7-7.7 mg/dl)[14], carbon electrode (2.0-11.0 mg/dl)[15], MWCNTs (0.3-3.8 mg/dl) [16], graphene nanoparticle (5.21-12.1 mg/dl)[17],Au electrode (12.5-700 mg/dl)[6].

Analytical recovery

In order to check the accuracy of the methods, the analytical recovery of added cholesterol in the serum samples was determined. The mean analytical recovery of added cholesterol (100 mg/dl and 200 mg/dl) in serum (n=5.0) was 94.73±0.92% and 96.33±0.73% for present electrode, which shows the high reliability of the biosensor.

Precision

To check the reproducibility and reliability of the methods, the cholesterol content of the sample in one run(Within batch) and after storage at -20^{o}C for one week (Between batch) were determined. The results showed that the cholesterol value of these determination agreed with each other and within batch and between batch coefficient of variation (CV) were <2% &<3% for present biosensor revealing itshigh reproducibility and repeatability.

Accuracy

In order to know the accuracy of present methods, the values of cholesterol in 10 serum samples were determined by commercial enzymic colorimetric method (x) and compared with those obtained by present methods(y). The serum cholesterol values obtained by biosensor employing present electrode showed a good correlation (r= 0.99) using regression equation(Fig.7)

Amperometric determination of total serum cholesterol

The total cholesterol content of sera of apparently healthy adults and persons suffering from various hyper cholesterolimic diseases in different age groups as determined by the present biosensor were in the range 156.64 to 224.69 mg/dl for male and 140.29 to 220.37 mg/dl for female and 240.08 to 350.67 mg/dl for male and 240.56 to 345.98 mg/dl for females respectively. Our serum total cholesterol values in apparently healthy adultswere in the normal established range.

Reuse and Storage of ChOxNPs+ChENPs/Au electrode

The electrode was used 200 times over a period of 3months, without any considerable loss of activity,when stored dry at 4^{0}C. This stability and reusability of the present biosensor is better than earlier reported biosensorbased on ChOxNPs(150 times, 50 % loss in 90 days)[6], carbon electrode[15] (70 days), MWCNTs (54 days)[16].

Conclusion The use of co-electrodeposited cholesterol esterase nanoparticles and cholesterol

oxidase nanoparticles onto Au electrode has resulted into improved amperometric cholesterol biosensor in terms of detection limit, working range and storage stability and reusability. Thus co-electrodeposited enzyme nanoparticles could be employed for improvement of other amperometricbiosensors.

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Table 1. A comparison of various analytic parameters of electrochemical cholesterol biosensors

Support used	Method of immobilization	Optimum Temperature (°C)	Optimum pH	Linearity (mg/dl)	Detection limit (mg/ dl)	Reference
ZnONPs	NR	25	6.8-7.6	0.319-3.0	0.37	[11]
[Poly(3-TPPA)]	Covalent	25	7.0	0-0.3	0.1	[12]
Alkylamine glass beads	Covalent	37	7.0	5.0-50	5.0	[13]
Alkyl amine glass beads	Covalent	32	6.6	2.0-11.0	2.14	[14]
Carbon electrode	Physical adsorption	25	7.0	0.7-7.7	0.02	[15]
MnO_2NPs/MWCNT	Physical adsorption	37	7.4	0.3-3.8	0.01	[16]
Graphene oxide nanoparicles	Physical adsorption	50	6.0-7.1	5.21-12.1	12.1	[17]
Au electrode	Physical adsorption	35	6.0	12.5-700	1.56	[6]
Au electrode	Physical adsorption	40	5.5	10.0-700	0.1	Present

ZnONPs: Zinc oxide nanoparticles; MnO_2NPs: Manganese oxide nanoparticles; Poly (3-TPAA): ChOxNPs; Cholesterol oxidase nanoparticle.

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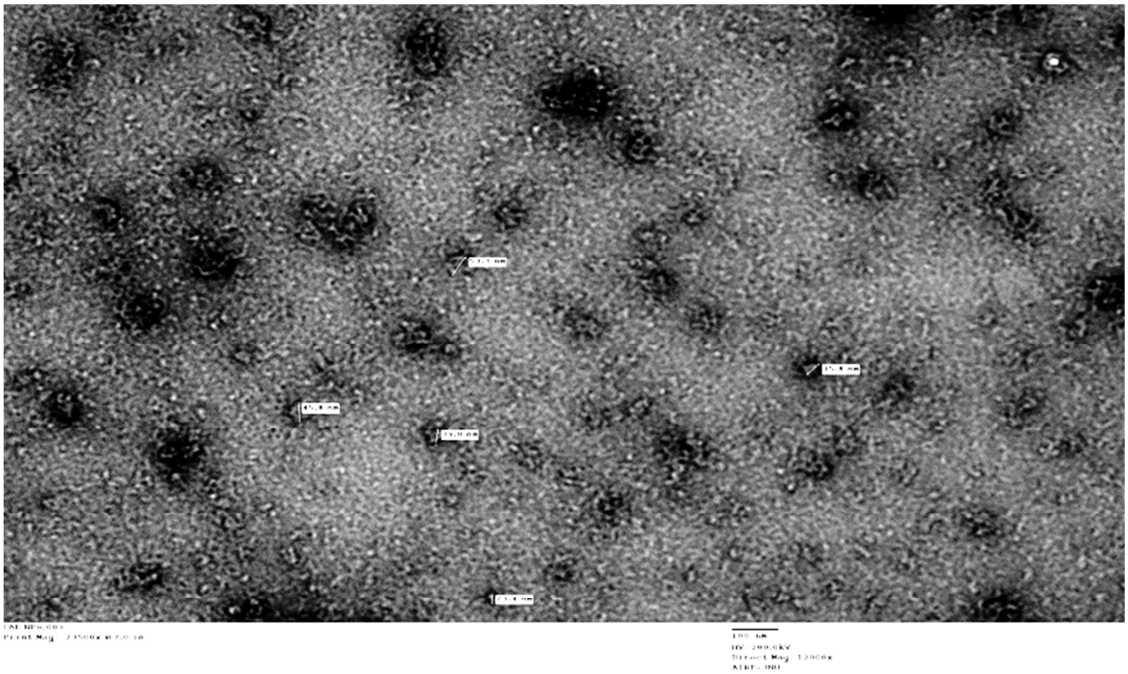

Fig. 1: TEM images of ChENPs

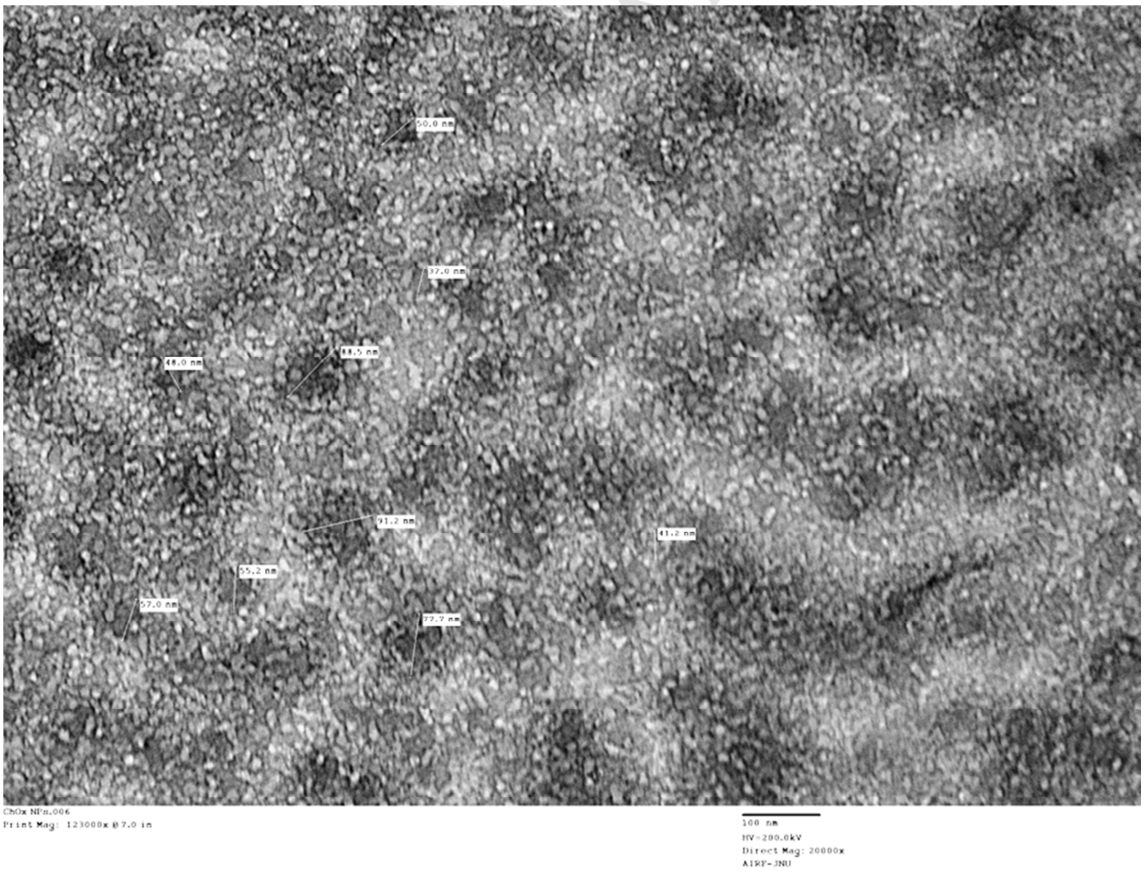

Fig.2: TEM images of ChOxNPs

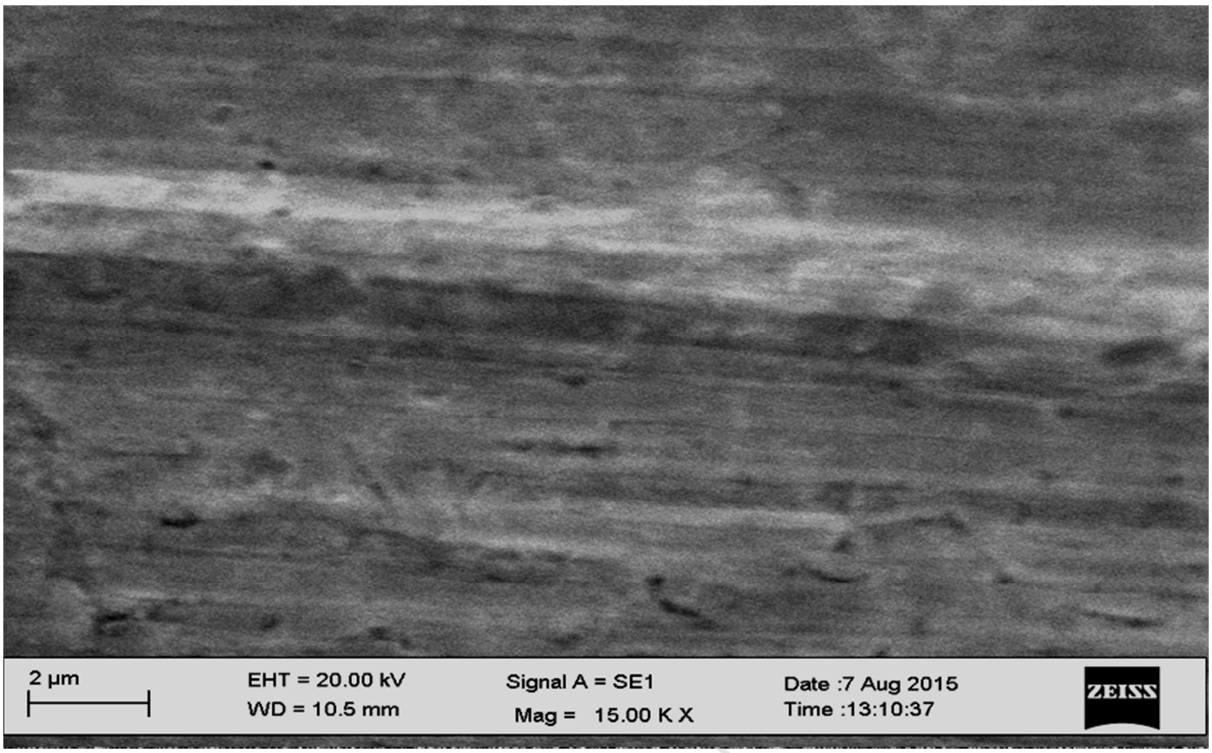

Fig. 3 (a): SEM images of bare electrode

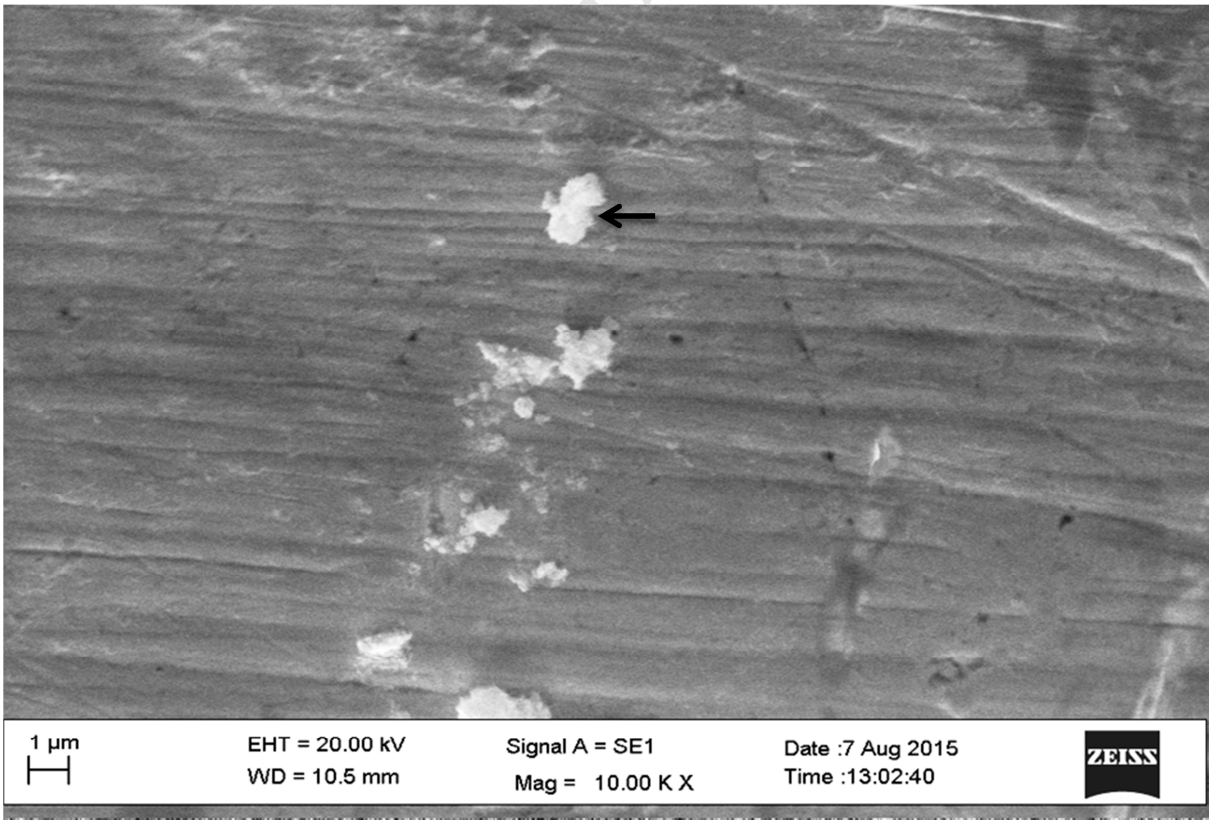

Fig. 3 (b): SEM images of Au electrode with immobilized ChENPs+ChOxNPs aggregates

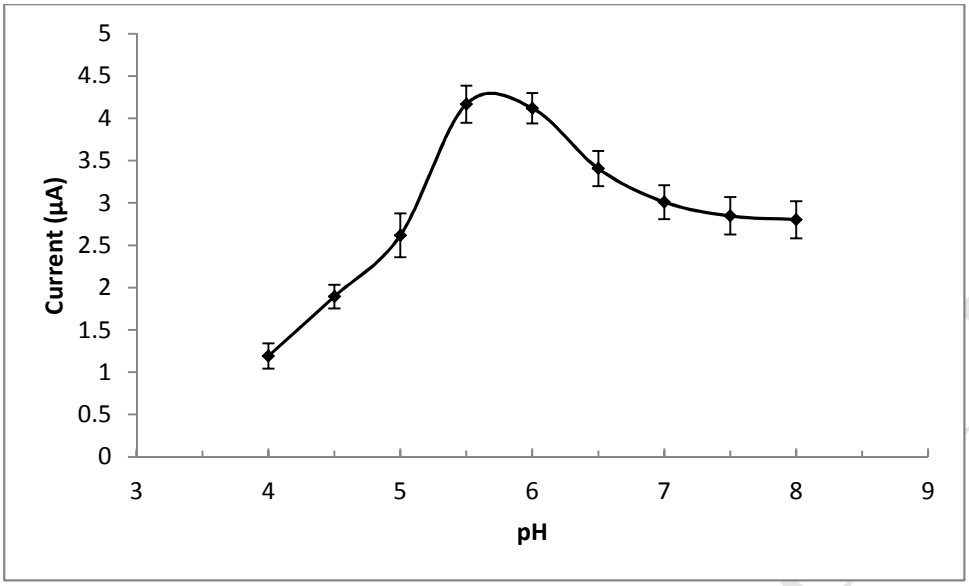

Fig. 4: Effect of pH on the current response of ChENPs+ChOxNPs modified Au electrode.

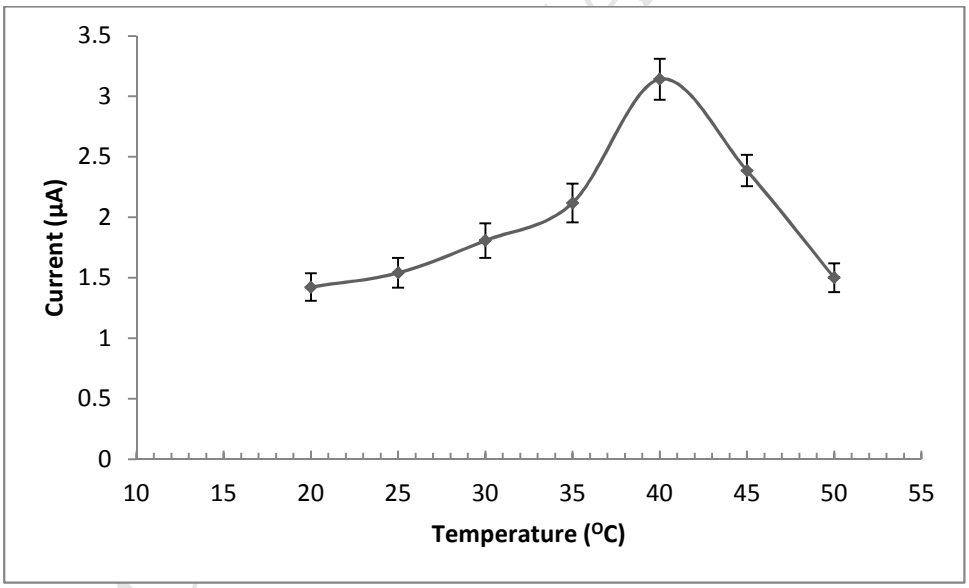

Fig. 5: Effect of temperature on current response of ChENPs+ChOxNPs modified Au electrode

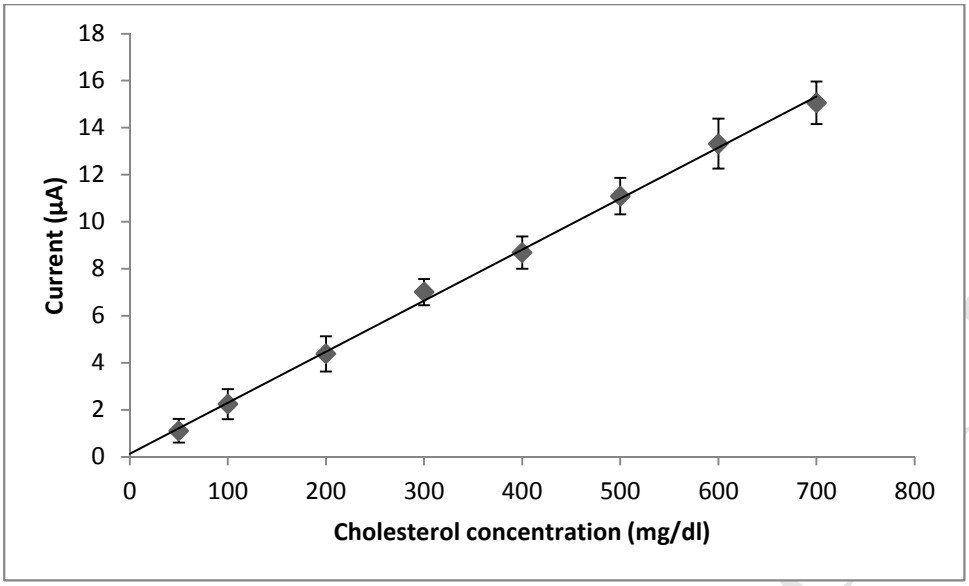

Fig. 6: Effect of cholesterol concentration on the current response of the fabricated cholesterol biosensor based on ChENPs+ChOxNPs/Au electrode

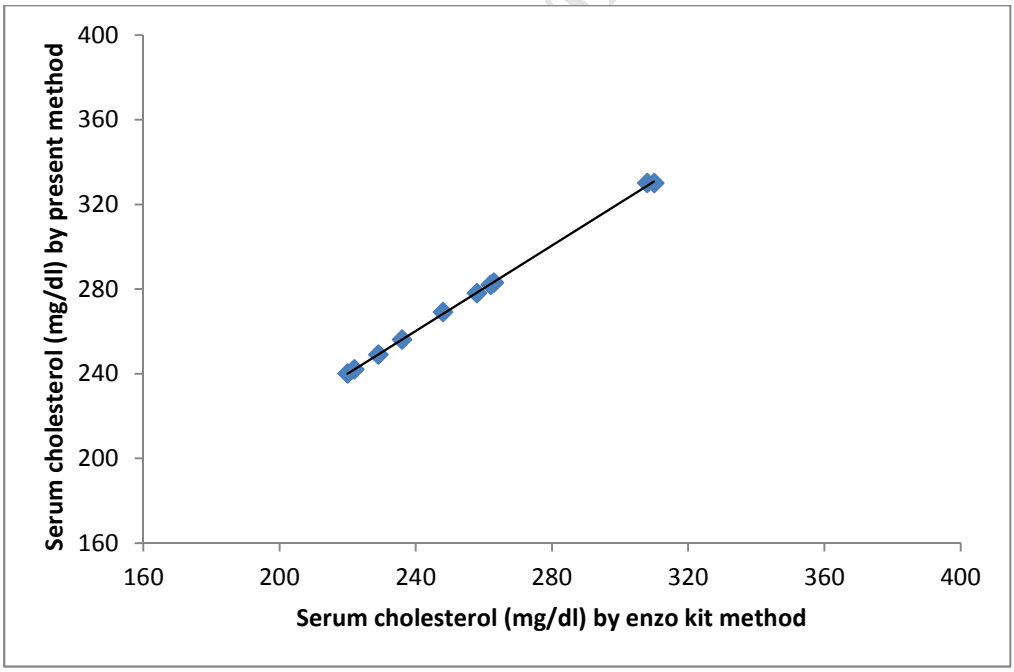

Fig. 7: Correlation between serum cholesterol value as determined by enzo kit method employing soluble enzymes (x axis) and present biosensor method (y axis) based on ChENPs+ChOxNPs/Au electrode.