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Enhanced electrocatalytic properties of Co₃O₄ nanocrystals derived from hydrolysed polyethylenimine in water/ethanol solvents for electrochemical detection of cholesterol

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Abstract

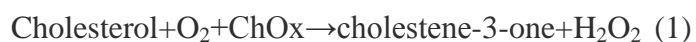
Developing of simple, low cost, sensitive, and selective cholesterol biosensors are essential for accurate monitoring of cholesterol to avoid the cardiovascular diseases. The present study describes the effect of hydrolysis of polyethylenimine in water/ethanol mixture on the morphology of the cobalt oxide (Co_3O_4), used as the main sensor component. The structure of the generated Co_3O_4 nanocrystals is consistent with a well-defined cubic phase crystallography, having only cobalt and oxygen elements. These nanocrystals exhibit large surface suitable for facile and high loading of cholesterol oxidase enzyme through physical adsorption method. Then the fabricated cholesterol oxidase/ Co_3O_4 nanocrystals composite was implemented for potentiometric detection of cholesterol in 10 mM phosphate buffer of pH 7.3. Importantly, the presented cholesterol biosensor revealed a wide linear range of 0.005 mM to 3.0 mM with a limit of detection (LOD) of 0.001 mM. Additionally, the sensitivity of biosensor was estimated around 60 mVdec^{-1} . The selectivity, stability, reproducibility and repeatability were also observed satisfactory. The dynamic response of proposed method demonstrated a fast response time of less than 1 s. Furthermore, the successive addition method confirmed remarkably stable response towards various cholesterol concentrations. Thus, the developed cholesterol oxidase/ Co_3O_4 nanocomposite may be used as an efficient alternative method to monitor low cholesterol concentrations from real samples.

Keywords: Cholesterol biosensor, Co_3O_4 nanocrystals, polyethylenimine, phosphate buffer solution

1. Introduction

Cholesterol represents an essential class of sterol that found in human body, particularly in the liver [1]. The function of cholesterol is to regulate bile acids, vitamin D and glucocorticoids including estrogen and progesterone. The presence of 70% of cholesterol in the human blood as an ester and approximately 30% is free cholesterol content in the human body, thus confirming the total concentration of cholesterol [2]. The physiological level of cholesterol in the human blood would be lower than 5.2 mM and the cholesterol concentration of 6.2 mM is

indicating the symptom of hypercholesterolemia. These high levels of cholesterol are causing the cardiovascular diseases, myocardial infarction and atherosclerosis. However, a low level of cholesterol known as hypercholesterolemia and becomes a cause of anemia and hepatopathy [3, 4]. Therefore, it is very essential to monitor the cholesterol level in order to avoid the health complications in the human body. The cholesterol has been determined by different analytical techniques including high performance liquid chromatography (HPLC) [5], colorimetry [6], spectrophotometry [7] and electrochemical routes [8]. The electrochemical techniques are more favorable for the cholesterol analysis due to their fast response, detection of cholesterol in low volume with acceptable stability and reproducibility. Several electrochemical biosensors have been designed for the detection of cholesterol using enzymatic approach [9]. The use of cholesterol enzyme enables the high sensitive and wide linear range detection of cholesterol concentration.



The oxidation of cholesterol in the presence of oxygen and cholesterol oxidase is resulting cholestene-3-one and H_2O_2 . The quantified amount of H_2O_2 is giving the indirect information about the concentration of cholesterol. Irrespective of high sensitivity of amperometric methods, they have a limitation of interference caused by vitamin C and uric acid at high anodic potential during the cholesterol determination [10]. Beside this, potentiometric method does not use any high potential it completely prevents the interference effect from ascorbic acid and uric acid during the sensing of cholesterol. The potentiometric method requires a highly active surface for the immobilization of cholesterol oxidase and consequently a strongly output potential should be produced. Further to add, the potentiometric method is capable to work within the human body cell for the monitoring of cholesterol level, therefore this electrochemical method is more suitable for the real biological environment [11]. A significant progress in the development of cholesterol biosensors has been made recently. However, there is still an ample space to design and fabricate fast and efficient electrochemical cholesterol biosensors via introducing of favorable nanostructured materials, which can act as an excellent host surface for the immobilization of cholesterol oxidase. The low dimension materials have proved to be very fast in response time, high selectivity and sensitivity towards the development of functional sensing devices [12]. The functional properties of nanostructured materials are mainly governed by the morphology, composition and size [13, 14, 15-19]. Among the various nanostructured materials,

cobalt oxide (Co_3O_4) is widely studied due to its local and tunable electronic properties. Moreover, the spinel structure of Co_3O_4 is allowing it to act as a notable material for the fabrication of novel electrochemical devices [20]. Therefore, Co_3O_4 has been utilized in different applications like heterogeneous catalysis, and electrochemical devices [21-27]. Numerous morphologies of Co_3O_4 have been prepared including nanowires/nanorods [28], nanotubes [29], nanocubes [30], nanospheres [31], and nanoplates [32]. These nanostructures were prepared by various growth methods. Importantly, Co_3O_4 exhibits a high isoelectric point value of approximately 8 and it can be increased at high temperature [33]. The high isoelectric point value allows the Co_3O_4 to be an excellent host material for the immobilization of enzymes/antibodies via strong electrostatic forces. The low isoelectric point value of cholesterol oxidase of 4.6 is revealing a strong bonding with the nanostructured surface of Co_3O_4 [18]. The oxidation reaction cholesterol substrate is slow, and it should be accelerated when enzyme based approach is used to determine cholesterol.

The proper immobilization of cholesterol oxidase onto desired metal-oxide surface could lead to a massive increase in the sensitivity, swiftness and selectivity towards cholesterol detection. For this purpose, we have produced nanostructures of Co_3O_4 using mixed solution of polyethylenimine in ethanol/water mixture. There were two aspects of using this approach for the growth of Co_3O_4 nanostructures: the first, polyethylenimine provided the additional amount of amine groups required for binding of cobalt ions during the growth in addition to the hydroxyl groups. Second, it controlled the growth kinetics of Co_3O_4 due to the use of organic/aqueous bisolvent system, thereby resulting in a well-defined morphology. To the best of our knowledge, using of Co_3O_4 nanocrystals for the development of sensitive and fast cholesterol biosensor has not been reported in the literature.

In this study, we have prepared nanocrystals of Co_3O_4 using polyethylenimine as an additional hydrolyzing agent during the wet chemical methods. These Co_3O_4 nanocrystals were employed as a host material for loading of cholesterol oxidase via electrostatic attraction and the resultant cholesterol oxidase/ Co_3O_4 nanocomposite was successfully used for the development of potentiometric biosensor. The linear range of proposed method was found between 0.005 mM to 3 mM with a Nernstian slope of 60 mV per decade. The limit of detection (LOD) was estimated about 0.001 mM. The selectivity and stability were also studied. The method is highly reproducible, repeatable and fast in response time. The overall obtained results attest that the

proposed cholesterol biosensor can be considered as an alternative tool for practical utilization in real samples.

2. Materials and methods

2.1. Used chemical reagents

Urea, cholesterol oxidase derived from *Streptomyces* sp. (≥ 20 units/mg), cholesterol, cobalt chloride hexahydrate, polyethylenimine, D-(+)-Glucose monohydrate (99.5%), L-ascorbic acid (99%), uric acid(99%), ethanol (99.8%), and 1-propanol (99.9%) were received from Sigma Aldrich Karachi, Pakistan. The deionized was employed as a solvent medium. The stock solution of cholesterol was made in 10 mM phosphate buffer solution of pH 7.3 by mixing certain amount of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), potassium dihydrogen phosphate (KH_2PO_4 99.9%), sodium chloride (NaCl 99.9%) and potassium chloride (KC 99.0%l in the deionized water. The pH of buffer solution was adjusted with 0.1M sodium hydroxide (NaOH 98%) and 0.1M hydrochloric acid (HCl 37%). The obtained chemicals were of high analytical grade and used without pretreatment.

2.2. The preparation of nanocrystals of Co_3O_4 by hydrothermal process

Prior to the synthesis of nanocrystals of Co_3O_4 , we have fabricated gold coated glass electrode according to our previous study [34]. Briefly, the cleaned glass substrates were kept in Evaporator machine, then at high vacuum the chromium metal of 10 nm were deposited as adhesive layers followed by the deposition of 100 nm thickness of gold layer. Afterwards, the nucleation processing for the deposition of nanocrystals of Co_3O_4 on the cleaned gold coated substrate was carried out according to the reported work [35]. The precursor solution was prepared by the dissolution of 0.1M cobalt chloride hexahydrate and 0.1M urea in 90 mL of deionized water. Then, 50 mg of polyethylenimine was dispersed in 10 mL of ethanol and sonicated for 20 minutes until a homogeneous dispersion was achieved. Later, the dispersed solution of polyethylenimine was added to cobalt precursor. The, seed layer coated gold coated glass substrate was vertically hanged in the beaker by exposing the seeded face towards precursor solution and the beaker was gently sealed and kept at 95 °C for 4 hours in electric oven. After the growth process, a pink product of cobalt hydroxide was deposited on the gold coated glass substrate and it was washed with deionized water followed by ethanol treatment. The cobalt hydroxide product was transformed into nanocrystals of Co_3O_4 by thermal

decomposition at 400 °C for 4 hours in air. The pure sample of Co₃O₄ nanostructures was synthesized in the absence of polyethylenimine by same process. Scheme 1 shows the detailed synthesis process. The crystalline phase of synthesized Co₃O₄ nanostructures was studied by powder X-ray diffraction (XRD) using source of X-rays as CuK α radiation ($\lambda = 1.5418 \text{ \AA}$) at 40 kV and 40 mA. The morphology and composition of Co₃O₄ nanostructures were investigated by low resolution scanning electron microscopy (SEM) at an accelerating voltage of 10 kV and energy dispersive spectroscopy (EDS). The biosensor signal as function of potential was measured via pH meter METTLER TOLEDO and the dynamic and successive addition stepwise responses were measured through the potentiostat supplied from Netherland. The impedance data were collected through electrochemical impedance spectroscopy using a frequency values from 100 KHz to 10 Hz at sinusoidal potential of 10 mV and zero bias potential in 0.5mM cholesterol solution prepared in 10 mM phosphate buffer solution of pH 7.3. The raw impedance data was analyzed through Z-view software with fitted equivalent circuit.

2.3. The use of physical adsorption for the immobilization of cholesterol oxidase onto nanocrystals of Co₃O₄

The physical adsorption was used for the immobilization of cholesterol oxidase onto nanocrystals of Co₃O₄ as described under. A 3 mg of cholesterol oxidase were added to 5 mL of 10 mM phosphate buffer solution of pH 7.3 and a 100 μ L of cross linker 5% glutaraldehyde was also added. Then, dip coating in the enzyme solution was carried out on the nanocrystals of Co₃O₄ for several times followed by drying at 25 °C. The potentiometric cell set up was based on two electrode configuration using enzyme immobilized nanocrystals of Co₃O₄ as working electrode and silver-silver chloride (Ag/AgCl) filled with 3.5 M KCl as reference electrode. A 10 mM concentration of cholesterol was prepared in the phosphate buffer solution of pH 7.3 and 1mL of 5% Triton solution. The triton was used as solubility agent for the cholesterol. Prior to the dissolution of cholesterol into buffer solution, it was solubilized with 2mL isopropanol at 55 °C. The dilution method was used to make fresh low concentration solutions of cholesterol in buffer solution of pH 7.3 before the potentiometric measurements. The desired concentration of interfering species was also prepared in 10 mM phosphate buffer solution of pH 7.3. The output potential experiments of presented cholesterol biosensor were done at 25 °C.

3. Results and discussion

3.1. Crystalline, composition and morphological implications

The powder XRD was employed to describe the crystallography of nanocrystals of Co_3O_4 prepared by hydrothermal method as shown in Figure 1. The pure sample of Co_3O_4 is well characterized by cubic phase as shown in Figure 1a and the diffraction patterns of sample are well matched with the standard card no: 01-080-1539. The nanocrystals of Co_3O_4 prepared in the presence of polyethylenimine were also studied by XRD technique and the observed diffraction patterns are enclosed in Figure 1b. The reflections were slightly shifted towards higher 2 theta angle which could be assigned to the long chain species of hydrolyzed product and polyethylenimine, and they might produce a stress during the crystal formation. The intensity of XRD diffraction patterns is indicating high quality of nanocrystals of Co_3O_4 prepared in this study. The XRD study has not shown any other phase or impurity in the prepared Co_3O_4 . The chemical composition in terms of elements was also studied by EDS, confirming the presence of only Co and O as the main elements in the prepared samples of Co_3O_4 as shown in Figure 2a-b. The EDS and XRD results are revealing the pure phase of prepared nanostructures of Co_3O_4 .

The morphology of pure Co_3O_4 and the polyethylenimine assisted Co_3O_4 was investigated by SEM as shown in Figure 3. The pure Co_3O_4 is defined by the self-assembly of big particles and shaping a platelet like structure as shown in Figure 3a,b. The size of particles is around 200 nm and the length of platelets is seen several microns. An addition of polyethylenimine dispersion in ethanol in the precursor of cobalt oxide has shortened the size of particles and the morphology of Co_3O_4 seems close to nanocrystals as shown in Figure 3c,d. The possible role of polyethylenimine might be the provision of additional nucleates which arranged the crystal to grow in the shape of nanocrystal. Also, the particles of Co_3O_4 are well controlled using

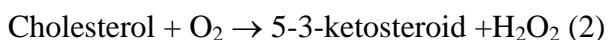
polyethylenimine due to slow down of growth kinetics and enable the crystal to grow slowly and properly as shown in Figure 3 c,d. The nanocrystals of Co_3O_4 are associated with large surface area and making a better contact with electrode, thus generating a strong output potential response. The large surface area allows the cholesterol oxidase to be heavily hosted by the Co_3O_4 nanocrystals through strong electrostatic interaction, thus proved itself a sensitive transducer for the generation of output signal and strongly favored the catalytic reaction of cholesterol oxidase towards the oxidation of cholesterol.

3.2. Potentiometric performance of cholesterol oxidase immobilized Co_3O_4 nanocrystals towards cholesterol determination

The presented cholesterol biosensor based on cholesterol oxidase immobilized Co_3O_4 nanocrystals was evaluated through the potentiometric method in 0.10 M phosphate buffer solution of pH 7.3[36]. The performance of cholesterol oxidase immobilized Co_3O_4 nanocrystals was compared with the pristine Co_3O_4 nanostructures as shown in Figure 4 a,b. It can be seen from Figure 4a that biosensing performance of cholesterol oxidase immobilized Co_3O_4 platelets is poor in terms of accuracy and precision, suggesting a poorly designed analytical device. However, the cholesterol oxidase immobilized Co_3O_4 nanocrystals were found highly efficient towards the detection of cholesterol in terms of wide linear range of 0.005 mM to 3 mM and low limit of detection of 0.001 mM as shown in Figure 4b. Also, the analytical behaviour of the proposed method is accurate as signified by the regression coefficient of 0.99. Additionally, the cholesterol oxidase immobilized Co_3O_4 nanocrystals have exhibited a Nernstian response as confirmed by the sensitivity value of 60 mVdec^{-1} . Furthermore, the successive addition method was used to develop the linear from 0.001mM to 3 mM for the cholesterol oxidase immobilized Co_3O_4 nanocrystals as shown in Figure 5.

This study verifies that the presented cholesterol biosensor has high sensitivity and stability towards the oxidation of cholesterol substrate during the measurement. It strengthened the results presented in Figure 4b. The presented cholesterol is well characterized by the wide linear range and an excellent sensitivity which are assigned to large surface area and excellent co-catalytic properties possessed by the Co_3O_4 nanocrystals. The Co_3O_4 nanocrystals exposed the unique

surface for the immobilization of cholesterol oxidase and also promoted the activity of enzyme being a co-catalyst. The enzymatic reaction processing on the cholesterol oxidase immobilized Co_3O_4 nanocrystals could be described by two different mechanisms. First, mechanism is followed by the selective activity of cholesterol oxidase towards cholesterol substrate. Second, mechanism is involving between cholesterol and the cholesterol oxidase immobilized Co_3O_4 nanocrystals which has more favourable kinetics than first reaction mechanism [37]. The superior performance of cholesterol oxidase immobilized Co_3O_4 nanocrystals could be indexed to synergetic effects [38]. Also, the hydrolysis of polyethylenimine in the organic and inorganic solvents may create the defects and electrical charges [39, 40]. The cholesterol reaction with cholesterol oxidase immobilized Co_3O_4 nanocrystals produced following substances [41].



The product 5-3-ketosteroid is short lived and it transformed into 4-3-ketosteroid due to the isomerism at trans double bond of 5-6 steroid ring through intermolecular shift of 4-6 β sites.



The potentiometric signal of presented biosensor is attributed to reaction mechanism described herein which enabled the generation of charged species floating on the surface of cholesterol oxidase immobilized Co_3O_4 nanocrystals. The performance of proposed method is describing the enhanced co-catalytic properties of Co_3O_4 nanocrystals for the cholesterol oxidase by providing the fast response, and direct electron transfer kinetics of different catalytic sites between cholesterol oxidase and Co_3O_4 nanocrystals. The inter-electrode study of 10 cholesterol oxidase immobilized Co_3O_4 nanocrystals electrodes was studied in 0.5mM cholesterol as shown in Figure 6.

The potentiometric response of each independent electrode was highly reproducible with less than 5% deviation indicating an excellent activity of cholesterol oxidase immobilized Co_3O_4 nanocrystals. This study was very vital to fabricate the practical cholesterol oxidase immobilized Co_3O_4 nanocrystals-based configurations at large scale. Also, the repeatability of same cholesterol oxidase immobilized Co_3O_4 nanocrystals electrode was investigated by conducting the three repeating measurements of cholesterol at alternative days in terms of linear range and limited of detection as shown in Figure 7.

The repeatability experiment has verified the results reported in Figure 4b, revealing the outstanding performance of cholesterol oxidase immobilized Co_3O_4 nanocrystals due to strong and favourable microenvironment for the cholesterol oxidase to retain activity for long term use. The storage life of the cholesterol oxidase immobilized Co_3O_4 nanocrystals was also evaluated for the period of three weeks as given in Table 1.

It is clear that a slight variation in limit of detection was notice, however the linear range and sensitivity of proposed were preserved. The storage life is related to the environmental conditions in addition to the composition of electrode and effectiveness of enzyme immobilization. In our study, we kept the modified electrode at 4 °C in phosphate buffer solution after the use. The dynamic response of presented cholesterol oxidase immobilized Co_3O_4 nanocrystals was also studied in 0.5 mM cholesterol as shown in Figure 8. The biosensor has shown a fast response time less than 1s suggesting the sensitivity of cholesterol substrate towards the cholesterol oxidase immobilized Co_3O_4 nanocrystals. The selectivity of present cholesterol biosensor was monitored under the common interfering species as depicted in Figure 9.

The selected interfering species were urea, dopamine, fructose, ascorbic acid, uric acid and glucose and they were used with 0.05mM concentration and the output potential signal is negligibly altered on addition to 0.01 mM and 0.05mM cholesterol solution as shown in Figure 9. However, same concentration of 0.1mM of cholesterol has produced output potential by interacting with cholesterol oxidase immobilized Co_3O_4 nanocrystals as shown in Figure 9, confirming an excellent selectivity of proposed method. The analytical features cholesterol oxidase immobilized Co_3O_4 nanocrystals towards detection of cholesterol were monitored by the % recover method as given in Table 2. The estimated performance has shown an excellent performance of presented cholesterol biosensor and enable it to be consider as a potential modified electrode for the practical aspects.

The fitted circuit for measuring the impedance values of different materials is enclosed as inset in Figure 10 describing the distinctive Nyquist plots. The impedance study is confirming the role of nanocrystal with fast charge transport compare to platelets structure, however a slight increase in impedance was noticed due to insulating nature of cholesterol oxidase and glutaraldehyde. The obtained impedance behavior and values are closely related to reported works [47, 48]. The

estimated impedance values from simulated EIS data are given in Table 3. It is obvious from these values that cholesterol oxidase immobilized Co_3O_4 nanocrystals confirmed an excellent charge transfer for increasing the performance of proposed cholesterol biosensor. The performance evaluation of cholesterol oxidase immobilized Co_3O_4 nanocrystals based cholesterol biosensor was compared with the well-established methods using enzymatic approach in the existing literature as given in Table 4. It is obvious from the Table 4 that different materials have been used for the cholesterol detection [35, 42-46]. The presented approach based on cholesterol oxidase immobilized Co_3O_4 nanocrystals is simple, accurate, sensitive and cost-effective and more likely has high potential to quantify the cholesterol under the biological matrix.

4. Conclusions

In summary, we have studied the effect of polyethylenimine hydrolysis in ethanol/water bisolvent system on the morphology of Co_3O_4 nanostructures using hydrothermal method. The prepared Co_3O_4 nanocrystals exhibited a large surface area which was successfully capitalized by the massive loading of cholesterol oxidase enzyme to fabricate a sensitive and selective potentiometric cholesterol biosensor. The Co_3O_4 nanocrystals have exhibited a cubic crystal geometry and the composition is mainly retained by cobalt and oxygen. The fabricated cholesterol oxidase/ Co_3O_4 nanocrystals were found to be highly efficient in generating the output potential in 10 mM phosphate buffer solution of pH 7.3 using a potentiometric method. Significantly, the cholesterol biosensor displayed a wide linear range of 0.005 to 3 mM and LOD of 0.001 mM. The sensitivity of the biosensor was found 60 mVdec^{-1} , indicating an excellent Nernstian response. The fast response of less than 1 s is further justifying the efficiency and ultra-sensitivity towards trace level cholesterol substrate. The storage life has confirmed the long term feasibility of the proposed cholesterol biosensor. The percent recovery method was also used to evaluate the practical aspect of proposed method and it was observed satisfactory. Based on the obtained results, we believe that the prepared Co_3O_4 nanocrystals can be of prime consideration for other related electrochemical applications.

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Conflict of interest statement

Authors declare there are not conflict of interest in this research work

5. References

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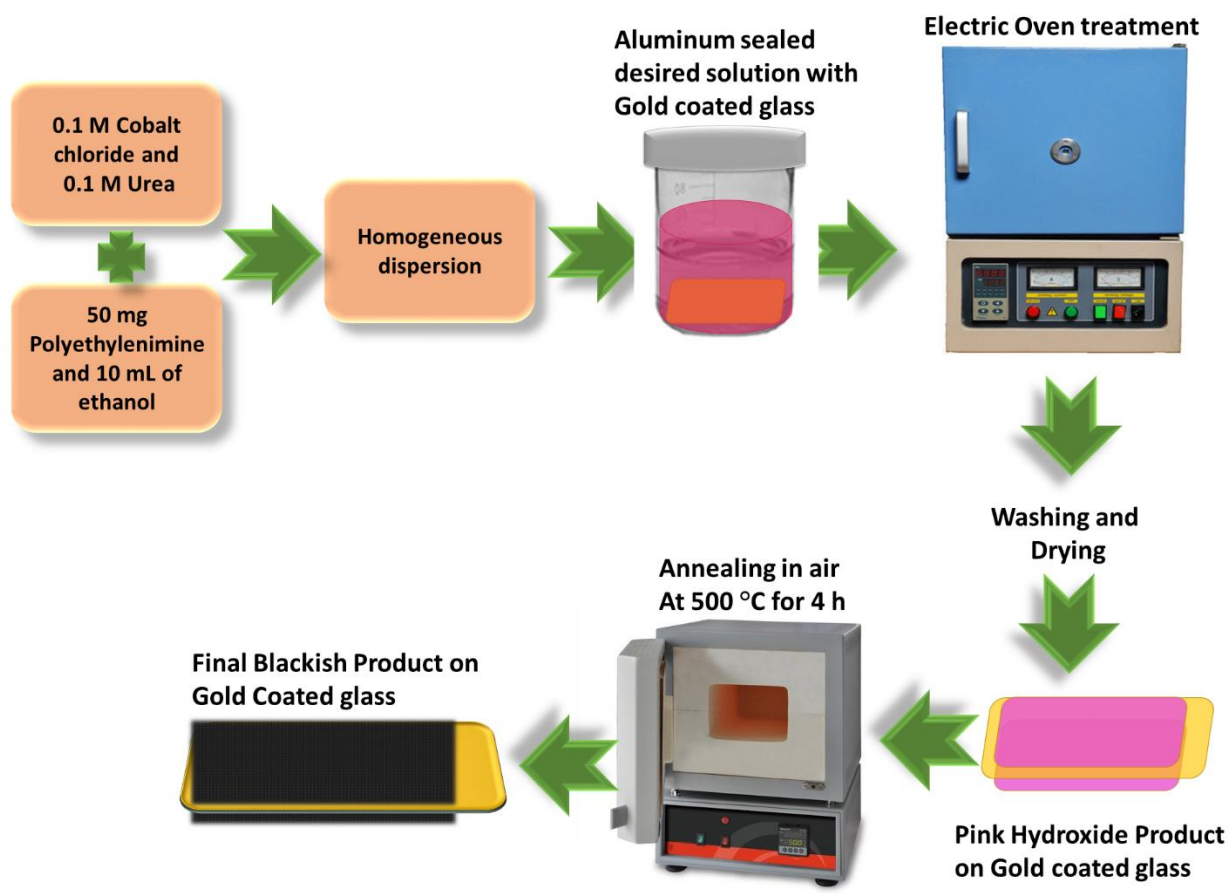
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Figure Captions



Scheme 1: Represents the synthesis process for the preparation of nanocrystals of Co_3O_4

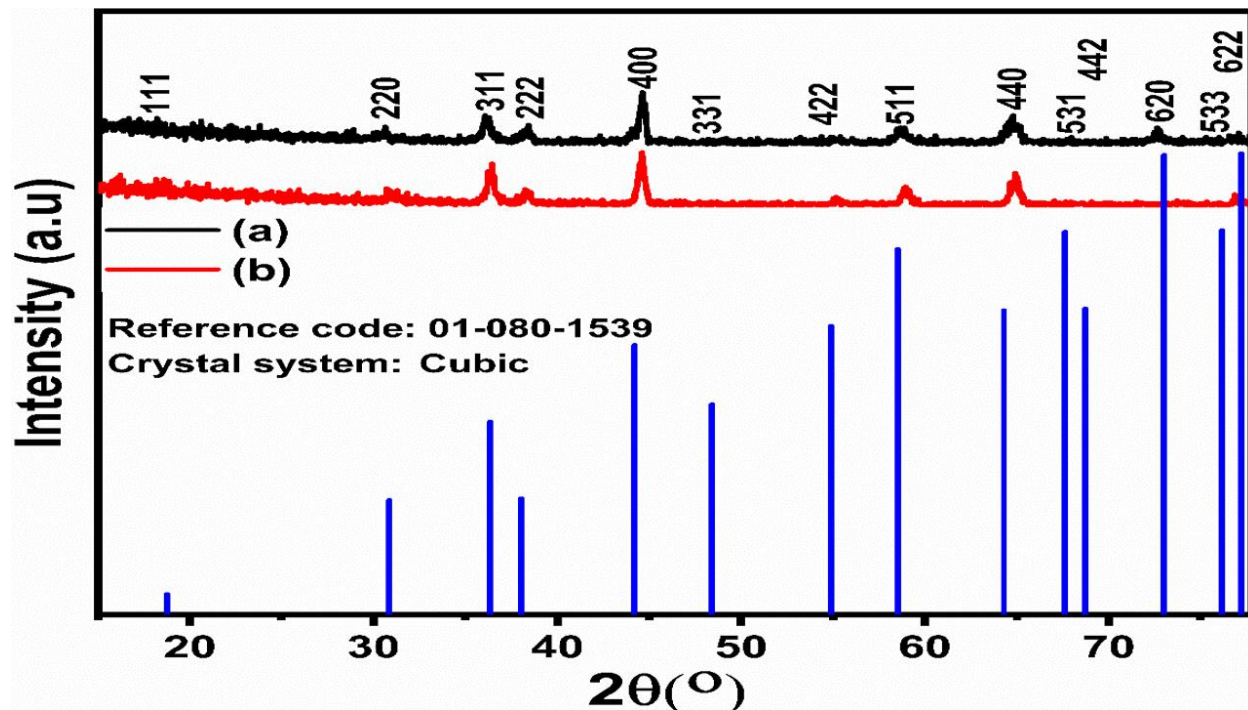


Figure 1: (a) XRD patterns of (a) pure Co_3O_4 , (b) Co_3O_4 nanocrystals prepared with polyethylenimine

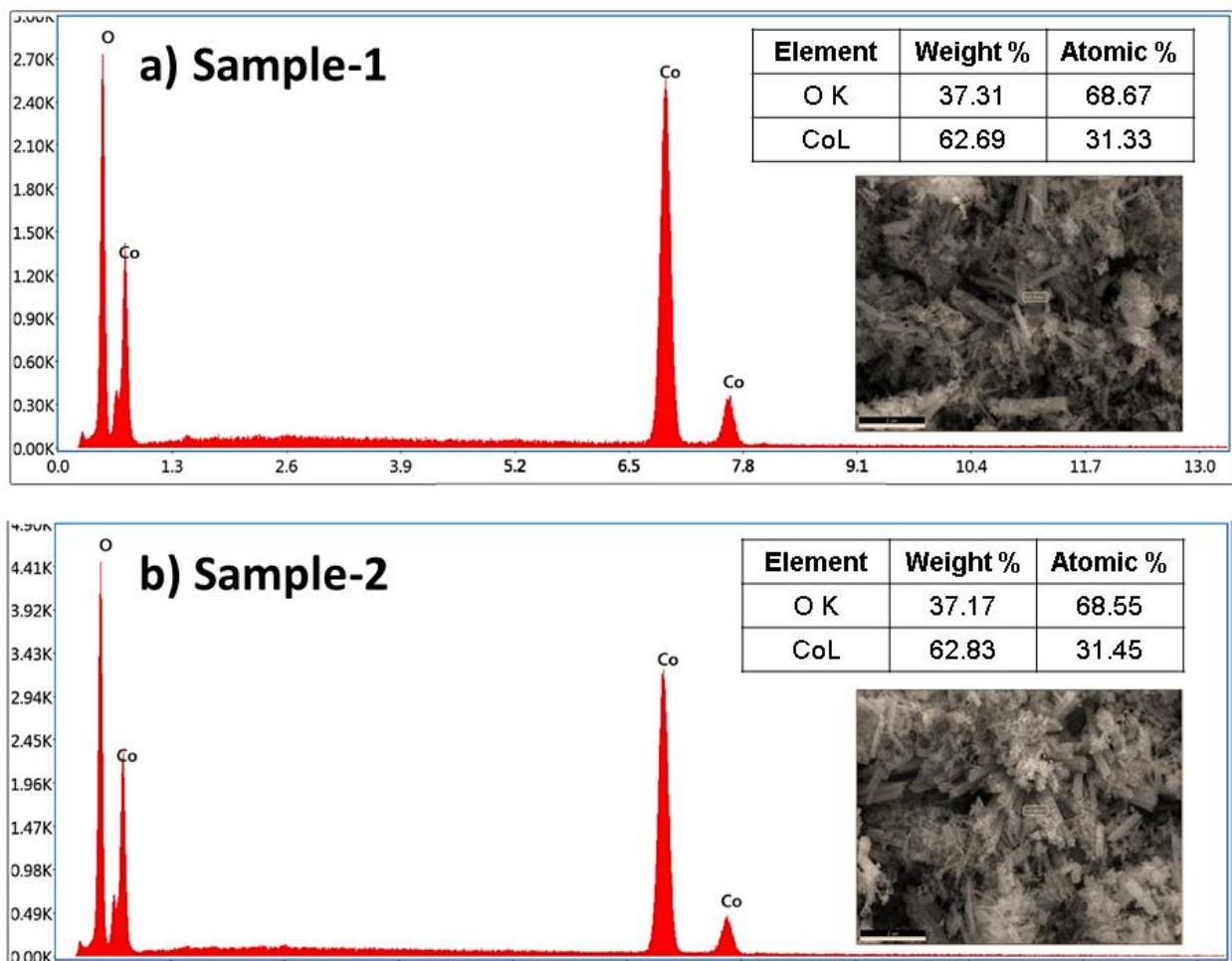


Figure 2: EDS spectra (a) pure Co_3O_4 , (b) Co_3O_4 nanocrystals prepared with polyethylenimine

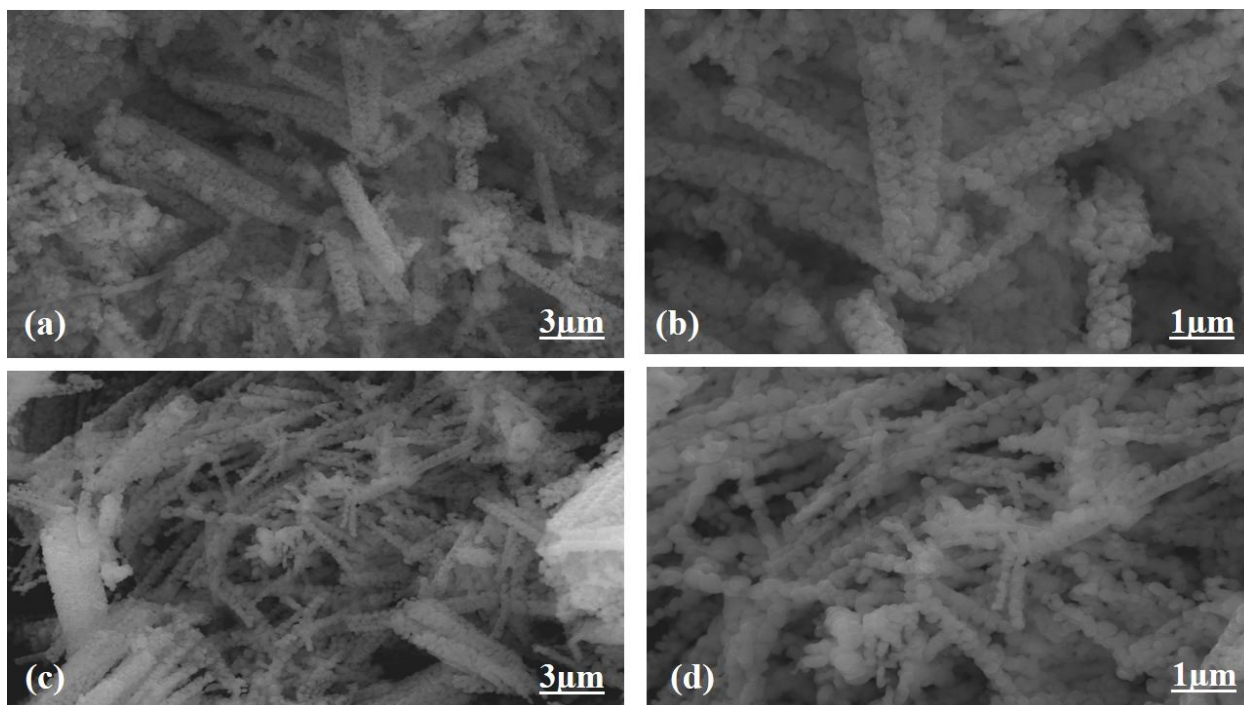


Figure 3: Distinctive SEM images (a) pure Co_3O_4 , (b) Co_3O_4 nanocrystals prepared with polyethylenimine

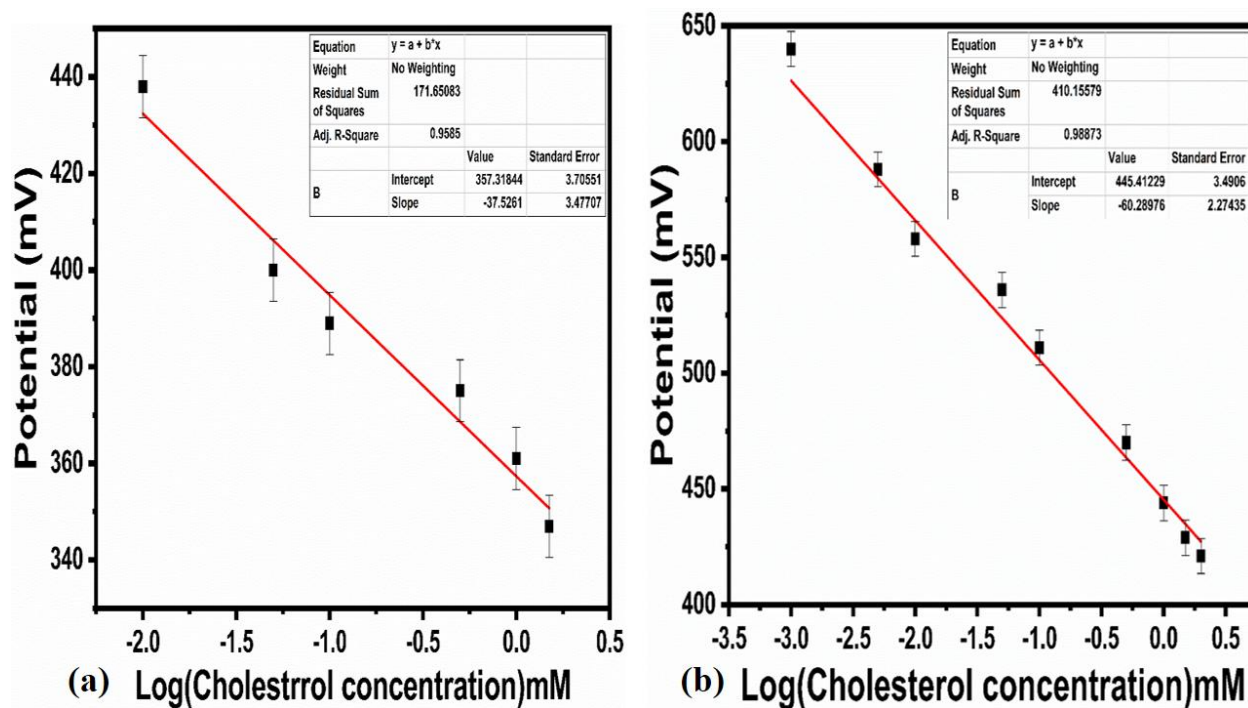


Figure 4: (a) potentiometric response of cholesterol oxidase immobilized pure Co_3O_4 platelets (b) cholesterol oxidase immobilized Co_3O_4 nanocrystals in 10 mM phosphate buffer solution of pH 7.3

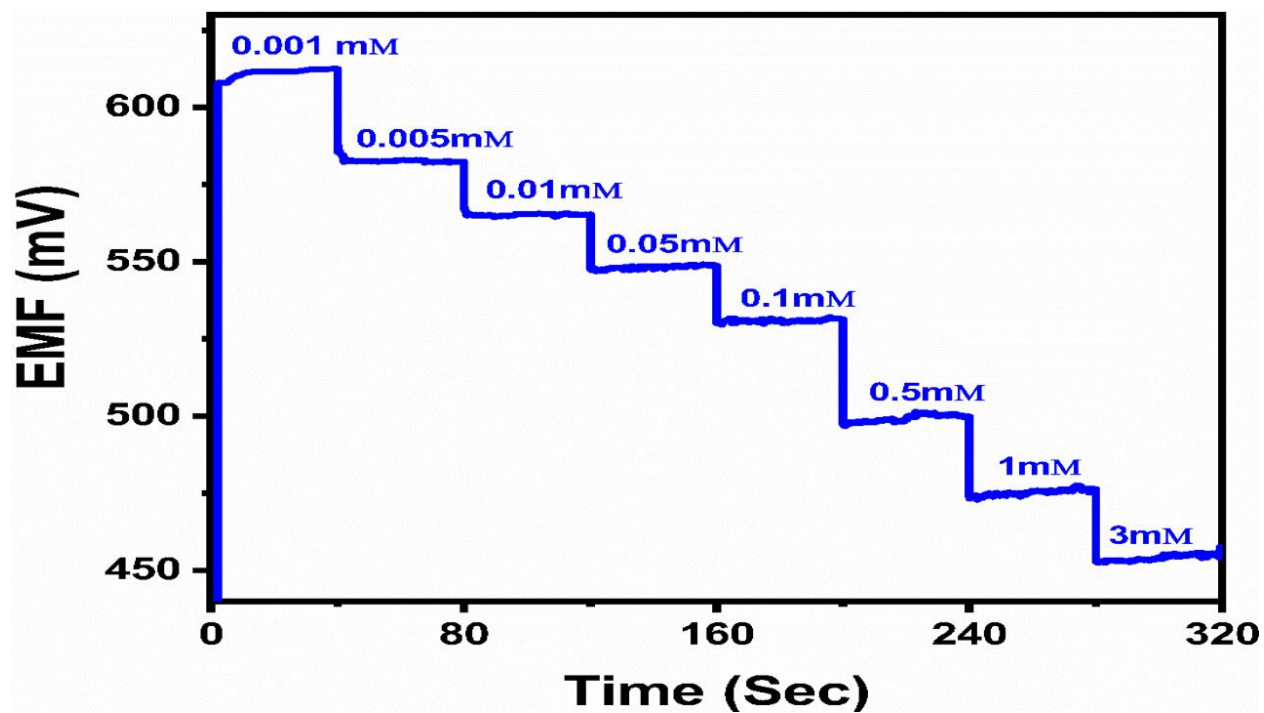


Figure 5: Sequential addition of cholesterol response of cholesterol oxidase immobilized Co_3O_4 nanocrystals in 10 mM phosphate buffer solution of pH 7.3

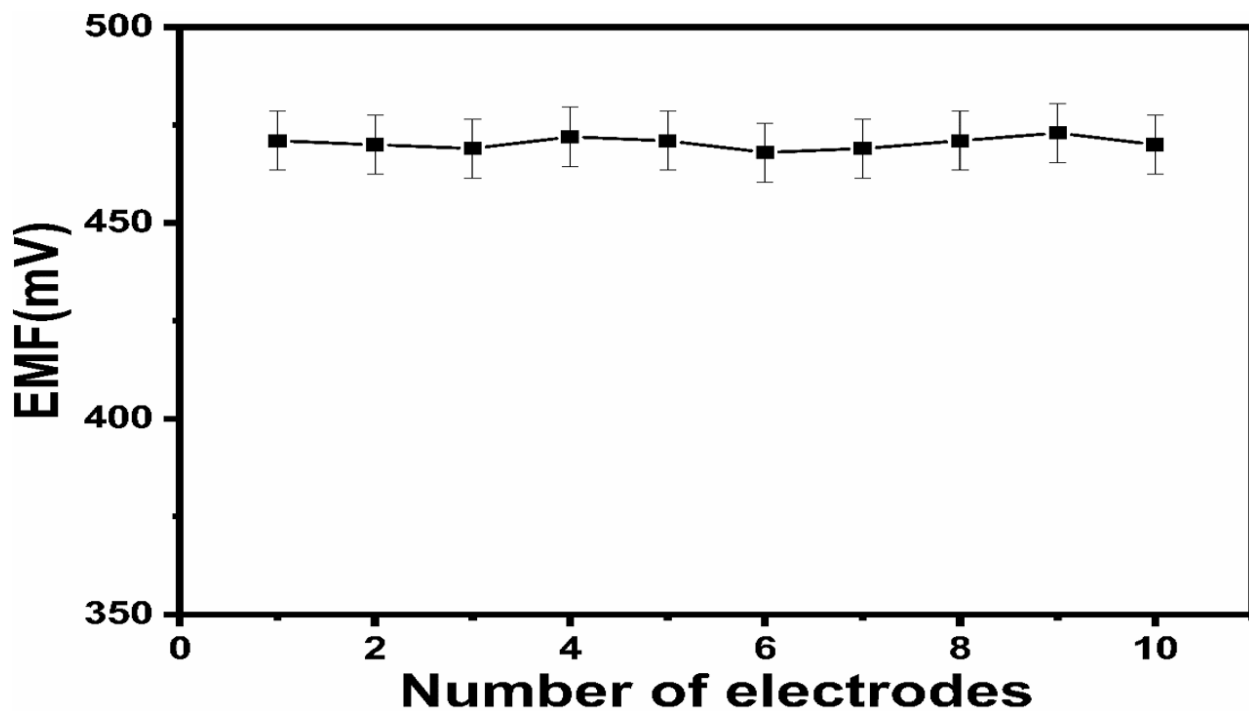


Figure 6: Inter electrode study of cholesterol oxidase immobilized Co_3O_4 nanocrystals in 10 mM phosphate buffer solution of pH 7.3 in 0.5mM cholesterol concentration

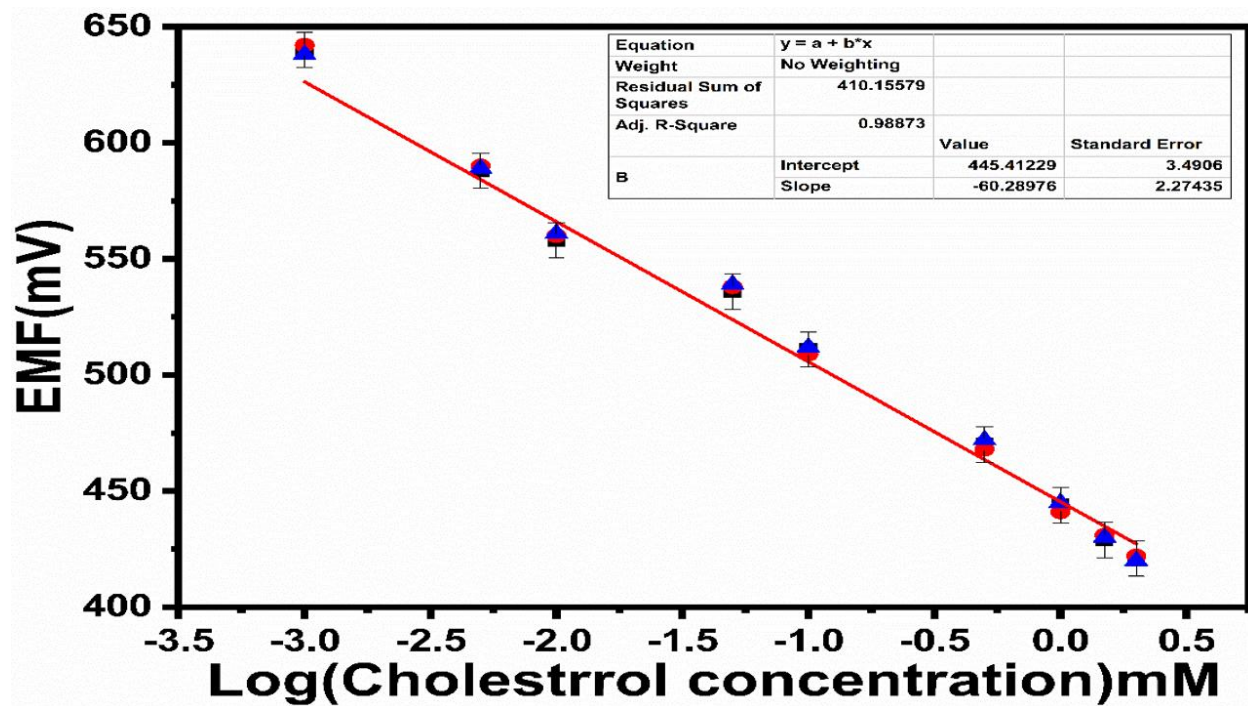


Figure 7: Repeatability study of cholesterol oxidase immobilized Co_3O_4 nanocrystals in 10 mM phosphate buffer solution of pH 7.3

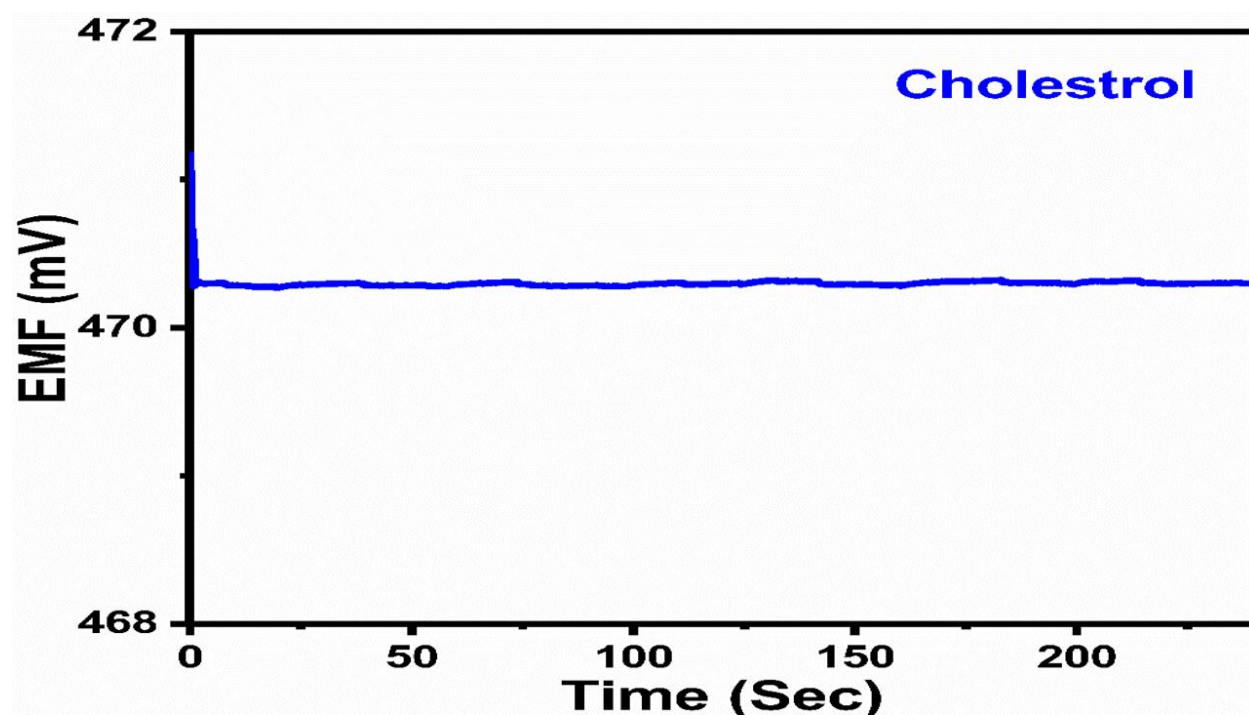


Figure 8: Dynamic response investigation of cholesterol oxidase immobilized Co_3O_4 nanocrystals in 10 mM phosphate buffer solution of pH 7.3 in 0.5mM cholesterol concentration

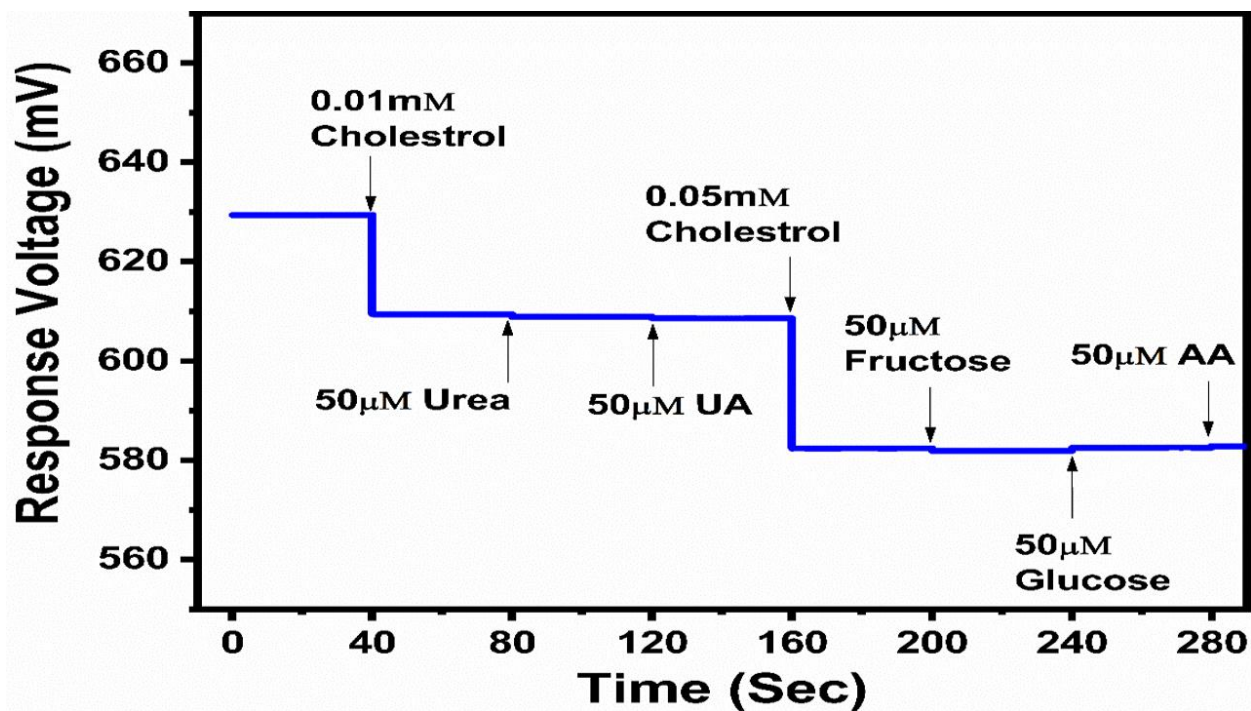


Figure 9: Selectivity response of cholesterol oxidase immobilized Co_3O_4 nanocrystals in 10 mM phosphate buffer solution of pH 7.3 in 0.01 and 0.05mM cholesterol concentration with addition of 0.05mM of common interfering substances

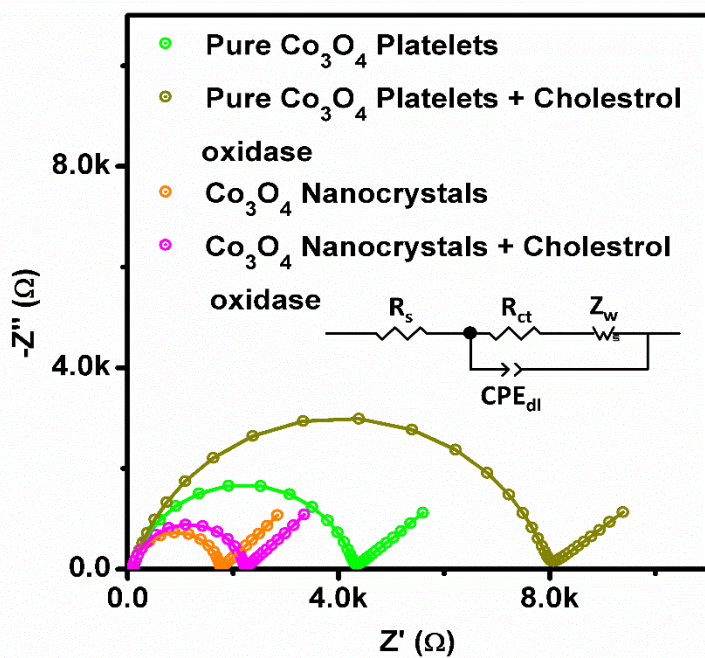


Figure 10: EIS analysis of Pure Co_3O_4 platelets and Co_3O_4 nanocrystals with and without cholesterol oxidase in 10 mM phosphate buffer solution of pH 7.3 containing 0.5mM cholesterol

Table 1: Storage life of cholesterol oxidase immobilized Co_3O_4 nanocrystals

No of weeks	Linear range (mM)	Slope (mVdec ⁻¹)	Limit of detection (mM)
1	0.005-3	60	0.001
2	0.004-3	60	0.003
3	0.006-3	60	0.002

Table 2: Percent recovery response of cholesterol oxidase immobilized Co_3O_4 nanocrystals

Experiment no:	Added (mM)	Found (mM)	% recovery
1	1	1.02	102
2	2	1.97	98.5
3	3	2.99	99.6

Table 3: Impedance values from EIS for various presented materials in this study

Materials	Charge transfer (R _{ct})
Pure Co_3O_4 platelets	4.1×10^3 Ohms
Pure Co_3O_4 platelets + Cholesterol oxidase	7.8×10^3 Ohms
Co_3O_4 nanocrystals	1.6×10^3 Ohms
Co_3O_4 nanocrystals + Cholesterol oxidase Enzyme	2.1×10^3 Ohms

Table 4: Comparative analysis of cholesterol oxidase immobilized Co_3O_4 nanocrystals with reported works

Electrode	Experimental	Linear range	Limit of	Sensitivity	Response
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Material	method	(mM)	detection (mM)	(mVdec ⁻¹)	time (s)
ChOx/ Co ₃ O ₄ nanowires/Au [35]	Potentiometric	1 x 10 ⁻⁷ – 1 x 10 ⁻³	0.035 x 10 ⁻⁷	-94.031	10
ChOx/ Iron nanoparticles [42]	Spectrophotometric	1.3 x 10 ⁻³ – 5.2 x 10 ⁻³			
ChOx / ZnO [43]	Electrochemical	0.65x 10 ⁻³ – 10.34 x 10 ⁻³			
ChOx/ cobalt oxide nanomaterials [44]	Flow injection analysis	4.2 x 10 ⁻⁶ – 50 x 10 ⁻⁶	4.2 x 10 ⁻⁶	43.5 nA mM ¹ cm ⁻²	15
ChOx/ chitosan-tin oxide nanobiocomposite [45]	Amperometric	0.26 x 10 ⁻³ – 10.36 x 10 ⁻³	0.13 x 10 ⁻³	34.7 mA/mg dL ⁻¹ cm ²	5
ChOx/Fe ₃ O ₄ nanoparticles [46]	Amperometric	1.3 x 10 ⁻³ – 5.2 x 10 ⁻³	0.5 x 10 ⁻³		50
ChOx/Co ₃ O ₄ nanocrystals= [Present work]	Potentiometric	5x10 ⁻⁶ – 3 x 10 ⁻²	1x10 ⁻⁶	60 mV/decade	Less 1 s