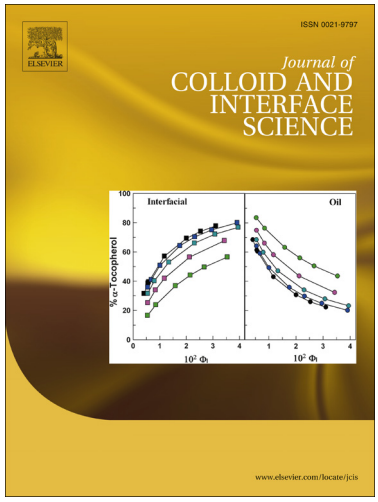


Nano interfaced Biosensor for Detection of Choline in Triple Negative Breast Cancer Cells

Vignesh Thiagarajan, Madhurantakam Sasya, Swaminathan Sethuraman, John Bosco Balaguru Rayappan, Uma Maheswari Krishnan

PII: S0021-9797(15)30259-9
DOI: http://dx.doi.org/10.1016/j.jcis.2015.10.014
Reference: YJCIS 20800

To appear in: *Journal of Colloid and Interface Science*

Received Date: 20 August 2015
Revised Date: 5 October 2015
Accepted Date: 6 October 2015

Please cite this article as: V. Thiagarajan, M. Sasya, S. Sethuraman, J.B.B. Rayappan, U.M. Krishnan, Nano interfaced Biosensor for Detection of Choline in Triple Negative Breast Cancer Cells, *Journal of Colloid and Interface Science* (2015), doi: http://dx.doi.org/10.1016/j.jcis.2015.10.014

Nano interfaced Biosensor for Detection of Choline in Triple Negative Breast Cancer Cells

Vignesh Thiagarajan[1,2], Madhurantakam Sasya[1,2], Swaminathan Sethuraman[1,2], John Bosco Balaguru Rayappan[1,3], Uma Maheswari Krishnan[1,2,*]

[1]Centre for Nanotechnology & Advanced Biomaterials (CeNTAB)

[2]School of Chemical & Biotechnology

[3]School of Electrical & Electronics Engineering

SASTRA University

Thanjavur – 613 401, India

---

*Corresponding Author

Prof. Uma Maheswari Krishnan Ph. D.

Deakin Indo–Australia Chair Professor

Associate Dean for the Departments of Chemistry, Bioengineering & Pharmacy

Centre for Nanotechnology & Advanced Biomaterials (CeNTAB)

School of Chemical & Biotechnology

SASTRA University, Thanjavur – 613 401

Tamil Nadu, India.

Ph.: (+91) 4362 264101 Ext: 3677

Fax: (+91) 4362 264120

E–mail: umakrishnan@sastra.edu

ABSTRACT

Choline, a type of Vitamin B, is an important nutrient in the human body and is involved in key metabolic pathways. Abnormal levels of choline leads to diseased conditions. The levels of choline and its associated compounds are found to be elevated in triple negative breast cancer (TNBC) patients. The choline level ranges from 0.4 to 4.9 mmol/kg in TNBC. Thus the detection of choline levels in cells can aid in diagnosing breast cancer. The present work aims to develop a nano-interfaced electrochemical biosensor for the rapid detection of choline in cancer cells. For electrochemical detection, glassy carbon electrode coated with a zinc oxide nano-interface was used as the working electrode. Zinc oxide synthesized by hydrothermal method was characterized using SEM and XRD. The choline oxidase (ChOx) enzyme was immobilized on the nano-interface by drop-casting. Choline oxidase (ChOx) converts choline to betaine and H_2O_2 in the presence of oxygen. The H_2O_2 produced was determined amperometrically. The amount of H_2O_2 produced is directly proportional to concentration of choline present. The sensitivity, selectivity, stability and concentration studies were carried out and quantification of choline in TNBC was also carried out. The results demonstrate that this biosensor has the potential to be developed as a clinical tool for breast cancer detection.

INTRODUCTION

Choline (trimethyl-beta-hydroxyethyl ammonium) is a quarternary ammonium compound that plays a vital role in the human metabolism and is a water-soluble essential nutrient [1]. It is required in our daily diet for maintaining body functions [2]. Choline plays a major role in the synthesis of phospholipids, especially phosphatidylcholine [3], and acts as a precursor of acetyl choline [4], which is a neurotransmitter in the central nervous system. Choline metabolism impairment leads to either decrease or increase in choline levels. Choline and acetylcholine have been recognized as biomarkers for disease diagnostics, especially in the case of Alzheimer's and Parkinson's disease [5][6][7]. Choline is also an anti-inflammatory agent [8]. In contrast, increasing levels of choline have been found to cause DNA damage and enhances risk of cancer [9]. Quantitative measurement of both choline and acetylcholine is therefore becoming important in clinical diagnosis.

Cancer is a fateful disease that results from mutation of the genes that control the cell cycle [10] [10]. In India, breast cancer is the major type of cancer that affects the population and is topping the charts among the fatal diseases [11]. Several biomarkers have been identified that can be helpful in diagnosis as well as treatment of breast cancer. Triple negative breast cancer is a unique type of cancer where absence of all the 3 major receptors namely hormone epidermal growth factor receptor 2 (HER-2), estrogen receptors (ER) and progesterone receptors (PR) has been observed [11]. Recent reports have indicated that triple negative breast cancers exhibit increased levels of choline ranging from 0.4 to 4.9 mmol/kg [12]. Therefore, detection of choline may serve as a tool for diagnosing the highly aggressive triple negative breast cancer at an early stage. Currently, ^{1}H NMR spectroscopy is the major technique used to determine the choline levels in breast cancer[13]. Choline biosensors have been majorly employed for determining choline levels in food samples [15]. The choline biosensors reported in literature are mostly electrochemical [14][4]. Other detection methods like fluorescence and chemiluminescence have also been reported [15][16]. Electrochemical detection has several advantages other methods. They are cost-effective, simple and consume less time for detection. Moreover, electrochemical biosensors are highly sensitive and selective towards the substrate [17][18][19][20][21] .

A paradigm shift in the field of biosensors is in the introduction of nano-dimensional interfaces at the electrode surface that facilitate enhanced electron transfer. Several nano-interfaces that have been successfully used in electrochemical sensors include nanostructures prepared from metals, their oxides and carbon structures [22]. Among these, ZnO possess several characteristics such as good catalytic property, high surface area to volume ratio and high isoelectric point (IEP) [23]. They are non-toxic and biocompatible. The isoelectric point of ZnO is 9.5 that helps in immobilization of ChOx with lower IEP (4.1) [26]. Structurally different ZnO structures can be prepared thereby enabling tuning of the sensing characteristics for specific enzymes [24][24] . Few reports of nanointerfaced choline biosensors are available in literature. Graphene [25], multi-walled carbon nanotubes [4], manganese oxide nanoparticles [14] and platinum nanoparticles [26] have been used as nanointerfaces. However, the maximum concentration of choline that was detected in these sensors was below 2 mM. The present work aims to use ZnO nanostructures to immobilize the enzyme choline oxidase and evaluate its performance for detection of choline from breast cancer cells.

Materials and methods

Materials

Choline chloride and choline oxidase were purchased from M/s Sigma Aldrich Ltd (India). Zinc nitrate, sodium hydroxide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, chitosan, acetic acid, ascorbic acid, citric acid and uric acid were purchased from Merck (India). MDA-MB-231 triple negative breast cancer cell line was purchased from NCCS, Pune. Dulbecco's modified Eagles' medium (DMEM) medium, phosphate buffered solution (PBS), fetal bovine serum (FBS), Penicillin/streptomycin, trypsin (0.05%), Radio immunoprecipitation assay (RIPA) buffer were purchased from Invitrogen. Distilled water was used for the preparation of all solutions.

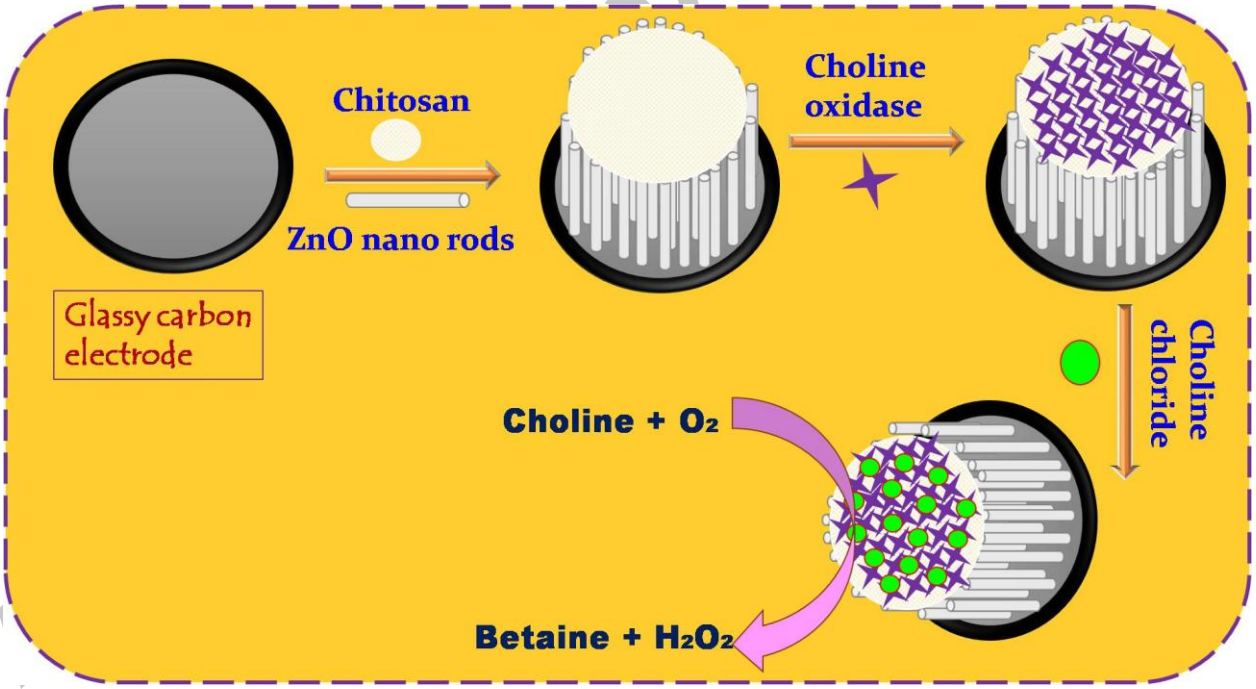
Preparation of ZnO nanoparticles

ZnO nanoparticles were synthesized using hydrothermal method. A 1:10 ratio of 0.1M $Zn(NO)_3$. $6H_2O$ and 0.2M NaOH solutions were mixed and the pH of the solution was adjusted to 14. The resulting mixture was placed in an autoclave at 60ºC for 6 h. The sample was cooled and the

samples were dried in an oven at 100ºC for 12 h. Field Emission Scanning Electron Microscopy (FE–SEM, JSM6701F, JEOL, Japan) was used to analyze the surface morphology of the synthesized nanoparticles. X-Ray Diffractometer (XRD) (D8 Focus, Bruker, Germany) was used to determine the structural characteristics of ZnO nanoparticles. The analysis was carried out within a 2θ range of 30º and 60º.

Modification of the working electrode

The glassy carbon electrode (GCE) was polished using alumina slurry of various grades. 1.0 mg of ZnO nanoparticles was mixed with 250 µL of chitosan (5 wt % in acetic acid). The above mixture was sonicated for 20 min. To this mixture, 20 µL of the choline oxidase (ChOx) stock solution (4 mg/ml in PBS) was added. Then, 3.0 µL of the prepared ZnO-ChOx solution was coated on to the surface of working electrode and allowed to dry. This prepared GCE/ZnO/ChOx was used as the working electrode. Stepwise modification of working electrode is shown in scheme 1.



Scheme 1: The schematic of stages involved in the modification of glassy carbon working electrode for developing choline biosensor.

Electrochemical measurements

Electrochemical workstation (CHI600, CH Instruments, USA) was used to study electrochemical measurements of a three-electrode system. Modified glassy carbon electrode (GCE/ZnO/ChOx) was used as the working electrode, Platinum wire was used as the counter electrode and Ag/AgCl (saturated in 0.1 M KCl) served as the reference electrode. Cyclic voltammetry (CV) and amperometric i-t curves were recorded in PBS solution at pH 8 where the ChOx activity was maximum. Cyclic voltammograms were recorded at a scan rate of 0.01V/s in pH 8.0 phosphate buffered solution (0.1M).

Cell culture studies

MDA-MB-231 triple negative breast cancer cells were cultured in T-75 mL flask and then trypsinized. The cells were counted and seeded with different densities (1, 1.5, 2, 2.5, 3 and 3.5 $\times 10^5$ cells) in T 25 mL and T 75 mL flasks containing DMEM medium with 10% FBS and 1% penicillin/streptomycin. The flasks were incubated in 5% CO_2 at 37^{o}C. After cells attained confluency, the media was removed, washed with ice cold PBS followed by addition of ice cold RIPA buffer and kept in ice for 5-10 min. The cells were then scrapped gently using cell scraper and the suspension was transferred into a micro centrifuge tube. The suspension was centrifuged at 16000 rpm for 20 min at 4^{o}C. After centrifugation, the supernatant was used for analysis.

Results and Discussion

The morphology of the zinc oxide nanoparticles prepared by hydrothermal method was investigated by scanning electron microscopy (Figure 1A). Rod-shaped nanostructures with an average width of 50 nm were observed. The x-ray diffraction pattern of the ZnO nanoparticles shown in Figure 1B exhibits sharp peak intensities that indicates the polycrystalline nature of the sample. The 2θ values of the peaks matches those for ZnO reported in literature (JCPDS card no: 36-1451). The peaks for the planes (100), (002), (101), (102), (110) in the sample confirm that the ZnO nanorods exist in the hexagonal wurtzite crystalline form. The grain size calculated using Scherrer's formula for the ZnO nanorods was 45.72nm.

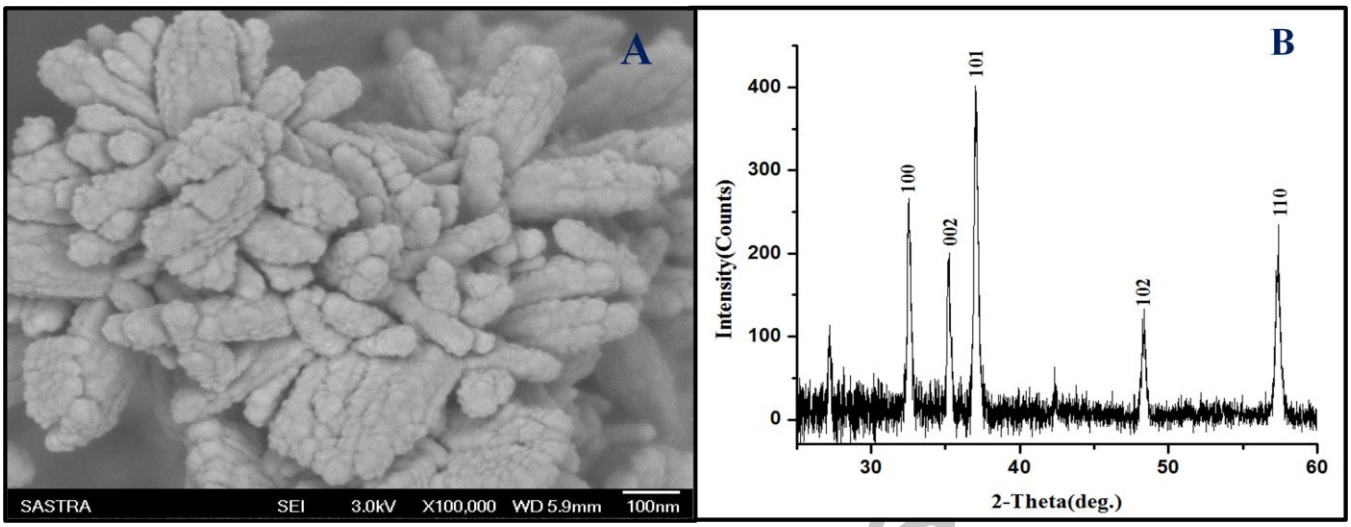


Figure 1: A) Scanning electron micrograph of ZnO nanoparticles; B) X-ray diffractogram of ZnO nanoparticles

Electrochemical measurements

The influence of the ZnO nanointerface and enzyme immobilization on the electrochemical oxidation of choline at the glassy carbon electrode (GCE) was studied using cyclic voltammetry. Figure 2.A shows the cyclic voltammograms recorded using bare GCE, ZnO coated GCE, Choline oxidase (ChOx) coated GCE and ChOx immobilized ZnO coated GCE in PBS at a scan rate of 0.01 V/s.

Interface study was conducted at a scan rate of 0.1V/s in PBS of pH 8. The introduction of the nanointerface showed a peak at the potential of -0.712 mV. The enzyme modified electrode without the nanointerface showed a peak potential at -0.698 mV and a slightly higher current flow of 3.18×10^{-6}A. The combination of the enzyme immobilized on the ZnO nanointerface displayed the maximum current flow with the oxidation peak appearing at -0.694 mV. The increase in current for GCE/ZnO and GCE/ZnO/ChOx electrodes may be attributed to the enhanced electron transfer property of ZnO nanoparticles. Figure 2.B shows the cyclic voltammograms recorded using ZnO coated GCE, Choline oxidase (ChOx) coated GCE and ChOx immobilized ZnO coated GCE in the presence of 0.3mM of choline chloride in PBS at a scan rate of 0.1 V/s.

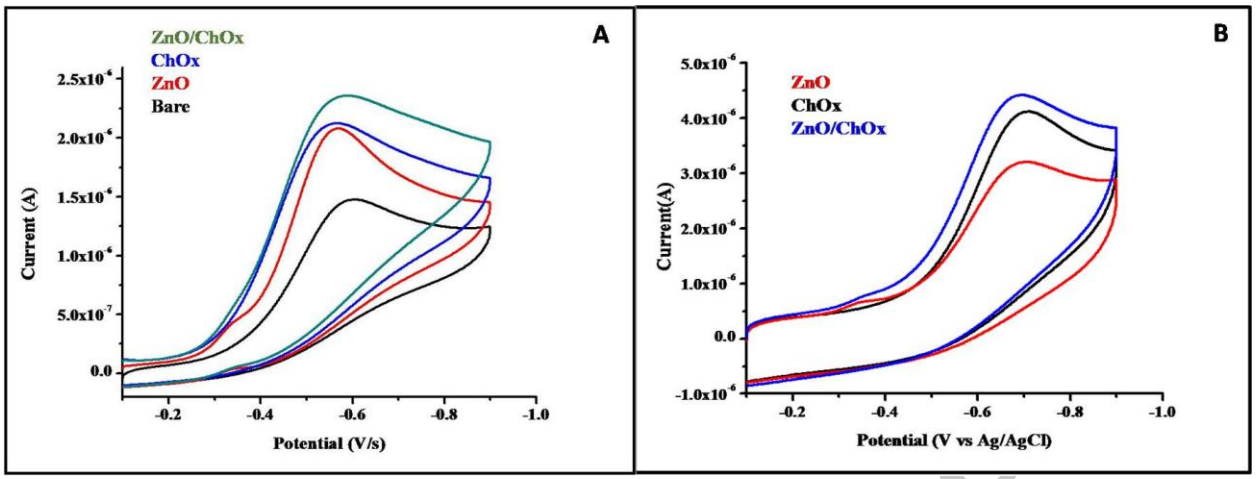


Fig.2Cyclic Voltammogram recorded for bare GCE, GCE/ZnO, GCE/ChOx, and GCE/ZnO/ChOx electrode in the absence of choline (A), GCE/ZnO, GCE/ChOx and GCE/ZnO/ChOx electrode after the addition of 0.3mM Choline Chloride in PBS (0.1 M, pH 8)

The active surface coverage in the working electrode was calculated using the following equation:

$$\text{Surface coverage } (\Gamma) = \frac{Q}{nFA}$$

where 'Q' is the charge, 'n' is the number of electrons transferred, 'F' is the Faraday constant and 'A' is the area of the electrode.

The active surface coverage of the ChOx coated GCE electrode was 0.41×10^{-9}M/cm^2 while it was 0.6835×10^{-9}M/cm^{2}for the ZnO coated GCE. The active surface coverage for the enzyme immobilized ZnO coated working electrode was calculated to be 0.8033×10^{-9}M/cm^2. The increase in the surface coverage for the GCE/ZnO/ChOx electrode may be a key contributor to the observed increase in the peak current. Details of shift in peak current, peak potential values and surface coverage of all modified electrodes is shown in Table 1.

Modified Electrode	Potential (Epc)	Current (Ipc)(10^{-6}A)	Surface Coverage (Γ) (10^{-9} M/cm^2)
GCE/ZnO	-0.712	1.67	0.6835
GCE/ChOx	-0.698	3.18	0.41
GCE/ZnO/ChOx	-0.694	4.14	0.8033

Table 1: Comparision of Epc, Ipc, surface coverage of modified electrodes

Cyclic voltammograms were recorded for the GCE/ZnO/ChOx working electrode between the scan rate of 0.01 V/s and 0.1 V/s in an electrochemical cell containing 0.1M PBS and 0.3 mM choline chloride and the results are presented in Figure 3. A progressive increase in current was observed with increasing scan rate indicating an enhanced flux of the electroactive species to the electrode surface at higher voltage scan rates. However the peak potential exhibited a shift from -0.763 mV at 0.01 V/s to -0.857 mV at 0.1 V/s. The plot of cathodic peak current versus scan rate was linear with a regression coefficient (R^2) of 0.97 (Figure 3-inset). As the lower scan rates showed lesser negative potential values, 0.01 V/s was chosen for further studies.

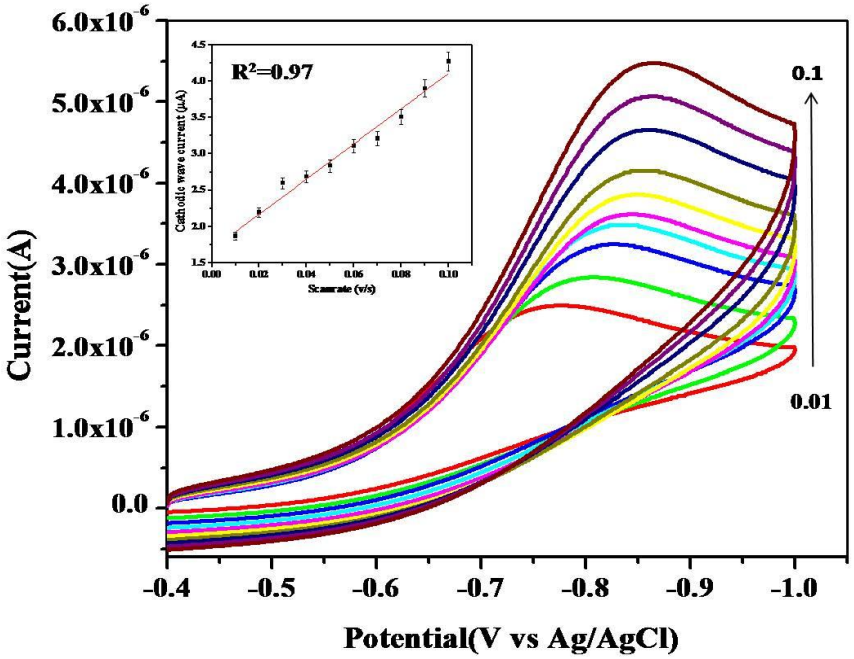


Fig.3 Cyclic Voltammogram recorded for the GCE/Chit/ZnO/ChOx electrode at various scan rates from 0.01V/s to 0.1V/s in PBS of pH 8 containing 0.3mM Choline chloride. Inset: Plot of cathodic wave current (n=3) vs scan rate

The concentration range of choline for which a linear relationship could be obtained was investigated by recording the cyclic voltammograms of the GCE/ZnO/ChOxworking electrode at a scan rate of 0.01 V/s at various concentrations of choline at pH 8.0. As the concentration of choline was increased from 0.3 mM to 3 mM, a progressive decrease in the peak current was observed along with a slight shift in the peak potential. This shift is due to e^- transfer limitation. A plot of peak current versus concentration of choline exhibited a linear trend between the concentration range of 0.3 mM and 3 mM with a regression coefficient of 0.94 (Figure 4).

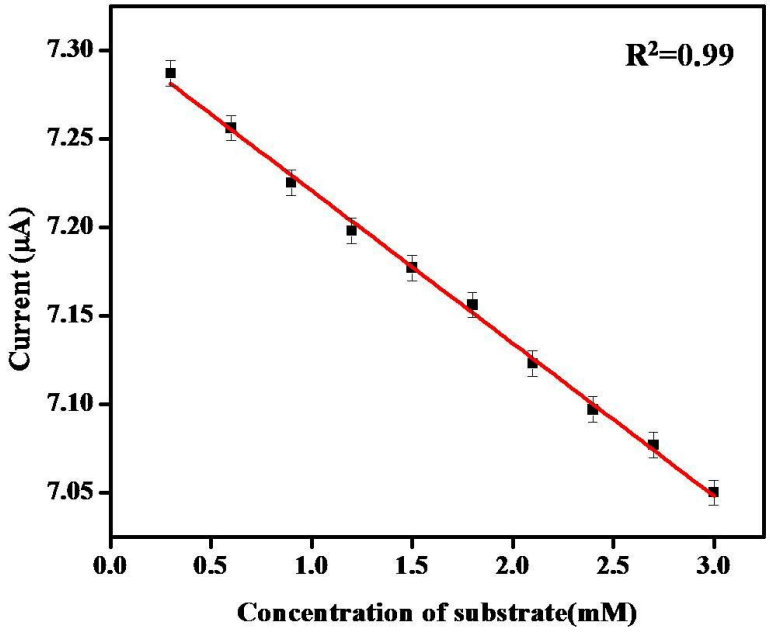


Fig. 4 Plot of peak current (n=3) vs concentration of choline.

Amperometric studies were performed at a potential of -0.76V in an electrochemical cell containing 10 mL 0.1M PBS, pH 8.0 for increasing concentrations of choline and the results are

shown in Figure 5. A stepwise decrease in the current was observed with increasing concentrations of choline. Choline Oxidase, the enzyme used in this reaction converts choline to Betaine and H_2O_2 in the presence of oxygen.

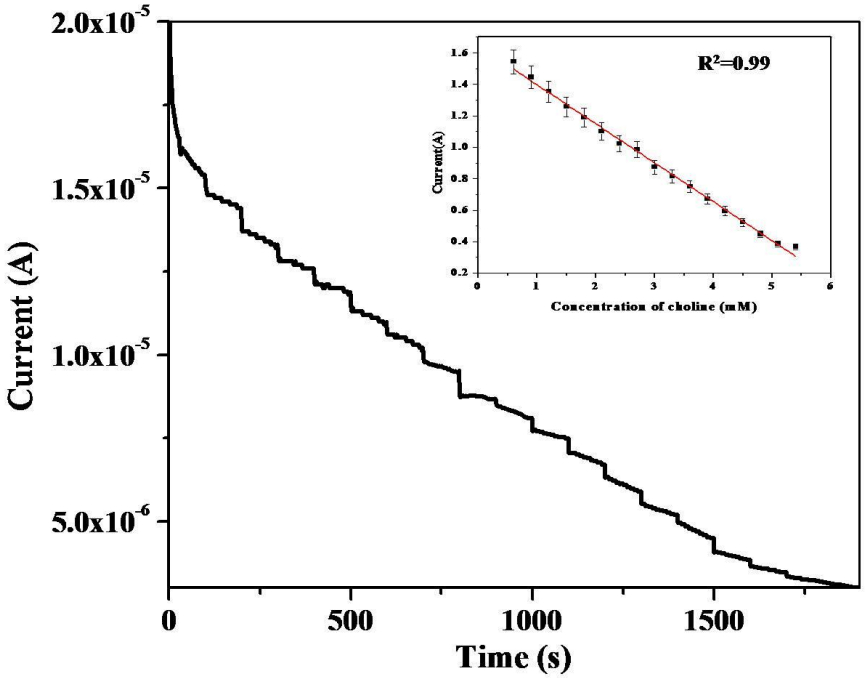


Fig.5 Amperometric response of the GCE/Chit/ZnO/ChOx electrode to successive addition of 0.3mM choline chloride to PBS at a constant potential of -0.76 V/s vs Ag/AgCl without stirring. Inset: Plot of Amperometric current vs choline concentration.

The decrease in current is due to the competition for electrons by O_2. The step-like pattern indicates that the choline molecules diffuse from the electrolyte into the surface of the GCE/ZnO/ChOx electrode through the chitosan layer and bind to the active sites of the immobilized enzyme. A linear response was obtained for the choline concentrations ranging

from 0.3 mM to 5.1 mM. The regression coefficient, R^2, for the amperometric measurements carried out under unstirred conditions was found to be 0.99.

The same study was performed with constant stirring of the electrolyte between the time intervals. The current versus time pattern was distinctly different from that recorded under unstirred conditions (Figure 6). An initial current spike was observed on addition of choline which then decreased and stabilized to a constant value. Progressive additions of choline however decreased the current flux. The appearance of the current spike is due to the convection-induced transport of the charged choline moieties to the electrode surface. The choline then gets oxidized to betaine due to the catalytic action of the immobilized choline oxidase on the electrode surface resulting in a decrease in the current that attained constancy within 200s. Therefore, the time interval between successive additions of choline was fixed as 200 s for subsequent trials. The linear range of detection under stirred conditions was from 0.3 mM to 5.7 mM with a regression coefficient of 0.97. As the linear response obtained in both stirred and unstirred conditions was similar, further trials were conducted in unstirred conditions as it exhibited higher linearity.

The sensor exhibited a rapid response to the addition of choline, which is evident from the very low response time of <4s. This quick response time is superior to several reports available in literature (Table 2).

Limit of detection and limit of quantification for the choline biosensor was determined using the following equations:

Limit of Detection = 3× SD/ S

Limit of Quantification = 10× SD/ S.

where ‘SD’ represents standard deviation and ‘S’ represents sensitivity of the sensor.

The sensor parameters calculated for detection of choline under stirred and unstirred conditions are summarized in Table 2.

Sensor parameter	Unstirred condition	Stirred condition
Limit of detection (mM)	0.647	0.978
Limit of quantification (mM)	1.934	2.96
Response time (s)	4	< 4
Linear range (mM)	0.3 - 5.1	0.3-5.7
Sensitivity (µA/mM)	0.28	0.64

Table 2: Characteristics of designed choline biosensor

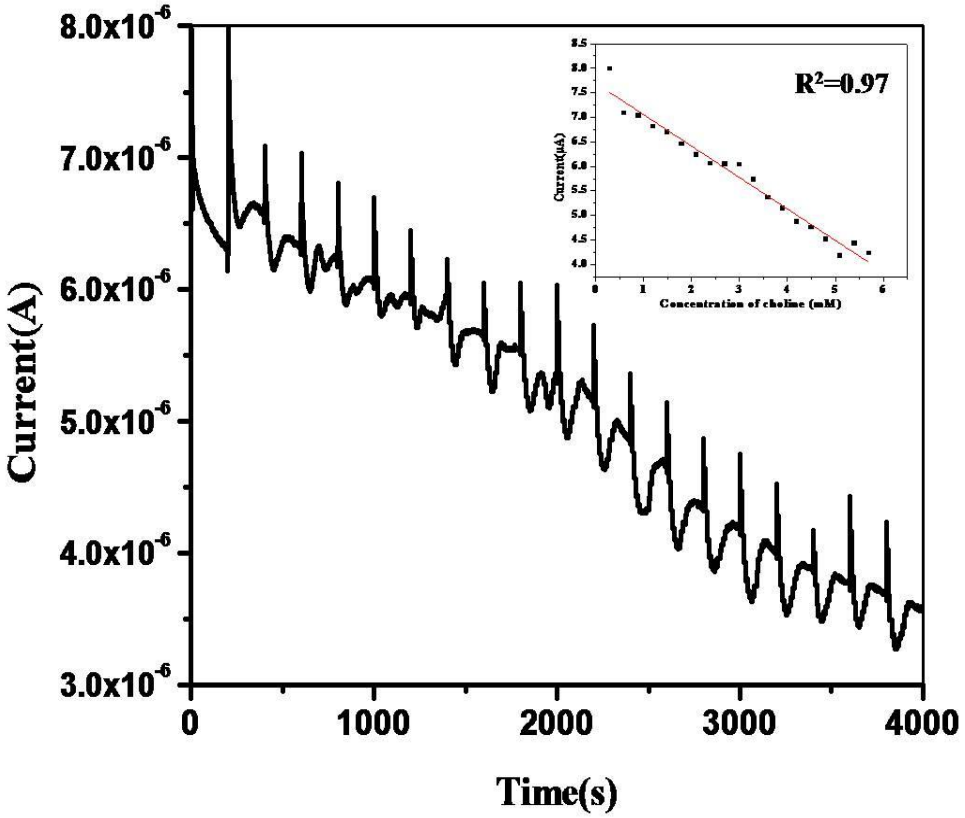


Fig.6 Amperometric response of the GCE/Chit/ZnO/ChOx electrode to successive addition of 3 mM choline chloride to PBS at a constant potential of -0.76 V/s vsAg/AgCl with stirring. Inset: Plot of Amperometric current vs choline concentration.

Stability and Precision

The stability of the working electrode under dry and wet conditions was investigated daily for a period of 2 weeks using cyclic voltammetry. For wet stability studies, the electrode was stored in 0.1 M PBS (pH 8) at 4ºC while the electrode was stored in a dry condition at 4^{o}C for dry stability studies. The wet stability was found to be 11 days and dry stability of the electrode was 12 days beyond which significant decrease in the current flow was observed. The repeatability of the current values obtained for a given set of conditions was determined by recording the cyclic voltammograms at 0.01 V/s in the presence of 0.3 mM choline at different time intervals. The relative standard deviation (RSD) was found to be 4.4 % which reflects the good fabrication process of the electrode.

Effect of interferents on the sensor performance

The effects of common interferents like citric acid and uric acid on the sensor performance was investigated using amperometry. Decimolar quantities of each interferent were added during measurement. The percentage of inhibition was calculated and showed in Fig.7. It was less than 8% for ascorbic acid, uric acid and citric acid suggesting that the sensor exhibits good selectivity towards choline.

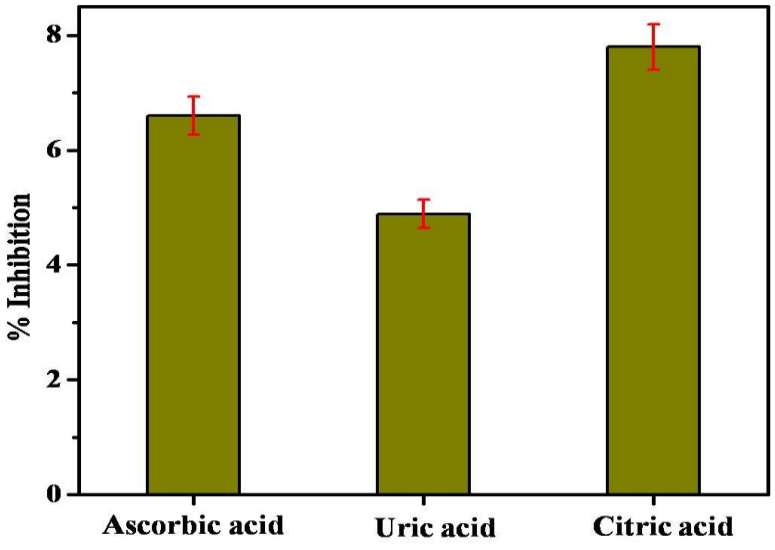


Fig.7 Interference study for the working electrode performed in 0.1M PBS (pH 8).

Working electrode	**Linear range (µM)**	**LOD**	**Stability (Days)**	**Response Time (Sec)**	**Sample**	**Ref.**
AchE-ChOx/MWCNT/ZrO_2	0.05-200	0.01	60	< 4	**Blood serum**	[4]
ChOx/MWCNT	5-100	0.1	30	<8	**Blood serum**	[30]
ChOx/CPME	1-50	0.1	40	<5		[31]
ChOx/HRP/CPME	1-80	0.4	55	<5		[31]
ZnO-NR	5-200		30		Milk	[15]
ChOx/HRP	50-700	10		80	**Rat cells**	[32]
AchE/ZnO/MWCNT	1-800	0.3	90	13		[33]
PDDA/ChOx/Au/MWCNT	1-500	0.3	30	7		[34]
This work	**300-5100**	**82.5 µM**	**14**	**3s**	**Breast cancer cells**	**This work**

Table 3: Comparision of characterictics of designed choline biosensor

Table 3 provides a comparison of the sensor characteristics of several choline sensors reported recently. A scan of literature reveals that reports on nano-interfaced choline sensors have employed carbon-based interfaces such as graphene and carbon nanotubes. In comparison, the zinc oxide interfaced choline oxidase working electrode reported in the present work exhibits more rapid response time, better sensitivity and can detect up to 5.1 mM concentration of choline compared to previous reports [4].

Cell culture studies

The nanointerfaced choline sensor was used to find the choline levels in triple negative breast cancer cells. The cells were lysed, centrifuged and the supernatant was analyzed for choline content using amperometry. Supernatants from different seeding densities of cells were used for the study and the results are shown in Figure 8. The amperometric plot depicts a step-wise decrease in the current for successive additions of samples from higher seeding densities suggesting an increased choline content in these cells.

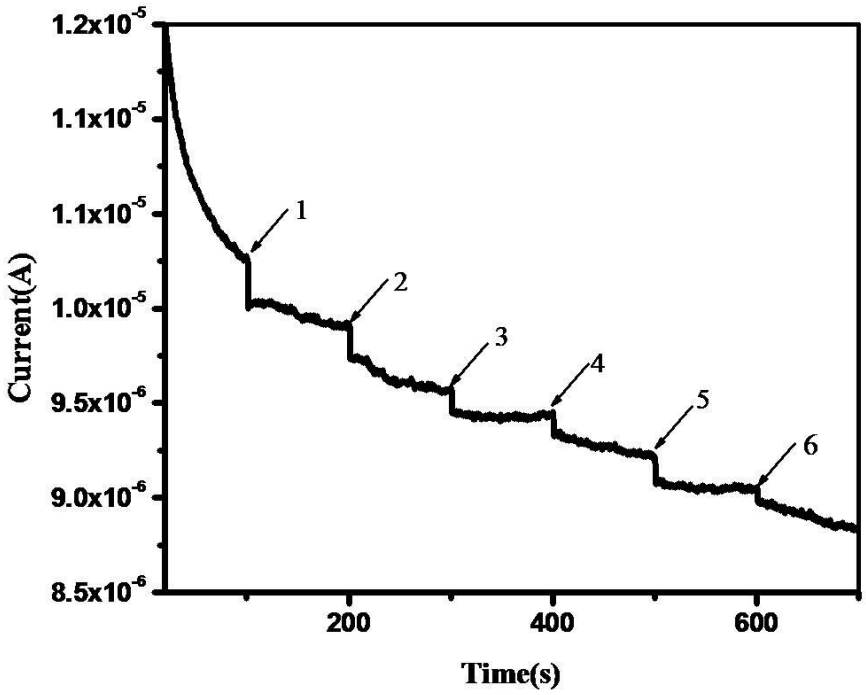


Fig.8 Amperometric response of the GCE/Chit/ZnO/ChOx electrode to successive addition of samples to PBS at a constant potential of -0.76 V/s vs Ag/AgCl. [Sample 1= 1 lakh cells, Sample 2= 1.5 lakh cells, Sample 3= 2 lakh cells , Sample 4= 2.5 lakh cells, Sample 5= 3 lakh cells, Sample 6= 3.5 lakh cells]

The concentration of choline in the cells was calculated from the calibration plot obtained using standard choline concentrations is shown in Supplementary Figure 1 and the results are tabulated in Table 4 and the plot for the same is shown in Supplementary Figure 2.

Cell density (x10^5)	Amperometric current (μA)	Substrate concentration (mM)
1.0	10.28	2.148
1.5	9.918	2.287
2.0	9.580	2.428
2.5	9.454	2.481
3.0	9.217	2.580
3.5	9.055	2.648

Table 4: Calculated concentration of choline using amperometric response for different cell densities.

The linear trend observed with increasing cell densities suggests that this sensor can be explored in future as a diagnostic tool for diagnosis of triple negative breast cancer which has been reported to display significantly high levels of choline when compared to other forms of breast cancer.

Conclusion

We have successfully designed and developed a ZnO interfaced biosensor by immobilizing ChOx for the detection of choline in breast cancer cells. Cyclic voltammetric studies reveal superior electron transfer capacity between ZnO and enzyme when compared to the enzyme-coated electrode. The developed biosensor shows a linear range of 0.3 mM to 5.1 mM. The detection limit (LOD) and quantification limit (LOQ) were 0.58 mM and 1.93 mM respectively. The developed biosensor exhibited a rapid response of 4s towards choline. The biosensor was further used to determine choline levels in triple negative breast cancer cells at various seeding densities. A linear increase in choline levels was obtained with increasing cell densities of TNBC. As TNBC is an aggressive form of breast cancer, the designed biosensor can facilitate its rapid and early detection through measurements of choline levels – a facet that can be explored as a diagnostic tool.

Acknowledgements

The authors wish to acknowledge the financial and infrastructural support from SASTRA University. Authors also wish to thank DST-FIST (SR/FST/LSI-453/2010) for funding towards

cell culture facility. Sasya Madhurantakam is thankful to Department of Science and Technology (DST) for the INSPIRE fellowship (DST/IF/120812).

[1] S.-B. He, G.-W. Wu, H.-H. Deng, A.-L. Liu, X.-H. Lin, X.-H. Xia, W. Chen, Biosensors & Bioelectronics 62 (2014) 331–6.

[2] S.H. Zeisel, J.K. Blusztajn, Annual Review of Nutrition 14 (1994) 269–296.

[3] L. Pinotti, Vitamin-like Supplementation in Dairy Ruminants: The Case of Choline, INTECH Open Access Publisher, 2012.

[4] S. Pundir, N. Chauhan, J. Narang, C.S. Pundir, Analytical Biochemistry 427 (2012) 26–32.

[5] J.O. Rinne, T. Myllykyla¨, P. Lo¨nnberg, P. Marjama¨ki, Brain Research 547 (1991) 155–158.

[6] J. Apelt, A. Kumar, R. Schliebs, Brain Research 953 (2002) 17–30.

[7] M. Klingner, J. Apelt, A. Kumar, D. Sorger, O. Sabri, J. Steinbach, M. Scheunemann, R. Schliebs, International Journal of Developmental Neuroscience 21 (2003) 357–369.

[8] T.J. Rowley, A. McKinstry, E. Greenidge, W. Smith, P. Flood, British Journal of Anaesthesia 105 (2010) 201–207.

[9] X. Xu, M.D. Gammon, S.H. Zeisel, Y.L. Lee, J.G. Wetmur, S.L. Teitelbaum, P.T. Bradshaw, A.I. Neugut, R.M. Santella, J. Chen, The FASEB Journal 22 (2008) 2045–2052.

[10] É. Ledoux, E. Cloutier, Relations Industrielles 67 (2012) 171.

[11] S. Cleator, W. Heller, R.C. Coombes, The Lancet. Oncology 8 (2007) 235–44.

[12] J.-H. Chen, G. Agrawal, B. Feig, H.-M. Baek, P.M. Carpenter, R.S. Mehta, O. Nalcioglu, M.-Y. Su, Annals of Oncology 18 (2007) 2042–2043.

[13] P.J. Bolan, S. Meisamy, E.H. Baker, J. Lin, T. Emory, M. Nelson, L.I. Everson, D. Yee, M. Garwood, Magnetic Resonance in Medicine 50 (2003) 1134–1143.

[14] Y.-H. Bai, Y. Du, J.-J. Xu, H.-Y. Chen, Electrochemistry Communications 9 (2007) 2611–2616.

[15] Z. Chen, X. Ren, X. Meng, D. Chen, C. Yan, J. Ren, Y. Yuan, F. Tang, Biosensors & Bioelectronics 28 (2011) 50–5.

[16] H. Li, Y. Guo, L. Xiao, B. Chen, Biosensors & Bioelectronics 59 (2014) 289–292.

[17] M. Pohanka, C. Republic, Methods 6 (2008) 57–64.

[18] S. Park, H. Boo, T.D. Chung, Analytica Chimica Acta 556 (2006) 46–57.

[19] R.M. Town, H. Kipton J. Powell, Analytica Chimica Acta 279 (1993) 221–233.

[20] B. Wang, B. Li, Q. Deng, S. Dong, Analytical Chemistry 70 (1998) 3170–3174.

[21] S. Madhurantakam, S. Selvaraj, N. Nesakumar, S. Sethuraman, J.B. Balaguru Rayappan, U. Maheswari Krishnan, Biosensors and Bioelectronics 59 (2014) 134–139.

[22] W. Chen, S. Cai, Q.-Q. Ren, W. Wen, Y.-D. Zhao, The Analyst 137 (2012) 49–58.

[23] W. Zhao, Y. Ni, Q. Zhu, R. Fu, X. Huang, J. Shen, Biosensors and Bioelectronics 44 (2013) 1–5.

[24] J. Xie, H. Wang, M. Duan, L. Zhang, Applied Surface Science 257 (2011) 6358–6363.

[25] D. Sun, H. Zhang, Microchimica Acta 158 (2007) 131–136.

[26] S. Bin He, G.W. Wu, H.H. Deng, A.L. Liu, X.H. Lin, X.H. Xia, W. Chen, Biosensors and Bioelectronics 62 (2014) 331–336.

[27] Z. Song, J.D. Huang, B.Y. Wu, H. Bin Shi, J.I. Anzai, Q. Chen, Sensors and Actuators, B: Chemical 115 (2006) 626–633.

[28] M.A. Rahman, D.S. Park, Y.B. Shim, Biosensors and Bioelectronics 19 (2004) 1565–1571.

[29] S. Pal, M.K. Sharma, B. Danielsson, M. Willander, R. Chatterjee, S. Bhand, Biosensors and Bioelectronics 54 (2014) 558–564.

[30] S.S. Razola, S. Pochet, K. Grosfils, J.M. Kauffmann, Biosensors and Bioelectronics 18 (2002) 185–191.

[31] L. Zhang, J. Chen, Y. Wang, L. Yu, J. Wang, H. Peng, J. Zhu, Sensors and Actuators, B: Chemical 193 (2014) 904–910.

[32] X. Qin, H. Wang, X. Wang, Z. Miao, L. Chen, W. Zhao, M. Shan, Q. Chen, Sensors and Actuators, B: Chemical 147 (2010) 593–598.

Graphical abstract: