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A miniaturized nanobiosensor for choline analysis

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ABSTRACT

A novel reusable chemiluminescence choline nanobiosensor has been developed using aligned zinc oxide nanorod-films (ZnONR). The chemically fashioned ZnONR were synthesized by hybrid wet chemical route onto glass substrates and used to fabricate a stable chemiluminescent choline biosensor. The biosensor was constructed by co-immobilization of the enzymes choline oxidase and peroxidase. The covalent immobilization of the enzymes on the ZnONR was achieved using 16-phosphonohexadecanoic acid as a cross-linker. The phosphonation of the ZnONR imparted significant stability to the immobilized enzyme as against physisorbed enzyme. A lower value of Michaelis–Menten constant (K_m), of 0.062 mM for the covalently coupled enzyme over the physisorbed enzymes facilitated enhanced stability of ZnONR nanobiosensor. The ZnONR-choline biosensor has been investigated over a wide range of choline from 0.0005 mM to 2 mM. Importantly, the recovery of choline in milk samples was close to 99%. Using the developed biosensor, choline was measurable even after 30 days with 60 repeated measurements proving the stability of the sensor (Intraday RSD%=2.83 and Interday RSD%=3.51).

1. Introduction

Milk, containing high level of choline, is an essential nutritional food for all age group (Phillips, 2012). Choline, as “vitamin-like”, is synthesized in the human body, but dietary supplementation is necessary to maintain proper function (Blusztajn, 1998; Zeisel, 2000). Choline has essential roles in brain development and memory function for infants as well as adults. It also has an important role to maintain the central nervous system and numerous metabolic functions in the body such as (a) methyl donor, (b) a precursor of the signaling lipids, platelet-activating factor and sphingosylphosphoryl choline and (c) as a precursor for acetylcholine, phosphatidyl choline and sphingomyelin biosynthesis (Dietary Reference Intakes, 1998). The adequate intake for choline ranges from 125 mg/day in infants to 550 mg/day in males over age 14 years and in breast-feeding women (Michel et al., 2006). Neurodegenerative disorders such as Alzheimer's, Parkinson's diseases and an increased risk of lethal prostate cancer have been implicated owing to abnormal metabolism of choline (Zeisel and Blusztajn, 1994; Richman et al., 2012). Since infant's nutritional intake is limited to a single source in general milk, choline supplementation is critically important. Therefore, the need for the development of a sensitive and efficient method for the estimation of the choline level in milk is extremely important.

Enzymatic choline biosensors based on thermal (Deshpande et al., 2011), colorimetric (Takayama et al., 1977), fluorimetric (Chen et al., 2011; Li et al., 2013) and electrochemical detection (Qin et al., 2010; Shimomura et al., 2009) have been reported for choline analysis. The reported choline biosensors use the enzyme choline oxidase (COD) that catalyzes choline in the presence of oxygen and produces hydrogen peroxide. Thus, one is able to measure choline by quantifying hydrogen peroxide using chemiluminescence technique. Owing to low background, high sensitivity, low cost of instrumentation and suitability for miniaturization in analytical chemistry, chemiluminescence (CL) has been recognized as one of the most useful analytical techniques (Dodeigne et al., 2000).

Recently, nanostructured metal oxides have been extensively reported for the construction of novel biosensors. The nanostructured materials provide upper limits in balancing the key factors that determine the efficiency of biocatalysts including surface area, mass transfer resistance and effective enzyme loading (Solanki et al., 2011). Among the reported nanostructured materials, zinc oxide (ZnO) has attracted considerable attention owing to wide band gap, strong excitation binding energy, esthetic morphologies

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and multifunctional properties (Singh et al., 2007). The nanostructured ZnO also possesses several advantages for biosensing owing to the wide optical emission spectra, high aspect ratio, polar surface along on the top of the rods, good electron communication and nontoxicity. Notably, the isoelectric point (IEP) of ZnO is as high as about 9.5 i.e. suitable for immobilization of the enzymes and proteins (Topoglidis et al., 2001). So far, various ZnO nanostructures such as nanoparticles, porous films, nanocombs and nanorods have been deployed for development of biosensors (Ahmad and Zhu, 2011; Arya et al., 2012). ZnO nanostructures have also been demonstrated to detect cytochrome *c* (Topoglidis et al., 2001), protein (Huang and Lee, 2008), uric acid (Zhang et al., 2004), glucose (Wang et al., 2006; Wei et al., 2006), phenol (Gu et al., 2008), urea (Solanki et al., 2008) and cholesterol (Israr et al., 2011).

Enzyme immobilization on the transducer surface is a critical step in the design of biosensors as enzyme molecules must retain their activity after the immobilization in order to achieve enhanced stability and shelf life. Self-assembled monolayers (SAMs) provide versatility and novel properties to surfaces and interfaces by conjugating biomolecules to facilitate a low-cost method of fabricating miniaturized biosensor devices with improved enzyme activity, stability and selectivity (Ulman, 1996; Mateo et al., 2007; Samanta and Sarkar, 2011). A unique approach to utilizing CL technique for the detection of acetylcholine has been reported using enzyme coupled multiple-branched nanostructures (Risveden et al., 2010). Moreover, carboxylic acid, phosphonic acids, alkyl phosphonic acids and phosphoric acids (Laibinis et al., 1989; Textor et al., 2000; Pawsey et al., 2002) have been reported to form favorable functionalized surfaces of metal oxides. Alkyl phosphonates and phosphonic acid can form well-packed SAMs with excellent thermal and hydrolytic stability. Moreover, these SAMs are reported to exhibit much improved binding over carboxylic acids to a wide range of metal oxides (Zhang et al., 2010).

Herein, we present for the first time, construction of a sensitive chemiluminescence choline nanobiosensor using bi-enzyme coupled zinc oxide nanorod-films (ZnONR) via SAMs of 16-phosphonohexadecanoic acid (16-PHA). The developed choline nanobiosensor exhibited good selectivity, sensitivity, molecule capturing efficiency and stable output response over the other reported biosensors (Razola et al., 2003; Chen et al., 2011; Li et al., 2013). The remarkable feature of the presented biosensor is its excellent stability and reusability over the period of 30 days with 78% retention of enzyme activity. Moreover, the presented biosensor also provided a much broader working range for choline analysis (0.0005–2 mM). The application of the developed nanobiosensor for analysis of choline in milk is successfully demonstrated.

2. Materials and methods

2.1. Materials and instrumentation

Choline oxidase (EC 1.1.3.17) from Alkaligenes sp. (COD), peroxidase (EC 1.11.1.7) from Horseradish (HRP), choline chloride (ChCl), Zinc nitrate hydrate ($Zn(NO_3)_2$, xH_2O), Sodium hydroxide (NaOH) 99.99% trace metals basis, 16-phosphonohexadecanoic acid (16-PHA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), *N*-hydroxy succinimide (NHS) Tween-20 and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were purchased from Sigma Chemical Co. (USA). Sodium phosphate dibasic, sodium phosphate monobasic and other chemicals were of GR grade, Merck (Germany). Centrifugation, shaking and filtration of the samples were done by Spinwin mini centrifuge, Spinix shaker purchased from Tarsons (India). White 384 well polystyrene microtiter plates were purchased from Nunc (Denmark). A Rigaku MiniFlex X-ray diffraction (XRD) using Cu K_α radiation was used to obtain the structural information. Raman spectra were recorded using Renishaw inVia micro-Raman spectrometer using 514 nm Argon laser. Fourier Transform Infrared (FT-IR) spectra were recorded using IRAffinity-1 (SHIMADZU, Japan) with attenuated total reflectance (ATR) attachment Specac Diamond ATR AQUA. For chemiluminescence measurement, Victor™ X4 2030 Opti-plate reader Perkin Elmer (USA) was used. Water produced in a reverse osmosis system (arium61316, Sartorius, Germany) was used for preparing all the solutions. Certified ultra-high pure nitrogen (99.9%), pH meter (Seven Multi Mettler Toledo, 8603, Switzerland) were used. Commercial milk samples of different fat contents were purchased from the local supermarket of Goa, India.

2.2. Sample preparation

Phosphate buffer (PB) 100 mM, pH 7.4 was prepared by mixing 100 mM of sodium di-hydrogen phosphate monohydrate and 100 mM of disodium hydrogen phosphate monohydrate in deionized (DI) water. PB was degassed before analysis. A stock solution of 100 mM ChCl was prepared by dissolving 0.139 g in 10 mL of PB (100 mM, pH 7.4). Working solution of ChCl was prepared freshly before daily use. Stock enzyme solutions were prepared in 100 mM PB (pH 7.4). Luminol solution was prepared by dissolving 4 mg of luminol in 2 mL 100 mM NaOH and making up the volume to 20 mL by 100 mM PB, pH 7.4.

For the determination of free choline a 5 mL aliquot of raw milk was added with 15 μL of Tween-20 (to give a final concentration of 0.3% v/v). To minimize the matrix effect, the solution was centrifuged for 10 min at 12 000 rpm at room temperature. A 1:4 (v/v) final dilution with PB was made prior to analysis so that the amount of choline lies in the calibration range. The developed protocol using dilution/centrifugation/filtration with 0.22 μM filter (Whatman, USA) we used to separate the milk fat.

2.3. Preparation of ZnONR film

Vertically aligned ZnONR films were deposited by hybrid wet chemical route at room temperature onto glass substrate by high pressure sputtering at ~30 Pa argon pressure with pre-deposited ZnO seed particles. The detailed method of preparation has been described elsewhere (Dalui et al., 2008). The ZnONR films thus produced were used in the construction of biosensor as shown in Fig. 1.

2.4. Fabrication of choline biosensor

Fabrication of choline biosensor is based on the efficient immobilization of bi-enzyme through SAMs. The glass containers used for monolayer preparation were cleaned with Piranha solution (a mixture of 98% H_2SO_4 and 30% H_2O_2, 7:3, v/v; caution: piranha solution reacts exothermally and strongly reacts with organics) for 1 h and rinsed exhaustively with deionized (DI) water and ethanol. ZnONR thin film was washed with ethanol, and dried under a stream of high purity nitrogen before use. These samples were immersed into 0.5 mM ethanolic solution of 16-PHA for 72 h to achieve self-assembly. The SAMs functionalized ZnONR were again rinsed in ethanol followed by DI water and dried under a stream of nitrogen. For enzyme immobilization, the carboxylic acid-terminated SAMs were modified using an aqueous equimolar solution of 100 mM EDC/100 mM NHS for 2 h at room temperature. The resultant NHS ester monolayers were reacted for 3 h in a solution of COD (1 IU/μL) and HRP (0.1 IU/μL) in PB (100 mM, pH~7.4). The covalently coupled bi-enzyme (COD/HRP) ZnONR thin film was taken out from solution and washed as described

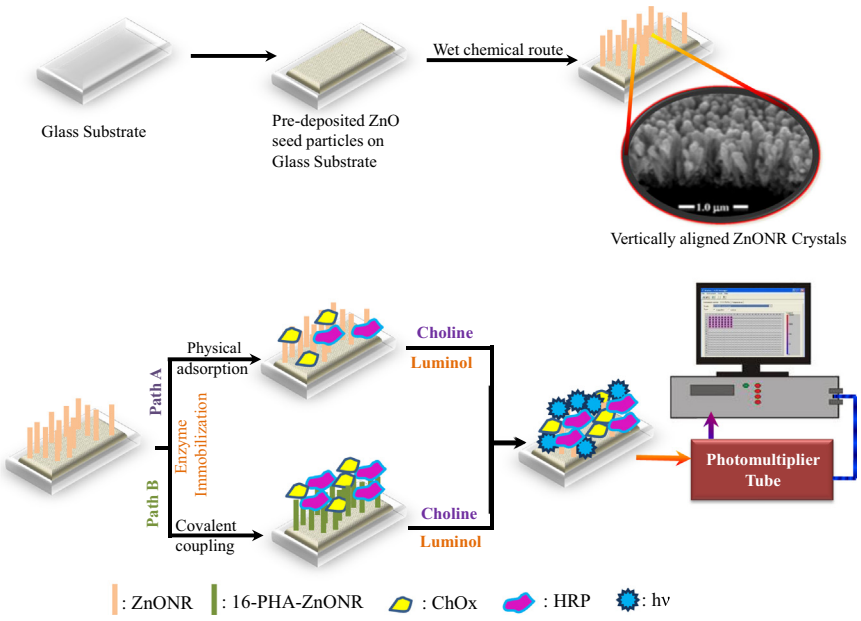

Fig. 1. Schematic pathway for construction along with working mechanism of the ZnONR choline biosensor during measurements for the attachment of bi-enzyme (COD/HRP) through physisorption (Path A) and covalent coupling (Path B) on ZnONR.

earlier. Similarly, same amount of the enzyme was also incubated on the ZnONR for 12 h to prepare physisorbed COD/HRP ZnONR films. The schematic of the enzyme immobilization procedure is shown in Fig. 1.

In the presented biosensor, COD provides the specificity and acts as a catalyst to initiate the bi-enzyme reaction. The enzyme catalytic reaction between COD and substrate ChCl solution produces the betaine aldehyde and hydrogen peroxide (H_2O_2) as product of this reaction. The produced H_2O_2 in the reaction is highly susceptible to undergo spontaneous reaction with second enzyme i.e. co-immobilized HRP. H_2O_2 thus produced was quantified using CL technique wherein, the numbers of photons emitted in presence of luminol were counted using a sensitive photomultiplier system Victor™ X4 2030.

3. Results and discussions

3.1. Characterization of ZnONR thin films

The XRD measurements (Supplementary Fig. S1) confirmed the polycrystalline nature of ZnONR with preferential [002] direction orientation. Raman spectroscopy offers a non-destructive tool for obtaining information about both the local and the non-local vibrational states related to the structure of ZnO. The reported Raman spectra of ZnONR film are shown in the inset of Supplementary Fig. S1. The spectrum is dominated by the presence of a strong sharp peak located at ~438 cm^{-1} identifying the good crystalline quality of our ZnONR films. The peak at ~438 cm^{-1} may be identified as high frequency branch of E_2 mode of ZnO. The broad and asymmetric nature of this peak is typical of the Raman active mode specially observed in wurtzite structure. The peak is preceded by peaks at ~332 cm^{-1} and ~378 cm^{-1} as reported [for ZnO films (Dalui et al., 2008). There is only one peak at ~577 cm^{-1} in the higher wave number region. The peak at ~577 cm^{-1} could be attributed to the A_1 longitudinal optical (LO) mode of ZnO. Origin of the above mode may be due to Zn interstitials present in the films. Raman spectroscopy results of ZnONR thus help us to conclude that these nanorod-films are of good crystalline quality with some Zn interstitials present in the film.

This technique is essentially a hybrid technique where we easily manipulate the size and distribution of the ZnO seed crystallites that in turn modulate the growth of ZnONRs. Thus, the ZnONR used in this work are [002] aligned polycrystalline as can be concluded from our XRD and Raman measurements.

3.2. Spectroscopic characterization by FT-IR

FT-IR spectroscopy was applied to measure the surface properties of the bi-enzyme COD/HRP coupling through SAMs via the carbodiimide cross-linking reaction on ZnONR surfaces. For the ZnONR samples, FT-IR ATR spectra were collected at a resolution of 2 cm^{-1} (128 scans). The FT-IR spectra recorded for ZnONR coupled enzyme (COD/HRP) via 16-PHA crosslinker are shown in Fig. 2(a). Fig. 2(a) (i), FT-IR spectrum of the bare ZnONR. Fig. 2(a) (ii), FT-IR spectra 16-PHA functionalized ZnONR. Fig. 2(a) (iii), FT-IR spectrum of covalently coupled enzyme on ZnONR.

Fig. 2(a) (ii) shows the asymmetric (ν_{as}(C–H)) and symmetric methylene stretches (ν_s(C–H)) of 16-PHA-modified ZnONR that appear at 2925 cm^{-1} and 2853 cm^{-1} respectively. These bands of 16-PHA modified ZnO suggest methylene chains during monolayer growth.

The C=O stretch at 1724 cm^{-1} on ZnONR associated with asymmetric and symmetric COO^- stretches at 1647 cm^{-1} and 1542 cm^{-1} respectively are characteristics of an organic carboxylic compound. The additional IR peaks at 1455 cm^{-1}, 1294 cm^{-1} and 1216 cm^{-1} are ascribed to C–H deformation and C–O stretch respectively. In the P–O stretching region (Fig. 2(a) (ii)), the binding mode information of phosphonic acids onto metal oxides is not easy to obtain. The peaks in the P–O stretching region appear between 1300 cm^{-1} and 800 cm^{-1} and the presence of several different binding modes in phosphonic acid monolayers is indicated by the presence of residual P=O and P–O–H sites. The peak located at 945 cm^{-1} in the spectrum of 16-PHA is assigned to a P–OH band that is observed only after binding to the ZnO surface. This indicates that the phosphonic acids bind to the surface Zn–OH group via condensation reactions. The linkage to the ZnO surface mode should be a tridentate chelating binding

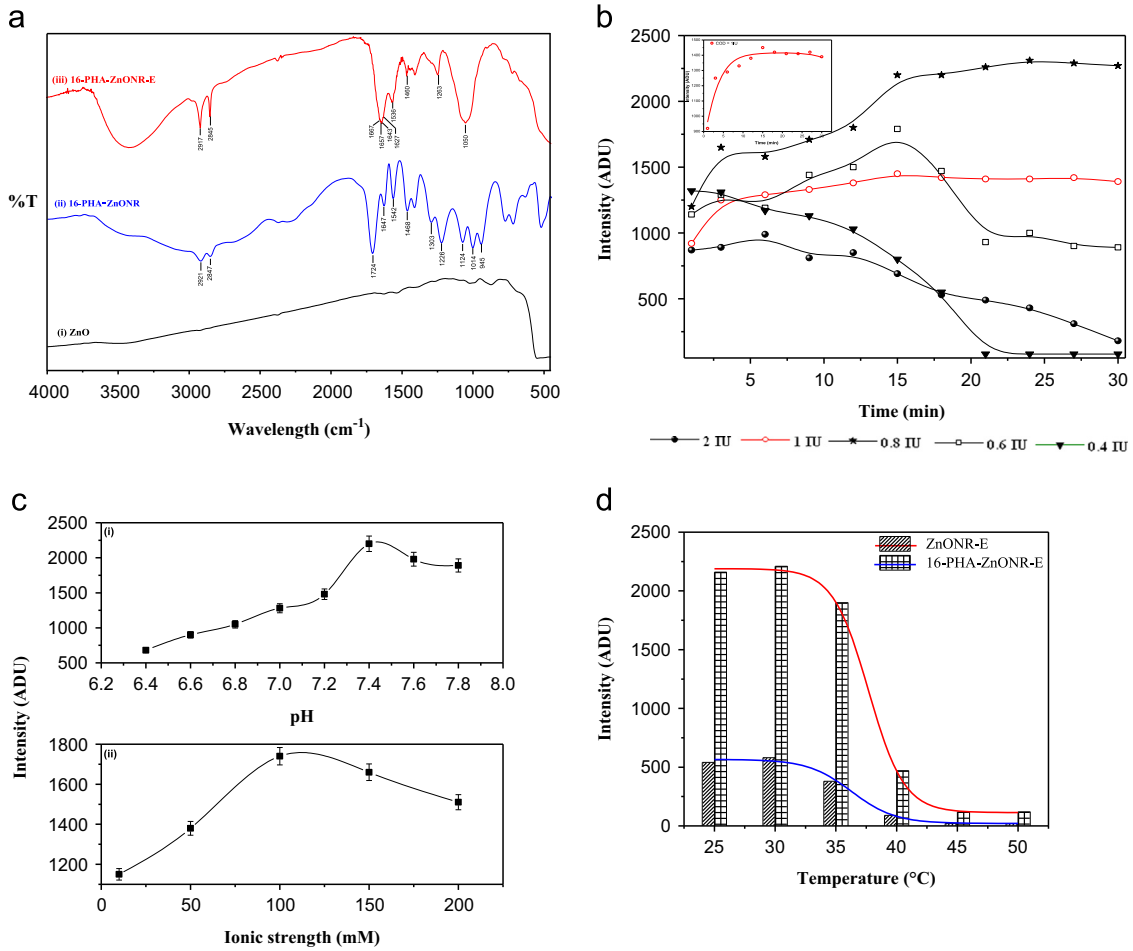

Fig. 2. (a) The ATR FT-IR spectrum of (i) the ZnONR film, (ii) ZnONR treated with 16-PHA, (iii) Enzyme (COD/HRP) coupled through 16-PHA functionalized ZnONR. Spectra were acquired using 128 scans at 2 cm^{-1} resolution collected under vacuum conditions. (b) Influence of COD concentration; 0.4 IU/μL, 0.6 IU/μL, 0.8 IU/μL, 1.0 IU/μL and 2 IU/μL (all solutions are in 100 mM, pH∼ 7.4 PB; [ChCl]=1 mM) (Inset: The intensity change for [COD]=1 IU/μL). (c) (i) Effect of ionic strength of the PB (10, 50, 100, 150 and 200 mM) on the response of ZnONR choline biosensor for 1 mM ChCl; (ii) influence of the PB pH (6.4–7.8) on the response of ZnONR choline biosensor for 1 mM ChCl. (d) Influence of temperature on physisorbed ZnONR and 16-PHA-ZnONR in the range of 25–50 °C; 100 mM, pH∼7.4 PB; 1 mM ChCl.

mode or a bidentate binding moiety rather than the monodentate surface binding mode. The existence of the peak at 1124 cm^{-1}, which is characteristic of the P=O stretching mode, is a possible evidence for bidentate binding modes. The observation of P–OH related modes indicates that the binding of 16-PHA on ZnONR occurs mainly through the PO_3H_2 groups. Similar coordination of binding modes of organic phosphonic acids on ZnO was also confirmed by Zhang et al., 2010.

The enzyme COD/HRP coupling through 16-PHA SAMs via the carbodiimide cross-linking reaction on ZnONR surfaces is presented as Fig. 2(a) (iii). Upon immobilization of COD/HRP in Fig. 2 (a) (iii) new absorption bands appeared; three IR bands at 1643 cm^{-1}, 1536 cm^{-1}, and 1263 cm^{-1} are ascribed to the absorption by the amide group that links COD/HRP to 16-PHA functionalized ZnONR. Also the bands at 1667 cm^{-1}, 1657 cm^{-1} and 1627 cm^{-1} are for the amide I region which is the common structural unit for all biomolecules (Nakano et al., 2007).

3.3. Optimization of influential parameters of the choline biosensor system

CL biosensors based on luminol involve appropriate enzymatic reaction between substrate and enzyme and the subsequent reaction between luminol and enzymatically produced H_2O_2. A minor change in enzyme concentration, pH, ionic strength and temperature can sometimes be responsible to lose the biological activity, as immobilized enzymes are very much sensitive towards environmental changes.

3.3.1. Optimization of the COD units

As COD is the main enzyme that imparts selectivity for choline, thus it is needed to optimize the amount of COD used in the construction of ZnONR biosensor. Various concentrations of COD were tested in order to get a stable response of ZnONR biosensor keeping other parameters like temperature, pH, ionic strength and amount of HRP constant.

The optimal concentration of COD used in the CL ZnONR biosensor system was investigated by measuring the CL intensities in the presence of different concentration of COD and same quantity of the other reagents. Immobilized COD amount on the biosensor response was studied by varying the concentration of COD from 0.4 to 2 IU/μL keeping the HRP units constant (0.1 IU/μL). From Fig. 2 (b), it was found that the effect was enhanced with the increasing quantity of COD up to 1 IU/μL. The inset figure clearly shows the stable CL intensity measured after 15 min. A stable intensity for the 0.8 IU/μL and 1 IU/μL COD was found using 1 mM ChCl. However, the stability of signal intensity extended to varying concentrations of ChCl only when 1 IU/μL COD was used. Thus, in order to realize the system effectively, 1 IU/μL COD was used for further experiment.

Further, the reaction time of COD on ZnONR choline biosensor was optimized (Fig. 2(b)). The CL intensity of the mixture gradually increased to a maximum value within 15 min. The result confirmed that the catalyzed oxidation process of choline was finished in 15 min. Thus, 15 min was adopted as the incubation time for further experiments.

3.3.2. Optimization of ionic strength and pH

The influence of the ionic strength and pH of PB on the activity of the enzyme was studied. Measurements were carried out on ZnONR system with various ionic strength (10, 50, 100, 150 and 200 mM PB) and pH (6.4–7.8) for 1 mM ChCl. The results are presented in Fig. 2(c). It is evident from Fig. 2(c) (i) that with increase in the ionic strength of PB, response signal increases up to 100 mM, and then decreases. Thus, further experiments were carried out with 100 mM PB. It is also clear from Fig. 2(c) (ii) that COD/HRP exhibit maximum activity at pH 7.4. On the other hand, it is known that the ideal pH balance for a healthy body is slightly more alkaline than acid measuring. Therefore, the pH of 7.4 was selected as a good compromise for our experiment. Milk and PB were mixed in different ratios (1:1–1:12) for the analysis of pH and ionic strength. The pH gets stabilized for milk:PB at 1:4 dilutions.

3.3.3. Optimization of temperature

The activities of free and immobilized enzyme were measured at different temperatures in the range 25–50 °C to determine optimal temperature for enzyme activity. The optimum temperature was determined for free and immobilized enzymes and presented as Fig. 2(d). While the physisorbed enzymes showed low signal response in the studied temperature range (25–50 °C), the co-immobilized enzymes exhibited significantly higher activity in the same temperature range. Enzymes immobilized through 16-PHA exhibited the highest change over the free enzyme activity. Both physisorbed and immobilized enzymes loose activities in the temperature range 40–50 °C. The residual activity was 3.7% for the physisorbed enzymes and 5.5% for enzymes immobilized at the same temperature (50 °C). Above 35 °C, the enzyme activity dropped significantly due to the thermal inactivation.

3.3.4. Kinetic parameters

The enzyme activities were determined at different substrate concentrations under the optimum conditions. Michaelis–Menten constant (K_m) (Eq. 1) values of the immobilized enzymes were determined using LineWeaver–Burk plot:

$$1/V = K_m/V_{max} \times 1/[S] + 1/V_{max} \quad (1)$$

where V, V_{max}, [S] and K_m are initial velocity, maximum velocity, initial substrate concentration and Michealis–Menten constant respectively.

3.4. Effect of surface modification

The stability of the bienzymes (COD/HRP) coupled to the ZnONR was evaluated when enzymes were attached on the surface via covalent coupling as against the physically adsorbed COD/HRP. The kinetic intensity profile of the resulting signal was recorded as shown inFig. 3(a). The signal reached a stable value (95% of the steady state) after 15 min (which is also supported by Fig. 2(b)). The covalently attached enzyme showed significantly higher signal intensity over the physically adsorbed enzymes. The order of the stable signal intensity recorded for COD/HRP functionalized ZnONR was found to be 7880 ADU (via phosphonation) and 1070 ADU (physisorbed i.e. non-specifically adsorbed COD/HRP) respectively. The signal intensities were found to be almost seven times higher for phosphonated ZnONR as against physisorbed COD/HRP nanorods.

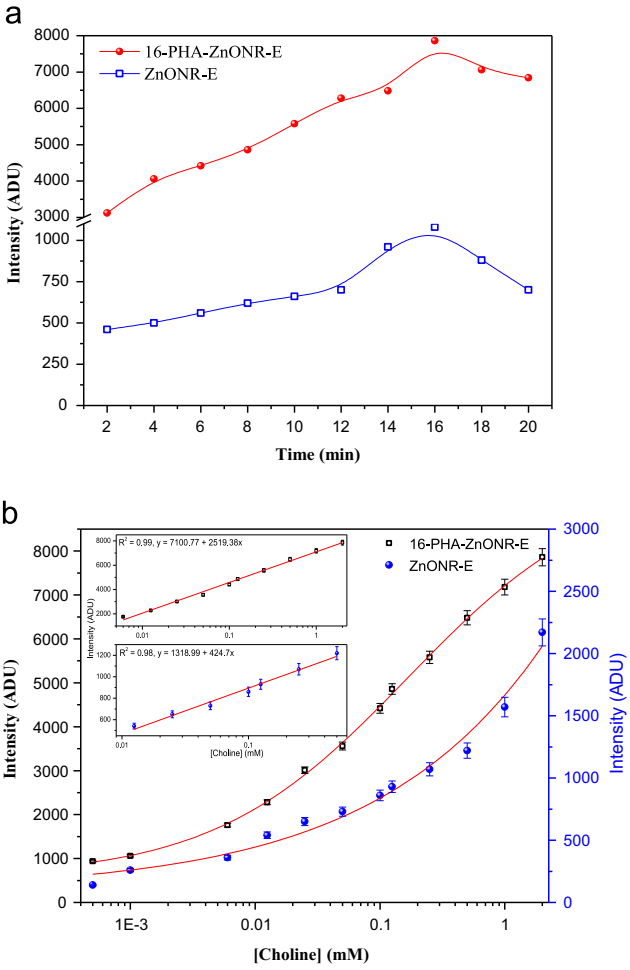

Fig. 3. (a) Comparison of the kinetic response of COD/HRP (covalently coupled against physically adsorbed) on ZnONR film. (b) Calibration curve for different choline concentrations obtained using 16-PHA-ZnONR coupled enzyme and physisorbed enzyme on ZnONR (Inset: the linear calibration graph).

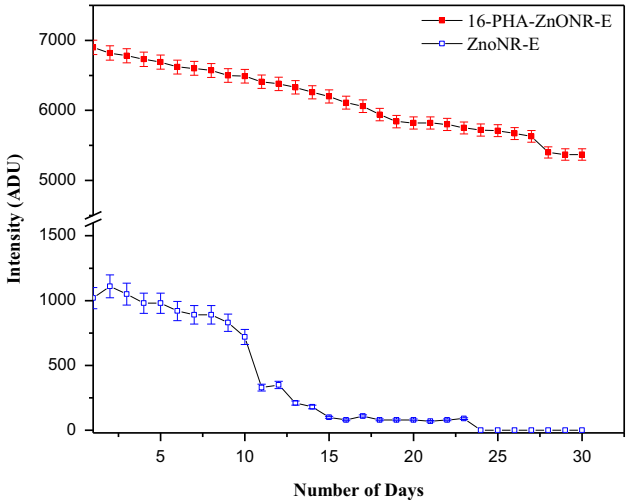

Fig. 4. The storage stability of ZnONR thin film modified with COD/HRP via16-PHA and physisorbed enzyme on ZnONR in a 100 mM, pH ~ 7.4 phosphate buffer solution over 30 days using choline biosensor each day showing its storages stability at 4 °C.

These important features of the presented biosensor play an important role to enhance efficacy and specificity towards the rapid signal transfer rate and bio-recognition properties.

3.5. Calibration of choline nanobiosensor

A calibration curve for choline (0.0005–2 mM), measured in triplicate, using ChCl as substrate is shown in Fig. 3(b). The calibration was found to be linear in the range 0.006–2 mM ($R^2=0.99$, $n=5$) with a relative standard deviation (RSD) of 4.1% $\pm$0.5 for the 16-PHA modified ZnONR (Fig. 3(b) Inset). The linear equation was $y=7100.77+2519.38x$ (mM). Limit of detection (LOD) was 0.0005 mM and limit of quantitation (LOQ) was 0.006 mM by analyzing replicate sets of 16-PHA modified ZnONR with good sensitivity about 193 ADU change per decade. Whereas for physisorbed enzyme ZnONR, the linearity was found in the range 0.5–0.0125 mM ($R^2=0.98$, $n=5$) with an RSD of 6.9% $\pm$ 0.8 (Fig. 3(b) Inset). The linear equation was $y=1318.99+424.7x$ (mM). LOD and LOQ were determined as 0.0125 mM by analyzing replicate sets with very low sensitivity about 19 ADU change per decade. Lower LOD, LOQ value, R^2 and sensitivity indicate that 16-PHA-ZnONR is much superior over physisorbed enzyme ZnONR. The reported linear range for the presented biosensor covers the free choline concentration (125 mg/day) found in infant formula, and thus the method can be adopted for routine measurements. Moreover, the method can also be easily applied to analyse the normal concentrations of dietary choline intake in milk by mere centrifugation and dilution of samples.

3.6. Operational stability

The stability and reusability of 16-PHA-ZnONR choline biosensor were evaluated against physically adsorbed choline biosensor by performing a series of repeated experiments over 30 days by using a single biosensor. The storage stability of developed biosensor was found excellent even after 60 measurements (Intraday RSD%=2.83 and Interday RSD%=3.51). The choline biosensor was stored under appropriate environmental conditions e.g. at 4 °C before and after the measurements. The response of the biosensor was found consistent for the first 10 days and after 30 days the biosensor response decreased to 78%. The decrease in response may be due to the reduction of enzyme activity and denaturation of the peroxidase. It was found that 16-PHA modified choline biosensor holds good storage stability, sensitivity and reusability for longer duration than physisorbed enzyme ZnONR surface as represented in Fig. 4.

The enhanced stability of the presented biosensor can be explained in terms of the stability imparted by SAM of 16-PHA crosslinker. The results of the experiment revealed good consistency in the calibration traces even as the surfactant residuals of the solution from 16-PHA-ZnONR choline biosensor was detached by washing in PB solution before each measurement. Repeated measurements on physically adsorbed choline biosensor revealed an unstable and non-reproducible response.

The ZnONR is coupled with the phosphate group of the 16-PHA chain on one side. The other end the 16-PHA is covalently coupled to the Enzyme COD/HRP via activation of the –COOH group. Thus, the 16-PHA SAMs provide strong binding to the either side of the chain. The improved stability was confirmed by washing the surface for both physisorbed and chemisorbed ZnONR sensor using PB. It was observed that the chemisorbed enzyme was held strongly even after 15 repeated wash as evident from the FT-IR spectra (Supplementary Figs. S2 and S3). The characteristic peaks present due to enzyme coupled SAMs were retained even after the multiple washing steps. In contrast, for the physisorbed enzyme, these peaks were found absent after the washing step in the FT-IR spectra.

The immobilization of enzyme is a well-accepted approach towards stabilization of enzyme (Mateo et al., 2007). An important feature that account for enzyme stability is the improvement obtained in the value of the Michaelis–Menten constant (K_m). The K_m value obtained using LineWeaver–Burk plot was estimated as 0.062 mM $\pm$ 0.06 for COD/HRP coupled via phosphonated ZnONR and 0.2 mM $\pm$ 0.09 for COD/HRP coupled via physisorbed enzyme ZnONR. The lower K_m value (almost 3.2 times) reflects higher enzymatic affinity of COD/HRP-coupled via phosphonated ZnONR towards choline as the affinity of phosphate group of 16-PHA is high with ZnO. The figures of merit of choline biosensor compared with other recent reported methods and presented in Table 1.

3.7. Recovery studies and real sample analysis

Recovery studies were performed on 16-PHA modified ZnONR for three different fat % milk samples (0.5, 1.5 and 3.5). The study was repeated with milk samples spiked with standard choline solution (1, 0.1 and 0.01 mM) to evaluate sensor performance for inter-batch and intra-batch reproducibility. The summaries of findings are tabulated in Table 2 with mean recovery intraday

Table 2
Recovery of choline from different fat % milk sample as determined by ZnONR choline biosensor to assess the recovery efficiency using developed assay.

Milk samples with different fat (%)	Choline spiked (mM)	Intraday		Interday	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Milk-1 (0.5%)	1	98.1 $\pm$ 0.30	0.33	97.3 $\pm$ 0.21	0.64
	0.1	97.4 $\pm$ 0.65	0.49	94.0 $\pm$ 0.57	0.75
	0.01	101.0 $\pm$ 0.23	0.57	96.1 $\pm$ 0.60	1.04
Milk-2 (1.5%)	1	97.1 $\pm$ 0.60	0.70	93.3 $\pm$ 0.37	0.84
	0.1	103.1 $\pm$ 0.78	0.67	97.5 $\pm$ 0.41	0.93
	0.01	98.3 $\pm$ 0.43	0.42	96.0 $\pm$ 0.69	1.10
Milk-3 (3.5%)	1	95.2 $\pm$ 0.29	0.28	94.7 $\pm$ 0.47	0.92
	0.1	97.9 $\pm$ 0.71	0.78	91.3 $\pm$ 1.01	1.05
	0.01	98.6 $\pm$ 0.56	0.53	97.0 $\pm$ 1.09	1.07

Table 1
Comparison of various established biosensing techniques for the analysis of choline.

Biosensor configuration	Transducer	Linearity (mM)	Detection limit (mM)	K_m (mM)	Stability (biosensor response retained)	Reference
Chemiluminescence 16-PHA-ZnONR	Optical	0.006–2	0.0005	0.062 $\pm$ 0.06	78% after 1 month	Our work
Flow Injection Analysis-Enzyme immobilized on CPG support	Thermal	0.03–0.5	0.03	0.45	more than 85% after 15 months	Deshpande et al. (2011)
ChOx and different concentrations of choline solution in CdTe quantum dots	Optical	0.005–0.15	0.0001	0.20	n.r.	Chen et al. (2011)
Fluorescent conjugated polymer and an enzyme-coupled assay	Optical	0.0001–0.02	0.00005	n.r.	n.r.	Li et al. (2013)
Amperometric ChOx /HRP immobilized solid carbon paste electrode	Electrochemical	0.00005–0.007	0.00001	n.r.	1 month	Razola et al. (2003)

RSD% 0.53 ± 0.17 and mean interday RSD% was calculated as 0.93 ± 0.16.

4. Conclusion

In conclusion, vertically aligned ZnONR-films with an average diameter of ~80 nm and length ~780 nm (film thickness) has been successfully demonstrated for the construction of a stable and reproducible choline nanobiosensor. The developed biosensor is demonstrated for choline analysis in milk and it can be easily adopted for routine analysis of choline content in milk and infant formula. The conjugation with COD/HRP has been carried out to prepare a choline biosensor to harvest the substantial advantages of large surface area and smooth optical signal communication in ZnONR. Stability, reproducibility, sensitivity, and selectivity of the biosensor have been investigated over a large dynamic concentrations ranging from 0.0005 mM to 2 mM of choline solution and good sensitivity is achieved with total analysis time of 15 min at room temperature. The enhanced stability of the enzyme on ZnONR is achieved via phosphonation. The SAMs on the ZnONR also facilitates the protection of ZnO material in aqueous media. The presented biosensor could easily be extended for routine analysis in milk/milk powder and dietary supplements.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.11.057.

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