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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