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High-performance electrochemical biosensor for the detection of total cholesterol

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ABSTRACT

We report on a highly sensitive electrochemical biosensor for the determination of total cholesterol. The novel biosensor was fabricated by co-immobilizing three enzymes, cholesterol oxidase (ChO_x), cholesterol esterase (ChE) and horseradish peroxidase (HRP), on nanoporous gold networks directly grown on a titanium substrate (Ti/NPAu/ChO_x–HRP–ChE). The morphology and composition of the fabricated nanoporous gold were characterized by scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS) and X-ray diffraction spectroscopy (XRD). The electrochemical behaviour of the Ti/NPAu/ChO_x–HRP–ChE biosensor was studied using cyclic voltammetry (CV), showing that the developed biosensor possessed high selectivity and high sensitivity (29.33 $\mu A\,mM^{-1}\,cm^{-2}$). The apparent Michaelis–Menten constant, K_M^{app} of this biosensor was very low (0.64 mM), originating from the effective immobilization process and the nanoporous structure of the substrate. The biosensor exhibited a wide linear range up to 300 $mg\,dL^{-1}$ in a physiological condition (pH 7.4), which makes it very promising for the clinical determination of cholesterol. The fabricated biosensor was further tested using real food samples margarine, butter and fish oil, showing that the biosensor has the potential to be used as a facile cholesterol detection tool in food and supplement quality control.

1. Introduction

Cholesterol is made by the liver as well as being part of a healthy dietary intake of fats. Cholesterol and triglycerides are important building blocks in the structure of cells and are used in making hormones and vitamin D, and producing energy. However, having a high total cholesterol (the sum of free cholesterol and cholesterol esters) level, particularly those of the low density lipoprotein type, can cause blood vessel damage and resulting diseases such as coronary heart disease and peripheral vascular disease. High cholesterol has also been linked to nephrosis, diabetes milletus, myxedema and jaundice. On the other hand, a low cholesterol level may result in hyperthyroidism, anaemia and maladsorption (Program, 1988). Most recent studies have shown that high cholesterol can affect macrophages which are part of the innate immune system that typically gobble up pathogens and clear away dead cells (Becker et al., 2010). The desired total plasma cholesterol for an individual is less than 5.2 mM (200 $mg\,dL^{-1}$), with a high level being considered as greater than 6.2 mM (240 $mg\,dL^{-1}$) (Program, 1988). Thus, the industrial and clinical determination of cholesterol is of great interest (Adanyi and Varadi, 2003; Aravamudhan et al., 2007a; Brahim et al., 2001; Arya et al., 2008; Lee and Park, 2010).

Electrochemical biosensors offer several distinct advantages. These devices are uniquely qualified for meeting the small size, cost effectiveness, low volume and power requirements of decentralized testing and show great promise for a wide range of biomedical and environmental applications (Wang, 2005; Kafi and Chen, 2009). In the development of electrochemical biosensor, facilitation of the electron transfer between the enzyme and the electrode is a great challenge because of the deeply embedded redox-active center of the metalloenzyme. On one hand, great efforts have been made to enhance the electron transfer in sensor design by using mediators, promoters or other special materials such as peroxidases for the construction of enzyme-based biosensors (Liu and Chen, 2005; Lu et al., 2007; Reilly and Aust, 1997). On the other hand, with the use of nanomaterials, higher sensitivity and the intimate attachment of enzymes are achievable due to the nanomaterials' high surface roughness as well as their unique physical, electronic and chemical properties (Carrara et al., 2008; Li et al., 2010; Chen and Holt-Hindle, 2010). Applications of nanostructured materials have been attracting great attention in the development of high-performance electrochemical biosensors (Saxena et al., 2011; Wisitsoraat et al., 2010; Kafi et al., 2010). Among them,

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gold nanomaterials have gained particular interest due to their ease of synthesis and functionalization, high chemical stability, low inherent toxicity (biocompatibility), and tunable optical and electronic properties (Eustis and El-Sayed, 2006; Ofir et al., 2008). It has been reported that the above mentioned properties of Au nanomaterials can effectively facilitate the electron transfer in the design of an electrochemical biosensor (Ahmadalinezhad et al., 2009; Safavi and Farjami, 2011; Sperling et al., 2008). Few biosensors have been reported for cholesterol determination compared with those reported for glucose; thus, developing biosensors based on gold nanostructures for the detection of total cholesterol may have a significant impact on both the clinical diagnostics and the food industry.

To develop the electrochemical cholesterol biosensor, Aravamudhan et al. attached the enzymes to the surface of aligned-gold nanowires through direct physical adsorption (Aravamudhan et al., 2007a). Their studies showed the fabricated biosensor with the sensitivity of 0.85 $\mu A\,mM^{-1}$ and the Michaelis–Menten constant of 17.1 mM. To improve the sensitivity, Gopalan et al. recently reported on the fabrication of a biosensor for the detection of free cholesterol by combining the advantageous features of MWNT (multi-walled carbon nanotubes), Au nanoparticles, chitosan and ionic liquid (Gopalan et al., 2009). Chitosan, a polysaccharide composed mainly of β-(1,4)-linked 2-deoxy-2-amino-D-glucopyranose units, is the deacetylated product of chitin, poly (N-acetyl-D-glucosamine). Chitosan has been used for the fabrication of biosensing devices because of its biocompatibility, biodegradability, multiple functional groups, as well as its solubility in acidic aqueous medium (Ahmadalinezhad et al., 2009; Tiwari and Gong, 2008). Given the huge medical implications, the industrial and clinical determination of cholesterol is increasingly important. Since 70% of cholesterol exists in ester form and 30% as free form in a blood sample, detection of the total cholesterol is desirable. In the present work, for the first time, we co-immobilized three enzymes, cholesterol oxidase (ChO_x), cholesterol esterase (ChE) and horseradish peroxidase (HRP), on the nanoporous Au networks with the aid of chitosan for the detection of the total cholesterol. The nanoporous Au networks were directly grown on a titanium (Ti) substrate using the hydrothermal method. The fabricated biosensor was free of any other promoters or special materials toxic to the environment and human. Our electrochemical measurements show that the fabricated cholesterol biosensor has high sensitivity, a very low Michaelis–Menten constant, a wide linear range, low detection limit, high selectivity and excellent stability. The cholesterol biosensor developed in this study was further tested using real food samples, indicating the promising applications in both clinical diagnostics and the food industry.

2. Experimental

2.1. Reagents and materials

Cholesterol oxidase (from *Streptomyces* species), cholesterol esterase (from hog pancreas), cholesterol, glucose, and Triton® X-100 (t-octylphenoxypolyethoxyethanol) were purchased from Sigma, 4-cholesten-3-on from Aldrich, titanium plates (1.25 cm × 0.8 cm × 0.5 mm, 99.2%) from Alfa Aesar and lactic acid from Fluka. Horseradish peroxidase, ascorbic acid, uric acid, lactic acid, sodium phosphate dibasic and sodium phosphate monobasic were used as received from Sigma–Aldrich. Nanopure water (18.2 MΩ cm) was used to prepare all solutions. All other chemicals were analytical grade and were used as received from commercial sources. A stock solution of cholesterol was prepared by dissolving cholesterol in isopropanol, then adding Triton X-100 and finally the phosphate buffer (pH 7.4) in a volume ratio of 10:4:86.

2.2. Preparation of the cholesterol biosensor

The hydrothermal method used in fabricating the nanoporous gold networks is similar to the previous studies (Koczkur et al., 2007; Wang et al., 2008; Peng et al., 2004). Briefly, titanium plates (1.25 cm × 0.8 cm) were washed in acetone, followed by Nanopure water, and then etched in a solution of 18 wt% hydrochloric acid at 85 °C for 10 min to remove the oxide layer and roughen the Ti surface. The etched Ti substrates were transferred into Teflon-lined autoclaves containing 10 mL of an aqueous mixture of inorganic metal precursor and a reducing agent. A 1.0 M ammonium formate solution was used as the reducing agent and the metal precursor was $HAuCl_4$. The autoclaves were sealed and heated at 180 °C for 10 h. The Ti plates coated with Au were dried and annealed under argon at 250 °C for 2 h. After final rinsing with pure water, the Ti plates coated with nanoporous gold were ready for further surface analysis and for the immobilization of enzymes. To immobilize the enzymes, a mixture of 20 μL of 2 mg mL^{-1} of cholesterol oxidase (ChO_x), 10 μL of 2 mg mL^{-1} horseradish peroxidise (HRP), 10 μL of 2 mg mL^{-1} of cholesterol esterase (ChE) and 10 μL of 2 mg mL^{-1} of chitosan was cast onto the nanoporous Au electrode (Ti/NPAu/ChO_x–HRP–ChE). Chitosan was used as a glue to enhance the stability of the biosensor (Khan and Dhayal, 2008). All prepared biosensors were stored at 4 °C when not in use.

2.3. Instruments and electrochemical experiments

Surface morphology and composition of the synthesized samples were characterized using scanning electron microscopy (SEM) (JEOL JSM 5900LV) equipped with an energy-dispersive X-ray spectrometer (EDS) (Oxford Links ISIS). Surface elemental compositions based on quantitative EDS analysis were reported in average values of readings taken at five different spots on each sample surface. All electrochemical experiments were performed using an electrochemical workstation (CHI660B, CH instrument Inc.), connected with an in-house-built, three-electrode glass cell (30 mL). A platinum coil was used as a counter electrode and was flame-annealed before each experiment. An Ag/AgCl (saturated KCl) electrode was used as the reference electrode. The fabricated Ti/NPAu/ChO_x–HRP–ChE electrodes were used as the working electrode. All potentials reported in this paper are referred to the Ag/AgCl (saturated KCl) reference electrode. Cyclic voltammetric measurements of cholesterol were carried out in a 0.1 M phosphate buffer solution (pH 7.4) at selected potential ranges. All measurements were conducted in a 30 mL solution and at room temperature (22 ± 2 °C).

3. Results and discussion

3.1. Surface morphological studies

Fig. 1A and B present a typical SEM image and EDS spectrum of the nanoporous gold (Ti/NPAu) electrode, respectively, fabricated using the hydrothermal method. The SEM image shows that nanoporous gold structure was formed and covered the substrate and that the thickness of the nanoporous gold layer was around 0.4 μm estimated from the depth of the pores. The diameter of the formed randomly porous structures varies from tens to hundreds of nanometers. Only Au and Ti peaks appear in the EDS spectrum, and no discernible carbon or oxygen signals are seen in the figure, showing that the synthesized nanoporous Au is free of surface organic impurities. The XRD pattern of the as-synthesized Ti/NPAu surface is shown in Fig. 1C. All diffraction peaks originate from the Au film and the Ti substrate. The lattice constant calculated from the pattern was 4.079 Å, consistent with the litera-