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Amperometric cholesterol biosensor based on the direct electrochemistry of cholesterol oxidase and catalase on a graphene/ionic liquid-modified glassy carbon electrode

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ABSTRACT

Cholesterol oxidase (ChOx) and catalase (CAT) were co-immobilized on a graphene/ionic liquid-modified glassy carbon electrode (GR–IL/GCE) to develop a highly sensitive amperometric cholesterol biosensor. The H_2O_2 generated during the enzymatic reaction of ChOx with cholesterol could be reduced electrocatalytically by immobilized CAT to obtain a sensitive amperometric response to cholesterol. The direct electron transfer between enzymes and electrode surface was investigated by cyclic voltammetry. Both enzymes showed well-defined redox peaks with quasi-reversible behaviors. An excellent sensitivity of 4.163 mA mM^{-1} cm^{-2}, a response time less than 6 s, and a linear range of 0.25–215 μM ($R^2 > 0.99$) have been observed for cholesterol determination using the proposed biosensor. The apparent Michaelis–Menten constant (K_M^{app}) was calculated to be 2.32 mM. The bienzymatic cholesterol biosensor showed good reproducibility (RSDs < 5%) with minimal interference from the coexisting electroactive compounds such as ascorbic acid and uric acid. The CAT/ChOx/GR–IL/GCE showed excellent analytical performance for the determination of free cholesterol in human serum samples.

1. Introduction

Cholesterol is an essential lipid which found in the cell membranes of all animal and human cells and is the precursor for other biological materials such as steroid hormones. Normally, the concentration of free cholesterol in human serum is in the range 1.0–2.2 mM (Zhao et al., 2008). However, high cholesterol accumulation in blood due to excessive ingestion results in fatal diseases, such as arteriosclerosis, cerebral thrombosis, myocardial infarction, coronary diseases and lipid metabolism dysfunction, etc. (Ahmadalinezhad and Chen, 2011; Sugano and Beynen, 1990). The measurement of blood cholesterol concentration is a routine practice in medical screening or diagnosis. Various methods have been reported for the analysis of cholesterol in biological fluids including colorimetric, spectrophotometric, HPLC, and electrochemical methods (Krug et al., 1994; Nakaminami et al., 1997; Yao et al., 1995; Wong et al., 1994; Hojo et al., 2011). Among these, some methods often present certain disadvantages, such as lack of specificity and selectivity because of the interfering reactions and use of unstable and corrosive reagents (Brahim et al., 2001). However, because of their excellent specificity, enzymatic procedures have been practically preferred over chemical methods for the determination of cholesterol in biological samples (Gopalana et al., 2009).

Electrochemical biosensors have emerged recently as alternative tools for rapid and real-time analysis of clinically relevant analytes (Xu and Wang, 2012). These biosensors are rapid, easy to handle and are of low cost. Most of these devices are based upon the amperometric detection of hydrogen peroxide as the product of the enzymatic reactions. For example most of the cholesterol biosensors are based on the use of cholesterol oxidase (ChOx), a flavin adenosine dinucleotide (FAD)-containing enzyme, which catalyzes the oxidation of cholesterol to H_2O_2 and cholest-4-en-3-one in the presence of molecular oxygen (Ahn and Sampson, 2004). The electrochemical quantification of H_2O_2 would be convenient if the transducer can facilitate the redox process at a favorable potential without any interference from other coexisting analytes. However, previous studies show that at common solid electrodes the kinetic of electron transfer of H_2O_2 is sluggish and amperometric detection requires high applied potentials (Iannlello and Yacynych, 1981) that may induce simultaneous oxidation/reduction of other electroactive species in samples which may lead to false positive signals (Zhao et al., 2008). Hence, acceleration of redox process of H_2O_2 is necessary to develop any electrochemical biosensor which is based on the monitoring of enzymatically generated H_2O_2. Recently, many researchers have used nanostructured materials such as metal nanoparticles and carbon nanotubes

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(CNTs) with catalytic effects to improve biosensor sensibility and to decrease the overpotential applied to the amperometric detection of H_2O_2 (Hall et al., 2000). However, many electrochemical techniques are based on the reduction of H_2O_2 by the catalysis of immobilized enzymes (peroxidases) to construct unmediated biosensors, which are based on the direct electron transfer between an electrode and immobilized enzyme (Ferapontova et al., 2001; Liu and Ju, 2002). It has been reported that proteins containing heme groups, such as hemoglobin, myoglobin and catalase (CAT) possess peroxidase like catalytic activity, which can reduce H_2O_2 due to the electroactive heme center (Gu et al., 2001; Liu et al., 2004) and have also been used for the preparation of H_2O_2 biosensors (Chen and Lu, 2006).

Graphene is a two-dimensional (2-D) sheet of carbon atoms in a hexagonal configuration with atoms bonded by sp^2 bonds (Novoselov et al., 2004). Graphene shows many advantages for electrochemical applications when compared to graphite or to carbon nanotubes. Graphene exhibits a surface area of 2630 $m^2 g^{-1}$, which is much greater than that of graphite ($\sim$10 $m^2 g^{-1}$) and even that of carbon nanotubes (1315 $m^2 g^{-1}$) (Pumera et al., 2009). The electrical conductivity of graphene is excellent. Electrodes made from graphene have significantly more uniform distribution of electrochemically active sites than do those made from graphite (Tan et al., 2010; Zhou et al., 2009). Therefore, graphene has exhibited potential applications in synthesizing nanocomposites and fabricating various electrical devices. Recently, its applications in biological and electrocatalytic aspects have boomed (Kang et al., 2009; Li et al., 2009).

Ionic liquids (ILs) are molten salts with the melting point close to or below room temperature. The good solvating properties, high conductivity, non-volatility, low toxicity, large electrochemical window and good electrochemical stability, make ILs suitable for many applications such as electrodeposition, electrosynthesis, electrocatalysis, electrochemical capacitors and lithium-ion batteries (Hapiot and Lagrost, 2008). Also ILs has been used as the modifier of the electrode or the binder to fabricate IL-carbon composites, typically for instance carbon ionic liquid electrode (CILE) (Safavi et al., 2009), IL-functionalized carbon nanotubes (Xiao et al., 2009), GR–IL hybrids (Yang et al., 2009), etc. Among these, the GR–IL based electrochemical sensors and biosensors have been recently reported for direct electron transfer and detection of different types of targets (Shan et al., 2010, 2009). These results suggest that GR–IL can remarkably increase the sensitivity and facilitate electron transfer of various redox biomolecules. Moreover, the combination of graphene sheets and IL via physical or chemical interactions can provide a more favorable micro-environment for the immobilization of enzymes/proteins, and IL can enhance their catalytic activity. The unique properties of both graphene and ionic liquid make this nanocomposite a promising platform for the construction of biosensors (Tunckol et al., 2012).

In this article, we attempt to propose a novel design for the bienzymatic cholesterol biosensor based on the co-immobilization of ChOx and CAT at the surface of a GR–IL modified GCE. The stable adsorption and direct electrochemistry of the two enzymes were observed at GR–IL/GCE. Through cooperation of the two enzymes, as well as the synergic effect of GR and IL, improved sensitivity and selectivity for cholesterol detection were achieved.

2. Experimental

2.1. Reagents and chemicals

ChOx (EC 1.1.3.6), CAT (EC 1.11.1.6), cholesterol and the ionic liquid 1-Butyl-3-methylimidazolium hexafluorophosphate ([bmim][PF_6]) were obtained from Sigma-Aldrich (Madrid, Spain). All other chemicals used were of analytical grade and used without further purification. Graphene oxide (GO) was prepared from graphite (Flakes) by the improved Hummers' method (Marcano et al., 2010). The GR–IL hybrid was prepared according to the literature (Yang et al., 2011a; Choi and Park, 2012). A mixture of GO/IL (95/5 w/w%) in 10 mL of deionized water was prepared. After sonication of the mixture for 60 min, the homogeneous solution was treated with 38 wt% hydrazine solution for reduction at 90 °C for 2 h. The resulting mixture was washed with deionized water several times and centrifuged to obtain GR–IL hybrid.

2.2. Instrumentation

Voltammetric measurements were carried out with an AUTOLAB (Eco Chemie B. V.) PGSTAT30 potentiostat/galvanostat. The electrochemical cell was assembled with a saturated Ag/AgCl reference electrode, a Pt wire auxiliary electrode, and the prepared working electrodes. The surface morphology of modified electrodes was characterized with a scanning electron microscope (SEM) (KYKY-EM3200).

2.3. Preparation of working electrodes

Glassy carbon electrodes were polished to a mirror-like surface with 1.0 and 0.3 μm alumina slurry, and then sonicated in water and ethanol, respectively. The ChOx/CAT/GR–IL/GCE was prepared as follows: briefly, a 10 μL of 1.0 mg/mL GR–IL suspension was spread on the surface of the electrode and dried at room temperature to form GR–IL modified GCE (GR–IL/GCE). After that, the GR–IL/GCE was separately incubated in 6 mg/mL CAT and 8 mg/mL ChOx in 0.01 M PBS (pH 7.4) each for 6 h at 4 °C to obtain ChOx/CAT/GR–IL/GCE. The mono-enzyme electrodes, i.e. CAT/GCE, ChOx/GCE, ChOx/GR–IL/GCE and CAT/GR–IL/GCE, were prepared in a similar way except that each electrode was only incubated in its corresponding enzyme solution. Finally, the electrodes were thoroughly rinsed with 0.01 M PBS (pH 7.4) to remove weakly adsorbed enzyme molecules.

2.4. Preparation of real samples

Free cholesterol in human serum samples was determined with the proposed cholesterol biosensor. A 0.5 mL serum sample was diluted to 10 mL with 0.01 M PBS (pH 7.4) containing 0.2% (v/v) Triton X-100 and 0.5% (v/v) isopropanol to make the cholesterol oncentrations fall in the linear range of the biosensor. The concentration of cholesterol was determined using standard addition method.

3. Results and discussion

3.1. Characterization of the modified electrode

Scanning electron microscopy (SEM) was employed to study the surface morphology of the modified electrode. Fig. 1A clearly shows that the GR–IL film has a typical crumpled and wrinkled structure of graphene sheet which provides a large rough surface for further immobilization of enzymes. When the electrode was incubated in the enzyme solution, the enzyme molecules adsorbed on the surface of GR–IL tended to aggregate as nanoparticles (white spots) throughout the surface which indicates the successful immobilization of enzymes on the GR–IL/GCE (Fig. 1B). Such behavior has been previously observed when glucose oxidase (GOD) and horseradish peroxidase (HRP) were immobilized on GR sheets (Yang et al., 2011a; Choi and Park, 2012). Non-covalent interactions such as π–π, π-cationic, ionic–ionic and/or hydrophobic–hydrophobic interactions