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Performance comparison between multienzymes loaded single and dual electrodes for the simultaneous electrochemical detection of adenosine and metabolites in cancerous cells

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ABSTRACT

The analytical performance of the multi enzymes loaded single electrode sensor (SES) and dual electrode sensor (DES) was compared for the detection of adenosine and metabolites. The SES was fabricated by covalent binding of tri-enzymes, adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), and xanthine oxidase (XO) along with hydrazine (Hyd) onto a functionalized conducting polymer [2,2:5,2-terthiophene-3-(p-benzoic acid)] (pTTBA). The enzyme reaction electrode in DES was fabricated by covalent binding of ADA and PNP onto pTTBA coated on Au nanoparticles. The detection electrode in DES was constructed by covalent binding of XO and Hyd onto pTTBA coated on porous Au. Due to the higher amount (3.5 folds) of the immobilized enzymes and Hyd onto the DES than SES, and the lower Michaelis constant (Km) value for DES (28.7 μM) compared to SES (36.1 μM), the sensitivity was significantly enhanced for the DES (8.2 folds). The dynamic range obtained using DES was from 0.5 nM to 120.0 μM with a detection limit of 1.43 nM ± 0.02, 0.76 nM ± 0.02, and 0.48 nM ± 0.01, for adenosine (AD), inosine (IN), and hypoxanthine (Hypo) respectively. Further, the DES was coupled with an electrochemical potential modulated microchannel for the separation and simultaneous detection of AD, IN, and Hypo in an extracellular matrix of cancerous (A549) and non-cancerous (Vero) cells. The sensor probe confirms a higher basal level of extracellular AD and its metabolites in cancer cells compared to normal cells. In addition, the effect of dipyridamole on released adenosine in A549 cells was investigated.

1. Introduction

Enzymes are versatile biological catalysts with high stereo and regioselectivity with high turnover rate. However, free enzymes are relatively unstable, cannot be recovered, and reused efficiently (Kress et al., 2002). To overcome these limitations and broaden their applicability, enzyme molecules are usually attached to an inert, insoluble material via immobilization. Due to their catalytic roles, enzymes still an active area of research in various analytical fields and more recently, they have been extensively used in the fabrication of electrochemical biosensors serving as specific chemical transducers, translating an analyte into a substance capable of being detected by a chemically or physically sensitive detector. To date, various types of enzymes based sensor have been developed using one, two or even multi enzyme immobilized onto an electrode surface (Ispas et al., 2012). However, the cascade reactions catalyzed by immobilized multienzymatic systems is less popular compared to a single enzyme immobilization due to the complicated and sophisticated procedures required in the fabrication of multienzyme sensors. Hence, the fabrication of multienzymes sensor is a challenging task, which in turn, will open new range of clinical and pharmaceutical applications.

For instance, adenosine (AD) is a naturally occurring purine nucleoside, which breaks down in the body through a series of ectoenzymes producing inosine (IN), hypoxanthine (Hypo), and H_2O_2 by adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), and xanthine oxidase (XO), respectively (Israelsen and Vander Heiden, 2010; Paton, 2013). Further, AD and its metabolites (IN, and Hypo) are important markers for ischemia, cardiovascular, and cancer progression. However, the clinical relevance and the basal release of extracellular AD, IN, and Hypo remain controversial especially in cancer (Mei et al., 1996). To enhance our current understandings about the basal release of AD, IN, and Hypo in cancer and normal cells, a selective and sensitive detection without any additional manipulations or derivatization of sampled biological fluids is needed.

Recently, AD and its metabolites are linked with cancer etiology. The elevated level of AD in the tumor microenvironment promotes

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cancer by the interference or inhibition of killer T-cells activation (Stagg and Smyth, 2010). Similarly, IN binds to AD receptors and initiate intracellular signaling pathway affecting cell proliferation through a direct or indirect activation of AD receptors (Soares et al., 2015). Hypo acts indirectly in cancer propagation by producing the reactive oxygen species (Schwab, 2008). Therefore, the development of a sensitive and simultaneous detection system is of a great interest for clinical and pharmaceutical industry, especially in drug screening, therapeutic efficacy, and biomedical applications.

Over the past few years, several analytical methods have been employed for the simultaneous detection of AD, IN and Hypo, such as high-performance liquid chromatography and gas/liquid chromatography-mass spectrometry (Farthing et al., 2007; Childs et al., 1996; Huang et al., 2004). Although these methods have numerous advantages but they are not easily adoptable for field analysis, expensive instruments, time consuming, require tedious preparations, and lack the miniaturization ability for point-of-care bioanalysis. In contrast, electrochemical biosensors are robust, user-friendly, and capable of miniaturization for on-site medical diagnosis. To date, various type of sensors for AD and metabolites have been reported employing different strategies like direct oxidation of the target molecule or enzyme based sensors. Fast-scan cyclic voltammetry (FSCV) was reported for AD detection by direct oxidation (Swamy and Venton, 2007). In this case, two oxidation peaks of adenosine were observed at 1.0 V and 1.4 V. Due to the higher oxidation potential of this technique, the main drawback of this method are related to its stability and the interference effect of the electroactive biological molecules present in blood such as, dopamine, NADH etc. Alternatively, enzyme reaction-based detection can overcome these issues; by monitoring the electrochemical signal that generated by the catalytic reduction of H_2O_2, which is a common enzymatic end product for adenosine and metabolites. Most of the reported electrochemical sensors to detect AD, IN, and Hypo are based on the immobilization of three enzymes (ADA, PNP and XO) onto a single electrode (Frenguelli et al., 2003; Llaudet et al., 2003; Henderson and Griffin, 1984). However, they suffer from low sensitivity, short-term stability, and narrow dynamic range. These shortcomings are due to the lack of an effective immobilization of the enzymes or a catalyst onto the single electrode, which results in inappropriate ratio of the enzymes. One of the effective way to overcome these disadvantages is by dividing the immobilization of three enzymes and a catalyst onto two separate electrodes; hence, it will enhance the amount of each enzyme, and enzyme kinetics consequently, improving sensor performance parameters.

To achieve stable immobilization of enzymes without loss of their biological functions, choice of substrate material play a critical role in the design of biosensors. Of those substrate materials, conducting polymers (CPs) and its composites are popular in the fabrication of biosensors. Polyterthiophene are highly stable compared to that of polyaniline, and polypyrrole (Lee and Shim, 2001). In addition, polyterthiophene are non-toxic and robust sensor probe substrate (Shiddiky and Shim, 2007; Kim et al., 2016). Further, presence of the functional groups like -COOH or $–NH_2$ in CP enables stable immobilization of biomolecule such as, enzymes through the amide bond formation. To enhance the conductivity of the CPs, often they composited with various nobel metals like Au, Ag, Pt nanoparticles or more recently dendrites (Noh et al., 2012a, 2012b). Of them, Au/CP composites have drawn more attraction due to their biocompatibility as compared to other composites. Similarly, porous Au dendrites were reported for sensitive detection of H_2O_2 (Naveen et al., 2016) further, the catalytic reduction of H_2O_2 can be enhanced by the presence of an additional catalyst on the electrode surface such as hydrazine (Hyd).

In the present study, the analytical performances of the SES and DES were compared in terms of sensitivity and enzyme kinetics parameter, Michaelis constant (Km) for the detection of adenosine and metabolites. Further, for the separation analysis of the target molecules, we tried to run the experiment using an electrochemical potential modulated microchannel (EPMM) coupled with DES for the first time. The DES consists of two separate screen-printed carbon electrodes (SPCEs), named reaction and detection electrode; both electrodes were modified separately and attached at the end of EPMM. The reaction electrode was fabricated by covalent bonds formation between ADA, PNP and carboxylic acid groups of functionalized CP [2,2:5,2-terthiophene-3-(p-benzoic acid)] (pTTBA) on the AuNPs layer. Similarly, the detection electrode was constructed by covalent binding of XO and Hyd onto pTTBA layer that formed on pAu dendrites. The H_2O_2 produced by the sequential enzymatic reaction was reduced at the detection electrode, which generated the electrochemical signal. At optimized conditions, the coupled system was applied for the separation and simultaneous detection of extracellular release of AD, IN, and Hypo in cancerous (A549) and non-cancerous (Vero) cells. In addition, the inhibitory effect of dipyridamole (DPM) on released adenosine was investigated in A549 cells, where the drug concentration profile was obtained employing same cell lines.

2. Experimental section

2.1. Materials and instruments

The detail of the required materials and instruments used in the experiments, can be found in Supplementary information under Section 1.1.

2.2. Fabrication of the dual electrode sensor (DES)

The dual electrode sensor composed of two SPCEs named reaction and detection electrode. The reaction electrode was fabricated by electrodeposition of AuNPs onto the SPCE before the polymerization of TTBA in a 0.5 M H_2SO_4 solution containing 0.001% HAuClO4, using linear sweep voltammetry (LSV) from + 1.5 to + 0.4 V. The electrodeposition conditions as follows: 60.0 s deposition time, − 0.6 V deposition potential, and 100 mV/s scan rate (Shiddiky et al., 2007; Kim et al., 2009). Thereafter, 1.0 mM of freshly prepared TTBA monomer was dissolved in a mixture solution of di (propylene glycol) ethyl ether and tri (propylene glycol) ethyl ether (1:1 vol ratio, 5 ml) and drop-casted on AuNPs layer and allowed to dry at room temperature. Afterwards, TTBA was electropolymerized by cycling the potential twice from 0.0 to + 1.4 V (vs. Ag/AgCl) at 100 mV/s in 0.1 M PBS. Further, the SPCE/ AuNPs/ pTTBA modified probe was immersed in 10 mM EDC/ NHS solution for 6 h. to activate the –COOH groups of pTTBA. After activation, the modified electrode was incubated in PBS solution containing 0.2 mg/ml of ADA for 75 min at 4 °C to immobilize ADA, followed by incubation in PBS solution containing 0.15 mg/ml of PNP, for 75 min at 4 °C to immobilize PNP and termed as reaction electrode. The detection electrode was fabricated by formation of AuNi alloy using chronoamperometery by applying a potential at − 0.8 V for 200 s. Afterward, pAu-dendrites layer was obtained by dealloying (etching) the nickel from AuNi alloy surface by cycling the potential between 0.0 and + 1.6 V in solution containing 0.5 M H_2SO_4 (at a scan rate of 50 mV/s for 30 cycles). For pTTBA formation and activation of –COOH groups, similar procedure was applied as mentioned for reaction electrode. After activation, the modified electrode first immersed in PBS solution containing 1 mg/ml of XO for 50 min at 4 °C followed by incubation in PBS solution containing 1.5 mg/ml of Hyd and the modified electrode was termed as detection electrode. Finally, the reaction and detection electrodes were combined in a sandwich (microfluidic) structure, facing each other to allow the sequential enzymatic reactions in the microenvironment created between both electrodes. The signal was monitored at the detection electrode by H_2O_2 reduction, due to the catalytic activity of Hyd. The SES was fabricated by turn wise immobilization of ADA, PNP, XO, and Hyd onto pTTBA layer after pAu layer formation and its performance was compared with DES.