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Quantification of Bax protein on tumor cells based on electrochemical immunoassay

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ABSTRACT

Bax protein is a pro-apoptotic member of Bcl-2 family proteins on cells and exhibits a close relationship with drug resistance of tumor cells. Its quantification can provide significant information for cancer treatment and research. A valid and sensitive electrochemical method for quantitative analysis of Bax protein on tumor cells was developed using thionine–graphene composites (TH–GN) based on a competitive immunoreaction. Bax protein and horseradish peroxidase were assembled on the TH–GN modified electrode to construct an electrochemical biosensor. The binding of Bax antibody could decrease the responses of the developed biosensor due to the strong steric hindrance effect and this effect was weakened in the presence of tumor cells or free Bax protein. Under optimal conditions the peak current change derived from the differential pulse voltammetry (DPV) measurements (ΔI_{DPV}) was proportional to the cell concentration from 2.5×10^3 to 1.6×10^5 cells mL^{-1} with a detection limit of 800 cells mL^{-1}. The average amount of Bax protein on single MCF-7 cell and MCF-7/ADR cell were calculated to be $(1.2 \pm 0.2) \times 10^{11}$ molecules and $(7.2 \pm 1.1) \times 10^{10}$ molecules, respectively. It indicates that the decrease of Bax protein is one of important reasons resulting in drug resistance of tumor cells.

1. Introduction

Chemotherapy is one of widely used methods of treatments for cancer. At a cellular level, anticancer drugs trigger cell cycle arrest and apoptotic or non-apoptotic cell death [1,2]. However, the repeated use of chemicals often leads to their invalidity due to the onset of resistance or tolerance by the target tumor cells. Drug resistance can occur at many levels, including increased drug efflux, drug inactivation, alterations in drug target, processing of drug-induced damage, and evasion of apoptosis [3]. Apoptosis is an active cell death program. It occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Disruption of normal apoptotic process is related to a variety of human diseases [4,5]. An imbalance between pro- and anti-apoptotic proteins leads to accumulation of cells and inability to respond correctly to apoptotic stimuli in many forms of cancer. Bcl-2 family proteins are important regulators of cell apoptosis. Their three-dimensional structures consist of two central, predominantly hydrophobic α-helices surrounded by six or seven amphipathic α-helices of varying lengths. These proteins are functionally divided into two groups including anti-apoptotic members (Bcl-xL, Bcl-2, KSHV-Bcl-2, Bcl-w) and pro-apoptotic members (Bax, Bid). Bax, which is a 21 kDa partner protein and shares 21% of amino acid identity with Bcl-2, is a strong promoter of programmed cell death. When Bax is in excess and Bax homodimers dominate, cells are susceptible to apoptosis [6,7]. Some researches have successfully confirmed the close relationship between Bax-deficiency in cancer cells and drug resistance. The investigated cells include human leukemic T cell line CCRF-CEM cells [8], human colorectal cancer cells [9], B cell chronic lymphocytic leukemia (B-CLL) cells [10], human breast cancer MCF-7 cells [11], human gastric adenocarcinoma SGC7901 cells [12], and so on. These studies indicate that some tumor cells, in which functional Bax genes were decreased or deficient, were resistant to the apoptotic effects of chemotherapeutic agents. In addition, sensitive cancer cells often express a higher Bax level than their resistant variants. Thus, the research on expression of Bax protein on tumor cells is essential in differentiation between tumor cells and drug-resistant tumor cells, in understanding of the drug-resistant mechanism and in appraisal of the validity of chemotherapy. Enzyme-linked immunosorbent assay (ELISA) and flow cytometry (FCM) are often employed in the comparison between the expression of Bax protein on cancer cells and that on drug-resistant cancer cells. However, to the best of our knowledge, the accurate content of Bax protein on one cell is still

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poorly understood, which demands to develop a valid and sensitive method.

Literatures report that various carbon based materials have been employed as modifiers in fabrication of electrochemical sensors [13–15]. Graphene, a novel class of 2D carbon-based nanomaterial in which sp^2 bonded carbon atoms are arranged into a honeycomb structure, has attracted a great deal of interest due to its unique physical and chemical properties including high surface area [16], strong mechanical strength [17], excellent thermal conductivity [18] and electric conductivity [19]. Graphene has found its potential application in many fields such as electronics, energy storage and conversion, fuel cells, solar cells, electromechanical resonators and sensitive sensors [20,21]. It is reported that graphene exhibits a lower charge-transfer resistance than graphite and glassy carbon electrodes and a comparable wide electrochemical potential window [22]. In recent years, one can find many reports on the graphene-based electrochemical sensors and biosensors owning excellent electric conductivity and electrocatalytic activity. Graphene is widely used in the construction of enzyme electrode [23], direct electrochemical measurement of some small biomolecules [24], DNA sensing [25], environmental detection [26], immunoassay [27], and so on. Recently, graphene-based composite materials have received increased attention due to synergistic contribution of two or more functional components and many potential applications [28]. The combination of graphene with different nanoscaled inorganic materials including metals (such as Au [29], Pt [30] and Cu [31]) and metal oxides (such as RuO_2 [32], ZnO [33], TiO_2 [34], Co_3O [35], MnO_2 [36] and CeO_2 [37]) provides an electrochemical sensing platform with enhanced electronic and catalytic properties, resulting in the development of a multifunctional nanoassembly system. In addition, the extended long-range π-conjugation structure of graphene is beneficial to immobilization of some organic molecules with phenyl ring based on the π–π stacking interaction. For example, polyaniline [38], prussian blue [39], methylene green [40] and thionine [41] can be uniformly dispersed on the plane of graphene, leading to the rapid and sensitive current responses in the detection of H_2, glucose, NADH, DNA and alpha fetoprotein, respectively.

In the present work, thionine–graphene sheet nanocomposites were prepared and characterized by transmission electron microscope, UV–vis and infrared spectrophotometry. This nanohybrid, Bax protein and horseradish peroxidase were immobilized on glassy carbon electrodes. The modified electrode participated in an electrocatalytic reduction of H_2O_2, exhibiting an obviously increased cathodic peak current of thionine, which was subsequently reduced by the addition of Bax antibody. Tumor cells and free Bax protein could contend with Bax antigens on the electrode for Bax antibodies, prohibiting the decrease of the current. A valid method for the quantification of Bax protein on tumor cells was developed based on the immunoreaction and the competitive binding.

2. Experimental

2.1. Materials and apparatus

Anti-Bax mouse antibody was purchased from Merck Millipore (USA). Horseradish peroxidase (HRP) and Bax ELISA Kit containing Bax protein were obtained from Beijing Biosynthesis Biotechnology Co. Ltd. (China). Graphene (GN) was purchased from Nanjing CJNANO Tech Co. Ltd. (China). Thionine (TH) was obtained from Sinopharm Chemical Reagent Co. Ltd. (China). Doxorubicin hydrochloride (adriamycin, ADR) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. (China). The buffer for the assay was 0.1 M phosphate buffered saline (PBS), prepared by mixing stock standard solution of Na_2HPO_4 and KH_2PO_4. Other chemicals were of analytical reagent grade and all aqueous solutions were prepared in Milli-Q ultrapure water.

Electrochemical measurement experiments were performed with a CHI660C electrochemical workstation (CH Instruments, China) by using a three-electrode electrolytic cell. Glassy carbon electrode (GCE, 3 mm in diameter) acted as the working electrode. A KCl saturated calomel electrode served as the reference electrode. A platinum plate served as the counter electrode. Absorption spectra were recorded on a UV-2450 UV-VIS spectrophotometer (Shimadzu, Japan). A Nicolet Nexus 670 FTIR spectrometer (Nicolet, USA) was employed for the infrared spectral measurements. The sizes of graphene and thionine–graphene composites were characterized ex situ by a Tecnai G^2 20ST transmission electron microscope (TEM, FEI, USA). The cell-modality observation was performed with an inverted optical microscope (OLYMPUS CKX41, Japan).

2.2. Preparation of thionine–graphene (TH–GN) composite suspension

2 mg graphene was added into 1 mL water and dispersed by ultrasonication for 40 min at room temperature. Next, 1 mL of 1.5 mg mL^{-1} thionine aqueous solution was added into the above solution and stirred vigorously for at least 12 h. The mixed solution was centrifuged at 10,000 rpm for 20 min and washed with 1 mL PBS for three times. Then the precipitate was dispersed in 1 mL of PBS and mixed with 1 mL of 0.2% chitosan (CS) solution. After gently stirring for overnight, the stable dark TH–GN–CS suspension was prepared. It was stored in a brown glass bottle at 4 °C for further use.

2.3. Cell culture

Human breast cancer MCF-7 cells and adriamycin-resistant human breast cancer MCF-7 cells (MCF-7/ADR) were obtained from XiangYa Central Laboratory of Central South University, China. They were cultured using RPMI-1640 growth medium (from Gibco) supplemented with 10% newborn calf serum in the absence and presence of 1 μmol L^{-1} ADR, respectively, in an incubator (5% CO_2, 37 °C).

2.4. Fabrication of biosensor and measurement procedures

As shown in Scheme 1, the developed method is based on an indirect competitive immunoassay, which is performed as follows. Prior to modification, GCE was polished with 0.05 μm α-Al_2O_3 power slurries until a mirror shiny surface appeared, and it was sonicated sequentially in acetone, HNO_3 (1:1, v/v), NaOH (1 mol L^{-1}) and double distilled water for 3 min. The treated electrode was scanned between −1.0 and 1.0 V versus SCE in 0.5 mol L^{-1} H_2SO_4 aqueous solution for sufficient cycles to obtain reproducible cyclic voltammograms. After the electrode was thoroughly rinsed with doubly distilled water and dried with a stream of nitrogen gas, 3 μL of the prepared TH–GN–CS suspension was dropped on the GCE surface and allowed to dry at room temperature. The nanocomposite-modified electrode was then immersed in 2.5% glutaraldehyde aqueous solution for 1 h. Subsequently, 5 μL of 50 μg mL^{-1} Bax protein (antigen, Ag) solution was cast on the electrode surface and allowed to dry in refrigerator for overnight. Finally, 5 μL of 4 mg mL^{-1} HRP solution was employed to block the non-specific site on the Bax-modified electrode. The prepared electrode was marked as Ag-HRP/TH–GN–CS/GCE and preserved in a refrigerator at 4 °C before use. The electrode was gently washed with PBS and then nitrogen-dried after every assembly process.