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Ultrasensitive detection of drug resistant cancer cells in biological matrixes using an amperometric nanobiosensor

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ABSTRACT

Multidrug resistance (MDR) is a key issue in the failure of cancer chemotherapy and its detection will be helpful to develop suitable therapeutic strategies for cancer patients and overcome the death rates. In this direction, we designed a new amperometric sensor (a medical device prototype) to detect drug resistant cancer cells by sensing "Permeability glycoprotein (P-gp)". The sensor probe is fabricated by immobilizing monoclonal P-gp antibody on the gold nanoparticles (AuNPs) conducting polymer composite. The detection relies on a sandwich-type approach using a bioconjugate, where the aminophenyl boronic acid (APBA) served as a recognition molecule which binds with the cell surface glycans and hydrazine (Hyd) served as an electrocatalyst for the reduction of H_2O_2 which are attached on multi-wall carbon nanotube (MWCNT) (APBA-MWCNT-Hyd). A linear range for the cancer cell detection is obtained between 50 and 100,000 cells/mL with the detection limit of 23 ± 2 cells/mL. The proposed immunosensor is successfully applied to detect MDR cancer cells (MDR_{CC}) in serum and mixed cell samples. Interferences by drug sensitive (SKBr-3 and HeLa), noncancerous cells (HEK-293 and OSE), and other chemical molecules present in the real sample matrix are examined. The sensitivity of the proposed immunosensor is excellent compared with the conventional reporter antibody based assay.

1. Introduction

Multidrug resistance (MDR) is a major factor in the failure of cancer chemotherapy. Several mechanisms have been convinced to play vital roles in the development of the MDR in cancer cells (Krishna and Mayer, 2000). Among them, a major form is mediated by a cell membrane transporter "Permeability glycoprotein, (P-gp)" which is encoded by the MDR1 gene in human cells. It includes two nucleotide-binding and two membrane-spanning domains, acts as energy dependent pump that decreases intracellular drug concentrations lower than the effective therapeutic levels (Bellamy, 1996; Gottesman et al., 2002). This phenomenon leads to the development of drug resistance in cancer cells. The early detection of MDR in cancer cells can lead to the adequate chemotherapy of cancer patients and overcome the mortality. Thus, it is extremely important and clinically significant to detect MDR_{CC} at the early stage. This will help the clinicians to develop alternate therapeutic strategies for cancer patients. Until now, several biological methods have been developed to detect P-gp expression for the diagnosis of MDR in cancer such as; polymerase chain reaction (Murphy et al., 1990), immunohistochemistry (Chan et al., 1990), flow cytometry (Ludescher et al., 1992), and microarray (Gillet et al., 2004). Although these methods can be used to detect MDR_{CC}, they are less sensitive, require highly trained professionals, and have no ability to be miniaturized for the point-of-care applications. Thus, few biosensor-based diagnostic methods have also been reported to detect MDR_{CC}. These methods, however, are indirect, nonselective, and less sensitive (Du et al., 2005; Zhang et al., 2011, 2014). Thus, the development of a sensitive, robust, and alternate method for the detection of MDR_{CC} directly in the biological fluids is desirable and has great clinical importance. To achieve a highly sensitive detection method for MDR_{CC}, a sandwich type-electrochemical immunosensor composed of P-gp monoclonal antibody (AntiP-gp) as a detection probe coupled with a ligand tagged-non-enzymatic catalyst as a reporter probe is worthy to attempt due to their selective and highly sensitive characteristics, respectively.

The stable immobilization of biomaterials including antibodies on the sensor probe is extremely critical in the biosensor fabrication (Rahman et al., 2008). Electrochemical biosensors composed of conducting polymers-AuNPs composite are considered to be highly stable and ultrasensitive because biomolecules can be covalently immobilized on the polymer backbone possessing –COOH or $–NH_2$ groups (Chandra et al., 2011a, 2013; Koh et al.,

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2011; Kim and Shim, 2013; Lee et al., 2010). Compare to the reporter antibody based conventional sandwiched type electrochemical immunosensor (Malhotra et al. 2012; Wu et al., 2015; Zhu et al. 2014), other ligands are worthy to attempt for the *in vitro* detection of cancer cells. This is important because it has been reported that the concentration of antigen (target molecule) on the cancer cell surface is low (Beck et al., 1996). This may result in low antigen–antibody binding and consequently poor detection of cancer cells. Thus, it is worthy to target molecules that are present on the cancer cell surface in abundance. Recent studies have clearly indicated existence of high concentration of glycans onto the cancer cell membrane (Dube and Bertozzi, 2005; Zhang et al., 2010) due to which numerous diol-groups are present on the cell surface. Arising from the unique capacity of boronic acids to form boronic esters with these diols (Das et al., 2011), it would be interesting to attempt boronic acid as an alternative recognition molecule (reporter probe) to detect cancer cell surface glycans.

To show the signal in an electrochemical immunosensor, an electrochemical indicator is needed, such as a bioconjugate composed of enzyme or nonenzymatic catalyst. Compared to expensive and easily denaturable enzyme, Hyd attached on a bioconjugate can be used due to its small size, stability, and high catalytic activity towards hydrogen peroxide (H_2O_2) reduction (Zhu et al., 2013). Thus, in this work we developed a sandwich immunoassay approach having a chemo-nano-conjugate composed of APBA, MWCNT, and Hyd instead of enzyme linked reporter antibody. In this conjugate, APBA and Hyd are used a reporter molecule and electrocatalyst for signal generation, respectively.

In the present study, a novel amperometric immunosensor for the detection of MDR_{CC} has been tried to develop for the first time through the detection of P-gp. The immunosensor probe is fabricated by forming covalent bonds between AntiP-gp and carboxylic acid group-functionalized conducting polymer layer on AuNPs deposited electrode surface. The nanocomposite was characterized by atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), electrochemical impedance spectroscopy (EIS), scanning electron microscopy (SEM), and the AntiP-gp immobilization was confirmed by quartz crystal microbalance (QCM) and XPS. The sandwich immunosensing approach was adopted where APBA-MWCNT-Hyd was reacted with the MDR_{CC} captured by the GCE/AuNPs/pTTBA/AntiP-gp probe. The immunoreaction was monitored in term of catalytic activity of Hyd towards H_2O_2 reduction. The experimental parameters were optimized and the detection limit of the MDR_{CC} was determined. Direct detection of MDR_{CC} in serum and in the mixed cell samples were performed to evaluate the real clinical value of sensor. The selectivity of the biosensor was also examined toward various non target cells, and chemicals present in the real sample matrix. The developed method was also compared with the conventional reporter antibody based method.

2. Material and methods

2.1. Materials

2,2′:5′, 2″-terthiophene-3′(*p*-benzoic acid) (TTBA) was synthesized through the Paal–Knorr pyrrole condensation reaction (Koh et al., 2011). Tetrabutylammonium perchlorate (TBAP, electrochemical grade) was purchased from Fluka (USA) and purified according to a general method, followed by drying under vacuum at 1.33×10^{-3} Pa (Noh et al., 2012). 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDC), *N*-Hydroxysuccinimide (NHS), dichloromethane (99.8%, anhydrous), trisodium citrate, sodium tetrahydridoborate, $HAuCl_4 \cdot 3H_2O$, bovine serum albumin (BSA), and Hydrazine sulfate were purchased from sigma Aldrich (USA). Monoclonal p-GP antibody, amino-phenylboronic acid, indium tin oxide (ITO) glass, and H_2O_2 (33%) were purchased from sigma Aldrich (USA). MWCNTs (4–6 nm diameter, 95%) were obtained from Iljin Nanotech (South Korea). phosphate buffer saline solutions (PBS) were prepared with 0.1 M of disodium Hydrogen phosphate, 0.1 M of sodium dihydrogenphosphate, and 0.9% sodium chloride. cell culture medium, fetal bovine serum (FBS), trypsin-EDTA, penicillin/streptomycin, hank's balance salt (HBS) solution, were purchased from Sigma-Aldrich (USA). All other chemicals were of extra pure analytical grade and used without further purification. all aqueous solutions were prepared in ultrapure water, which was obtained from a Milli-Q water purifying system (18 M Ω cm).

2.2. Apparatus

All electrochemical experiments were performed in a three electrode cell. The modified glassy carbon electrode (GCE) (dia. 3.0 mm), Ag/AgCl (in saturated KCl), and a platinum (Pt) wire were used as working, reference, and counter electrodes, respectively. Voltammograms and chronoamperograms were recorded using a potentiostat/galvanostat, Kosentech, model KST P-2 (South Korea). A Multimode AFM device (Veeco Metrology) equipped with a Nanoscope IV controller (Veeco) was used at ambient conditions to get the images. The QCM experiment was performed using a SEIKO EG&G model QCA 917 and a PAR model 263A potentiostat/galvanostat (USA). An Au-coated working electrode (area: 0.196 cm^2; 9 MHz; AT-cut quartz crystal) was used for the QCM experiment. The impedance spectra were obtained using a EG&G Princeton Applied Research PARSTAT2263 at an open circuit voltage from 100.0 kHz down to 100.0 mHz and a sampling rate of five points per decade (AC amplitude: 10.0 mV). XPS was performed using a VG Scientific XPSLAB 250 XPS spectrometer and a monochromated Al Kα source with charge compensation at KBSI (Busan). The SEM images were obtained using a Cambridge Stereoscan 240. A JEOL JEM-2010 electron microscope (Jeol High-Tech Co., Japan) with an acceleration voltage of 200 kV was used to obtain TEM images.

2.3. Preparation of APBA-MWCNT-Hyd conjugate

Firstly, MWCNTs were functionalized according to previously reported methods (Goldman and Lellouche, 2010; Piran et al., 2009). Briefly, 100 mg of MWCNT was treated with mixture of concentrated 12.0 M HNO_3 and 36.0 M H_2SO_4 (90 °C, 2.0 h) followed by multiple rinsing with deionized water until no acid was detected. After drying at 80 °C under vacuum overnight, a black powder was obtained. Next, the treated MWCNT (3.0 mg) was dispersed into 1.0 mL PBS (pH 7.0) containing 10.0 mM EDC/NHS solutions and incubated at room temperature for 6 h to activate the –COOH on the MWCNTs. The resulting mixture was separated by centrifugation and the precipitate was washed three times. Meanwhile, an optimized 1.0 mg/mL hydrazine sulfate solution and 10.0 mM APBA was prepared in PBS. Subsequently, the activated MWCNTs were mixed with hydrazine sulfate and APBA solution. The mixture was stored in a refrigerator overnight at 4 °C followed by centrifugation. The resulting deposit was washed five times with PBS to remove any free APBA and Hyd. Finally, the prepared composite was dispersed in 1.0 mL PBS and stored in a refrigerator at 4 °C for further use.

2.4. Fabrication of immunosensor probe

The construction of the sensor is shown in Scheme 1. A layer of the polymer of the TTBA monomer was formed on the GCE/AuNPs surface through electropolymerization of 1.0 mM TTBA monomer