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# Ultrasensitive dual probe immunosensor for the monitoring of nicotine induced-brain derived neurotrophic factor released from cancer cells

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) was detected in the extracellular matrix of neuronal cells using a dual probe immunosensor (DPI), where one of them was used as a working and another bioconjugate loading probe. The working probe was fabricated by covalently immobilizing capture anti-BDNF (Cap Ab) on the gold nanoparticles (AuNPs)/conducting polymer composite layer. The bioconjugate probe was modified by drop casting a bioconjugate particles composed of conducting polymer self-assembled AuNPs, immobilized with detection anti-BDNF (Det Ab) and toluidine blue O (TBO). Each sensor layer was characterized using the surface analysis and electrochemical methods. Two modified probes were precisely faced each other to form a microfluidic channel structure and the gap between inside modified surfaces was about 19 μm. At optimized conditions, the DPI showed a linear dynamic range from 4.0 to 600.0 pg/ml with a detection limit of 1.5 ± 0.012 pg/ml. Interference effect of IgG, arginine, glutamine, serine, albumin, and fibrinogene were examined and stability of the developed biosensor was also investigated. The reliability of the DPI sensor was evaluated by monitoring the extracellular release of BDNF using exogenic activators (ethanol, $K^+$, and nicotine) in neuronal and non-neuronal cells. In addition, the effect of nicotine onto neuroblastoma cancer cells (SH-SY5Y) was studied in detail.

## 1. Introduction

Neurotrophins (NTs), a family of small and structurally related signaling proteins, are responsible in the survival, growth, and development of the mammalian nervous system. Various types of NTs have been discovered, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and NT-4/5 (Nagahara et al., 2011). Among them, BDNF has been widely studied and known to be expressed in the central and peripheral nervous systems through ligation with tropomyosin receptor kinase B (TrKB). The regulated synthesis and secretion of BDNF plays an important role in learning and memory, neuronal growth, and synaptic transmission as well as plasticity of neurotransmitters (Su et al., 2014; Nagahara et al., 2011; Egan et al., 2003). Several biological, pharmacological, and genetic studies have suggested that BDNF is one of the significant factors involves in the major neurological and psychiatric disorders, such as Parkinson's, Alzheimer's, Huntington's, amyotrophic lateral sclerosis, stroke, bipolar disorder, depression, and stress (Novartis Foundation, 2008; Suzuki et al., 2014). Interestingly, it has also been reported that the level of BDNF increases under the external stimulators, such as $K^+$, nicotine, and alcohol (Balkowiec et al., 2000; Serres et al., 2006; Gärtner et al., 2002). Of them, nicotine is one of the most widely abused addictive alkaloids and has been shown to produce some roles in neurodegenerative disorders. Thus, the study of secretory BDNF level in the presence of these stimuli especially nicotine would be significant in clinical, pharmaceutical, and biomedical fields thereby opening wide range of applications like drug screening, therapeutics, and clinical treatments.

So far, BDNF has been analyzed in various neuronal cells with the developed techniques employing enzyme-linked immunosorbent assay (ELISA) (Lever et al., 2001; Elfving et al., 2010), electrochemiluminescence (Newton et al., 2005), fluorescence (Nakajima et al., 2008), or high-performance liquid chromatography (HPLC) (Lyons et al., 1999). These techniques are useful, however, they are time consuming, requiring large sample volumes and lacks of miniaturization for onsite analysis. On the other hand, electrochemical methods are considered valuable and promising ones due to their robustness, cost-effectiveness, and miniaturization ability (Hussain et al., 2017). To date, electrochemical immunosensors have been developed on single electrode system and investigated towards their target molecules (Ronkainen et al., 2010; Hussain et al., 2016; Chikkaveeraiah et al., 2012). However, they may suffer from the low sensitivity due to

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the lack of effective immobilizations of mediators/catalysts and bioconjugate together on a single electrode, and most importantly, they cannot be translated for *in vivo* application. To overcome these issue, one of the possible way is to immobilize the indicator molecule onto the second electrode; thereby formation of immune complex occurs without external incubation. Thus, the fabrication of DPI comprising of a nano-bioconjugate/catalysts on one probe and reaction antibody immobilized onto other probe containing mediator surface would be an effective analytical approach for *in vitro* as well as *in vivo* applications. Such immunosensors containing the nano-bioconjugate particles and antibody onto two different probes have not been reported to date. Therefore, we developed an amperometric immunosensor in a microfluidic architecture using dual probe assembly to detect BDNF and evaluated for the exogenic effect of nicotine on BDNF secretion from nerve cells for the first time.

To fabricate a robust electrochemical biosensor, it is crucial to stably immobilize biomaterials on a sensor surface. Using functionalized conducting polymer (CP) formed on the probe surface is one of the excellent ways for stable sensor fabrication (Lee et al., 2001; Moon et al., 2017; Skotheim and Reynolds, 2007). However, only CP layer may not be able to enhance the electron transfer during the electrocatalytic process due to the lack of enough conductivity, therefore, incorporating CP with gold nanoparticles (AuNPs) is considered to be the most preferred way to enhance sensitivity, catalytic activity, structural integrity, and better conductivity (Naveen et al., 2017; Saha et al., 2012). Thus, polyterthiophene based CP [2,2:5,2-terthiophene-3-(p-benzoic acid)](pTTBA) containing (–COOH) group was electropolymerized onto AuNPs modified surface, where the stable immobilization of the antibody on the sensor surface was achieved through the amide bond formation between amine groups of antibody and –COOH groups of CP.

For signal generation in immunoreactions, different types of indicators have been used for sensing devices to generate a fluorescence (Qiu et al., 2017), photoelectrochemical (Lin et al., 2017; Zhang et al., 2018) or an electrochemical signal (Hermanson, 2013; Zhou et al., 2018). In case of most electrochemical immunosensors, bioconjugate is composed of enzyme or hydrazine to generate the signal, which limit the lifetime of the sensor system. Hence, it is worth to explore a stable molecules for electrochemical signal generation. One of the candidate is toluidine blue O (TBO), which is small, stable, inexpensive molecule and can be used as a redox indicator in electrochemical sensing without adding additional molecule like $H_2O_2$ to generate the electrochemical signal. Therefore, TBO can be one of ideal substitutes for enzymes in the synthesis of bioconjugate.

In the present study, a microfluidic immunosensor comprising of a pair of screen printed carbon electrodes (SPCE) has been developed to detect BDNF. The working probe was fabricated by covalently immobilizing capture anti-BDNF (Cap Ab) on the AuNPs/CP composite layer. The bioconjugate loading probe (bioconjugate probe) was fabricated by drop casting of the bioconjugate particles composed of detection anti-BDNF (Det Ab) and TBO immobilized on self-assembled 4′-([2,2′:5′,2"-terthiophen]-3′-yl)-[1,1′-biphenyl]-4-carboxylic acid (pTTBPA) at AuNPs. After the modification of both working and bioconjugate probes, they were precisely attached and wrapped. The detection mechanism is based on the microfluidic sandwiched approach, where the bioconjugate particles are readily available onto the bioconjugate probe for signal generation. Therefore, making the detection method simpler compared to the conventional immunosensor approaches, which require additional incubation steps. Effect of various exogenous activators (ethanol, $K^+$, and nicotine) on the release of BDNF from various cancer cells was also studied to show the prospective importance of sensing system in drug screening and therapeutics. Moreover, the effect of nicotine on the induced-release of BDNF from neuroblastoma cancer cells (SH-SY5Y) was confirmed using the proposed sensor in terms of time and concentrations profiles.

## 2. Experimental

### 2.1. Reagents

Lyophilized BDNF antibody (Cap Ab and Det Ab) were purchased from Thermo Fisher Scientific. Lyophilized BDNF (expressed in *E. coli, 27* kDa), TBO, sodium phosphate dibasic, sodium phosphate monobasic, RPMI-1640 medium, bovine serum albumin (BSA), trypsin-ethylenediaminetetraacetic acid (EDTA), penicillin/streptomycin, PBS solution, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC), N-hydroxysuccinimide ester (NHS), gold(III) chloride trihydrate ($HAuCl_4$), trisodium citrate, sulfuric acid ($H_2SO_4$), sodium chloride, hydrogen peroxide ($H_2O_2$, 30 wt% in water), di (propylene glycol) ethyl ether, tri (propylene glycol) ethyl, and phosphate-buffered saline (PBS) solution were purchased from Sigma-Aldrich (USA) and used as received. Cancer cell lines SH-SY5Y, PC-12, and noncancerous Vero cell lines were purchased from the American Type Culture Collection (USA). Monomer, [2,2:5,2-terthiophene-3-(p-benzoic acid)](pTTBA) bearing a carboxylic acid group, was synthesized according to the previous report (Kim et al., 2012).

### 2.2. Instruments

The electrochemical experiments were performed using an all-in-one screen-printed carbon electrode (SPCE), consist of working, reference (Ag/AgCl), and counter electrodes, respectively. Kosentech PT-1 model and an EG & G PAR 273A model galvanostat was used to Cyclic voltammetry (CV) and chronoamperometry. The impedance spectra were obtained with the EG&G Princeton Applied Research, PARSTAT 2630 multimode. An AFM device developed by Veeco metrology and equipped with a Nanoscope IV controller (Veeco), was used to take the AFM images of the fabricated sensor probe. XPS experiments were performed in KBSI (Busan) using a VG Scientific ESCA Lab 250 XPS spectrometer coupled with a monochromatic Al Kα source with charge compensation and XPSPEAK41 software was used to analyze the XPS data. QCM experiments were performed at an Au-coated working electrode (area: 0.196 $cm^2$; 9.0 MHz; ATcut quartz crystal) using a SEIKO EG&G model QCA 917 and a PAR model 263A potentiostat/galvanostat (USA).

### 2.3. Preparation of bioconjugate particles

Bioconjugate particles were prepared by immobilizing Det Ab and TBO chemically attached using EDC/NHS onto a self-assembled pTTBPA at AuNPs. To do this, SAM of pTTBPA and AuNPs composite were synthesized using the previously reported method (Zhu et al., 2012). At first, 50 ml of $HAuCl_4$ (0.01 wt%) was dissolved in $H_2O$ and mixed with 1 ml of 38.8 mM trisodium citrate. After 1 min, 0.5 ml of $NaBH_4$ (0.1 M) solution was added drop wise under gentle stirring. The color of resulting solution turned to pink violet upon adding $NaBH_4$, which showed the formation of AuNPs. Next, the pTTBPA was self-assembled onto the AuNPs 1:1 ratio (*v/v*) by mixing 1 ml of pTTBPA (1 mM) and incubating the reaction solution for 12 h, followed by successive centrifugation and washing steps. The pTTBPA at AuNPs was obtained in the final step (Koh et al., 2011) and was treated with 10 mM of EDC/NHS for 6 h to activate the –COOH groups of pTTBPA. Finally, 1 ml of the self-assembled monolayer (SAM) was mixed with 0.2 ml Det Ab and 0.2 ml of TBO (0.5 mM) and incubated for 12 h at 4 °C (optimized). After incubation, the solution mixture was centrifuged and washed with 0.1 M PBS to remove the unbound Det Ab and/or TBO. Finally, the designed bioconjugate particles (Det Ab/ TBO-SAM) was stored at 4 °C until further use