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Electrochemical Detection of Bisphenol A – Induced Neuronal Toxicity Using RGD Peptide Modified ITO Electrode Cell Chip

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An ITO electrode based neuronal cell chip was developed to electrochemically monitor the effect of environmental toxins on PC12 cell neurite outgrowth. PC12 cells were immobilized on an ITO electrode coated with a synthetic oligopeptide, which increased the cellular attachment and stability on the electrode surface. The undifferentiated PC12 cells on the ITO electrode were treated with the nerve growth factor to induce neurite outgrowth. The differentiation level of PC12 cells were determined by confocal microscopy. The effects of Bisphenol A on the cells were examined by cyclic voltammetry. We found that cyclic voltammetric peak current decreased with the concentration of Bisphenol A because of the decreased cell viability. This study has a number of potential applications in cell based biosensors and neuronal prosthetic devices.

Keywords Cyclic voltammetry; environmental toxin; nanobiochip; neurite growth; PC12 cells

1. Introduction

Cell behavior is not represented well by only gene and protein expression levels because a cell is much more complicated than the sum of its parts [1,2]. A cell based sensor array [3] and a label-free electrochemical methods have been tried for the rapid and precise detection of cell behavior [4,5]. Tailoring proper substrates is important in various research fields from microarray to molecular electronics for designing new bioengineering [6]. The most common materials for bio-interfacial studies are glass and gold surfaces. The glass surface has been widely used as biomaterial in cell biology related research because it can be easily modified by surface chemistry or patterning technology. In addition, the glass surface is very biocompatible and optical transparent [7]. However, the glass lacks electro-conductive

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property and dynamic interfacial property of biomolecules on the surface. On the other hand, the gold surface has good electro-conductive property and surface binding property for tailoring molecules. However, the gold surface is limited because of its non-transparent characteristics in cell biology related study [8]. Therefore, a new biomaterial or microarray is required for cellular adhesion to study cellular behaviors. Indium oxide (In_2O_3) doped with tin oxide (SnO_2), or Indium tin oxide (ITO), is an optically transparent and electrically conductive material. It have been commonly used as thin film layers on a glass substrate for touch panel contacts, electrodes for liquid crystal, plasma displays, gas sensors, and solar cells. The benefits of both electrically conductive and optically transparent properties are important at the dynamic examination of living cells under various environments [9].

A cell based assay has been developed by directly immobilizing living cells on substrate because a cell microarray is efficient and convenient for monitoring various samples in parallel [10–13]. Immobilization of living cells as *in vitro* is an important step in the fabrication of a cell based arrary [14]. However, there still exist barriers such as keeping the cell concentration, viability, and natural physiological state of cells immobilized on a substrate. Surface functionalization with biocompatible materials can be a reliable candidate for cellular adhesion without loss of viability. Hence, surface modification with extracellular matrices such as peptides, proteins, conducting polymers and other biologically active materials has been suggested to improve cell-surface adhesion for establishing physiological-like condition *in vitro*. In the previous reports [15,16], we intensively investigated RGD-MAP-C peptide as cellular adhesion molecules which enhance cell immobilization on a gold substrate for electrochemical study in a cell chip. However, peptide modification on the substrate, which is optically transparent and electrically conducting, has not been tried to optically and electrically monitor neuronal cells simultaneously. Gold is well known conducting material which has been widely used in cell based research because of its non-toxicity, but its non-transparent characteristics that prevent practical use of common inverted microscope were also a barrier for studying cell's behavior intensively.

In this study, the synthetic oligopeptides (RGD-MAP-C) were immobilized on an Indium Tin Oxide (ITO) surface for cell immobilization and subsequent biological toxicity detection. The immobilization level of RGD-MAP-C peptide was confirmed by AFM. Undifferentiated PC12 cells were cultured on the RGD-MAP-C coated ITO electrode and further treated with nerve growth factor (NGF) to induce neurite outgrowth. Finally, the effects of an environmental toxin, Bisphenol A, on the differentiated PC12 cells were investigated electrochemically.

2. Experimental Details

2.1. *Materials*

RGD-MAP-C was synthesized by Peptron (Daejeon, South Korea). Bisphenol A was purchased from Sigma-Aldrich (Germany). Nerve growth factor (NGF 2.5S, mouse) was purchased from Millipore (MA, USA). All other chemicals were of reagent grade.

2.2. *Peptide Immobilization*

The developed chip is composed of a layer of ITO deposited on the glass surface (1.1 mm thickness, 1200 Å coating thickness, and 15 ohm resistance) as a working

electrode. The RGD-MAP-C peptide was deposited on a glass substrate to form a thin film by adding 0.1 μg/ml peptide solution on the substrate at 4°C for about 12 hours. And then, the peptide modified ITO substrate was rinsed with PBS and dried under N_2 gas. The surface topography of the bare ITO or RGD-MAP-C modified ITO substrate was investigated by AFM (Nanoscope® III, Digital instruments, CA, USA).

2.3. *Cell Culture*

A PC12 cell line was cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2. To induce neurite outgrowth, the cells were treated with NGF.

2.4. *Confirmation of Neurite Outgrowth*

PC12 cells were cultured on an ITO electrode (0.5 cm^2) in a 96 well plate and treated with 100 ng/ml of NGF to induce neurite outgrowth. The cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then dried under N_2 gas. For confocal study, cells were investigated by NTEGRA spectra Scanning Confocal Raman Spectrometer (NTMDT, Russia) mounted on an inverted optical microscope (Olympus IX71).

2.5. *Electrochemical Behavior of PC12 Cell Neurite Outgrowth by Cyclic Voltammetry*

Cyclic voltammetry experiments were performed to determine the electrical properties of living cells and the effect of environmental toxins on cellular behaviors in normal laboratory conditions using a CHI660 instrument (Warminster, PA, USA) controlled by General Propose Electrochemical System (GPES) software. A home-made three electrode system consists of a cell-based chip as the working electrode, a platinum wire as the auxiliary electrode, and Ag/AgCl as the reference electrode. PBS buffer (10 mM, pH 7) was used as an electrolyte at a scan rate of 0.1 V/s.

3. Results and Discussion

3.1. *AFM Topological Analysis of a RGD Modified ITO Surface*

Figure 1 demonstrates the surface topological AFM images of a bare (a) or RGD-MAP-C deposited (b) ITO electrode, respectively. In these images, the peptide modified electrode exhibited less rough surface topology with reduced mean roughness value of 2.8 nm, compared to the bare ITO electrode with that of 2.6 nm. It indicates the immobilization of RGD peptide on ITO electrode surface. Because the Arg–Gly–Asp (RGD) peptide sequence is known as a cell recognition site for numerous adhesive proteins present in the extracellular matrix (ECM), the self-assembly of RGD containing peptide on ITO surface for electrochemical study enhance the affinity between the cell surface and ITO surface and can increase the voltammetric signal from the target cell. The sulfhydryl group of cysteine, which is the last amino acid sequence of the RGD-MAP-C peptide, attaches to the ITO surface easily by

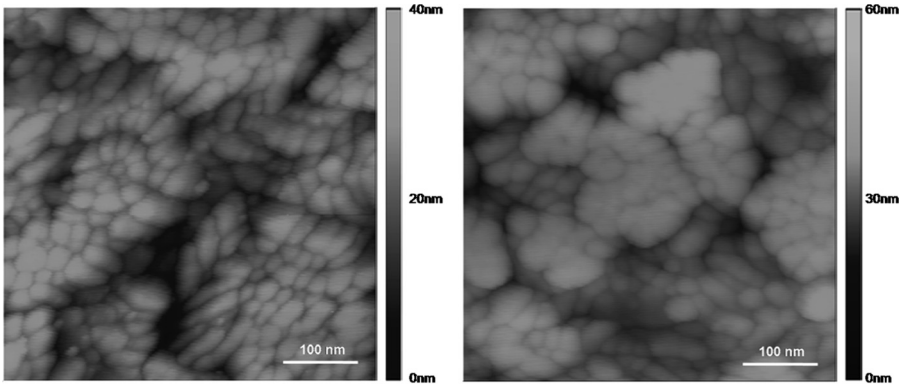


Figure 1. Surface topological AFM images: (a) bare ITO electrode and (b) RGD-MAP-C peptide deposited ITO electrode. The mean roughness values are 2.8 nm for bare ITO and 2.6 nm for RGD-MAP-C coated ITO surface.

thiol-metal bonding and form self-assembled, nano-scaled peptide film. However, the binding affinity of the peptide on the ITO surface is known to be weaker than on the gold surface [17,18].

3.2. *NGF Induced Neurite Outgrowth in a PC12 Cells*

To confirm the possible abnormal activity of neural cells on artificial surface or reduced neural cell differentiation activity which can be measured by microscopic images, the neurite of PC12 cells were investigated by confocal laser microscope equipped with an inverted optical microscope. The neurite outgrowths were successfully observed in NGF-differentiated PC12 cells; however, not in undifferentiated PC12 cells (Fig. 2). The length of the longest neurite in the differentiated PC12

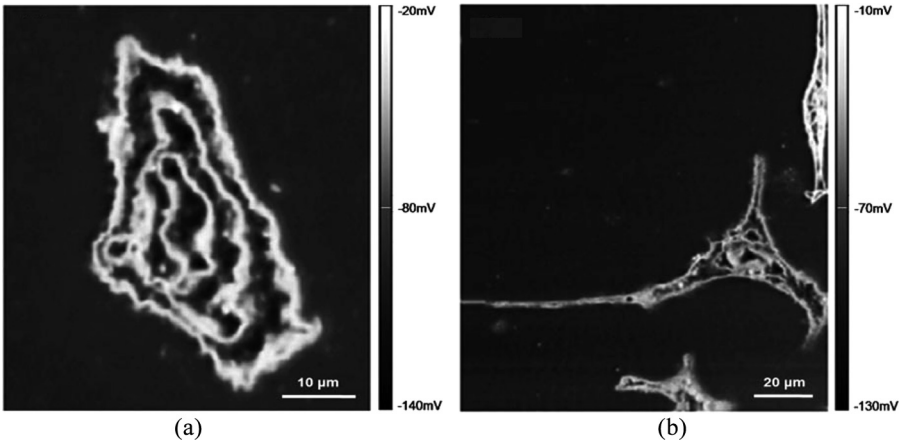


Figure 2. Confocal microscopy images of (a) undifferentiated and (b) differentiated PC12 cells on an ITO surface.

cells was measured to be approximately 65 μm which is two-fold longer than the cell body (25 μm) (Fig. 2b). These results mean that RGD-MAP-C modified ITO substrates provide excellent environmental conditions that enable precision electrochemical monitoring of neurite outgrowth of PC12 cells with outstanding transparent characteristics.

3.3. *Electrochemical Investigation of PC12 Cells on an ITO Electrode*

Figure 3a shows the voltammetric behavior of the differentiated PC12 cells immobilized on the RGD-MAP-C peptide modified ITO electrode within the potential range from −0.2 V to +0.5 V (verses Ag/AgCl). PC12 cells exhibits unreversible behavior on the reduction potential peak at about −0.05 V. The cyclic voltammetry of PC12 cells at different scan rates (80, 100, 120, 140, 160, 180, and 200 mV/s) was determined as in Figure 3b. The electrical current under a cyclic voltammetric curve increased with the scan rate. In addition, the peak current ratio at different scan rates was $i_{pa}/i_{pc} \neq 1$, which indicates a distinct quasi-reversible character of the cell

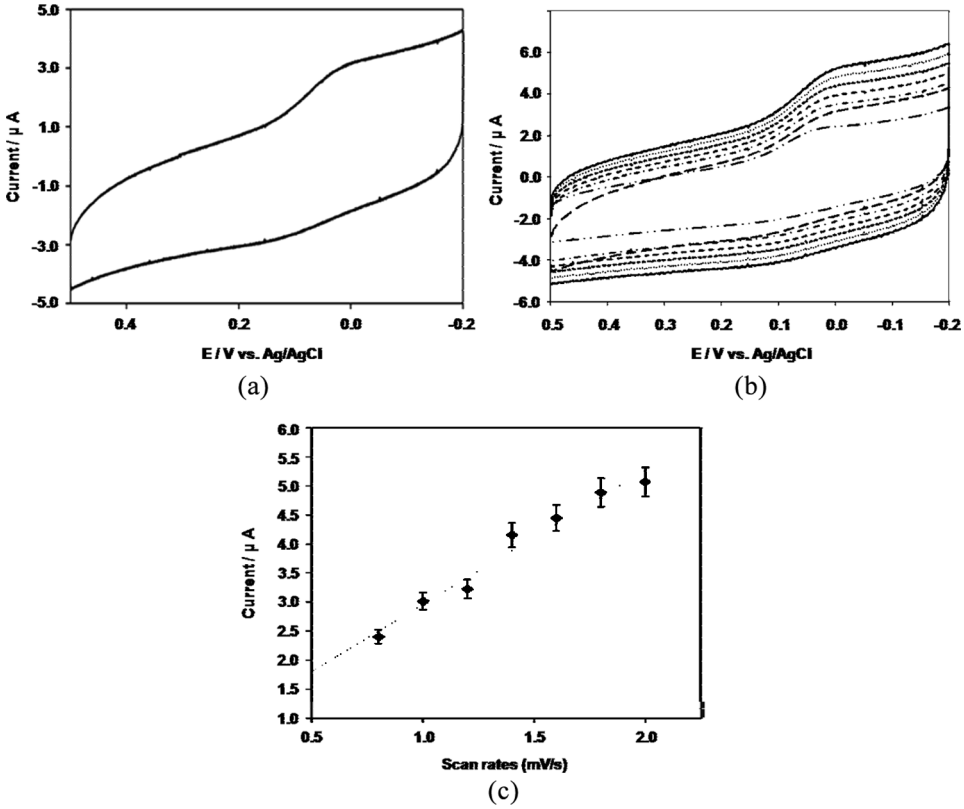
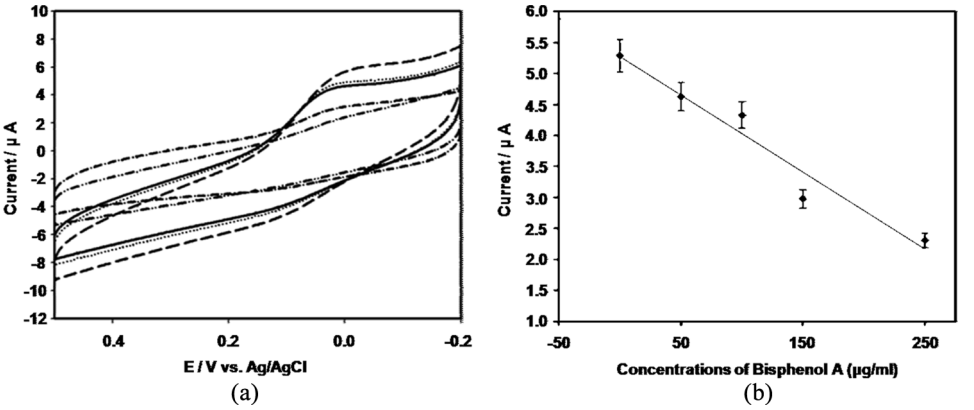


Figure 3. Cyclic voltammogram of (a) PC12 cell on RGD-MAP-C peptide coated ITO electrode (b) different scan rates (·-·-) 80, (- -) 100, (-··-) 120, (-·-·) 140, (·····) 160, (·····) 180, (——) 200 mV/s. (c) Linear plot between the reduction peak current and the scan rates at (80, 100, 120, 140, 160, 180, and 200 mV/s).



(a) (b)

Figure 4. (a) Cyclic voltammetry of PC12 cells treated with different concentrations of Bisphenol A (-·) 0, (——) 50, (·····) 100, (-··-) 150, and (- - -·) 200 µg/ml. (b) Linear relationship between the reduction peak current and the concentration of Bisphenol A.

electrode process. Figure 3c demonstrates the linear relationship between the scan rate and reduction peak current of PC12 cells.

3.4. *Cyclic Voltammetric Study of the Effect of Environmental Toxins on PC12 Cell Neurite Outgrowth*

Bisphenol A (BPA) is an organic compound with two phenol functional groups. It has been used in the synthesis of polysulfones and polyether ketones as an antioxidant in some plasticizers and a polymerization inhibitor in PVC. It is a key monomer in products of polycarbonate plastic and epoxy resins [19]. However, BPA has estrogenic properties and neurotoxicity. BPA is known to inhibit neural cell differentiation and proliferation during development as an endocrine disruptor [20]. To investigate the effect of environmental toxins on PC12 cell neurite outgrowth, the inoculated cells were immobilized on a RGD peptide coated ITO substrate for 24 hours. The cells were treated with NGF for approximately 2 days and then incubated with different concentrations of BPA in serum free media for 4 hours. Finally, the cells were investigated with cyclic voltammetry. Figure 4a shows cyclic voltammetric responses of differentiated PC12 cells to different concentration of BPA (0 to 250 µg/ml). The peak current decreased dramatically as the concentration of BPA. Figure 4b shows the inverse linear relationship between the current peak and the concentration of BPA. The decrease in the current peak might be due to the decreased cell viability with increasing BPA concentration.

4. Conclusion

In the current study, we developed a cell chip by modifying an ITO electrode with RGD-MAP-C peptide and immobilizing cells on the ITO electrode. Using the developed cell chip, we monitored the effect of an environmental toxin on differentiated PC12 cells electrochemically using cyclic voltammetry. We found that cyclic voltammetric peak current decreased with the concentration of BPA because of

the decreased cell viability. In conclusion, the developed cell chip allowed us to detect the neuronal toxicity effect of environmental neurotoxins.

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