

Fabrication of ITO/Gold nanoparticle/RGD peptide Composites to Enhance Electrochemical Signals from Human Neural Stem Cells

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ABSTRACT

Gold nanoparticles (GNP) and RGD peptide modified indium tin oxide (ITO) electrode was fabricated to enhance the electrochemical signals from neural stem cells (HB1.F3). Aminopropyltrimethoxylane (APTMS) was self-assembled on ITO electrode surface to immobilize GNP and its topological characteristics were confirmed by scanning electron microscopy (SEM). Thereafter, cysteine containing RGD peptide (RGD-MAP-C) was self-assembled on GNP immobilized surfaces via strong Au-S bond. Cells were seeded on the fabricated surface, and their electrochemical characteristics were analyzed by cyclic voltammetry (CV). As a result, ITO/GNP/RGD peptide composite was found to give highest redox signals compared to the bare ITO, ITO/RGD peptide and ITO/GNP substrate. Finally, the cell proliferation on different substrates were also analyzed by trypan blue assay to verify the effects of ITO/GNP/RGD peptide composites on human neural stem cells. Our newly fabricated substrate can be usefully applied for both electrochemical and optical study of stem cells.

Keywords: ITO electrode, gold nanoparticle, RGD peptide, electrochemical method, stem cell chip

1 INTRODUCTION

A cell chip was recently introduced to increase the reliability and sensitivity of *in vitro* assay by detecting redox behavior of living cells¹. A variety of electrochemical tools have been employed to detect the electrochemical response of living cells, such as open circuit potential at the cell/electrode interface², electric cell-substrate impedance sensing (ECIS)³, cyclic voltammetry (CV)⁴ and differential pulse voltammetry (DPV)⁵. All these electrochemical methods were proven as efficient tool for the determination of cell viability with high sensitivity and accuracy. However, the working electrodes mainly composed of gold (Au) or other metals used for the sensitive detection of electron-transfer or generation of living cells were found to

be improper for the integration of other valuable techniques such as confocal microscopy, Raman spectroscopy and optical microscopy²⁻⁵. A transparent conducting material, indium tin oxide (ITO)-deposited glass substrate, was utilized as a working electrode to replace the gold electrode for the integration of other optical techniques⁶. Unfortunately, the sensitivity of cell chip composed of ITO working electrode was found to be significantly decreased due to the low electrochemical activity of ITO surface that sacrificed huge advantage of electrochemical method.

Besides the electrochemical activity and resistance of working electrode in cell chip, surface modification of electrode surface is another important issue for the enhancement of sensitivity of cell chip. Since the sensitivity of cell chip depends on the electron transfer between cell and electrode surface generated by the redox characteristics of cells, the surface modification of working electrode for establishing *in vivo*-like condition is very important step for the increase of cell adhesion, proliferation and spreading on electrode surface that directly affects the sensitivity of cell chip. ECM or its components are well-known biomaterials which have excellent ability for increasing cell adhesion on artificial surface via integrin receptor-based linking⁷. Consequently, a variety of ECM proteins or its components (e.g., fibronectin, collagen, PLL, RGD etc.) were modified on cell chip to attach living cells to electrode surface by chemical or physical adsorption that showed remarkable performance for cell adhesion⁸⁻⁹. However, the uncontrolled thickness of ECM proteins decreased the electrochemical sensitivity of working electrode and caused the decrease of sensitivity¹⁰. Hence, advanced technique which can enhance cell adhesion without decreasing the electrical sensitivity of electrode is needed.

We have previously reported a cell chip modified with newly developed RGD peptide that entails the cysteine residues at the end of its sequence¹¹. By using cysteine-containing RGD peptide, RGD peptide could be easily modified on the electrode surface by simple self-assembly technique that showed significant enhancement of the redox peaks from cells on electrode surface compared to the other

ECM proteins. This cysteine-containing RGD peptide was further applied to fabricate RGD peptide nanopatterned surface that showed higher performance than mono-layered surface with respect to the electrochemical signals, cell adhesion strength and the sensitivity of fabricated cell chip⁵. One disadvantage of this technology is the complex method for the fabrication of nanoporous alumina mask which is laborious and time-consuming.

Here, a cell chip composed of ITO, gold nanoparticle (GNP) and RGD peptide composites was fabricated to enhance electrochemical signals from undifferentiated human neural stem cell (HB1.F3) and to increase cell proliferation. GNPs with diameter of 20 nm and 60 nm were self-assembled on amine-functionalized ITO electrode and its structural characteristics were confirmed by scanning electron microscopy (SEM). Cysteine-containing RGD peptide (RGD-MAP-C) was further modified on ITO/GNP complex by self-assembly technique. Therafter, the electrochemical signals obtained from HB1.F3 cells on different substrate (bare ITO, ITO/GNP, ITO/GNP/RGD-MAP-C) were compared by cyclic voltammetric tool. Finally, trypan blue assay was conducted to compare the proliferation rate of undifferentiated human neural stem cell on different kinds of substrate.

2 EXPERIMENTAL METHODS

2.1 Materials and reagents

RGD-MAP-C peptide was designed by our group and synthesized by Peptron (Daejeon, Korea). Gold nanoparticles with different diameter were purchased from BBInternational (South wales, UK). Aminopropyl trimethoxylane (APTMS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's phosphate buffered saline (D-PBS) (pH 7.4, 10mM) was purchased from STEMCELL technologies. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics were obtained from Gibco (Invitrogen, Grand Island, USA).

2.2 Cell culture

An immortalized human neural stem cells (HB1.F3) were kindly donated by Seung U. Kim (Chungang University, Korea). HB1.F3 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained under common cell culture conditions at 37 °C in an atmosphere of 5% CO₂.

2.3 Fabrication of ITO/GNP/RGD peptide composites

ITO-coated glass substrates were cleaned by sonication for 20min using sopy water, deionized water (DIW) and ethanol sequentially. A cell chamber was then attached to clean ITO surface using polydimethylsiloxane (PDMS) and baked for 6h. Basic piranha solution (1:1:5, H₂O₂:NH₄OH:H₂O) was added to chamber attached ITO surface and maintained for 30min at 80°C. After washing with DIW and dried under N₂ stream, 5% APTMS solution was added to each chamber and incubated for 24h. After exhaustive rinsing with ethanol and DIW, APTMS coated ITO suface were heated at 100°C for 10min to remove losely bound organosilane molecules. Next, GNPs with diameter of 20nm and 60nm each were added and incubated for 24h at 4°C. Finally, RGD-MAP-C peptide solution (0.05mg/mL in PBS) was applied and kept for 12h at 4°C, followed by washing twice with PBS buffer. The fabrication steps were shown in figure 1.

2.4 Electrochemical measurements

All electrochemical experiments were conducted using a potentiostat (CHI-660, CH Instruments, USA). A three-electrode system composed of an ITO/GNP/RGD peptide composites bottom as a working electrode, a platinum wire as the auxiliary electrode and Ag/AgCl as the reference electrode. Measurements were performed to detect the electrochemical properties of living stem cells. PBS (10 mM, pH 7.4) was used as an electrolyte at a scan rate of 50 mV/s.

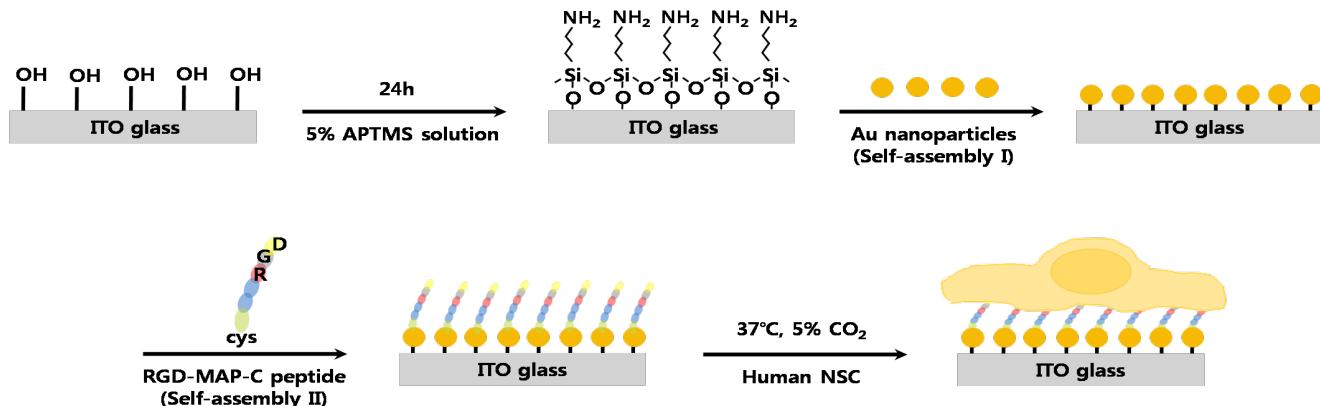


Figure 1. Schematic diagram of fabrication process of ITO/GNP/RGD peptide composites electrode.

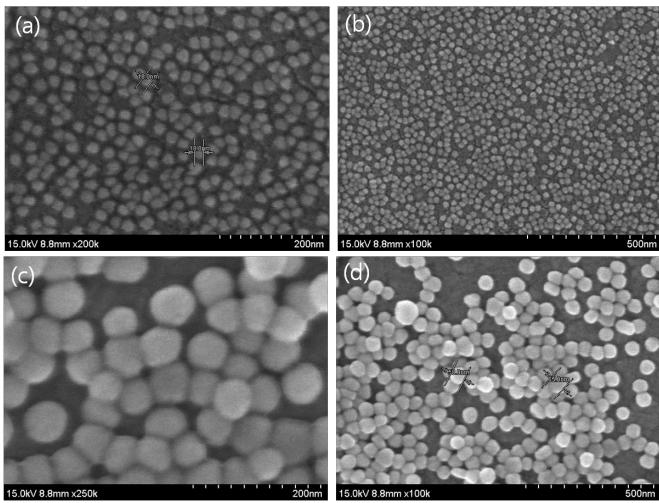


Figure 2. SEM images of (a) 20nm and (b) 60nm GNPs immobilized on APTMS functionalized ITO surface. (c) and (d) are high magnification images of (a) and (b), respectively.

3 RESULTS & DISCUSSION

3.1 Characterization of composite electrode

GNPs with 20nm and 60nm in diameter were attached on ITO surface by self-assembly technique via strong chemical bond. APTMS was selected for the immobilization of GNPs due to the fact that GNPs can be more efficiently attached on amine-functionalized surface than mercapto-functionalized surface¹². Figure 2 shows the SEM images of GNPs attached on ITO surface with well distributed geometry regardless of its diameter (20nm and 60nm). The immobilization of RGD-MAP-C on ITO/GNP surfaces was further confirmed by Raman spectroscopy which is not available by SEM images. Moreover, SERS effect was also detected due to the nanostructures fabricated by GNPs on ITO surface (data not shown).

3.2 Detection of electrochemical signals from human neural stem cell

Approximately 600 μ L of 3×10^4 cells/mL were added for each cell chamber attached on different kinds of substrate. After 72h of incubation, cyclic voltammetry was performed to detect the electrophysiological characteristics of human NSCs immobilized on various kinds of electrode. The exact mechanism of electrochemical signals from cells is still uncovered; however, intracellular redox system and the mitochondrial energy system have been considered as the origin of the cellular redox peaks which can be used as indicator of cell viability¹³. As shown in figure 3a, clear cathodic and anodic peaks from HB1.F3 cells were detected at -60mV and 110mV, respectively. HB1.F3 cells were also found to attach on bare ITO and ITO/RGD-MAP-C electrode; however, no redox peaks could be obtained from both fabricated electrodes at the same concentration of cells

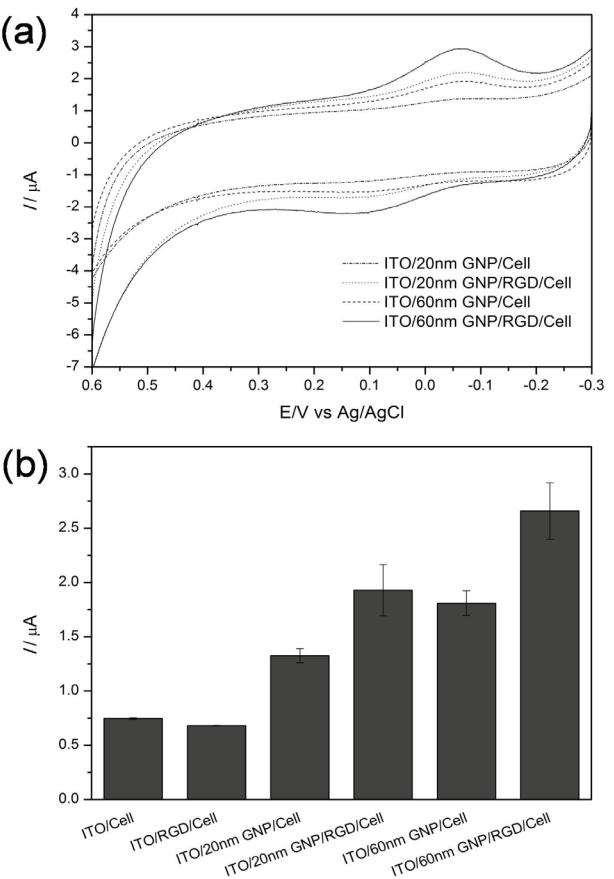


Figure 3. (a) Cyclic voltammogram of human NSCs on different substrate and (b) Current intensities of cathodic peak at -60mV from cells attached on different kinds of electrode. Data are the mean \pm standard deviation of three different experiments.

due to the low electrochemical sensitivity of ITO electrode (data not shown). Figure 3b shows the current intensities from cells on different kinds of electrode, indicating that modification of GNPs on ITO electrode enhances the electrochemical signals from cells. The redox peaks from cells were found to be more increased in 60nm GNP than 20nm GNP. Remarkably, RGD-MAP-C peptide modified ITO/GNP surfaces significantly increased the current intensities of cathodic peaks compared to the that of bare ITO/GNP surface. The signal enhancement induced by RGD-MAP-C peptide modification was evident at ITO/20nm GNP/RGD peptide electrode that showed more higher current intensity than ITO/60nm GNP substrate, while bare ITO/20nm GNP electrode showed weak intensity compared with ITO/60nm GNP electrode. Doxorubicin, a representative chemotherapeutic agent, was then added to HB1.F3 cells on ITO/60nm GNP/RGD peptide composites and its adverse effects on human NSCs were successfully assessed by differential pulse voltammetry (data not shown). Since the intensities of redox peaks directly affect the sensitivity of cell chip based on electrochemical tool, our newly fabricated composite electrode may contribute to the fabrication of highly sensitive stem cell chip.

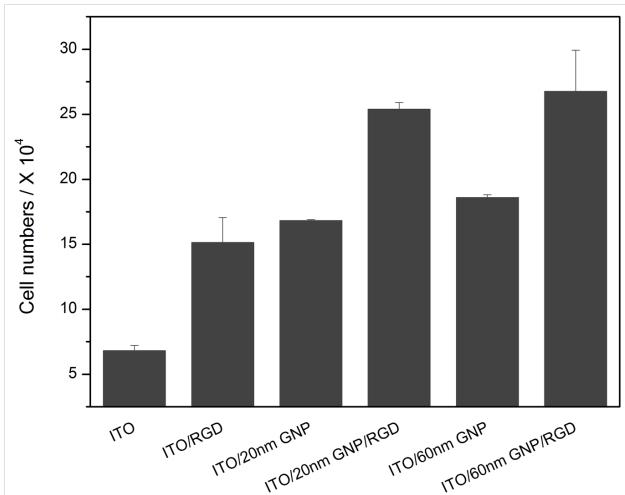


Figure 4. Number of human NSCs after 3days of incubation with different kinds of substrate. Data are the mean \pm standard deviation of three different experiments.

3.3 Stem cell proliferation on different substrate

Since cell proliferation largely contributed by the cell adhesion on artificial surfaces and can affect the intensities of peak current, the proliferation rate of human NSCs was confirmed by trypan blue assay. Figure 4 shows the number of HB1.F3 cells seeded on different kinds of substrate after 3 days of incubation. The results indicate that GNPs, RGD peptide and GNP/RGD peptide composite all increase the proliferation of neural stem cells compared with bare ITO surface. Among the various kinds of surface, the ITO/GNP/RGD peptide composites was found to incredibly increase the proliferation rate of HB1.F3 cells regardless of the sizes of GNPs. Previous study reported that the nanostructures on bottom surface tend to increase cell binding affinity and proliferation rate because cells prefer three-dimensional exterior than two-dimensional surface which is similar to *in vivo* environments¹⁴. We also have reported that RGD nanopatterned surface increase cell adhesion force of rat neural cancer cells (PC12) compared with the bare gold and RGD mono-layered surface⁵. Since GNPs attached on ITO surface realize three-dimensional space and also induce the strong attachment of RGD-MAP-C peptide by Au-S covalent bond, GNPs immobilized on ITO surface can be automatically contributed to the nanopatterning of RGD peptide and may provide three-dimensional environment for human NSCs. Hence, the fabricated ITO/GNP/RGD peptide composites was proven as effective substrate for the enhancement of electrochemical, as well as for the establishment of *in vivo*-like condition.

4 CONCLUSION

In this study, GNP and RGD peptide (RGD-MAP-C) modified ITO electrode was fabricated by simple two-step

self-assembly technique for the enhancement electrochemical signals from human NSC (HB1.F3). GNP attached on APTMS functionalized ITO surface showed well-distributed geometry which was verified by SEM images. Among the various kinds of fabricated electrodes, ITO/50nm GNP/RGD peptide composites was found to give highest electrochemical signals than any other substrate. This composite electrode also showed remarkable performance for the increase of stem cell proliferation which was confirmed by trypan blue method. Our newly fabricated substrate can be usefully applied for both electrochemical and optical study of stem cells.

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