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ITO/gold nanoparticle/RGD peptide composites to enhance electrochemical signals and proliferation of human neural stem cells

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Abstract

A cell chip composed of ITO, gold nanoparticles (GNP) and RGD-MAP-C peptide composites was fabricated to enhance the electrochemical signals and proliferation of undifferentiated human neural stem cells (HB1.F3). The structural characteristics of the fabricated surfaces were confirmed by both scanning electron microscopy and surface-enhanced Raman spectroscopy. HB1.F3 cells were allowed to attach to various composites electrodes in the cell chip and the material-dependent effects on electrochemical signals and cell proliferation were analyzed. The ITO/60 nm GNP/RGD-MAP-C composite electrode was found to be the best material in regards to enhancing the voltammetric signals of HB1.F3 cells when exposed to cyclic voltammetry, as well as for increasing cell proliferation. Differential pulse voltammetry was performed to evaluate the adverse effects of doxorubicin on HB1.F3 cells. In these experiments, negative correlations between cell viability and chemical concentrations were obseved, which were more sensitive than MTT viability assay especially at low concentrations (<0.1 μg/mL).

From the Clinical Editor: In this basic science study, a cell chip composed of ITO, gold nanoparticles and RGD-MAP-C peptide composites was fabricated to enhance electrochemical signals and proliferation of undifferentiated human neural stem cells (HB1.F3). The ITO/60 nm GNP/RGD-MAP-C composite electrode was found to best enhance the voltammetric signals of the studied cells.

Key words: Neural stem cells; Composites electrode; Electrochemical method; Nanobiochip; Doxorubicin

The cell is the basic building block of living organisms and provides a variety of invaluable information to whole tissues, organs and even whole organisms including animals and human beings.[1] These characteristics of living cells have led to the development of in vitro assays that can monitor the effects of drugs or chemicals easily and rapidly, which is not possible using molecules, DNA- or protein-based analysis. Various kinds of optical/fluorescence methods have been utilized to detect the cellular responses and cell viability, which is essential for efficient drug screening and toxicity assessment of chemicals or toxins. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), trypan blue and DAPI (4′,6-diamidino-2-phenylindole) exclusion assays are representative tools for the validation of cell viability.[2–4] However, techniques that utilize an optical source for the detection of cell viability may have signal errors or variations due to light interference or photo-bleaching effects, which can influence the determination of cell viability.

A cell chip was introduced as a reliable candidate to overcome the disadvantages of optical techniques and to increase the reliability and sensitivity of in vitro assays by detecting the redox behavior of living cells.[5,6] 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 interface,[7] electrochemical impedance spectroscopy (EIS),[8] cyclic voltammetry (CV)[9] and differential pulse voltammetry (DPV).[10] All these electrochemical methods were shown to be efficient tools for the

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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 could not be easily integrated with other valuable techniques such as confocal microscopy, Raman spectroscopy and optical microscopy.[11] A transparent conducting material, indium tin oxide (ITO)-deposited glass substrate, was utilized as a working electrode to replace the gold electrode for integration with other optical techniques.[12] Unfortunately, the sensitivity of the cell chip containing an ITO working electrode was found to be significantly decreased due to the low electrochemical activity of the ITO surface, which is the primary advantage of the electrochemical method.

Besides the researches for enhancing the electrochemical activity and for decreasing the resistance of working electrode in cell chip, surface modification technologies using biomaterials are another important issue in regards to enhancing the sensitivity of cell chips.[13] Since the sensitivity of cell chips strongly depends on electron transfer between cell and electrode surface generated by the redox characteristics of cells, surface modification of the working electrode to establish in vivo-like conditions is very important to increase cell adhesion, proliferation and spreading, all of which directly affect the sensitivity of cell chip.[10,14] Attachment of ECM or its components has been shown to increase cell adhesion on artificial surfaces via integrin receptor-based linking.[15] Consequently, a variety of ECM proteins or its components (e.g., fibronectin, collagen, laminine, PLL, etc.) have been applied on cell chips to improve attachment of living cells to the electrode surface by chemical or physical adsorption and these modified surface resulted in remarkable cell adhesion.[16,17] However, the uncontrolled thickness of ECM proteins or its components was found to decrease the electrochemical sensitivity of working electrodes and caused a decrease in the sensitivity of cell chips. Hence, other techniques that can enhance cell adhesion without decreasing the electrical sensitivity of electrode are essential for the development of highly sensitive electrochemical cell-based chips. We have previously reported a cell chip containing a newly-developed arginine–glycine–aspartic acid (RGD) peptide that consists of cysteine residues at the end of its sequence.[9,11,18] By using the cysteine-containing RGD peptide, an RGD peptide mono-layered Au surface could be easily fabricated by self-assembly on the electrode surface, which showed significant enhancement of redox peaks from cells when compared to other ECM proteins. This cysteine-containing RGD peptide was further applied to fabricate an 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 chip.[10] One disadvantage of this technology is the complex method required to fabricate the nanoporous alumina mask, which is both laborious and time-consuming.

Herein, we developed a stem cell chip composed of ITO, gold nanoparticle (GNP) and RGD peptide composites to enhance the electrochemical signals from undifferentiated human neural stem cell (HB1.F3) and increase cell proliferation on the electrode surface. The ITO/GNP/RGD peptide composites fabricated by a simple two step self-assembly technique were evaluated by scanning electron microscopy (SEM) and surface-enhanced Raman spectroscopy (SERS) via the GNPs structures formed on the ITO surface. The effects of GNPs, RGD-MAP-C peptide and the nano-array size on the electrochemical signals and proliferation of neural stem cell on chip surface were investigated by CV and the trypan blue exclusion assay. Finally, the adverse effects of doxorubicin (Dox) on HB1.F3 cells were monitored by DPV.

Methods

Materials

GNPs 20 nm and 60 nm in diameter were obtained from BB International (New York, United Kingdom). Aminopropyltrimethoxysilane (APTMS) was purchased from Sigma-Aldrich (Germany). Synthesized peptides (RGD-MAP-C) were designed by our group and synthesized by Peptron (Korea). The phosphate-buffered saline (PBS; pH 7.4, 10 mM) solution consisting of 136.7 mM NaCl, 2.7 mM KCl, 9.7 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and antibiotics were purchased from Invitrogen. Other chemicals used in this study were obtained commercially and were of reagent grade.

Cell culture

An immortalized human neural stem cell line (HB1.F3) was 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% CO_2.

Fabrication of cell chip composed of ITO/GNP/RGD peptide composites

ITO-coated glass substrates were first cleaned by sonication for 20 min using soapy water (0.1% Triton™ X-100), deionized water (DIW) and ethanol sequentially. The ITO surface was incubated in a basic piranha solution (1:1:5, H_2O_2:NH_4OH:H_2O) for 30 min at 80 °C. After washing with DIW and drying under N_2 stream, 5% APTMS solution was added to the basic piranha-treated ITO electrode and incubated for 24 h. After exhaustive rinsing with ethanol, the APTMS coated ITO surfaces were heated at 100 °C for 10 min to remove loosely bound organosilane molecules. Next, a 1 cm × 2 cm × 0.5 cm (length × width × height) plastic chamber (Lab-Tek(R), Thermo fisher scientific, USA) was attached on the APTMS-functionalized ITO surface to fabricate cell chip chamber using polydimethylsiloxane (PDMS). GNPs with diameters of 20 nm and 60 nm were then added to cell chip and incubated for 24 h at 4 °C, followed by washing with DIW and dried under N_2 stream to remove unbound GNPs. The fabricated cell chip chamber was sterilized by 70% ethanol and UV for 2 h. Finally, RGD-MAP-C peptide solution (0.05 mg/mL in PBS) was applied and incubated for 12 h at 4 °C, followed by washing twice with PBS buffer. The whole process of fabrication was presented

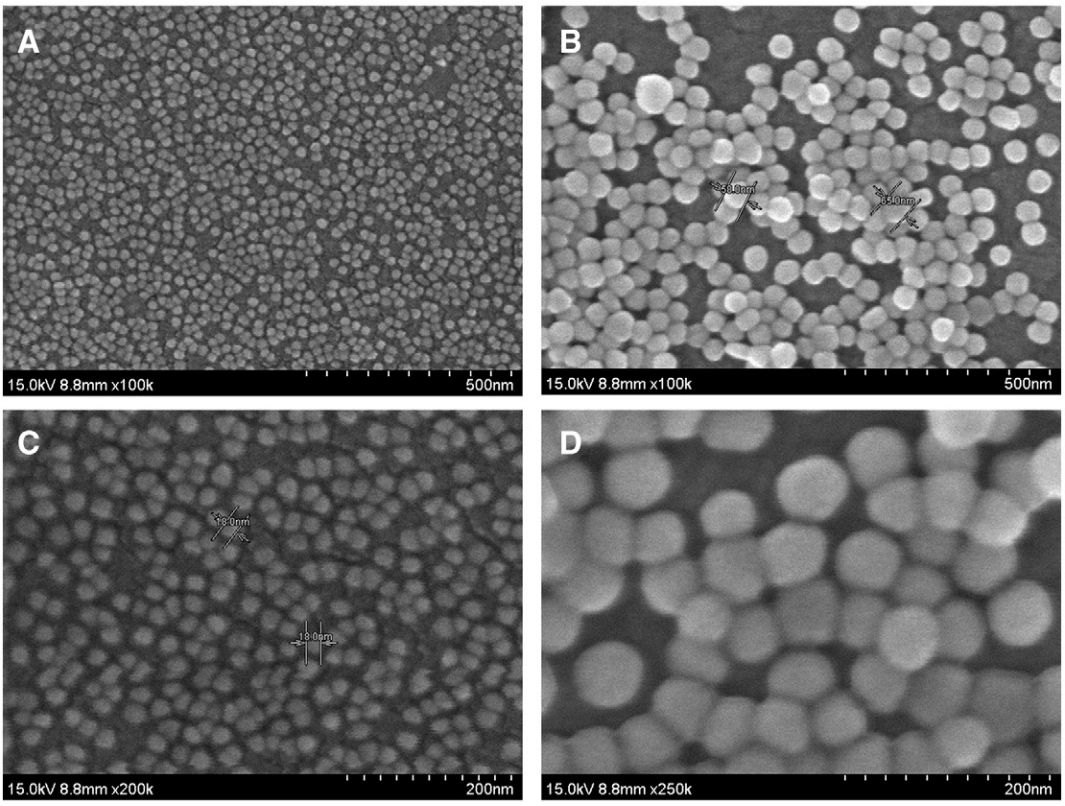


Figure 1. SEM images of **(A)** 20 nm and **(B)** 60 nm GNP immobilized ITO electrode. **(C)** and **(D)** are images of **(A)** and **(B)**, respectively, at high magnification.

in supplementary information (Supplementary Figure S1, available online at http://www.nanomedjournal.com).

Electrochemical measurements

All electrochemical experiments were conducted using a potentiostat (CHI-660, CH Instruments, USA). A three-electrode system composed of ITO/GNP/RGD peptide composites as a working electrode, a platinum wire as the auxiliary electrode and Ag/AgCl as the reference electrode. For electrochemical measurements of the cells on the chip surfaces, approximately 600 μL of 3×10^4 cells/mL solution was added to each cell chamber attached to different kinds of substrates. After 72 h of incubation, cells were washed with PBS (0.01 M, pH 7.4) and the redox characteristics at the cell-electrode interface were assessed by CV and DPV. The scan rate for all of the voltammetric measurements was 50 mV/s.

Raman spectroscopy

RGD-MAP-C peptide immobilized on GNP was investigated by Raman spectroscopy using Raman NTEGRA spectra (NT-MDT, Russia). The resolution of the spectrometer in the XY plane was 200 nm and along the Z axis was 500 nm. Raman spectra were recorded using NIR laser emitting light at a wavelength of 785 nm.

Trypan blue proliferation assay

Approximately 3×10^4 cells/mL were seeded on the surface of different fabricated chips under common cell culture conditions. After 72 h of incubation, the cells were detached from the substrate and mixed with trypan blue/serum-free DMEM solution (1:10). Viable cells were counted using a common hemocytometer.

MTT viability assay

Approximately 1×10^4 cells were seeded in a 96-well microtiter plate to investigate the mitochondrial activity of the cells treated with different concentrations of Dox using the MTT viability assay. After 24 h of incubation, medium was removed and replaced with Dox-free media. Then, 20 μL of stock MTT (5 mg/mL) solution was added to each well, followed by incubation for 3 h at 37 °C and 5% CO_2. Media were discarded, cells were lysed, and formazan was dissolved with DMSO. Absorbance was measured at 540 nm using a Benchmark microplate reader (Bio-Rad, Mississauga, ON, Canada). All measurements were carried out in triplicate in three or more independent experiments.

Results

Confirmation of ITO/Gold nanoparticles/RGD peptide composites

GNPs with diameters of 20 nm and 60 nm were attached onto the ITO surface using a self-assembly technique. APTMS was intentionally selected for the immobilization of GNPs since GNPs can be more efficiently attached on an amine-functionalized surface than mercapto-functionalized surface.[19,20] The SEM

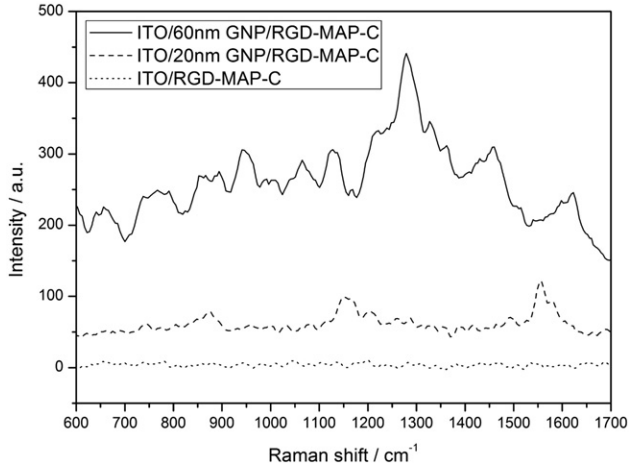


Figure 2. Raman spectra of various kinds of composite surfaces. Backgrounds were subtracted from the Raman spectra obtained from the surfaces without RGD-MAP-C peptide modification.

images in Figure 1 show that the GNPs attached onto the ITO surface with well distributed geometry regardless of its diameter (20 nm and 60 nm). RGD-MAP-C peptide containing quadruple branches of repetitive RGD sequences and cysteine residues at the end of its terminal was self-assembled on GNP/ITO surface to provide a cell-friendly environment. The immobilization of the RGD-MAP-C peptide on ITO/GNP surfaces was confirmed by Raman spectroscopy. Strong Raman signals were obtained from GNP modified ITO electrode surface due to the well-known SERS effects (Figure 2). The Raman signals were more strongly enhanced from the 60 nm GNP modified ITO surface than the 20 nm GNP/ITO surface, which showed clear Raman peaks at 1620 cm^{-1} (amide I), 1460 cm^{-1} (C–H bend), 1280 cm^{-1} (amide III), 1060 cm^{-1} (rocking NH_2), 940 cm^{-1} (str. C–C) and 640 cm^{-1} (wagging COO^-).

Electrochemical properties of ITO/GNP/RGD peptide composites

CV was performed to examine the electrochemical characteristics of bare ITO, APTMS modified ITO, RGD-MAP-C absorbed ITO, ITO/20 nm GNP, ITO/20 nm GNP/RGD-MAP-C, ITO/60 nm GNP and ITO/60 nm GNP/RGD-MAP-C in the presence of 2 mM $Fe(CN)_6^{4-}$ redox couple in 0.1 M KNO_3 solution. Clear redox peaks were detected from all substrates regardless of the presence of GNPs or RGD-MAP-C peptide. The peak separation for the bare ITO electrode was 94 mV while the $|E_{pc}-E_{pa}|$ of the APTMS modified ITO electrode was 58 mV, indicating that $Fe(CN)_6^{4-}$ was more reversible on the APTMS functionalized ITO surface due to the high affinity between the anionic redox probe and polycationic layers (Figure 3, *A*).[19] The I_{pa} and I_{pc} values were also higher on the APTMS modified ITO electrode than the bare ITO surface (20.1% and 17.5%, respectively) (Figure 3, *B*). GNPs and RGD-MAP-C peptide produced no remarkable effects due to the fact that $Fe(CN)_6^{4-}$ was strong enough to prevent any changes in the surface charge or resistance caused by the immobilization of GNP and/or RGD-MAP-C peptide even though GNP is highly electrocatalytic and RGD-MAP-C is a resistive material.

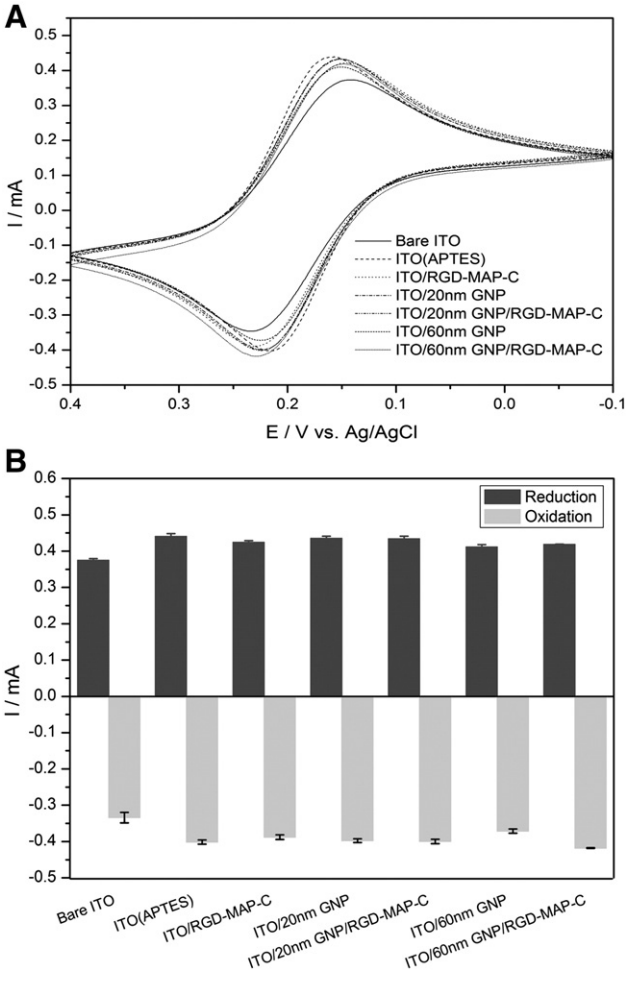
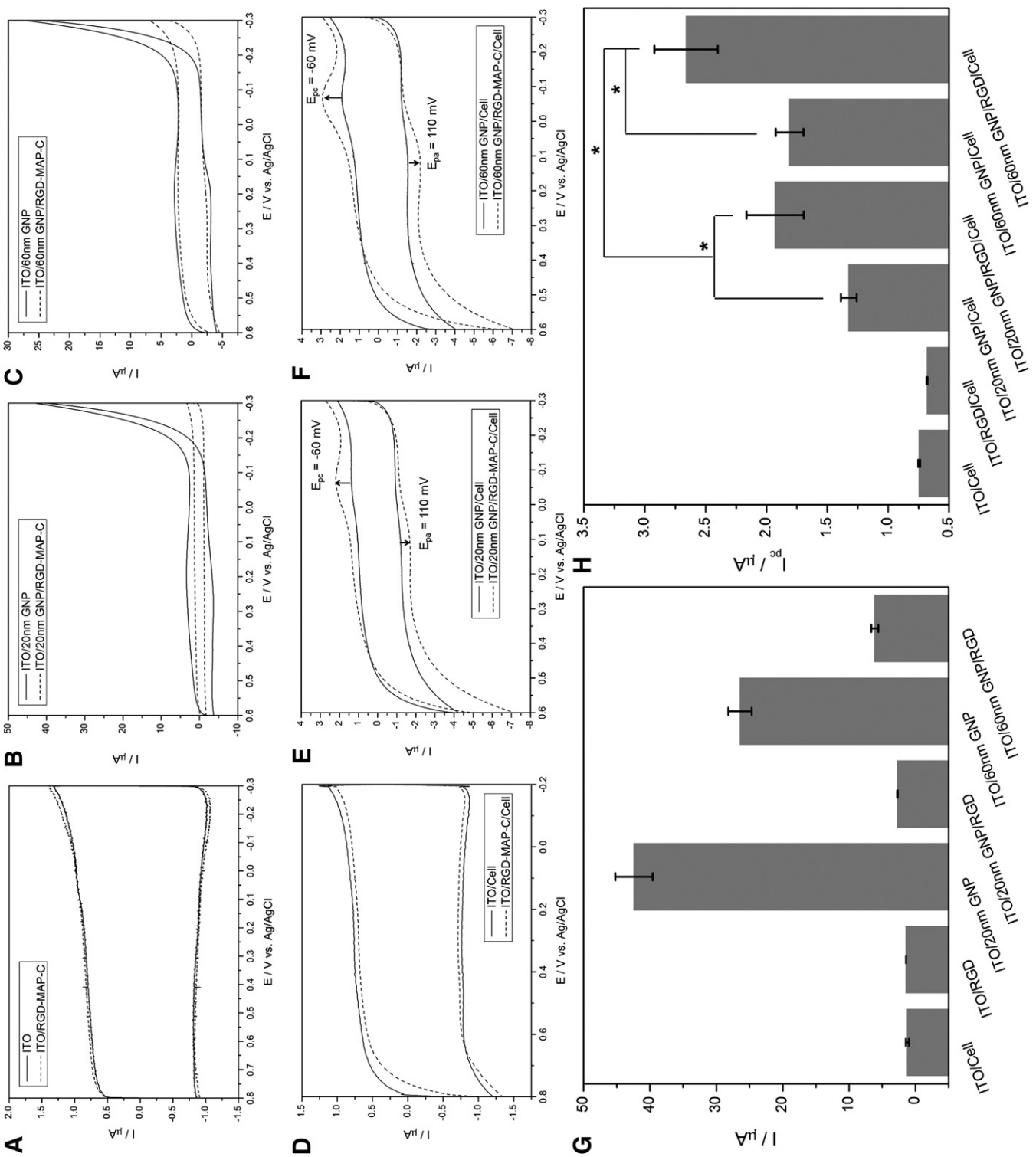


Figure 3. **(A)** Cyclic voltammogram of ITO, ITO/RGD, ITO/20 nm GNP, ITO/20 nm GNP/RGD, ITO/60 nm GNP and ITO/60 nm GNP/RGD peptide electrode in the presence of 2 mM $Fe(CN)_6^{4-}$ redox couple in 0.1 M KNO_3 solution. All ITO surfaces were functionalized with APTMS except 'Bare ITO'. **(B)** Current intensities of the cathodic (I_{pc}) and anodic (I_{pa}) peak obtained from **(A)**.

Electrochemical properties of undifferentiated human neural stem cells on ITO/GNP/RGD peptide composites

Figure 4, *A-C* shows the cyclic voltammograms of different surfaces in the presence of 10 mM PBS (pH 7.4) without cells. The ITO electrode produced very stable currents regardless of the addition of the RGD-MAP-C peptide (Figure 4, *A*). After the modification of GNPs, the current intensities and capacitance were dramatically increased due to the addition of the GNPs, which have a high electrocatalytic property (Figure 4, *B-C*). The current intensity at E = −0.3 V (vs. Ag/AgCl) was the highest for the ITO/20 nm GNP electrode due to the higher surface to volume ratio than ITO/60 nm GNP (Figure 4, *G*). However, the current intensity significantly decreased after the addition of the RGD-MAP-C peptide due to the self-assembly of cysteine containing-peptide on the surface of GNPs, which blocks


A
ITO
ITO/RGD-MAP-C
B
ITO/20nm GNP
ITO/20nm GNP/RGD-MAP-C
C
ITO/60nm GNP
ITO/60nm GNP/RGD-MAP-C
D
ITO/Cell
ITO/RGD-MAP-C/Cell
E
ITO/20nm GNP/Cell
ITO/20nm GNP/RGD-MAP-C/Cell
E_{pc} = -60 mV
E_{pa} = 110 mV
F
ITO/60nm GNP/Cell
ITO/60nm GNP/RGD-MAP-C/Cell
E_{pc} = -60 mV
E_{pa} = 110 mV
E / V vs. Ag/AgCl
I / μA
G
ITO/Cell
ITO/RGD
ITO/20nm GNP
ITO/20nm GNP/RGD
ITO/60nm GNP
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H
I_{pc} / μA
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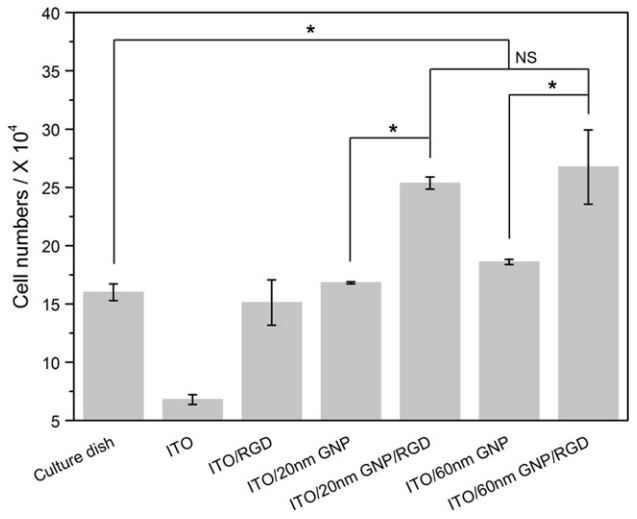

Figure 5. Proliferation of HB1.F3 cells seeded on different types of substrate. Approximately 2.1×10^4 cells were seeded on ITO, ITO/RGD peptide, ITO/20 nm GNP, ITO/20 nm GNP/RGD peptide, ITO/60 nm GNP and ITO/60 nm GNP/RGD peptide surfaces. Cells were counted by trypan blue proliferation assay after 72 h of incubation in a common incubator. Asterisk (*) means the increase in cell numbers was significant ($P < 0.05$, Student's t test) while 'NS' means the difference between two groups (ITO/20 nm GNP/RGD and ITO/60 nm GNP/RGD) was not significant ($P > 0.05$, Student's t test). Error bars are the mean±standard deviation of three different experiments.

electron transfer between GNPs and electrolyte. In contrast, the voltammogram for the cell immobilized electrode was completely different than the voltammogram observed for the electrode without cells (Figure 4, *D-F*). Undifferentiated human neural stem cell (hNSC) on the chip surface showed clear cathodic (E_{pc}) and anodic peaks (E_{pa}) at −60 mV and 110 mV, respectively. The peak separation $|E_{pc}-E_{pa}|$ between the anodic and the cathodic peaks was approximately 170 mV, and the peak current ratio I_{pa}/I_{pc} was greater than 1, which is representative of the distinct quasi-reversible characteristics of the cell. However, the I_{pc} value for the ITO/60 nm GNP/RGD-MAP-C composites electrode was higher than the ITO/20 nm GNP, ITO/20 nm GNP/RGD-MAP-C and ITO/60 nm GNP electrode (Figure 4, *H*). This is remarkable because the immobilization of RGD-MAP-C peptide on ITO/GNP significantly decreased current intensities at E=−0.3 V (vs. Ag/AgCl) and remarkably increased the electrochemical signals from hNSCs. ITO/20 nm GNP/RGD-MAP-C composites electrode also had a higher I_{pc} value than ITO/20 nm GNP and ITO/60 nm GNP, while ITO/60 nm GNP produced a higher current intensity than ITO/20 nm GNP. These results indicate that the immobilization of RGD-MAP-C peptide is very important for enhancing the electrochemical signals from hNSCs, which may affect the cell binding affinity to the artificial electrode surface via the formation of RGD peptide nanopatterned arrays. The fabricated ITO/60 nm GNP/RGD-MAP-C peptide electrodes were found to be effective for enhancing the electrochemical signals of a human breast cancer cell (MCF-7) that proves the generality of our composites electrode (Supplementary Figure S2).

Stem cell proliferation on different substrate

To confirm the superior biocompatibility of the fabricated surface, a trypan blue assay was conducted to count cell numbers after 72 h of incubation. The results of this analysis indicate that GNPs, RGD peptide and GNP/RGD peptide composite all increased the proliferation rate of hNSC when compared with the bare ITO surface (Figure 5). Differential interference contrast (DIC) images were also achieved to confirm the positive effects of GNP/RGD peptide composites on the stem cell growth (Supplementary Figure S3). Among the various kinds of surface, the ITO/GNP/RGD-MAP-C peptide composite was found to be best in regards to increasing the proliferation rate of HB1.F3 cells. The proliferation rate of HB1.F3 cells was not affected by the size of the GNP since there were no significant differences in cell numbers between ITO/20 nm GNP and ITO/60 nm GNP surface, as well as ITO/20 nm GNP/RGD-MAP-C and ITO/60 nm GNP/RGD-MAP-C substrates, respectively. The biocompatibility of both the ITO/60 nm GNP/RGD-MAP-C and ITO/20 nm GNP/RGD-MAP-C substrates was similar with respect to the cell proliferation rates, while the I_{pc} value of ITO/60 nm GNP/RGD-MAP-C composites was 37.8% higher than ITO/20 nm GNP/RGD-MAP-C electrode (Figure 4, *H* and Figure 5). The I_{pc} value of the ITO/60 nm GNP electrode without the RGD-MAP-C peptide was also 37.1% higher than the ITO/20 nm GNP electrode at the same numbers of hNSCs. These results indicate that hNSCs (HB1.F3 cells) prefer specific sized nanopatterned arrays and attached more strongly to the 60 nm GNP immobilized ITO surface than the ITO/20 nm GNP surface. We have previously reported that cells prefer a three-dimensional exterior over a two-dimensional surface, which is similar to in vivo environments. In this report, we also found that hNSCs preferred the 3D environment with respect to the size of the nanopatterned array. Hence, the fabricated 60 nm-sized GNP with RGD-MAP-C peptide on ITO electrode could be an effective substrate to enhance the electrochemical properties, as well as to establish an vivo-like condition, which will enhance cell adhesion and proliferation on artificial electrode surfaces.

Electrochemical determination of negative effects of doxorubicin on human neural stem cells

hNSCs (HB1.F3 cells) on an ITO/60 nm GNP/RGD-MAP-C composites electrode were treated with 0.01 μg/mL-1 μg/mL of Dox to assess the toxicity of Dox. After 24 h incubation, differential pulse voltammetry (DPV) was performed to measure the dose-dependent toxicity of Dox by analyzing the

Figure 4. **(A-C)** CV for comparing the electrochemical signals using ITO, ITO/RGD, ITO/20 nm GNP, ITO/20 nm GNP/RGD, ITO/60 nm GNP and ITO/60 nm GNP/RGD peptide electrode without cells. **(D-F)** CV for comparing the electrochemical signals using ITO, ITO/RGD, ITO/20 nm GNP, ITO/20 nm GNP/RGD, ITO/60 nm GNP and ITO/60 nm GNP/RGD peptide electrode with HB1.F3 cells. **(G)** Reduction current values of various substrates at E=0.3 V (vs. Ag/AgCl). **(H)** I_{pc} values of HB1.F3 cells on each substrates. CV was measured using PBS (0.01 M, pH 7.4) as an electrolyte at a scan rate of 50 mV/s and all experiments were conducted at a temperature of 27±1 °C using Pt and Ag/AgCl as a counter and reference electrode, respectively. Asterisks (*) mean the increase in the peak current was significant ($P < 0.05$, Student's t test). Error bars are the mean±standard deviation of three different experiments.

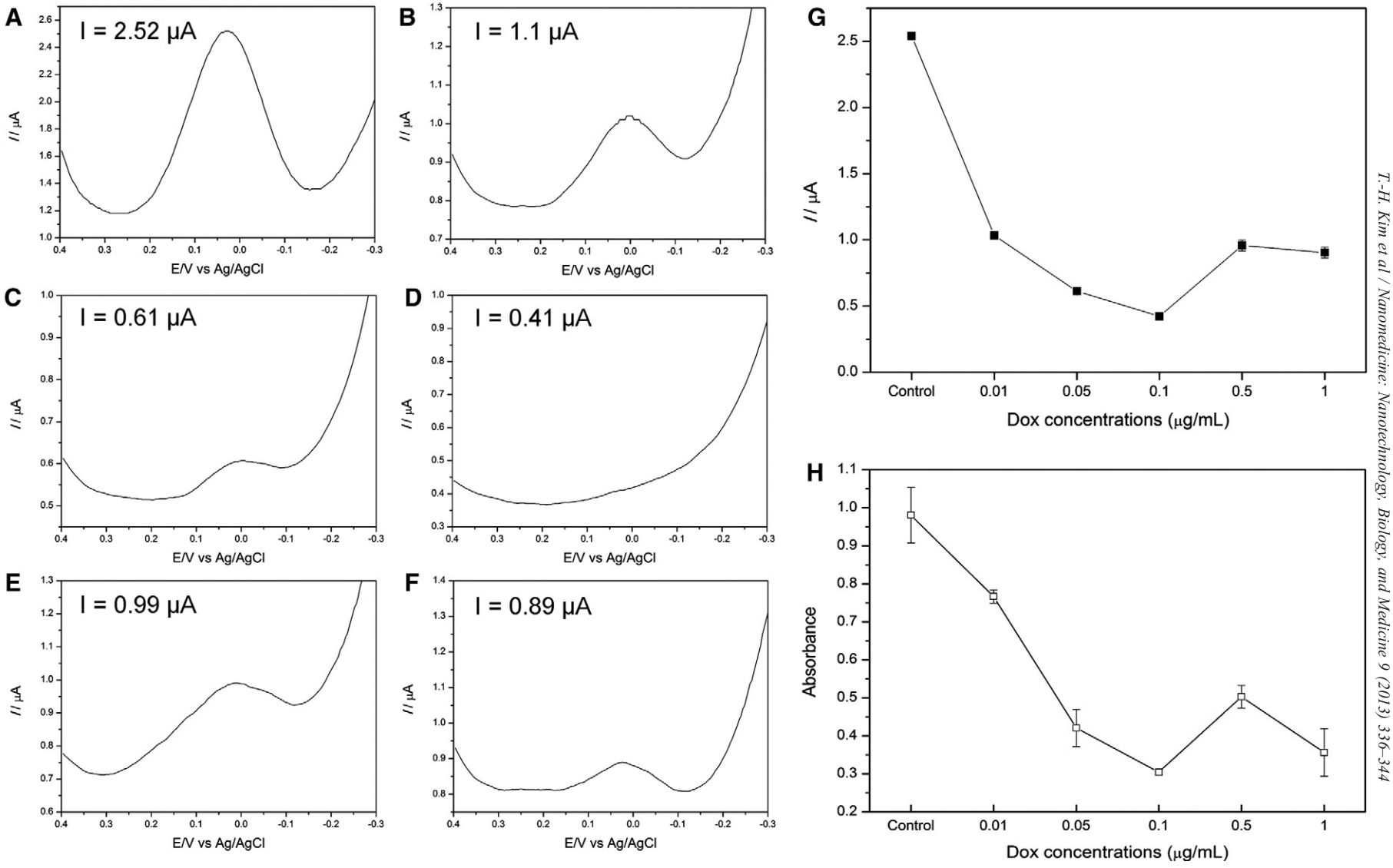

Figure 6. Effects of doxorubicin on HB1.F3 cells: The changes of DPV current peak for HB1.F3 cells on ITO/60 nm GNP/RGD-MAP-C treated with **(A)** 0 μg/mL, **(B)** 0.01 μg/mL, **(C)** 0.05 μg/mL, **(D)** 0.1 μg/mL, **(E)** 0.5 μg/mL and **(F)** 1 μg/mL doxorubicin. **(G)** Correlations between the different doses for doxorubicin treated HB1.F3 cells and its corresponding peak current obtained from **(A-F)**. Pulse amplitude and pulse width were 50 mV and 50 ms, respectively. **(H)** Correlations between the different doses of doxorubicin treated HB1.F3 cells and its corresponding absorbance values obtained using the conventional MTT viability assay. Absorbance values were measured at 540 nm. Data are the mean±standard deviation of three different experiments.

electrochemical signals from Dox-treated HB1.F3 cells. A strong peak current was achieved from cells at 28 mV (vs. Ag/AgCl) that were not treated with Dox (Figure 6, *A*); however, the intensities of the peak current decreased with an increase in the Dox concentrations up to a concentration of 0.1 μg/mL (Figure 6, *B-D*, *G*). MTT viability was conducted to confirm the electrochemical findings and similar results were observed (Figure 6, *H*). However, the DPV signals decreased by 59.5% when the cells were treated with 0.01 μg/mL Dox, while absorbance values obtained by MTT assay only decreased by 15.9% at the same concentration of Dox. From 0.01 μg/mL to 0.1 μg/mL Dox, the decrease in cell viability as measured by the DPV and MTT method was 58.9% and 61.7%, respectively, indicating that our electrochemical technique show a similar performance at high Dox concentrations. These results indicate that a cell chip composed of the ITO/60 nm GNP/RGD-MAP-C composite can be used to assess the potential cytotoxicity of various types of anticancer drugs or toxins at very low concentrations with high sensitivity and reproducibility. At 0.5-1.0 μg/mL Dox, the electrochemical signal from the HB1.F3 cells increased and was much higher than the signal observed for 0.05 μg/mL Dox-treated cells (Figure 6, *E-F*). Since the electrochemical signal from the cells directly represents the cell viability, an increase in cell viability was unexpected at these higher Cox concentrations; however, this phenomenon was also confirmed by the MTT assay. Thus, the increase of cell viability at high Dox concentrations (0.5-1.0 μg/mL) should be further investigated.

Discussion

Electrochemistry of cells on the chip surface can be affected by three major factors; electrocatalytic properties of the electrode used for cell immobilization, cell binding affinity to the electrode and the number of cells on the electrode surface (cell proliferation rate).[10,12,21] ITO is a well-known transparent electrode, which shows a stable background current with low electrocatalytic property. Combining novel metals or polymers such as gold nanoparticles or polyaniline/polypyrrole onto the ITO surface has been shown to be an excellent technique to enhance the electrochemical sensitivity of the ITO electrode for use as bio-sensors or biochips.[22,23] Since ITO is slightly toxic to neural cells and gold is a well-known biocompatible material, this simple technique can be more effectively applied for the fabrication of a neural cell chip. In addition, we previously found that RGD-MAP-C peptide significantly increases electrochemical signals and proliferation of various kinds of cancer cells on gold electrode surfaces.[18] Moreover, when the RGD-MAP-C peptide was fabricated as nanopatterned arrays, the cell adhesion affinity to the artificial electrode surface was shown to be enhanced, which is one of most important factors influencing the sensitivity of electrochemical signals of cells.[10] This positive effects of three dimensional nanopatterned array was found to be related with the gene that controls the cell adhesion protein known as P-Cofilin. The three dimensional surface induces the increased phosphorylation of cofilin in cells grown on topographic RGD-modified surface, leading to the enhancement of cell adhesion on the artificial surface and increase of cell growth. However, the process used to fabricate the nanoporous anodic aluminum oxide (AAO) mask to create the homogeneous peptide nanopatterned surface was found to be laborious and time-consuming. Since the RGD-MAP-C peptide easily attached to the surface of GNPs by self-assembly and then formed a peptide nanopatterned surface on the ITO/GNP electrode, the drawback of AAO-assisted peptide nanopatterned array can be simply overcome by using the ITO/GNP/RGD-MAP-C composite electrode. Neural stem cells used in this study showed no preference for the size of the RGD-MAP-C modified GNP with respect to its proliferation rate, which was 4 times higher than that on the bare ITO surface; however, the electrochemical signals achieved from NSCs on different kinds of substrate showed the remarkable differences of current intensity with respect to the size of GNP, as well as the immobilization of RGD-MAP-C peptides. The electrochemical phenomena was generally governed by the Randles–Sevcik equation,

$$i_{pc} = 2.69 \times 10^8 n^{3/2} A D^{1/2} v^{1/2} C$$

where i_{pc}=peak current, n=number of electrons involved, A=electrode area (m^2), D=diffusion coefficient (m^2/s), v=scan rate (V/s) and C=concentrations of analytes (mol/L). Since the numbers of cells on ITO/20 nm GNP/RGD-MAP-C and ITO/60 nm GNP/RGD-MAP-C were similar to each other as confirmed by 'Student *t* test' ($P < 0.05$), the concentrations of analytes (C) and number of electrons involved (n) were almost same. The electrode area (A) of ITO/20 nm GNP/RGD-MAP-C is 3 times higher than that of ITO/60 nm GNP/RGD-MAP-C due to the surface to volume ratio, indicating that i_{pc} value of ITO/20 nm GNP/RGD-MAP-C also should be 3 times higher than ITO/60 nm GNP/RGD-MAP-C theoretically. However, i_{pc} from ITO/60 nm GNP/RGD-MAP-C/Cell composites electrode was 37.5% higher than that of the ITO/20 nm GNP/RGD-MAP-C/Cell, indicating that diffusion coefficient (D) of ITO/60 nm GNP/RGD-MAP-C/Cell composites electrode was much higher than that of ITO/20 nm GNP/RGD-MAP-C/Cell composites electrode. Since the diffusion coefficient (D) in electrochemistry of cells is partially dependent on the cell adhesion affinity to the electrode surface (cell–electrode interfaces), it is significant that undifferentiated hNSCs prefer a particular nanopatterned array size and attached more strongly on the 60 nm GNP immobilized ITO surface than the ITO/20 nm GNP surface that contributed to the large enhancement of redox signals from cells. Hence, this peptide composites electrode can easily satisfy the transparency, electrochemical sensitivity and biocompatibility essential for fabricating a neural stem cell chip, all of which contribute to superior characteristics when compared to peptide functionalized gold, ITO or other polymer composites.

The fabricated surface (ITO/60 nm GNP/RGD-MAP-C) was for then used to evaluate the effects of a well-known chemotherapeutic agent, Dox, on undifferentiated hNSCs. Dox has been widely used as anticancer drug for cancer therapy; however, many kinds of adverse effects have been reported including nausea, vomiting, neutropenia and heart arrhythmias due to its non-targeting property.[24] Neural stem cells were

recently found to exist in the adult human brain in an undifferentiated state and may be important to the regeneration of neural and glial cells.[25] Hence, there is a need to assess the effects of Dox on undifferentiated human neural stem cells. Electrochemical signals obtained from cells decreased approximately 3.5 times more than the absorbance values of MTT viability assay when the cells were treated with 0.01 μg/mL Dox. This demonstrates that the newly fabricated composites electrode can be used to accurate assess the toxicity of chemotherapeutic agents especially at low concentrations.

In summary, the ITO/GNP/RGD peptide composite was easily fabricated by two step self-assembly technique that enhanced both electrochemical signals and proliferation of undifferentiated hNSCs, especially in ITO/60 nm GNP/RGD-MAP-C peptide composites electrode. The fabricated surface also showed excellent performance for the assessment of adverse effects of Dox on undifferentiated hNSCs which was superior to conventional MTT assay. Moreover, the fabricated surface containing the 60 nm GNP/RGD-MAP-C peptide composite was found to be most suitable for SERS study, which produced stronger Raman peaks than any other materials. Hence, our fabricated substrate may be used to assess the cytotoxicity of anticancer drugs at low concentrations, as well as for electrochemical and optical studies of stem cells.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2012.08.006.

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