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Monitoring *in vitro* neural stem cell differentiation based on surface-enhanced Raman spectroscopy using a gold nanostar array

Neuro-cell chip based on gold nanostars modified substrate was developed for *in situ* non-invasive monitoring of neural stem cell differentiation process using SERS technique.

Monitoring in vitro neural stem cell differentiation based on surface-enhanced Raman spectroscopy using a gold nanostar array

Waleed Ahmed El-Said, Seung U. Kim and Jeong-Woo Choi

The development of neurochips for non-invasive monitoring of neural stem cell stimulation is highly desirable and can enable the efficient optimization of tissue development protocols. Traditional methods, including cell staining and sorting, have long been used, but these techniques are time-consuming and may damage cells. Here, we have developed a cell-based chip to monitor the in vitro stepwise differentiation process of isolated mouse neural stem cells, the one-step differentiation of adult human neural stem cells (HB1.F3), and the electrochemical stimulation of rat pheochromocytoma PC12 cells. Results showed that each cell line exhibited a different behavior during differentiation. The DNA contents changed irregularly during the differentiation of HB1.F3 cells, while the percentage of proteins increased. In addition, the results revealed that the electrochemical stimulation of PC12 cells induced changes in the synthesis of DNA and proteins. The differentiation of isolated mouse neural stem cells showed a decrease in some peaks corresponding to the DNA content and an increase in the percentage of protein, in addition to the irregular behavior of some peaks related to both nucleic acids and proteins. The increase in protein percentage could indicate local variations in protein structure and a maturation shift. These results demonstrate that the SERS technique allows for more rapid biological sample analysis without time-consuming staining, enabling researchers to monitor engineered tissues and optimize culture conditions in a near real-time manner.

Introduction

Embryonic stem cells have the ability to differentiate into one of more than 200 cell types, such as neurons, cardiomyocytes, hepatocytes, islet cells, skeletal muscle cells, and endothelial cells. Stem cell therapy could change the treatment of intractable human diseases such as Parkinson’s disease, ischemic heart diseases, and diabetes. Neural stem cells (NSCs) have considerable therapeutic potential for the treatment of neurological disorders. The differentiation of NSCs is an important process during the development of the central nervous system. Therefore, monitoring the differentiation of NSCs is important in fields such as regenerative medicine and transplantable tissue, and in its use as an alternative source of donor tissue neural precursors.

Further applications include the repair of pathological processes and the repair of neurodegenerative processes, including Parkinson’s and Alzheimer’s diseases. In addition, understanding the mechanisms of in vitro stem cell differentiation will allow the development of rational approaches to systematically manipulate cell fates. Common methods reported for monitoring the differentiation of neural cells include reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry, immunocytochemistry, colorimetry, or sorting techniques. However, these techniques have a number of disadvantages, such as being labor-intensive, multistep, and time-consuming processes; being endpoint assays that do not offer the mechanism details; or being destructive methods requiring biomarkers or labels, thereby limiting potential application. The development of an efficient, high-throughput, rapid, simple, sensitive, nondestructive and label-free technique for in vitro monitoring of the differentiation of NSCs on a single-cell level is therefore needed. This would have an impact on biomedicine as well as on the development of effective stem cell sensors.

Cell-based chips using optical or electrochemical detection systems hold great promise as a cell-based detection method. Electrochemical cell-based chips allow for easy monitoring and analysis of the cell signals of living cells arising from redox reactions, utilizing several electrochemical techniques such as...
cyclic voltammetry (CV), differential pulse voltammetry, electric cell-substance impedance sensing (ECIS), scanning electrochemical microscopy (SECM), electrochemical impedance spectroscopy (EIS), and the oxygen electrode. In our previous studies, the voltammetric behaviors of different cancer cells were determined using modified electrodes. These studies reported on the effect of anti-cancer drugs on a cell chip using CV and potential stripping analysis methods. However, the electrochemical behavior of the living cells represented only the cell viability based on electron transfer between the cell and the electrode surface, and the mechanism of the stem cell differentiation could not be monitored using these electrochemical methods.

Cell-based chips for optical detection have the distinct advantage of allowing visualization of changes in cell status. However, although white light imaging (phase contrast or differential interference contrast) can reveal the approximate level of in situ differentiation, it is a qualitative technique. Fourier transform infrared (FT-IR) spectroscopy can be used to monitor the single-cell differentiation of fixed or dried stem cells, but it is not suitable for monitoring the differentiation of living cells. Although Raman spectroscopy has demonstrated the ability to monitor the differentiation of living NSC, it is too slow to characterize a sufficient number of individual cells to be a reliable clinical technique. Nonetheless, Raman spectroscopy could be used extensively in biomedical research.

The enhancement factors in surface-enhanced Raman spectroscopy (SERS) can be as high as $10^5$–$10^{15}$, which allows this technique to be sensitive enough to detect single molecules. In addition, SERS has 2 to 3 times the sensitivity compared to fluorescence. As such, the increased sensitivity of Raman scattering has generated tremendous interest in the nanomaterial, spectroscopy, and analytical chemistry communities. SERS offers an exciting opportunity to overcome the critical disadvantages of conventional Raman spectroscopy, allowing for relatively lower laser intensity, longer wavelengths, and rapid signal acquisition times. Consequently, near infrared (NIR) SERS is becoming a useful tool for biological applications with high selectivity and sensitivity, which could extend Raman utility to a wide variety of interfacial systems previously inaccessible to conventional Raman spectroscopy. In addition, the SERS technique gives effective, molecule-specific information regarding immobilized molecules on the metal surface in situ and in aqueous solutions. At present, many researchers continue to demonstrate the great potential of SERS applications in the fields of biochemistry, biophysics, and molecular biology. Average SERS-enhanced spectra is one of two strategies to acquire the SERS signals, which are obtained from an ensemble of colloidal particles and aggregates giving a relatively low signal especially before aggregation. Several studies have reported on the use of colloidal metallic (gold (Au) or silver (Ag)) nanoparticles (NPs) or nanorods to perform SERS on living cells and various important biological species such as DNA and protein. Au NPs of different diameters were used as SERS-active agents for monitoring chemical changes during the differentiation of isolated mouse neural stem cells. However, the non-homogeneity of the NP aggregates caused a dramatic change in the enhancement of Raman signal from one point to another on the cell surface. During NP preparation, surfactants such as CTAB (cetyltrimethylammonium bromide) or PVP (polyvinylpyrrolidone) were used. The existence of these species on the metal NP surface results in fewer active sites, and the SERS signal generated by these species could severely interfere with the SERS signals of the target molecules. Samanta et al. (2014) reported on the fabrication of specific targeting SERS nanotags based on the modification of Au NPs with three active Raman reporters and antibodies for the identification of differentiated mouse neural stem cells. The antibody-conjugated metal NPs overcome the non-homogeneity limitations of non-targeting NPs. However, the presence of antibodies was reported to cause unwanted SERS signals that were barely distinguishable from the Raman signals originating from target molecules inside the cell. In addition, nuclear-targeted Au NPs have been used as intracellular probes to monitor the differentiation of isolated mouse neural stem cells using SERS. This was a challenge due to the existence of several cellular barriers limiting the delivery of SERS-active colloidal NPs to the cell nucleus. Moreover, Au NP-targeting of the cell nucleus has been reported to influence cellular function, causing DNA damage, cell death, and apoptosis. The development of SERS-active surfaces has been based on the existence of particular hot spots (Ag or Au nanostructures) on modified substrates, which permits the detection of a few molecules with fluctuating spectral characteristics. However, the fabrication of SERS-active substrates has been found to create a number of problems, including poor signal enhancement, uniformity, and reproducibility. In previous work conducted by our group, we developed different SERS-active surfaces to monitor anticancer effects as well as to analyze intracellular states. An advanced method for the fabrication of SERS-active surfaces is still required for more effective enhancement of Raman signals.

The present work represents the development and application of an Au nanostructure-modified ITO substrate as a SERS-active surface, cell culture system, and working electrode, as shown in Scheme 1. This provides a quantitative, label-free, biocompatible, and noninvasive optical spectroscopy technique for monitoring the in vitro stepwise differentiation process of isolated mouse neural stem cells, the one-step differentiation of adult human neural stem cells (HB1.F3), and the electrochemical stimulation of rat pheochromocytoma PC12 cells. Thus, the approach described in this paper plays a vital role in identifying the cell types in biomedical stem cell research, overcoming the limitations of the SERS-NP-based approach. Moreover, this technique is a promising noninvasive tool to monitor the changes in the chemical composition of NSCs during their differentiation, and it has potential applications in high-throughput analysis, drug screening, and induced pluripotent stem cell generation.

**Experimental section**

**Materials**

Hydrogen tetrachloroaurate(III)trihydrate (HAuCl₄·3H₂O, 99.9 + %) and phosphate buffered saline (PBS) (pH 7.4, 10 mM) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neurite growth factor (NGF) was obtained from Millipore. Polyethylene glycol-200
(PEG) \( (M_W = 200) \) was obtained from Yakuri Pure Chemicals Co. Ltd (Osaka, Japan). All other chemicals used in this study were commercially obtained as reagent-grade chemicals. All aqueous solutions were prepared using deionized water (DIW) from a Millipore Milli-Q water purifier operating at a resistance of 18 M\(\Omega\) cm.

**Cell culture**

Rat neural PC12 cells and HB1.F3 were cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% antibiotics (Gibco). The cells were maintained under standard cell culture conditions at 37 \( ^\circ \)C in an atmosphere of 5% CO\(_2\). The medium was changed every two days. The number of cells was determined by the trypan blue assay with a hemacytometer. Primary mouse neural stem cell was isolated and cultured from embryonic (E13.5) mouse brains according to the method reported previously.

**Electrochemical measurements**

All electrochemical experiments were performed using a potentiostat (CHI-660, CH Instruments, USA) controlled by general-purpose electrochemical system software. A homemade three-electrode system consisting of cell/Au nanostars/ITO as the working electrode, a platinum wire as the counter electrode, and Ag/AgCl as the reference electrode was set up. Measurements were carried out to study the electrical properties of living cells under normal laboratory conditions. PBS (10 mM, pH 7.4) was used as an electrolyte at a scan rate of 50 mV s\(^{-1}\).

**Raman spectroscopy**

The biochemical compositions of the control PC12, HB1.F3 and isolated mouse neural stem cells, as well as the effects of one-step differentiation on PC12 cells and HB1.F3 cells and of stepwise differentiation of isolated mouse neural stem cells were investigated based on the SERS technique using Raman NTEGRA spectra (NT-MDT, Russia) equipped with an inverted optical microscope and a liquid nitrogen-cooled CCD detector. The maximum scan-range, \( XYZ \), was 100 \( \mu \)m \( \times \) 100 \( \mu \)m \( \times \) 6 \( \mu \)m; the resolution of the spectrometer was 200 nm in the \( XY \) plane and 500 nm along the \( Z \) axis. Raman spectra were recorded using an NIR laser-emitting light at a wavelength of 785 nm, with an irradiation laser power of 3 mW on the sample plane. The medium was removed, and the cells were washed three times with PBS, which was used during the SERS measurements to eliminate the effect of the medium on SERS signals. Ten scans of 5 s from 600–1750 cm\(^{-1}\) were recorded, and the mean result was used. A blank spectrum was acquired prior to each step, which allowed the absorbance to be subsequently measured.

**Fabrication of gold nanostar array-modified ITO substrate**

Au nanostar arrays were electrochemically deposited onto ITO substrates (20 mm \( \times \) 10 mm) using different concentrations of
a HAuCl₄ aqueous solution containing 20 µL mL⁻¹ of PEG-200 as a surfactant, according to our previously reported method.⁵⁷ Then, ITO-coated glass substrates were typically cleaned by sequential sonication in 1% Triton X-100 solution, DIW, and ethanol for 15 min, followed by sonication in basic piranha solution (1:1:5, H₂O₂:NH₃:H₂O) for 30 min at 80 °C. Finally, the substrates were rinsed with DIW and dried under N₂ stream to obtain a clean ITO surface. Au nanostar arrays were electrochemically deposited onto the ITO substrates by using different concentrations of HAuCl₄ (0.3, 0.5 and 0.7 mM) under a constant potential of −1.3 V (vs. Ag/AgCl), and the deposition temperature was controlled and maintained at 25 °C in an electric, heated, thermostatic water bath. To remove any trace surfactants adsorbed on the Au nanostar-modified ITO surface, the substrates were rinsed in DIW and then boiled for 5 min with isopropyl alcohol under N₂ stream. The active area for electrochemical deposition of the Au nanostars was 10 × 10 mm, and their surface morphologies were analyzed using a scanning electron microscope (SEM) (ISI DS-130C, Akashi Co., Tokyo, Japan), as shown in Fig. 1.

**SERS monitoring of isolated mouse neural stem cells differentiation**

To monitor the differentiation of isolated mouse neural stem cells, approximately 2 × 10⁴ isolated mouse neural stem cells were assembled on an Au nanostar/ITO substrate surface (20 mm × 10 mm) for 28 days. The cells were examined at 14 time points, beginning with the Raman spectrum for adhered cells (day 0), and then every 48 h after the cell had adhered to the surface of the substrate. SERS mapping was conducted for each cell, and the Raman spectrum was extracted from 20 points. The mean Raman spectrum of each observed time point during isolated mouse neural stem cells differentiation is offset and overlaid in Fig. 6.

**In vitro PC12 electrochemical stimulation**

Electrochemical stimulation of the neurite outgrowth of PC12 was developed based on a previously reported method.²⁸ Typically, PC12 cells were assembled onto an Au nanostar/ITO substrate at a density of 2 × 10⁴ cells per cm², then incubated for 24 h to permit attachment and spreading. The PC12 cells were subjected to a steady potential of 100 mV for 2 h. For electrical stimulation, the Au nanostar/ITO served as the anode, an Au wire placed at the opposite end (along the length) of the well served as the cathode, and an Ag wire served as a quasi-reference electrode. The cells were maintained in a CO₂ incubator for the duration of the electrical stimulation. After electrical stimulation, the cells were incubated for an additional 24 h (for a total of 48 h from the start of the experiment). Using SEM, the cell neurite lengths of the stimulated cells were compared with controls to estimate the extent of differentiation.

**Results and discussion**

**Development of gold nanostar/ITO substrate**

Au nanostars are biocompatible nanoconstructs that represent a promising platform for various biomedical applications such as SERS,²⁹ photodynamic therapy,³⁰ photothermal therapy,³¹ photoacoustic imaging,³² and biosensing.³³ In this study, Au nanostar-modified ITO substrates were developed by the reduction of HAuCl₄ using electrochemical deposition in the presence of PEG-200 as a structure-directing agent. Fig. 1a–c show SEM images of the Au nanostar/ITO substrates prepared using different concentrations of HAuCl₄, revealing the formation of Au 3D

![Fig. 1](image-url) SEM image of an Au nanostar-modified ITO substrate fabricated using a HAuCl₄ aqueous solution of different concentrations: (a) 0.3 mM, (b) 0.5 mM and (c) 0.7 mM containing 20 µL mL⁻¹ of PEG-200 under a constant potential of −1.3 V (vs. Ag/AgCl), with the deposition temperature maintained at 25 °C. (d) UV-vis spectra of the three Au nanostar/ITO substrates. Scale bar 100 nm.
nanostructures 40 nm in diameter. The nanostar morphology consists of multiple perpendicular branches within the same plane, but at slightly different angles. In addition, the density of Au nanostructures is increased by increasing the concentration of HAuCl₄, as shown in Fig. 1a–c. Fig. 1d shows the UV-vis spectra of three Au nanostar/ITO substrates ranging from 350–800 nm. The absorption spectra of the Au nanostar/ITO substrates demonstrate that Au nanostars have two broad surface plasmon absorption peaks: one at around 553 nm, resulting from transverse electronic oscillation, and the other in the NIR region (704 nm), due to the longitudinal oscillation of conduction band electrons. These two plasmon bands result from the hybridization of the plasmon from branches that act as “hot spots” due to the “lightning rod” effect.³⁴ Because of the heterogeneous branch morphology, nanostar ensembles probably enable a wider range of LSPR modes, which explains the broadening of the extinction spectra. In addition, the plasmon peak intensity increased with increasing density of the Au nanostars, as the intensity depends on the branch number/length.³⁴

SERS monitoring of the electrical stimulation of PC12 cells

The rat pheochromocytoma PC12 cell line has been reportedly used as a model system for neuronal differentiation by electrical stimulation or treatment with nerve growth factor.³⁵ In this study, we used the PC12 cell line as a model system for neuronal differentiation by electrical stimulation, by applying a steady potential of 100 mV for 2 h to the cells. The SEM technique was used to confirm the differentiation of PC12. Fig. 2b shows the SEM image of control PC12, and Fig. 2c and d show the SEM images of PC12 after cell stimulation, which demonstrate the changes in morphology and the apparent enlargement of neurons extending from the cell body after cell stimulation. The Raman spectrum of the control PC12 is shown in Fig. 2a, which shows Raman peaks at 775 cm⁻¹ (Trp, U, C and T), 1001 cm⁻¹ (Phe), 1092 cm⁻¹ (PO₂⁻), 1205 cm⁻¹ (Phe and Trp), 1230 cm⁻¹ (amide III and T), 857 cm⁻¹ (Tyr), 1490 cm⁻¹ (G and A) and 1620 cm⁻¹ (C=C Trp and Tyr str.).³⁶ Moreover, the peak at 1270 cm⁻¹ corresponds to the presence of amide III and catecholamines such as dopamine (DA). The Raman peak at 1557 cm⁻¹ was assigned to C==C str. of a phenyl group. These data correspond to earlier published cell spectra.³⁷ On the other hand, the Raman spectrum of the differentiated PC12 cells exhibit changes in several Raman peaks. These changes include a decrease in the Raman peak intensities at 1001 cm⁻¹ (Phe) and 1170 cm⁻¹ (pro, C–C/C–N str.). Conversely, the intensities of the Raman peaks at 775 cm⁻¹ and 1097 cm⁻¹ (PO₂⁻ of nucleic acids) increased after cell differentiation. Moreover, new Raman peaks appeared at 857 cm⁻¹ (Tyr), 875 cm⁻¹ (lipids, C–C–N str. and carbohydrate C–O–C ring), 1450 cm⁻¹ (carbohydrate CH₂), and 1490 cm⁻¹ (nucleic acids, G and A). On the other hand, the Raman peak at 1470 cm⁻¹ (pro. def. CH) disappeared after the differentiation of PC12. These results illustrate the ability of the SERS-active surface to monitor the differentiation of PC12 cells. In addition, the electrical stimulation of the PC12 cells induces changes in DNA and protein synthesis.

SERS monitoring the differentiation of HB1.F3 cells

The SERS technique was also applied to monitor the differentiation of HB1.F3 cells as shown in Fig. 3. Fig. 3 exhibits typical Raman peaks, including characteristic peaks corresponding to nucleotide and sugar-phosphate backbone vibrations, such as the peaks at 690 cm⁻¹ (DNA, G), 796 cm⁻¹ (PO₂⁻ in DNA, U, C and T), 1310 cm⁻¹ (A) and 838 cm⁻¹ (PO₂⁻ in RNA). The protein contents were dominated by Raman peaks corresponding to

Fig. 2 Electrical stimulation of PC12: (a) Raman spectrum for (1) undifferentiated and (2) differentiated PC12 cells within the range of 600 cm⁻¹ to 1750 cm⁻¹, (b) SEM image of the undifferentiated PC12 cell, (c and d) SEM images of the differentiated PC12 cell.
amide II (1540 cm\(^{-1}\)), 913 (Ring str. CC), 955 (str. CC \(\alpha\)-helix), 1120 (CN str.), 1155 (CC/CN str.), 1214 cm\(^{-1}\) (amide III \(\beta\)-sheet) and 1284 cm\(^{-1}\) (amide III) vibrations. Amino acids could be identified by peaks corresponding mainly to phenyl groups, such as Phe (1001 cm\(^{-1}\)), Trp (730 and 755 cm\(^{-1}\)), Trp vibrations (1540 cm\(^{-1}\)), Tyr vibrations (838 cm\(^{-1}\)) and 1617 cm\(^{-1}\) (C\(\equiv\)C Trp, Try). The Raman peaks of lipids were present at 730 cm\(^{-1}\), 1452 cm\(^{-1}\) (CN str.), 1388 cm\(^{-1}\) (CH def.), and 1097 cm\(^{-1}\) (hydrocarbon chain vib.). Carbohydrates were detected by the identification of Raman peaks for sugars, especially the COC vibrations of sugar rings (1047 cm\(^{-1}\) and 1284 cm\(^{-1}\)), and peaks at 1440 cm\(^{-1}\) (dCH\(_2\)), 1400 cm\(^{-1}\) (dCOO), and 1120 cm\(^{-1}\) (CO str. carbohydrate). In comparison to the undifferentiated HB1.F3 cells, the Raman spectrum for differentiated HB1.F3 cells (Fig. 3) demonstrated a decrease in Raman peak intensities at 755 cm\(^{-1}\) (A and Trp), 838 cm\(^{-1}\) (Tyr and PO\(_2\) in RNA/DNA), 913 cm\(^{-1}\) (pro, Ring str. CC), 955 cm\(^{-1}\) (pro, str. CC \(\alpha\)-helix), 1155 cm\(^{-1}\) (pro, CC/CN str.), 1440 cm\(^{-1}\) (carbohydrate, dCH\(_2\)) and 1540 cm\(^{-1}\) (Trp and amide II). Raman peak intensities at 690 cm\(^{-1}\) (DNA, G) and 1120 cm\(^{-1}\) (CO str. carbohydrate) increased after cell differentiation. These results indicate that some DNA contents (A and PO\(_2\) in DNA) decrease during the differentiation of stem cells, while G/DNA increases. On the other hand, the percentage of most protein contents (Trp, Tyr, pro, Ring str. CC, str. CC \(\alpha\)-helix, str. pro. CC/CN) decrease, indicating local variations in protein structure.

**Cyclic voltammetry monitoring the differentiation of HB1.F3 cells**

In addition, a CV technique was used to monitor the differentiation of HB1.F3 cells. The cyclic voltammetric behavior of undifferentiated HB1.F3 cells over the potential range of +0.6 to −0.2 V exhibited a quasi-reversible redox process with a cathodic peak at +0.13 V and an anodic peak at +0.25 V. The voltammetric behavior of the cells was recorded for 25 cycles. The results demonstrated that an increase in the number of CV cycles caused no change in the potential peaks, but there was a slight decrease in peak current (Fig. 4a). In addition, the differentiated HB1.F3 cells exhibited quasi-reversible redox behavior with a cathodic peak at +0.13 V and an anodic peak at +0.21 V. The voltammetric behavior of the cells showed a decrease in the peak current with an increase in number of cycles (Fig. 4b). It is notable that the differentiated HB1.F3 cells showed a higher current peak than the undifferentiated cells. The peak-peak separation of
undifferentiated HB1.F3 cells (120 mV) is more than that of the differentiated HB1.F3 cells (80 mV), indicating higher reversibility of the differentiated HB1.F3 cells than of the undifferentiated HB1.F3 cells (Fig. 4c). The high reversibility of differentiated HB1.F3 cells is related to the nature of neurons, in which the membrane potential very rapidly undergoes a large change (hyperpolarization or depolarization). This is in contrast to non-excitable cells, in which the membrane potential is held at a relatively stable value (resting potential).

SERS analysis of undifferentiated living isolated mouse neural stem cells

The differentiation of neural isolated mouse neural stem cells is characterized by its progression over a long period. Thus, the differentiation process was monitored over time as a stepwise process. Fig. 5 shows optical images of isolated mouse neural stem cells during differentiation over 28 days, which illustrate the changes in the morphology and appearance of neurons that extend from the cell body. The length of the neurons increased with increased differentiation time.

Fig. 6a shows the Raman spectrum of undifferentiated isolated mouse neural stem cells, exhibiting characteristic Raman peaks corresponding to nucleotide and sugar-phosphate backbone vibrations. These include the Raman peaks at 1097 cm\(^{-1}\) (PO\(_2^-\) bonds in DNA, U, C and T) and 824 cm\(^{-1}\) (PO\(_3^-\) bonds in RNA). Different protein contents were dominated by Raman peaks corresponding to amide II (1570 cm\(^{-1}\)), amide III \(\beta\)-sheet (1214 cm\(^{-1}\)) and amide III (1284 cm\(^{-1}\)). Moreover, amino acids could be identified by peaks corresponding mainly to phenyl groups, such as Phe 1001 cm\(^{-1}\) (C–C ring breathing: Phe/protein), Trp (724 and 762 cm\(^{-1}\)), and Tyr vib. (838 and 1570 cm\(^{-1}\)). The Raman peaks of lipids present at 724 cm\(^{-1}\), 1452 cm\(^{-1}\) (CN str.), 1388 cm\(^{-1}\) (CH def.), and 1097 cm\(^{-1}\) correspond to vibrations of the hydrocarbon chains. Carbohydrates were also detected by identification of the Raman peaks of sugars, especially the COC vibrations of sugar rings (1051, 1097 and 1284 cm\(^{-1}\)) and CH def. at 1388 cm\(^{-1}\). The peak assignments of the spectrum are presented in Table 1.

SERS real-time monitoring of isolated mouse neural stem cells differentiation

Ami et al. (2008) and Zelig et al. (2010) have reported that nucleic acids in undifferentiated cells were decreased in comparison to differentiated cells,\(^4\) while Schulze and his group (2010) have shown high nucleic acid content in undifferentiated cells.\(^38\) On the other hand, some studies have demonstrated that the protein composition\(^39,40\) increased compared to differentiated cells.\(^4\) However, such increases have also been associated with the differentiation process.\(^38\)

The SERS technique was used as a real-time tool to monitor the differentiation of isolated mouse neural stem cells. The SERS results of isolated mouse neural stem cells during differentiation (Fig. 6b) demonstrated that the intensities of some Raman peaks changed. New peaks appeared while others disappeared during the differentiation of isolated mouse neural stem cells. The change in the Raman spectrum at several Raman shifts represents varying amounts of biochemical components, including nuclear material, DNA/RNA bases, the DNA backbone, amino

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![Fig. 5](image_url)  
Optical images of control isolated mouse neural stem cells at day 0 (a) and after 2 (b), 4 (c), 6 (d), 8 (e), 10 (f), 12 (g), 14 (h), 16 (i), 18 (j), 20 (k), 22 (l), 24 (m), 26 (n) and 28 (o) days of differentiation process.
acids, proteins, and lipids, as indicated by changes in the intensity of the peaks at 650 (Tyr), 738 (Trp), 828 (Trp and DNA/RNA PO2), 850 (Trp), 1001 (Phe), 1160 (pro, C–N/C–C str.), 1220 (DNA/RNA bases T and A), 1340 (bases of DNA/RNA G and A), 1580 (pro, (C=O Trp and Tyr)) and 1617 cm\(^{-1}\) (prot, C=O Trp and Tyr).

The behavior of the Raman intensity changes at each peak was monitored. Peaks at 650 cm\(^{-1}\) (Tyr) and 1160 cm\(^{-1}\) (pro, CN/CC star.) appeared after the cells had adhered to the substrate for two weeks, and the intensity increased with culture time, as shown in Fig. 7a and b. Fig. 7c and d demonstrate that the intensities of the Raman peaks at 738 cm\(^{-1}\) (Trp) and 1617 cm\(^{-1}\) (pro, (C=O Trp and Tyr)) increased with culture time, which indicates that the percentage of these protein components increased as culture time increased. However, these peaks disappeared after two weeks. Similarly, Fig. 8a shows that the intensity of the Raman peak at 828 cm\(^{-1}\) (Trp and to DNA/RNA PO2) increased with culture time for two weeks and then disappeared. The Raman peak intensities at 850 cm\(^{-1}\) (Trp), 1220 cm\(^{-1}\) (bases of DNA/RNA (T and A) and proteins (amid III)), and 1340 cm\(^{-1}\) (bases of DNA/RNA G and A) changed irregularly with culture time, as shown in Fig. 8b–d. In addition, the Raman peak intensity at 1001 cm\(^{-1}\) (Phe) decreased with culture time (Fig. 9a). In contrast, Fig. 9b demonstrates that the intensity of the Raman peak at 1580 cm\(^{-1}\) (bases of DNA/RNA (G and A)) increased with culture time.

Remarkably, the SERS spectra of the differentiated isolated mouse neural stem cells demonstrated a significant increase in Raman intensities corresponding to some protein components, such as amino acids, Trp, Tyr, and CN/CC (650, 738 and 1617 cm\(^{-1}\)). On the other hand, the percentage of Phe (1001 cm\(^{-1}\)) decreased during the differentiation process. The percentage of the bases of DNA/RNA (G and A) (1580 cm\(^{-1}\)) increased with an increase in the differentiation time. In contrast, it was observed that the intensities of the Raman peaks of proteins and DNA were changed in an irregular manner.

Furthermore, Fig. 9c and d show that the Raman intensities of nucleic acids (DNA–RNA composites) at 1580 cm\(^{-1}\) were divided by protein-related bands, such as 1001 cm\(^{-1}\) (Phe) and 1160 cm\(^{-1}\) (CN/CC star.). These results indicate that the Raman peaks for the protein/nucleic acid intensity increased, while the peaks for the protein components decreased.

The percentage of some protein components in the differentiated isolated mouse neural stem cells increased, while others...
decreased, as compared to the undifferentiated cells. This was also observed in the percentage of DNA/RNA components, as DNA is the original template for protein synthesis. Thus, a change in the DNA may affect synthesis of the protein. Although previous studies have shown an increase in nucleic acids and proteins in differentiated cells as compared to undifferentiated cells, others have shown opposite results. Our results demonstrated an increase in some nucleic acids and proteins, a decrease in some components, and irregular trends in other components during the differentiation process, which is consistent with previous studies.

To verify our results, the change in the total protein content during isolated mouse neural stem cell differentiation was evaluated based on a colorimetric method using a Diamond GPT kit following the manufacturer’s instructions. The culture medium was removed, the adhered cells were washed three times with PBS, and the culture medium was replaced with fresh medium containing serum-free growth medium. The adhered cells were then incubated for 24 h.

Fig. 7 Changes in the intensities of some Raman peaks that characterize the protein content with culture time at different Raman shifts: (a) 650 cm⁻¹ (Tyr), (b) 1160 cm⁻¹ (CN/CC star.), (c) 738 cm⁻¹ (Trp), and (d) 1617 cm⁻¹ (C=CC Trp and Tyr). Data represent the mean ± standard deviation of ten different points.

Fig. 8 Changes in the intensities of some Raman peaks that characterize the nucleic acid percentages with culture time at different Raman shift values: (a) 828 cm⁻¹ (Trp and to DNA/RNA PO₂), (b) 850 cm⁻¹ (Trp), (c) 1220 cm⁻¹ (bases of DNA/RNA (T and A) and proteins (amid III)), and (d) 1340 cm⁻¹ (bases of DNA/RNA G and A). Data represent the mean ± standard deviation of ten different points.
times with ice-cold PBS buffer, and the PBS was drained off. 100 μL per 10^7 cells in chilled RIPA buffer were then added, the adherent cells scraped off, and the cell suspension kept for 1 h. Finally, the cell lysate was collected by centrifuging the suspension at 12 000 rpm for 20 min at 4 °C. The total protein concentration of the lysate was determined based on a colorimetric test, in which a standard solution was used. The intensity of the specimen was measured against the reagent blank at 546 nm wavelength after 5 min. Fig. 10 shows the change in the total protein content as a function of differentiation time, demonstrating that the total amount of protein increased with increasing differentiation time, which is in agreement with the SERS results.

These findings indicate that there is a change in the percentage of total Raman-active biomolecular components during the differentiation of isolated mouse neural stem cells.

Conclusions

In summary, these results demonstrate that the Raman technique can be used for real-time monitoring of the differentiation of neural cells (PC12 cells, embryonic stem cells and adult stem cells). Each cell line behaves differently during differentiation. The differentiation of neural isolated mouse neural stem cells could be analyzed with time, as it is a stepwise process, which is a challenge in the tissue engineering filed. These results also demonstrate that the DNA content seems to change irregularly during the differentiation of stem cells. The percentage of most proteins components increased, while that of some protein components decreased. The increase in the amount of proteins reflects local variations in protein structure and a maturational shift. The results also indicate that the electrostimulation of PC12 cells induces changes in the synthesis of DNA and proteins. The differentiation of isolated mouse neural stem cells showed irregular changes in DNA content and an increase in protein percentage, in addition to the irregular behavior of some peaks related to both nucleic acids and proteins. Furthermore, the differentiated HB1.F3 cells showed higher reversibility than the undifferentiated HB1.F3 cells. Therefore, the SERS analysis technique allows for a more rapid sample analysis without time-consuming staining.
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