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RESEARCH PAPER

In-situ Detection of Neurotransmitter Release from PC12 Cells Using Surface Enhanced Raman Spectroscopy

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Abstract Rat pheochromocytoma PC12 cells have frequently been used as a dopaminergic neuron model due to their various functions, including the synthesis, storage, and secretion of catecholamines. Furthermore, PC12 cells release a measurable amount of dopamine (DA) in response to some chemicals. PC12 cells are thus considered to be one of the most common *invitro* models for studying neurotransmitter release. Here, we applied Surface-enhanced Raman Spectroscopy (SERS) to determine with high sensitivity the *in-situ* short-time effects of cisplatin (cis-diamine-dichloroplatinum), bisphenol-A, and cyclophosphamide on the extracellular DA level released from PC12 cells. In addition, using the SERS technique, changes in the biochemical composition of the PC12 cell lysates were investigated to determine the intracellular DA level. Gold nano-patterned substrates were fabricated based on electrochemical deposition of Au nanorods onto ITO substrates; these substrates were then used as SERS-active surfaces. The Raman spectroscopy results demonstrated that the changes in the Raman spectra depending on the treatment agent were in agreement with the HPLC results on the extracellular DA level. Therefore, the SERS technique can overcome the limitations of other detection techniques, and can be used with cellular nanoarrays to study the effect of a wide range of chemicals.

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1. Introduction

Rat pheochromocytoma PC12 cells have been commonly used as *in vitro* dopaminergic neuron models for studying neurotransmitter release, due to their intrinsic functions, which include the synthesis, storage, and secretion of catecholamines [1-4]. In addition, PC12 cells have been reported to release a measurable amount of dopamine (DA) in response to some chemicals, such as cisplatin, cyclophosphamide, and glucocorticoids [5]. DA is an excitatory chemical neurotransmitter that plays a critical role in the function of the central nervous, hormonal, and cardiovascular systems. It has been reported that a decrease in DA level may result in serious diseases such as Parkinson's disease [6]. The DA release process in PC12 cells is rapid, taking place within milliseconds, and is largely confined to the synapses [7]. Detection techniques therefore need to have high spatial and temporal resolutions. Consequently, much effort has gone into the development of highly sensitive techniques that are capable of monitoring the level of neurotransmitter released [8-10]. To date, electrophysiological techniques and fluorescent probe labeling are the most commonly used techniques [11]. However, all of these techniques possess disadvantages. On the other hand, Raman spectroscopy is a non-invasive technique that provides detailed molecular information [12] and can be used under aqueous conditions. Recently, Raman spectroscopy and its derived applications have been tested for their efficacy in measuring neurotransmitter secretion. Schulze *et al.* showed that artificial neural networks are capable of accurately assigning the Raman spectra of aqueous solutions of different neurotransmitters [13]. However, the low

scattering cross-section of Raman spectroscopy limits its application in trace analysis. Recently, SERS has been reported as a valuable detection technique, as it offers an opportunity to overcome the critical disadvantages associated with conventional Raman spectroscopy [14]. The associated techniques for Raman signal enhancement, which make use of a structured metal surface, could be applied for the effective detection of ultra-low concentrations of biological materials, by using low laser power with a short signal acquisition time [15-20]. Therefore, a great deal of attention has been focused on the synthesis of shape-controlled SERS structures with different morphologies, including spherical gold (Au) NPs, nanospheres, nanorods, and nanostars. In addition, the Raman enhancing capabilities of these structures have been investigated, as they are greatly influenced by their shape [21]. However, the fabrication of SERS-active substrates was found to have a number of problems, including poor signal enhancement, poor uniformity or reproducibility, and the need for a further process to remove the template and byproducts. Therefore, an advanced method for developing new SERS-active surfaces is still required for more effective enhancement of Raman signals. The purpose of this study is to determine the short-time effects of cisplatin, bisphenol-A (BPA), and cyclophosphamide (Fig. 1) treatments on the extracellular neurotransmitter release process, as well as changes in the intracellular DA level. Here, we have reported a single-step and template-free method for the fabrication of a highly sensitive Au nanorods/ITO substrate, and its application in the monitoring of the effects of three different chemicals on the amount of DA released from PC12 cells. The Au nanorods/ITO substrate was developed based on electrochemical deposition of Au onto the ITO surface in the presence of cetyltrimethylammonium bromide (CTAB) as a surfactant. This Au nanorods/ITO substrate has exhibited significant Raman signal enhancement; therefore, the effects of low concentrations of different chemicals on the amount of DA released from PC12 cells could be successfully detected using the SERS technique, and the results were validated using an HPLC method.

2. Materials and Methods

2.1. Materials

Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.9+ %), phosphate buffered saline (PBS) (pH 7.4, 10 mM) solution, dopamine hydrochloride, BPA, and CTAB were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cisplatin and cyclophosphamide monohydrate were purchased from Calbiochem (Germany). All other chemicals used in this study were obtained commercially in the reagent grade. All aqueous solutions were prepared using deionized water (DIW) deionized with a Millipore Milli-Q water purifier operating at a resistance of 18 MΩ cm.

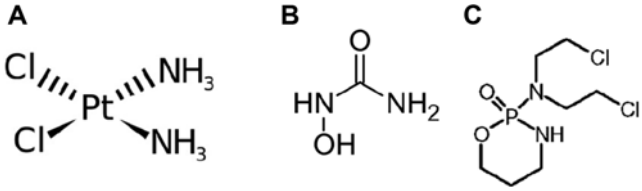

Fig. 1. Molecular structures of (A) cisplatin, (B) bisphenol-A, and (C) cyclophosphamide.

2.2. Cell culture

The rat neural cell line that was collected from the Adrenal medulla of a PC12 rat was 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°C in an atmosphere of 5% CO_2. The medium was changed every two days.

2.3. Electrochemical measurements

Fabrication of the Au nanorods/ITO substrate and all electrochemical measurements were performed using a potentiostat (CHI–660, CH Instruments, USA) controlled by general–purpose electrochemical system software. A homemade three–electrode system was used, and consisted of the ITO substrate as the working electrode, a platinum wire as the auxiliary electrode, and Ag/AgCl as the reference electrode.

2.4. Fabrication of gold nanorod/ITO substrate

The Au nanorod-modified ITO substrates were developed according to the following deposition protocol that was previously reported by our team [14,16]: Au nanorod arrays were electrochemically deposited onto cleaned ITO substrates (2 cm × 1 cm) using 0.1 mM $HAuCl_4$ aqueous solution containing CTAB (20 μg/mL) as a surfactant. The potential was maintained at - 0.9 V (*vs.* Ag/AgCl), and the deposition temperature was kept constant at 25ºC in an electric-heated thermostatic water bath. In order to remove any trace of surfactant that may have been adsorbed onto the Au nanorod/ITO surface, the substrates were rinsed with DIW and then boiled in isopropyl alcohol for 5 min. The active area for the electrochemical deposition of the Au nanorods measured 1 cm × 1 cm, and the surface morphology was analyzed by a scanning electron microscope (SEM) (ISI DS-130C, Akashi Co., Tokyo, Japan).

2.5. Raman spectroscopy

The effects of cisplatin, BPA, and cyclophosphamide on the extracellular and intracellular DA from PC12 cells were investigated by Raman spectroscopy using Raman NTEGRA spectra (NT-MDT, Russia). The maximum scan-range, XYZ, was 100 μm × 100 μm × 6 μm, and the resolution of the spectrometer in the XY plane was 200 nm, and 500 nm along the Z axis. Raman spectra were recorded using an NIR laser emitting light at a 785 nm wavelength. Ten scans of 5 s from 600 to 1,750/cm were recorded, and the mean of these scans was used.

3. Results and Discussion

3.1. Development of gold nanorod-modified ITO substrate

Au nanorod arrays were fabricated by the electrochemical deposition of Au nanorods from an aqueous solution of Au^{3+} ions in the presence of CTAB as a surfactant. Fig. 2A shows an SEM image of the Au nanorod/ITO surface, representing a uniform distribution of Au nanorods with an average length of 200 nm, a diameter of 51 nm, and an aspect ratio of 4. In our previous work, we reported that the absorption spectrum of the Au nanorods/ITO substrate demonstrated a weak surface plasmon absorption band within the visible region at 520 nm [16]. In addition, Orendorff *et al.* have reported on the application of different Au or Ag nanostructures as SERS active surfaces, and have demonstrated that the enhancement factor depends on the size, shape, and aspect ratio of the nanostructure used [22]. Here, the modified Au nanorod/ITO substrate exhibited two surface plasmon absorption bands (Fig. 2B), a sharp peak at around 485 nm (in the visible region) contributed by transverse electronic oscillation, and a broad peak at 738 nm (in the near-IR region) due to longitudinal oscillation of the conduction band electrons [23]. Therefore, the Au nanorod-modified ITO substrate could potentially strongly absorb energy and scatter the electromagnetic field, as the enhancement of the surface electric field depends on the surface plasmon excitation [24]. Moreover, the longitudinal absorption band of the Au nanorod/ITO substrate (738 nm) overlaps with the excitation wavelength from a diode laser (785 nm), which could be an additional enhancement factor. Therefore, the Au nanorod/ITO substrate could eventually lead to strong enhancement of Raman signals, as the enhancement of the surface electric field depends on the surface plasmon excitation.

3.2. Monitoring of the intracellular and extracellular release of DA from PC12

The release of DA from PC12 cells was verified with the HPLC technique, as shown in Fig. 3. The HPLC result of dopamine hydrochloride dissolved in PBS (pH 7.4, 10 mM) is shown in Fig. 3A, which indicates the presence of two peaks at a retention time of about 2 and 4 min, corresponding to the solvent and dopamine, respectively. In addition, the extracellular release of DA from PC12 cells was investigated as follows: PC12 cells with a density of 2.1×10^5 cells/mL were cultured and allowed to grow for 2 days, followed by removal of the culture medium and rinsing of the cell wells with PBS. 5 mL of fresh PBS buffer with an excess of glucose was then added to cells, and incubated for 10 min at 37°C. Following that, the presence of DA in PBS was investigated using HPLC, as shown in Fig. 3B, revealing the release of extracellular DA. In addition, the presence of intracellular DA was confirmed by HPLC analysis of the chemical composition of the PC12 cells lysate obtained after treatment with PBS buffer for 10 min (Fig. 3C).

3.3. Monitoring the effects of three chemicals on the level of intracellular and extracellular DA released from PC12

The efficiency of three different chemicals (BPA, cisplatin, and cyclophosphamide) on the release of neurotransmitter from PC12 cells was assessed by estimating the amount of DA released from PC12 cells, as induced by these

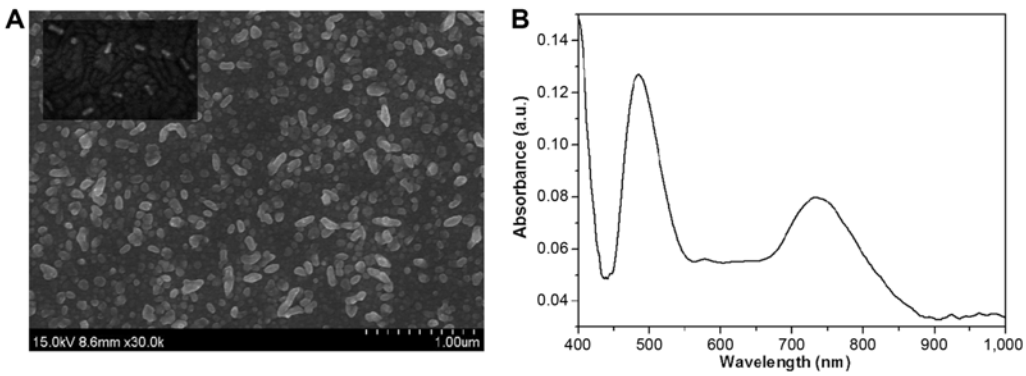

Fig. 2. (A) SEM topography of the Au nanorods/ ITO substrate. (B) UV–vis spectra of the Au nanorods/ITO substrate.

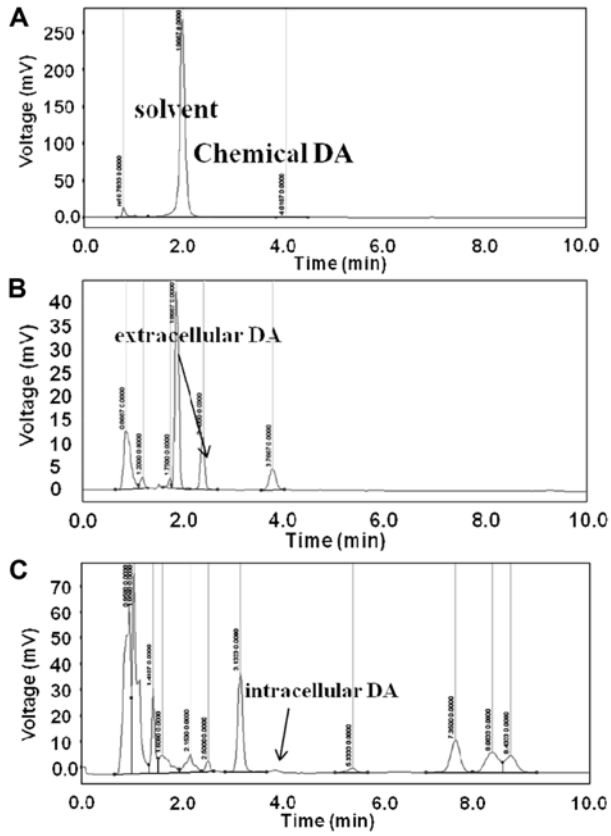

Fig. 3. HPLC results of (A) a solution of chemical dopamine in PBS, (B) extracellular dopamine from PC12 cells, and (C) intracellular dopamine from PC12 cells.

chemicals, as well as the intracellular DA levels, as shown in Fig. 4. Platinum analogue cisplatin is one of the most widely used cytotoxic drugs, due to its direct binding cross-linking to DNA, which causes apoptosis in rapidly dividing cells [25], as the neurons are post-mitotic. Cisplatin-induced neurotoxicity includes sensory and autonomic neurotoxicity; moreover, cisplatin has been reported to induce apoptosis of PC12 cells, depending on the dose and time, and this can be identified by DNA fragmentation [26]. On the other hand, incubation of PC12 cells with cisplatin, carboplatin, and cyclophosphamide has been reported to show a significant increase in the release of DA from PC12 cells. Moreover, cisplatin has shown the greatest effect among these three chemicals [27].

In this study, the effects of three different chemicals on the amount of DA released from PC12 cells was investigated as follows: PC12 cells with a density of 2.1×10^5 cells/mL were cultured and allowed to grow for 2 days, followed by removal of the culture medium and rinsing of the cell wells with PBS. 5 mL of fresh PBS buffer containing 1 mM of BPA, cisplatin or cyclophosphamide was then supplied to the cells and incubated for 10 min at 37°C. Fig. 4A shows a significant increase in the amount of DA released following treatment of PC12 cells with these three drugs. In addition, the maximum release of DA from PC12 cells among the three drugs tested in this study was induced by treatment with cisplatin, as shown in Fig. 4A. Furthermore, the effect of these three drugs on the level of intracellular DA in treated cells was investigated based on analysis of the cells lysates after cell treatment for 10 min. Fig. 4B shows the changes in the level of intracellular DA obtained from HPLC, which demonstrates a decrease in the intracellular DA level. It was also found that cisplatin has the greatest effect on the amount of DA released, as compared to BPA and cyclophosphamide.

In addition, the SERS technique using Au nanorods/ITO as the surface-enhanced active substrate was used to confirm the effects of the three drugs on the neurotransmitter released from PC12 cells. Fig. 5 shows the Raman spectra of control PC12 cells as well as PC12 cells treated with 1 mM of BPA, cisplatin or cyclophosphamide for 10 min at 37°C. This figure exhibits a series of Raman peaks at 775/cm (Trp, U, C and T), 1001/cm (Phe), 1092/cm (PO_2^-), 1205/cm (Phe and Trp), 1230/cm (amide III and T), 857/cm (Tyr), 1270/cm (amide III and catecholamines, *e.g.* DA), 1557/cm

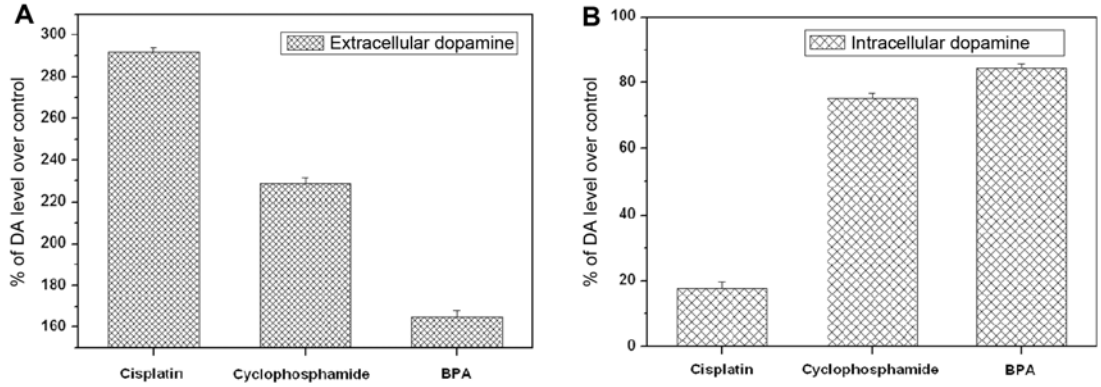

Fig. 4. HPLC results on the effect of various chemicals on (A) extracellular dopamine from PC12 cells and (B) intracellular dopamine from PC12 cells.

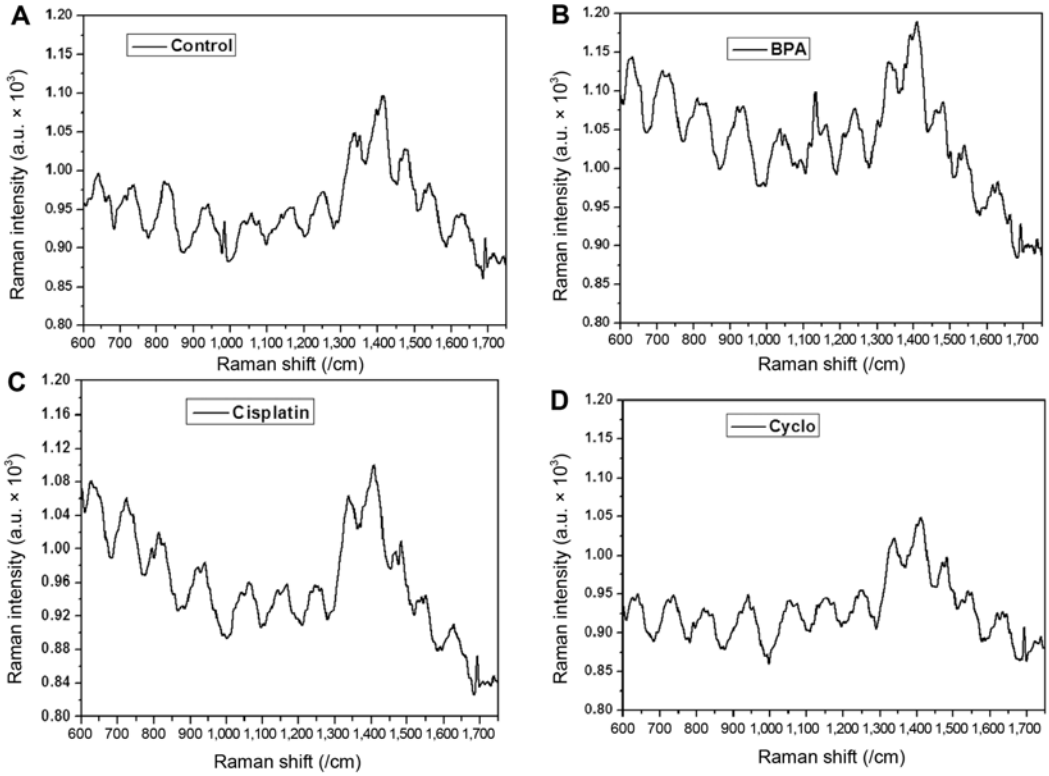

Fig. 5. Effects of various chemicals on the extracellular dopamine from (A) control PC12 cells, (B) PC12 cells treated with 1 mM of BPA, (C) PC12 cells treated with 1 mM of cisplatin, and (D) PC12 cells treated with 1 mM of cyclophosphamide. All cells groups were treated for 10 min at 37°C.

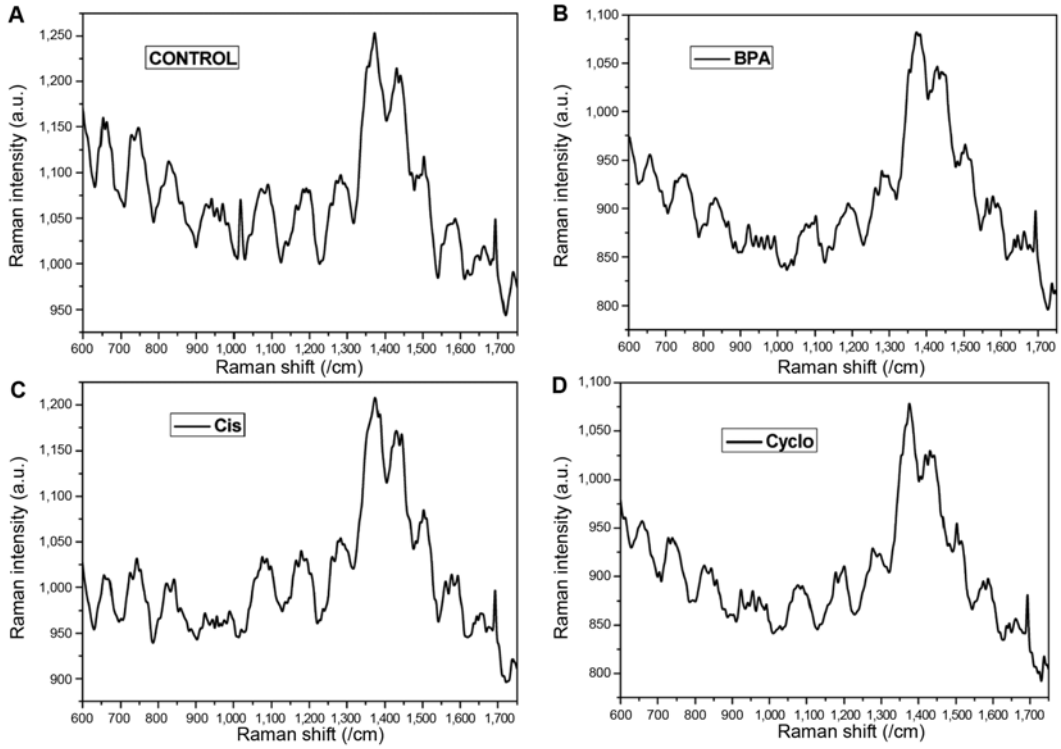

Fig. 6. Effects of various chemicals on the intracellular dopamine from (A) control PC12 cells, (B) PC12 cells treated with 1 mM of BPA, (C) PC12 cells treated with 1 mM of cisplatin, and (D) PC12 cells treated with 1 mM of cyclophosphamide. All cells groups were treated for 10 min at 37°C.

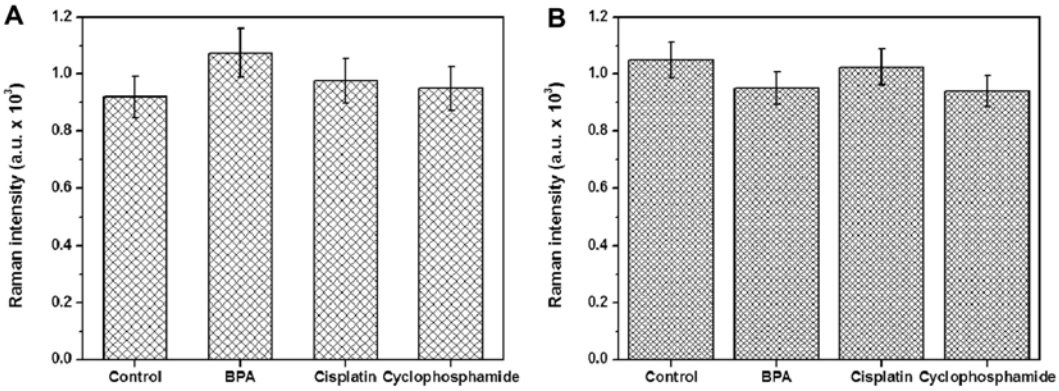

Fig. 7. Effects of various chemicals on the intracellular dopamine from (A) control PC12 cells, (B) PC12 cells treated with 1 mM of BPA, (C) PC12 cells treated with 1 mM of cisplatin, and (D) PC12 cells treated with 1 mM of cyclophosphamide. All cells groups were treated for 10 min at 37°C.

(C=C str. of phenyl group), 1490/cm (G and A), and 1620/cm (C=C Trp and Tyr str.). These results are in agreement with previously reported studies [16,24,28,29]. These results also indicate that the intensity of the Raman peak at 1270/cm can be used as an indication of the DA level; Fig. 7A demonstrates that the intensity of the Raman peak at 1270/cm for the PC12 cells treated with cisplatin shows the highest intensity, confirming the release of the greatest amount of DA among the treated cells.

Raman spectra of the lysates of the control and treated PC12 cells show a series of Raman peaks (Fig. 6), including the following peaks, 671/cm (T and G), 760/cm (Trp), 854/cm (Trp), 923/cm (pro CC str.), 975/cm (C-C str. ß-sheet =CH bend), 1001/cm (Phe), 1125/cm (prot. str. CN), 1134/cm (Prot), 1270/cm (DA and amide III), 1450/cm (Prot), and 1625/cm (C=C Tyr and Trp). This result indicates that treatment of PC12 cells with BPA, cisplatin or cyclophosphamide results in very few changes, such as those within the range from 900/ to 1000/cm. In addition, Fig. 7B indicates that the intensity of the Raman peak corresponding to DA (1270/cm) decreased after the PC12 cells were treated with 1 mM of BPA, cisplatin or cyclophosphamide.

3.4. Effects of the drugs on cell viability

The effects of the treatment of PC12 cells with the three drugs on cell viability as well as cell morphology were determined using optical microscopy and an MTT assay. The cytotoxicity test involves an initial supply of 1 mM of the drug for 10 min, followed by removal of the culture medium and rinsing of the cell wells with PBS. Fig. 8 shows the morphologies of the control PC12 cells as well as the treated cells, indicating that treatment of the PC12 cells with 1 mM of cisplatin, BPA or cyclophosphamide

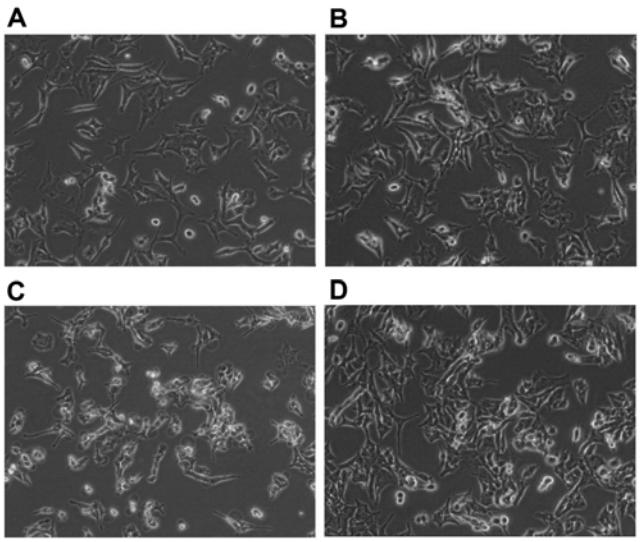

Fig. 8. Microscopy images of (A) control PC12 cells, (B) PC12 cells treated with BPA, (C) PC12 cells treated with cyclophosphamide, and (D) PC12 cells treated with cisplatin. All treatments were carried out for 10 min.

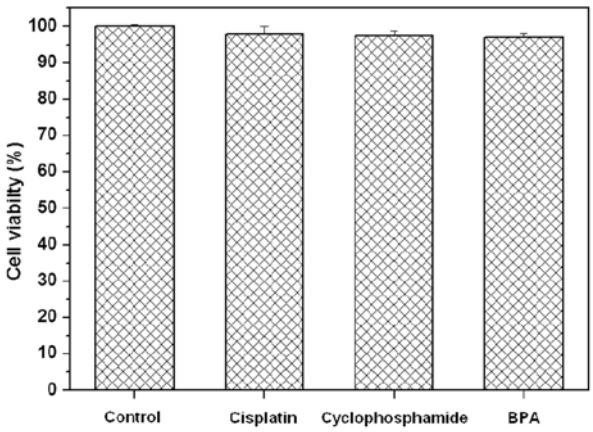

Fig. 9. MTT assay of the cytotoxicity for (A) control PC12 cells, (B) PC12 cells treated with BPA, (C) PC12 cells treated with cyclophosphamide, and (D) PC12 cells treated with cisplatin. All treatments were carried out for 10 min.

for 10 min did not have any significant effect on the cell morphology. In addition, Fig. 9 shows the cytotoxicity effects of the three drugs on the PC12 cells, based on an MTT assay. Considering the cell viability of the control cells as 100%, the results indicate that incubating the cells with 1 mM of cisplatin, BPA or cyclophosphamide for 10 min leads to approximately 97% cell viability. Due to the short treatment time, no significant damage to the cells could occur following incubation with 1 mM of cisplatin, BPA or cyclophosphamide.

4. Conclusion

The present work shows the feasibility of using the SERS technique to investigate the effects of three different drugs on the amount of DA released from PC12 cells. The results establish that the SERS method allows for the identification of chemical changes in cell lysates. In addition, the short treatment time of the PC12 cells with these drugs did not have any effect on the cell morphology or cell viability. Therefore, the proposed technique may be applicable for monitoring, diagnosis, and drug detection.

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