Raman-Based in Situ Monitoring of Changes in Molecular Signatures during Mitochondrially Mediated Apoptosis

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Supporting Information

ABSTRACT: Obtaining molecular information from inside cells is an important topic to understand the outcome of molecular interactions between potential drug molecules and biomolecules inside cells. To envision this goal, we investigated the surface-enhanced Raman scattering-based single-cell spectroscopic method to monitor changes in intracellular molecular signatures during mitochondrially mediated apoptosis in real time. Triphenylphosphine-modified gold nanoparticles were localized successfully to the mitochondria and greatly enhanced to obtain the intrinsic Raman scattering spectrum of mitochondria and cytochrome c in the live cell. Photothermally induced apoptosis showed a moderate decrease in the disulfide bond and a sharp increase in β-sheet structures depending on the input-laser power, along with morphological changes. However, chemical drug induced-apoptosis showed more subtle changes in the disulfide bond, as well as changes in Raman peaks corresponding to cytochrome c, and the appearance of a new peak at 1420 cm⁻¹, which enabled us to study the molecular interactions within the mitochondria in real time from a single cell, following treatment with a novel pyruvate dehydrogenase kinase inhibitor.

INTRODUCTION

The development of cell biology strongly relied on fluorescence-based microscopy, which uses organic fluorescent molecules, fluorescent proteins, or quantum dots to monitor cellular functions, morphology, and the expression levels of specific markers. Raman scattering is a promising technology for use in label-free biological imaging applications because of its intrinsic ability to obtain spectroscopic information of interest without interference from water molecules. Unfortunately, the main limitations of Raman scattering are its extremely low signal intensity, which requires the use of a special optical system for label-free imaging. Another way to overcome this limitation of Raman scattering is to use the strong ability of gold or silver nanoparticles to plasmonically enhance the Raman scattering, which has greatly improved the ability to detect and obtain molecular information in single living cells. Recently, Austin et al., demonstrated the use of plasmonically enhanced Raman scattering-based single cell imaging spectroscopy to demonstrate the effect of an anticancer drug using nuclear-targeted gold nanoparticles (AuNPs). The same group also demonstrated the capability of plasmonically enhanced Raman scattering to monitor the entire cell cycle, showing changes in the bands around 511, 658, 844, and 1322 cm⁻¹, corresponding to the −S−S− vibration, guanine, sugar−phosphate and adenine, and guanine and RNA, respectively. Both studies used nuclear-targeting of AuNPs to monitor molecular changes occurring inside a single cell. Systemic studies for other important subcellular organelles, such as mitochondria, will also require the use of this Raman-based single cell spectroscopic technology, for example, to study the interaction of novel drug molecules with mitochondria. Mitochondria are the power plants of the cell and play a central role in metabolic tasks and cellular proliferation regulation. The damage and subsequent dysfunction in mitochondria is an important factor in a range of human diseases. There are currently a large number of anticancer drug molecules that have been developed, or are under development, that can target the mitochondria through a diverse array of mechanisms. In this study, we focused on the spectroscopic analysis of the molecular signal changes that occur in the mitochondria during either photothermally or chemically induced apoptosis (Figure 1). To generate plasmonically enhanced Raman scattering in mitochondria, we used mitochondrially targeted AuNPs. For cellular imaging,
the use of AuNPs instead of silver nanoparticles provides substantial benefits because of their excellent biocompatibility and strong plasmonic enhancement using near-infrared light (785 nm), which enabled us to avoid the high autofluorescence background often found in cell imaging studies.19

RESULTS AND DISCUSSION

Preparation and Characterization of the Triphenylphosphine-AuNPs. We selected triphenylphosphine (TPP) as the ligand for mitochondria targeting.20,21 To prepare TPP-modified AuNPs (TPP-AuNPs), 1 μL of TPP solution in dimethylformamide (0.1 mg/10 mL) was added to 1 mL of tannic acid modified AuNPs (TA-AuNPs, 50 nm). After shaking for 2 h at room temperature, the solution was centrifuged at 3400 rcf for 15 min. Following this, the precipitate was redispersed in distilled water (1 mL). Transmission electron microscopy (TEM), UV–visible spectroscopy, Raman spectroscopy, and zeta potential analysis all demonstrated the successful modification of TA-AuNPs into TPP-AuNPs, as shown in Figure 2. The TPP-AuNPs were well dispersed in distilled water as shown by the TEM and UV–visible spectra (Figure 2A,B). The Raman spectroscopy analysis confirmed the change in the surface state of the TPP-AuNPs compared to the TA-AuNPs. The TA-AuNPs showed no noticeable Raman spectrum (black line in Figure 2C), but TPP-AuNPs (red line) showed a noticeable Raman spectrum originating from the TPP molecule. Because of TPP binding to the AuNPs via the Au–P bond, the Raman spectrum of the TPP-AuNPs was different from that of the TPP powder. The TPP powder showed an intense Raman
A peak at 1000 cm$^{-1}$ which can be ascribed to an in-plane C−C−C angle deformation vibration for the phenyl rings in TPP, but this peak intensity at 1000 cm$^{-1}$ was significantly decreased in the case of the TPP-AuNPs (Figure 2C).

The zeta potential analysis showed the change in the surface charge as a result of the TPP modification. The TA-AuNPs had a strong negative surface charge (−50.01 mV) at neutral pH, but upon changing TA to TPP, the TPP-AuNPs showed a more positive surface charge (−30.04 mV). At a low pH (pH 2.3), the TPP-AuNPs showed a positive surface zeta potential indicating the successful modification of the TA-AuNPs with TPP molecules (Figure 2D).

**Analysis of Intracellular Distribution of TPP-AuNPs.**

To evaluate the mitochondria-targeting capability of the TPP-AuNPs, TPP-AuNPs (0.1 nM) and TA-AuNPs (0.1 nM) were incubated with HSC-3 cells, and then the particle distribution inside the cells was monitored using bright-field (B/F) and dark-field (D/F) microscopy. Figure S1 shows the time-dependent particle uptake efficiency. Although the TPP-AuNPs were not efficiently internalized at an early time point (3 h), the TPP-AuNPs were well internalized into the HSC-3 cells after 6 h. Based on this result, we set the incubation time as 6 h to allow the TPP-AuNPs sufficient time to localize to the mitochondria. More importantly, in order to obtain molecular signals derived from the mitochondria, the exact localization of the plasmonic nanoparticles into the mitochondria is critical. To further examine this, we compared the distribution of TA-AuNPs and TPP-AuNPs in a single cell with B/F and D/F microscopy, single cell Raman imaging, and a spectrum analysis (Figure 3A,B for TA-AuNPs, 3C,D for TPP-AuNPs). The cells were stained with a mito-tracker to obtain molecular signals derived from the mitochondria, the exact localization of the plasmonic nanoparticles into the mitochondria is critical. To further examine this, we compared the distribution of TA-AuNPs and TPP-AuNPs in a single cell with B/F and D/F microscopy, single cell Raman imaging, and a spectrum analysis (Figure 3A,B for TA-AuNPs, 3C,D for TPP-AuNPs). The cells were stained with a mito-tracker to obtain molecular signals derived from the mitochondria (Figure 3A(iii)) and a 2.98 mW/cm$^2$ input laser power (785 nm).

Interestingly, the Raman spectra obtained from the cells incubated with the TA-AuNPs were not identical to each other because of a protein corona that formed around the TA-AuNPs. The spectra displayed in Figure 3D showed no significant difficulty in analyzing the Raman spectrum as following. The Raman shift peaks located at 505, 840, and 1000 cm$^{-1}$ were assigned to the $\sim$S−S$\sim$ stretching vibration,13 symmetrical lipid −O−C−C−N$\sim$ stretches and tyrosine,15 and a benzene ring stretching vibration (phenyl alanine),25 respectively. The Raman shift peaks located at 750, 1127, 1313, and 1581 cm$^{-1}$ were assigned to the vibrational modes typical for cytochrome c (Cyt C),26,27 (Figure 3D). In the case of Hela cells, Raman scattering at 750 and 1581 cm$^{-1}$, which were assigned to the pyrrole breathing modes in Cyt C, were clearly observed in a label-free Raman
analysis, but the current in situ surface-enhanced Raman scattering (SERS)-based analysis showed a relatively small Raman scattering intensity at 750 cm$^{-1}$. The difference can be expected from the difference of the Raman scattering mechanism between the normal Raman scattering and SERS mechanism. The Raman shift peaks located at 1225 cm$^{-1}$ were assigned to the amide III vibrational mode of $\beta$-sheets. The same Raman spectral patterns were observed from other types of cells (human fibroblast cell, data not shown here). Therefore, it is believed that the Raman spectra displayed in Figure 3D were close to the Raman spectra of Cyt C in the mitochondria of a cell (see Supporting Information Table S1 for detailed assignment).

**Time-Dependent Changes during Photothermally Induced Apoptosis.** Based on these Raman spectra (Figure 3D) as a starting point, we followed the changes in the molecular signal during apoptosis induced by either photothermal (PTT) damage or by drug molecules that would be expected to interact with the kinases present in the mitochondria. First, we followed the Raman signal changes over time after applying a focused 785 nm laser for 1 s with different input-laser powers (0, 12, and 24 mW) to a single cell. The input-laser power was focused on the area inside cells through a 60× objective lens, and then acquired the Raman signal changes using the same objective lens. Figure 4A shows that there were significant changes in the cell morphology using both B/F microscopy, D/F microscopy, and Raman imaging 30 min after exposure to a high input laser (24 mW), indicating that the cell was undergoing apoptosis. Figure 4B shows typical changes in the Raman spectra at 5 min intervals during apoptosis (24 mW). The disulfide Raman peak at 505 cm$^{-1}$ slowly decreased with time because of the reduction of disulfide residues of protein by glutathione, which is one of the defense mechanisms that occurs following cell stress. The Raman peak at 840 cm$^{-1}$ was relatively unchanged over time. The Raman peak related to Cyt C at 1127 cm$^{-1}$ did not change significantly. However, the Raman shift corresponding to $\beta$-sheets (1225 cm$^{-1}$), and the Raman shift at 1585 cm$^{-1}$ significantly increased after 20 min, as shown in Figure 4B. This is because of significant structural changes in proteins present in the mitochondria as a result of hyperthermia-induced apoptosis. The moderate decrease in the disulfide bond at 505 cm$^{-1}$ agrees well with previously reported results, where HSC-3 cells laden with nuclear targeted AuNPs were induced to undergo apoptosis following light illumination. The appearance of the amide III $\beta$-sheet Raman shift at 1225 cm$^{-1}$ is related to the significant changes in the $\alpha$-helical structure of proteins (1300–1400 cm$^{-1}$).

These spectral changes were found to be clearly correlated with the input laser power, as shown in Figures 4C & S2. If there was no photothermal damage to the cells, the Raman shifts were not changed significantly (Figures 4C-0 mW & S2A). However, when stronger photothermal damage was applied to the cell, more significant changes in the Raman signal intensity were observed over time (Figures 4C and S2B-12 mW, 24 mW). Much faster and clearer intensity changes were observed at high input laser powers (Figures 4C (12 vs 24 mW)). Therefore, it is believed that Raman-based single cell spectroscopy is a promising way to monitor the molecular signal changes inside cells.

**Time-Dependent Changes during Dichloroacetate Induced Apoptosis.** Next, we monitored the molecular

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**Figure 4.** (A) Photothermal damage induced apoptosis in HSC-3 cells (785 nm, 24 mW, 1 s exposure). (B) Time-dependent changes in Raman spectra during apoptosis (24 mW). (C) The intensity changes of specific Raman shifts at 505 cm$^{-1}$ (–S–S–), 1127 cm$^{-1}$ (Cyt C), 1225 cm$^{-1}$ ($\beta$-sheet), and 1585 cm$^{-1}$ (pyrrole ring) after applying with 0, 12, and 24 mW laser illumination.
signal changes during apoptosis induced by a chemical drug molecule such as dichloroacetate (DCA) sodium, which is a well-known inhibitor of phosphate dehydrogenase kinase (PDHK) that is located in the mitochondrial matrix. The LC_{50} for DCA in HSC-3 cells was determined to be 500 mM, as determined by a cell viability assay (Figure S3). Based on this, we investigated the effect of different DCA concentrations (5, 50, and 500 mM) on HSC-3 cells using Raman scattering. Figure 5A shows the changes in the single cell morphology induced by the addition of DCA (500 mM). Compared to PTT-induced apoptosis, the apoptotic cell morphology changes caused by DCA were not clearly evident, even after 30 min. However, the Raman spectra were found to change significantly, as shown in Figure 5B. The disulfide peak at 505 cm\(^{-1}\) disappeared very rapidly, but the peak at 840 cm\(^{-1}\) was unchanged, both of which were observed for PTT-induced apoptosis (Figure 5B). The Raman shift at 1127 cm\(^{-1}\) changed significantly, splitting into two peaks (1116 and 1141 cm\(^{-1}\)). The Raman shift at 1313 cm\(^{-1}\) also changed significantly decreasing in intensity after 10 min. As the Raman scattering at 1313 cm\(^{-1}\) decreased further after 15 min, a new peak at 1420 cm\(^{-1}\), corresponding to the \(-\text{CH}_2-\) bending mode for proteins and lipids, was observed. Because the addition of DCA does not induce physical damage inside the cell, there were no significant increases in the amide III/β-sheet Raman shift at 1225 cm\(^{-1}\). Time-dependent monitoring of the peaks at 505, 1127, 1225, and 1585 cm\(^{-1}\) showed DCA concentration dependent changes, as shown in Figure 5C. At a low DCA concentration (5 mM), the Raman scattering at 505 cm\(^{-1}\) was not significantly changed, but small changes in the Raman shift at 1127 cm\(^{-1}\), corresponding to Cyt C, were observed (Figures 5C & S4A). When the DCA concentration was increased to 50 mM, the Raman peaks at 505 and 1127 cm\(^{-1}\) showed a more significant decrease with time compared to at low DCA concentration. At 500 mM DCA, the Raman peaks at 505 and 1127 cm\(^{-1}\) changed very rapidly (Figures 5C & S4B) and a new peak at 1420 cm\(^{-1}\) was observed, which indicates a significant change in the intracellular environment, Cyt C, and the membrane structures of the mitochondria.

**Time-Dependent Changes during Potential Inhibitor Induced Apoptosis.** Both results in Figures 4 and 5 demonstrate the strong capability of single cell Raman spectroscopy to monitor molecular changes in the mitochondria. To further envision the capability of this method to screen for potential drug molecules that can inhibit the mitochondrial function, we performed the same experiment with a novel small molecule inhibitor of PDHK (2,4-dihydroxy benzamide derivative to VER-246608), which showed a strong potency in HSC-3 cells (Figure S5). The LC_{50} for the novel PDHK inhibitor on HSC-3 cells was determined to be 0.4 mM (Figure S5).

Based on this, we investigated molecular signal changes in mitochondria following the addition of the novel PDHK inhibitor (50 μM, 500 μM, and 5 mM). Figure 6A shows the morphological changes in HSC-3 cells after treatment with 5 mM of the novel PDHK inhibitor. Although little changes were observed in the single cell by B/F microscopy, D/F microscopy, and Raman mapping images, clear changes in the Raman scattering spectra, could be observed, as shown in Figure 6B. The disulfide peak at 505 cm\(^{-1}\) decreased very rapidly after 10 min, but the peaks at 840 and 1000 cm\(^{-1}\) did not change over time. Similar to DCA-induced apoptosis, the
Raman scattering peaks at 1127 and 1313 cm$^{-1}$ showed a decreased intensity with peak splitting. Unlike DCA-induced apoptosis, a new peak at 1420 cm$^{-1}$ was not found in this case. Figure 6C shows the quantitative changes in specific Raman shifts at 505 cm$^{-1}$ (S-S), 1127 cm$^{-1}$ (Cyt C), 1225 cm$^{-1}$ ($\beta$-sheet), and 1585 cm$^{-1}$ (pyrrole ring) after treatment with 50 $\mu$M, 500 $\mu$M, and 5 mM of the new PDHK inhibitor.

Figure 6. (A) Novel PDHK inhibitor (5 mM) induced apoptosis in HSC-3 cells. (B) Time-dependent changes in Raman spectra during novel PDHK inhibitor-induced apoptosis (5 mM). (C) Intensity changes in specific Raman shifts at 505 cm$^{-1}$ (S-S), 1127 cm$^{-1}$ (Cyt C), 1225 cm$^{-1}$ ($\beta$-sheet), and 1585 cm$^{-1}$ (pyrrole ring) after treatment with 50 $\mu$M, 500 $\mu$M, and 5 mM of the new PDHK inhibitor.

**CONCLUSIONS**

In summary, we have demonstrated the use of a single cell spectroscopic tool that can monitor changes in molecular signals in mitochondria during apoptosis using mitochondrially targeted plasmonic nanoparticles (TPP-AuNPs) and Raman microscopy coupled with B/F microscopy, D/F microscopy, and fluorescence imaging. Characteristic changes in the Raman signals could be identified for both photothermally and chemically induced apoptosis. The Raman scattering at 505 cm$^{-1}$ can be used as an indicator of cellular homeostasis. The Raman scattering at 840 cm$^{-1}$ can be used as an internal standard peak because its intensity does not change during apoptosis, regardless of how the apoptosis was initiated. The intensity changes in the signature peaks related to the Cyt C or $\beta$-sheet showed a close dependence on the concentration of potential drugs or input laser power, indicating that there is a strong potential for this single cell Raman-based spectroscopic tool for use in future drug screening applications.

**EXPERIMENTAL SECTION**

**Materials.** TA-stabilized gold colloids (50 nm) were purchased from BBI Solutions (Madison, WI, USA). TPP was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s phosphate buffered saline was purchased from Mediatech, Inc (Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone (Waltham, MA, USA). Antibiotic solution and 0.25% trypsin-ethylene diamine tetraacetic acid were purchased from Gibco (Waltham, MA, USA). Mito Tracker Green FM was obtained from Invitrogen, Inc (Carlsbad, CA, USA). The cytotoxicity assay kit with water soluble tetrazolium salts was purchased from DOGEN (EZ-CytoX, Seoul, South Korea).

**Instruments for Characterization.** A transmission electron microscope (H-7100, Hitachi, Tokyo, Japan) was
used for the TEM analysis. Extinction spectra were obtained with a UV–vis spectrometer (SCINCO, South Korea). Zeta potential analysis was performed with a zeta potential and particle size analyzer (ELSZ-1000, Otsuka Electronics, Tokyo, Japan).

B/F and D/F images were obtained using a microscope (Olympus IX73, Tokyo, Japan) equipped with a D/F condenser [U-DCD (NA 0.8–0.92), Tokyo, Japan].

Raman spectra of single cells were acquired using an inverted Raman microscope (NOST, South Korea) with a 60x objective (NA 0.7) (Olympus, Tokyo, Japan). The sample was excited with a diode laser (785 nm, IPS, USA). The scattered Raman signal was detected with a confocal motorized pinhole (100 μm) directed to a spectrometer (FEX-MD, NOST, South Korea) (600 g mm⁻¹ grating) and finally to the spectroscope CCD [Andor (DV401A-BVF), Belfast, North Ireland]. EzScan (NOST, South Korea) software was used for the acquisition of the Raman images. Spectra were recorded in 0.5 μm X/Y steps for all samples. The laser beam diameter was 684.07 nm [785 nm, 60x objective (NA 0.7)]. The laser power was set to 3 mW and an integration time of 0.03 s was chosen to ensure fast mapping and to avoid cell damage. The required time to obtain a single cell Raman image (35 × 35 μm) was 196 s (total pixel number: 4900, integration time/pixel = 0.03 s, time to move next pixel = 0.01 s).

**Cell Culture.** Human oral squamous carcinoma (HSC-3) cells were used as the cancer cell model, and cultured on a glass bottom dish. The diameter of the glass was 1.5 cm. The cells were cultured in DMEM supplemented with 10% v/v FBS and 1% v/v antibiotic. The cell cultures were maintained in a 5% CO₂ atmosphere in a humidified 37 °C incubator during Raman analysis.

**Data Analysis.** Five Raman spectra were obtained from a single cell by illuminating with a 785 nm laser (3 mW, 0.03 s exposure time) every 5 min and then the average Raman intensity of the selected Raman scattering peaks at 505, 1127, 1225, and 1585 cm⁻¹ were obtained. Data fitting was performed with a nonlinear curve fit and Gaussian fitting methods in the Origin program.

**ASSOCIATED CONTENT**

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00629.

Intracellular distribution of AuNPs, Raman spectrum assignment, and time-dependent Raman spectrum (PDF)

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Notes

The authors declare no competing financial interest.

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