Research article

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Insights into the intracellular behaviors of black-phosphorus-based nanocomposites via surface-enhanced Raman spectroscopy

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Abstract: As one of the prospective two-dimensional nanomaterials, black phosphorus (BP), which has excellent physical and chemical properties, has witnessed quick development in theranostic applications. The more recent advances in combining BP nanosheet (NS) with nanoparticles exhibit new opportunities to develop multifunctional nanocomposites. However, more effort should be devoted to elucidate the nanomaterial-cell interaction mechanism before the bio-applications of BP-nanoparticle hybrids. Herein, the intracellular behaviors of BP-gold nanoparticles (BP-Au NSs) are first investigated using the surface-enhanced Raman scattering (SERS) technique. The presence of Au nanoparticles on the surface of a BP sheet allows nanohybrids with excellent SERS activity to enhance the intrinsic Raman signals of cellular components located around the NSs. Data from an endocytosis inhibitor blocking assay reveal that the nanohybrids are mainly taken up by macropinocytosis and caveolae-dependent endocytosis, which are energy-dependent processes. Associated with colocalization experiments,

nanohybrids are found to internalize into lysosomes and the endoplasmic reticulum. Moreover, the SERS difference spectrum is extracted after Raman-fluorescence colocalization statistical analysis to distinguish the molecular structural differences in the biochemical components of the two organelles. These findings supply a definite cellular mechanistic understanding of the nano-biointeractions of nanocomposites in cancer cells, which may be of great importance to the biomedical applications of nanotechnology in the future.

Keywords: black phosphorus-gold nanocomposites; surface-enhanced Raman spectroscopy; endocytosis mechanism; intracellular localization.

1 Introduction

Black phosphorus (BP), a metal-free layered semiconductor that possesses predominant physicochemical characteristics [1–3], has been extensively applied in the biomedical field, such as biosensors, therapeutics, diagnostics, bioactive scaffolds and medical imaging [4–10]. Bare BP can slowly react with oxygen to form nontoxic phosphate and phosphonate, which are prevalent in the human body and are easy to metabolize, which allows it to have good biocompatibility and biodegradability [11, 12]. As a two-dimensional inorganic nanomaterial, BP possesses a large surface area that enables it to serve as the nanocarrier for drug delivery [13, 14]. This nanomaterial can also be used as a photothermal agent and photosensitizer for cancer therapy because of its broad absorption across the infrared region and the ability to produce reactive oxygen species [15–19]. Moreover, recent reports have illustrated that the combination of BP with nanoparticles, such as metal nanostructures, polymer and upconversion nanoparticles, offers superior or additional physicochemical properties to achieve high-performance and multimodal theranostic applications [20–24]. Yang et al.

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reported an image-guided cancer therapy based on multifunctional $\text{BPs@Au@Fe}_3\text{O}_4$ nanocomposites that integrated the photothermal and photodynamic effects derived from BP sheets with the plasmonic photothermal feature from Au and the magnetic resonance contrast enhancement effect from Fe_3O_4 nanoparticles [20]. BPs@Au nanohybrids could also generate more singlet oxygen under ultrasound irradiation compared to that of bare BP nanosheets (NSs), allowing more effective sonodynamic cancer therapy [24]. However, to date, work rarely has been carried out to investigate the intracellular behaviors of BP-nanoparticle composites.

Endocytosis is the process of transferring extracellular substances into the cell through the deformation of the plasma membrane [25]. As a universal entry mechanism of various nanomaterials, traditional endocytic pathway can be divided into four intracellular channels: clathrin- and caveolae-independent endocytosis, caveolae- or lipid-rafts-mediated endocytosis, macropinocytosis and clathrin-mediated endocytosis [26, 27]. After the nanomaterials are swallowed into the cells, they will be metabolized and acted within cytoplasm and shuttle in different organelles for transportation. Surface-enhanced

Raman spectroscopy (SERS) has been proven to be a powerful bioanalytical technique for the noninvasive single-cell analysis, biochemical component identification and biomedical imaging [28]. The molecular patterns of the cellular microenvironment around metal-like nanostructures can be monitored in a label-free manner by SERS. For example, Huefner et al. explored the endo-lysosomal pathway during the endocytosis of AuNPs using Raman combined with chemometric methods. In addition, the molecular composition inside the endocytic compartments were well characterized by the reporter-free SERS technique [29]. SERS seems to be an effective tool to study both the intracellular trace of metal-based nanomaterials and the molecular changes during nanomaterial-cell interaction.

Herein, we investigate the intracellular behaviors of a kind of BP-metal nanohybrids using the SERS technique. BP-Au NSs are fabricated via a one-step facile synthetic method (Scheme 1), whose product exhibits high-near infrared SERS activity to acquire the Raman fingerprints of cellular composition located around the nanohybrids. This feature also allows the SERS technique to monitor the intracellular trajectory of BP-based nanohybrids.

Scheme 1: Schematic illustration of synthetic process of BP-Au NSs and the endocytosis pathways in cancer cells.

In view of this, the endocytosis mechanism of BP-Au NSs is explored via counting the SERS signals inside the cells in the presence of various endocytic inhibitors. The double fluorescence colocalization experiments are then carried out to reveal the subcellular localization of the nanohybrids. The precise hyperspectral characterization of the organelles is well distinguished after the settling down of BP-Au NSs.

2 Results and discussion

2.1 Synthesis and characterization

Single- or few-layered BP NSs were synthesized by a modified mechanical exfoliation method from BP crystal powders in organic solvent [30–32]. As shown in Figure 1A, the transmission electron microscopic (TEM) image reveals an apparent flake structure of BP NSs with an average lateral size of about 300 nm, which reflects an appropriate size for efficient endocytosis [33]. The TEM image shows that the surface of BP NSs is coated with a great deal of globular Au nanosphere whose average size is about 30 nm (Figure 1B). These data are consistent with

the result measured by dynamic light scattering analysis, which indicates that the average diameters of AuNPs and BP-Au NSs are 37 nm and 342 nm, respectively (Figure S1, Supporting Information). In the enlarged view (Figure 1C), a distinct lamellar structure can be observed at the edge of nanohybrids, which confirms the successful connection of Au nanoparticles with BP NSs. A lot of AuNPs are confined to the NS, forming nanoaggregates, which have been proven to be an excellent SERS substrate due to the abundant “hot spots” existing in the nanostructure [34]. The energy-dispersive X-ray (EDX) spectral mapping shows three elemental mapping images (Au, P and O) with good overlap, further verifying the successful fabrication of BP-Au NSs (Figure 1D). Compared to bare BP, BP-Au NSs display significantly improved absorption in the ultraviolet (UV)-visible region and show a characteristic absorption peak at 540 nm, which could attest the presence of Au nanoparticles (Figure 1E). The Raman characterization of the nanohybrids (Figure 1F) exhibits three primary Raman bands, one out-of-plane phonon mode (A_g^1) and two in-plane modes (B_{2g} and A_g^2), which are located at about 358, 431 and 459 cm^{-1} , respectively [35–37]. Compared with the Raman spectrum of BP NSs, no significant change is observed from that of BP-Au NSs.

Figure 1: (A) TEM image of BP nanosheets. (B) Overall and (C) enlarged TEM images of BP-Au NSs. (D) EDX elemental mapping of BP-Au nanocomposites. (E) UV-Vis absorption spectra of the BP and BP-Au NSs at the same concentration. (F) Raman spectra of BP NSs and BP-Au NSs.

2.2 SERS activity of BP-Au NSs

To illustrate the SERS activity of BP-Au NSs, the SERS experiments were carried out using human hepatocellular carcinoma (HepG2) cells as the model. After treatment with the nanomaterials and fresh medium for 4 h, Raman signals of cells from different regions were detected. As shown in Figure 2A and B, cells display faint signals

Figure 2: Raman mapping and corresponding spectra of HepG2 cells incubated with (A) fresh medium, (B) BP NSs and (C) BP-Au NSs. Scale bar: 15 μm .

whether in Raman single-point measurement or overall imaging after incubation with fresh medium or BP NSs. On the contrary, ubiquitous intensive SERS signals from 620 to 1720 cm^{-1} can be observed from almost all of the intracellular regions when the cells are incubated with BP-Au NSs (Figure 2C).

The discovery of the excellent SERS activity of BP-Au NSs endows them with the capability for ultrasensitive detection of various cellular components. The stretching vibration peaks of DNA mainly display at 835 cm^{-1} (asymmetric O-P-O stretching vibration), 1316 cm^{-1} (guanine) and 1375 cm^{-1} (thymine and adenine). The characteristic bands of proteins basically appear at 655 cm^{-1} (C-C twisting of tyrosine), 820 cm^{-1} (tyrosine ring breath), 1004 cm^{-1} (phenylalanine ring symmetrical breathing) and 1597 cm^{-1} (C-C bending vibration of phenylalanine and tyrosine). Raman peaks at 1130 cm^{-1} (C-O/C-C stretching vibration in disaccharide) and 1271 cm^{-1} (lipid CH_2 deformation mode) represent the existence of carbohydrates and lipids in cells. These data indicate that the BP-Au NSs can be used as a SERS-active substrate to detect the intracellular complex biological components. Detailed assignments are summarized in Table S1 (Supporting Information).

2.3 Time course of cellular uptake of BP-Au NSs

We further investigated the process of cellular uptake of BP-Au NSs by the confocal Raman spectrometer. The SERS signals of HepG2 cells were acquired after the cells were incubated with BP-Au NSs for 1, 2, 4, 6, 8 and 12 h. As shown in Figures 3 and S2 (Supporting Information), visible Raman peaks originating from intracellular components confirm that the nanohybrids can be rapidly internalized into the cells within an hour. As time progresses, a moderate increase in the intensity of Raman signals is found over an incubation period of 6 h, meaning that the nanohybrids gradually accumulate inside the cells. Afterward, the intensity of Raman signals emerges a downward trend with elongation of incubation time. During the entire endocytosis process, the strongest Raman signals of cancer cells induced by BP-Au NSs appear at 6 h, which may be chosen as the best time point for further SERS investigation. There is a nonlinear relation between the incubation time of nanohybrids and the intensity of Raman signals. The main reason for the decrease in SERS intensity is probably related to the excessive irreversible aggregation of nanohybrids, which could not maintain appropriate inter-nanoparticle spacing on

Figure 3: Average SERS spectra of HepG2 cells incubated with BP-Au NSs for 1, 2, 4, 6, 8 and 12 h, respectively.

plasmon resonance, resulting in a reduction in the inter-particle hot spots [38, 39].

2.4 Mechanism of endocytosis of BP-Au NSs

SERS spectroscopy has become a viable alternative to fluorescence-based techniques due to its narrow spectral bandwidth, label-free feature and resistance to autofluorescence and photobleaching. For cellular mechanism study on the internalization of nanohybrids, the cells were coincubated with BP-Au NSs and various endocytosis inhibitors, such as amiloride (Ami, macropinocytosis inhibitor), methyl- β -cyclodextrin (M β CD; caveolae inhibitor), chlorpromazine (CPZ; clathrin inhibitor) and sodium azide (NaN₃; adenosine triphosphate-depleting agent). These endocytosis inhibitors were administered at concentrations that could not affect cell viability (Figure S3, Supporting Information). SERS images of HepG2 cells were acquired by integrating the signals from 620 to 1720 cm⁻¹, which indirectly reflected the distribution of BP-Au NSs within the cross-section of the cells. As shown in Figure 4A, intense SERS signals can be observed in the cells incubated with BP-Au NSs in the absence of any endocytosis inhibitor. In contrast, the Raman signals of HepG2 cells are hardly measured when the cells were pretreated with NaN₃, showing that the endocytosis of BP-Au NSs is energy

dependent. Strong Raman signals also appear in the cells coincubated with CPZ, indicating that the uptake of BP-Au NSs can essentially not be interrupted by inhibition of the clathrin-dependent pathway. In the case of cells exposed to Ami or M β CD, relatively hypointense SERS images are obtained, which implies that the nanohybrids are mainly ingested into the cells by macropinocytosis and caveolae-dependent endocytosis.

For quantitative estimation of the uptake of nanohybrids into cells, 150 Raman spectral lines per group were acquired from the cells coincubated with different endocytosis inhibitors. The spectral line that could clearly report the fingerprint information of intracellular components was judged to be the positive signal. As shown in Table 1, CPZ has little impact on the enhanced Raman signals of HepG2 cells containing BP-Au NSs, with about 84% positive spectral lines being detected, whereas the ratios of valid SERS spectral lines obtained from the cells pretreated with Ami, M β CD and NaN₃ are 44.7%, 48% and 38.7%, respectively. Moreover, the rate of positive signals decreases dramatically when multiple pathways of endocytosis are inhibited. The endocytosis mechanism of BP-Au NSs was also verified using traditional fluorescence microscopy. The fluorescent (FL) signals of BP-Au NSs were acquired by grafting an exogenous fluorescence dye, Rh B, onto the NSs. As shown in Figures 4B and S4 (Supporting Information), the most distinct FL signals of BP-Au

Figure 4: (A) Raman mapping of HepG2 cells incubated with BP-Au NSs after treatment with different endocytic inhibitors (scale bar: 10 μm). (B) Fluorescence images HepG2 cells incubated with BP-Au/Rh B in the presence of various endocytosis inhibitors. Scale bar: 200 μm .

Table 1: Effect of inhibitors on the uptake of BP-Au NSs into HepG2 cells measured by SERS spectroscopy.

Inhibitor	Number of spots for SERS detection	Positive signals	Positive ratio (%)
Control	150	142	94.7
CPZ	150	126	84
M β CD	150	67	44.7
Ami	150	72	48
NaN ₃	150	58	38.7
M β CD + Ami	150	32	21.3
M β CD + NaN ₃ + Ami	150	14	9.3

NSs are observed in the cells coincubated with or without CPZ, while cells treated with nanohybrids in the presence of Ami, M β CD or NaN₃ only emit obscure fluorescence,

which are consistent with the data assessed by the SERS technique, further demonstrating that energy-dependent macropinocytosis and caveolae-mediated endocytosis are the main endocytosis pathways of BP-Au NSs. A similar mechanism of endocytosis of BP NSs has been demonstrated [13], implying that the introduction of Au NPs has no significant impact on the process of cellular uptake.

2.5 Subcellular organelle localization

To investigate the intracellular fate of nanohybrids after endocytosis, fluorescence colocalization imaging experiment was performed. The subcellular structures of lysosomes, mitochondria and the endoplasmic reticulum (ER) were stained with FL organelle-specific trackers

Figure 5: Fluorescence co-localization analysis of BP-Au NSs with the (A) ER, (B) mitochondria and (C) lysosome at different time points. Scale bar: 20 μ m. (D–F) Colocalization coefficients between nanohybrids and different organelles.

to facilitate colocalization analysis [40–42]. Nanoparticles and other extracellular substances taken up by macropinocytosis and caveolae-dependent endocytosis could be enclosed in multiple organelles [43–45]. As shown in Figure 5, the fluorescence ascribable to BP-Au NS nanohybrids does not match that of mitochondria tracker but coincided with that of lysosomes and ER stains during the entire incubation period. These results indicate that BP-Au NS nanohybrids are mainly distributed in the ER and lysosomes. To accurately evaluate the subcellular distribution of the internalized nanohybrids, we calculated the colocalization coefficient of BP-Au NSs with ER tracker, lysotracker and mitochondria tracker (Figure 5D–F). With increasing incubation time, the amount of nanohybrids internalized by macropinocytosis within lysosomes gradually decreased (Figure 5F), indicating the possibility of either intracellular degradation of BP-Au NSs or transportation of the hybrids to extracellular and intracellular regions. The colocalization coefficient value for BP-Au NSs with the ER exhibits an increase at 2 h, followed by a slight decline at 4 h (Figure 5D). These results demonstrate that prolonged retention of BP-Au NSs inside living cells can be achieved when the nanohybrids are localized in the lysosome and ER via multi-endocytosis pathways.

The exact subcellular localization of BP-Au NSs in the lysosome and ER makes it possible to identify the fingerprints of these two organelles through Raman-fluorescence dual-mode mapping using Raman microspectroscopy. The fluorescence images of the two

organelle-specific probes were acquired under 514.5 nm laser excitation in the 515–600 nm range (Figure S5, Supporting Information). The SERS images of HepG2 cells mediated by BP-Au NSs were obtained using 785 nm laser excitation. Figure 6A displays the Raman-fluorescence dual imaging of the cancer cells, where a good overlap between the Raman signals and the FL images of the two organelles (ER and lysosomes) is observed, which is in accordance with the data detected by fluorescence colocalization assay. The overlap analysis between the two imaging modes was further performed using Matlab software, with the best anastomotic pixels being separated (Figure 6B). The overlap rates in the lysosome and ER regions are 52% and 61%, respectively. Then, the SERS spectra of cellular components were extracted from these overlapped pixel points, which could reflect the molecular information on the ER and lysosome. As shown in Figure 6C, the mean SERS spectra of the ER and lysosome exhibit similar spectral patterns but notable differences in some Raman peaks, indicating the diversity of biochemical components in the two organelles. For example, Raman bands at 1160 cm^{-1} (C-C/C-N stretching of protein), 1214 cm^{-1} (amide III β -sheet), 1353 cm^{-1} (CH_2/CH_3 wag of protein), 1399 cm^{-1} (COO^- symmetrical stretching of protein), 1471 cm^{-1} (C-H deformation/bending of protein) and 1570 cm^{-1} (amide II) mainly appear in the mean Raman spectrum of the ER [46, 47]. However, the dominant Raman peaks in the spectral lines of lysosome are located at 1135 cm^{-1} (C-C stretching mode of protein, chain C-C stretching of lipid or C-O/C-C in disaccharide),

Figure 6: (A) SERS-fluorescence dual-mode imaging analysis of the subcellular localization of BP-Au NSs. Scale bar: $20\text{ }\mu\text{m}$. (B) Overlap analysis of the FL and SERS images. (C) SERS spectra extracted from the ER and lysosome of HepG2 cells.

1284 cm^{-1} (Amide III, C-O-C vibrations of sugar rings), 1448 cm^{-1} (CH_2/CH_3 deformations in proteins and lipids) and 1519 cm^{-1} (Amide II) [47–49]. Not only the distributions of biochemical molecular species but also those of active lysosomes and ER in the cells were successfully visualized via the SERS technique. It may pave new insights for synthesizing functional targeted BP-based nanomaterials and the understanding of the interaction between cells and nanomaterials.

3 Conclusions

In this work, for the first time, a novel Raman active substrate based on BP-Au NSs has been developed for the detection of cellular uptake activity of BP-based nanohybrids by SERS spectroscopy. Enhanced Raman signals can be obtained by virtue of the fabricated SERS substrate, which makes it favorable for acquiring more detailed and rich fingerprint information of the intracellular biological components. The label-free SERS imaging is conducted to investigate the endocytosis mechanism of BP-Au NSs. The results demonstrate that the nanohybrids are mainly internalized via macropinocytosis and caveolae-dependent endocytosis, followed by transportation to the lysosome and ER. These data provide a guidance for essential understanding of the cellular behaviors of BP-based nanocomposites when applied to cancer research. Furthermore, the SERS-fluorescence dual mode imaging, coupled with organelle-specific probes, enables us to distinguish the unique molecular information of intracellular compartment from complex biological systems. In summary, this study elaborated the endocytosis mechanism and intracellular distribution of BP-Au NS nanohybrids using the SERS technique, which could provide an insight into the understanding of the interaction between cells and nanohybrids and further inspire the application of nanomaterial-assisted cancer therapies.

4 Experimental section

4.1 Materials

The BP crystal powders were purchased from a commercial supplier (XFNANO, Nanjing, China) and stored in a dark Ar glovebox. Chloroauric acid ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$) was bought from Sinopharm Chemical Reagent Co., Ltd.

(Shanghai, China). Ami and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-methyl-2-pyrrolidone (NMP), CPZ, dimethyl sulfoxide (DMSO), M β CD, NaN_3 and Rh B were provided by Aladdin biological technology Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and trypsin were obtained from GIBCO (Grand Island, NY, USA). Lyso-Tracker Green, Mito-Tracker Green, ER-Tracker Green and ER-Tracker Blue-White DPX were obtained from Yesen Biotechnology Technology Co., Ltd. (Shanghai, China). Deionized distilled water (Milli-Q System, Millipore, Billerica, MA, USA) was used throughout the experiment.

4.2 Synthesis of BP NSs and BP-Au NSs

The single-layered BP NSs were prepared by a liquid exfoliation method according to a previously reported protocol. Typically, the bulk BP crystal (25 mg) powers were immersed in NMP (25 ml) and kept sonicated with a sonic tip for 6 h at a power of 600 W (on/off cycle: 4 s/2 s). Subsequently, the mixture solution was ultrasonicated for 12 h at 4°C using a power of 400 W. The dispersion was dealt with centrifugation (7000 rpm, 10 min) to collect the sediment containing BP NSs and then resuspended in NMP (5 ml). For the synthesis of BP-Au NSs, the BP (150 μl , 5 mg ml^{-1}) and HAuCl_4 (300 μl , 10 mM) solutions were successively dispersed in boiling water (20 ml) for 2 min until the color of the finished product was altered to purple red. Afterward, the solution was concentrated by centrifugation at 7000 rpm for 10 min. For fluorescence microscopy imaging, the BP-Au NSs and Rh B (20 mg) solutions were stirred overnight in a dark room and precipitation of BP-Au/Rh B was obtained after centrifugation (7000 rpm, 10 min).

4.3 Characterization

TEM images and energy dispersive spectrometer mapping were characterized by a 120 kV JEM-2100HR transmission electron microscope (JEOL, Japan) equipped with an EDX spectrometer. UV-vis absorption spectra were taken on a spectrophotometer (UV-6100S, MAPADA, China). Raman spectra were measured by a high-resolution confocal Raman spectrometer (Derbyshire, England) equipped with excitation wavelengths of 514.5 nm and 785 nm. The hydrodynamic diameters of the Au nanoparticles and BP-Au NSs were measured by a Malvern Mastersizer 2000 (Zetasizer Nano ZS90, Malvern Instruments Ltd., UK).

4.4 SERS analysis of cancer cells

For SERS bioanalysis, the HepG2 cells were cultured in high-glucose DMEM medium with 10% FBS under humidified 5% CO₂ at 37°C. Cells precultured on the quartz slide were incubated with fresh medium, BP NSs and BP-Au NSs for 4 h. Then, the samples were washed with phosphate buffer solution (PBS) several times for SERS measurement. To study the process of uptake of BP-Au NSs, HepG2 cells were incubated with 50 µM of BP-Au NSs for 1, 2, 4, 6, 8 and 12 h, respectively. After rinsing with PBS for three times, 30 cells were measured per time point by the Raman spectrometer under the nm laser excitation.

4.5 Cytotoxicity assay of endocytosis inhibitors

HepG2 cells were cultured in a 96-well plate at a density of 4×10^4 cells/well overnight, then the medium was replaced by fresh medium containing Ami (80 µg ml⁻¹), CPZ (15 µg ml⁻¹), MβCD (8 mM) and NaN₃ (40 mM). After 24 h of incubation, 10 µl of MTT (5 mg ml⁻¹) was added and incubated in the dark for another 4 h, then MTT was replaced by DMSO to completely dissolve the purple formazan and detected the absorbance at 490 nm.

4.6 Fluorescence imaging and SERS measurement in the presence of endocytosis inhibitors

The HepG2 cells in the logarithm growth period were seeded in a culture dish at a density of 3×10^5 cells per well for 10 h. After that, Ami (80 µg ml⁻¹), CPZ (15 µg ml⁻¹), MβCD (8 mM) and NaN₃ (40 mM) were pre-incubated cells for 2 h and then substituted with BP-Au/Rh B (50 µM) for another 6 h. Fluorescence imaging studies were performed with a fluorescence microscope. For SERS measurement, the cells were incubated with BP-Au NSs (50 µM) and detected under the Raman microspectrometer.

4.7 Confocal fluorescence imaging

To study the subcellular localization of nanohybrids, HepG2 cells were seeded in 20-mm glass-bottom dishes and then treated with BP-Au NSs (50 µM) for 1, 2 and 4 h, respectively. After washing with PBS and fixing by 4% paraformaldehyde for 10 min, the cells were incubated with the primary FL probe: Lyso-Tracker Green, Mito-Tracker

Green and ER-Tracker Blue-White DPX. Finally, the cells were washed to eliminate excess dyestuffs and observed under the confocal microscopy (LSM-710).

4.8 Acquisition of Raman spectrum from organelles

For fluorescence Raman imaging study, the HepG2 cells were incubated in fresh medium containing BP-Au (50 µM) for 2 h. Soon afterward, Lyso-Tracker Green and ER-Tracker Green were added for 15 min. Raman imaging and fluorescence imaging were performed under Raman microspectroscopy with excitations at 514.5 nm (FL) and 785 nm (Raman).

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