SUPPLEMENT METHODS

The use of advanced spectral imaging to reveal nanoparticle identity in the biological samples

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Materials and Methods

Materials. LL-CPK1 (ATCC® CL101.1TM) porcine renal epithelial cells from proximal tubule were purchased from American Type Culture Collection (ATCC; Manassas, VA). Trypsin, penicillin-streptomycin solution (lot# 04619001), phosphate-buffered saline (PBS; lot# 05319001), and Dulbecco's Modified Eagle Medium (DMEM) (lot# 20818006) were purchased from Corning (Manassas, VA). Fetal bovine serum (FBS) was obtained from Seradigm (Logan, UT), paraformaldehyde (PFA) from Electron Microscopy Services (Hatfield, PA), Mounting Media HistoChoice® from Amresco, lysis buffer from Thermo scientific (Rockford, IL), protease inhibitor cocktail from Roche (Mannheim, Germany), Nitric Acid TraceMetal Grade (lot# 1119040) and Hydrochloric Acid TraceMetal Grade (lot# 4119080) from Fisher Scientific (Fair Lawn, NJ 07410), and Silver Staining Kit from Invitrogen (Cat no. LC6070).

Cell culture. LL-CPK1 cells were cultured to a confluent monolayer in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2 and 95% humidity. Cells were trypsinized (using a 0.05% solution of trypsin) regularly for passage when 70-90% confluence was reached. For our experiments, cells were cultured to reach confluence before treatment with vehicle or different types of NPs.

Synthesis of Ag-NPs and Au-NPs. For the synthesis of Ag(s)-NPs, silver nitrate ($AgNO_3$) and for gold nanoparticles (Au-NPs), chloroauric acid ($HAuCl_4$) were purchased from Millipore-Sigma, USA and was used without further purification. Ag and Au-NPs synthesis were carried out by taking 5 mL of betel leaf broth (*Piper betle* L.) and adding 90 mL of 1×10^{-3} M aqueous $AgNO_3$ and $HAuCl_4$ solutions individually at room temperature, followed by exposing the reaction mixtures to direct sunlight irradiation at Chapman University School of Pharmacy, CA, USA (latitude 33°N) in June 2019 from the time period between 11:00 a.m. and 2:00 p.m. under clear sky conditions with different time periods ranging from 5 min to 1 h. The Ag and Au-NPs colloidal solutions thus obtained were purified by repeated centrifugation (by using a Thermofisher ultra microcentrifuge) at 20,000 rpm for 10 min followed by redispersion of the pellets of Ag and Au-NPs into 20 mL of ultrapure water. To further purify the NPs, the centrifuging and redispersion process was repeated for five times. The bio-reduction of the Ag and Au ions in solutions were monitored by periodic sampling of aliquots of the reaction mixtures and measuring the UV–vis spectrum of the solutions individually. Silver nanoparticles (no-shell) were also synthesized by chemical methods in order to compare with silver (core-shell NPs). Briefly, Ag NPs (no-shell) were prepared by reducing 180 mL of $AgNO_3$ (1×10^{-3} M) using 20 mL of $NaBH_4$ (0.04 mg/mL). The loose dark pellet was collected from chemically synthesizing Ag NPs through centrifugation at 20,000 rpm at 26 °C for 20 min in a Thermo ultra-microcentrifuge. The chemically synthesized Ag NPs were further purified by the centrifuging and redispersion process was repeated for three times.

UV-VIS Spectrum Detection. Spectrophotometer (SpectraMax M5 Microplate Reader) was used to read the ultraviolet-visible (UV-VIS). The UV-VIS wavelength of Ag-NP and Au-NP clusters were read at concentrations of 0.025, 0.05, 0.125, 0.25, 0.375, 0.5 mg/mL diluted in deionized water, which was filtered through Milli-Q (MilliporeSigma, Burlington, MA). The wavelength range of 200-800 nm was used to read the UV spectrum for all the chemicals.

DLS and FTIR. NP characterizations were carried as previously described[1, 2] (**Supp Fig. 3**). The DLS study exhibited broad distribution of particles for Au-NPs (100.6±27.0 nm) and Ag(s)-NPs (107.7±30.3 nm). This broad distribution of particles is often found for bio-synthesis of nanoparticles using plant extract. On the other hand, comparatively sharp distribution of particles was observed for chemically synthesized Ag-NPs (113.7±15.8 nm). The FTIR spectra revealed some evident peaks of Betel leaf extract at 3271 cm^{-1} (O-H stretching of alcohols and N-H stretching of amines), 2919 cm^{-1} (H-C-H stretching; aliphatic), 1570 cm^{-1} (aromatic C-C=C stretching and N-H bending of amides), 1379 cm^{-1} (O-H bending of carboxylic acids), and 1049 cm^{-1} (C-O stretching of esters). Similar peaks were also observed in the FTIR spectra of Au-NPs and Ag(s)-NPs. However, their peaks are slightly shifted in many cases in comparison with that of Betel leaf extract, which might be due to the interaction of nanoparticles with the phytochemicals of Betel leaf extract. The results overall indicate that different polyphenols (O-H stretching; aromatic C-C=C stretching), carboxylic acids (O-H bending) and protein molecules (N-H stretching; N-H bending) present in Betel leaf extract could be adsorbed on the surface of Au-NPs and Ag(s)-NPs during their synthesis, thereby stabilizing those nanoparticles. On the other hand, FTIR spectra of chemically synthesized Ag-NPs revealed peaks at 3314 cm^{-1} and 1636 cm^{-1} which can be assigned to O-H stretch and H-O-H scissor of water molecules (associated with Ag-NPs), respectively. Evidently, the FTIR spectra of biosynthesized Ag(s)-NPs and chemically synthesized Ag-NPs are completely different.

Cell Treatment. Cells were seeded on sterilized 22x22 mm coverslip (Globe Scientific), in 6 wells plate (Greiner bio-one Cellstar®, the total volume of 2 mL at each well) under normal growth conditions until reached 70-80% confluency. The cells then incubated with 100 µL of the selected chemical at different concentrations of NPs for 18 hrs. Then, the cells washed three times by PBS, fixed the cells for 10 min in fixing solution (2.5 mL PFA, 7.5 mL PBS and 0.2 g sucrose) at room temperature. Afterward, the coverslip was placed on the slide that contains 25 µL of Mounting Media overnight at room temperature.

Spectral Imaging. Spectral imaging instrument (Applied Spectral Imaging's GenASIs™ Hyperspectral Imaging System) and Olympus microscope (Model BX61) were used in this research. Images were manually acquired with 60X magnification objective. Xenon arc lamp was used as our light source in transmission mode through the sample. Contrast was produced through the absorption of light in dense areas of the sample. Ag-NPs and Au-NPs cluster wavelengths were identified by utilizing the brightfield filter. Optical Density (OD) spectra were extracted and used to view and classify spectra.

Our system used a previously described standard microscopy set-up[3]. This set-up was widely available in most laboratories. Hyperspectral system was based on a Sagnac interferometer. The beam splitter split the light originating from the selected area in the sample into two beams. A set of mirrors led the beams down two paths of various lengths. At the end of the paths, the two beams are combined and superimposed on the sensor. The total intensity of these two superimposed beams at each point of the sensor is a function of the spectrum of this point on the sample and the difference in the distance between the two paths. This difference is called the Optical Path Difference (OPD). The intensity of the merged beams is captured by a Charged-Coupled Device (CCD) camera. Each measurement is called a frame, which is a gray level image measured by the CCD camera. To extract a hyperspectral image a set of frames are acquired, each corresponds to

slightly different OPD. This process arose simultaneously for all pixels in the image. The vector of intensities at each pixel, collected from the set of images with shifted OPD's, is called an interferogram. The Hyperspectral image is derived by Fourier Transformation of the interferograms of all pixel.

For each NP type, we defined wavelengths, which represent areas that we selected within the image in order to compare their spectra and to build a spectral library after background subtraction. For instance, inside the chemical image, to compare the chemical substance spectrum with the background spectrum, we defined wavelengths in the area that contained NP clusters, and in the area that contains nothing (background). This background wavelength also represented an empty cover treated the same way but without the addition of NPs. We then displayed the two spectra (NPs and background). The NP spectra were obtained by subtracting the background spectra. After that, we saved these NP spectra in libraries for further cell analysis. At least 10 images were captured randomly from the cells that were treated with NPs. Afterward, we analyzed the captured images by using spectral libraries of the NPs and scanned all the areas to obtain the location that only matched with the NP libraries. For the Ag-NPs and Au-NPs samples, we used the brightfield SUN analysis (within the SpectraView software, Applied Spectral Imaging), which performs Spectral UNmixing, separating an image into layers that corresponded to the absorption spectra (libraries). This led us to quantify the amount and pinpoint the location of material according to its absorption spectra.

At a resolution of 9 nm (**Supp Fig. 1**), we were able to collect 45 unique datapoints for each spectrum in each pixel of an image. Each of the datapoint was converted to a binary value, and it thus contained 2 sets of information; wavelength-intensity for a human operator and relative-position for the computer analysis (**Supp Fig. 2**). To generate self-validation on the analysis, confirmation was acquired among those 45 points; each point self-validated toward the rest of 44 other points. This resulted in 990 unique interactions (${}_2C_{45}$). If this self-validation did not produce a confidence coefficient of 99%, the system will generate the next levels of interactions (${}_2C_{990}$, ${}_2C_{489,555}$, ${}_2C_{119,831,804,235}$, etc.). The analysis was incredible accurate at 99.99% when 10 spectral libraries or more were used.

Time-lapse imaging. LLCPK cells were grown on six wells plate and treated with 0.1 mg NPs in 2 mL media. Living cells were imaged using a spectral imaging system, every 20 minutes for 8 hrs.

Transmission Electron Microscopy. The cells were treated with Ag-NPs and Au-NPs and incubated for 18 hours. Afterward, the cells were washed three times for 5 minutes with PBS. Then, the cells were trypsinized and fixed for 60 min in primary fixation (2% paraformaldehyde and 2.5% glutaraldehyde in 1% PBS buffer) at room temperature, followed by three washing with PBS. The resulting samples were postfixed with 1% osmium tetroxide for 60 min, followed by three-time washing with buffer and three-time washing with water. Afterward, the cells were dehydrated in a series of alcohol, then embedded in epoxy resin. Ultrathin sections of 120 nm were then stained with uranyl acetate and lead citrate and observed by TEM. The slices were examined under a JEM-2100F transmission microscope (JEOL) and the images were recorded on Gatan Oneview CCD as an image montage with the aid of SerialEM software. For TEM image, the

microscope was operated at 200kV and the images were taken at a minimum magnification of 10,000.

Animal Studies. All animal procedures were performed according to Chapman University Animal Care and Use Committee Guidelines. A total of four groups of mice were injected intravenously with saline solution (vehicle control), Ag(s)-NPs, Ag-NPs or Au-NPs for a total treatment time of 24 hours. The total NPs were injected at 1 mg/kg in 45-days old wild-type C57BL6 Black with averaged body weight of 27.5±2.8 g. Both male and female mice were used and randomly selected for the treatments. Then, the mice were euthanized to collect liver, kidney, liver, heart, and spleen. Tissues were taken for spectral imaging or ICP-MS analyses.

ICP-MS measurement. Approximately 0.1 g of tissue (liver, kidney, heart, and spleen) weighed and added into 5 mL a mixture of concentrated nitric acid (HNO_3) and hydrochloric acid (HCl) (4: 1) in digestion vessels. All the vessels were immediately shaken by hand and covered overnight for pre-dissolution. Afterward, the vessels were placed in oil and heated to about 100 °C for around 3 hours. The Au samples were diluted with 1% (v/v) HNO3 and 1% (v/v) HCl; and the Ag and Ag (s) samples with 1% HNO3.

Measurement with ICP-MS. All prepared standards and tissue samples were measured by the ICP-MS system (Thermo Scientific iCAP RQ ICP-MS). Quantification was carried out within elements (100-0.5 ppb) internal standard correction. The main operating conditions for ICP-MS were as follows: the radio frequency (RF) power 1550 W; argon gas flow rates for the plasma, auxiliary, and nebulizer flow were 14 L min^{-1}, 0.8 L min^{-1}, and 1.07 L min^{-1}, respectively.

Hematoxylin and eosin staining. The tissue processing of the organs was performed by STP 120 Spin Tissue Processor (Thermo Fisher Scientific, USA). The dehydration step was performed by immersing the organs in a series of alcohol, 70% for 30 minutes, 80% for 30 minutes, 95% for 45 minutes, and 100% for 45 minutes. The clearing step was performed for 1.5 hours by using xylene. The last step of processing was the infiltration by paraffin for 1.5 hours. The tissues were then sectioned for H&E staining and observed under spectral imaging (**Table 1**).

Table 1. H&E staining steps

Compound	**Time**
Xylene	17 min
100% Ethanol	2 min
90% Ethanol	1 min
75% Ethanol	1 min
Tap water	3 min
Hematoxylin	3 min
Tap water	30 sec 3x
Dip (fast) in acid alcohol (200ml of 70% alcohol + 500 µl of HCl)	
DI water	15 min
Eosin	2 min
Tap water	2 min 2x
90% Ethanol	1 min
100% Ethanol	1 min

Xylene	2 min
Mounting media & coverslip	

Silver Staining. The distribution and localization of Ag-NPs and Au-NPs in the cell nucleus were validated with a silver staining Kit following manufacturer's protocol (Invitrogen Co., Thermo Fisher Scientific, Cat. LC6070). Briefly, after sample fixation, 30 % of ethanol was added to the samples for 10 minutes. The sensitizing solution was added, followed by 30 % ethanol, 10 minutes each. The samples were washed with ultrapure water and incubated in a staining solution for 15 minutes. The samples were washed again with ultrapure water for 20-60 seconds and incubated in the developing solution for 4-8 minutes. Lastly, stopper solution was added and gently agitated for 10 minutes, followed by washing with ultrapure water for 10 minutes. The resultant deposits of metallic silver around NPs were visualized using the spectral imaging system.

Statistics. Most of our statistical analysis was conducted by using Spectral Imaging software GenASI™ SpectraView version 7.2.7.34276 and GraphPad Prism software version 8.4.2. Microsoft Excel software version 16.37 was also used for linear regression analyses to obtain a standard calibration curve and linear equation. We used Student t-test to compare 2 groups and ANOVA followed by Tukey's posthoc test to compare 3 or more groups. A minimum of 3 independent studies was performed, and a more accurate repeat (*N*) was indicated in each figure by the dot plots and/or figure legend. Whenever possible, our studies were conducted in pairs by including control groups in each experimental group. The correlation analyses were performed by using Pearson correlation coefficient test. All data were reported as mean ± standard error of mean (SEM). While $P<0.05$ was considered significant, the level of significance was indicated accurately in each graph and figure legend.

Reference

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