

Intracellular Gold Nanoparticle Aggregation and their Potential Applications in Photodynamic Therapy

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Electronic Supplementary Information

Materials:

Chloroauric acid (HAuCl₄), sodium citrate, dimethyl sulfoxide (DMSO), methylthiazoltetrazolium (MTT), protoporphyrin IX (PpIX), 5-aminolevulinic acid (5-ALA), Dihydrorhodamine-123 (DHR123), and L-15 medium (Leibovitz) were obtained from Sigma-Aldrich (Saint Louis, MO). Branched polyethyleneimine (BPEI) was obtained from Polysciences, Inc. (Warrington, PA). Fetal bovine serum (FBS) was purchased from American Type Culture Collection (ATCC, Manassas, VA). All other reagents and solutions were obtained from Invitrogen (Carlsbad, CA) except as indicated.

Synthesis of Au NPs and characterization

Negatively charged Au NPs (Au NPs (-)) were synthesized by citrate reduction of HAuCl₄. Briefly, 20 mL of 4 mM sodium citrate and 20 mL of 4 mM HAuCl₄ solutions were mixed and stirred for 2 min, and then irradiated with a 400 W metal-halide UV lamp (Cure Zone 2) under continuous stirring for 1 hr in an ice bath. The resultant gold colloid solution was reddish. The ice bath was changed every 20 min during synthesis. To prepare positively charged Au NPs (Au NPs (+)), a mixture of 20 mL of 1.4 mg/mL branched polyethyleneimine (BPEI, with a molecular weight of 10,000 g•mol⁻¹) and 20 mL of 4 mM HAuCl₄ solutions were stirred for 5 min in an ice bath and reacted for 1 hr

under UV irradiation. The size and zeta potential of Au NPs were measured by laser Doppler electrophoresis using a Nano-ZS Zetasizer (Malvern Instruments Ltd. Malvern, UK). Equal volume (500 μ L) of Au NPs (+) and Au NPs (-) at the same concentration (2 mM) was mixed to form Au NP aggregates. Au NPs and Au NP aggregates were immobilized on oxidized silicon substrates and observed using scanning electron microscopy (SEM, Carl Zeiss SMT Inc., Peabody, MA, USA), respectively. The plasmon resonance of prepared Au NPs and Au NP aggregates was determined by measuring the UV-visible absorption spectra with 1 nm resolution using a multi-mode microplate reader (SynergyTM HT, BioTek Instruments, Inc. Winooski, VT).

Surface-Enhanced Raman spectroscopy (SERS) Measurement

Au NPs and Au NP aggregates were deposited on the silicon wafer to achieve SERS-active substrates for measurements of 10^{-7} M Rhodamine 6G (R6G). Raman measurements were carried out with an in-house built Raman imaging and spectroscopy system. The system involved 785 nm laser excitation, 300 groove/mm grating spectrometer (Acton series spectrograph SP2300, focal length 300 mm) attached with spectroscopy grade CCD (Princeton instruments, SPEC-10), and high sensitivity imaging grade CCD (Princeton Instruments, PIXIS 1024 BR). The same 100 \times Nikon CFI60 microscope objective was used for laser excitation as well as collection of the Raman signal.

Cell-free reactive oxygen species (ROS) measurement

Colloidal solutions (50 μL) of single and aggregated Au NPs (2 mM) were mixed with 50 μL of 10 μM protoporphyrin IX (PpIX) to study their effects on ROS formation, respectively. Dihydrorhodamine-123 (DHR123, nonfluorescent) was used as a universal ROS tracking agent. Oxidation of DHR123 by ROS resulted in the formation of fluorescent Rhodamine 123 (R123). Then, 50 μL of 10 μM DHR123 was subsequently added to the mixture under the dark condition. Final concentrations of 0.67 mM Au NPs, 3.33 μM DHR123, and 3.33 μM PpIX were maintained constant for all measurements. Samples ($n=3$) in a total volume of 150 μL in 96-well plates was irradiated either by a broadband light source of broadband light or 600 nm and 700 nm long pass (LP) filtered light for different time durations. The fluorescence measurements were done after 1-min irradiation using a multimode microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) at an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm.

Cell culture and biocompatibility test of Au NPs

Human breast cancer cell line MDA-MB-231 (ATCC) was cultured in L-15 medium (Leibovitz) (Sigma-Aldrich, Saint Louis, MO) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) at 37°C without CO₂. To evaluate the cytotoxicity of Au NPs, MDA-MB-231 cells at the seeding density of 8,000 cells/well in 96-well plate were incubated with media containing Au NPs (-) and Au NPs (+) at the same concentration (40 μM) for 24 and 48 hrs, and cell viability was determined by measuring the metabolic activity using the MTT assay (ASTM E2526-08 standard

method for estimating of cytotoxicity). Briefly, cultures treated with different Au NPs were incubated with medium containing MTT (0.5 mg/mL) for 2 hrs and then the reaction product of formazan crystal was extracted with DMSO. Absorbance of the extract (OD value) was measured at 570 nm with the Synergy™ multi-mode microplate reader. Cultures without Au NPs were used as control. Cell viability was calculated as

$$\text{Cell viability (\%)} = \frac{\text{OD Value treated} - \text{blank}}{\text{OD Value control} - \text{blank}} \times 100\%.$$

Cellular photodynamic therapy (PDT)

MDA-MB-231 cells were seeded (8,000 cells/well) and cultured in 96-well plates for 24 hrs prior to PDT. Cells were incubated with the precursor of protoporphyrin IX (PpIX), *i.e.* 5-aminolevulinic acid (5-ALA) of 0.2 mM in serum free medium for a total time of 24 hrs. During the last 8 hrs, to induce the intracellular formation of Au NPs, cells were firstly incubated with Au NPs (+) for 4 hrs, and then Au NPs (-) of same concentration were added into cells for another 4 hrs incubation. Cells treated with sequence of Au NPs (-) and Au NPs (-), Au NPs (+) and Au NPs (+) were also studied for comparison. The total concentration of Au NPs added into cells is 40 μ M for all treatment groups. During the PDT treatment, cells were refreshed with Hank's Balanced Salt Solution (HBSS) and then put under light irradiation. The cells were irradiated either with a broadband light source of a 150 W halogen lamp (100 mW/cm² at the location of the electrode, Dolan-Jenner Fiber-Lite MI-150) for 1 min or with the same light source but filtered through 600 nm and 700 nm long pass (LP) filters (Thorlab, FEL600 and FEL 700) for 20 min and 30 min, respectively. After irradiation, cells were further cultured with the complete medium for 24 hrs before MTT assay.

To confirm MTT assay, MDA-MB-231 cells after various PDT treatments were also fluorescently stained for viability using a Live/Dead Viability/Cytotoxicity kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, cells cultured on glass cover slips after various PDT treatments were washed with HBSS and incubated with 2 μ M calcein acetoxymethyl (Calcein AM, 0.05%) and 0.5 μ M ethidium homodimer-1 (EthD-1, 0.2%) in HBSS for 30 min in the incubator. Viable cells were stained fluorescent green by Calcein AM, while the nuclei of dead cells were stained fluorescent red by EthD-1 in case of membranolysis. The stained cells were examined under a Nikon Eclipse 80i epi-fluorescence microscope.

Intracellular singlet oxygen species measurement

For measuring the intracellular singlet oxygen species, the Singlet Oxygen Sensor Green (SOSG) reagent was prepared at the final concentration of 1.33 μ M in HBSS. MDA-MB-231 cells treated with 5-ALA with/without different Au NPs and Au NP aggregates were incubated with the SOSG solution for 2 hrs before light irradiation. Then the cells in each well were refreshed with 100 μ L of HBSS for light irradiation. After irradiation, the well plate was further incubated for 20 min at 37 $^{\circ}$ C and then the fluorescence intensity was measured using the microplate reader with the excitation at 485/20 nm and the emission at 528/20 nm.

Cellular uptake of Au NPs and intracellular localization of Au NPs

To determine the uptake of Au NPs by MDA-MB-231, cells seeded at the same density were respectively treated with Au NPs (-)-Au NPs (-), Au NPs (+)-Au NPs (-),

and Au NPs (+)-Au NPs (+) in serum-free medium as described in the PDT treatment. After incubation, loose particles were removed by rinsing the culture with HBSS at least three times. The cells were trypsinized, centrifuged and gently washed with HBSS before collection. The collected cells were then lysed with an alkaline buffer consisting of sodium dodecyl sulfate (SDS) and 1N sodium hydroxide. The released Au NPs were then quantified by spectrometric assay.

To determine the intracellular destination of Au NPs and Au NP aggregates, MDA-MB-231 cells were treated with different Au NPs in the same sequence and the same incubation time as what was done in the PDT treatment as described above. Cells were rinsed with HBSS and then trypsinized, washed and centrifuged into cell pellet. Cell pellet was fixed with 4% paraformaldehyde (EM Sciences, Hatfield, PA) for 2 hrs at room temperature, post-fixed and stained with 1% osmium tetroxide for 1 hr, dehydrated in a graded series of ethanol solutions and then embedded in epoxy resin. Ultrathin sections (~70 nm) were obtained and examined with a transmission electron microscope (TEM, Philips CM20, 120 kV).

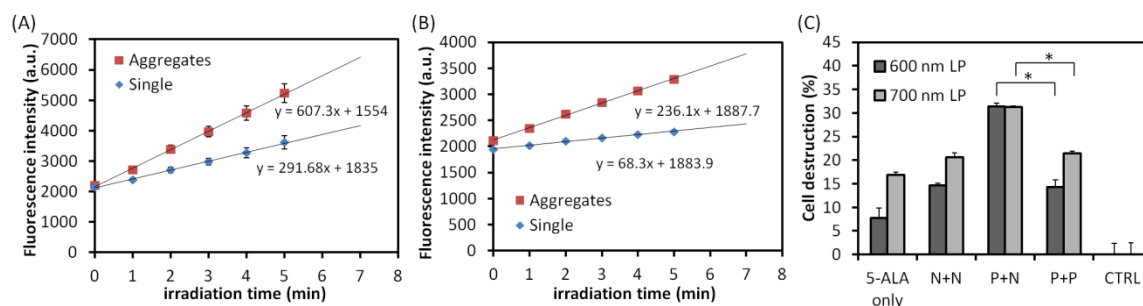


Fig. S1. Time-resolved kinetics of ROS formation from PpIX in the presence of single Au NPs and Au NP aggregates under irradiation with (A) 600 nm long pass filtered light and (B) 700 nm long pass filtered light (N=3). ROS was detected using DHR123 probe. (C) Cell destruction of MDA-MB-231 cells after various PDT treatments irradiated with 600 nm LP filtered light for 20 min and 700 nm LP filtered light for 30 min. (N: Au NPs (-); P: Au NPs (+)) * statistically significant, $p < 0.05$

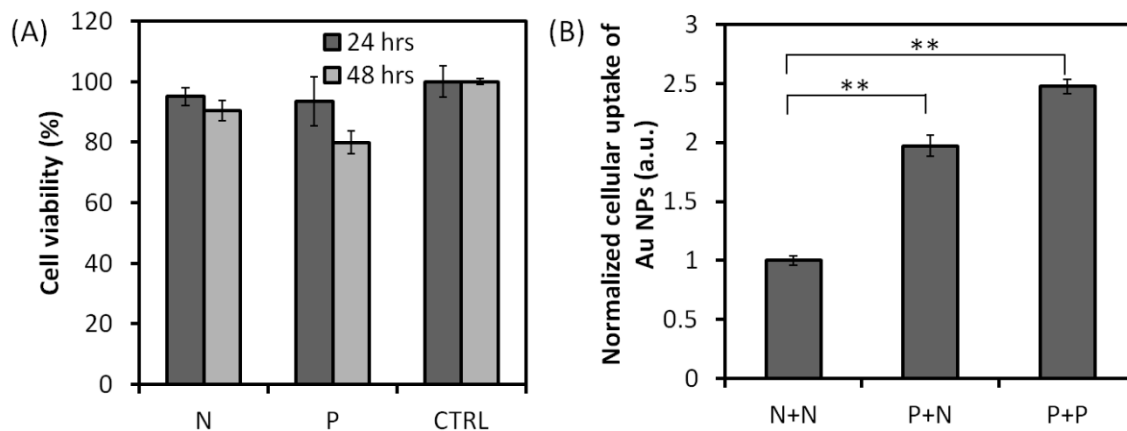


Fig. S2 (A) Cell viability of MDA-MB-231 cells after 24- and 48-hr incubation with Au NPs (-) and Au NPs (+). Culture without Au NPs was used as controls. (B) Normalized cellular uptake of Au NPs by MDA-MB-231 cells following different sequence of incubation with Au NPs with opposite surface charge. N: Au NPs (-); P: Au NPs (+). ** statistically significant, $p < 0.001$

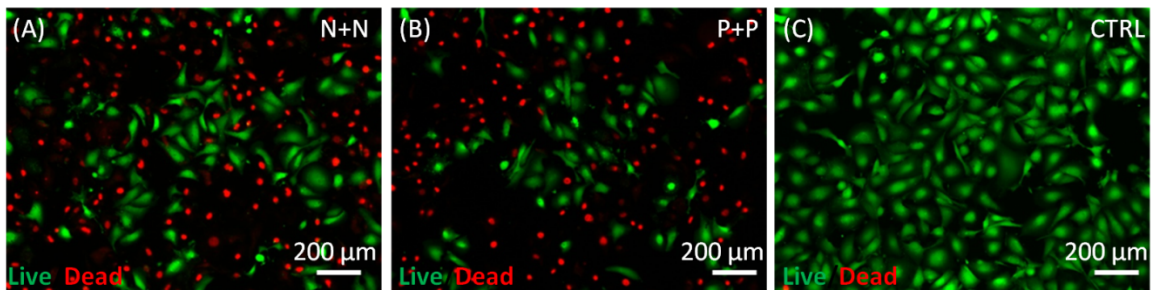


Fig. S3. Representative fluorescence images of MDA-MB-231 cells stained with a Live/Dead kit after various PDT treatments with 1-min broadband light irradiation. Cells after incubation with 5-ALA were treated with (A) Au NPs (-)-Au NPs (-), (B) Au NPs (+)-Au NPs (+), and (C) control group (no 5-ALA and Au NPs). Live cells were stained green with calcein-AM and dead cells stained red with EthD-1. N: Au NPs (-); P: Au NPs (+). Scale bar: 200 μm .

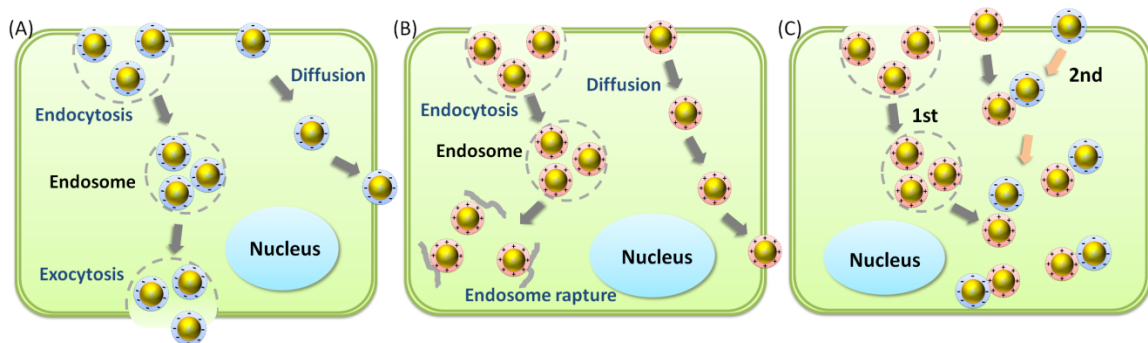


Fig. S4. Intracellular trafficking pathways of (A) Au NPs (-), (B) Au NPs (+), and (C) hypothetical mechanism for intracellular formation of Au NP aggregates.

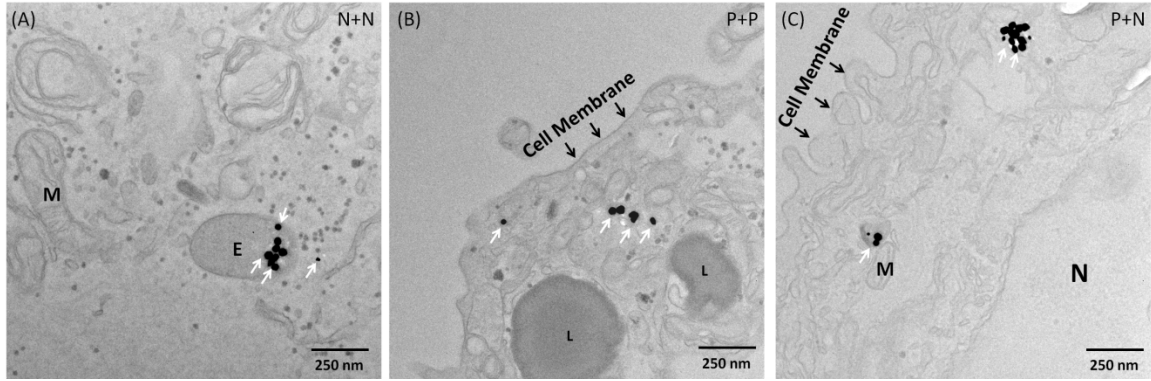


Fig. S5. Representative TEM micrographs of intracellular localization of Au NPs in MDA-MB-231 cells. Cells were incubated with (A) Au NPs (-) only (N+N) and (B) Au NPs (+) only (P+P), and (C) Au NPs (+)-Au NPs (-) combination (P+N). N: Au NPs (-); P: Au NPs (+). Scale bar: 250 nm. Au NPs were indicated by white arrows. E: endosome; M: mitochondria; L: lysosome; N: nucleus.

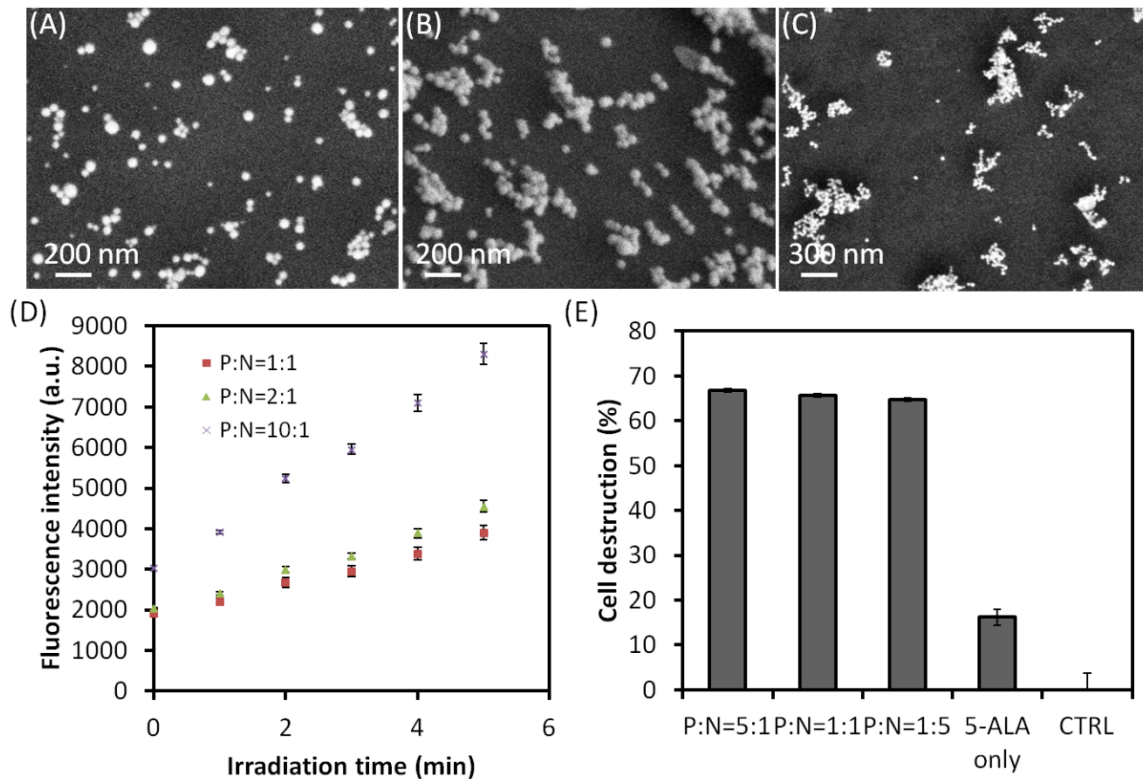


Fig. S6. SEM images of Au NP aggregates formed by mixing Au NPs (+) and Au NPs (-) at the ratio of (A) 1:1, (B) 2:1 and (C) 10:1. (D) Time-resolved kinetics of ROS formation from PpIX upon broadband light irradiation in the presence of Au NP aggregates formed by mixing Au NPs (+) and Au NPs (-) at different ratios. ROS was measured by using DHR123 probe. (E) Cell destruction upon various PDT treatments with 1-min broadband light irradiation in the combination of 5-ALA and Au NP aggregates at different ratios between Au NPs (+) and Au NPs (-). Culture without treatment was used as control (CTRL). N: Au NPs (-); P: Au NPs (+).