

Mitochondria-targeting Gold Nanoparticles for Improved Therapeutic Efficacy of Photodynamic Therapy of Breast Cancer

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ABSTRACT

In recognition of the unique contribution of gold nanoparticles (Au NPs) and critical role of mitochondria in photodynamic reaction, mitochondria-targeting Au NPs are prepared and employed to photodynamic therapy (PDT) of breast cancer. Au NPs with positive surface charge are synthesized and modified with functional groups (triphenylphosphonium, TPP). The integration of positive surface charge with mitochondrion-targeting domain (*i.e.*, TPP) onto Au NPs allow their selective accumulation in the mitochondria of breast cancer cells, leading to elevated reactive oxygen species (singlet oxygen $^1\text{O}_2$, in particular) formation and significant improved destruction of breast cancer cells by 5-aminolevulinic acid (5-ALA)-assisted PDT under light irradiation. These findings provide guidance for the improved design of intracellular organelle-targeted therapeutic nanoparticles for PDT.

Keywords: Gold nanoparticles, photodynamic therapy, mitochondria, triphenylphosphonium, reactive oxygen species

1 INTRODUCTION

Photodynamic therapy (PDT), which combines a photosensitive drug (*i.e.* photosensitizer, PS) with light irradiation, is an emerging minimally invasive therapeutic modality for a variety of oncologic diseases. Despite its benefits, PDT has not yet become a mainstream cancer therapeutic technique partially due to limited efficacy and poor selectivity of commercial PSs. There is still a great need for further enhancing the therapeutic effectiveness and specificity of current PDT. In recognition of their superior optical and surface plasmonic properties, as well as good biocompatibility and robust versatility in surface functionalization, gold nanoparticles (Au NPs) can be utilized as the enhancers for PDT by promoting the generation of reactive oxygen species (ROS), mainly composed of singlet oxygen ($^1\text{O}_2$), under light irradiation. Our recent findings have shown that Au NPs can enhance 5-aminolevulinic acid (5-ALA)-induced ROS formation and the enhancement is size-dependent, which suggest that

plasmonic Au NPs may amplify photonic energy absorbed and then transfer the energy to neighboring PSs for enhancing ROS formation. Compared to conventional PDT, the use of Au NPs as an enhancer and mediator to elevate ROS formation represents a promising PDT strategy [1-4].

In the meantime, given that mitochondria are the primary site for preferential accumulation of PS and $^1\text{O}_2$ production, together with their crucial roles in regulating cell apoptosis and cancer metabolism, they are of particular interest as potential target for improved PDT efficiency. In fact, some PSs such as protoporphyrin IX (PpIX) and phthalocyanine preferentially accumulate in mitochondria [5]. In this regard, targeted accumulation of Au NPs into mitochondria is expected to further enhance the generation of $^1\text{O}_2$. Various approaches have been explored to target mitochondria using targeting moieties, among which lipophilic cationic triphenylphosphonium (TPP) has been widely applied in drug design or surface modification for targeting mitochondria. Combinations of phosphonioalkanethiol and triphenylphosphonium have shown a remarkable ability to pass through mitochondrial membranes and accumulate inside mitochondria [6]. In the present study, mitochondria-targeting Au NPs were fabricated by conjugating TPP functional groups onto Au NPs with positive surface charge. It is hypothesized that the combination of positive surface charge with mitochondria-targeting domain (*i.e.*, TPP) onto Au NPs can selectively accumulate into mitochondria of breast cancer cells. Consequently, ROS formation will be elevated under light irradiation, efficient and selective destruction of breast cancer cells by 5-ALA enabled PDT will be better achieved.

2 MATERIALS AND METHODS

2.1 Synthesis of Au NPs, surface modification with TPP and characterization

Positively charged Au NPs (Au NPs (+)) were prepared by reducing chloroauric acid (HAuCl_4) with branched polyethyleneimine (BPEI) under UV irradiation as described in our previous study [1]. Briefly, a mixture of BPEI with a molecular weight of 10,000 $\text{g}\cdot\text{mol}^{-1}$ and HAuCl_4 solutions was stirred for 5 min in an ice bath and

reacted for 1 hr under UV irradiation. Mitochondria-targeting TPP molecules with thiolated ends were synthesized and conjugated onto Au NPs surface to form the TPP-Au NPs *via* the high affinity of thiol groups (SH-) to Au [6]. Physicochemistry properties of prepared Au NPs were characterized by UV-visible spectrophotometer, dynamic light scattering and laser Doppler electrophoresis, accordingly, and TPP conjugation onto Au NPs was confirmed by Surface-enhanced Raman spectroscopy (SERS) and Fourier transform infrared spectroscopy (FTIR).

2.2 Cell culture and cellular PDT

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. Human mammary epithelial cell line MCF-10A (ATCC) was maintained in DMEM/F-12 media supplemented with 5% horse serum, 1% pen/strep, EGF (20 ng/mL), hydrocortisone (0.5 mg/mL), cholera toxin (100 ng/mL) and insulin (10 µg/mL) in a humidified 5% CO₂ atmosphere at 37 °C.

Cellular PDT treatment was performed according to the procedures described in our previous study [1]. Briefly, cells were seeded (8,000 cells/well) and cultured in 96-well plates for 24 hrs and then the cells were incubated with serum-free cell culture medium containing 1 mM 5-ALA with/without Au NPs for 4 hrs. Then the cells were carefully washed with HBSS and refreshed with 100 µL HBSS for each well. The cells were irradiated with a broadband light source using a halogen lamp (100 mW/cm² at the location of the electrode, Dolan-Jenner Fiber-Lite MI-150, Dolan-Jenner Industries, MA) for 1 min. After irradiation, the cells were further cultured with the complete medium for 24 hrs before cell viability evaluation.

2.3 Singlet oxygen measurement

To measure the intracellular ROS level and specifically ¹O₂ in the PDT treatment, Singlet Oxygen Sensor Green reagent (SOSG, Molecular Probes), was used. Briefly, SOSG reagent was prepared at a final concentration of 1.33 µM in HBSS. MDA-MB-231 and MCF-10A cells treated with 5-ALA with/without different Au NPs were incubated with SOSG reagent at working concentration for one hour before light irradiation. Then the cells were washed with PBS to remove excess SOSG reagent and 100 µL of HBSS was added into each well for irradiation. The fluorescence intensity was measured using the SynergyTM fluorescence microplate reader with an excitation BP filter at 485/20 nm and emission BP filter at 528/20 nm. Since SOSG detected the singlet oxygen free radicals from both PDT treatment and intrinsic baseline, the fluorescence intensity was subtracted with that of the sham control (treated in the same way but no 5-ALA and Au NPs).

2.4 Cell viability evaluation after various PDT

After various PDT treatments, cell viability was determined by thiazolyl blue tetrazolium bromide (MTT) assay and Live/Dead Viability/Cytotoxicity staining. For MTT assay, cultures after different treatments were incubated with cell culture medium containing MTT (0.5 mg/mL) for 2 hrs and then the reaction product of formazan crystal was extracted with dimethyl sulfoxide (DMSO). Absorbance of the extract was measured at 570 nm with the SynergyTM multi-mode microplate reader. Cultures without 5-ALA and Au NPs under the same light irradiation condition were used as control.

For Live/Dead staining, MDA-MB-231 cells cultured on glass cover slips after PDT treatment were gently washed with HBSS and then incubated with the Live/Dead Viability/Cytotoxicity solution for 30 min. Viable cells were stained green by calcein acetoxymethyl (Calcein AM, 0.05%), while the nuclei of dead cells were stained red by ethidium homodimer-1 (EthD-1, 0.2%).

The PDT results were also evaluated based on a co-culture system with the presence of both breast cancer cells and normal cells. MDA-MB-231 cells were first labeled with Cell TrackerTM Green CMFDA for 30 min. Both MDA-MB-231 cells and MCF-10A cells of the same amount were suspended, pooled and co-cultured in the medium composed of 1:1 original cell culture medium for each cell type. The co-cultured cells were conducted for PDT with various Au NPs (40 µM) similarly to that described above. The fluorescence images merged with bright field images of cells were captured before PDT and 24 hrs after PDT using a Nikon Eclipse 80i epifluorescent microscope.

2.5 Mitochondrial localization of TPP-Au NPs

To determine the intracellular localization of different Au NPs after uptake, MDA-MB-231 cells incubated with various Au NPs as described above were rinsed with HBSS to remove any free particles, trypsinized, washed and centrifuged into cell pellet. Cell pellets were fixed with 4% paraformaldehyde (EM Sciences, Hatfield, PA) for 2 hrs at room temperature, post-fixed 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).

2.6 Statistical analysis

All quantitative results were obtained at least in triplicate. Data were expressed as the mean ± SD. Unpaired student's t-test was used for statistical significance analysis. A value of p<0.05 was considered statistically significant.

3 RESULTS AND DISCUSSION

After conjugation of TPP molecules onto Au NPs, positively charged TPP-Au NPs (45 ± 10 mV) with diameter of 50 ± 16 nm showed negligible cytotoxicity to both breast cancer and normal cells. TPP-Au NPs were preferably taken up by breast cancer cells, as much as about two times of that ingested by normal cells. It is most likely due to a lower membrane potential with cancerous cells that provides an intrinsic selective uptake of positively charged TPP-Au NPs in cancer cells.

In PDT treatment, selective destruction of breast cancer cells is a synergy between preferred uptake of TPP-Au NPs and subsequently elevated ROS ($^1\text{O}_2$, in particular) formation by TPP-Au NPs with the presence of PS under light irradiation. As shown in Figure 1A, the highest $^1\text{O}_2$ formation occurred in MDA-MB-231 cells treated with TPP-Au NPs. With the presence of Au NPs, a significantly higher level of $^1\text{O}_2$ was always measured in MDA-MB-231 cells compared to MCF-10A cells.

With the increase of intracellular $^1\text{O}_2$ level in PDT, higher cell destruction was expected. Indeed, the presence of Au NPs caused a significant increase in cell killing by PDT. The highest cell destruction was observed with TPP-Au NPs, which is consistent with the $^1\text{O}_2$ measurement. Compared to 5-ALA-PDT only, more MDA-MB-231 cells were killed with TPP-Au NPs (Figure 1B).

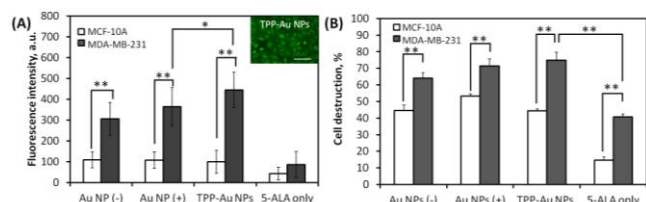


Figure 1 (A) Elevated generation of singlet oxygen species ($^1\text{O}_2$) in MCF-10A and MDA-MB-231 cells treated by various Au NPs in combination with 5-ALA or 5-ALA only after PDT treatments. Inset: Representative fluorescence images of MDA-MB-231 cells after PDT treatments with TPP-Au NPs. Singlet Oxygen Sensor Green (SOSG) probe inside of cells with green fluorescence in the presence of intracellular $^1\text{O}_2$. Scale bar: 100 μm . (B) Cell destruction of MCF-10A and MDA-MB-231 cells after PDT treatments with various Au NPs. * statistically significant, $p < 0.05$; ** statistically significant, $p < 0.001$.

To further confirm the MTT result, MDA-MB-231 cells after various PDT treatments were also fluorescently stained for viability (live/dead staining). The staining revealed that the presence of TPP-Au NPs caused the most cell death (Figure 2A), consistent with MTT results.

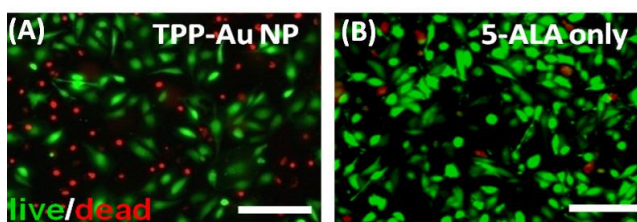


Figure 2 Representative fluorescent images of MDA-MB-231 cells stained with a Live/Dead kit after various PDT treatments. Live cells were stained green with Calcein-AM and dead cells stained red with EthD-1. Cells were treated with (A) 5-ALA and TPP-Au NPs and (B) 5-ALA only after light irradiation and further cultured for 24 hrs. Scale bar: 200 μm .

In the study on the selectivity of PDT, a co-culture system was used to emulate the *in vivo* circumstances where cancer cells coexist with normal cells. As shown in Figure 3, examination of the co-culture by fluorescence microscopy showed that a significant decrease of MDA-MB-231 cells (green-labeled) after PDT treatment in the TPP-Au NP-assisted groups. To quantify the difference, fluorescently labeled cells from 5 randomly selected regions were counted before and after PDT treatment. It was found that about 70% MDA-MB-231 cells were killed in TPP-Au NPs group compared to control group. A significant decrease of breast cancer cells was observed after TPP-Au NP-enabled PDT, while normal cells remaining their normal spindle shape and became confluent after culture for 24 hrs (Figure 3A). Clearly, under the same treatment, more breast cancer cells were destroyed than normal cells, implying the potential selectivity of TPP-Au NP-enabled PDT.

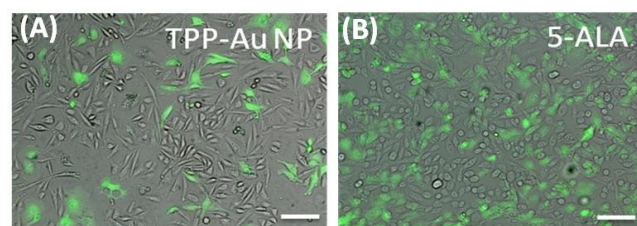


Figure 3 Selective destruction of MDA-MB-231 cells in the co-culture experiment. Merged bright field and fluorescence microscopic images of co-cultured MCF-10A (non-labeled, bright field) and MDA-MB-231 (green, fluorescence). Cells were treated with (A) 5-ALA and TPP-Au NPs and (B) 5-ALA only after light irradiation and further cultured for 24 hrs. Scale bar: 200 μm .

To determine the intracellular destination of ingested Au NPs, MDA-MB-231 cells loaded with Au NPs were sectioned and examined by TEM. As shown in Figure 4, after incubation for 4 hrs, TPP-Au NPs mainly accumulated in mitochondria, more specifically in the mitochondrial intermembrane space.

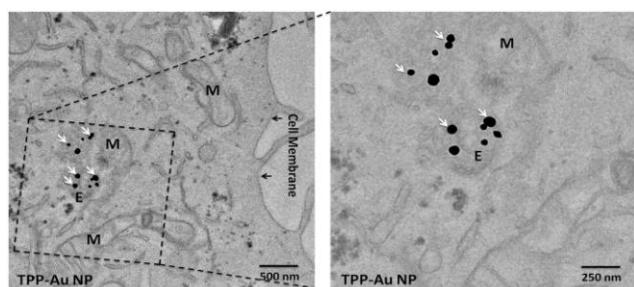


Figure 4. Representative TEM micrographs of intracellular localization of TPP-Au NPs in MDA-MB-231 cells. Au NPs were indicated by white arrows. E: endosome; M: mitochondrion.

Clearly, TPP-Au NPs yielded the most efficient killing of breast cancer cells with less damage to normal cells. This enhancement might be attributed to the specific delivery of TPP-Au NPs into mitochondria. Conjugation of TPP lipophilic cation, a commonly used mitochondriotropic molecule, onto Au NPs is expected to guide the Au NPs into mitochondria. Recognizing that mitochondria are the primary sites for converting 5-ALA into PpIX and forming $^1\text{O}_2$ during photodynamic reaction, over 90% of the total body oxygen is consumed by mitochondria, and localization of PSs in the mitochondria is more efficient to kill cells, thus, accumulation of Au NPs in mitochondria would be beneficial for enhancing the formation of $^1\text{O}_2$. Indeed, taking advantage of their mitochondria targeting feature, TPP-Au NPs showed the elevated generation of ROS and $^1\text{O}_2$ and resulted in much higher killing of breast cancer cells (74.8% cell destruction) than the cells treated with 5-ALA alone under the same PDT condition. Taken together, the enhanced PDT efficiency by TPP-Au NPs is a combined effect of 1) superior optical and plasmonic properties of Au NPs and 2) the localization of Au NPs in mitochondria for enhanced ROS formation.

4 CONCLUSION

Conjugation of Au NPs with mitochondria-targeting TPP leads to targeted delivery of Au NPs into mitochondria of breast cancer cells for further enhanced PDT efficacy but with minimal damage to normal cells. TPP-Au NPs, combining its mitochondria-targeting domain with positive surface charge, represent a promising strategy to significantly enhance therapeutic efficacy in 5-ALA-assisted PDT and selective destruction of breast cancer cells. Targeted delivery of Au NPs into mitochondria provides an important guidance and critical evidence on the effectiveness of intracellular targeting for further optimization of drug delivery and improvement of therapeutic efficacy. Understanding the cellular uptake and intracellular destination of Au NPs in relation to their physicochemical properties is of particular importance for their potential applications as a functional therapeutic agent.

5 REFERENCES

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