Supporting Information

Targeting Mitochondria with Au-Ag@Polydopamine Nanoparticles for Papillary Thyroid Cancer Therapy

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Materials. $HAuCl_4$ and $AgNO_3$ (99.8%) were purchased from Sinopharm Chemicals Reagent Co. Ltd. Sodium citrate (SC, 99.5%), hydrogen peroxide solution (30 wt.% in H_2O) and HCl (36%-38%) were purchased from Beijing Chemical Works. Tannic acid (TA), dopamine hydrochloride (DA, 99.0%), tris-(hydroxymethyl) aminomethans (Tris), NaN_3 (≥99.5%), chlorpromazine hydrochloride, nystatin, genistein (≥98%), amiloride hydrochloride, nocodazole (≥99%), 2',7'-dichlorofluorescin diacetate (DCFH-DA, ≥97%), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. 2,2-diphenyl-1-(2,4,6,-trinitrophenyl)hydrazyl (DPPH) was purchased from Alfa Aesar. tris-buffered saline and tween 20 (TBST) solution, sodium dodecyl sulfate (SDS), acrylamide, ammonium persulfate, and N,N,N',N',-tetramethylethylenediamine (TEMED) were purchased from Aladdin. Dulbecco's modified Eagle's medium with high glucose (H-DMEM), and fetal bovine serum (FBS) were purchased from Gibco. JC-1 kit, annexin V-FITC/PI kit, Cell Cycle kit, bicinchoninic acid (BCA) protein assay kit, and enhanced chemiluminescence (ECL) kit were purchased from Vazyme Biotech Co. Ltd. Cell mitochondria isolation kit were purchased from Beyotime. Propidium iodide (PI) was purchased from Invitrogen. p53 (DO-1), dihydroorotate dehydrogenase (DHODH, E-8) antibodies were purchased from Santa Cruz Biotechnology Inc. β-actin, LC3 antibodies were purchased from Abcam. Inc. Alcohol was analytical grade and used as received. In all preparation, deionized water was used.

Characterization. UV-visible (UV-vis) absorption spectra were obtained by a Shimadzu 3600 UV-vis-NIR spectrophotometer at room temperature under ambient conditions. Transmission electron microscopy (TEM) was measured using a Hitachi H-800 electron microscope at 200 kV acceleration voltage with a CCD camera. High-resolution TEM (HRTEM) imaging and the energy-dispersive X-ray spectroscopy (EDX) element mapping were implemented by a JEM-2100F electron microscope at 200 kV. The X-ray powder diffraction (XRD) investigation was carried out on a Rigaku X-ray diffractometer using Cu K radiation (λ=1.5418 Å). Fourier-transform infrared (FTIR) spectra were obtained by Brucker IFS66V. Thermogravimetric analysis (TGA) was measured on an American TA Q500 analyzer under N_2 atmosphere with the flow rate of 100 mL/min. The laser confocal fluorescent microscopy images of the cells cultured on the confocal dishes were taken by using the laser scanning confocal microscope OLYMPUS BX81 (FluoView FV1000). X-

ray photoelectron spectroscopy (XPS) was investigated using a VG ESCALAB MKII spectrometer with a Mg KR excitation (1253.6 eV). Dynamic light scattering (DLS) measurements were obtained using a Zetasizer NanoZS (Malvern Instrument). Inductive coupled plasma emission spectrometer (ICP) was carried out with Perkin Elmer Optima 3300DV analyzer. Flow cytometric analysis of apoptosis and cell cycle were implemented by beckman coulter FC500. The bright field and fluorescent images were operated by an Olympus IX51 inverted fluorescence microscope.

Preparation of Ag seeds. A monodisperse citrate-stabilized Ag seeds were prepared on the basis of the previous method.[1] A 100 mL aqueous solution containing SC (5 mM) and TA (0.01 mM) was heated in a three-neck rounded-bottom flask. After boiling, 1 mL of $AgNO_3$ (0.1 M) was added to this solution and kept boiling for 15 min. The diameter of as-prepared Ag seeds was about 23.2±2.4 nm. Finally, the seed solution was purified by centrifugation at 8500 rpm for 10 min and redispersed in deionized water for further use. The final Ag atom concentration measured by ICP was 1 mM.

Preparation of Au-Ag@PDA NPs. The preparation of Au-Ag@PDA NPs was operated under room temperature using sacrificial Ag seeds, $HAuCl_4$ aqueous solution, and the reductant of DA. In a typical preparation, 100 μL of 0.1 M $HAuCl_4$ and 400 μL of 1 mM Ag seeds solution were mixed in 50 mL deionized water at room temperature. After mixing for 3 min, the formation of branched structures was trigged by adding 600 μL of 0.03 M DA. After 10 min, the formation of PDA shell was trigged by adjusting the solution pH to 8.5 by adding 50 mL of 10 mM Tris-HCl buffer solution. After 3 h, the Au-Ag@PDA NPs were purified by 3 times centrifugation at 5000 rpm for 5 min, and then redispersed in deionized water for further use.

Cellular internalization and exclusion of Au-Ag@PDA NPs detected by ICP-AES. Human papillary thyroid cancer cell line (TPC-1) was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). TPC-1 cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells/well and incubated for 24 h and then the cells were incubated with 80 μg/mL Au-Ag@PDA NPs for 0.5, 1, 3, 6, and 24 h. For the exocytosis experiments, medium containing Au-Ag@PDA NPs was removing after incubating for 24 h and the cells were washed with PBS (pH=7.4) twice. After that, the fresh medium was replaced every 24 h for a total of 3 times. The cells were washed with PBS (pH=7.4) twice and

harvested at each time point. After centrifugation, the cells were treated with aqua regia for 4 h and then diluted to detect Au concentration by ICP-AES. Each group was measured in 3 wells and repeated 3 times.

Cell mitochondria isolation and detect the endocytotic Au-Ag@PDA NPs by ICP-AES. Mitochondrial separation was carried out by using a cell mitochondria isolation kit from Beyotime in accordance with the manufacturer's instructions. Briefly, TPC-1 cells were seeded in 10cm-culture dishes at an initial density of 1.5×10^7 cells/well and incubated for 24 h and then the cells were incubated with 80 μg/mL Au-Ag@PDA NPs for 0.5, 1, 3, 6, and 24 h. For the exocytosis experiments, medium containing Au-Ag@PDA NPs was removing after incubating for 24 h and the cells were washed with PBS (pH=7.4) twice. After that, the fresh medium was replaced every 24 h for a total of 3 times. The cells were washed with PBS (pH=7.4) twice and harvested at each time point. Then the cells were washed with ice-chilled PBS (pH=7.4) again. After that, they were incubated in mitochondria isolation agent at 4 ºC environment and homogenized with a glass homogenizer. The whole solution was centrifuged at 800 rpm for 5 min to remove unbroken cells and the supernatant was further centrifuged at 11000 rpm for 5 min at 4 ºC. The resulting pellet contained the mitochondria, mitochondrial fragment and the endocytotic Au-Ag@PDA NPs in mitochondria. Finally, the pellet were treated with aqua regia for 4 h and then diluted to detect Au concentration by ICP-AES. Each group was measured in 3 wells and repeated 3 times. It is mentioned that the cellular endocytosis and exocytosis in 10cm-culture dishes were also carried out at the same time but without the separation of mitochondria.

Mechanism of cellular uptake pathway of Au-Ag@PDA NPs. TPC-1 cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells/well and incubated for 24 h. Then the inhibitors were first added into serum-free H-DMEM and the cells were incubated for 1 h (control group without inhibitors, low temperature group was under 4 ºC environment). Next, the medium was replaced by a fresh medium containing 10% FBS and 80 μg/mL Au-Ag@PDA NPs for another 3 h. Then the cells were washed and harvested to detect the Au concentration by ICP-AES. Each group was measured in 3 wells and repeated 3 times.

Subcellular localization of Au-Ag@PDA NPs by TEM observation. TPC-1 cells were seeded in flasks (25 cm^2) at an initial density of 1×10^6 cells/well and incubated for 24 h and then the cells were incubated with 80 μg/mL Au-Ag@PDA NPs for

different time. For the exocytosis experiments, medium containing Au-Ag@PDA NPs was removing after incubating for 24 h and the cells were washed with PBS (pH=7.4) twice. After that, the fresh medium was replaced every 24 h. At the determined time, the cells were washed and harvested. Then fixed with 2.5% glutaradyhyde at 4 ºC for 24 h, post-fixed with 1% OsO_4 in PBS for 1 h, dehydrated in a grades series of ethanol, acetone, propylene oxide, and embedded in Epon. After slicing, stained with uranyl acetate and lead citrate and observed by TEM.

Mitochondrial membrane potential and cellular ROS level. TPC-1 cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells/well and incubated for 24 h. Then the cells were incubated with different concentration of Au-Ag@PDA NPs for 6 h or 80 μg/mL Au-Ag@PDA NPs for different time. For JC-1 staining, the cells were washed with PBS (pH=7.4) twice, harvested and resuspended in medium, then adding the JC-1 dye according to the JC-1 kit. After removing the dye, the cells were analyzed by using Beckman coulter FC500 flow cytometer. The method of laser confocal imaging is as above, but for the use of confocal dishes. For ROS detection, the cells were washed with PBS (pH=7.4) twice, harvested and resuspended in PBS, then adding 10 μM DCFH-DA dye. After removing the dye, the cells were analyzed by using Beckman coulter FC500 flow cytometer. Each group was measured in 3 wells and repeated 3 times.

Antioxidant activity test. The antioxidant activity of Au-Ag@PDA NPs was evaluated by their ability to reduce the concentration of the stable free radical DPPH, namely Au-Ag@PDA NPs reacted with DPPH and converted it to 1,1-diphenyl-2-(2,4,6,-trinitrophenyl)hydrazine. First, the stock solution of DPPH was prepared at 0.1 mmol/L in ethanol and stored at -20 ºC. Second, 1 mL Au-Ag@PDA NPs aqueous solution with specific concentration was added into 1 mL DPPH solution in a quartz cell and the absorbance at 526 nm (Abs_{samlpe}) of the mixture solution was measured by UV-vis spectrophotometer at the time point of 30 min. (Note: The kinetics curves were measured in a model of dynamics at 1 s intervals up to 300 s). Thirdly, the absorbance at 526 nm of control solution ($Abs_{control}$) (1 mL DPPH mixing with 1 mL H_2O) and blank solution (Abs_{blank}) (1 mL Au-Ag@PDA NPs aqueous solution with specific concentration mixing with 1 mL alcohol) were also measured. The scavenging percentage was calculated using the following formula:[2]

$$DPPH\ Scavenging\ effect = (1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}) \times 100\%$$

Annexin V apoptosis assay. TPC-1 cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells/well and incubated for 24 h and then incubated with different concentration of Au-Ag@PDA NPs for 24 h. After the treatment, the cells were harvested and washed with precooling PBS (pH=7.4) twice. The cells were resuspended in 100 μL binding buffer and then adding 5 μL annexin V-FITC and 5 uL PI staining solution. Blending gently and then avoid light reaction at room temperature for 10 min. Finally, adding 400 μL binding buffer and the samples were detected using Beckman coulter FC500 flow cytometer.

Cytotoxicity assay of Au-Ag@PDA NPs *in vitro* and the detection of cell proliferation rate. TPC-1 cells were seeded in 96-well culture plates at an initial density of 4000 cells/well and incubated for 24 h and then the cells were incubated with different concentration Au-Ag@PDA NPs for 24 h. The cell viability for TPC-1 was evaluated by a standard methyl thiazolyltetrozolium (MTT) assay on 96-well plates. The optical density was measured at 490 nm for the MTT assay. The Au-Ag@PDA NPs in each concentration were assayed in five wells and the assay was repeated three times. The cell proliferation rate (g_t (%)) was detected by MTT assay and calculated using equation:

$$g_t = \frac{v_t - v_0}{v_0} \times 100\%$$

where v_0 is the optical density measured immediately for the TPC-1 cells without further incubation and v_t is the optical density measured for the TPC-1 cells after incubation with different concentration of Au-Ag@PDA NPs.

Cell cycle analysis. TPC-1 cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells/well and incubated for 24 h and then incubated with different concentration of Au-Ag@PDA NPs for 24h. After the treatment, the cells were harvested and washed with precooling PBS twice. Then the cells were fixed in 70% ice-chilled alcohol at 4 ºC for 24 h. Fixed cells were centrifuged for collecting and washed with PBS twice. Cells were further centrifuged and resuspended in 0.5 mL of staining solution containing 50 μg/mL PI, 100 μg/mL RNase A, 0.2% Triton X-100, and then avoid light incubation for 30 min. Cells were then analyzed using Beckman coulter FC500 flow cytometer.

Western blot analysis. TPC-1 cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells/well and incubated for 24 h and then incubated with different concentration of Au-Ag@PDA NPs for 24 h. After treatment, the cells were washed and lysed by ice-cold lysates. After centrifugation of the cell lysates at 12500 rpm, 4 ºC for 20 min, the total protein extracts were determined by performing the BCA protein assay. The equal amounts of protein (about 25 μg) were loaded on to 12%-15% SDS-polyacrylamide gel electrophoresis and the separated proteins were transferred onto membranes. After blocking the membranes with 5% w/v skim milk (in TBST solution) for 1 h, the membranes were washed and probed with desired primary antibodies overnight at 4 ºC. The membrane was washed 5 times with TBST, then incubated with secondary antibodies for 1 h. After washing 5 times with TBST, the target proteins were detected by ECL kit.

Photothermal effect of Au-Ag@PDA NPs *in vitro*. TPC-1 cells were seeded in 96-well culture plates at an initial density of 4000 cells/well and incubated for 24 h. The cells were incubated with different concentration Au-Ag@PDA NPs for 24 h, then they were irradiated by an 808 laser at different power density for 10 min. As for the control groups, the cells were not incubated with Au-Ag@PDA NPs but irradiated by laser at corresponding power density for 10 min. At last, the samples were analyzed for cell viability by MTT assay. Each laser power density was assayed in five wells and repeated three times.

Apoptosis staining. For in vitro photothermal therapy, TPC-1 cells were seeded in 12-well culture plates at an initial density of 2×10^5 cells/well and incubated for 24 h and then incubated with 80 μg/mL Au-Ag@PDA NPs for 4 h. Each well was irradiation by an 808 laser with different power density for 10 min. Then the TPC-1 cells were cultured for 30 min and stained with 50μg/mL PI.

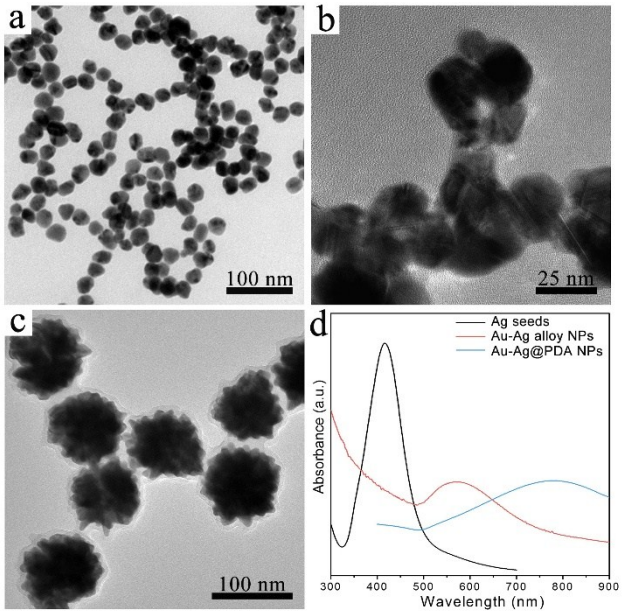

Figure S1. TEM images (a-c) and corresponding UV-vis absorption spectra (d) of Ag seeds (a), Au-Ag alloy NPs (b), and Au-Ag@PDA NPs (c).

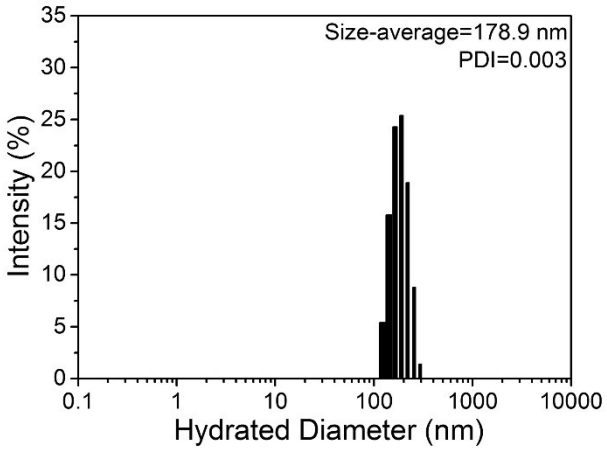

Figure S2. Hydrated diameter statistics of Au-Ag@PDA NPs.

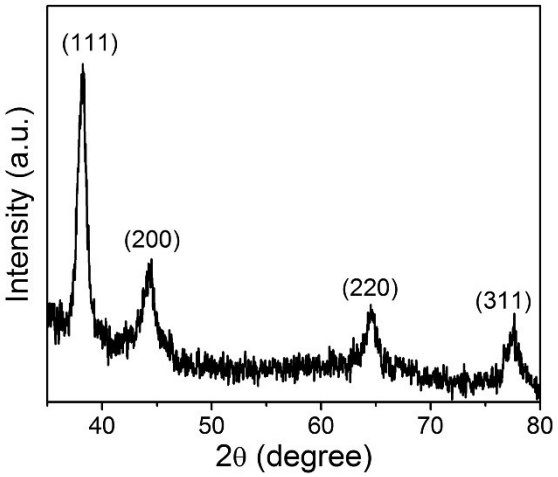

Figure S3. XRD pattern of Au-Ag@PDA NPs.

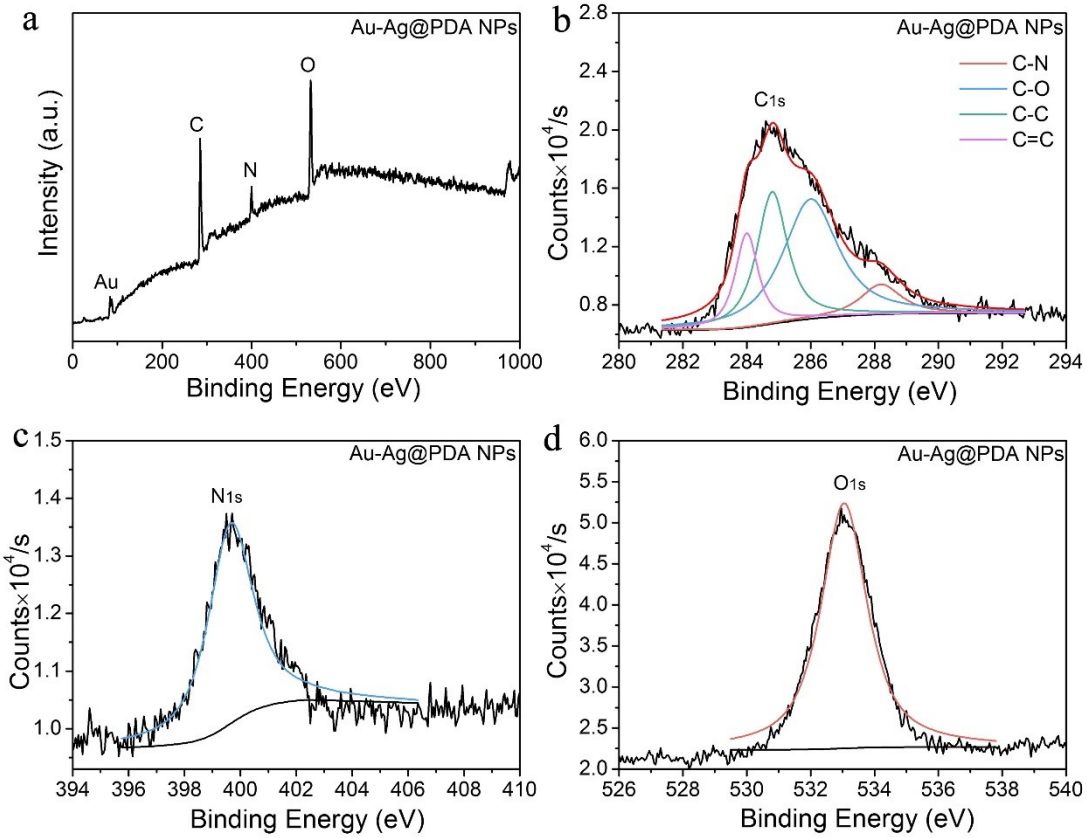

Figure S4. XPS survey spectrum (a), C 1s (b), N 1s (c), and O 1s (d) spectra of Au-Ag@PDA NPs.

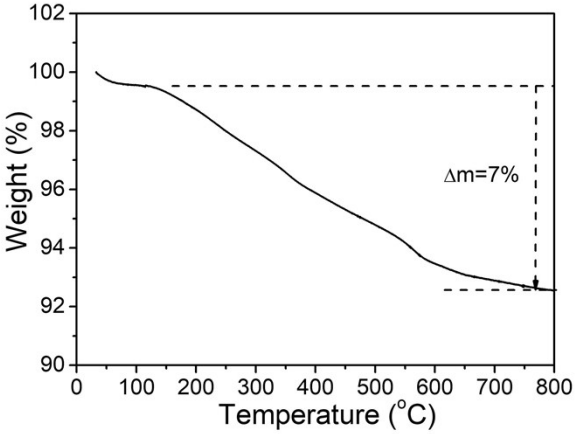

Figure S5. TGA data of Au-Ag@PDA NPs. The mass fraction of PDA shell was calculated as 7 wt % of the total mass of the Au-Ag@PDA NPs.

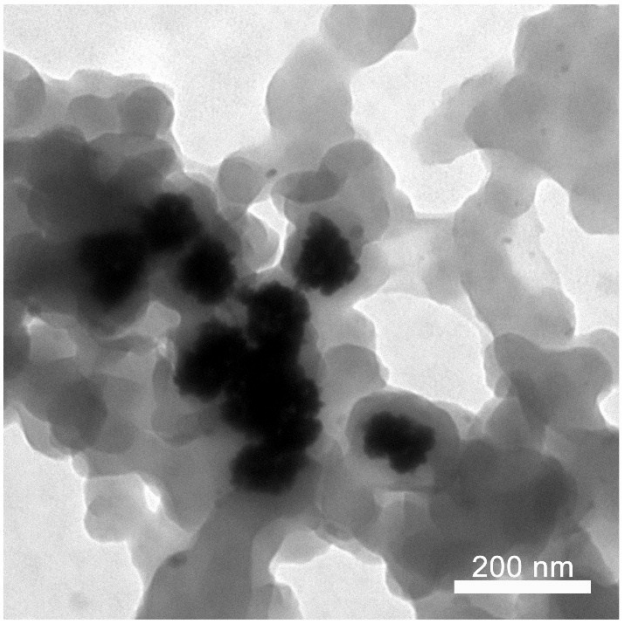

Figure S6. TEM image of Au-Ag@PDA NPs without adjusting the solution pH. As shown in the figure, the NPs aggregated together owing to the strong intermolecular hydrogen bonding in the acid environment.

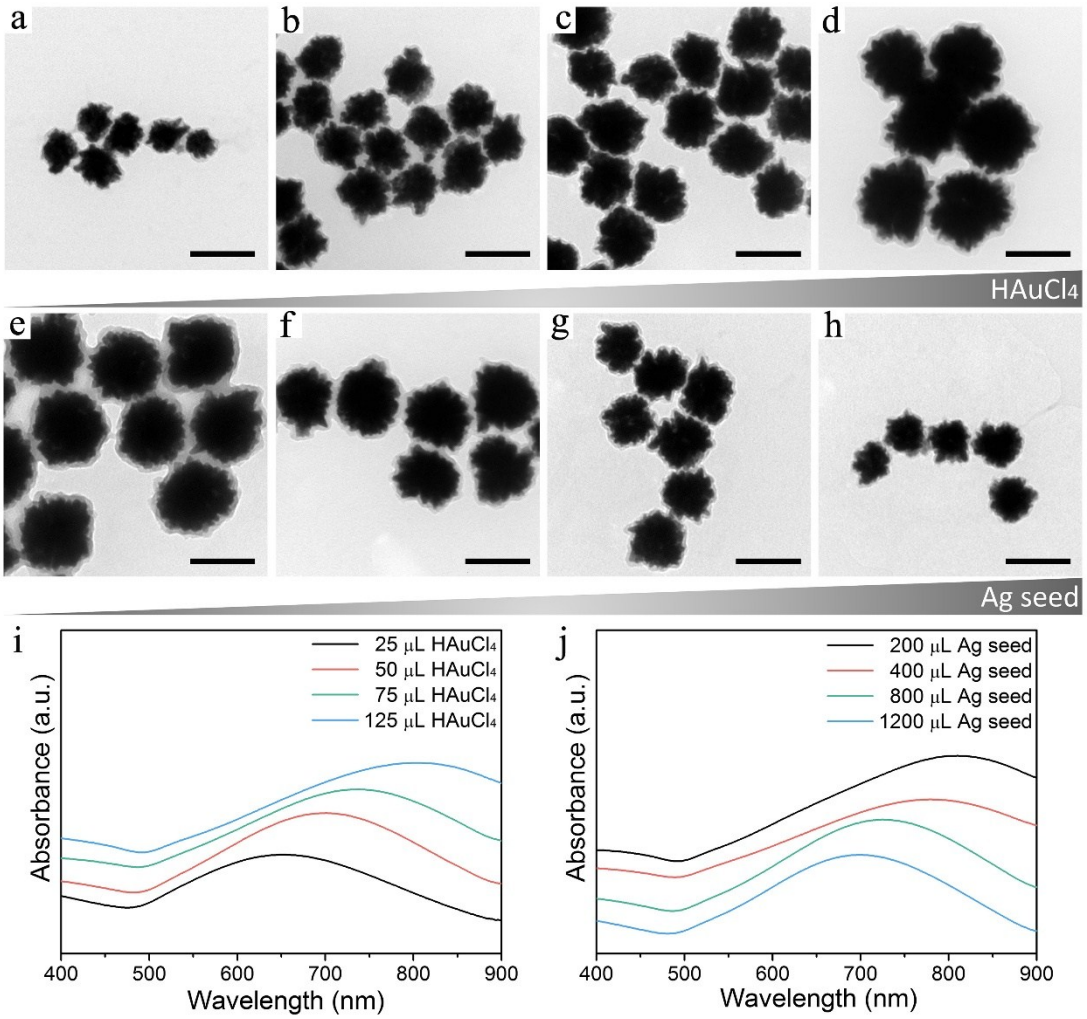

Figure S7. TEM images of Au-Ag@PDA NPs prepared at constant Ag seed amount of 400 μL and different $HAuCl_4$ amount of 25 (a), 50 (b), 75 (c) and 125 (d) μL and at a constant $HAuCl_4$ amount of 100 μL and different Ag seed amount of 200 (e), 400 (f), 800 (g), and 1200 (h) μL. (i, j) UV-vis-NIR absorption spectra of Au-Ag@PDA NPs influenced by the $HAuCl_4$ amount (i) and Ag seed amount (j). The amount of DA and solution pH were fixed at 600 μL and 8.5. The scale bar is 100 nm.

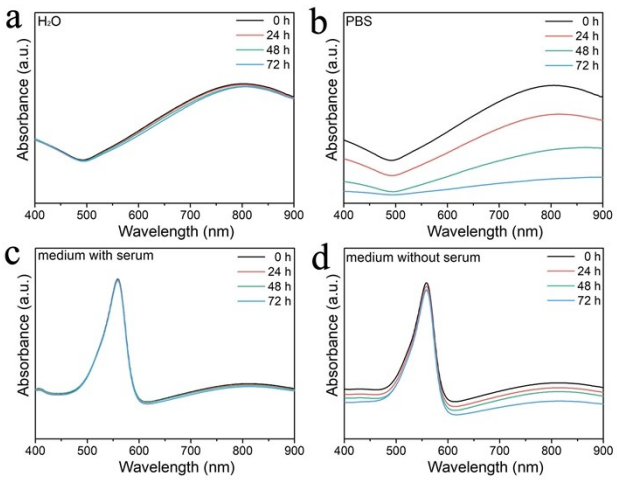

Figure S8. The UV-vis absorption spectra of Au-Ag@PDA NPs after 72 h storage at room temperature in H_2O (a), phosphate-buffered saline (PBS, 0.1M, pH=7.4) (b), medium with (c) and without (d) 10% serum.

Table S1. The ICP-AES measurement of Au and Ag element.

	Control	H_2O	PBS	Medium with serum	Medium without serum
Au/ppm	0.0483	0.0456	0.0219	0.0142	0.0206
Ag/ppm	0.0229	0.0218	0.0375	0.0214	0.0275
SD (Au)	0.0055	0.0020	0.0022	0.0006	0.0056
SD (Ag)	0.0009	0.0022	0.0073	0.0087	0.0032

Note: SD means standard deviation.

It is used to indicate if there is the leakage of Au and Ag element from Au-Ag@PDA NPs after incubating in H_2O, phosphate-buffered saline (PBS, 0.1M, pH=7.4), medium with and without 10% serum. The experimental detail is, after incubating with 200 μg/mL Au-Ag@PDA NPs for 3 days, the four solutions are centrifuged and the supernatant are taken for ICP-AES characterization. In addition, the Au and Ag elements from deionized water are also measured as a control group. As shown in Table S1, the content of Au and Ag elements from the Au-Ag@PDA NPs in four kinds of solution is almost same as that in deionized water. This result demonstrates there is no leakage of Au and Ag elements from the Au-Ag@PDA NPs in four kinds of solutions.

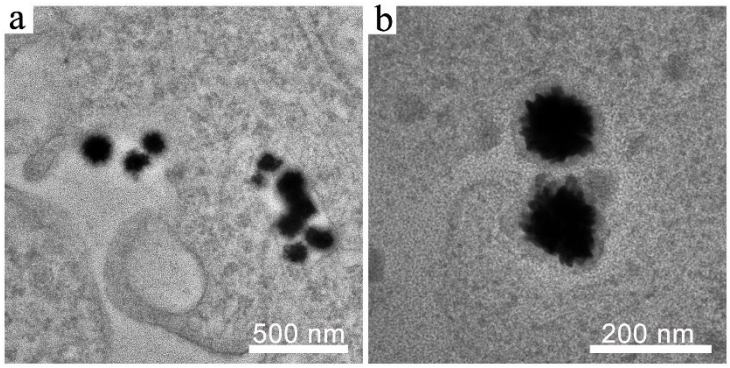

Figure S9. TEM images of the endocytosis process of Au-Ag@PDA NPs in TPC-1 cells. The uptake moment of macropinocytosis (a) and caveolae-mediated endocytosis (b).

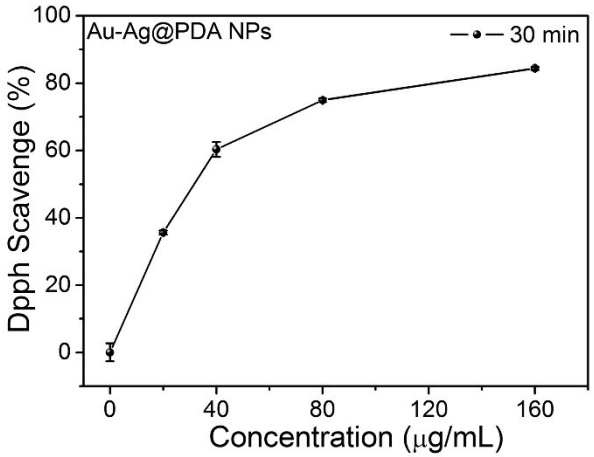

Figure S10. DPPH scavenging effects within 30 min as a function of the concentration of Au-Ag@PDA NPs. The error bars indicate the standard errors of three independent experiments.

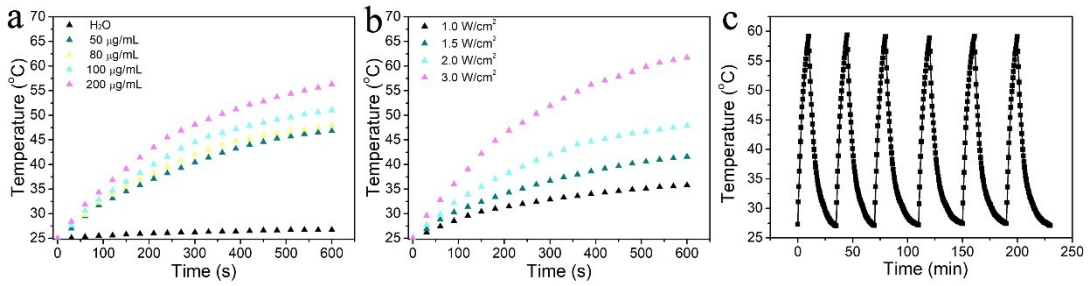

Figure S11. The dependence of temperature increment on the concentration of Au-Ag@PDA NPs (a) and power density of 808 nm laser (b), in which the power density and concentration is fixed at 2.0 W/cm^2 and 80 μg/mL, respectively. The irradiation time is 10 min. (c) The temperature variation of 80 μg/mL Au-Ag@PDA NPs suspension that is irradiated by 3.0 W/cm^2 808 nm laser for 6 cycles with 10 min irradiation each time.

The photothermal performance of Au-Ag@PDA NPs is studied. As shown in Figure S9a, the temperature of 50 μg/mL Au-Ag@PDA NPs solution increases from 25.0 to 46.8 ºC after 2.0 W/cm^2 808 nm laser irradiation for 10 min. Further increasing the concentration of Au-Ag@PDA NPs, this increment becomes more obvious, which is attributed to the collective heating effect of Au-Ag@PDA NPs solution with high concentration.[3] The photothermal property of Au-Ag@PDA NPs also depends on the laser power density. High power density leads to obvious temperature increment, because more energy is absorbed by the solution (Figure S9b). The photothermal stability of Au-Ag@PDA NPs is test by repeatedly irradiating the Au-Ag@PDA NPs solution for 10 min. As shown in Figure S9c, the temperature increament is nearly unchanged after 4 cycles irradiation. This result indicates the good photothermal stability of the Au-Ag@PDA NPs.

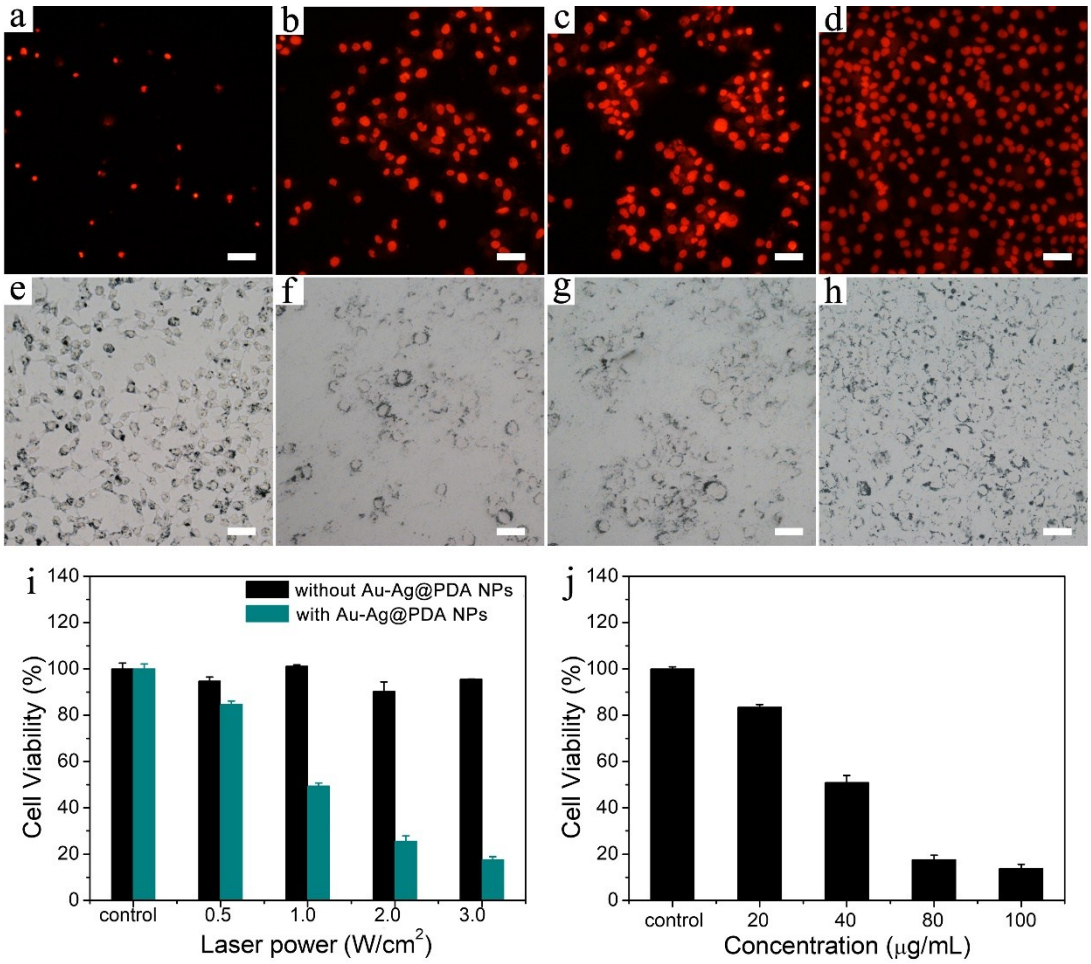

Figure S12. Fluorescent (a-d) and bright field (e-h) images of TPC-1 cells incubated with 80 μg/mL Au-Ag@PDA NPs after irradiated by an 808 nm laser with the power density of 0.5 (a, e), 1.0 (b, f), 2.0 (c, g), and 3.0 (d, h) W/cm^2. The dead cells are stained by PI with red color. The scale bar is 50 μm. (i) The cell viability of TPC-1 cells incubated with 80 μg/mL Au-Ag@PDA NPs after 808 nm laser irradiation with different power density for 10 min. (j) The cell viability of TPC-1 cells incubated with different concentration Au-Ag@PDA NPs after 2.0 W/cm^2 808 nm laser irradiation for 10 min.

The photothermal effect of Au-Ag@PDA NPs *in vitro* is directly monitored using fluorescence microscope. As shown in Figure S10a-h, the dead cells are stained by PI which exhibit red emission. With the increase of laser power indensity, the cell death rate increases. The *in vitro* photothermal therapy effect is also studied by comparing the cell viability of TPC-1 cells under 808 nm laser irradiation in the presence and absence of Au-Ag@PDA NPs. In the presence of Au-Ag@PDA NPs, the cell viability after 10 min irradiation decreases dramatically as increases the laser power

density from 0.5 to 3.0 W/cm^2 (Figure S10i). When the laser power density reaches 3.0 W/cm^2, only 17.6 % of TPC-1 cells survives. As above mentioned, the photothermal performance of the Au-Ag@PDA NPs is concentration dependent. With increasing the concentration of Au-Ag@PDA NPs, the cell viability ratio decreases significantly, which is 13.8 % at the concentration of 100 μg/ml (Figure S10j). Tvhis result shows the non-invasive and enhanced thermal ablation of TPC-1 cells can be achieved by photothermal therapy.

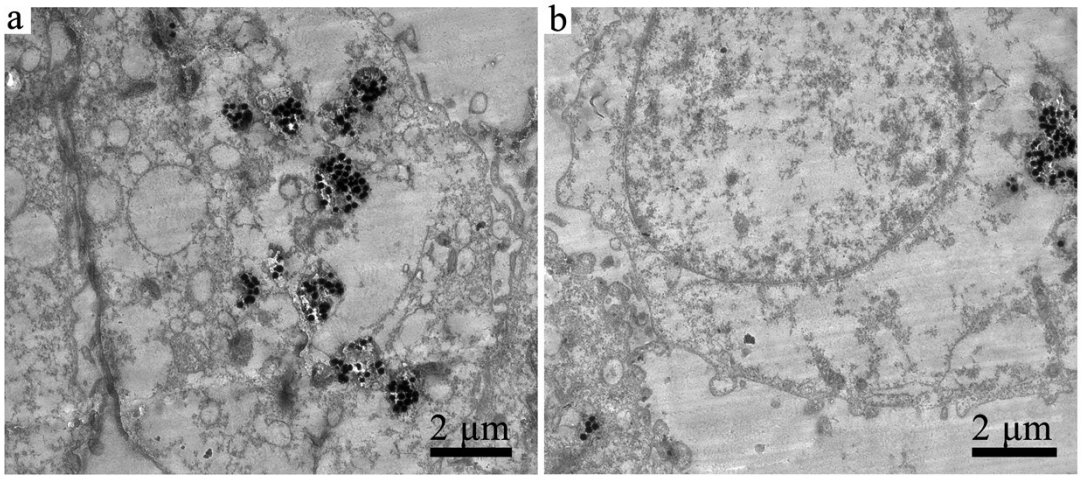

Figure S13. TEM images (a, b) of TPC-1 cells incubated with 80 μg/ml Au-Ag@PDA NPs after 0.8 W/cm^2 808 nm laser irradiation for 10 min. The cells are incubated with 80 μg/mL Au-Ag@PDA NPs for 24 h and then irradiated by 0.8 w/cm2 808 nm laser. As shown in Figure S13a-b, the cells become swollen, and the cell membrane and organelle membrane dissolve and rupture.

REFERENCE

1. Bastús, N. G.; Merkoçi, F.; Piella, J.; Puntes, V. Synthesis of Highly Monodisperse Citrate-Stabilized Silver Nanoparticles of up to 200 nm: Kinetic Control and Catalytic Properties. *Chem. Mater.* **2014**, *26*, 2836−2846.

2. Sanna, V.; Pala, N.; Dessì, G.; Manconi, P.; Mariani, A.; Dedola, S.; Rassu, M.; Crosio, C.; Iaccarino, C.; Sechi, M. Single-Step Green Synthesis and Characterization of Gold-Conjugated Polyphenol Nanoparticles with Antioxidant and Biological Activities. *Int. J. Nanomed.* **2014**, *9*, 4935−4951.

3. Jiang, R.; Cheng, S.; Shao, L.; Ruan, Q.; Wang, J. Mass-Based Photothermal Comparison Among Gold Nanocrystals, PbS Nanocrystals, Organic Dyes, and Carbon Black. *J. Phys. Chem. C* **2013**, *117*, 8909−8915.