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Supporting Information for

Stimulus-responsive surface-enhanced Raman scattering: a "Trojan horse" strategy for precision molecular diagnosis of cancer

Cai Zhang,[a] Xiaoyu Cui,[a] Jie Yang,[a] Xueguang Shao,[a] Yuying Zhang,[b] Dingbin Liu [a]*

[a]College of Chemistry, Research Center for Analytical Sciences, State Key Laboratory of Medicinal Chemical Biology, and Tianjin Key Laboratory of Molecular Recognition and Biosensing, Nankai University, Tianjin 300071, China

[b]School of Medicine, Nankai University, Tianjin 300071, China

*Corresponding author: Dingbin Liu (email: liudb@nankai.edu.cn).

Chemicals

All chemicals used in this work were at least of analytical grade. Ultrapure water purchased from Hangzhou Wahaha Group Co. Ltd was used throughout this work. Potassium permanganate ($KMnO_4$) was purchased from Sinopharm Chemical Reagent Beijing, Co., Ltd. Chloroauric acid ($HAuCl_4$), trisodium citrate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl), N-hydroxysuccinimide purum (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), HEPES, acetic acid and sodium acetate were obtained from Sigma-Aldrich. Amino polyethylene glycol (PEG-NH_2, molecular weight 1000), poly (allylamine hydrochloride) (PAH), polyacrylic acid (PAA) were obtained from J&K Scientific.

Synthesis of AM NPs

Gold nanoparticles were prepared following the classic sodium citrate reduction method. Briefly, 0.25 mM $HAuCl_4$ solution (295.5 mL) was boiling and refluxed for 5 min, then 4.5 mL of sodium citrate solution (0.5% w/v) was rapidly added and the mixture was kept boiling for 15 min to yield AuNPs. In order to fabricate AM NPs, 100 mL of the as-prepared Au NPs were put into a round-bottomed flask, and then 0.5 mL of $KMnO_4$ solution (22 mg/mL) was dropwise added into the solution under vigorous stirring. 5 min later, 1 mL PAH solution (25 mg/mL) was introduced by dripping slowly. The AM NPs were obtained when the color of the mixture turned from purple to brown. The reaction time was fixed for approximately 1 h. Finally, the fabricated AM NPs were purified by centrifugation at 10000 rpm for 10 min, and the final products were stored at 4^{o}C for further use.

For *in vivo* applications, the AM NPs were modified with PEG through a layer-by-layer coating strategy. In brief, 75 mL of the as-prepared AM NPs were concentrated to 25 mL and mixed with 40 mg PAA under magnetic stirring. Then, 6 mg EDC and 8 mg NHS were added to the mixture, followed by adjusting the pH to 8. After stirring for 6 h, the free PAA was removed by centrifugation. Next, 30 mg NH_2-PEG was dissolved in 1 mL water and then dropwise added to the above solution under magnetic stirring. After the addition of 6 mg EDC and 8 mg NHS into the mixture, the pH of the solution was adjusted to 8. Subsequently, the reaction solution was ultrasonicated for 1 h and then stirring for 5 h. Excess PEG was removed by centrifugation. The final products were stored at 4 °C for further use.

Characterizations of the AM NPs

The as-prepared AM NPs were characterized by X-ray photoelectron spectroscopy (XPS) analysis which was performed on an Axis Ultra DLD spectrometer fitted with a monochromated Al Kα X-ray source (hν = 1486.6 eV), hybrid (magnetic/electrostatic) optics, a multichannel plate and a delay line detector (Kratos Analytical, Manchester, UK). The (Hitachi, HT7700) field emission transmission electron microscopy (TEM) was used to study the morphology and structure of AM NPs. A dilute solution of AM NPs was deposited into a 230-mesh Cu grid and dried by water dispersion to prepare the samples for HAADF-STEM imaging and EDX elemental mapping (Philips Tecnai G2 F20). Fourier transform infrared (FT-IR) spectra were detected on a Nicolet IR AVATAR-360 spectrometer (Nicolet, USA) with pure KBr as the background. UV-vis absorption spectra were monitored by a UV-vis spectrophotometer (Shimadzu, Japan). The DLS and zeta-potential measurements were performed on a Zetasizer Nano ZS90 instrument (Malvern, UK). The hydrodynamic diameter and zeta-potential values were obtained directly based on its own software with special mathematical model and correlation function. A scattering angle of 90 degrees is used to measure particle size and molecule size; Laser Doppler Micro-electrophoresis is used to measure zeta potential. An electric field is applied to a solution of molecules or a dispersion of particles, which will then move with a velocity related to their zeta potential. This velocity is measured using a patented laser interferometric technique called M3-PALS (Phase analysis Light Scattering), which enables the calculation of electrophoretic mobility for the zeta potential measurement of a wide range of sample types and dispersion media.

Acidic pH/H_2O_2-dependent AM NPs decomposition

We utilized the color change and corresponding UV-vis absorption spectra of AM NP solutions to characterize the decomposition of MnO_2 shells. The color of the AM NPs (0.02 nM) was recorded before and after treating with acidic pH/H_2O_2 solution (pH 6.5, H_2O_2: 100 μM) for 30 min. As a contrast study, the same set of AM NPs was mixed with acidic buffer solution (pH 6.5), neutral PBS solution (pH 7.4), or neutral H_2O_2 solution (pH 7.4, H_2O_2: 100 μM), and then the corresponding UV-vis absorption spectra were recorded under the same conditions. The AM NPs were incubated in different media, and their absorbance at 380 nm was measured at different time points. Furthermore, TEM of the AM NPs treated with the acidic pH/H_2O_2 solution, pure acidic solution or neutral pH/H_2O_2 solution were also collected to validate the decomposition of the MnO_2 shells.

Acidic pH/H_2O_2-activated turn-on MRI of AM NPs

MR imaging of AM NPs (with different Mn^{2+} concentrations) was conducted after incubating in acidic buffer solutions (pH 6.5) or neutral buffer solutions in the presence or absence of H_2O_2 (0.5 mM) for 30 min. The signal intensity values of the samples were also measured by a 3.0-T clinical MRI scanner. Relaxation rates r_1 (1/ T_1) were calculated from T_1 values at different Mn^{2+} concentrations as determined by ICP-OES.

Acidic pH/H_2O_2-activated Raman enhancement of AM NPs

To evaluate the isolation effect of MnO_2 shells to block the interactions between the Raman dyes and the Au NP core, the Raman spectrum of the mixture of R6G and AM NPs was detected. Briefly, 0.2 μM of R6G was mixed with 0.02 nM of AM NPs and vortexed for 30 min. Then, the Raman spectrum of the mixture was recorded by a confocal Raman spectrometer using a 633 nm (3 mW) laser excitation with an acquirement time of 10 s. As a control, the Raman spectrum of the mixture containing the same concentration of R6G (0.2 μM) and the same colloidal concentration of Au NPs (0.02 nM) was collected under the same procedures and data acquisition conditions. Raman spectrum of 2 mM R6G solution was also recorded under the same testing conditions. To assess the Raman enhancement ability of the AM NPs after decomposition of the MnO_2 shells, Raman spectra of various biomolecules (protein, glucose, nucleic acid, NADH, ATP, and amino acid) were collected after mixing with AM NPs in different media. In detail, AM NPs were first treated with a neutral buffer solution (pH 7.4) or acidic pH/H_2O_2 solution (pH 6.5, H_2O_2: 100 μM) for 30 min. Then, the mixtures were concentrated by centrifugation (6000 rpm, 2 min). Afterward, the concentrated AM NPs were mixed with biomolecules at diverse concentrations, and the Raman spectra of the samples were collected on a confocal Raman spectrometer using 633 nm (3 mW) laser excitation with acquirement time of 10 s.

Animal models

All animal studies were performed in compliance with the institutional guidelines made by the Tianjin Committee of Use and Care of Laboratory Animals and the overall regulations approved by the Animal Ethics Committee of Nankai University. Female Balb/c mice (20–22 g) bought from Beijing HFK Bioscience Co. Ltd were used for building tumor models. In brief, a mouse was injected subcutaneously in the inguinal region with 4T1 cells (2×10^6) that were suspended in 60 μL of PBS. The tumor models at different growth periods were applied for further *in vivo* and *ex vivo* experiments (the early growth group was less than 4 days and the late growth group was more than 14 days after transplantation of tumor cells).

In vivo and ex vivo experiments

100 μL of PEGylated AM NPs (1 mg/mL) was injected into the tumor models intratumorally or subcutaneously to study the sensitivity and specificity of the PEGylated AM NPs in tumor tissue. We paid particular attention to the SR-SERS effect of the naked Au NPs decomposed from PEGylated AM NPs in the tumor microenvironment. MR imaging of the tumor models was also obtained by a 3.0-T clinical MRI scanner. Meanwhile, the Raman signals of the tumors and normal tissues were detected by a confocal Raman spectrometer using 633 nm (10 mW) laser excitation, and the data acquirement time was fixed to be 3 s.

To test the activated MRI and SERS signals after intravenous injection of the probes, 200 μL of PEGylated AM NPs solution was intravenously injected into the tumor model mice. MR imaging was implemented by a 3.0-T clinical MRI scanner at different time points. Next, the mice were sacrificed and the tumor plus surrounding normal tissues were cut out at 4 h post-injection when the T_1 MR intensity of tumor reached the maximum values. Then, the whole tissues were sliced and fixed on glass slides. The Raman spectra and mapping images of the tissue slices were recorded by a confocal Raman microscope (n = 7). The Raman mapping was performed using a 633 nm excitation source, 3 mW laser power, 50 × objective lens, and an acquisition time of 8 s. All the Raman maps were generated and analyzed through WiRE 4.2 software (Renishaw).

For further studying the activated MR whitening ability and SERS capability of the degradable PEGylated AM NPs in the tumor tissues, the tumor mice bearing tumors at different growth periods were used for MR imaging and SERS detection (n = 7). The difference of the biomolecules distributed in the tumor tissues was evaluated by SERS spectra. In detail, each tumor was sliced into several layers, from which the Raman spectra of three slices near the middle of the tumor were collected. For each slice, we measured 20 different single spectra randomly.

For investigating the classification of the samples, PCA was applied using the SERS spectra. To further verify the accuracy of the Raman results, untargeted metabolomics profiling analysis of tumor tissues at different growth periods was performed on XploreMET platform (Metabo-Profile, Shanghai, China) using gas chromatography–time-of-flight mass spectrum (GC-TOF/ MS) (6 repeats per set). The sample preparation procedures are referred to in previously published methods.[1]

Biodistribution of PEGylated AM NPs in vivo

200 μL of PEGylated AM NPs (1 mg/mL) was injected intravenously into tumor mice (n = 7) and the tumor mice without any treatment were set as the control. Then the mice were sacrificed at different time points after injection (4 h, 1 d and 7 days). Subsequently, the major organs (heart, liver, spleen, kidney, and brain) and tumors of the mice were harvested and weighed. The tissue samples were digested in aqua regia for 7 days and the samples were mediated to an appropriate volume and acid concentration by water. Finally, the samples were analyzed by inductively coupled plasma optical emission spectrometer (ICP-OES) (SpectroBlue, Germany).

Assessment of in vivo toxicity

To study the biocompatibility of PEGylated AM NPs *in vivo*, weight changes of Kunming mice after intravenously injected with PEGylated AM NPs or PBS were monitored for 30 days. Besides, the mice intravenously injected with PEGylated AM NPs or PBS were sacrificed on the 1st and 7th days. Next, the major organs (heart, liver, spleen, lung, kidney, brain and thymus) of the mice were collected for H&E-staining. The biochemical analysis of blood was also performed. The liver function markers include serum albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (T-Bil), direct bilirubin (D-Bil) and gamma-glutamyl transferase (GGT), while the kidney function markers include blood urea nitrogen (BUN), creatinine (CR) and uric acid (UA). All the markers were measured on the 1st and 7th days after injection of the PEGylated AM NPs. The mice without any treatment were set as control (n = 7).

To study the effect of the AM NPs on the immune system, the immune cells in the blood including lymphocyte, monocyte and neutrophil granulocyte were collected for cell counting after injection of the PEGylated AM NPs to the mice for 4 h and 1 day (n = 7). Besides, the cytotoxic effect of the PEGylated AM NPs on peripheral blood mononuclear cells was performed by fluorescent-activated cell sorting (FACS). In detail, the peripheral blood mononuclear cells were isolated from the blood, after exposure to the PEGylated AM NPs for 4 h and 1 day. Then the cells were incubated with propidium Iodide (PI) and Annexin V-FITC in the dark. The cell necrosis and apoptosis were analyzed by flow cytometry (BD FACSVerse, USA).

To further investigate the neurotoxicity of the PEGylated AM NPs, the brain tissues of the mice were collected after injection with the PEGylated AM NPs for 1 and 7 days. The mice without any treatment were set as the control. These brain tissues were fixed in 4%

paraformaldehyde and then sliced. Apoptotic cells in brain sections were identified by the TUNEL (TdT-mediated dUTP nick end labeling) staining kit (Roche). In detail, the sections were treated with Terminal Deoxynucleotidyl Transferase and then incubated with FITC-12-dUTP at 37 °C for 3 h. After that, the sections were washed with PBS three times and then stained with DAPI for 10 min at room temperature to label the nucleus. Finally, the sections were viewed and photographed under a fluorescence microscope.

References

1. Y. Ni, Y. Qiu, W. Jiang, K. Suttlemyre, M. Su, W. Zhang, W. Jia and X. Du, *Anal. Chem.*, 2012, **84**, 6619-6629.

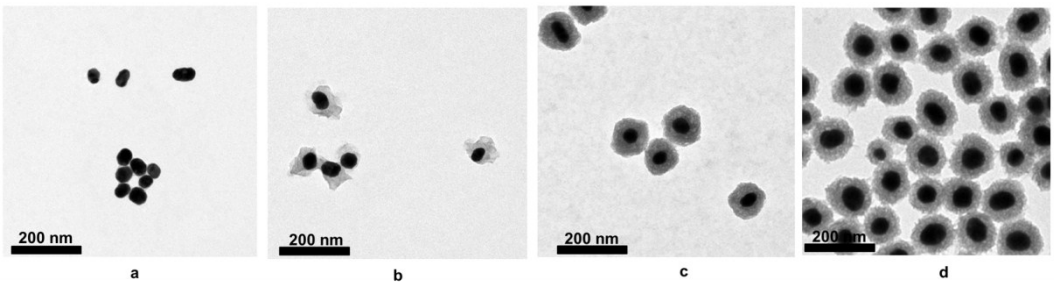


Fig. S1. TEM of AM NPs with different shell thickness prepared from different concentrations of $KMnO_4$ and PAH. 100 mL AuNPs (0.02 nM) were prepared as the core, and the thickness of the MnO_2 shell increased with the addition of the $KMnO_4$ and PAH. **(a)** 2.2 mg KMnO4 and 5 mg PAH. **(b)** 4.4 mg KMnO4 and 10 mg PAH; **(c)** 11 mg KMnO4 and 25 mg PAH; **(d)** 22 mg KMnO4 and 50 mg PAH solution; At low concentrations of the $KMnO_4$ and PAH, the MnO_2 shell appears to be ultra-thin or comprised of patchy protrusions around the surface of the Au NP cores. When increased the amounts of the $KMnO_4$ and PAH to be 11 mg and 25 mg respectively, the MnO_2 shell evolves into a relatively well-defined core-shell nanostructure.

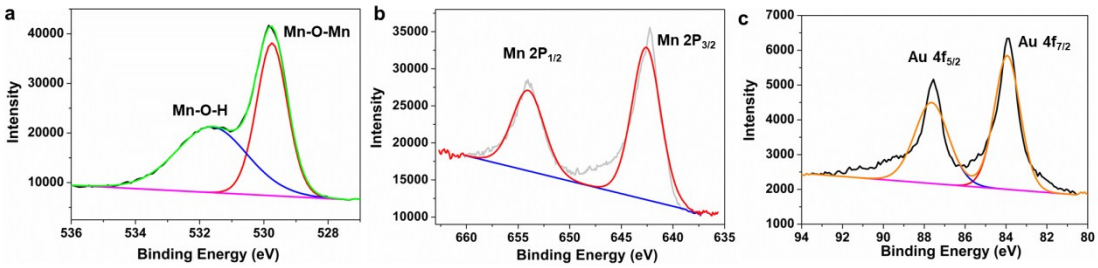


Fig. S2. XPS survey of the O 1s, Mn 2p, Au 4f spectra for AM NPs.

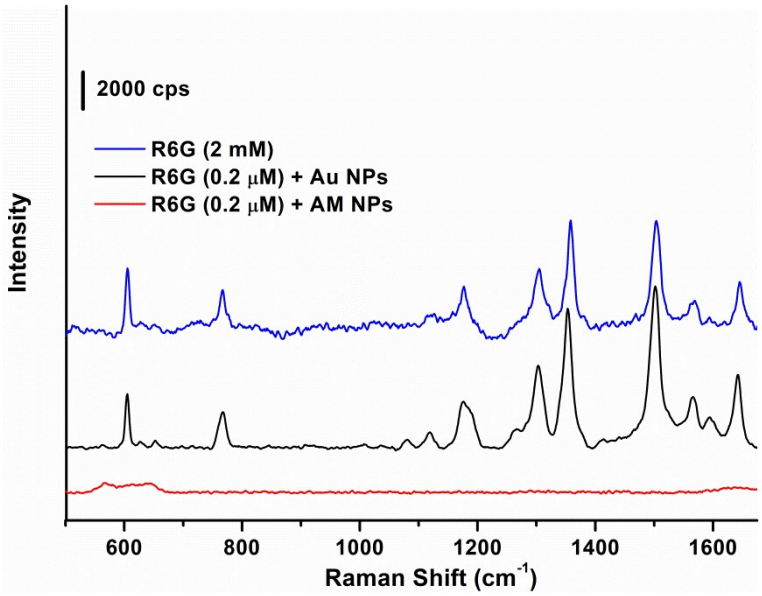
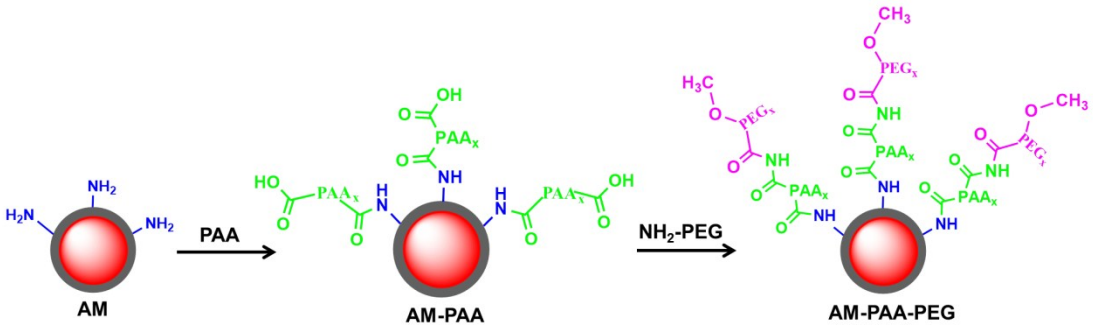


Fig. S3. Raman spectra of pure rhodamine 6G (R6G, 2 mM), a mixture of Au NPs (55 nm in diameter, 0.02 nM) with R6G (0.2 μM), and a mixture of AM NPs (0.02 nM) with the same concentration (0.2 μM) of R6G. Laser power: 3 mW (633 nm); Data acquirement time is 10 s.



Scheme S1. Schematic illustration of synthetic procedure of the PEGylated AM NP via a layer-by-layer strategy.

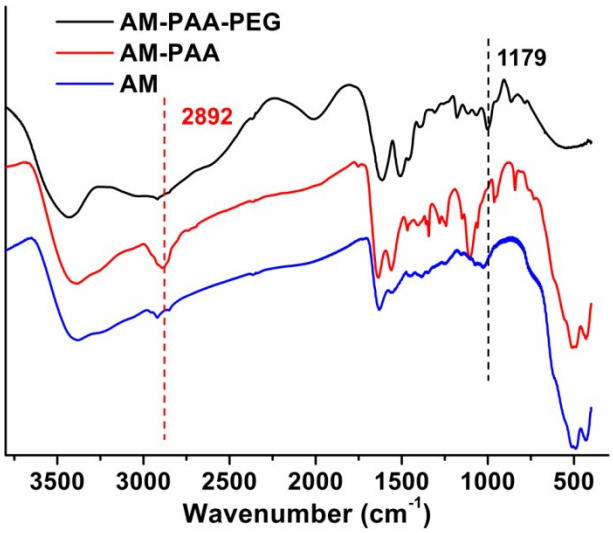


Fig. S4. FT-IR spectra of AM, AM-PAA and AM-PAA-PEG NPs. The red dashed line at 2892 cm^{-1} corresponds to the O-H bond of PAA, and the black dashed line at 1179 cm^{-1} is assigned to the ether bonds (C-O-C) of PEG.

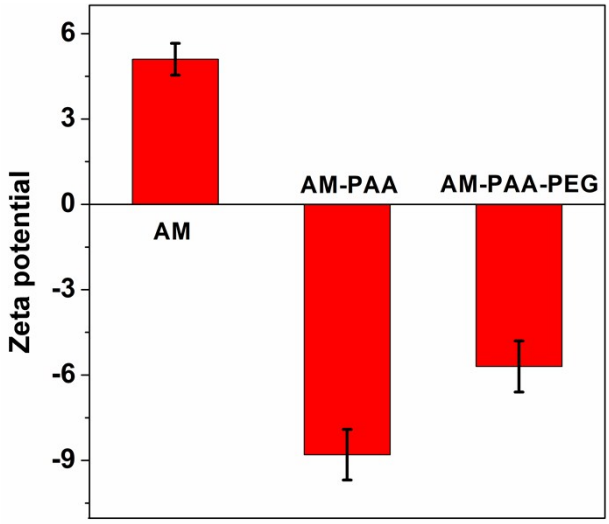


Fig. S5. Zeta potential of AM, AM-PAA and AM-PAA-PEG NPs. Error bars represent the standard deviations between three parallel experiments.

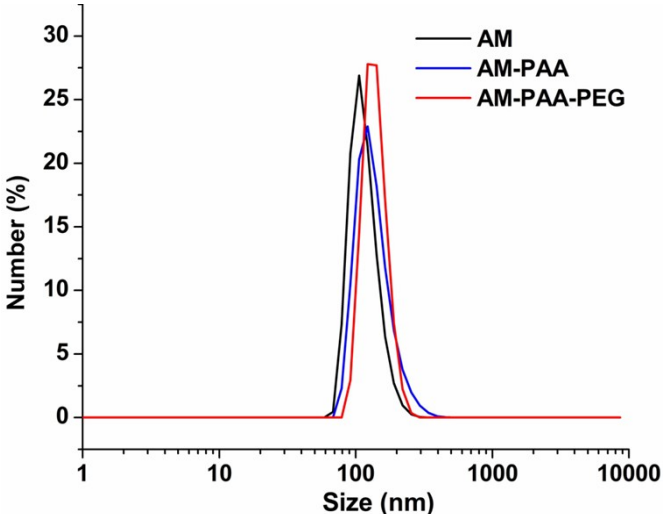


Fig. S6. DLS analysis of AM, AM-PAA, and AM-PAA-PEG NPs.

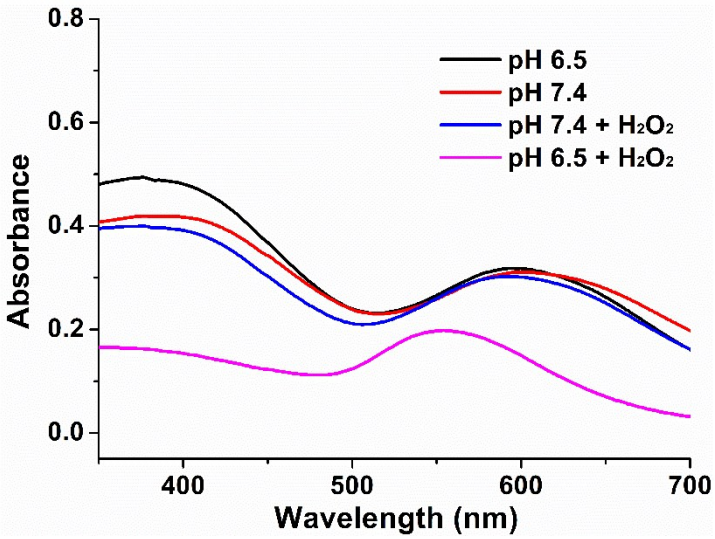


Fig. S7. UV-vis spectra of AM NPs treated with neutral, acidic, neutral H_2O_2 or acidic H_2O_2 solution for 30 min.

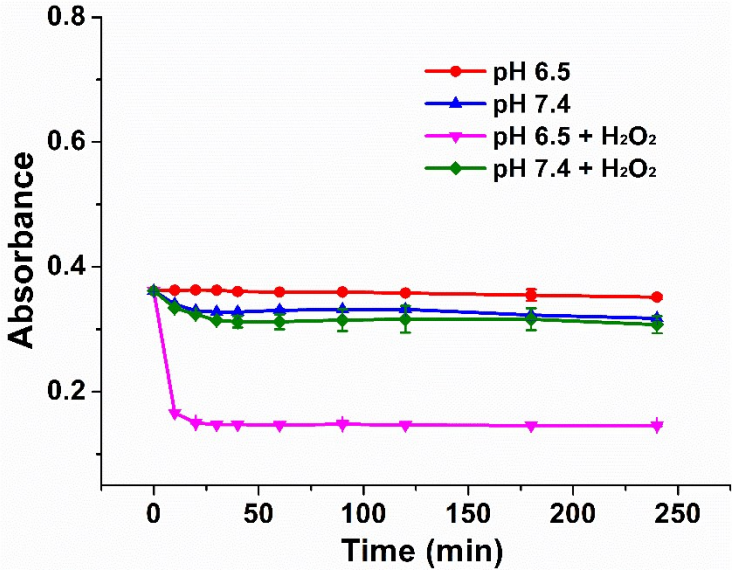


Fig. S8. Dynamic changes of UV-vis absorption of the AM NPs at 380 nm in different conditions. Error bars represent the standard deviations between three parallel experiments.

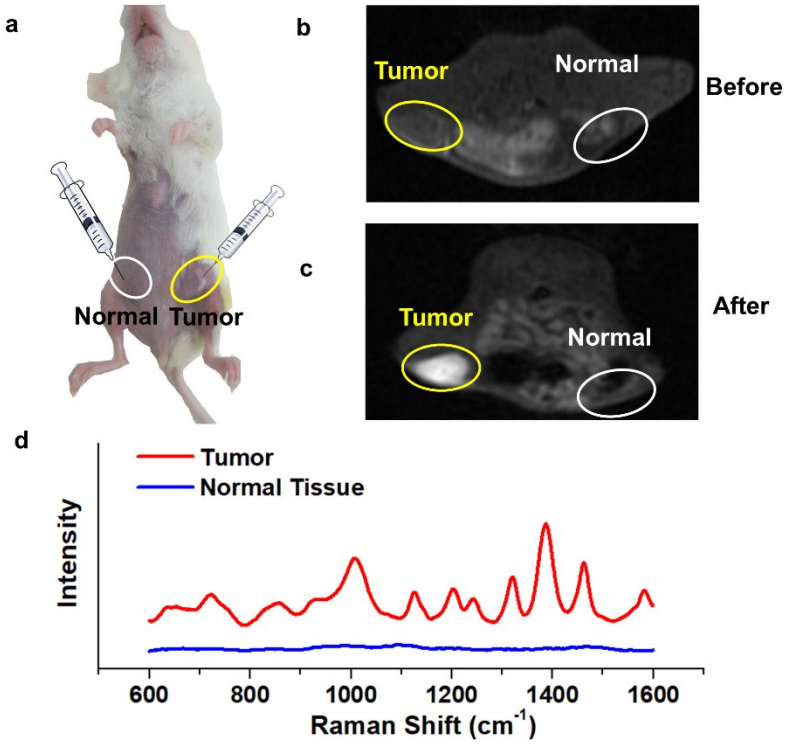


Fig. S9. *In vivo* MR imaging and Raman spectra of tumors and normal subcutaneous tissues after local injection of PEGylated AM NPs. (**a)** Schematic illustration of a tumor-bearing mouse injected with the PEGylated AM NPs. MR imaging of the same mouse before (**b**) and after (**c**) injecting the same amount of PEGylated AM NPs (100 µL, 1 mg/mL) into the tumor and normal subcutaneous tissue, respectively. (**d)** Raman spectra of the probe-injected tumor and normal subcutaneous tissues (n=7) using 633 nm (10 mW) laser excitation (acquirement time 3s). The mean spectra for the tumor and normal signals are displayed in red and blue, respectively.

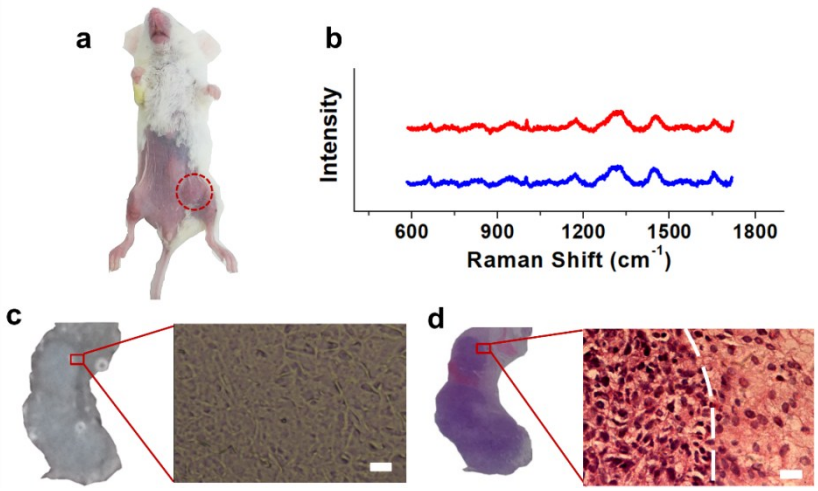


Fig. S10. Raman spectra and mapping image of the tumor-bearing mouse without probe treatment. (**a**) Photo and (**b**) Raman spectra of a tumor-bearing mouse without probe treatment using 633 nm (3 mW) laser excitation (acquirement time 8 s). The mean spectra for the tumor and normal signals are displayed in red and blue, respectively. (**c)** Raman mapping image of the tissue slice based on the same channels in **Fig. 3c** and (**d**) corresponding H&E-staining image. Scale bars: 10 μm.

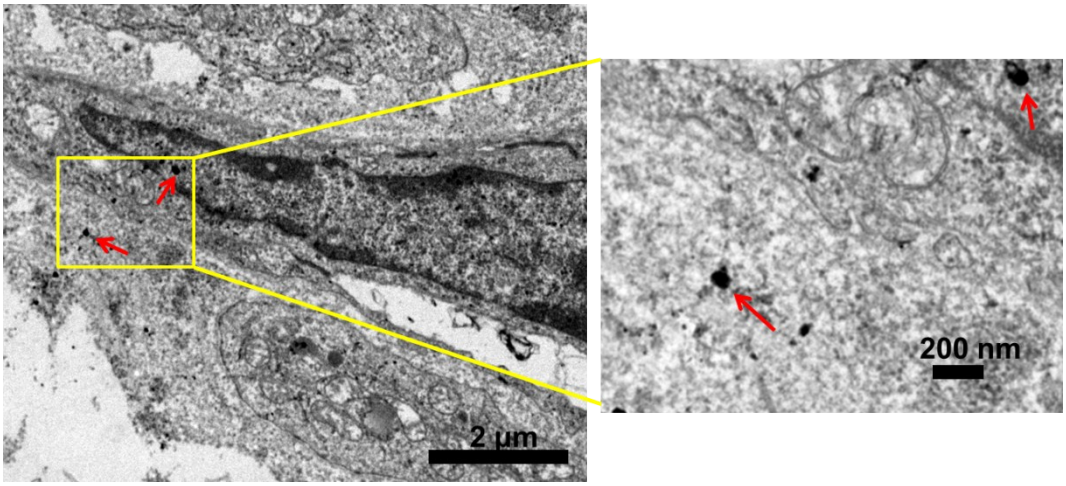


Fig. S11. TEM images of tumor tissue sections that were derived from the tumor mice intravenously injected with PEGylated AM NPs (200 μL, 1 mg/mL). The insets show the enlargement of the Au NPs transformed from AM NPs

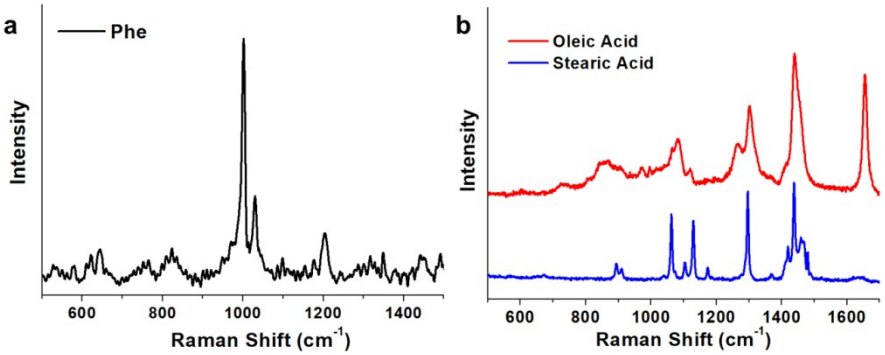


Fig. S12. SERS spectra of phenylalanine (a) and fatty acids (b).

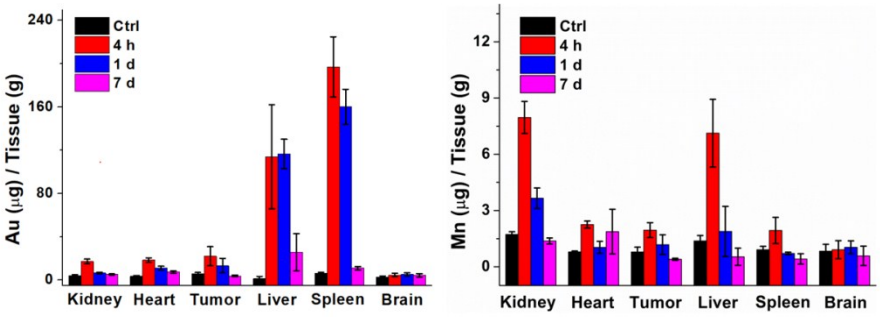


Fig. S13. Biodistribution of the Au and Mn elements in the major organs and tumors of the tumor-bearing mice injected intravenously with PEGylated AM NPs (200 μL, 1 mg/mL) for 4 h, 1 d, and 7 d and that without any treatment. The Au and Mn amounts were determined by ICP-OES. Error bars represent the standard deviations between seven parallel mice.

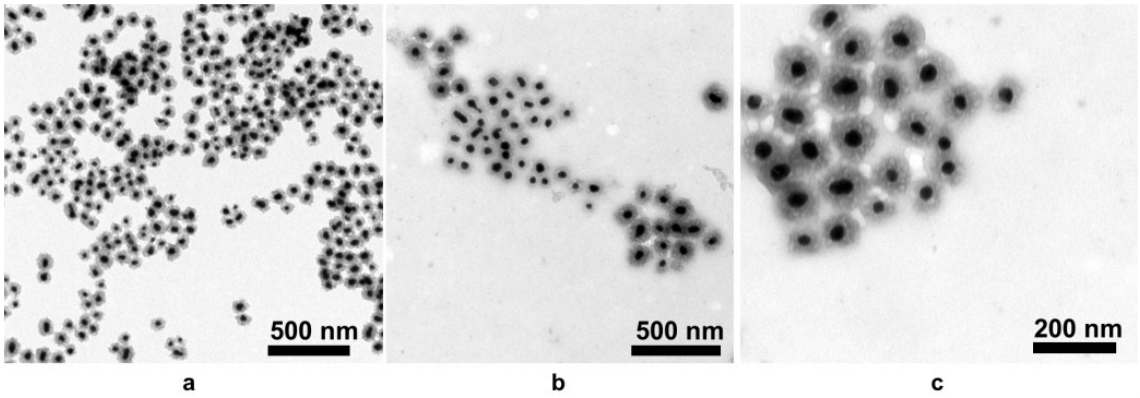


Fig. S14. TEM images of PEGylated AM NPs before (**a**) and after incubated with mice serum for 6 h (**b**, **c**).

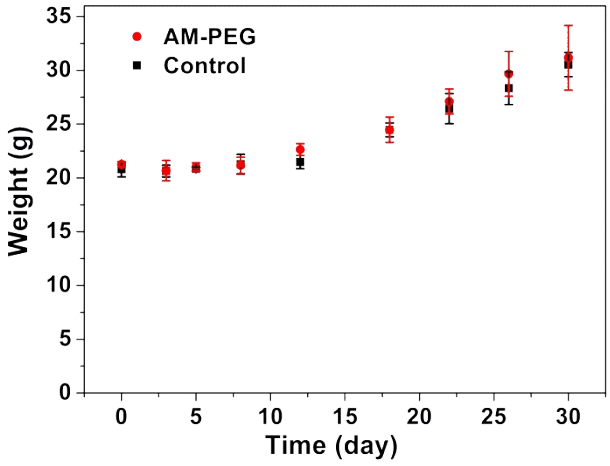


Fig. S15. Body weights of the Kunming mice treated with or without PEGylated AM NPs (200 μL, 1 mg/mL) for 30 days. Error bars represent the standard deviations between three parallel mice.

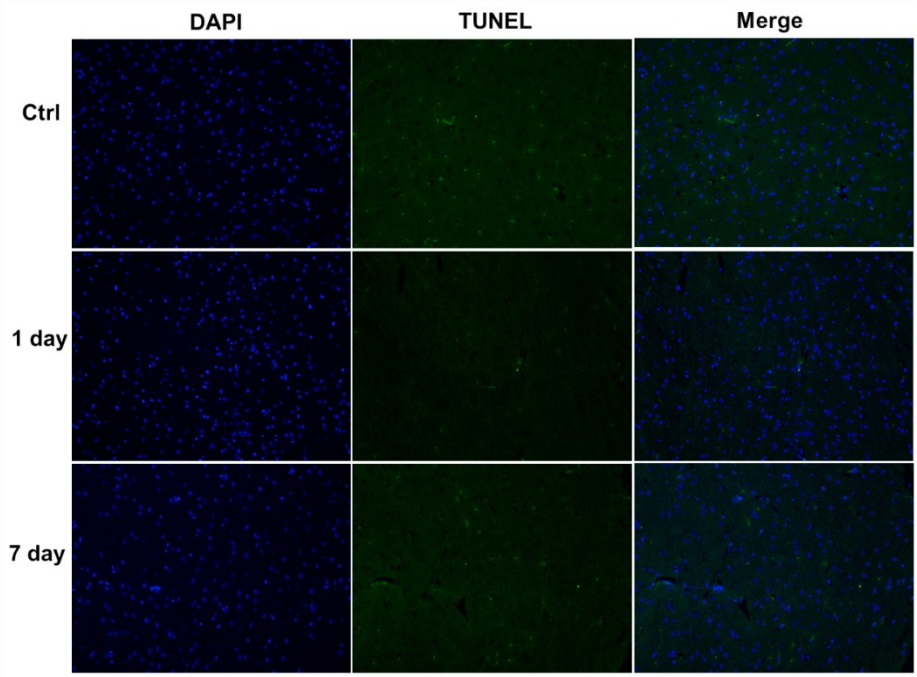


Fig. S16. TUNEL (TdT-mediated dUTP Nick-End Labeling) staining of the brain tissues of the mice after intravenous injection of the PEGylated AM NPs (200 μL, 1 mg/mL) for 1 day and 7 days and compared with the control without any treatment. Blue and green represent nucleus and apoptotic cells respectively.