Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supporting information

Near-infrared emitting Gold-silver Nanoclusters with Large Stokes Shift for Two-photon In Vivo Imaging

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EXPERIMENTAL SECTION

Reagent and materials

All chemicals were the type of analytical grade and were used without further purification. Tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$), Sodium hydroxide, 6-aza-2 thiothymine was purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Silver nitrate (99.9%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Rhodamine B was bought from Solarbio Science & Technology Co., Ltd. (Beijing, China). Deionized water (18.2 MΩ cm^{-1}) was used during all experiment steps.

Characterization

TEM was conducted by Talos F200X G2. Fluorescence excitation and emission spectra were recorded by SF2000 (Shimadzu, Japan). UV–vis spectra was recorded by UV-1800 spectrophotometer (Shimadzu, Japan), DLS (dynamic light scattering) measurements were conducted by nanoparticle size analyzer (OMNI, USA), and X-ray photoelectron spectroscopy (XPS) spectra were obtained by using AXIS Ultra DLD (Shimadzu, Japan). Fluorescence Lifetime was detected by Edinburgh Instruments FLS1000 transient optical lifetime detector. Inductively Coupled Plasma Mass spectrometry (ICP-MS) was recorded on iCAPQ of Thermo scientific.

One pot synthesis and purification of ATT-Au/AgNCs

Briefly, $AgNO_3$ solution (0.44 mL, 10 mM) was mixed with $HAuCl_4$ solution (0.56 mL, 10 mM) under gentle stirring at room temperature. After 2 min, ATT (0.4 mL, 80 mM) containing 200 mM NaOH was added into the mixture and stirred for 5 hours at 60 ℃. The crude products were filtered with a 220 nm-filter membrane to remove the large-sized AgCl sediments. Then the final resultant was obtained by ultrafiltration centrifugation (MWCO 10 KDa) and stored at

4 ℃ in the dark for further characterization and application. The ATT-AuNCs and ATT-AgNCs were synthesized under same conditions expect replacing the precursor with 1 mL 10 mM $HAuCl_4$ and 1 mL 10 mM $AgNO_3$ solution respectively.

Stability test in different media

The colloidal stability was tested in three different conditions: water, phosphate-buffered saline solution, RPMI 1640 medium containing bovine serum albumin. The fluorescence intensity was recorded every twelve hours.

Cell culture

The 4T1 (mouse breast cancer) cell line and 293T cells were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). The 4T1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin at 37 ℃ in 5% CO_2. The 293T cells were maintained in same conditions except replacing RPMI 1640 medium to DMEM medium.

MTT assay

MTT test was conducted to determine the cell viability under different ATT-Au/AgNCs concentrations. 4T1 cells and 293T cells were seeded in 96-well plate (200 μL per well) at a density of 6000 cells per well and cultured overnight. Then the culture media was replaced by 200 μL medium containing different concentrations of ATT-Au/AgNCs (0, 20, 40, 60, 80, 100, 120 μg/mL). After incubation for 24 hours, 10 μL of MTT solution (5 mg/mL) was added to every well and then was incubated for 4 h. Then the mixture was removed and 150 μL DMSO was added. Finally, the absorbance at 490 nm was recorded by a microplate reader.

***In vitro* two-photon fluorescence imaging**

To evaluate the ability of cellular imaging, 4T1 cells were incubated with 100 μg/mL ATT-Au/AgNCs for 1 h. Later, the cells were washed three times by PBS buffer and then fixed by 4% paraformaldehyde to avoid unnecessary changes in cell morphology. Next, the treated cells were stained by Hoechst 33258 for 30 min and then washed three times again. Finally, the two-photon fluorescence intensity was detected by Leica TCS SP8 with the two-photon IR Laser Chameleon Ultra Ⅱ at an excitation wavelength of 720 nm, and the output power is about 40 mW.

***In vivo* two-photon fluorescence imaging**

According to the NIH guidelines and relevant laws, all animal experiments were performed and authorized by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. The Balb/c Nude mice (4-5 weeks old) were purchased from Shanghai Jiao Tong university laboratory animal center. For *in vivo* two-photon imaging, the Balb/c nude mouse was anesthetized by intraperitoneal injecting 100 μL 1% pentobarbital sodium solution first and put it on a homemade plate. Then the mice were intramuscularly injected with 200 μL ATT-Au/AgNCs in the rear paw. Fluorescence images were taken by a Leica TCS SP8 with the two-photon IR Laser Chameleon Ultra Ⅱ at an excitation wavelength of 720 nm, and the output power is also about 40 mW.

Fluorescence Lifetime measurement

We use the double-exponential fitting function.

$$\tau = A_1 e^{\frac{-t}{\tau_1}} + A_2 e^{\frac{-t}{\tau_2}}$$

	Value / ns	Std. Dev / ns		Value / ns	Std. Dev / ns	Rel %
τ_1	170.38	6.99	A_1	96.81	2.23	13.55%
τ_2	1239.82	21.93	A_2	84.86	1.36	86.45%

Quantum yield

The QY of the nanoclusters was calculated by the following equation.

$$QY_s = QY_f * \frac{F_s}{F_f} * \frac{A_f}{A_s} * \frac{\eta_s^2}{\eta_f^2}$$

In this equation, F are the integrated areas of fluorescence intensity between 500nm and 850nm, A are the absorbance at 360nm, η are the solvent refractive index. The subscript "s" and "f" are "sample" and "reference" respectively. And the η_s is 1.33 (water) and the η_f is 1.36 (ethanol). The standard reference we chose is Rhodamine B dissolved in ethanol, and QY is about 71%.

	F	A	η	QY
Au-Ag/ATT	42029	0.05	1.33	5.4%
Rhodamine B	2557190	0.025	1.36	71%

Two-photon excitation and emission spectra measurement

The two-photon excitation and emission spectra were recorded by a two-photon fluorescence microscope. The method of two-photon excitation and emission spectra is similar with the one-photon. For the TPE emission spectra, firstly we fix the excitation wavelength and select different emission collection bands to collect images, analyzing the change of fluorescence intensity at the same position by graphs to get the strongest fluorescence

wavelength. On the other hand, the TPE excitation spectra is measured conversely. Fixed the emission wavelength and use different excitation light to excite the sample, so as to obtain excitation wavelengths of different intensities. On the set panel of the computer, the emission collection area can be set manually which will include the maximum emission wavelength of the sample and avoid the influence of the reflected light of the excitation light.

Two-photon absorption cross section

The two-photon absorption cross-section (TPACS) of the nanoclusters was calculated by the following equation.

$$\sigma_s = \frac{F_s}{F_f}\left[\frac{\phi_f C_f \eta_f}{\phi_s C_s \eta_s}\right]\sigma_f$$

where F_s and F_f are the two-photon fluorescence signals of ATT-Au/AgNCs and the reference dyes Rhodamine B respectively by using the above two-photon equipment. ϕ_s is the quantum yield of ATT-Au/AgNCs. ϕ_f is the quantum yield of Rhodamine B in ethanol (0.71). C_s is the concentration of ATT-Au/AgNCs (in terms of Au concentration obtained by ICP-MS), C_f is the concentration of Rhodamine B. η_s is the refractive index of the water. η_f is the refractive index of ethanol. σ_2 is the known TPA cross section of the reference dye Rhodamine B.

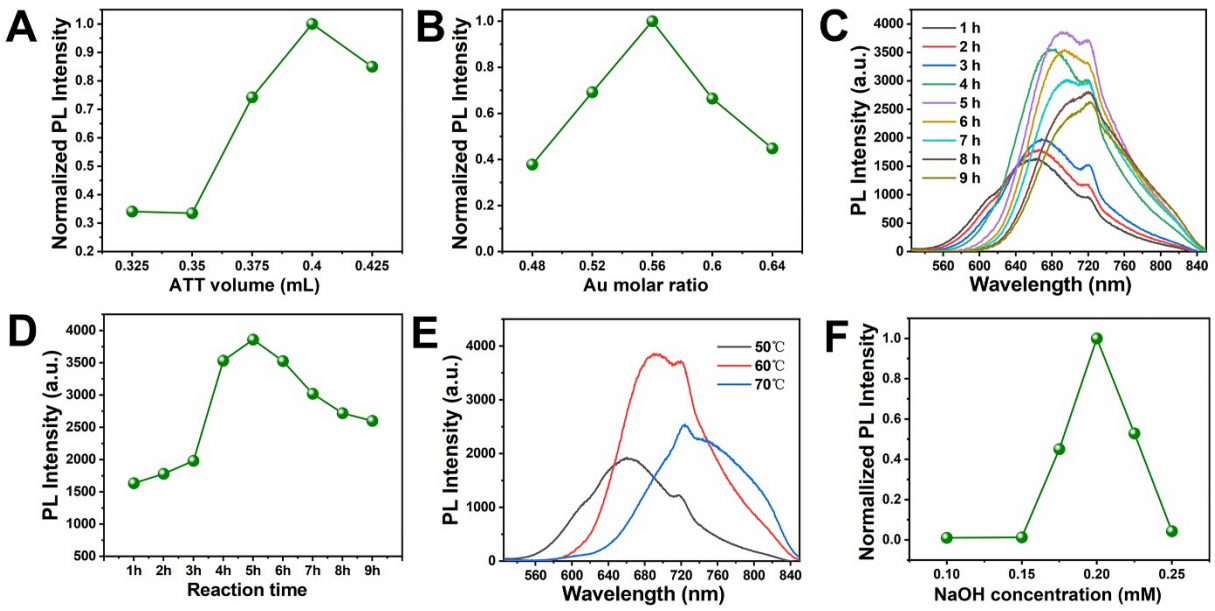

Fig. S1. Influence of the volume of ATT (A), the gold molar ratio (B), the reaction time (C) and (D), the temperature (E), the concentration of NaOH (F).

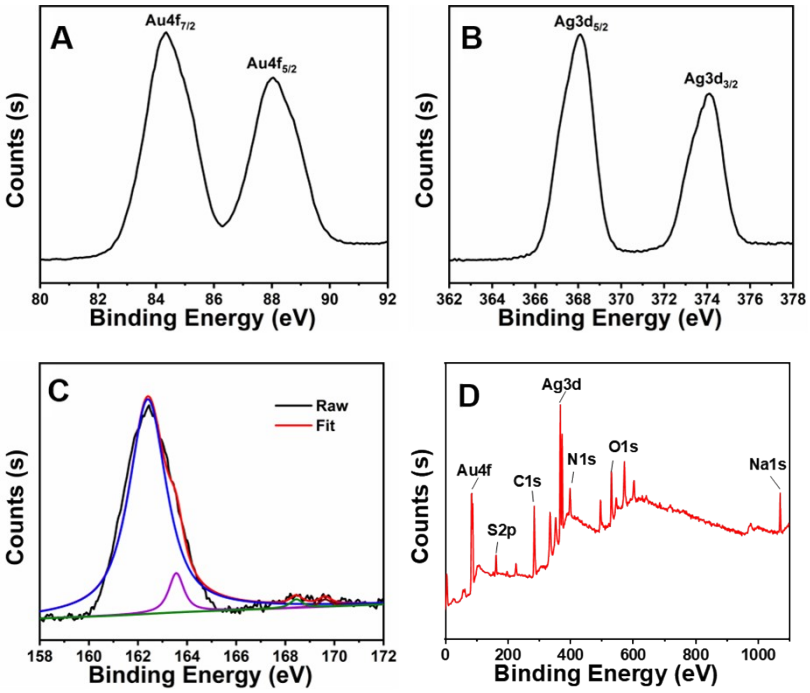

Fig. S2. XPS spectra of (A) Au4f (B) Ag3d and (C) S2p of ATT-Au/AgNCs and wide-scan XPS spectrum (D).

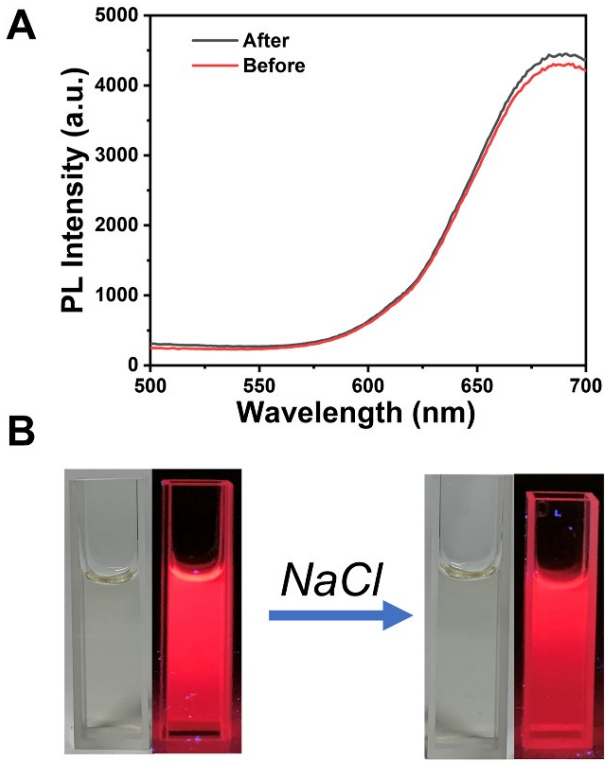

Fig. S3 (A) fluorescence intensity of ATT-Au/AgNCs before and after adding 100 μL 1M NaCl; (B) ATT-Au/AgNCs in room light and UV light before and after adding NaCl.

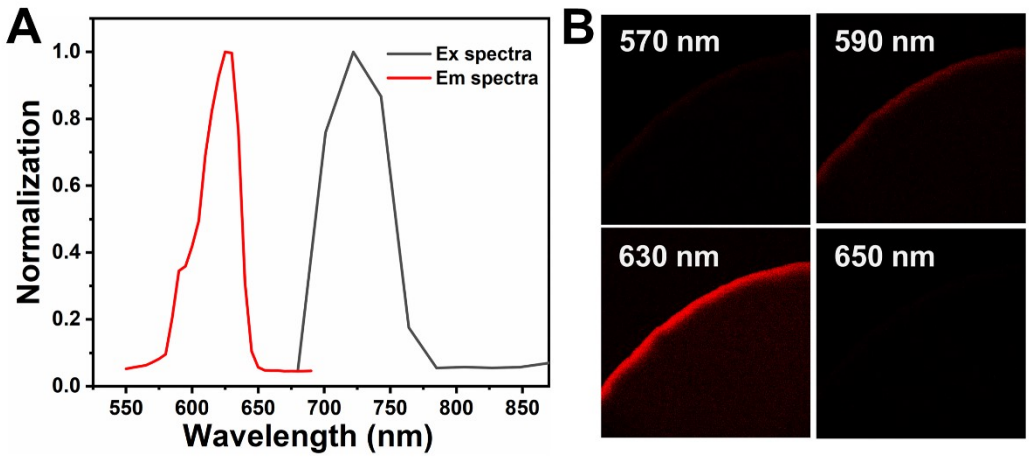

Fig. S4 (A) TPE spectra (red curve) and two photon fluorescence spectra (grey curve, λ_{ex} = 720 nm); (B) Two-photon fluorescence imaging of ATT-Au/AgNCs droplet at different emission wavelength (λ_{ex} = 720 nm).

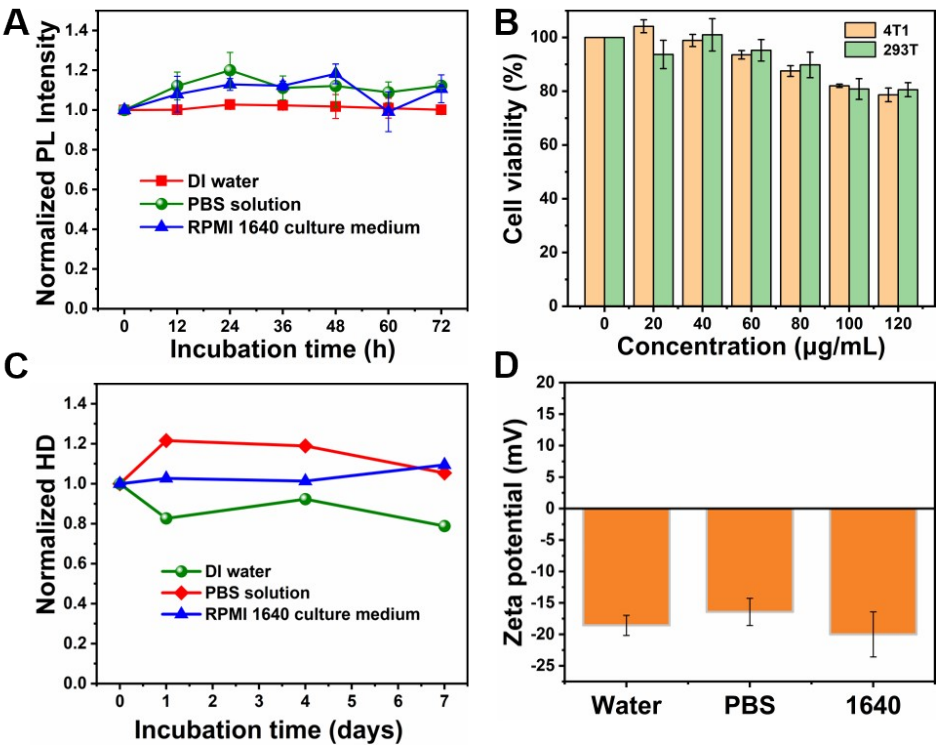

Fig. S5 (A) Colloidal stability of ATT-Au/AgNCs in water, PBS solution and RPMI 1640 culture medium; (B) Cell viability of 4T1 cells and 293T cells after 24 h of incubation with different concentrations of ATT-Au/AgNCs by a MTT assay. (C) The normalized hydrodynamic diameter at different incubation times in water, PBS solution and RPMI 1640 culture medium; (D) The Zeta potential of ATT-Au/AgNCs in water, PBS solution and RPMI 1640 culture medium.

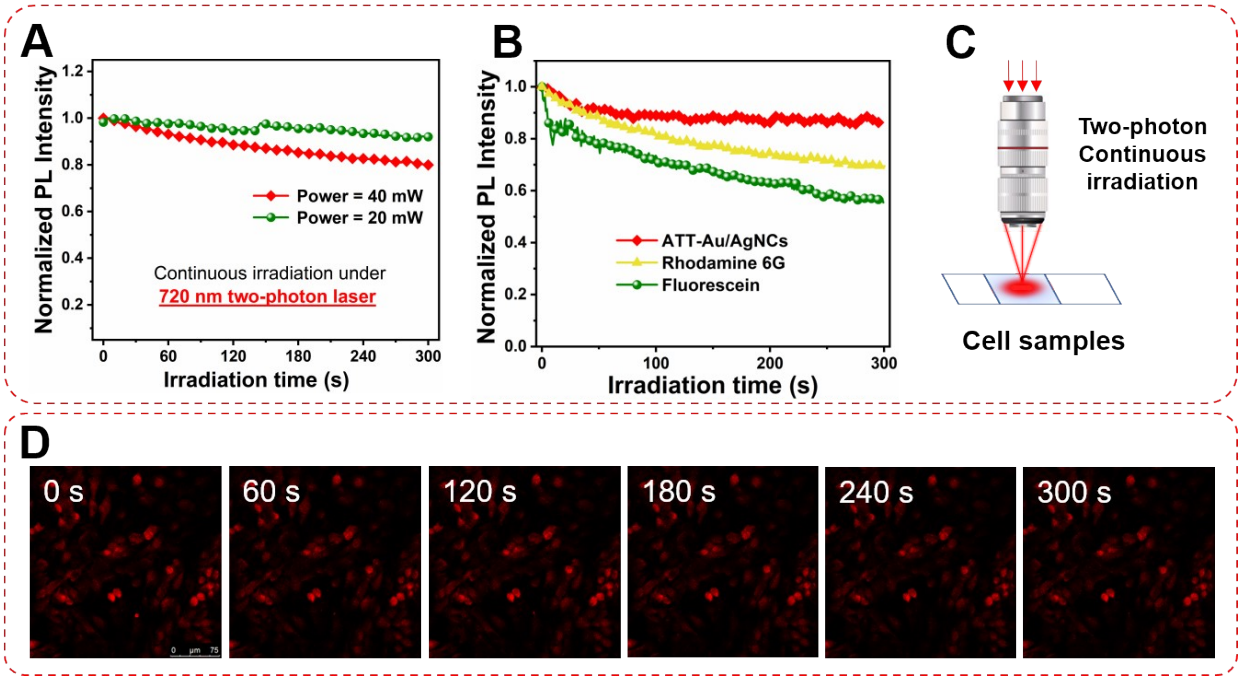

Fig. S6 (A) normalized PL intensity of ATT-Au/AgNCs in 4T1 cells under continuous irradiation of two-photon laser at different output power; (B) normalized PL intensity of ATT-Au/AgNCs, Rhodamine 6G and Fluorescein under continuous irradiation of two-photon laser at power of 20 mW; (C) schematic illustration of two-photon photostability test; (D) Two-photon fluorescent images of 4T1 cells under continuous irradiation of two-photon laser at different time. (Scale bar is 75 μm, output power is about 20 mW, λ_{ex} = 720 nm).