Electronic Supplementary Material (ESI) for Chemical Communications.

Fluorogen-free Aggregation Induced NIR Emission from Gold Nanoparticles

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Supporting Information

Experimental detail

Materials

For the gold nanoparticles, the chemicals gold (III) chloride trihydrate was purchased from Sigma-Aldrich, trisodium citrate dihydrate was purchased from Merck Chemicals, EDTA disodium salt dihydrate was purchased from HIMEDIA and sodium borohydride was purchased from Thomas Baker. All glassware were cleaned with aqua regia (mixture of 1:3 volume ratios of HNO_3 and HCl) and rinsed with millipore water prior to use. TEM images of the biological cells were recorded in Sophisticated Analytical Instrument Facility-DST at AIIMS, New Delhi. Fluorescence imaging experiments were carried out in Advanced Center for Material Science of IIT Kanpur.

Synthesis of Au-1

23.6 mg (0.05 mmol) of Gold (III) chloride trihydrate was dissolved in 200 mL of millipore water and boiled under reflux condenser with constant stirring. Then 20.5 mg (0.07 mmol) of trisodium citrate dihydrate was dissolved in 1.8 mL of millipore water and added to refluxing solution. After the addition, initially the solution was dark violet which quickly changed to red. The reaction was allowed to continue for 30 min. The final gold nanoparticles were cooled to room temperature.

Synthesis of Au-2_{ar}

23.6 mg (0.05 mmol) of Gold (III) chloride trihydrate was dissolved in 200 mL of millipore water and boiled under reflux condenser with constant stirring. Then 20.5 mg (0.07 mmol) of trisodium citrate dihydrate was dissolved in 1.8 mL of millipore water and added to refluxing solution. After the addition, initially the solution was dark violet which quickly changed to red. The reaction was allowed to continue for 30 min. The final gold nanoparticles were cooled to room temperature. Then 20 mL of 100 time diluted aqua regia was added to the solution and stirred for 30 min.

Synthesis of Au-3_{ar}

20 mg (0.05 mmol) of Gold (III) chloride trihydrate was dissolved in 200 mL of Millipore water and boiled under reflux condenser with constant stirring. Then 6.4 mg (0.02 mmol) of trisodium citrate dihydrate was dissolved in 640 µL of millipore water and added to refluxing solution. After the addition, initially the solution was dark violet which quickly changed to red. The reaction was allowed to continue for 30 min. The final gold nanoparticles were cooled to room temperature. Then 200 µL of aqua regia was added to the solution and stirred for 30 min. The red solution changed to blue within 24 h.

Synthesis of Au-4

23.6 mg (0.05 mmol) of Gold (III) chloride trihydrate was dissolved in 200 mL of Millipore water and boiled under reflux condenser with constant stirring. Then 57 mg (0.2 mmol) of trisodium citrate dihydrate was dissolved in 1.8 mL of millipore water and added to refluxing solution. After the addition, initially the solution was dark violet which quickly changed to red. The Reaction was allowed to continue for 30 min. The final gold nanoparticles were cooled to room temperature.

Synthesis of Au-5

23.6 mg (0.05 mmol) Gold (III) chloride trihydrate was dissolved in 250 mL of Millipore water and then 50 µL aliquots of freshly prepared 0.1 M sodium borohydride ($NaBH_4$) were added at room temperature to the slowly mixing solution of gold until a stable red-colored colloid was observed (1250 μL $NaBH_4$), and the reaction was left for 24 h at room temperature.

Synthesis of Au-6

23.6 mg (0.05 mmol) of Gold (III) chloride trihydrate was dissolved in 5 mL of Millipore water and the solution were well mixed into 1 mL of disodium ethylenediaminetetraacetate (Na_2H_2EDTA, 0.025 mmol) at room temperature, followed by an adjustment of the pH of the mixture solution at 5.1 with aqueous solution of NaOH (0.1 M). The volume of the mixture solutions was adjusted to 50 mL with water, followed by 1 min vigorous mixing. The mixture solution remained yellowish during mixing. Subsequently, the resulting mixture was very rapidly poured into 250 mL of boiling Millipore water under vigorous stirring, followed by further 2 h boiling and then cooled down to room temperature.

Transmission Electron Microscopy (TEM)

The TEM images of gold nanoparticles were performed using FEI, Technai G2 20 S-TWIN. ImageJ software was used to analyze the average diameter. Selective Area Diffraction (SAD) images were also captured by the same instrument. Gold nanoparticles were placed in carbon coated copper grids with 200 mesh obtained from Icon Analytical.

Absorption spectroscopy

Absorbance measurement was characterized using UV-Vis spectrometer (UV-1601, Shimadzu) within 400-800 nm wavelength range. The path length was 1 cm for all the measurements.

Calculation of molarity of gold nanoparticle solutions

Absorbance spectrum is used to calculate the concentration of gold nanoparticle solution. First the ratio of A_{spr}/A_{450} is calculated and then concentration is calculated according to the equation available in literature.[S1]

NIR Luminescence spectroscopy

Gold nanoparticle solutions were characterized by luminescence using FLS-980 Edinburgh Instrument. The luminescence measurement of gold nanoparticle solution was taken over 800-1500 nm. Excitation and emission band width of equipment were kept at 15.01 nm.

NIR Absolute quantum yield measurement

Absolute QY measurement was performed using FLS-980 Edinburgh Instrument. For the sample preparation, 15-40% absorption of the light was considered as ideal for the measurements. Excitation and emission band widths of the equipment were kept at 12 and 0.2 nm respectively and the maximum signal level was kept within 1 M cps. All the scans were

repeated thrice by the instrument for comparison. The sample was compared with millipore water as blank within the range 550 nm to 1500 nm.

Fluorescence measurement

Fluorescence of fluorescein was measured using Synergy Micro plate Reader (BIOTEK USA) Instrument, within the range of 470-700 nm with λ_{ex} = 435 nm. Excitation and emission band width of equipment were kept at 16.0 nm.

Relative quantum yield measurement

Fluorescein was dissolved in acetic acid to obtain 10 mM stock solution; this was then diluted to the desired final concentrations by gold nanoparticle solutions. The fluorescence quantum yield of fluorescein was esitmated by addition of diluted aqua regia, using a fluorescence standard, fluorescein in pH 4 (Q = 0.18)[S2] as a reference for comparison.

Absorbance kinetics measurement

The kinetic studies of aggregation process due to aqua regia addition was monitored using Synergy Micro plate Reader (BIOTEK USA) Instrument, at 650 nm with an interval of 2 s. The slope of the initial rate has been plotted against the amount of aqua regia. The fitting of straight line follow R^2 = 0.99.

Fluorescence life time measurement

Fluorescence decay of fluorescein was estimated using TCSPC system from Horiba Jobin Yvon FluoroHub Instrument, with λ_{ex} = 435 nm and λ_{em} = 520 nm. Data analysis was performed with DAS6 software. The decay time data were analyzed using a sum exponential, employing a nonlinear least-squares reconvolution analysis.[S3] Average fluorescence lifetimes were calculated as $\Sigma B_i\tau_i$ with normalized B_i. The radiative and nonradiative decay rate constants are calculated according to the equations available in literature.[S4]

DLS measurement

The hydrodynamic diameters of the gold nanoparticles were measured using a Zetasizer Nano ZS90 (Malvern Instruments). DTS applications 7.03 software was used to analyze the data. All sizes reported here were based on intensity average. For each sample, three DLS measurements were conducted with a fixed 11 runs and each run lasts 10 s.

Field Emission Scanning Electron Microscope (FE-SEM)

The FE-SEM (Carl ZEISS Ultra plus Gemini, Germany) was employed to analyze the morphological aggregation features where all the samples were prepared by drop casting the solution in silicon wafers at 30 kV.

Experimental detail with HepG2 cell

Cell Viability assay

To check the cell viability MTT assay was carried out. 5×10^3 HepG2 cells suspended in 200 μL of medium were plated in 96-well plates (Corning, NY, USA) and grown under normal

conditions. GNP solution at 0.5 nM concentration was added to the monolayer in triplicates for 1, 4 and 12 h. Cultures were then assayed by the addition of 20 μL of 10 mg/mL MTT and incubating it for another 4 h at 37 ºC. The MTT containing medium was then aspirated and 100 μL of DMSO was added to solubilise the water insoluble formazon. The absorbances of the lysates were determined on FLUO star optima microplate reader (BMG Labtech, Germany) at 570 nm.

To elucidate the mode of translocation of NPs in nucleus, ivermectin and importazole were used to inhibit importin α/β- and importin β-mediated nuclear import respectively. Briefly, 5×10^4 HepG2 cells were seeded onto each coverslips in 6 well plate and incubated for 24 h in CO_2 incubator at standard conditions. Thereafter, cells were pre-incubated with or without 25 μM ivermectin or 50 μM importazole for 1 h and further co-administered with 25 μM ivermectin or 50 μM importazole with 0.5 nM of NPs for next 1 hr. After incubation, cells were washed with PBS thrice and fixed with 4% formaldehyde. Thereafter, cells were counterstained with DAPI and slides were prepared for fluorescence microscopy.

Statistical analysis

Data are specified as mean ± standard deviation (SD) of triplicate independent experiments and statistically evaluated using one way ANOVA and followed by Tukey post hoc test using Graph Pad Prism 6 (Graph Pad Software, San Diego, CA, USA). A p-value of less than 0.05 was considered to be statistically significant.

Fluorescence Microscopy

Fluorescence microscopic images of cells were captured using Carl Zeiss LSM780 fluorescence microscope. Images were obtained via a high resolution peltier cooled monochrome camera with F set 20 wide field camera modes and 1.8 times magnification. The rhodamine filter and long pass red filter were used for excitation and emission purpose respectively. TV mode in ZEN software was used to acquire images.

Transmission Electron Microscopy in cells

Cells were treated with gold nanoparticles at 0.5 nM concentration for different time duration i.e. 1, 4 and 12 hours. After respective incubation, treated cells were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (pH 7.4). The cells were then post fixed with 1% OsO_4 in Sorensen phosphate buffer (0.05 M Sorensen phosphate buffer, 0.25 M glucose, 1% OsO_4) for 1 h followed by washing with distilled water twice and further pre-staining with solution of 2% uranyl acetate for 12 h, according to the Standardized protocol developed by ‘Sophisticated Analytical Instrument Facility’ of AIIMS, New Delhi, India. The cells were then finally analyzed by FEI, Technai 200 KV TEM. Images were analyzed by Si-Viewer Olympus soft imaging solution (Olympus, USA).

Supporting Figures

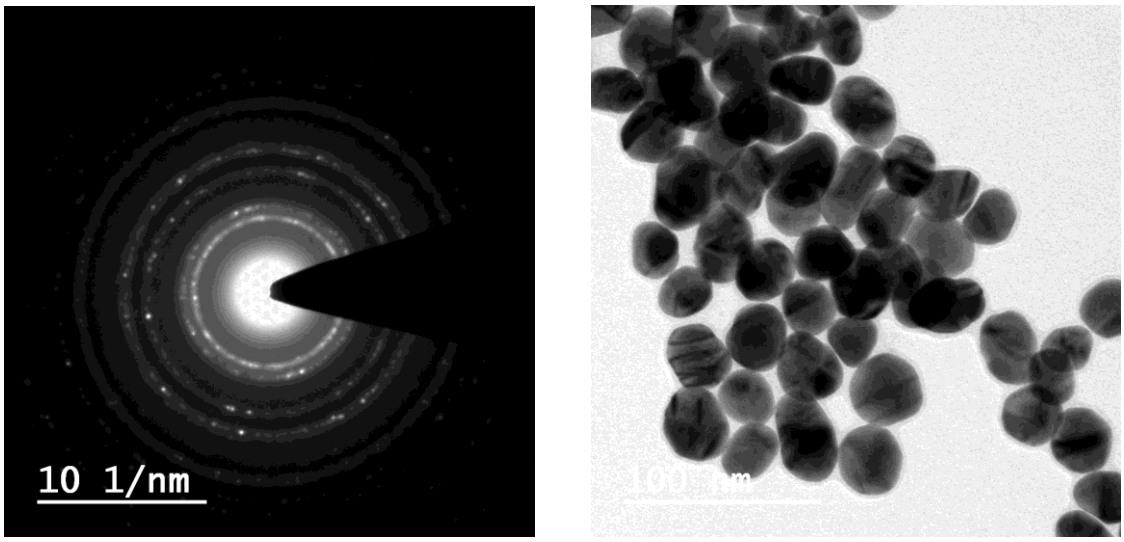

Figure S1. SAD (left) and TEM (right) image of **Au-1.**

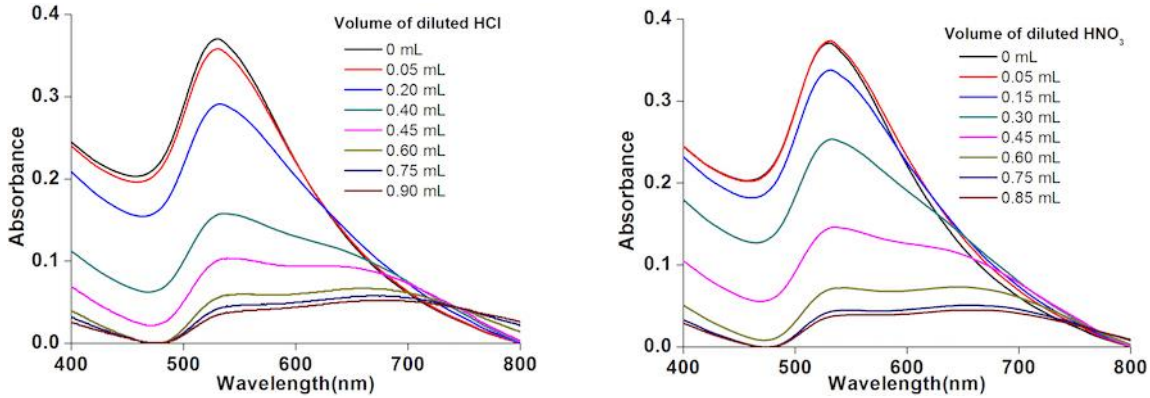

Figure S2. Absorbance spectra of **Au-1** due to addition of diluted hydrochloric acid (left) and diluted nitric acid (right).

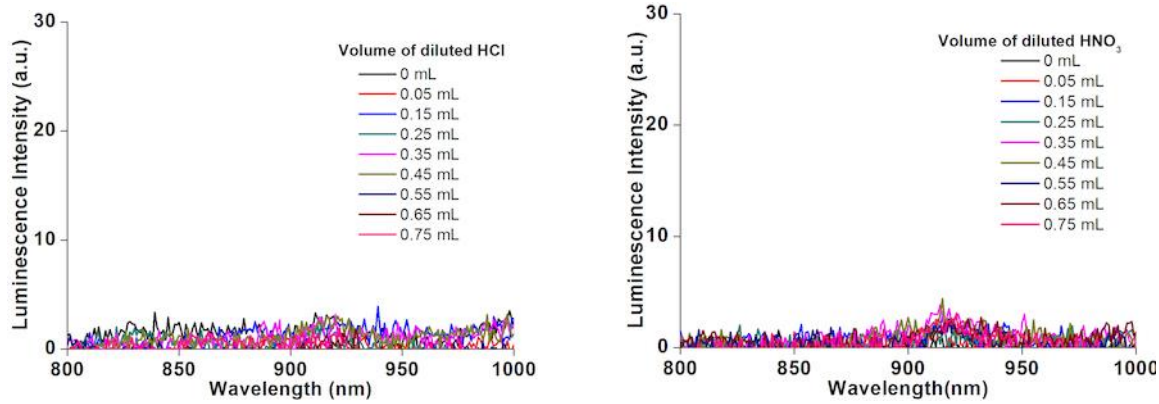

Figure S3. Luminescence spectra of **Au-1** due to addition of 100 time diluted hydrochloric acid (left) and nitric acid (right).

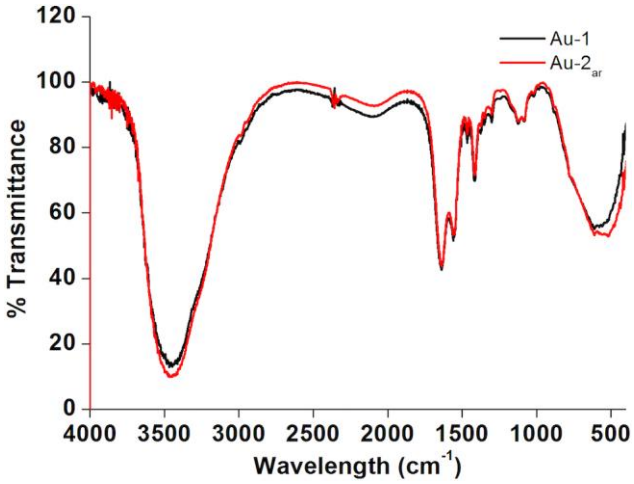

Figure S4. IR spectra of **Au-1** and **Au-2$_{ar}$**.

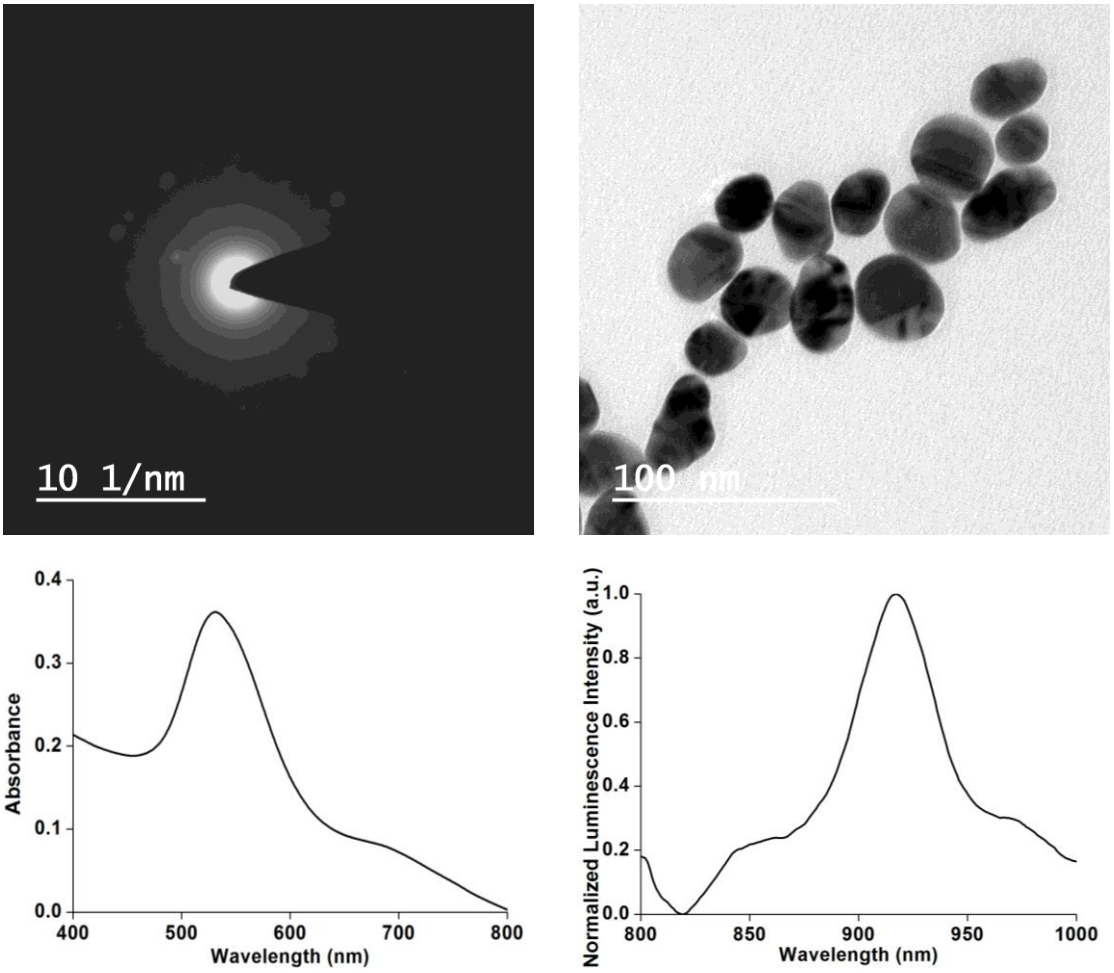

Figure S5. SAD Image (top left), TEM Image (top right), absorbance (bottom left) and luminescence (bottom right) of **Au-2$_{ar}$.**

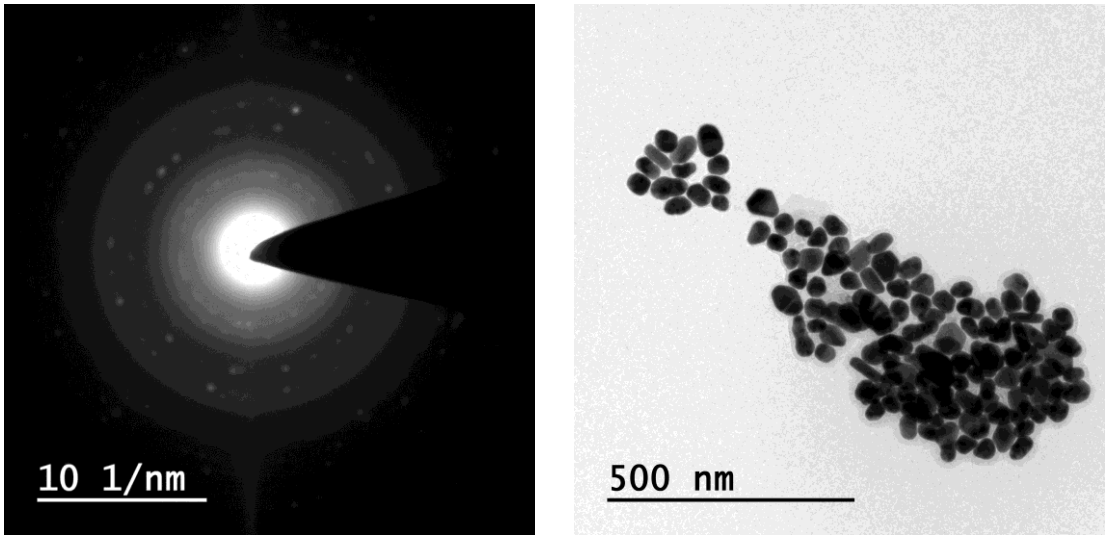

Figure S6. SAD (left) and TEM (right) Images of **Au-3$_{ar}$.**

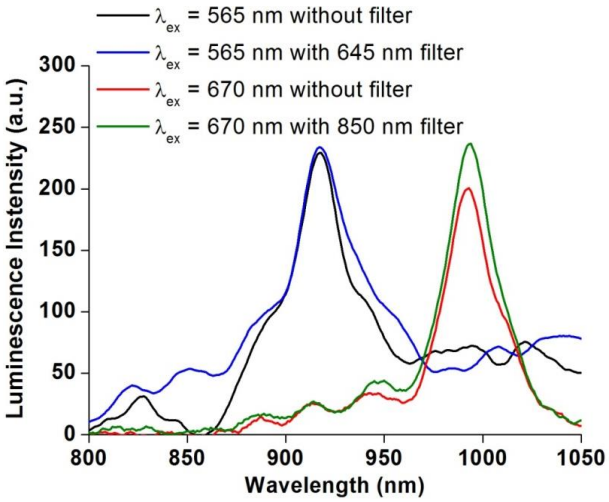

Figure S7. Luminescence of **Au-3$_{ar}$** with and without 645 nm and 850 nm filters.

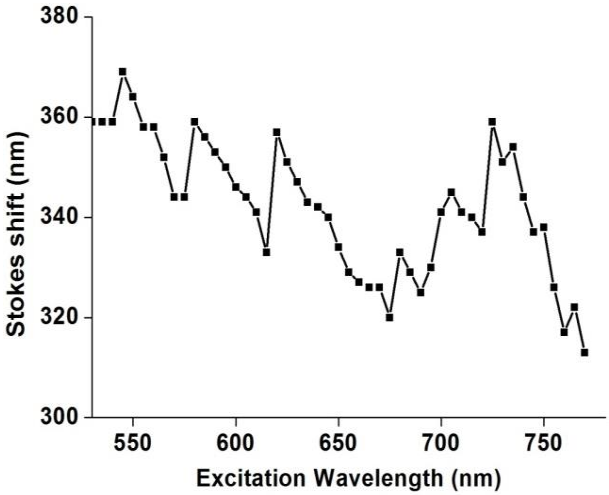

Figure S8. Stokes shift of **Au-3$_{ar}$** for excitation wavelength within 520 nm to 775 nm.

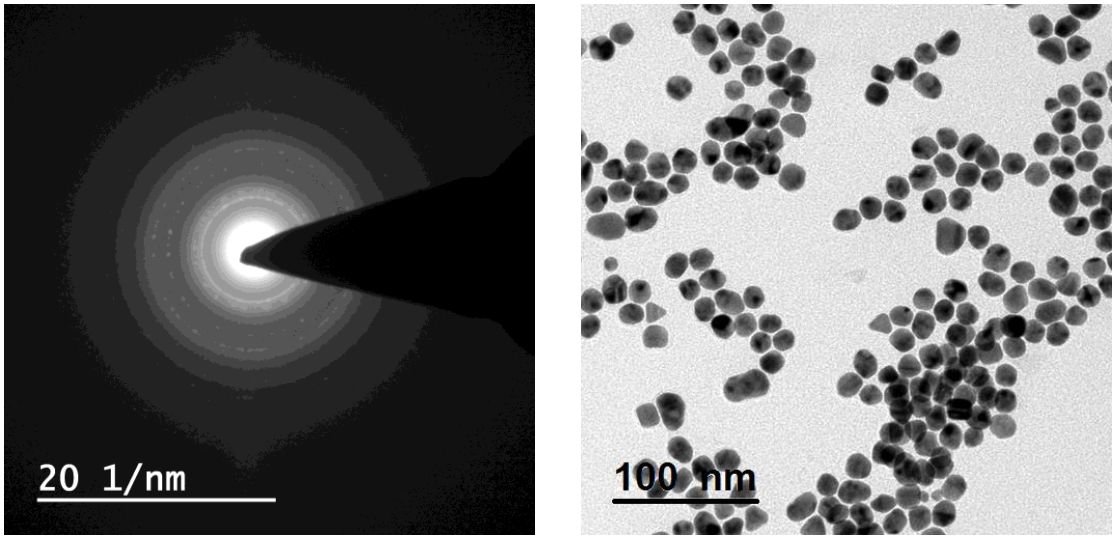

Figure S9. SAD (left) and TEM (right) images of **Au-4**

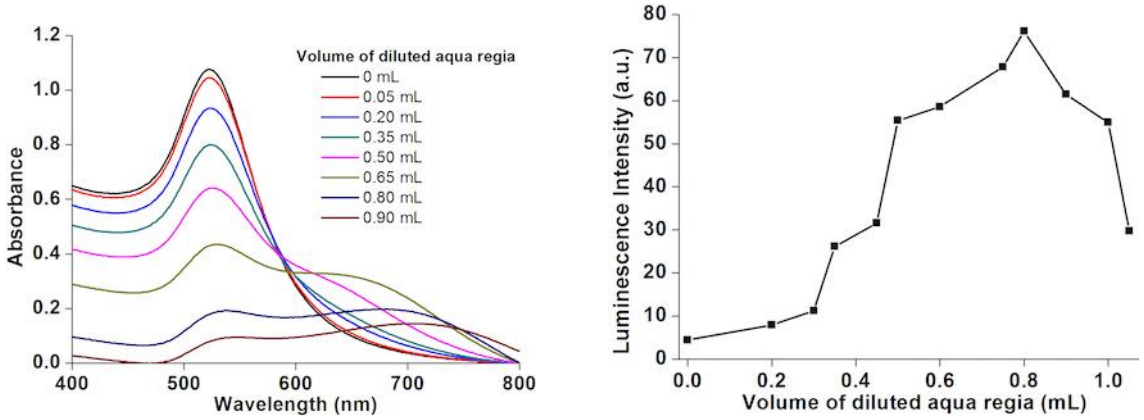

Figure S10. Absorbance (left) and luminescence intensities (right) at 916 nm (λ_{ex} = 560 nm) for **Au-4** due to addition of 100 time diluted aqua regia.

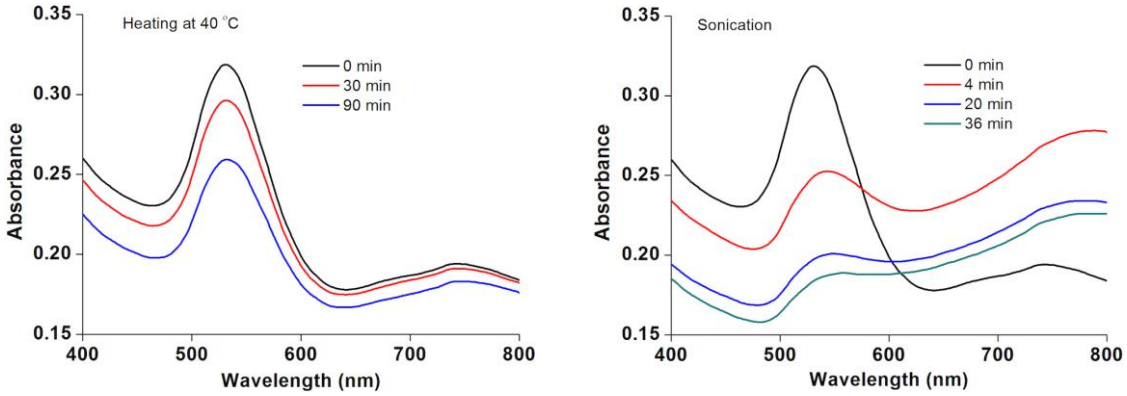

Figure S11. Absorption spectra for aggregated **Au-4** upon heating (left) and sonication (right) after different time interval.

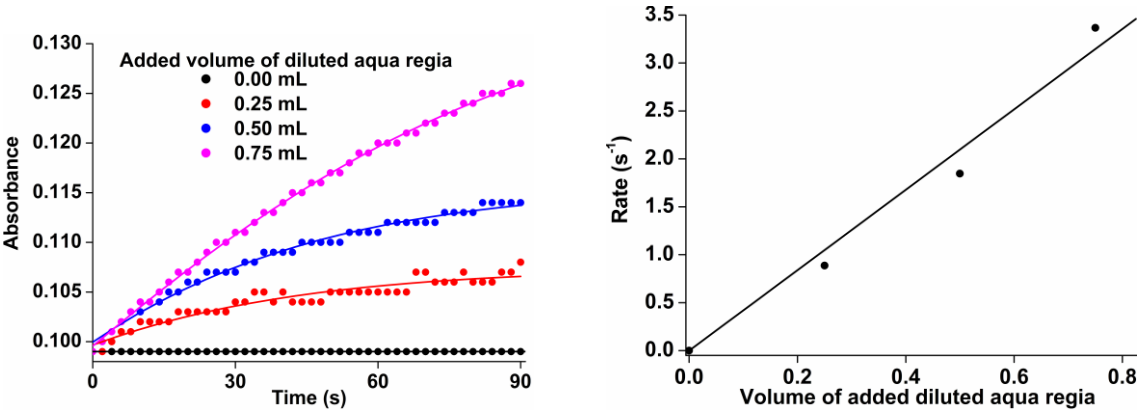

Figure S12. Absorbance kinetics at 650 nm (left) due to aggregation of **Au-4** due to addition of 100 time diluted aqua regia and pseudo first order rate ($R^2 > 0.99$, right figure) with respect to the concentration of aqua regia addition.

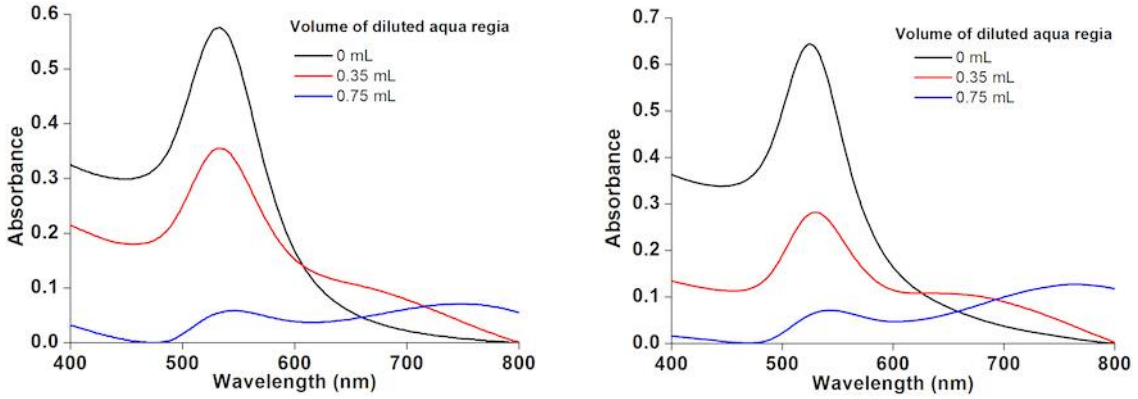

Figure S13. Absorbance of **Au-5** (left) and **Au-6** (right), monitored during addition of 100 time diluted aqua regia.

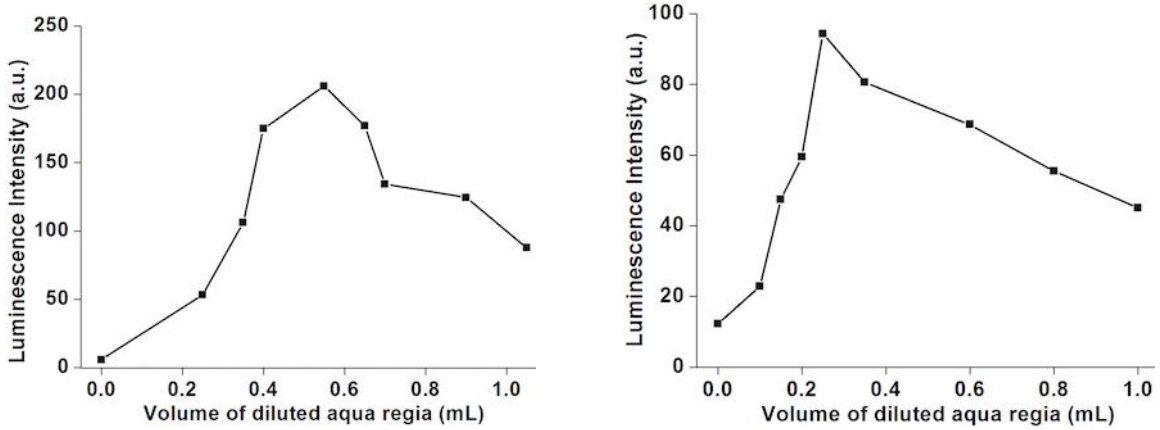

Figure S14. Change in emission intensities of **Au-5** (left) and **Au-6** (right) at 916 nm (λ_{ex} = 560 nm) due to addition of 100 time diluted aqua regia.

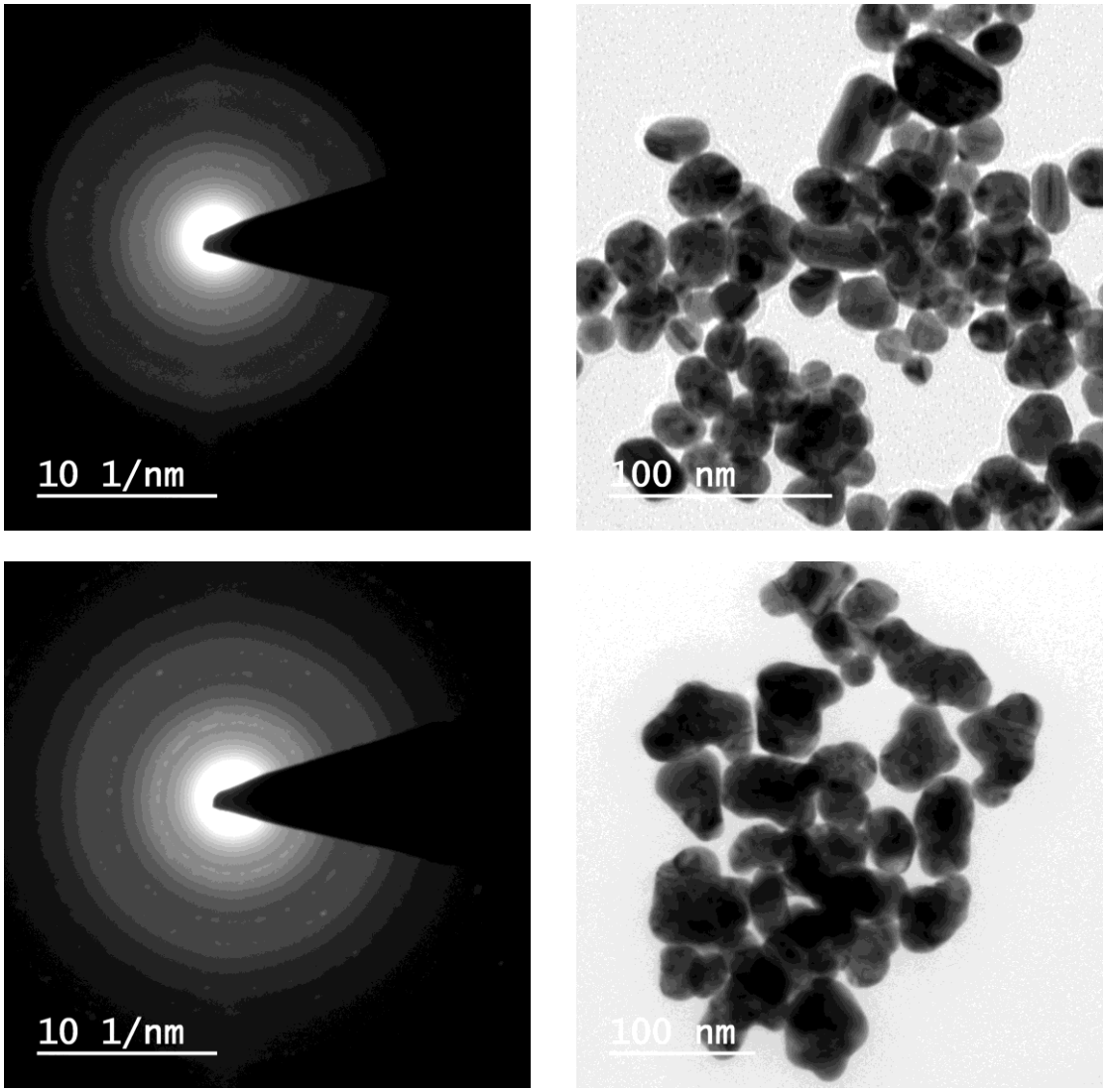

Figure S15. SAD and TEM Images for **Au-5 (**top left and top right) and **Au-6** (bottom left and bottom right).

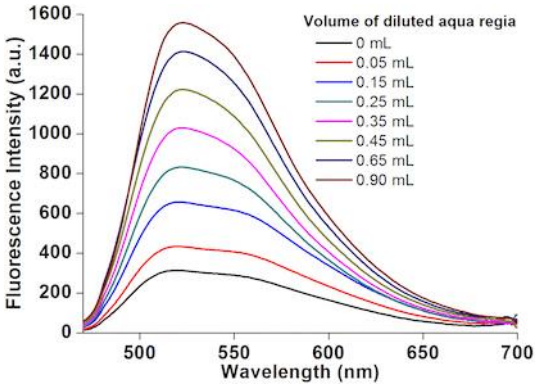

Figure S16. Change in the fluorescence of fluorescein in presence of **Au-4** due to addition of 100 time diluted aqua regia.

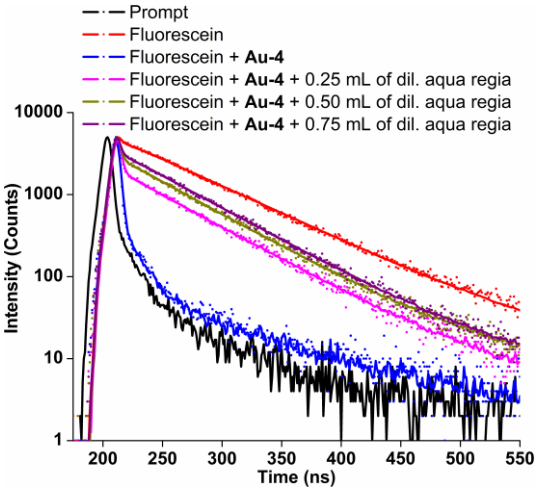

Figure S17. Fluorescence decay plot of fluorescein in presence of **Au-4** and treatment with different amount of diluted aqua regia.

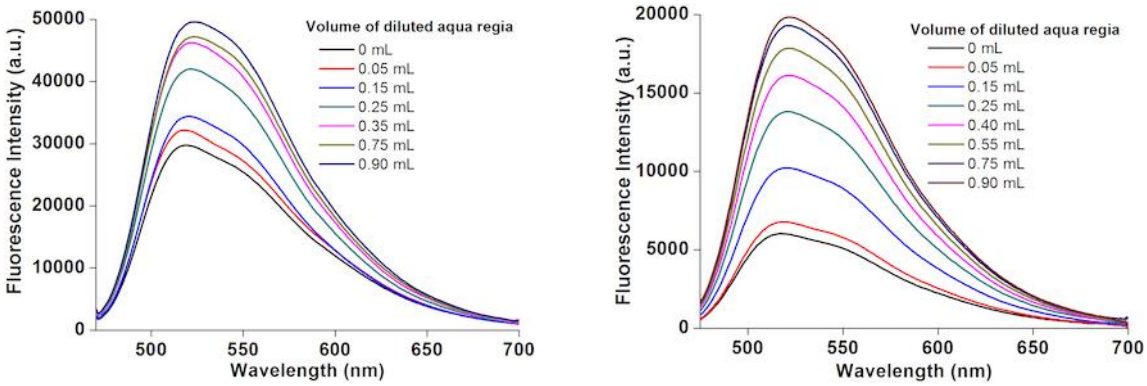

Figure S18. Change in fluorescence of fluorescein (λ_{ex} = 435 nm) in presence of (left) **Au-5** and (right) **Au-6**, during addition of diluted aqua regia.

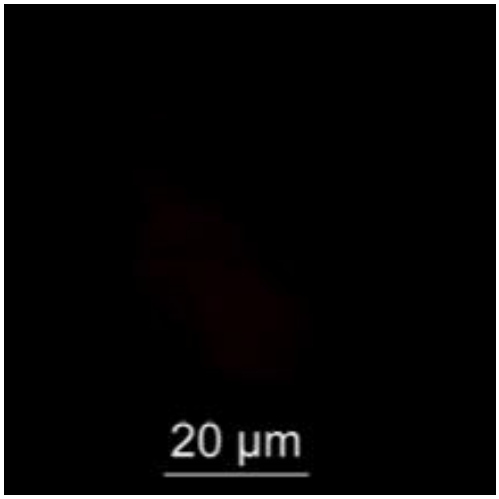

Figure S19. Fluorescence microscopic images of fixed human liver carcinoma HepG2 cells without any probe treatment. The cells were excited at 550-575 nm and emissions were monitored with long pass red filter.

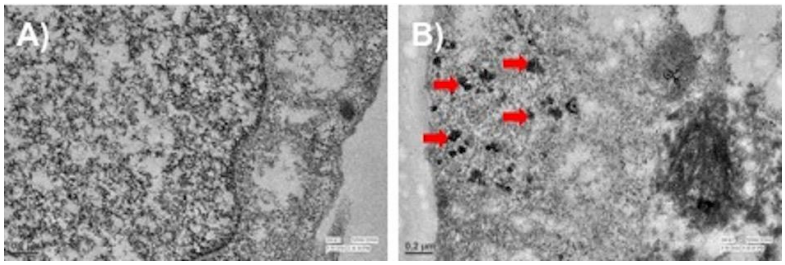

Figure S20. TEM images of fixed human liver carcinoma HepG2 cells (A) without any probe treatment and (B) after the treatment of **Au-3$_{ar}$** (0.5 nM) for 1 h.

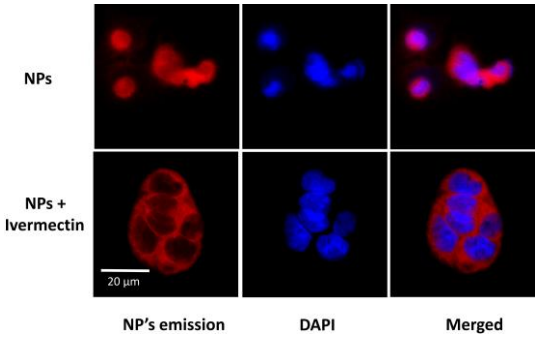

Figure S21. Fluorescence microscopic images of nanoparticles treated fixed human liver carcinoma HepG2 cells with and without ivermectin. The cells were excited at standard DAPI excitation and 550-575 nm for NIR imaging and emissions were monitored with standard DAPI filter and long pass red filter for NIR imaging.

Supporting Table

Table S1. Summary of diameters along major and minor axis of different nanoparticles **Au1-Au6**.

Gold Nanoparticle	Axis	Average diameter (nm)
Au-1	Major	39.58 (±7.5)
	Minor	29.56 (±4.8)
Au-2$_{ar}$	Major	32.79 (±6.6)
	Minor	25.35 (±3.0)
Au-3$_{ar}$	Major	58 (±13)
	Minor	40 (±5.0)
Au-4		16.5(±2.0)
Au-5	Major	30.11(±7.6)
	Minor	23.56(±5.5)
Au-6	Major	54.91(±19)
	Minor	37(±8.7)

Table S2. Fluorescence life-time data for fluorescein in **Au-4** and during addition of diluted aqua regia.

Treatment of diluted aqua regia to fluorescein in **Au-4**	**Quantum Yield**	**Average excited state life-time (ns)**	**Rate constant for radiative decay ($\times 10^9$ s^{-1})**	**Rate constant for nonradiative decay ($\times 10^9$ s^{-1})**
Control (fluorescein only)	0.18	3.14	0.32	1.46
0.00 mL aqua regia	0.008	0.19	5.29	656.08
0.25 mL aqua regia	0.04	0.80	1.26	30.12
0.50 mL aqua regia	0.14	1.14	0.87	5.37
0.75 mL aqua regia	0.16	2.16	0.46	2.44

Supporting References

S1. W. Haiss, N. T. K. Thanh, J. Aveyard and D. G. Fernig, *Anal. Chem.* 2007**,** **79**, 4215-4221.

S2. R. Sjöback, J. Nygren and M. Kubista, *Spectrochim. Acta Part A* 1995, **51**, L7-L21.

S3. G. Hungerford, A. Allison, D. McLoskey, M. K. Kuimova, G. Yahioglu, K. Suhling, *J. Phys. Chem. B* 2009, **113**, 12067–12074.

S4. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed. Springer Science + Business Media, LLC, 2006.