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# Rapid and Label-Free Cancer Theranostics Via In-Situ Bio-Self-Assembled DNA-Gold Nanostructures Loaded Exosomes

Farouk Semcheddine, [a,1] Nida El Islem Guissi, [b,1] Weiwei Liu, [a] Tayyaba, [a] Lv Gang, [c] Hui Jiang, [a] and Xuemei Wang [a]*

[a] State Key Laboratory of Bioelectronics (Chien-Shiung Wu Lab), School of Biological Science and Medical Engineering, Southeast University, Nanjing, Jiangsu 210096 (P. R. China)

[b] Department of Biomedical Engineering, College of Engineering and Applied Sciences, Nanjing University, Nanjing, Jiangsu 210093 (P. R. China)

[C] Mathematical & Physical Science School, North China Electric Power University, Baoding, 071003 (P. R. China)

[1] Farouk Semcheddine and Nida El Islem Guissi contributed equally to this work.

* Correspondence: xuewang@seu.edu.cn

## Materials and Methods

### Cell culture and animals

The human breast cell lines HeLa and SKBR3, blood cancer cell line K562, human lung carcinoma cell line A549 and the normal human hepatic cell line L02 were obtained from the American Type Culture Collection (ATCC). Hela cells were grown in ATCC-formulated Eagle's Minimum Essential Medium (EMEM) and SKBR3 cells in ATCC-formulated McCoy's 5A Medium Modified. A549 cells were cultured in DMEM supplemented with 2mM L-Glutamine. K562 and L02 cells were grown in RPMI-1640 medium. All media (Hyclone, Logan UT, USA) were supplemented with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA) and penicillin/streptomycin (37°C, 5% $CO_2$).

6-week-old male BALB/c nude mice (weighing 17–19 g; Qinglong Mountain Animal Breeding Center, Nanjing, China) were maintained under specific pathogen-free conditions, and had access to free access to food and water. The animal experiment was conducted in accordance with guidelines of the Animal Research and Ethics Board of Southeast University and approved by the National Institute of Biological Science and the Animal Care Research Advisory Committee of Southeast University.

### Cell viability assay

The in-vitro cytotoxicity of $HAuCl_4$ and ctDNA solutions were evaluated using MTT assay. After HeLa, SKBR3, K562, A549 and L02 cells were seeded in 96 well plates for 24 h, $HAuCl_4$ (1, 5, 10, 15, 20, and 25 μM) and ctDNA (100 μM), either separately or in combination, were incubated with the cells for 24 h. 10 μL of MTT solution (5 mg/ml) was then added to each well and incubated for 4 h. After discarding the medium, 200 μl of DMSO was added in each well, and plates were shaken for 10 minutes. The absorbance was measured at 490 nm using a microplate reader. MTT experiments were repeated three times. The cell viabilities were assessed as follows:

$$\text{Cell viability}\ (\%) = \frac{\text{OD test}}{\text{OD control}} \times 100\%$$

### In situ bio-self-assembled DNA-Au nanostructures

ctDNA aqueous solution was first sonicated for 30 seconds in order to obtain short DNA sequences. All cells (HeLa, SKBR3, K562, A549 and L02) were cultured in 75 $cm^2$ cell culture flask. The media used were supplemented with a low concentration of $HAuCl_4$ to take advantage of the cells surface prior to their attachment on the flask. HAuCl4 solution (15 μM) and ctDNA (100 μM) were added 4 h later and left incubated with cells for 20 h. The cells were then trypsinized, centrifuged, and washed three times with PBS. Isolation of the biosynthesized DNA-Au nanoclusters from cells was conducted via freeze-thaw method.

### Isolation and characterization of bio-self-assembled DNA-Au nanostructures

The DNA-Au nanoclusters were characterized by Atomic Force Microscopy (AFM) (Brucker icon AFM), Transmission Electron Microscopy (TEM) (JEOL, JEM 2100), X-ray photoelectric

spectroscopy (XPS) and energy dispersive spectroscopy (EDS) using a field-emission scanning electron microscope (Zeiss, Ultra Plus). Fluorescence spectra and Fourier Transform Infrared Spectroscopy (FTIR) were respectively obtained by a fluorescence spectrophotometer (LS-55, PerkinElmer Company, USA) and a Thermo Nicolet iS10 (KBr pellet).

**In-vitro fluorescence bioimaging**

HeLa, SKBR3, K562, A549 and L02 cells were cultured in confocal dishes for 12 h. HAuCl4 solution (15 μM), alone or in combination with ctDNA, was incubated with cells for 24 h. Subsequently, medium was discarded, cells were washed five times with PBS and fluorescence within the cells was imaged via a confocal laser fluorescence microscope (Nikon, Eclipse Ti), at magnification of 20 X.

**Isolation of exosomes**

The exosomes of all different cells were isolated using the ultracentrifugation method. Briefly. After incubating cells with $HAuCl_4$ and ctDNA solutions for 24 h, cells culture media were collected, centrifuged at 800 x g for 10 min, supernatant was then centrifuged at 2000 x g for 20 minutes, then the supernatant was centrifuged again at 10,000 x g for 30 minutes while keeping the whole centrifugation temperature at 4 $^{o}$C. At this point, a 0.2-μm filter was used to ensure the elimination of cell debris and other large vesicles. Finally, the supernatant was centrifuged at 120,000 x g at a temperature of 4 $^{o}$C for 70 minutes using an ultra-centrifuge (BECKMAN COULTER, OptimaTM L-100K Ultracentrifuge). The exosomes pellet was collected and stored at -20 $^{o}$C for further use. BCA protein assay kit (Thermo Scientific Inc.) was used to measure the total protein concentration in exosomes. Calculations were made by following the manufacturer instructions.

**Characterization of the isolated exosomes**

Scanning Electron Microscopy (SEM) (Zeiss Ultra PLUS) was used to visualize the isolated exosomes and Transmission Electron Microscopy (TEM) (JEOL, JEM 2100) was used to characterize the size, shape of exosomes as well as the bio-self-assembled DNA-Au nanoclusters inside the collected exosomes. For SEM analysis, 10 uL of PBS containing exosomes was mounted on a clean silicon chip, dried under a ventilation hood in order to immobilize them, then were visualized. For TEM analysis, same procedure was used, exosomes were mounted on carbon coated copper grids instead, and imaged using a JEOL JEM-2100 TEM. The DNA-Au nanoclusters were further analysed using X-ray photoelectron spectroscopy (XPS).

**Dynamic Light Scattering, Zeta Potential of exosomes and fluorescence bioimaging**

Our exosomes were further characterized using Dynamic Light Scattering and Zeta Potential analysis. 10 uL of the extracted exosomes was diluted in 1 mL PBS. The average size and zeta potential were assessed using a Malvern Zetasizer Nano ZS90. Experiments were performed in triplicates.

A simple sample preparation was used to visualize the fluorescence of exosomes released by SKBR3 and L02 cells previously incubated with DNA and Au solutions. A drop of the PBS

containing exosomes was placed on a rectangular microscope glass slide. A square coverslip was then slowly placed on top of the exosome sample to prevent forming and trapping of air bubbles. Samples were placed on a confocal fluorescence microscope (Nikon Japan) and fluorescence inside exosomes was detected at higher magnification (Oil emersion Lens, i.e. 60 X).

**Cellular uptake of exosomes by parent cells**

For this experiment, we evaluated the possibility whether isolated exosomes would be preferentially uptaken by their parent cell. To this regard, we investigated the uptake by HeLa and A549 cells of the exosomes isolated from HeLa cells. We selected these two cell lines as models due to their fast growth in vitro and in tumor xenograft.

HeLa and A549 cells were first cultured in 35 mm sterile confocal dishes for 24 h. After reaching a confluency of about 90 %, both cells were incubated with 10 uL of DNA-Au@Exo and directly visualised 5 min and 10 min after incubation. The cellular uptake was also evaluated 12 h after incubation with exosomes. Cells were then washed three times with PBS, then fixed with 4% paraformaldehyde for 10 min and stained using 4',6-diamidino-2-phenylindole (DAPI) for 15 min. Cells were then visualized using confocal laser scanning microscope TCS Sp8 (Leica, Germany), (Oil emersion Lens, Scale bar = 10 μm).

**Preparation and characterization of MTX loaded exosomes**

The drug was passively loaded into exosomes using the incubation with donor cell method. Briefly, HeLa cells were cultured in a 75 $cm^3$ dish for 24 h then treated with a low dose of mitoxantrone (2.5 ug/mL). 4h later, DNA and Au solutions were added into the medium. After another 20 h, mitoxantrone loaded DAN-Au@Exo (MTX@DNA-Au@Exo) were isolated from the medium using the above-mentioned procedure. Drug loading was estimated using UV spectrophotometer ((Thermo Scientific BioMate-3S) at wavelength of 660 nm. Particle size and zeta potential were also measured. MTX loaded exosomes (MTX@Exo and MTX@DNA-Au@Exo) were characterized by FTIR spectroscopy.

The in vitro MTX release from exosomes was investigated by placing the MTX@Exo in a dialysis bag and immersed in PBS containing 0.1% w/v Tween 80 at two different pH levels (pH 7.4 and pH 5). A temperature of 37°C and slow stirring were kept during the process. Samples were withdrawn at different time points (1, 2, 3, 4, 5, 10, 20 and 40 h), and were analyzed spectrophotometrically. The amount of MTX released from exosomes was expressed in terms of cumulative percentage of drug released as a function of time.

**In vitro tumor cell growth-inhibition and toxicity effect of exosomes**

The effect of free exosomes, free MTX solution, MTX@Exo, DNA-Au@Exo and MTX loaded DNA-Au@Exo on HeLa cancer cells was evaluated by MTT assay. Briefly, HeLa cells were seeded into 96-well plates and grown in MEM medium containing 10% fetal bovine serum for 24 h. Subsequently, the culture medium was replaced with fresh medium containing free MTX solution, MTX@Exo and MTX loaded DNA-Au@Exo, or PBS, and cultured for 12h, 24h and 48h respectively. All preparations used for this experiment contain the same drug concentration (5μg/mL). Then 10uL of MTT solution (5mg/mL) was added to each well and cell viability was

measured using a microplate reader at 490 nm wavelength.

**In-vivo bio-distribution and tumor targeting of MTX loaded DNA-Au@Exo**

In order to study the targeting ability and in-vivo biodistribution of intravenously administrated MTX@DNA-Au@Exo (isolated from HeLa cells), nude mice were subcutaneously injected below the armpit with HeLa cells ($10^7$ cells per mouse). When the tumors reached an average size of 100 $mm^3$, MTX@DNA-Au@Exo suspension (100 μg total protein) isolated from both HeLa and A549 cancer cells were directly administered via tail vein. Mice were then anesthetized for fluorescence imaging prior to each time point (0.5, 1, 4, and 12 h). To evaluate the tumor targeting ability and the bio-distribution of MTX@DNA-Au@Exo at a tissue level, mice were sacrificed 12 h post-administration, major organs or tissues (heart, lung, liver, kidney, spleen, and tumor) were collected, washed, and dried for fluorescence imaging.

**In-vivo antitumor activity of MTX loaded DNA-Au@Exo**

Human breast cancer cells (HeLa, $10^7$ cells in 200 mL PBS) were subcutaneously engrafted into the mammary fat pads of the mice, and allowed to grow. The mice were then randomly divided into four experimental groups (n=3): control (saline), MTX solution, MTX@Exo and DNA-Au-MTX@Exo. After the tumor size reached 0.1 $cm^3$, MTX solution, MTX@Exo and DNA-Au-MTX@Exo, at a dose corresponding to 3 mg/kg of MTX, were injected through tail vein at day 1, 4, 7, 10 and 13. During the treatment period, the anti-tumor activity and the body weight were monitored. Tumor volumes were assessed using the following formula: $V = (W^2 \times L)/2$, where L represents the length and W represents the width of the tumor.

**Histology evaluation of MTX loaded DNA-Au@Exo**

In order to investigate the possible damage of MTX-DNA-Au@Exo on major organ tissues, Haematoxylin-Eosin (HE) staining was used to stain heart, liver, lung, spleen, kidney, and brain tissues. Cellular apoptosis in tumor tissues induced by saline, free MTX, MTX-Exo and MTX-DNA-Au@Exo were evaluated by TUNEL assay and HE stain.

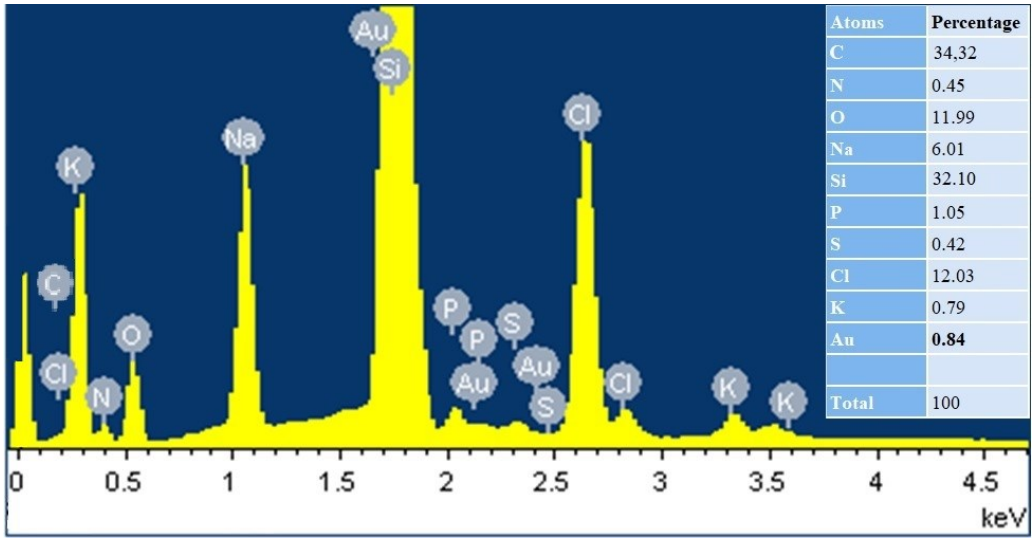
**Assessment of relevant biochemical parameters**

To further evaluate the toxicity of exosomes towards major organs (heart, liver, and kidney), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and blood urea nitrogen (BUN) were measured using the commercially available kits (Jiancheng Bioengineering Institute, China). 24 h after injection, blood samples were collected and centrifuged. The obtained serum was stored at −20°C before analysis.

**References**

[1] K. W. Witwer, E. I. Buzás, L. T. Bemis, A. Bora, C. Lässer, J. Lötvall, E. N. Nolte-'t Hoen, M. G. Piper, S. Sivaraman, J. Skog, C. Théry, M. H. Wauben, F. Hochberg, *J. Extracell. Vesicles*, 2013, **2**, 20360.

**Supplementary Figures**




| Atoms | Percentage |
|---|---|
| C | 34.32 |
| N | 0.45 |
| O | 11.99 |
| Na | 6.01 |
| Si | 32.10 |
| P | 1.05 |
| S | 0.42 |
| Cl | 12.03 |
| K | 0.79 |
| Au | **0.84** |
| | |
| Total | 100 |

**Figure 1S.** Energy-dispersive X-ray spectroscopy (EDS) of SKBR3 cells lysate, previously treated with Au and DNA solutions, confirms the presence of Au inside the cells.

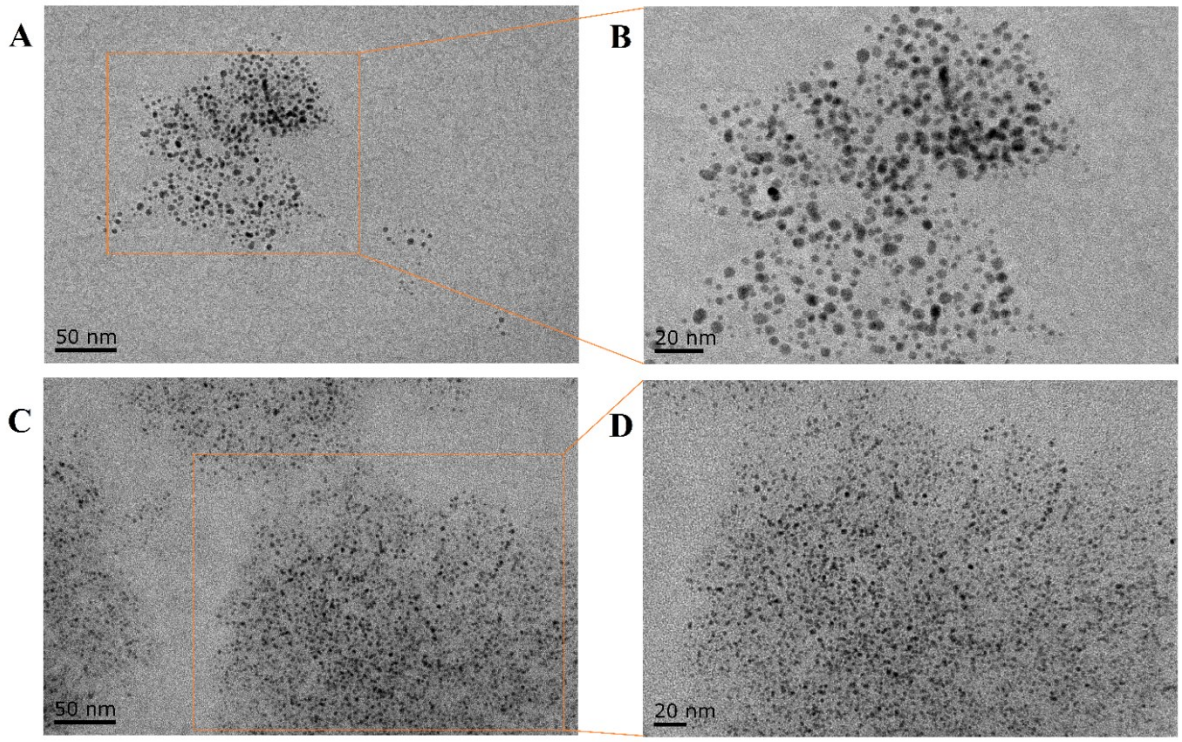



**Figure 2S**: High resolution (HR-TEM) images of the self-assembled DNA-Au nanostructures

extracted from K562 cells (A, B) and HeLa cells (C, D) (scale bar 50 nm and 20 nm).

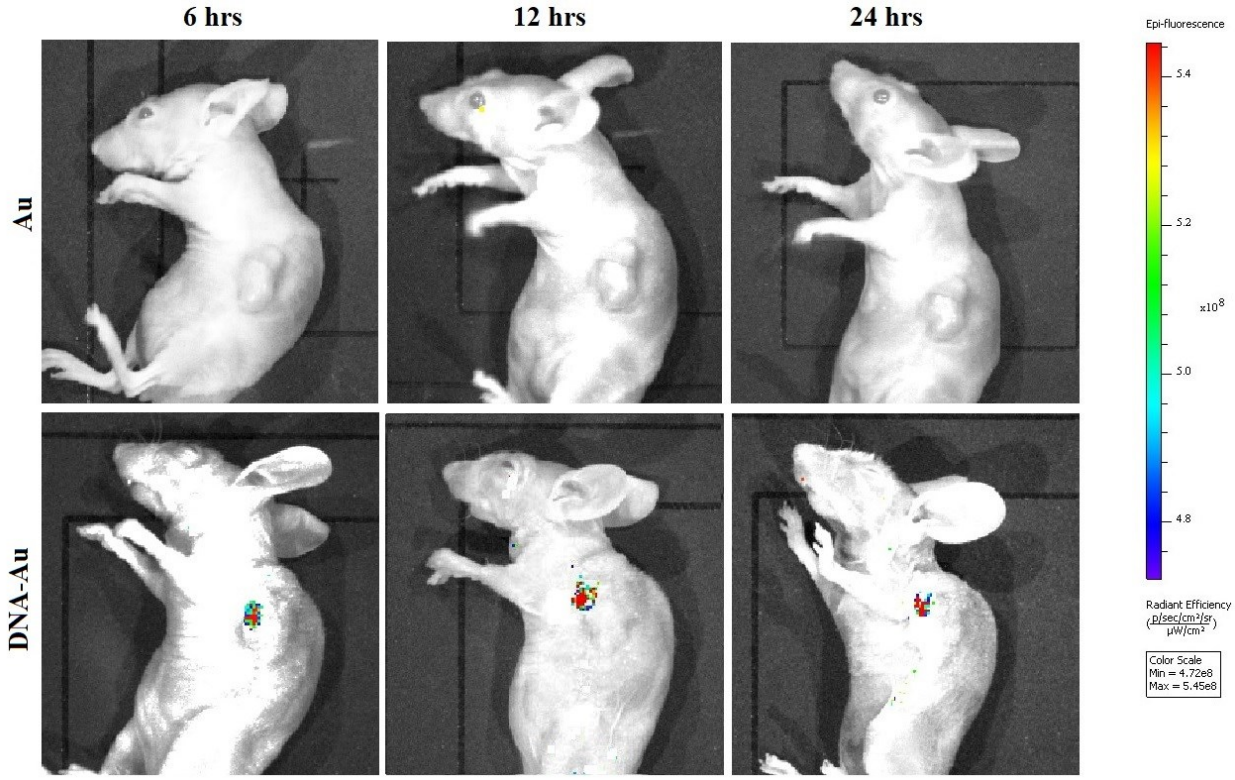



**Figure 3S.** In vivo fluorescence imaging of HeLa xenograft mice models intravenously injected with Au, and DNA-Au solutions. Images were taken 6, 12, and 24 h after tail injections.

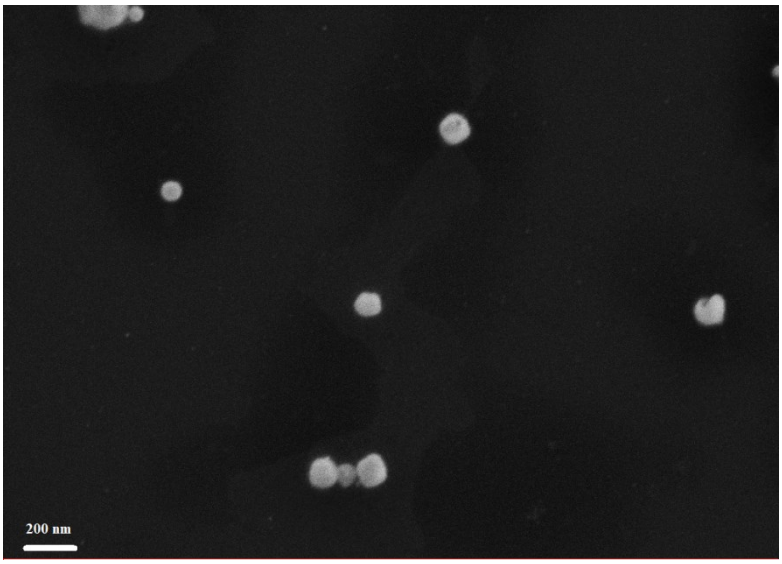



**Figure 4S**. Scanning electron microscopy (SEM) images of exosomes isolated from SKBR3 cells after incubation with both DNA and Au solutions (scale bar is 200 nm).

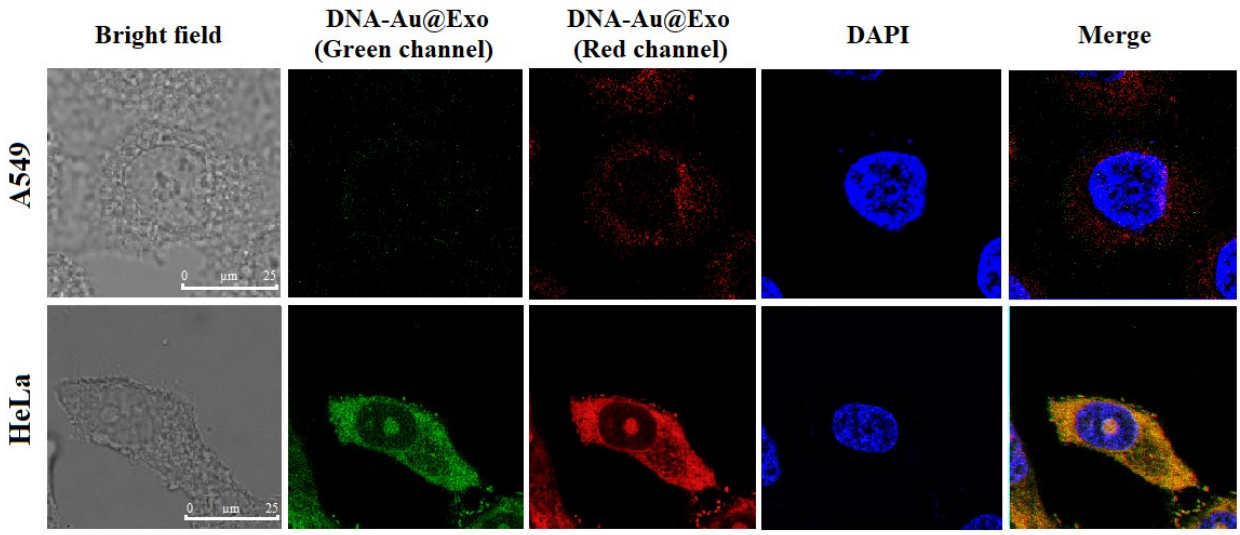



**Figure 5S.** Confocal microscopy images of HeLa-derived dye-free DNA-Au@Exo uptake by A549 and HeLa cells 24 h after incubation. HeLa and A549 cells were washed, fixed, stained with DAPI (blue), then visualized under confocal microscopy (scale bar 25 μm).

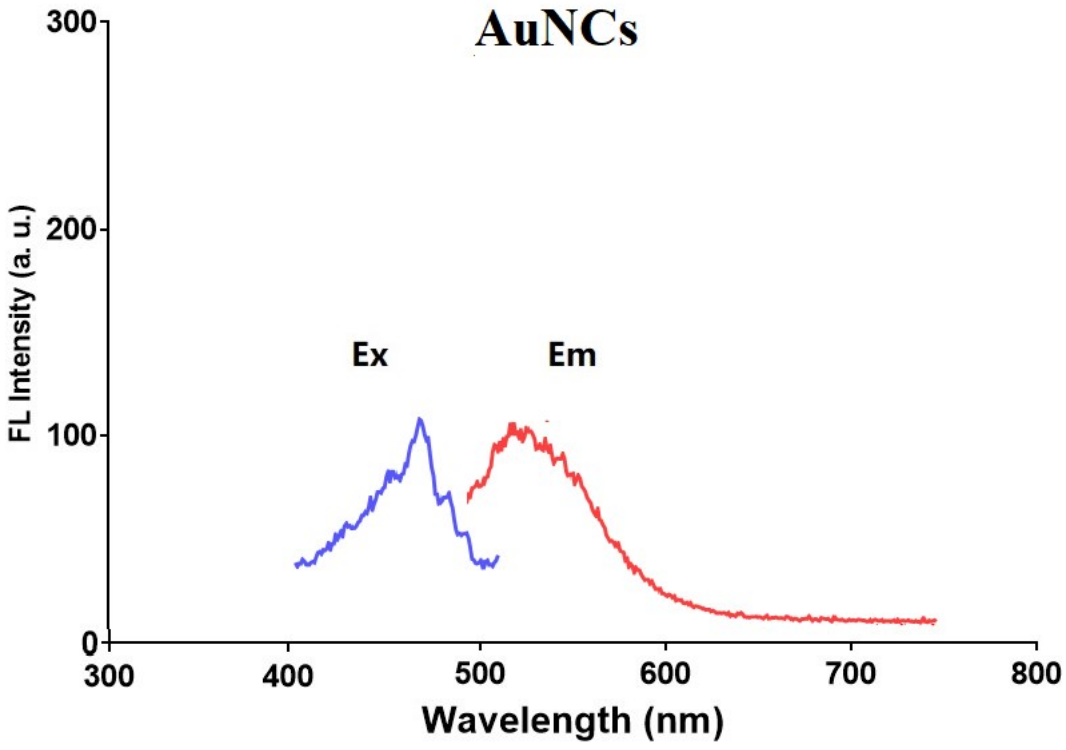



**Figure 6S.** Fluorescence spectrum of biosynthesized AuNCs obtained after the incubation of SKBR3 cells with Au ions for 24 h.