Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information SI1 : Flow cytometric analysis of cell cycle for untreated- and Au@DTDTPA(Gd)-treated U251 cells.

Flow cytometric analysis of cell cycle with propidium iodide DNA staining in untreated- or Au@DTDTPA(Gd)-treated U251 cells. Treated U251 cells were exposed to Au@DTDTPA(Gd) nanoparticles at 5 mM for 24 h. To remove Au@DTDTPA(Gd), cells were washed with PBS. Immediately (0 h), 8 h or 16 h after washing, cells were trypsinized, counted, fixated with -20°C absolute ethanol then, stained with propidium iodide (2,5 µg/mL). DNA content was quantified using flow cytometry (BD Accuri C6 plus flow cytometer, BD biosciences).

Table SI1: Impact of Au@DTDTPA(Gd) (24h exposure) on the percentage of cells in the G0/G1, S, and G2/M phase and on the proliferation index (PI). DNA content was determined immediately (0 h), 8 h or 16 h after nanoparticles' removal. Results are expressed as mean ± SEM (n = 3 independent experiments).

Times (Hours)	Treatment by Au@DTDTPA(Gd)	G_1%	S%	G_2/M%	PI (S% + G_2%)
0h	Ctrl	58.55 ± 2.02	4.69 ± 0.53	36.76 ± 1.52	41.45 ± 2.02
	5 mM	49.58 ± 2.13	7.80 ± 0.37	42.62 ± 1.77	50.43 ± 2.13
8h	Ctrl	77.67 ± 1.34	4.32 ± 0.73	18.02 ± 0.64	22.33 ± 1.34
	5 mM	60.79 ± 3.77	8.82 ± 1.98	30.39 ± 2.17	39.21 ± 3.77
16h	Ctrl	54.79 ± 0.66	20.10 ± 1.17	25.11 ± 1.67	45.21 ± 0.66
	5 mM	57.82 ± 2.62	16.20 ± 2.10	25.96 ± 0.51	42.18 ± 2.62

If exposure to Au@DTDTPA(Gd) for 24 h induced slight but insignificant changes in the distribution of the cell cycle during the first 8 h after elimination, the proliferation index of glioma cells U251 treated with gold nanoparticles remained unchanged compared to cells not treated at 16 h. Consequently, these results show little impact of the Au@DTDTPA(Gd) (24 h exposure) on the proliferation of U251 cells, which makes it possible to consider it as negligible when the effects of gold nanoparticles on migration/invasion are taken into account.

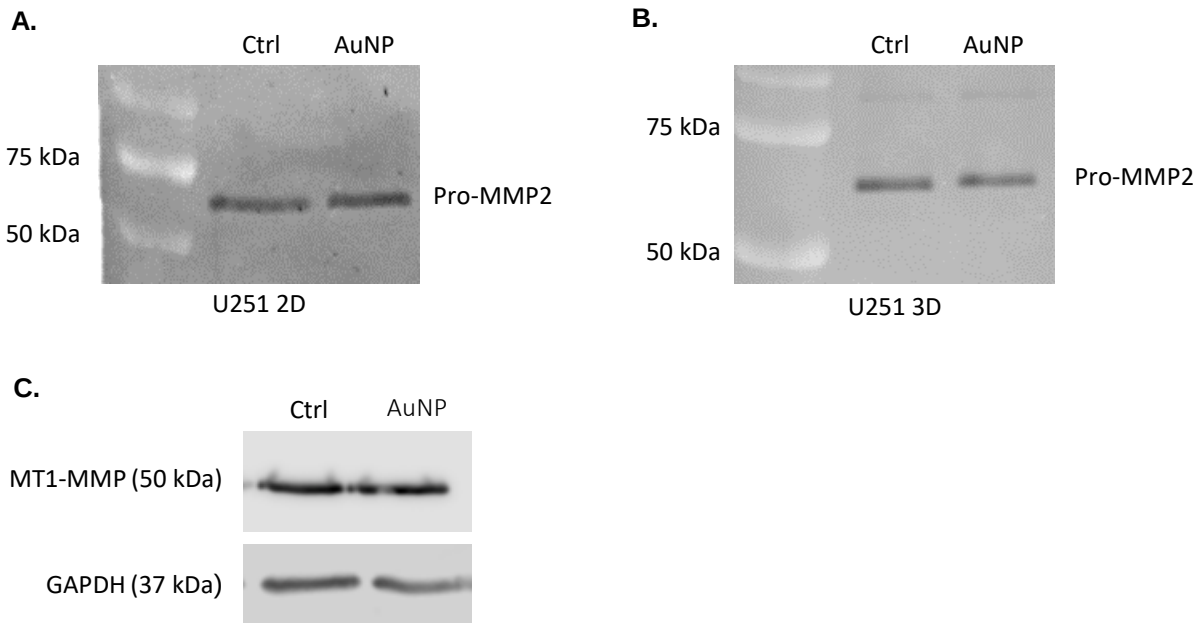
Supporting Information SI2: Matrix metalloproteinase proteolytic activity

U251 cells were seeded at 4 x 10^4 cells/mL in 25-cm^2 flasks for 3 days. Cells were treated with 5mM of Au@DTDTPA(Gd) for 24 h. Then, cells were gently washed with HBSS, then 6 ml of Opti-MEM® reduced serum media were added for 16 h. Finally, supernatants were harvested and centrifugated at 300G during 10 min at 4°C to remove cell debris. In parallel, number of cells in each flask was counted using a TC20.
Protein dosage in supernatants was performed using a colorimetric detection (Pierce BCA protein assay).

Gelatin zymography
Equal amounts of conditioned media (12 µg) were analyzed by gelatin zymography under non-reducing conditions using a 10% (v/v) polyacrylamide gel containing 1 mg/mL gelatin. After electrophoresis, gels were washed twice in 50 mM Tris–HCl pH 7.5, 5 mM CaCl2 and 2.5% (v/v) Triton X-100 for 30 min each wash, and then incubated in 50 mM Tris–HCl pH 7.5, 5 mM CaCl2 at 37°C for 48 h. Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue (G-250) dye in 10% (v/v) acetic acid and 10% (v/v) isopropanol, then destained in 10% (v/v) acetic acid and 10% (v/v) isopropanol until clear bands of MMP-2 and MMP-9 were visualized.

Cell surface biotinylation and western-blotting
Cells were washed with ice-cold PBS containing 20µM Na3VO4, then incubated with 0.5 mg/mL EZ-Link™Sulfo-NHS-LC-Biotin in PBS at 4°C for 30 min and the reaction was stopped by three washes with PBS. Cells were then incubated at 4°C for 15 min with 100 mM glycine in PBS, rinsed with PBS and lysed with NP40 (Nonidet P40), buffer [50 mM Na2PO4, 300 mM NaCl, 1 mM Na3VO4, EDTA10 mM, 1% (v/v) NP40] containing protease inhibitor cocktail (P830). Lysates were centrifuged (10,000G ,10 min, 4°C) and equal amounts of proteins were incubated with 40µL of ImmunoPure® immobilized streptavidin on agarose beads (Pierce, Rockford, USA) overnight at 4°C. After centrifugation (10,000G, 10 min, 4°C), the beads were washed with NP40 buffer and the biotinylated proteins were eluted by incubation with 10 mM D-Biotinin PBS overnight at 4°C. After centrifugation (10,000G, 30 sec, 4°C), the samples were boiled for 5 min in Laemmli sample buffer and analyzed by SDS-PAGE followed by western blot. Equal amounts of proteins (20 µg) were boiled for 5 min in Laemmli sample buffer and then analyzed by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were placed in blocking buffer 5% (w/v) non-fat dry milk in Tris-buffered saline/Tween 20 (50 mM Tris, pH7.5, 150 mM NaCl, and 0.1% (v/v) Tween 20) for 1 h at room temperature and incubated overnight at 4°C with primary antibody anti-MT1-MMP (AB815) from Calbiochem. After five washings with Tris-buffered saline/Tween 20, membranes were incubated for 1 h at room temperature in the presence of appropriate horseradish peroxydase-coupled secondary antibody. Immunocomplexes were detected by chemiluminescence and analyzed with ChemiDoc XRS™System (Biorad®).

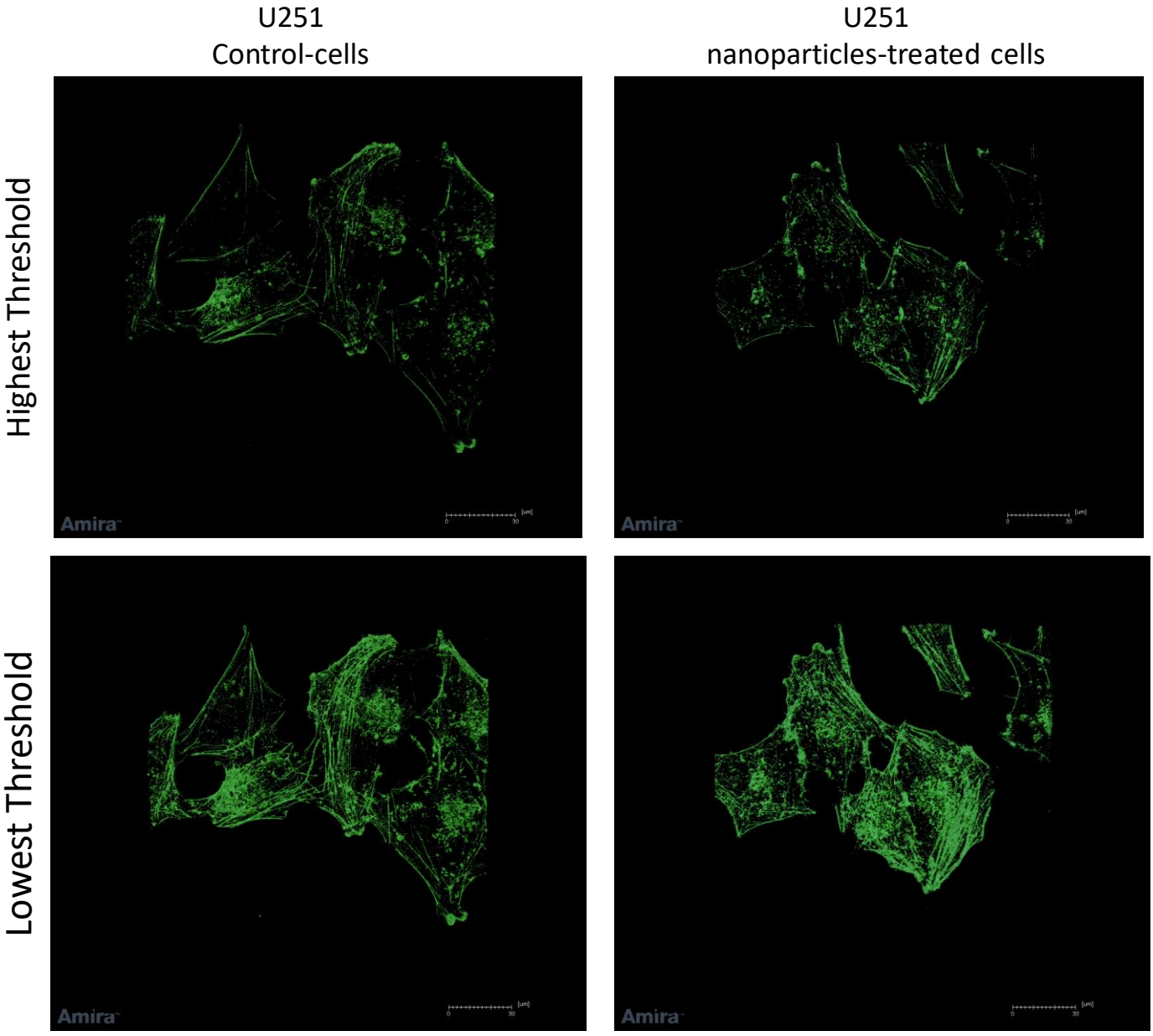
Supporting Information SI2: Matrix metalloproteinase proteolytic activity



(A and B) Analysis of MMP-2 activity by gelatin zymography for U251 cells. Metalloproteases were extracted from the supernatant of **(a)** monolayer cultures **(b)** spheroids cultures of U251 cells exposed or not to 5 mM Au@DTDTPA(Gd). Representative photos of 3 independent experiments are presented. Whatever the conditions considered, only pro-MMP-2 was detected, suggesting the lack of activation of MMP-2. **(C) Analysis of MT1-MMP expression by western-blot for U251 cells.** From monolayer cells culture, MT1-MMP were extracted after surface cells biotinylation. Housekeepping protein used to normalized MT1-MMP results is GAPDH (n = 3 independent experiments)

Supporting Information SI3: Analysis of actin cytoskeleton organization by confocal mcroscopy

Fluorescently labeled phalloidin has been used to study actin cytoskeleton organization by confocal microscopy. U251 cells were plated in 8-well Labtek II coated with 10 µg/mL of fibronectin and then exposed to 5 mM of Au@DTDTPA(Gd) for 24 h. After fixation with PFA 4% for 30 min, F-actin distribution, was visualized using Alexafluor-488-conjugated phalloidin (Ab176753, Abcam). Immunofluorescence-labeled cell preparations were analyzed using a Zeiss LSM 710 confocal laser scanning microscope with the 63X oil-immersion objective zoom 1x and Zeiss operating system (Carl Zeiss MicroImaging GmbH, Deutschland). Acquisitions were performed by exciting Alexafluor 488 with Argon laser. Emitted fluorescence was detected through the appropriate wavelength window. Twenty images were captured with a 0.25 µm z-step.



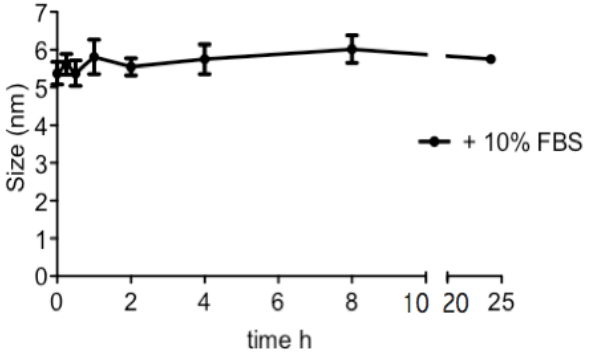
Supporting information SI3 presents the 3D isosurface reconstruction of cells using 2 different thresholds for untreated control cells or nanoparticles-treated cells. At the lowest threshold, a clear difference appears in the actin labeling between untreated and Au@DTDTPA(Gd)-treated cells. While only the actin cortex is visible in control cells, the Au@DTDTPA(Gd)-treated cells exhibit a well-developed intracellular stress fibers and thicker actin cortex. Adhesion structures appear clearly at the edge of U251 cells and actin patches with a highest threshold value (framed areas) are more present in nanoparticles-treated cells.

Supporting Information SI4 : Physicochemical characterization of Au@DTDTPA nanoparticles

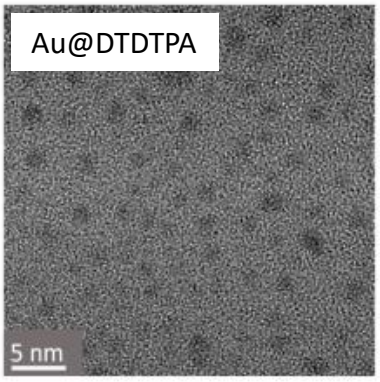
A.

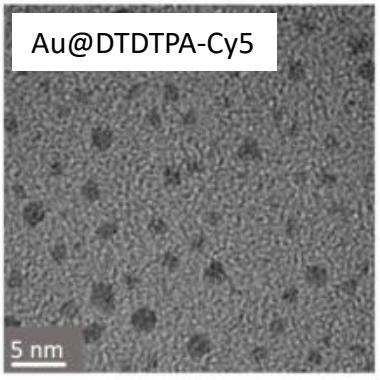
	Water	Cell culture medium
Au@DTDTPA-Cy5	5.10 nm	5.58 nm
Au@DTDTPA	4.80 nm	5.56 nm

B.

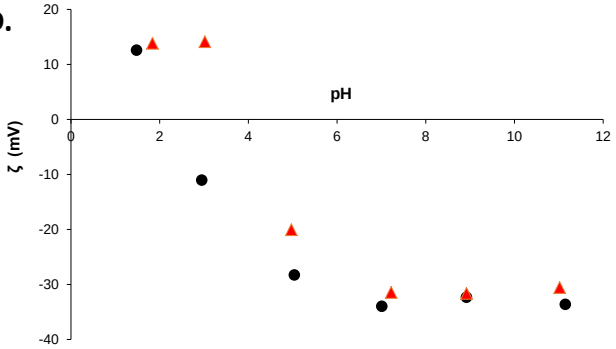


C.





D.



(A) Hydrodynamic diameter of Au@DTDTPA-Cy5 et Au@DTDTPA nanoparticles in water and in complete cell culture medium. **(B)** Au@DTDTPA hydrodynamic size following incubation in complete RPMI medium containing 10% FBS. Error bars represent SEM. **(C)** Transmission electron micrographs of nanoparticles. **(D)** Zeta potential of Au@DTDTPA (black circles) and Au@DTDTPA-Cy5 (red triangles) as a function of pH.

The hydrodynamic diameter of Au@DTDTPA and fluorescent Au@DTDTPA-Cy5 are similar, comprised between 4.8 and 5.6 nm whatever the medium. The hydrodynamic diameter of these nanoparticles is slightly higher in cell culture medium than in water (Table A).

Fig. B shows that the hydrodynamic diameter of Au@DTDTPA nanoparticles fluctuates between 5 and 6 nm when they are introduced in RPMI medium containing 10% FBS[1] while the size of the gold core is comprised between 2.0 and 3 nm (Fig. C)[1,2]. It can be concluded that the colloidal suspension of these gold nanoparticles is stable even in protein rich medium with a high ionic strength.

The colloidal stability of Au@DTDTPA and Au@DTDTPA-Cy5 is confirmed by the measurement of zeta-potential as a function of pH (Fig D). For pH > 5, zeta-potential of Au@DTDTPA and Au@DTDTPA-Cy5 is similar and strongly negative[2]. The colloidal stability is therefore ensured by the electrostatic repulsion between gold nanoparticles with the same charge.

[1]*Data from Butterworth, K. T et al. "Preclinical evaluation of gold-DTDTPA nanoparticles as theranostic agents in prostate cancer radiotherapy" Nanomedicine 2016, 11, 2035-2047.*

[2]*Data from Jiménez Sánchez, et al. "Fluorescent Radiosensitizing Gold Nanoparticles" International Journal of Molecular Sciences 2019, 20, 4618*