SUPPORTING INFORMATION

Effects of Cell Culture Media on the Dynamic Formation of Protein-NP Complexes and Influence on the Cellular Response

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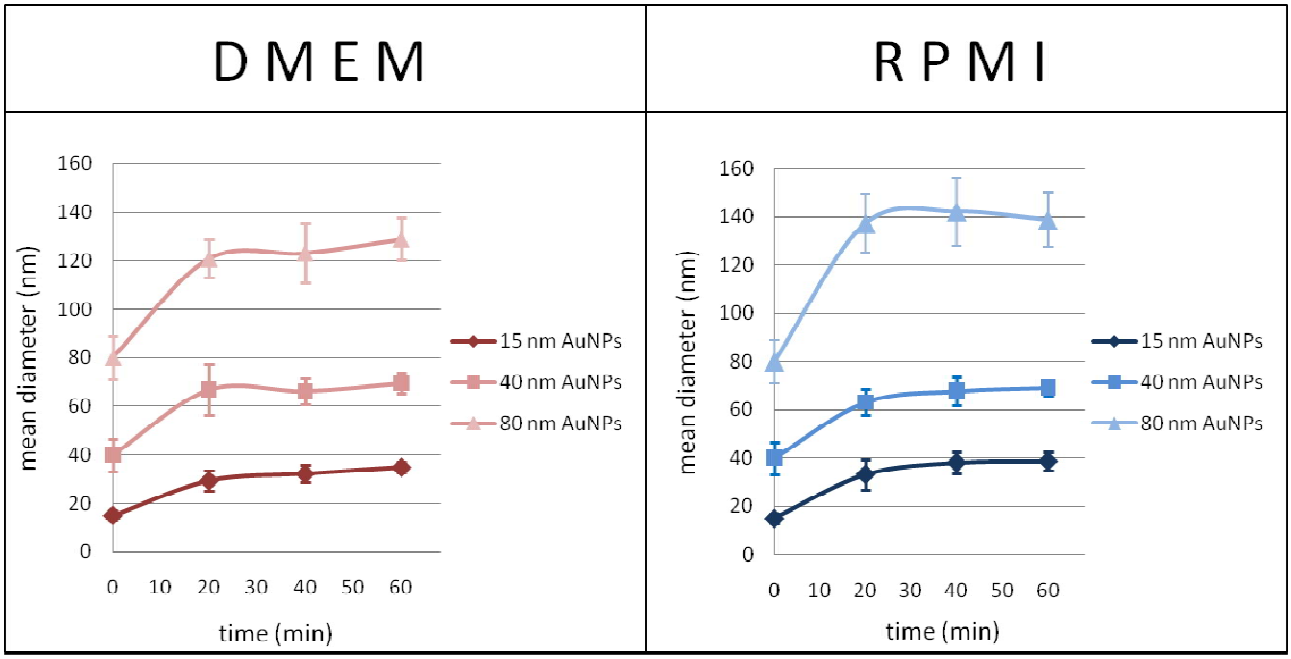

Figure S1. DLS analyses of 15, 40 and 80 nm AuNPs suspended in DMEM (left) and RPMI (right) with 10% FBS, at 37 °C, within the first hour of incubation.

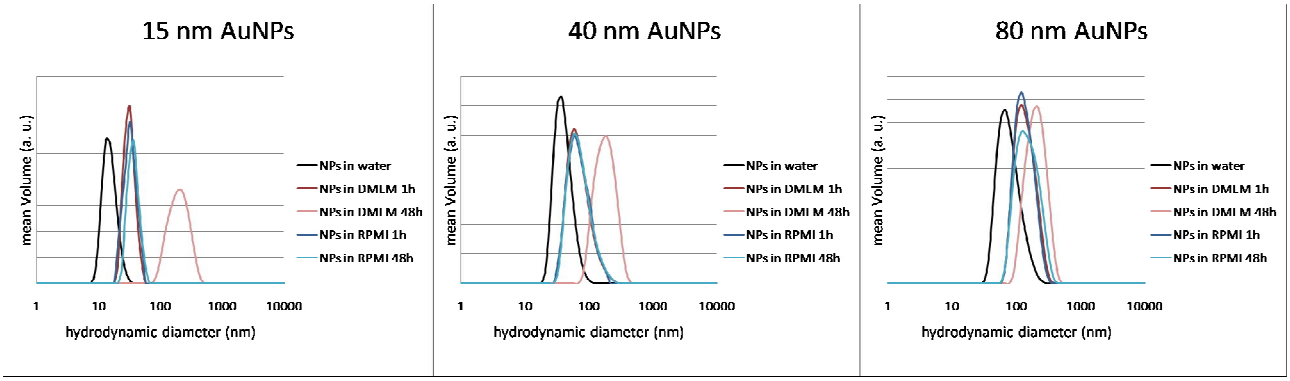

Figure S2. DLS analyses of 15, 40 and 80 nm AuNPs suspended in DMEM and RPMI (10% FBS), at 37 °C, over time (1 and 48 hours are shown). Graphs indicate NPs hydrodynamic diameter variation during protein corona formation, without any evidence of size polydispersion.

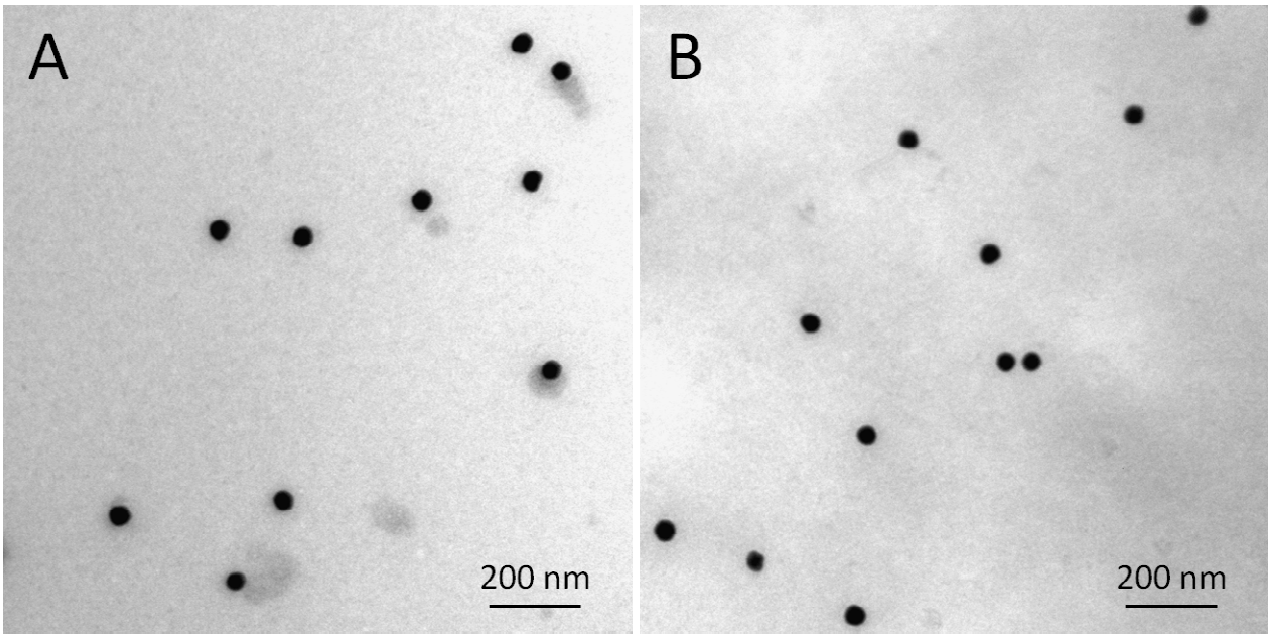

Figure S3. Representative TEM images of 40 nm AuNPs after 48 hours of incubation in DMEM (A) or RPMI (B) supplemented with 10% FBS, at 37 °C. TEM analyses typically show single and isolated NPs with a negligible presence of aggregates/agglomerates.

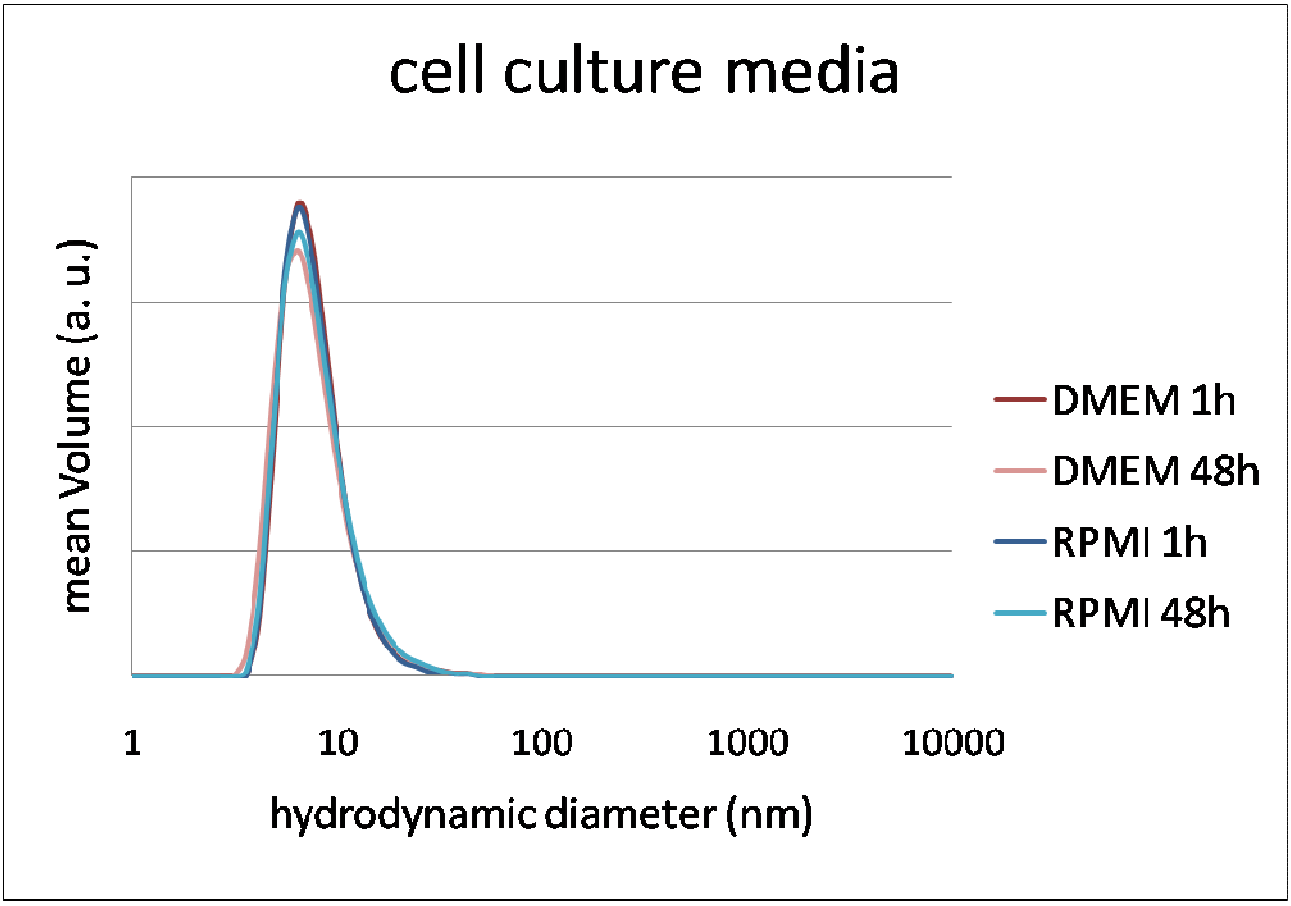

Figure S4. DLS analysis of DMEM and RPMI (10% FBS), at 37 °C, over time (1 and 48 hours are shown). Graphs show a sharp peak centered around 8 nm, accounting for the total proteins content in solution, and indicating no significant protein aggregation phenomena.

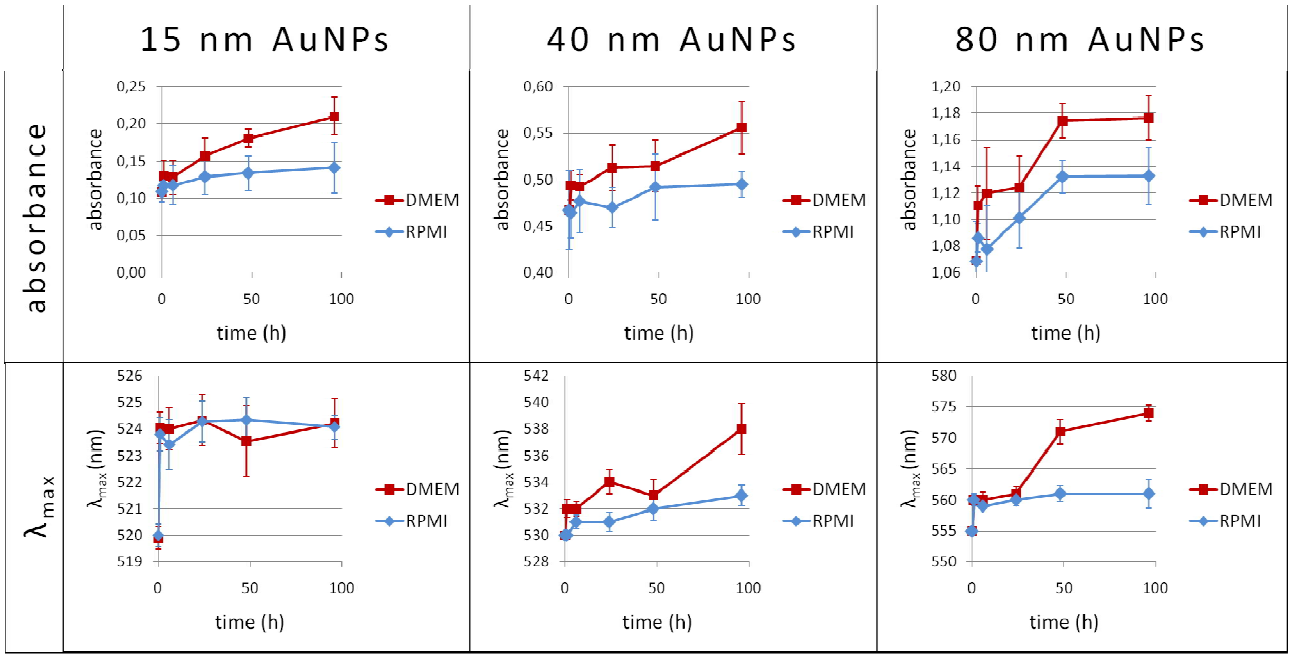

Figure S5. Temporal variations of LSPR peaks of 15, 40 and 80 nm AuNPs suspended in DMEM (red lines) and RPMI (blue lines) in terms of absorbance intensity (top row) and wavelengths of absorption maximum (bottom row).

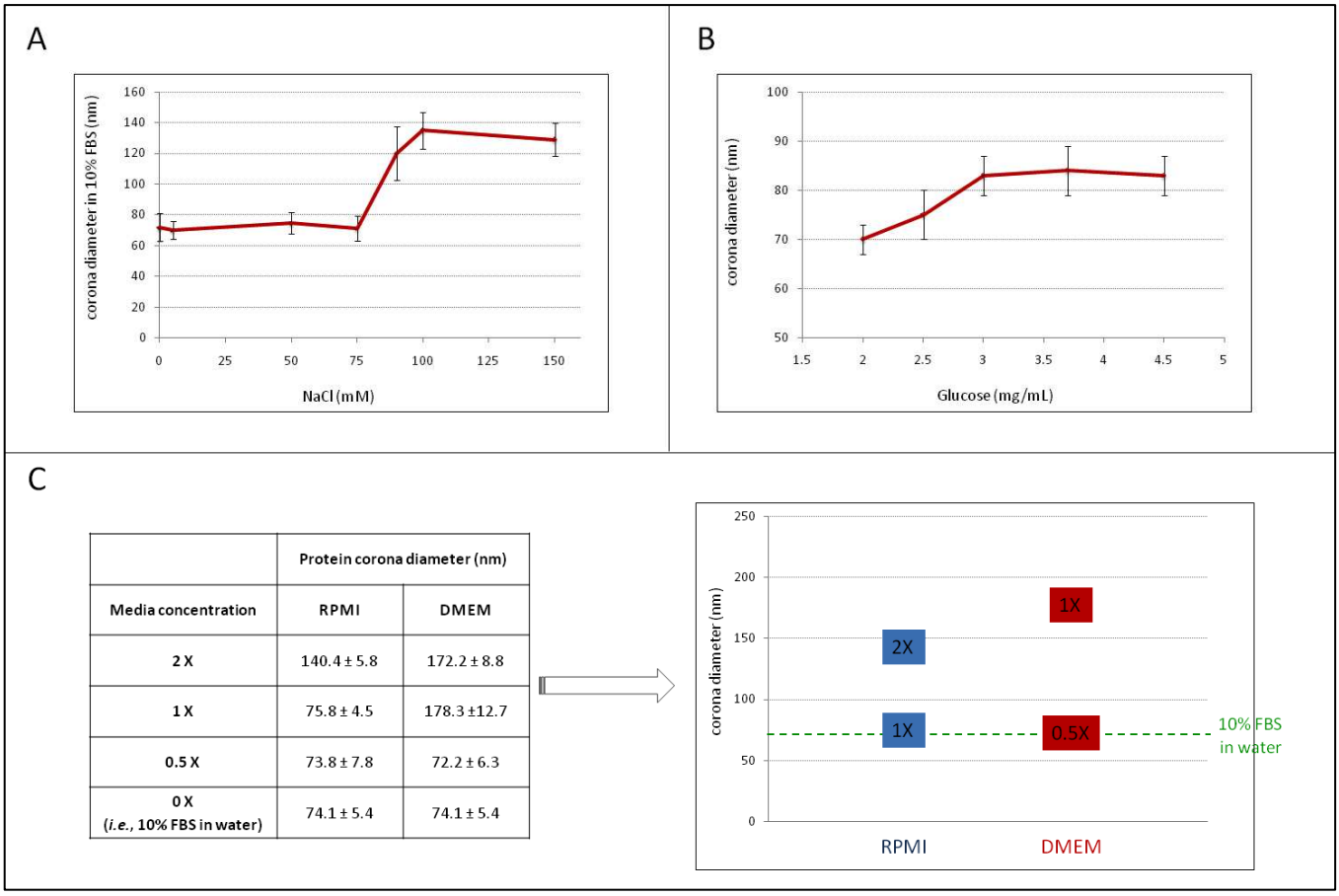

Figure S6. Investigation of parameters affecting protein corona formation. (A) Dependence of protein corona diameter on ionic strength (in 10% FBS in water). (B) Corona diameter in 1X RPMI (with 10% FBS) as a function of increasing concentration of glucose (2 mg/mL represents the standard glucose concentration in 1X RPMI, while 4.5 mg/mL is the concentration of glucose in 1X DMEM). (C) Variation of protein corona size as a function of media concentration (0X medium concentration corresponds to 10% FBS in water). The graph on the right highlights the similar values of protein corona diameter found in 1X RPMI and 0.5X DMEM (as well as in FBS in water). All these data were obtained by DLS analyses using 40 nm AuNPs, after 48h of incubation at 37 °C.

In order to get a deeper insight in the mechanisms governing protein corona formation that lead to the significant differences observed in DMEM and RPMI, we focused on the composition of the two media. In the vast complexity of the two mixtures, we found that two components, namely salts amount and glucose concentration, strongly varied between the two media, being significantly more abundant in DMEM. Therefore, we designed two model experiments, in which we studied the influence of ionic strength and glucose concentration on protein corona size. In the first experiment, 40 nm AuNPs were suspended in 10% FBS in water, with increasing concentrations of NaCl, and

protein corona size was evaluated by DLS after 48 h of incubation (Figure S6A). We observed that, at low ionic strength, corona diameter was ca. 70 nm (i.e., the same value obtained in 10% FBS in water), while, at high values of NaCl concentration (>100 mM), the corona size is characterized by a remarkable increase (~135 nm). On the other hand, the experiments performed in 1X RPMI with increasing glucose concentration (from 2.0 mg/mL , that is the typical glucose concentration in 1X RPMI, to 4.5 mg/mL, namely the concentration in 1X DMEM) revealed a slight increase (~85 nm) of corona size, likely due to a stabilizing effect of the sugar on the proteins in solution (Figure S6B). However, although both these parameters are found to affect corona formation, it is clear that they cannot account individually for the large difference in the corona size observed in the two media. This suggests a cooperative mechanism of multiple parameters, present in the complex mixtures, in governing proteins adsorption onto the nanomaterials. Such process is ruled, in fact, by several factors, such as protein-NPs, protein-protein, protein-media components, and NPs-media components interactions. The cooperative mechanism is experimentally evidenced by the variations observed in the protein corona size as a function of the media concentration (Figure S6C). In particular, we found that, by decreasing the concentration of DMEM to 0.5X, the corona size dramatically decreases (with respect to 1X DMEM), reaching the same value measured in RPMI 1X. Such corona size is the same obtained at lower concentrations of RPMI as well as in pure 10% FBS in water (Figure S4C). On the other hand, by increasing the concentration of RPMI to 2X, the corona dimension remarkably grows, approaching the typical values detected in 1X DMEM. This means that the complex composition of the cellular medium globally accounts for the multifaceted and intricate process of protein corona formation.

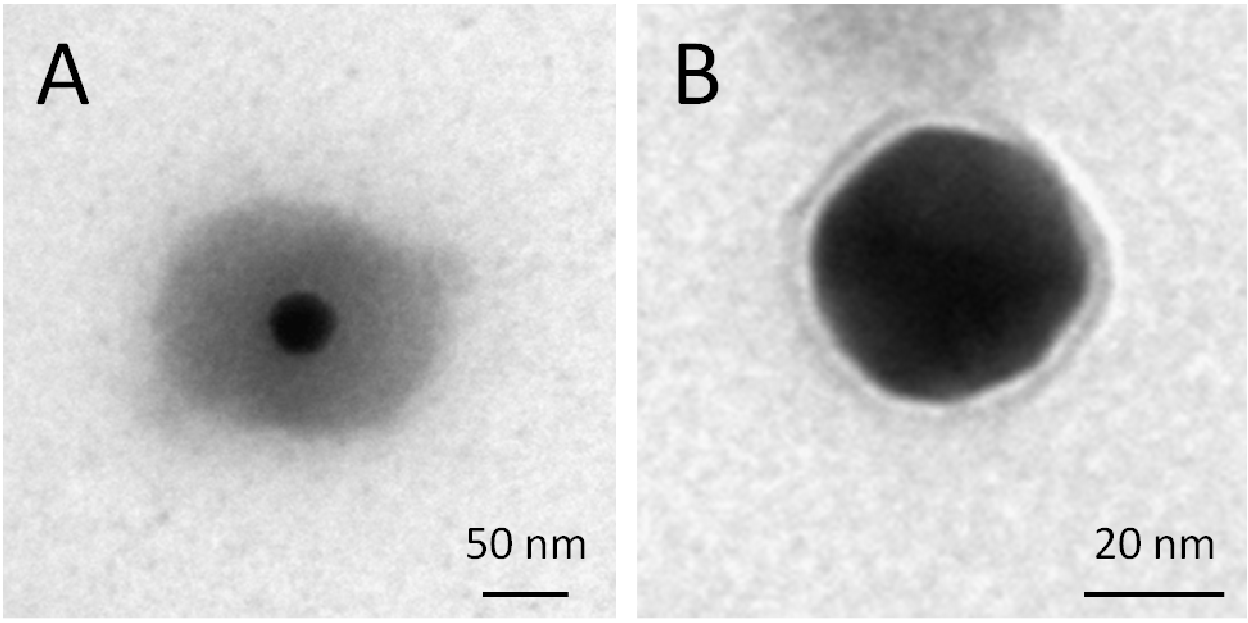

Figure S7. Representative TEM images of protein coronas formed around 40 nm AuNPs in DMEM (A) or RPMI (B) after 48 hours of incubation (after washing by centrifugation the excess of free proteins in solution). The protein layer appears like a grey shadow around the dark particles. A clear difference in the size of the protein corona layer surrounding NPs surface in the two media is evident, consistent with the spectroscopic data reported in the main text (although centrifugation and TEM imaging conditions slightly perturbed the protein corona size).

	UniProt a.n.	Protein Name	Biological process	M.W. (Da)
H.M.W.	gi/119900517*	Predicted: Talin 1	Cell-cell junction assembly, cellular movement, cytoskeletal anchorage	269780°
	Q28107	Coagulation factor V	Blood coagulation	248983
	Q2UVX4	Complement C3	Complement pathway, immune response, inflammatory response	187253
	A8E647	Alpha-2-macroglobulin	Endopeptidase inhibitor activity, protein binding	167576
	Q28085	Complement factor H	Complement alternate pathway, immune response, innate immunity	140374
	Q28178	Thrombospondin-1	Cell adhesion	129534
	A5D7R6	Inter-alpha globulin inhibitor H2 polypeptide	Hyaluronan metabolic process	106187
	P01030	Complement C4	Complement pathway, immune response, inflammatory response	101885
M.M.W.	Q2KJC7	Periostin, osteoblast specific factor	Cell adhesion, extracellular matrix organization, tissue development	86859
	gi/268607679*	Coagulation factor XIII chain A precursor	Blood coagulation	82675°
	Q3SX14	Gelsolin	Actin filament capping	80731
	Q29443	Serotransferrin	Ion transport, iron transport, transport	77753
	A5D7S8	Fibulin 1	Extracellular matrix organization	77530
	P00735	Coagulation factor II or prototrombin	Blood coagulation, acute phase	70506
	B0JYQ0	ALB protein	Transport	69293
	P02769	Serum albumin	Transport, homeostasis, maintenance of mitochondrion location	69293
	A6QQA8	QSOX1 protein	Cell redox homeostasis	62975
	A7E3W2	Galectin 3 binding protein	Cell adhesion	62127
	Q3ZBS7	Vitronectin	Cell-matrix adhesion, extracellular matrix organization	53575
	Q3MHN5	Vitamin D binding protein	Transport	53342
	P41361	Antithrombin III	Blood coagulation	52347
	P50448	Factor XIIa inhibitor	Blood coagulation	51723
	gi/2232299*	IgM chain constant region secreted form	Immune sistem	47915°
	P34955	Alpha-1 antiproteinase	Inhibitor of serine proteases	46104
	Q9N2I2	Protein C inhibitor	Blood coagulation	45297
	gi/76673754*	Predicted: similar to Pleckstrin	Actin cytoskeleton reorganization, platelet aggregation,	40057°
	P12763	Alpha-2-HS-glycoprotein	Mineral balance	38419
L.M.W	P15497	Apolipoprotein A-I	Cholesterol – lipid – steroid metabolism, lipid transport	30276
	P02081	Hemoglobin fetal subunit beta	Oxygen transport, transport	15859
	P01966	Hemoglobin alpha chain	Oxygen transport, transport	15184

Table S8. Identification of proteins from protein corona by means of LC-MS/MS analysis. Proteins are grouped in high molecular weight (H.M.W.), medium molecular weight (M.M.W.) and low molecular weight (L.M.W.), according to their theoretical molecular weight (M.W.) obtained from Uniprot database (http://www.uniprot.org/). Protein name and biological processes were also obtained from Uniprot database. (*) protein indicated with "gi" sequence identification number provided by NCBI database. (°) Theoretical molecular weight calculated from the aminaocid sequence by means of the free source ExPASy ProtParam tool (http://expasy.org/tools/protparam.html).

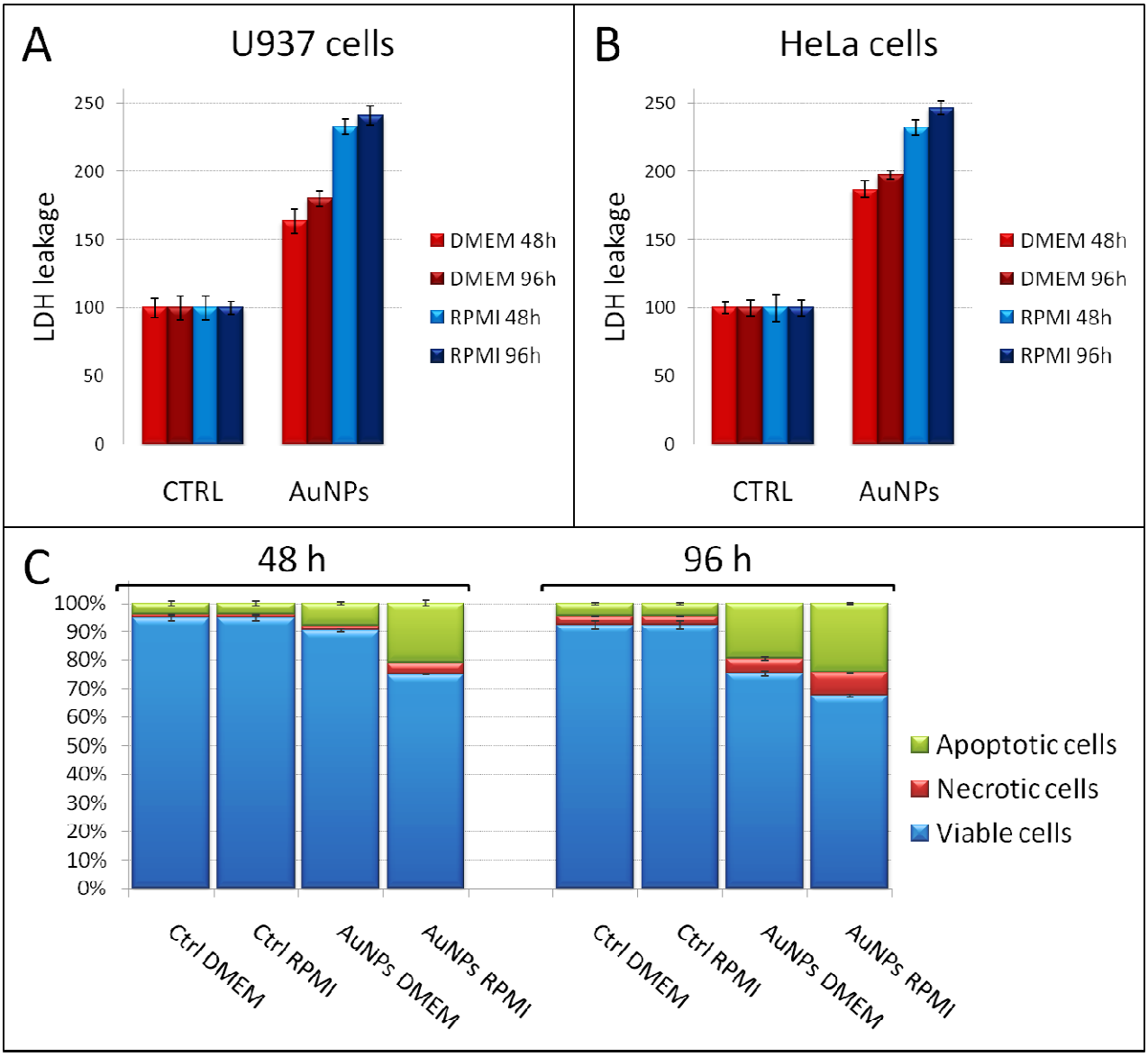

Figure S9. (A-B): LDH leakage assay performed on U937 cells (A) and HeLa cells (B) cultured for 48 and 96 h both in DMEM and RPMI, in the presence of 15 nm AuNPs (500 pM). Data are expressed in percentage with respect to the negative controls (indicated in the graphs as CTRL). Positive controls were performed by treating the cells with 0.9% Triton X-100, giving leakage values in the range of 700-800% (not shown in the graphs). (C) Annexin-V/Propidium iodide apoptosis detection by flow cytometry analyses of U937 cells treated with 15 nm AuNPs (500 pM) in DMEM or RPMI for 48 and 96 h.

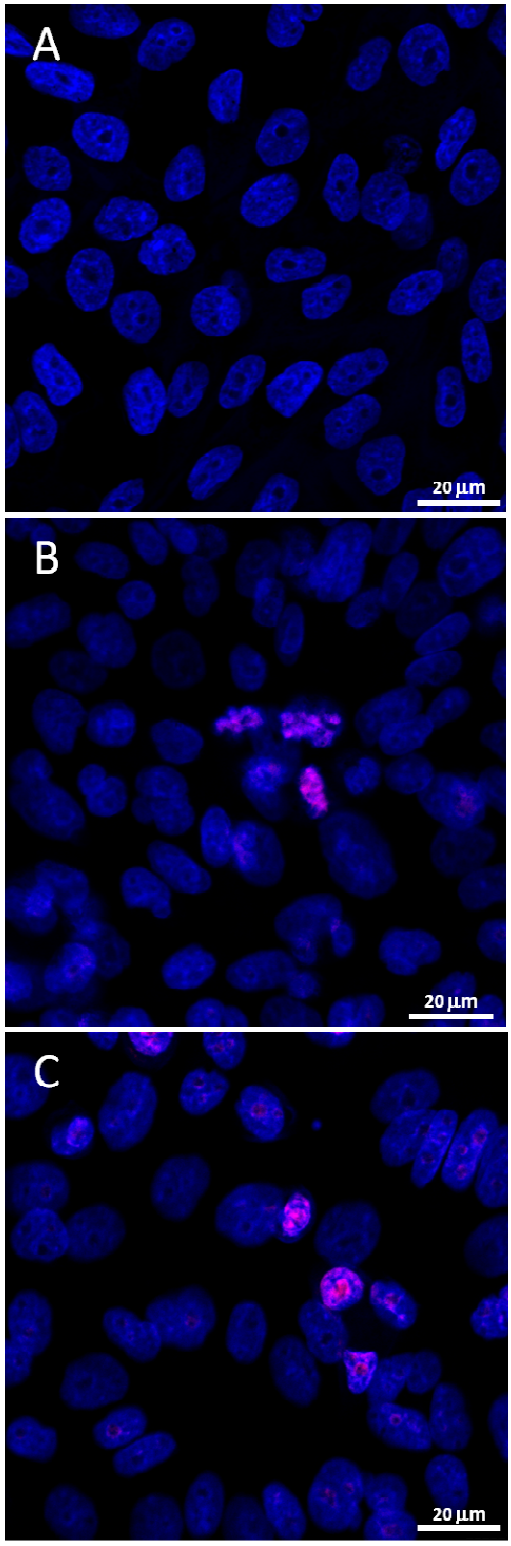

Figure S10. Representative confocal images of HeLa cells and detection of DNA damage by TUNEL assay. (A) HeLa cells cultured in DMEM (negative control). (B-C) HeLa cells incubated with 15 nm AuNPs (500 pM) for 48 hours in DMEM (B) and in RPMI (C). Nuclei are stained with Hoechst 33342 (blue) while cells containing DNA strand nicks are detected by TUNEL assay and fluoresce red.

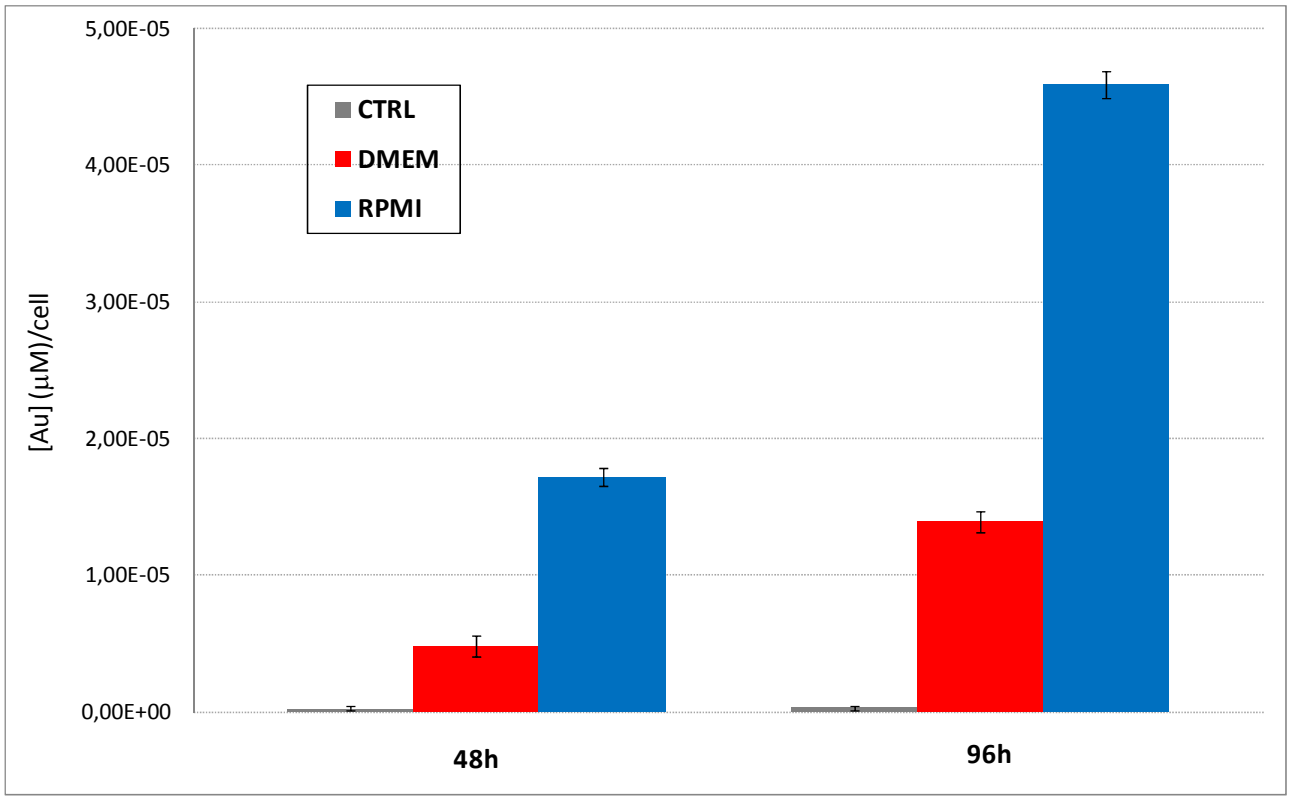

Figure S11. ICP-AES analyses of 15 nm AuNPs (500 pM) internalized by HeLa cells in DMEM and RPMI (at 37 °C, after 48 and 96 hours of incubation). Data are expressed as the amount of internalized gold (determined by ICP-AES) per cell, and indicate that the cellular uptake of AuNPs in RPMI is significantly higher than in DMEM.

EXPERIMENTAL PROCEDURES

LDH leakage assay

Cell cultured in DMEM or RPMI were seeded in 96-well microplates and treated with 15 nm AuNPs at a final concentration of 500 pM, following the procedures reported for the WST-8 assay (see Experimental in the main text). After 48 and 96 hours of cells-NPs interaction, the lactate dehydrogenase (LDH) leakage assay was performed onto microplates by applying the CytoTox-ONE Homogeneous Membrane Integrity Assay reagent (Promega), following the manufacturer's instructions. LDH released in the extracellular environment was measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into fluorescent resorufin ($560_{Ex}/590_{Em}$) by using Fluo Star Optima (BMG LABTECH) microplates reader. The fluorescence intensity is proportional to the number of lysed cells. As negative controls, we applied the same assay onto U937 and HeLa untreated cells. Results are normalized with respect to negative controls (expressed as 100%). Positive controls consisted in the treatment of such cells with 0.9% Triton X-100, that cause complete cell lyses, and so, the maximum release of the intracellular LDH enzyme. Data were expressed as mean ± SD. Differences in LDH leakage between cell treated with AuNPs and controls were considered statistically significant performing a t-student test with a p-value <0.05.

Flow cytometry analyses of cell fate by in vivo staining with FITC-conjugated annexinV and propidium iodide (PI)

U937 cells were treated with 15 nm AuNPs (500 pM) for 48 and 96 h in both DMEM and RPMI; 1 μg/ml Staurosporine (Sigma) treated cells were used as positive control. Controls as well as treated cells were harvested and washed with PBS. Cells were stained according to the manufacturer's instructions, by using Annexin V-FITC/PI Apoptosis Detection Kit (Sigma) and analyzed in a FACSAria II (BD Biosciences, NJ) flow cytometer. In brief, the cells were resuspendend in 1× Annexin V Binding Buffer at a concentration of 1×10^6 cells/mL. Then 5 μL of Annexin V-FITC and 5 μL of PI were added per 100 μL of this cell suspension (1×10^5 cells). After gentle vortexing, the cells were incubated for 15 min at room temperature in the dark. Again, 400 μL of 1× binding buffer was added to each tube before analyzing in the flow cytometer. The data were collected and analyzed with BD FACSDiva software and correspond to 20,000 cells/run. By this test, it was possible to distinguish and count apoptotic cells ($AnnexinV^+/PI^-$), necrotic cells ($AnnexinV^-/PI^+$), and viable cells ($AnnexinV^-/PI^-$).

Terminal dUTP Nick-End Labeling (TUNEL) Assay

HeLa cells cultured both in DMEM and RPMI and exposed to 15 nm AuNPs for 48 h, were fixed as described in the main text (Experimental Section, Confocal Microscopy Imaging paragraph). Then, cells were washed twice with PBS and stained by using Click-iT TUNEL imaging assays (Molecular Probes) as per manufacturer's instructions. Briefly, DNA strands cleaved or nicked by nucleases were labeled with EdUTP (dUTP modified with an alkyne) by terminal deoxynucleotidyl transferase (TdT) at the 3′-hydroxyl exposed ends. Detection is based on a click reaction between EdUTP and an azide modified AlexaFluor 647. Cells were finally counterstained with Hoechst 33342. Samples were imaged by confocal microscopy. TUNEL positive nuclei are stained both with Alexa647 and Hoechst 33342, TUNEL negative nuclei only with Hoechst 33342.

ICP analyses

To quantify the amount of internalized Au per cell by HeLa cells, we used elemental analysis by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Varian Vista AX spectrometer). Cells were seeded in 6 multi-well plates (Sarsted) at 10^6 cells/ml. Cell doping was performed with 500 pM AuNPs in both DMEM and RPMI, and ICP analyses were carried out after 48 and 96 h of incubation. Untreated cells were used as control. Five replicates were analyzed for each treatment. After incubation cells were detached by trypsinization and washed twice by centrifugation in PBS. The number of cells was determined by a hemocytometer and cell suspensions were standardized at 10^6 cell/ml. Samples were dissolved overnight in 1 mL of concentrated HCl/HNO_3 3:1 (v/v), diluted to 10 mL with ultrapure water, and the resulting solution directly analyzed by ICP.