Electronic Supplementary Information

Suppression of nanoparticle cytotoxicity approaching *in vivo* serum concentrations: limitations of *in vitro* testing for nanosafety

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Experimental Procedures

Cell culture
A549 cells (ATCC-CCL-185) were grown as monolayers in Minimum Essential Medium supplemented with 4, 10, 20 or 40 mg ml^{-1} of fetal bovine serum (FBS) or human blood serum (HS). Tissue culture reagents were purchased from GIBCO Invitrogen Corporation/Life Technologies Life Sciences (Carlsbad, CA, USA).

Human blood serum
For the HS, blood samples were collected from approximately 10 different volunteers into tubes containing clot activator, incubated at room temperature for 45 min and centrifuged for 15 min at 2000 RCF. The supernatant (serum) was collected and preserved in cryovials at -80 °C. When serum was used for experiments, it was allowed to thaw at room temperature and centrifuged for 3 min at 16.2 kRCF. It was never re-frozen or re-thawed. The blood donation procedure was approved by the Human Research Ethics committee at University College Dublin.

Nanoparticles
Fluorescently labeled amino-modified polystyrene nanoparticles (blue, 50 nm PS-NH_2, Sigma-Aldrich) and fluorescently labeled silica nanoparticles (green, 200 nm SiO_2, Kisker – Products for Biotechnology) were used without further modification or purification.

Nanoparticle uptake
Cells were incubated for different periods of time with freshly-prepared nanoparticle dispersions made in Minimum Essential Medium supplemented with the different concentrations of FBS or HS. For nanoparticle uptake measurements cells were harvested with trypsin after nanoparticle exposure, fixed with 4% formaline and resuspended in PBS before their analysis on a Dako CyAn-ADP cytometer.

Cell viability
Cell viability was studied by adding propidium iodide to a final concentration of 20 μg ml^{-1} (Sigma-Aldrich, USA) to nanoparticle-treated cells after being harvested and resuspended in PBS for their analysis by flow cytometry. Cell numbers were obtained by manual counting using a haemocytometer chamber.

Cell cycle analysis
Incorporation of the base analogue EdU was analyzed as a measurement of cell proliferation using the Click-iT EdU Flow Cytometry Kit (Invitrogen Corporation/Life Technologies Life Sciences, CA, USA), following manufacturer's instructions.

ATP intracellular content
Intracellular levels of adenosine triphosphate (ATP) were quantified with the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation, USA) after 24 h of incubation in the different conditions and according to the manufacturer´s recommendations.

Fluid phase endocytosis
Uptake levels were monitored with 10 kDa fluorescein isothiocyanate-labeled dextran (Invitrogen Corporation/Life Technologies Life Sciences, CA, USA). Cells were pre-incubated in the different media for 6, 24, 48 or 72 h, after which 0.25 mg ml^{-1} dextran was added to the corresponding medium for 1 h; cells were then harvested, fixed with 4% formaline and resuspended in PBS for their analysis by flow cytometry.

Nanoparticle characterization
Nanoparticle dispersions in medium at different protein concentrations were characterized by dynamic light scattering using Malvern Zetasizer Nano ZS90 after being aged in a tissue culture incubator at 37 °C and 5% CO_2 for 1 or 24 h. The apparent diameter of silica nanoparticle dispersions in medium at

different protein concentrations was determined by differential centrifugal sedimentation using a CPS Disc Centrifuge DC24000 after incubation for 1 h at 37 °C and 5% CO_2.

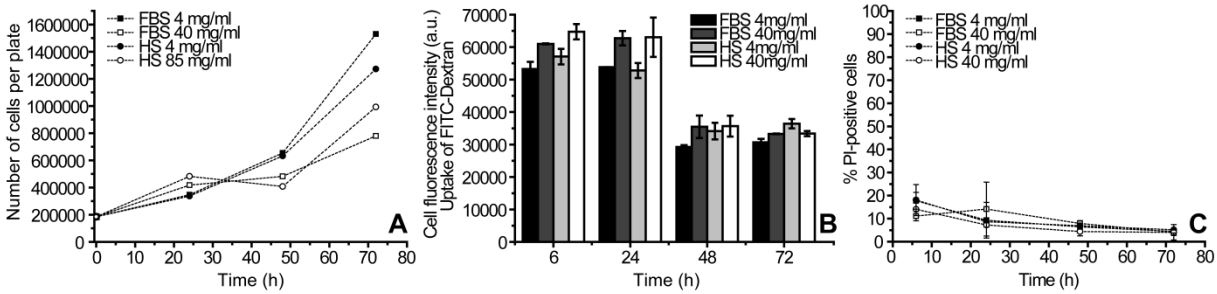

Figure S1. Cell proliferation and dextran uptake are largely unaffected by incubation in high protein concentrations of FBS and HS. (a) Cell numbers of cultures grown in 4, 40 or 85 mg ml^{-1} of FBS or HS are comparable during the first 48 h of incubation, during which time the differences reported in the main text are already visible. After 72 h differences in the number of cells arise. (b) Dextran uptake for cells grown in 4 or 40 mg ml^{-1} of FBS or HS for different amounts of time. Cells were exposed for 1 h to fluorescent 10 kDa dextran, a fluid-phase endocytotic marker, after being grown in the different media for the indicated times, and the cell fluorescence levels measured by flow cytometry. Results are presented as the average over roughly 10000 cells. Error bars indicate standard deviation of three replicas. (c) Incubation in high protein concentrations of FBS and HS does not alter cell viability. Staining with propidium iodide (PI) occurs upon damage to the plasma membrane and thus indicates loss of cell viability. Percentage of PI-positive cells remains low (<15%) and comparable among the different growth conditions. Error bars indicate standard deviation of three replicas.

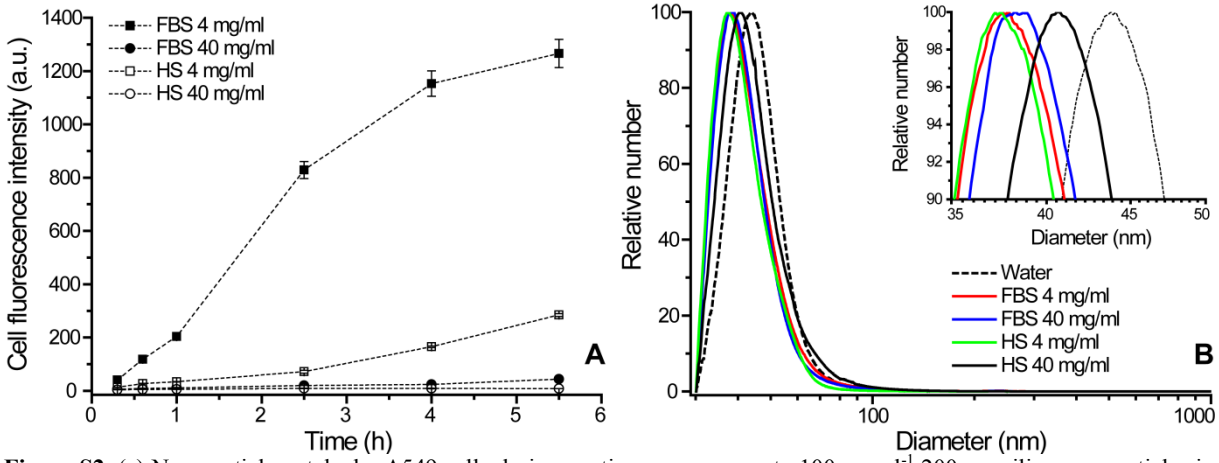

Figure S2. (a) Nanoparticle uptake by A549 cells during continuous exposure to 100 µg ml^{-1} 200 nm silica nanoparticles in the presence of different concentrations of serum proteins. The intracellular dose was assessed as the average nanoparticle fluorescence per cell. Uptake was higher in the presence of low amounts of serum (4 mg ml^{-1}) compared to high amounts of serum (40 mg ml^{-1}) for both FBS and HS. Error bars indicate standard deviation of three replicas. (b) Nanoparticle dispersion characterisation by differential centrifugal sedimentation after 1 h incubation under the different conditions used in panel a. The results show no major change in dispersion characteristics with serum concentration, neither for FBS nor for HS. Note that the decrease in apparent diameter comparing the nanoparticles in water with the nanoparticles in the presence of protein is consistent with adsorption of protein on the nanoparticle surface (see, e.g., ref 10 for details).

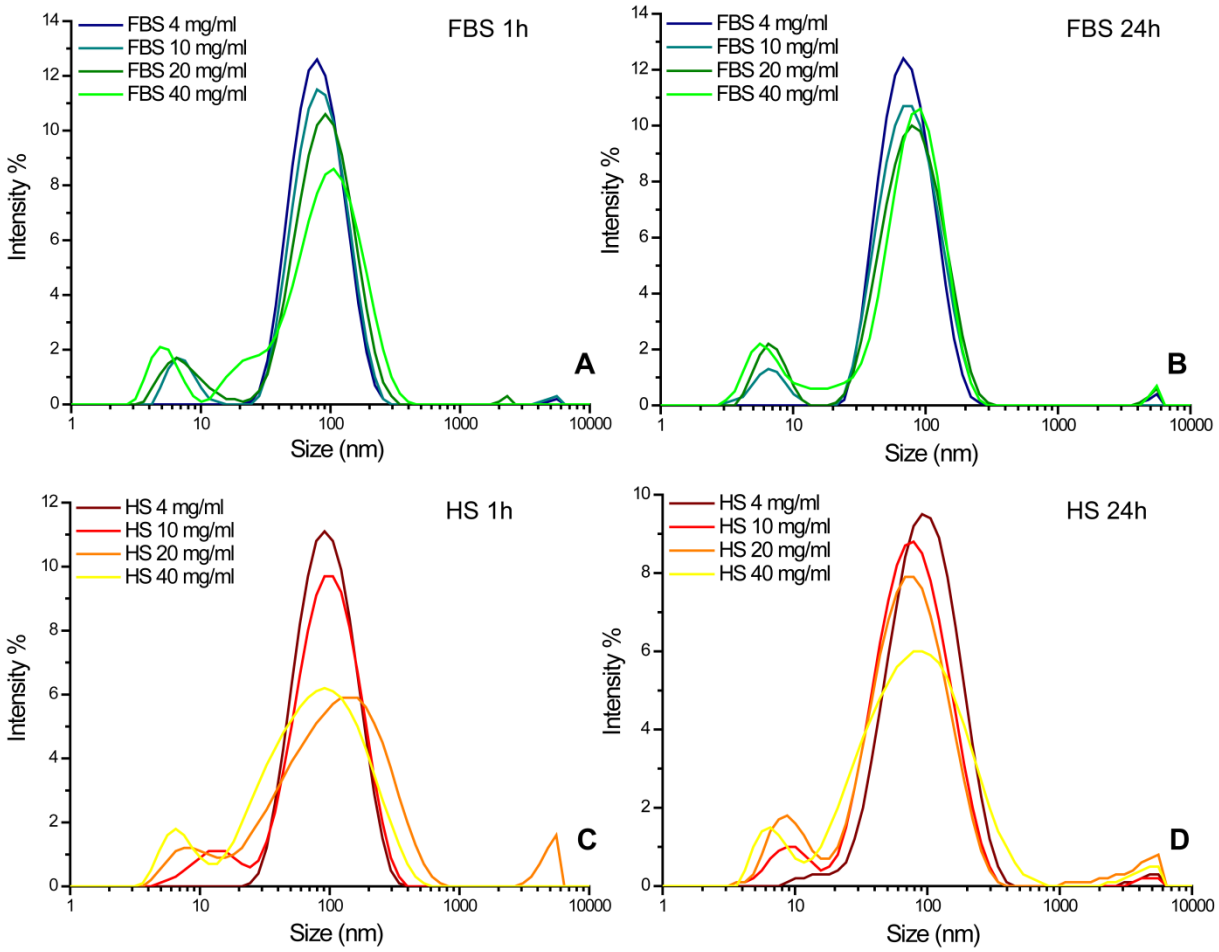

Figure S3. Characterisation by dynamic light scattering of amino-modified polystyrene nanoparticle dispersions in the different conditions used. Dispersions of the nanoparticles in 4, 10, 20 and 40 mg ml^{-1} of FBS, after incubation for (a) 1 h or (b) 24 h. Dispersions of the nanoparticles in 4, 10, 20 and 40 mg ml^{-1} of HS, after incubation for (c) 1 h or (d) 24 h. All dispersions were aged in a tissue culture incubator at 37°C and 5% CO_2 for the indicated times to mimic the conditions of the experiments with cell cultures. The results show the size distributions obtained by CONTIN analysis. The analysis is complicated by the high concentrations of proteins, but the results nevertheless suggest that the dispersion state is not radically different between low and high protein concentrations, especially considering the large difference in cellular impact (Figure 1). Furthermore, the dispersion state appears stable with time. The two peaks of lower intensity, one of smaller size (>10 nm) and one of larger size (~5000 nm) could be observed in all the dispersions, and are also present in the nanoparticle-free dispersant (Supplementary Figure S4).

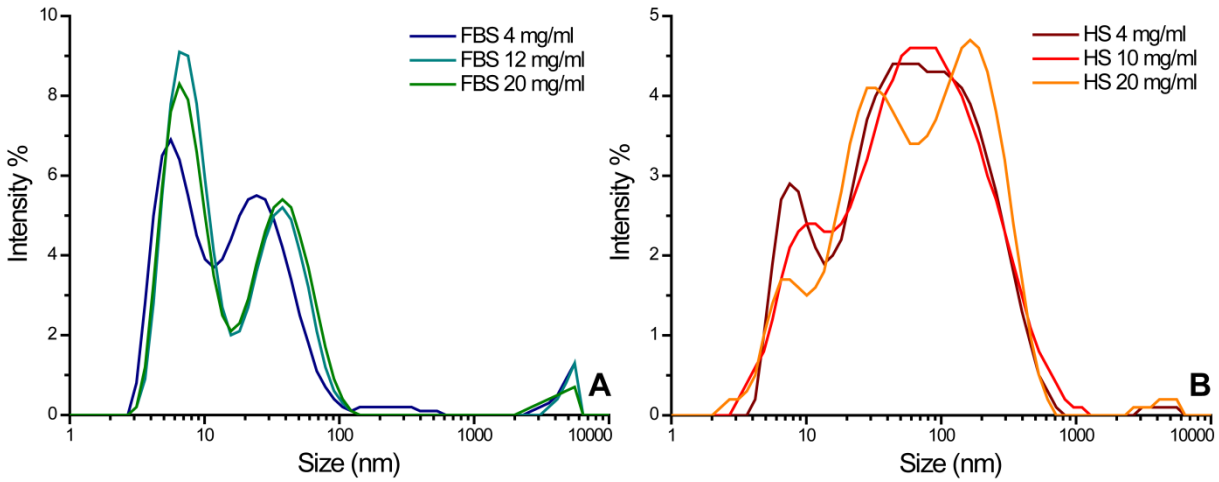

Figure S4. Characterisation of nanoparticle-free media by dynamic light scattering. The results show the size distributions obtained by CONTIN analysis. Analysis of (a) FBS and (b) HS at 4, 10, 12 or 20 mg ml^{-1}, incubated at 37˚C and 5% CO_2 for 1 h, showed that although of quite low intensity, the protein solutions without nanoparticles give a detectable signal. This signal is expected to contribute at least partly to the minor peaks observed in the nanoparticle dispersion size distributions shown in Supplementary Figure S3.