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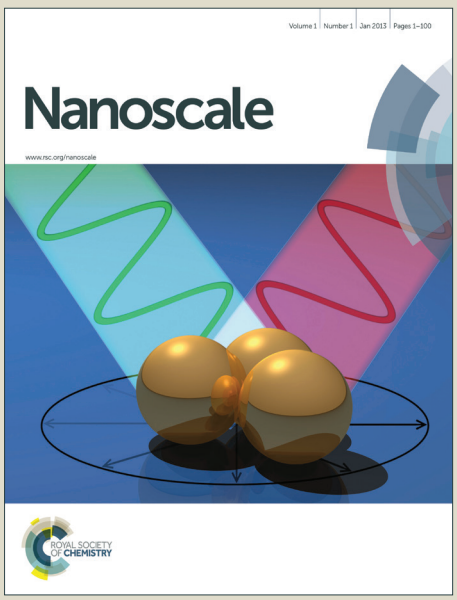

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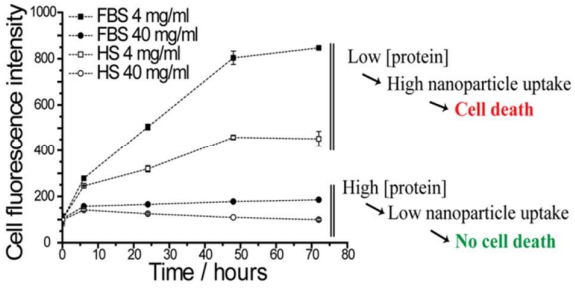
Graphical abstract

The adverse effects of cell death-inducing nanoparticles can be suppressed by increasing the serum concentration from typical *in vitro* to more realistic *in vivo* concentrations.



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COMMUNICATION

Suppression of Nanoparticle Cytotoxicity Approaching *In Vivo* Serum Concentrations: Limitations of *In Vitro* Testing for Nanosafety

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Nanomaterials challenge paradigms of *in vitro* testing because unlike molecular species, biomolecules in the dispersion medium modulate their interactions with cells. Exposing cells to nanoparticles known to cause cell death, we observed cytotoxicity suppression by increasing the amount of serum in the dispersion medium towards *in vivo*-relevant conditions.

Nanomaterials are currently being studied for a range of applications, including as novel drug-delivery vectors.[1, 2] Concerns regarding the potential toxicity of nanomaterials, intended for use in medicine or incidental, have however also been raised.[3] Identification of the parameters that play a role in the interaction between nanoparticles and biological systems has consequently been the objective of a great number of studies.[4-7] It is now emerging that intrinsic nanoparticle characteristics, such as their material and surface, are not the only relevant parameters, but that the identity of nanomaterials must also be defined with reference to the environment in which they are exposed to cells. Thus, nanomaterials in biological fluids typically associate to their surfaces a host of biomolecules, termed the 'biomolecular corona',[8, 9] the composition of which has been shown to vary with nanoparticle material,[10] size[11] and medium composition.[10, 12] For nanoparticles with high energy of the bare surface, the association is so strong that some of the corona biomolecules remain on the surface for hours,[11, 13] thereby constituting the *de facto* identity of the nanoparticle in the biofluid.[9] Indeed, nanoparticle uptake has been shown to be mediated by biomolecules in the corona,[14, 15] while passive uptake in most cases has been observed only for nanoparticles with specially designed surfaces and/or in absence of corona.[16] The presence of the corona in fact reduces the unspecific interaction between nanoparticles and the cell membrane,[17, 18] and subsequently can mitigate the direct damage that some nanomaterials induce when bare.[17, 19-21] Such damage that stems directly from exposing nanoparticles to cells in the absence of a corona clearly has little to do with the expected *in vivo* behavior, where nanoparticles are introduced in complex biological fluids.

Once recognized that the exposure conditions determine nanoparticle-cell interactions, it is a short leap to challenge the practice of using standardized culturing conditions for maintaining cell lines for *in vitro* studies. That is, cells used for *in vitro* studies are typically cultured in growth medium that is supplemented with lowered concentration of serum proteins as a source of nutrients. Usually, serum of bovine origin is used (also for cells of human origin) and is applied to cells in dilutions ranging from 2 to 10%. Such standards have been adopted, without alteration, in most studies on nanoparticle-cell interactions, including those addressing their potential hazards. However, while for most molecular species the serum concentration does not constitute a relevant parameter, we show here that for nanoparticle-cell studies this constitutes a crucial variable, making the difference between cell death and a benign interaction. Succinctly put, using a fixed concentration of toxic nanoparticles we show that we can obtain any result – from cell survival to cell death – simply by varying the protein concentration in the medium used to expose nanoparticles to cells.

In order to study the role of serum concentration on nanoparticle-cell interactions, we used amino-modified polystyrene particles of 50 nm diameter as a model cytotoxic nanoparticle, known to cause cell death by apoptosis at high doses[22, 23] and cell cycle arrest at lower.[24, 25] Extensive characterization of how these nanoparticles interact with cells (including subcellular localization data by immunofluorescence[21, 25] as well as electron microscopy,[23] and lack of export from cells[25]) has been performed previously as reported elsewhere.[21, 23, 25] In order to test the effect of exposure conditions on their impact on cells, the nanoparticles were dispersed at a concentration of 100 μg/ml in cell media supplemented with different concentrations of fetal bovine serum (FBS) or human blood serum (HS). The concentrations of FBS and HS ranged from the typical "10%" serum supplementation (corresponding to roughly 4 mg/ml proteins for typical commercial FBS) to 100% pure serum (40 mg/ml; in which case no growth medium can be added).

A549 cells (human adenocarcinomic alveolar basal epithelial cells) were then exposed to the different nanoparticle dispersions. In the absence of nanoparticles, cell growth, cell viability and internalization of fluorescently labeled dextran were largely unaffected by incubation at high concentration of FBS and HS in the time scales relevant for the study (Figure S1), thus excluding that the effects we examined were solely related to the cells being exposed to high concentrations of serum.

To quantify cell death upon exposure to the nanoparticles, we used propidium iodide (PI), a fluorescent molecule which does not enter through the plasma membrane of healthy cells (as exemplified in Figure S1c). In agreement with previous observations, exposure of cells to a high concentration of the

amino-modified polystyrene nanoparticles in typical cell growth medium (4 mg/ml FBS) caused cell death, as indicated by the increment in the percentage of PI-positive cells from <5% to 15% after 6 h of exposure, and up to 90% after 24 and 48 h (Figure 1a). Strikingly, when cells were exposed to the nanoparticles in medium at increasing protein concentrations, despite the fact that all dispersions contained the same dose of nanoparticles, a clear trend could be observed where an increase in protein concentration led to a decrease in cell death. In the extreme cases of 20 and 40 mg/ml of serum proteins in the medium, cell death levels were as low as in untreated control cells (Figure S1c). Though there are quantitative differences, the same trend was observed for exposure to nanoparticles in increasing concentrations of HS (Figure 1b).

The impact of the amino-modified polystyrene nanoparticles on cells was also analyzed in terms of cell energy, by measuring the intracellular levels of ATP after exposure to the nanoparticles for 24 h (Figure 1c). In line with the cell viability results (Figure 1a-b), ATP levels decreased when cells were exposed to the amino-modified nanoparticles in the presence of low protein concentrations (4 mg/ml), but were significantly less affected in the presence of high concentrations of protein (40 mg/ml). This tendency was again present for both FBS and HS.

Amino-modified polystyrene nanoparticles are known to arrest the cell cycle when exposed to cells at sub-lethal doses.[24, 25] Therefore, we also analyzed cell cycle progression after nanoparticle exposure (Figure 1d) by measuring cell proliferation with EdU (5-ethynyl-2'-deoxyuridine). This nucleoside analogue is only incorporated by healthy cells performing active DNA synthesis. As expected from previous studies, when cells were exposed to sub-lethal doses of the amino-modified nanoparticles in the presence of low concentrations of proteins (4 mg/ml) the proportion of cells synthesizing DNA (EdU-positive) decreased. When the same nanoparticle dose was applied in the presence of higher protein concentrations (40 mg/ml), DNA synthesis instead remained high, at levels comparable to untreated control cells. Once more, this conclusion holds for both FBS and HS.

Altogether, the results (Figure 1) suggest that the presence of high concentrations of proteins "protects" cells from the nanoparticle-associated cytotoxic effects observed in typical *in vitro* conditions (10% serum), in terms of cell death, ATP levels and cell proliferation. Furthermore, though modulated in magnitude by the origin (bovine or human) of the proteins, the general effect is the same for FBS and HS.

To investigate further these outcomes, we used flow cytometry to measure nanoparticle uptake under the different exposure scenarios. Interestingly, we found that the uptake levels of the amino-modified polystyrene nanoparticles decreased dramatically as the nanoparticles were administered in dispersions with increasing protein concentrations (Figure 2). Again, even though there were quantitative differences, the general outcome that higher protein concentration decreased uptake was independent of whether the proteins were FBS or HS. In order to determine if this effect was dependent on nanoparticle type, we performed the same experiment for fluorescently labeled silica nanoparticles. Lower uptake was observed at increasing content of proteins in solution also for silica nanoparticles (Figure S2), and a similar trend has recently been described for nanoparticles targeted for the transferrin receptor as well.[26] Thus, by simply increasing the amount of serum in the dispersion medium, the actual nanoparticle dose internalized by cells decreases.

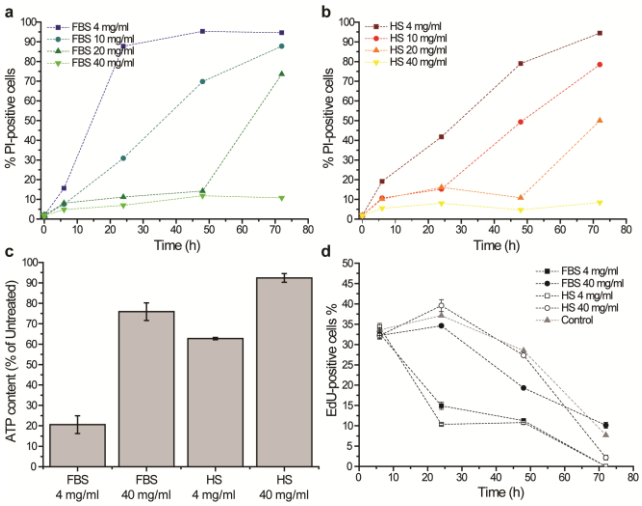

Fig. 1 Nanoparticle impact on cells depends on extracellular serum concentration. A549 cells were exposed to 100 µg/ml amino-modified polystyrene nanoparticles in the presence of different concentrations of serum proteins. (a-b) Cell death (quantified as the percentage of PI-positive cells) as a function of exposure time to nanoparticles in (a) FBS or (b) HS. Cell death is substantial when nanoparticles are exposed in low amounts of FBS or HS (4 mg/ml), but is much reduced in the presence of increasing serum protein concentrations (10, 20 and 40 mg/ml). (c) ATP content of cell cultures after 24 h of exposure to the nanoparticles, expressed in relation to control cells grown in the corresponding nanoparticle-free media. At low serum concentration (4 mg/ml), cell energy decreases substantially, but at higher serum concentrations (40 mg/ml) the decrease is much less substantial. (d) Effect of nanoparticle exposure on cell proliferation. DNA synthesis, quantified as the proportion of cells incorporating EdU, decreased only for cells exposed to nanoparticles in the presence of low concentration of proteins from FBS or HS. (At longer exposure times, DNA synthesis decreases also for control cells due to the cells being deprived of new nutrients and the cell culture reaching confluence.) Error bars indicate standard deviation of three replicas.

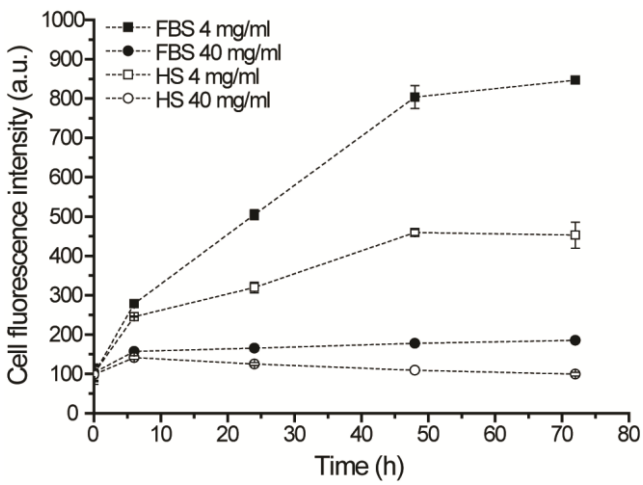

Fig. 2 Nanoparticle uptake depends on extracellular serum concentration. A549 cells were exposed continuously to 100 µg/ml amino-modified polystyrene nanoparticles in the presence of different concentrations of serum proteins, and the intracellular dose quantified in terms of the mean nanoparticle fluorescence per cell using flow cytometry. Error bars indicate standard deviation of three replicas.

To exclude that the observed effects were due simply to a difference in agglomeration, the nanoparticle dispersions obtained at the different protein concentrations were characterized (Figure S3-4). The results suggest no major difference in dispersion state between the different conditions, as well as stable dispersions for both the amino-modified polystyrene and the silica nanoparticles.

Importantly, these results overall showed that low intracellular nanoparticle doses (Figure 2) were achieved under the same conditions in which the nanoparticles induced a low cytotoxic response (Figure 1). Thus, the toxic effect induced by the amino-modified nanoparticles correlates with the dose achieved inside cells, and this, in turn, is determined by the serum concentration in the nanoparticle dispersion.

In summary, amino-modified polystyrene nanoparticles were utilized to determine the role of exposure conditions on their effect on cells, since these nanoparticles are known to be able to trigger cytotoxic responses. We have shown that by exposing cells to the same nanoparticle dose that under usual *in vitro* conditions induces cell death, one can essentially completely mitigate the cytotoxic response simply by increasing the concentration of serum proteins in the medium in which the nanoparticles are dispersed. The effect can be correlated to the lower nanoparticle internalization measured in the presence of higher serum concentration, an outcome that is independent of the origin of the serum protein and is observed also for other nanomaterials.

The origin of the observed lowered uptake still needs to be clarified in detail. Possible explanations of this may include competition of the free molecules in solutions for entry sites on cells, macromolecular crowding effects, different biomolecular coronae on the particles, different adhesion properties on the cell membrane and others. Detailed studies are needed to clarify whether these or other factors are the source of the observed effect on uptake. Nevertheless, the outcome cannot be ignored and implications are clear. From a broader perspective, it may be time for nanomaterial hazards testing to depart from the traditional *in vitro* conditions inherited from molecular studies, and move towards more realistic exposure conditions relevant to the system investigated. Again, we stress that these effects are connected to the unique properties of nanoscale materials, where the dispersion medium does not only serve the purpose of feeding the cells, but also confers new properties to the nanoparticles and how they interact with cells. Thus, for nanoparticle-cell interactions, future studies should clearly consider and report the serum concentration used for *in vitro* testing – in particular for comparative studies on the same material.

Conclusions

Overall, the implications of the reported effect are vast and span multiple levels. Further studies will be required to address these points fully, but we feel it is important to lay them out for consideration.

First, the effect of the medium in which nanoparticles are dispersed should be taken into consideration when comparing *in vitro* studies on cell lines cultured at different serum content, such as for instance cells grown at 2% or 5% serum rather than 10% serum, particularly in cases where different labs use different serum content to culture the same cell type. The different serum content applied could explain the sometimes poor correlation between different studies[27] and in part also disparate results on different cell types. Moreover, not only the total concentration but also the protein composition as well as the treatment imparted to the serum (such as heat inactivation) later used for nanoparticle dispersion affect nanoparticle uptake levels.[28] Proteomic characterizations are available,[29,30] and could be useful in future.

Second, for cells typically in contact with blood serum (such as endothelial cells), the use of lowered serum content should be questioned, as it does not represent a condition relevant to real *in vivo* exposure scenarios. Related to this, the origin of the serum similarly needs to be taken into account and the use of proteins of bovine origin for human cells can be questioned. Even though the homology of bovine and human proteins is rather high, the effect of biomolecules in the nanoparticle corona may be emphasised by switching to human serum (or plasma) on human cells, where affinities of human proteins for human receptors will necessarily be higher than bovine proteins. The results presented here already indicate that, even if the overall trend is the same when increasing protein content, there are quantitative differences between human and bovine serum, suggesting that the serum species indeed also plays a role. Furthermore, it is important to consider that most cell lines now used for *in vitro* testing, even when of human origin, have been isolated several years or even decades ago and have been grown in simplified *in vitro* conditions ever since. Thus, it will be important to determine the implications of this on potentially acquired behaviors, and assess if possible to simply switch now their growth conditions.

Third, from a broader perspective, culturing conditions may need to be standardized in order to mimic different exposure routes or applications, such as lung surfactant fluid, blood plasma and others. Thus, different fluids may be used when studying for instance nanoparticle interactions with lung epithelia or gut epithelia, rather than serum. Several clusters and consortia at European and international level have started to address this issue and it will be important to monitor the outcomes of such studies. On such a foundation, the translation of *in vitro* results into *in vivo* outcomes may be clarified and more robust *in vitro* platforms for alternative testing strategies may be developed.

Another important implication emerging from these results is that when moving towards biological fluids closer to *in vivo* conditions, the overall internalized nanoparticle dose can be strongly reduced and as a consequence of this, some of the effects observed under standard *in vitro* conditions could be mitigated – or even disappear. This suggests that such effects may be hard to be observed *in vivo*. This may also have implications in sub-lethal toxicity effects, including those in the ecotoxicology field.[31, 32] On the other hand, the effects observed under standard *in vitro* conditions may still be relevant in the case of biopersistence and bioaccumulation, making the question of nanoparticle degradation or export another central matter for the field. Cases of biopersistence and accumulation raises the difficult question on how to best measure dose in nanoparticle interaction studies. It may be that a dose-response may be better described in terms of a dose metric relating internalized nanoparticle dose (rather than applied dose) to biological response. Other parameters such as cell heterogeneity (for instance in terms of cell area or cell cycle

state) might need to be considered as well.[33, 34]

Finally, while we have focused here the discussion on implications for nanosafety testing, clearly several of these aspects are relevant also to nanomedicine and should be considered when testing nanoparticles designed for such applications. First examples have already emerged. Also for nanomedicine applications, poor correlation between *in vitro* and *in vivo* testing is often observed and particles which seem successful *in vitro* may lose their efficacy when tested *in vivo*.[26] The overall implications of exposure conditions on the uptake of these nanoparticles are the same as what has been described here in relation to nanosafety.

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Notes and references

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