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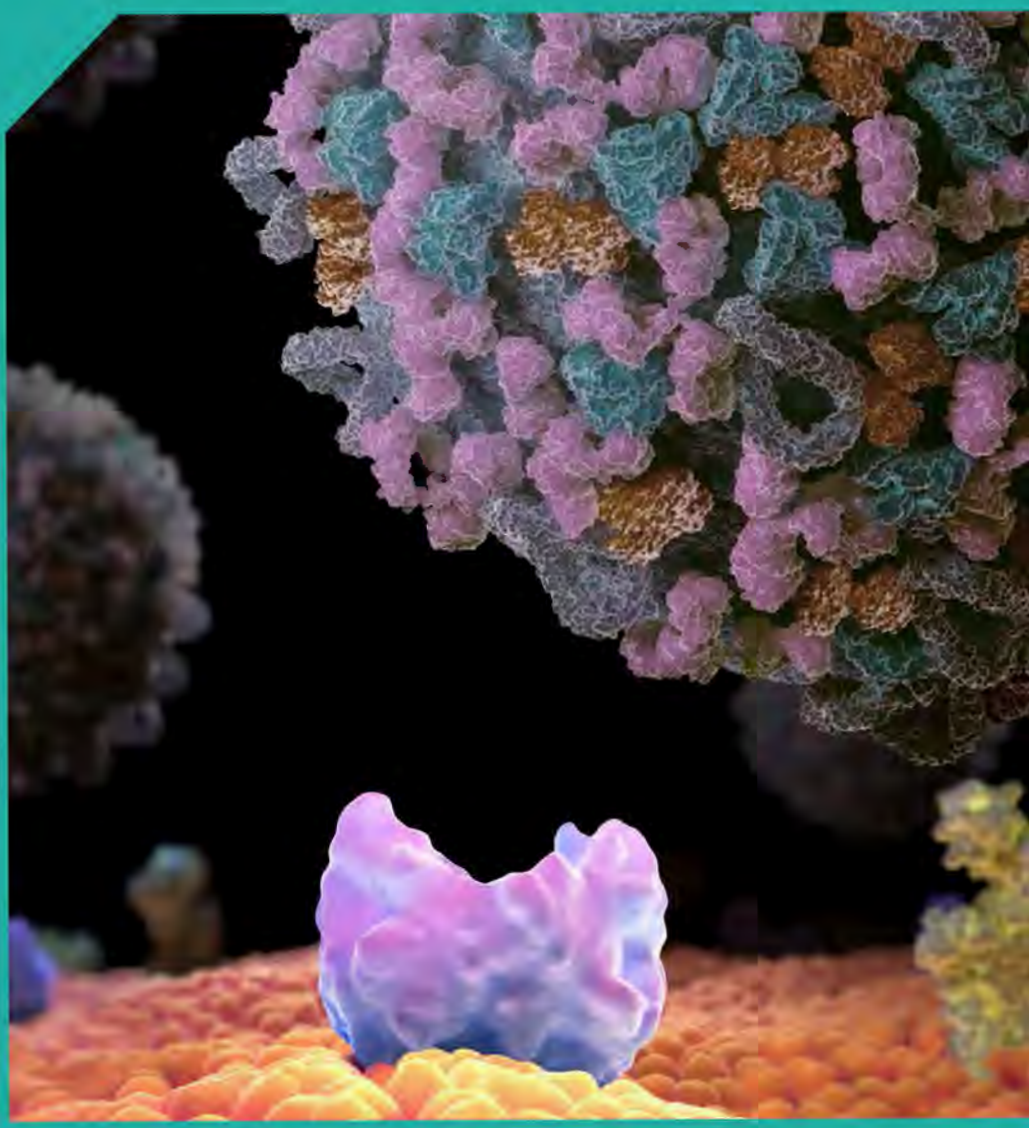
Visualisation and quantification of the interaction of fluorescent nanoparticles with ecotoxicologically relevant species

Authors: Louise Rocks, Sergio Anguissola and Kenneth A. Dawson



Comhshaol, Pobal agus Rialtas Áitiúil
Environment, Community and Local Government

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ENVIRONMENTAL PROTECTION AGENCY

The Environmental Protection Agency (EPA) is responsible for protecting and improving the environment as a valuable asset for the people of Ireland. We are committed to protecting people and the environment from the harmful effects of radiation and pollution.

The work of the EPA can be divided into three main areas:

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Advocacy: *We work with others to advocate for a clean, productive and well protected environment and for sustainable environmental behaviour.*

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- intensive agriculture *(e.g. pigs, poultry)*;
- the contained use and controlled release of Genetically Modified Organisms *(GMOs)*;
- sources of ionising radiation *(e.g. x-ray and radiotherapy equipment, industrial sources)*;
- large petrol storage facilities;
- waste water discharges;
- dumping at sea activities.

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- Office of Environmental Enforcement
- Office of Environmental Assessment
- Office of Radiological Protection
- Office of Communications and Corporate Services

The EPA is assisted by an Advisory Committee of twelve members who meet regularly to discuss issues of concern and provide advice to the Board.

EPA Research Programme 2014–2020

Visualisation and quantification of the interaction of fluorescent nanoparticles with ecotoxicologically relevant species

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EPA Research Report

Prepared for the Environmental Protection Agency

by

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DISCLAIMER

The EPA Research Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Executive summary

The present programme aimed to clarify the nature and consequences of the interaction between engineered nanoparticles and the animate and inanimate environment. Given its modest size, no attempt was made to include the very wide range of issues, choices of materials or organisms implied. Instead, we focused on the key questions and on the development of key methodologies and infrastructural support to allow nanosafety to be a strong, credible scientific field. The outcomes of the project were also significant in pathfinding subsequent larger-scale European Union programmes and in framing the advice and explanations given to the European Commission and the public. Furthermore, it had significant impact on the development of a new communication platform, currently available on the website of the Directorate-General for the Environment.

Some of the results of the project were striking in their implications. In accordance with most modern and emerging views, nanomaterials tend not to be acutely toxic, unless they are explicitly prepared to be so. There are some exceptions, easily identified. We did, however, recognise that all nanoparticles examined in a wide range of sizes tend to accumulate within cells, with limited (or no) potential to be cleared from them. We have therefore concluded that many (perhaps most) nanomaterials may be considered to be low-toxicity bioaccumulative materials and have suggested that future research should focus on their longer-term impact, rather than on simple toxicity questions. The project was also one of the first in Europe to show how known high-throughput approaches, such as high-content analysis, could be applied as a screening technique to evaluate the safety of nanomaterials, examining multiple endpoints and giving an overall snapshot not only of the acute toxicity but also of some of the more subtle signalling responses that may have implications for longer-term accumulation. This approach is now becoming widely accepted, and it is also the basis of a number of large European Union-funded projects.

This question of accumulation was considered for aquatic species. Zebrafish embryos were exposed to nanoparticles shortly after fertilisation for up to 120 hours and showed accumulation in the chorion, although nanoparticles were not observed inside the animal. *Daphnia magna*, a filter feeder, accumulated nanoparticles in the digestive tract. The microscopic nature of these accumulations (whether intracellular or not, for example) is not yet clear. However, once more, the key issue is associated with accumulation, rather than overt toxicity.

The project focused on one nanomaterial that is very widely used in the information technology industry in Ireland (nanosilica) and a model particle (polystyrene) as a reference state, as well as several other, less detailed, studies involving other inorganic oxides and carbon materials. The statements made above also pertain to those materials and to many others examined in less detail.

Other aspects of the project were geared more towards the building of systematic knowledge or enabling tools. Particular stress was placed on novel or state-of-the-art physico-chemical characterisation of the nanoparticles in the relevant media (environmental and other biological fluids), rather than only in their pristine form. Their stability and interactions with biomolecules contained in such fluids, and the assessment of their ability to enter cells and species relevant for environmental toxicology and exert biological activities, was explored. In particular, we have evidence for the influence of their interactions with environmental and biological fluids (how the environment modifies the nanoparticles) on their reported biological activity. Various aquatic species, widely accepted as appropriate sensors for environmental impact, including *Pseudokirchneriella subcapitata*, *D. magna*, *Thamnocephalus platyurus* and *Vibrio fischeri* were explored. These studies highlighted an issue that has been emerging over the last few years, that is, the importance of ensuring that both the characterisation, and all subsequent *in vitro* testing, of nanoparticles should be performed in media containing relevant molecules to ensure that the exposure is meaningful.

1 Introduction

1.1 Objectives

The fast-paced development of nanotechnology, with the introduction of many diverse particle types, is such that a detailed case-by-case analysis of safety will be difficult. Instead, early responses should focus on identifying the key questions to be asked in research and an effective screening safety testing approach that is rapid and inexpensive to apply. This was what we attempted to achieve with this project, which we consider acted as a pathfinding study with multiple outcomes and consequences at the European level.

In this project, we investigated the mechanisms of interactions of nanoparticles (NPs) with species relevant for environmental toxicology and *in vitro* cell cultures in order to elucidate the mechanisms of action of nanoparticles to gain a better understanding of their safety. We particularly wished to identify generic mechanisms that might allow us to generalise and elevate the type of questions that could be asked in future, such as mechanisms that might lead to impacts not evidently recognisable from acute and screening tests. In addition, we sought to develop (essentially for the first time) the (effective and realistic) use of high-content analysis (HCA) methods that could be used to identify acute toxicity. These could then be used to rapidly identify materials that have any obvious acute toxicity, and to prioritise relevant studies for those that do not but that could still raise subtle questions owing to longer-term accumulation.

We also aimed to innovate in terms of the development of the conditions of exposure and their role in drawing meaningful conclusions. This included the physico-chemical characterisation of the nanoparticles in the relevant media (environmental and other biological fluids), in terms of their stability and interactions with biomolecules contained in such fluids, and the assessment of their ability to enter cells and species relevant for environmental toxicology and exert biological activities. In particular, we have evidence for the influence of their interactions with environmental and biological fluids (how the environment modifies the nanoparticles) on their reported biological activity. We decided to make in-depth studies with two classes of materials and look in a more general manner at many others commonly available. The silicas (believed to be not acutely toxic) were of direct interest to Irish industry, where they are used at very high levels, as was polystyrene, which allows us to modify the surfaces and purposely introduce different effects to induce a range of toxicities, thereby enabling us to develop HCA around known materials of high toxicity. The positively charged polystyrene particles have subsequently become recognised as a positive control material for future HCA studies of other nanomaterials and are now used across Europe in that manner.

A broad range of silica and polystyrene nanoparticles of different sizes and with different surface modifications were either synthesised or obtained from commercially available stocks. The chosen nanoparticles demonstrated good size distribution and stability in biological and environmental fluids. The investigated panel of nanoparticles was tested using models of aquatic species, widely accepted as appropriate sensors for environmental impact, including *Pseudokirchneriella subcapitata*, *Daphnia magna*, *Thamnocephalus platyurus* and *Vibrio fischeri*.

1.2 Selection of model systems and materials

1.2.1 Nanoparticles

Several silica and polystyrene nanoparticles with sizes between 50 and 300 nm and different surface modifications, unlabelled and labelled with fluorescent dyes, were obtained from commercially available stocks to perform the tasks outlined in this project. In a number of cases, appropriate particles were not available, and these were synthesised for the project. The use of this panel of particles enabled highly accurate and reproducible measurements to be made in several parts of the study (e.g. cell uptake) that have subsequently become reference studies. Some nanoparticles were selected because they had been previously shown to have no biological impact on cells but to have stable and fixed (internal) dye labelling and, therefore, their interactions with aquatic species and cells could be monitored over extended periods of time. At the same

time, nanoparticles with specific surface modifications that confer a positive charge (amine groups – NH_2 – or polyethylenimine – PEI) or a negative charge (sulphonyl – OSO_3H – and carboxyl – COOH – groups) were also investigated to elucidate the effect of surface charge on the biological fate of nanoparticles, their uptake, localisation and eventual biological impact. A list of the commercial nanoparticles used is given in Table A1.1 (see Appendix 1).

In addition, the partners from University College Cork addressed a key challenge of the project: the development of nanoparticles with improved optical properties, optimised for *in vivo* studies. A range of reproducible yellow–green fluorescent and near-infrared (NIR) labelled silica nanoparticles, as shown in Table A1.2, were synthesised in the size range 20–300 nm (similar to the commercially available samples). The particles listed in both tables were tested in *in vitro* and *in vivo* assays. Efforts were made to ensure reproducible data of high quality at all stages. Extensive characterisation of all nanoparticles was undertaken to significantly reduce the opportunity for artefacts and anomalous results. One potential source of such artefacts is the leakage of free dye from nanoparticles labelled by embedding a fluorescent marker in the polymeric matrix (Tenuta et al., 2011). This leaching was overcome for materials developed specifically for the project, but it was recognised as a potentially difficult aspect of using commercially available materials. In any case, it can (and should) be checked for in all future studies.

1.2.2 Dispersion of nanoparticles

In order to investigate and correctly relate variation in biological behaviours to nanoparticle properties, it is necessary to comprehensively measure the physico-chemical features of nanomaterials and their dispersions. Both primary nanoparticle size and dispersion size in simple media and more realistic exposure conditions (such as *in vitro* cell culturing media and ecological exposure medium) have been investigated using dynamic light scattering and zeta potential measurements. Upon contact with serum, the particles are coated by proteins and some larger particle–protein complexes are formed (Cedervall et al., 2007). These dispersions were quite stable (as were the particle–protein coronas) for the duration of our experiments (see, for example, Walczyk et al., 2010).

Nanoparticles employed in biological and ecological fluids have been shown to be subject to powerful controlling features such as salt-induced screening/bridging and biomolecule adsorption/interaction. Such interactions can drive processes such as loss of ligand functionality through surface ligand exchange, leading to loss of functional groups, or through functionality shielding by corona adsorption.

1.2.3 Environmental toxicology models and assays

As a model for environmentally relevant toxicological effects, RTG-2 and PLHC-1 fish cell lines were utilised in conjunction with two cytotoxic indicator dyes, namely alamarBlue and Neutral Red. Furthermore, following exposure of nanoparticles to aquatic species, a number of standard and representative environmental toxicity tests were performed. These included microtox acute toxicity, algal growth inhibition, thamnotox acute toxicity and immobilisation tests. Zebrafish embryos (at 2 h post fertilisation) were also exposed to nanomaterials to assess development and mortality effects. These tests were focused on the effect of surface charge, and therefore negatively charged NIR SiO_2 nanoparticles synthesised at University College Cork and positively charged polystyrene (PS-PEI) sourced at University College Dublin were used for testing.

To qualitatively assess the uptake and clearance and/or bioaccumulation of nanoparticles over time, we exposed *D. magna* neonates to 50- and 100-nm fluorescently labelled nanoparticles. Confocal microscopy was used to image the neonates in localisation studies.

Zebrafish were employed as a vertebrate model to study developmental toxicity; the embryos are transparent, which allows for easy detection of morphological alterations and manipulation. To complement the uptake studies in cells *in vitro*, dispersions of both commercial SiO_2 (50 nm) and PS-OSO_3H (40 nm) nanoparticles at 20–50 ppb concentration were prepared, and zebrafish embryos were allowed to develop in these dispersions from 2 h to 72 h post fertilisation.

1.2.4 **In vitro** *toxicological models and assays*

A range of silica and polystyrene nanoparticles, 40–100 nm, labelled with fluorescent dyes and with no observable effect on cell viability, were used to assess the uptake and trafficking of nanoparticles in A549

(adenocarcinoma human alveolar basal epithelial) cells. To further understand the interactions between nanoparticles and the biomolecules that govern their uptake by cells, we compared uptake of nanoparticles as previously described but in the presence or absence of serum proteins, that is, serum-free conditions. Confocal and transmission electron microscopy (TEM) were combined to investigate nanoparticle intracellular localisation, together with flow cytometry to quantify fluorescence intensity due to nanoparticle uptake.

1.3 High-content analysis platform

In parallel with uptake and localisation studies by fluorescence imaging, electron microscopy and flow cytometry, and the assessment of nanoparticle impact on cells and aquatic species, this project also focused on the development and implementation of HCA automated epifluorescence microscopy as a platform for assessing nanoparticle impact on cells in a multi-parameter approach. With new materials being increasingly developed, there is the need to develop high-throughput screening approaches to be used for fast and effective safety assessment of nanomaterials. To this end, we used HCA to expose cell lines representative of different organs to the same positively charged polystyrene nanoparticles, known to induce toxic responses, and negatively charged nanoparticles, which acted as a negative control and have no acute toxicity.

1.3.1 Establishment of an HCA platform to identify the cellular pathways of toxicity

In order to establish HCA as an *in vitro* platform to assess the potential impact of nanomaterials on human health and identify the cellular pathways activated in response to nanoparticles, we selected a panel of cell lines as representative cells from the main routes of exposure, accumulation and clearance of nanoparticles from the body. A549 and R3/1 alveolar adenocarcinoma cells were selected to mimic the inhalation route, RAW 264.7 macrophages and hCMEC/D3 endothelial cells were used as model for the interaction of nanoparticles with the immune system and the endothelium that forms the blood vessels, respectively, HepG2 hepatocellular carcinoma cells were used to study eventual effects on the liver, and 1321n1 astrocytoma and SHSY5Y neuroblastoma cells were chosen to monitor potential neurotoxic effects. Finally, HEK293 embryonic kidney cells were selected to assess the potential impact on the kidneys as the main clearance route.

2 Key results

2.1 *In vitro* assessment of the environmental toxicology impacts of nanoparticles

Although the predicted environmental concentrations of nanoparticles are thought to be low – available exposure estimates for surface waters indicate that concentrations of silver nanoparticles range from 30 to 80 ng/l; metal oxide nanoparticles such as TiO_2 concentrations will range from 0.7 to 16µg/l; and concentrations of carbon nanotubes will range from 0.5 to 0.8 ng/l (Mueller and Nowack, 2008) – it is not yet realistic to study the effects of such low concentrations in acute toxicity testing. Therefore we have adopted the usual practices, working at higher concentrations and seeking to extrapolate. It is believed that environmental effects (if they are relevant at acute levels) will be also be manifest under more realistic exposure conditions. However, there is also the potential for "hotspots" of exposure in specific product value chains to arise, and these must also be considered. Furthermore, in addition to acute studies, we later stress the potential for nanomaterial accumulation, which must also be considered.

2.1.1 Exposure to fish cell lines

The negatively charged silica (SiO_2) nanoparticles synthesised at University College Cork and positively charged polystyrene (PS-PEI) sourced at University College Dublin (both at 55 and 110 nm) were used for *in vitro* cytotoxicity testing on two fish cell lines, RTG-2 and PLHC-1, with the aid of two cytotoxic indicator dyes, alamarBlue and Neutral Red. The assays indicated that both 55- and 110-nm NIR SiO_2 nanoparticles did not cause adverse effects, and a reduction in viability was only observed at high concentrations (1000 ppm) and following long exposures (96 h). Conversely, PS-PEI nanoparticles showed a time- and dose-dependent decrease in viability in both assays and for both cell lines.

In both assays, the 110-nm particles showed a slightly higher cytotoxicity than the 55-nm particles, suggesting that the effect observed could result from multiple contributing factors, the reactive functional groups on the nanoparticle coating and core size effects. It should be noted, however, that at concentrations of 1000 ppm for the silica particles, and 200 ppm for the PS-NH_2 particles, the particle size increased to over 200 nm and 3 µm, respectively, indicating considerable particle aggregation at these concentrations in the fish cell culture medium. This may result in significant depletion of the medium, leading to an indirect toxic effect, as observed in the case of carbon nanotubes in mammalian cells (Casey et al., 2008) and, furthermore, any interaction of such aggregates with the cells should be considered as an indirect nanoparticle effect.

2.1.2 Exposure to aquatic species

The same particles were also tested against several standard environmental toxicity tests (as mentioned in section 1.2.3). Silica nanoparticles showed no significant toxicity in any of the acute environmental toxicity tests performed on the different organisms for both diameters. In contrast, both 55- and 110-nm PS-PEI nanoparticles showed a significant toxic response, owing to their positive charge (Nemmar et al., 2003), in most of the acute toxicity tests performed, except for the microtox test, where a much weaker effect was observed. A summary of the half-maximal effective concentration (EC_{50}) values is shown in Figure 2.1, wherein a difference in the sensitivity for both nanoparticle diameters with the different organisms was observed as follows: *P. subcapitata* > *D. magna* > *T. platyurus* > *V. fischeri*. The difference in sensitivity observed was found to be in accordance with studies using other nanoparticles found in the literature, in which algae and crustaceans (daphnids) were the most sensitive organisms in aquatic exposure to nanoparticles (Kahru and Dubourgier, 2010). The effects observed were dependent on the nanoparticle size in the cases of *D. magna* and *T. platyurus*, indicating that the effect observed could be not only due to the reactive functional groups on the nanoparticle coating but also a possible core size effect, as, per unit mass/volume concentration, the 55-nm particles present a higher degree of surface functionality than the 110-nm particles. No difference in cytotoxicity was observed in the case of the algal test.

The same phenomenon was also observed in the case of the *D. magna*.

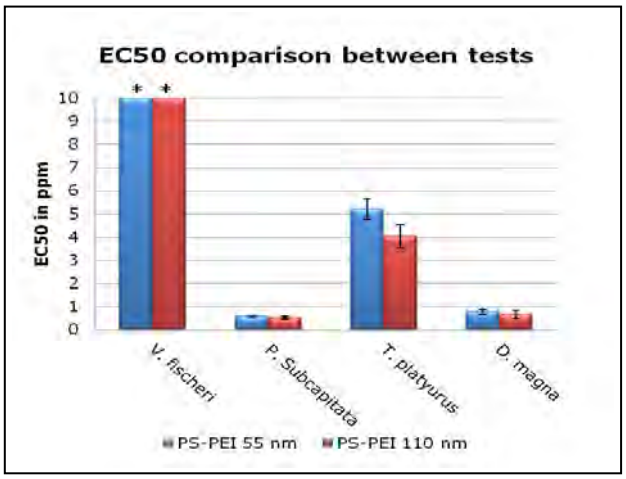

Figure 2.1. EC_{50} values for PS-PEI nanoparticles front toxicity test. *The EC_{50} value in the microtox test could not be determined using concentrations up to 1000 ppm.

Overall, these results show the importance of the surface charge and size of the nanoparticles on the different responses obtained and the different sensitivity of the organisms to these particles, probably as a result of their agglomeration, and hence bioavailability, when they were presented in the different exposure media.

2.1.3 Uptake and depuration studies on D. magna

To qualitatively assess the uptake and clearance and/or bioaccumulation of nanoparticles over time, we exposed *D. magna* neonates to fluorescently labelled nanoparticles and imaged using confocal microscopy for localisation studies. Results showed clear uptake of 50-nm SiO_2 nanoparticles along the gastrointestinal tract (Figure 2.2); no fluorescence was observed in non-exposed animals, as expected.

Confocal images of the depuration over time showed a reduction in fluorescence in the gastrointestinal tract that is consistent with time for both treatments, although some fluorescence was still apparent after 48 h depuration. This indicates that nanoparticles accumulate significantly in the gut, making it difficult for the neonates to fully remove the nanoparticles ingested over long periods of time. Although fluorescence was detected at all time points, the moulting of the neonates at the later stages made the detection more difficult,

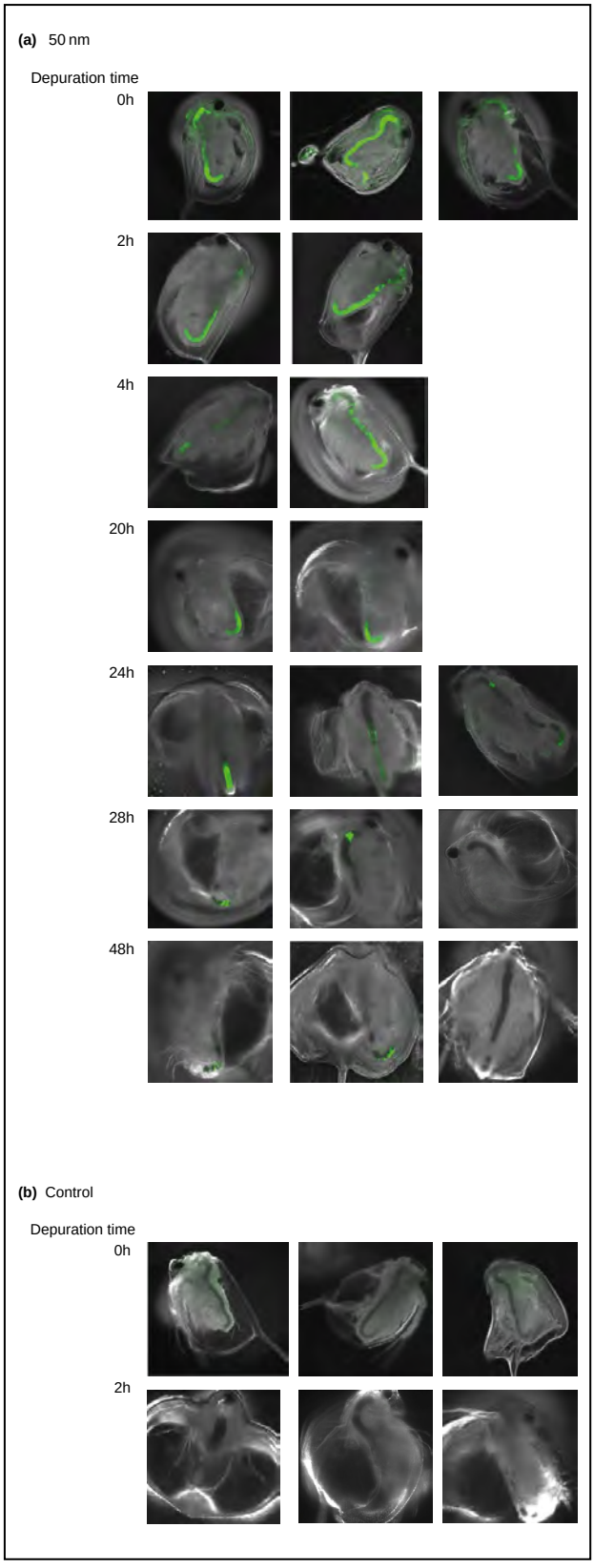

Figure 2.2. (a) Confocal images of *D. magna* neonates exposed to 50-nm fluorescent silica nanoparticles at different depuration times. (b) Unexposed neonates used as controls at different depuration times.

as the position of the neonate on the microscope slide was not compatible with complete observation of the gastrointestinal tract. Therefore, it is very likely that the fluorescence accumulation, and hence nanoparticle accumulation, is actually higher than observed.

It is suggested that outputs are best when oriented to study materials along value chains and outcomes reported in a manner relevant to the needs of each of the stakeholder communities, including specifically industry technology application developers and end users. Therefore, a tiered framework is needed to forecast releases, emissions and exposure, along the value chain and nanomaterial life cycle. Such investigations are ongoing under the umbrella of the University College Dublin co-ordinated FP7 project, FutureNanoNeeds.

2.2 *In vitro* assessment of the toxicological impacts of nanoparticles in human cell lines

2.2.1 Uptake of nanoparticles in human cell lines

Flow cytometry is an analytical procedure to quantitatively characterise several parameters of cells suspended in a stream of fluid. With flow cytometry, we can acquire physical parameters and fluorescence intensity for tens of thousands of cells, thereby obtaining a statistically robust representation of each sample analysed. Using well-characterised standardised procedures for nanoparticle dispersion and exposure to cells, quantitatively reproducible uptake curves can be obtained.

Cells exposed to fluorescent SiO_2 nanoparticles were analysed by flow cytometry and the fluorescence intensity was normalised to the number of nanoparticles in the dispersion and the average fluorescence per particle, allowing for quantitative measurements. The results indicated that SiO_2 nanoparticle uptake is less rapid as particle size increases, but even particles as large as 300 nm entered the cells, with little evidence of a dramatic change in the internalisation kinetics for sizes beyond 100 nm (Figure 2.3). Nanoparticle uptake was linear for the first few hours (Shapero et al., 2011).

Visualisation of nanoparticles in cells or multicellular organisms can be achieved indirectly by measuring the intensity of a fluorescent dye embedded in the nanoparticles. Nanoparticles can also be observed directly using TEM when their density is significantly different from the density of the tissue. Confocal microscopy and electron microscopy clearly showed that the final localisation of both the 50- and the 100-nm SiO_2 nanoparticles was in the lysosomes. This was particularly clear after long exposure times, as shown in Figure 2.4 after 24 h of uptake. Earlier events of uptake and trafficking in cells are more complex. Fluorescence microscopy showed that, after 2 h of exposure to SiO_2 nanoparticles, there was no clear evidence of co-localisation with endosomal structures, or lysosomes. However, even after 24 h of exposure, there was no evident co-localisation with endosomes. The time course of the TEM showed that in the first 10–30 min SiO_2 nanoparticles crossed the cell membrane and entered early endosomal structures. After 4 h, as shown in Figure 2.5, SiO_2 nanoparticles reached the lysosomes (Figure 2.5a and b; 50- and

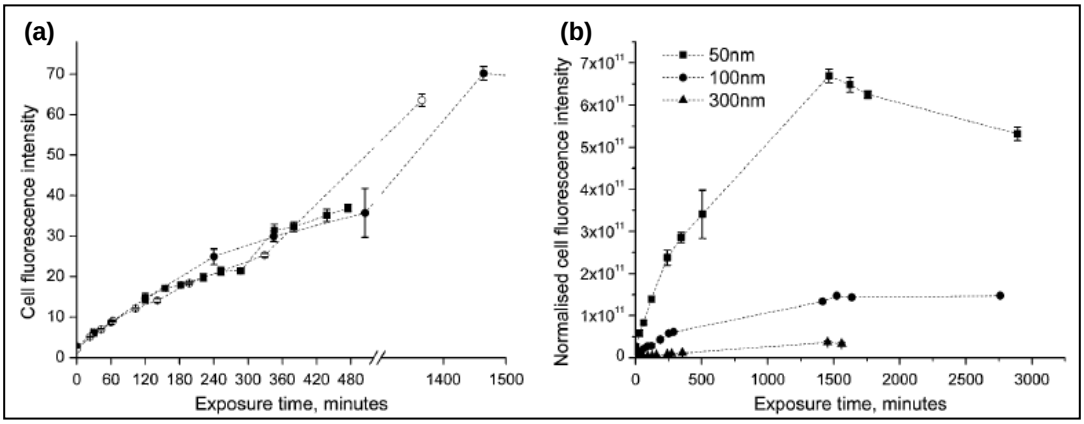

Figure 2.3. (a) Three replicas of a time-resolved uptake study of 50-nm SiO_2 nanoparticles exposed to A549 cells at 100 µg/ml. (b) Normalised uptake kinetics of 50-, 100- and 300-nm SiO_2 nanoparticles exposed to A549 cells at 100 µg/ml. The cell fluorescence intensity is normalised by the fluorescence intensity and the number of nanoparticles in the starting dispersion.

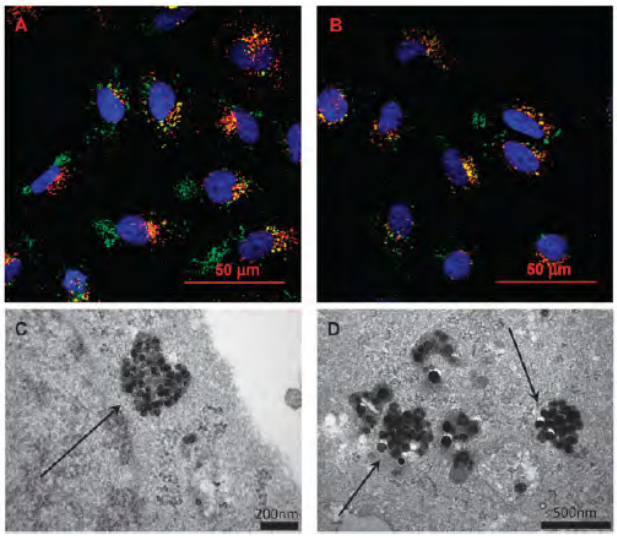

Figure 2.4. Co-localisation of green SiO_2 nanoparticles with lysosomes in A549 cells. Confocal and electron microscopy of A549 cells after 24 h of exposure to 100 µg/ml 50-nm (a and c) and 100-nm (b and d) green SiO_2 particles. Red: immunostaining of lysosomes with LAMP1 antibody (secondary Alexa-647 antibody). Blue: DAPI staining of nuclei. Arrows indicate the localisation of nanoparticles in the cells.

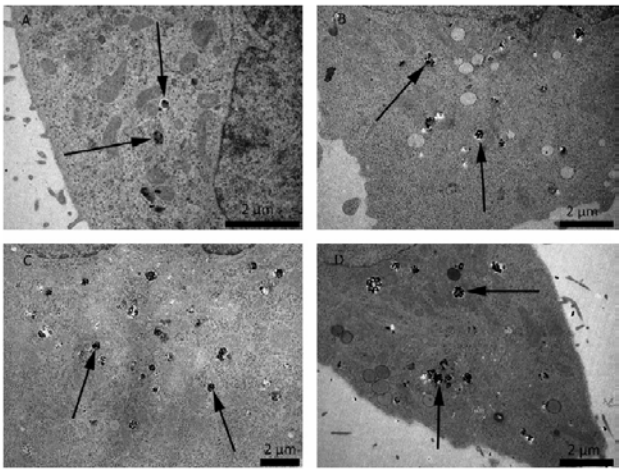

Figure 2.5. Electron microscope images of A549 cells exposed for 4 h (a and b) and 24 h (c and d) to 100 µg/ml 50- and 100-nm green SiO_2 particles (a, c and b, d, respectively), showing the later stages of uptake and nanoparticle sub-cellular localisation. Arrows indicate the localisation of nanoparticles in the cells.

100-nm particles, respectively), and at 24 h lysosomes were highly populated with particles (Figure 2.5c and d for both particle sizes).

The imaging studies indicated that all the tested nanoparticles accumulated in lysosomes. Therefore, we decided to investigate further the nature of uptake and to specifically investigate whether it was a passive or an energy-dependent mechanism. Two approaches are widely used to investigate energy-dependent mechanisms in cells: one uses a chemical agent, sodium azide, to shut down ATP production in the mitochondria; the second approach relies on lowering temperature to inhibit all active processes (enzymes are not active at this lower temperature). Depletion of cellular energy using sodium azide (which inhibits the respiratory chain in the mitochondria, thus impairing the production of ATP in the cell) or lowered cell culture temperature considerably reduced the rate of increase of intracellular fluorescence, corresponding to reduced particle uptake (Figure 2.6). Electron microscopy images of cells after 4 h of exposure to SiO_2 nanoparticles in the presence of sodium azide or at 4 °C confirmed that the uptake largely ceases with energy depletion (Lesniak et al., 2013). These results are in agreement with similar data from the literature regarding the interaction of mesoporous silica with pancreatic and hepatic cancer cells, and silica-coated nanoparticles with HeLa cells, where the authors also found the uptake to be energy dependent (Xing et al., 2005; Lu et al., 2007).

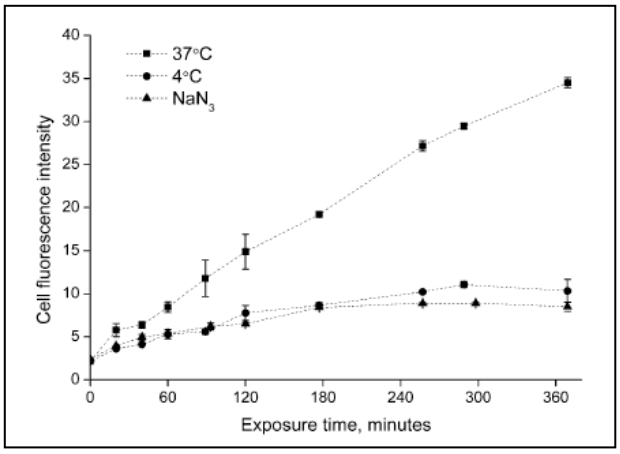

Figure 2.6. Energy dependence of endocytosis of 50- and 100-nm SiO_2 nanoparticles exposed to A549 cells at 100µg/ml at 4°C or with 5µg/ml NaN_3.

2.2.2 Presence or absence of the protein corona affects uptake and sub-cellular localisation of nanoparticles

To further understand the interactions between nanoparticles and biomolecules that govern their uptake by cells we compared uptake of nanoparticles as previously described, but in the presence of serum, that is, in complete minimum essential culture medium (cMEM, containing 10% v/v fetal bovine serum) or the absence of serum proteins, that is, serum-free (SF) conditions. Confocal and TEM were combined to investigate nanoparticle intracellular localisation, along with flow cytometry to quantify fluorescence intensity due to nanoparticle uptake. We found that nanoparticles were also internalised by the cells in the SF medium; however, in these conditions cell damage was also observed (Figure 2.7). Extensive TEM analysis was performed to compare intracellular load and location in cMEM and SF conditions. TEM imaging suggested that uptake in cells exposed to nanoparticles in SF conditions was higher than in cMEM conditions, at the same exposure times, as also quantified by flow cytometry. Moreover, while, in complete medium, nanoparticles were always seen enclosed in vesicles along the endolysosomal pathway (Allouni et al., 2009), in SF medium, along with nanoparticles engulfed in vesicles and in lysosomes, we also found nanoparticles that seemed to be free in the cytosol. We also noted that, after nanoparticle treatment in SF conditions, in many cases a large number of nanoparticles were clustered close to the plasma membrane.

With confocal microscopy we could confirm by immunostaining that the major final localisation of the nanoparticles was in the lysosomes, as in cMEM conditions (Allouni et al., 2009). This was particularly clear after long exposure times (24 h), where a high level of co-localisation could be seen with lysosomal marker (LAMP1)-positive structures, even though in these conditions not all of the nanoparticles were (yet) found there. This could also be related to the presence of nanoparticles that seemed free in the cytosol at TEM analysis. Moreover, confocal imaging confirmed the presence of residual clusters of nanoparticles out of the cells (and also on the glass slide) in SF conditions, as was also noted by electron microscopy. It also clearly confirmed the general observations of higher intracellular load in cells exposed to the nanoparticles in SF conditions.

Imaging and flow cytometry suggested that uptake of nanoparticles in the absence of corona on the nanoparticle surface (SF conditions) was higher, and

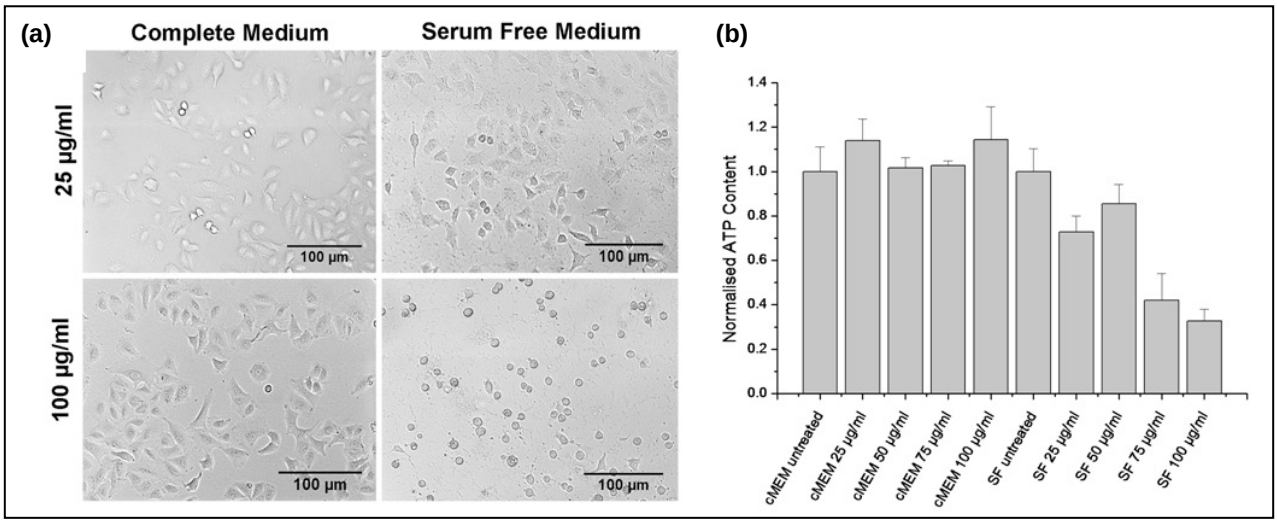

Figure 2.7. Cell viability after exposure to silica nanoparticles in the presence or absence of serum in the medium. A549 cells were exposed to different amounts of 50-nm SiO_2 nanoparticles in complete and serum-free (SF) medium for increasing times. (a) Phase contrast images of A549 cells exposed for 2 h to 25 and 100 µg/ml of 50-nm silica nanoparticles in cMEM and SF conditions. Several cells assumed a spherical shape after exposure to silica nanoparticles in SF medium, indicative of cell death in these conditions. (b) ATP content of cells after exposure to different doses of silica nanoparticles in complete and SF medium for 24 h. A decrease in cell viability could be detected when cells were exposed to silica in the absence of serum.

the interaction of bare surfaces seemed to induce cell damage. We hypothesised that a stronger adhesion on the cell surface in SF conditions could be the explanation (at least in part) for the higher degree of uptake and also the observed impact on cells. In order to evaluate and confirm this hypothesis, we developed a different method to be able to measure nanoparticle adhesion to the cell membrane in the presence or absence of a corona. This allowed us to study nanoparticle adhesion to the cell membrane (in conditions in which nanoparticle uptake is shut down) and its effect on nanoparticle internalisation efficiency, and to exclude the potential presence of residual nanoparticles outside the cell membrane, which could affect the flow cytometry fluorescence levels. The results confirmed that indeed nanoparticles in SF conditions had stronger adhesion to the cell membrane, while the presence of proteins on the nanoparticles, such as after incubation in cMEM conditions, strongly reduced the initial adhesion, and this resulted in a lower internalised dose. We found similar results when we exposed polystyrene nanoparticles to serum of different compositions, which resulted in different amounts of proteins being bound to the nanoparticle surfaces (Lesniak et al., 2010). Overall, this indicated that the presence of proteins adsorbed on the nanoparticle surface from the surrounding environment strongly affects nanoparticle–cell interactions. In the absence of this layer, adhesion of nanoparticles to the cell membrane is much stronger, and this can also have consequences for the impact of nanoparticles on cells (Lesniak et al., 2012).

2.2.3 Proteomic studies following particle exposure

As previously discussed, once nanoparticles are dispersed in a biological fluid, they lower their surface energy by adsorbing free biological species from the surrounding environment. This phenomenon, which has become a well-accepted paradigm, has been defined as “biomolecular corona”. Studying the formation of this biomolecular corona is important for a better understanding of the interactions between nanoparticles and biological systems.

The comparison of uptake of nanoparticles in the presence and absence of serum proteins clearly highlighted different kinetics of uptake and adhesion to the cell membrane. However, even in SF conditions we cannot fully exclude the presence of proteins on the nanoparticles, because of the very high surface energy of the bare material and the observed strong interactions with the cell membrane.

In order to address this question, cells were exposed to the nanoparticles in SF conditions, and, after only 1 h in contact with cells, the extracellular nanoparticles were recovered, in order to investigate whether proteins were already present on their surface after such a short time (Lesniak et al., 2012). Gel electrophoresis was used to detect the eventual presence of proteins on the nanoparticles recovered from cell cultures exposed to 50 nm SiO_2 nanoparticles in SF medium. Indeed proteins adsorbed on the nanoparticles could be found even on nanoparticles originally added to cells in SF conditions. In order to clarify their nature and origin, mass spectrometry was used for their identification. A list of the most abundant proteins that were recovered on the nanoparticles exposed to cells in SF medium is given in Table A1.3.

A corona of a very different nature was found on the particles recovered from cells exposed in the absence of serum in the medium. While in complete medium the major components of the corona were immunoglobulin, complement proteins and apolipoproteins, which are all typical proteins present in the serum used for cell culture, the most abundant proteins that adsorbed on the nanoparticles exposed to cells in SF conditions were mainly cytosolic proteins, components of the cytoskeleton, and proteins normally associated with the cell membrane. These results can be related to the strong adhesion of the bare silica on the cell membrane in SF conditions and can also be connected to the cell damage observed in these conditions. Interestingly, almost none of these proteins could be found on the nanoparticles exposed to cells in complete medium. Moreover, while the protein corona formed in serum is normally composed of only 200–300 different proteins (Cedervall et al., 2007; Lundqvist et al., 2008), it is interesting to note that for the particles recovered from cells in SF medium we could identify more than 800 proteins. This is probably due to the lower concentration of proteins in the recovered SF medium (compared with the protein concentration in serum or, in this case, the cMEM), thus less competition for the nanoparticle surface.

2.3 Development of a high-content screening platform

In parallel with uptake and localisation studies by fluorescence imaging, electron microscopy and flow cytometry, and the assessment of nanoparticle impact on cells and aquatic species, this project also focused on the use of HCA automated epifluorescence microscopy as a platform for assessing the impact of nanoparticles on cells in a multi-parameter approach (Anguissola et al., 2014).

2.3.1 Establishment of an HCA platform to identify the cellular pathways of toxicity

In order to establish HCA as an *in vitro* platform to assess the potential impact of nanomaterials on human health and identify the cellular pathways activated in response to nanoparticles, we selected a panel of cell lines as being cells representative of the main routes of exposure, accumulation and clearance of nanoparticles from the body. A549 and R3/1 alveolar adenocarcinoma cells were selected to mimic the inhalation route. RAW 264.7 macrophages and hCMEC/D3 endothelial cells were used as a model for the interaction of nanoparticles with the immune system and the endothelium that forms the blood vessels, respectively. HepG2 hepatocellular carcinoma cells were used to study eventual effects on the liver, and 1321n1 astrocytoma and SHSY5Y neuroblastoma cells were chosen to monitor potential neurotoxic effects. Finally, HEK293 embryonic kidney cells were selected to assess the potential impact on the kidneys as the main clearance route.

The Organisation for Economic Co-operation and Development (OECD) has compiled a list of recommended *in vitro* and *in vivo* assays to test for toxicity induced by chemicals (OECD, online). These tests are accepted by the scientific and regulatory community for the assessment of industry and consumer products being released onto the market. The same are currently being tested for their applicability to nanomaterials, and have been adjusted when nanomaterials show interference. With numerous new nanomaterials being introduced in industrial and consumer products, these OECD-recommended assays might not provide sufficient output. For this reason we decided to compare two parameters assessed by our HCA screening platform with their respective OECD-recommended *in vitro* assays, in order to assess whether HCA provided similar sensitivity with the advantage of measuring multiple parameters and multiple nanomaterials in the same experiment. Mitochondrial activity measured by tetramethylrhodamine methyl ester (TMRM) was compared with the MTS assay, while uptake of TOPRO-3 resulting from loss of plasma membrane integrity was compared with uptake of propidium iodide (PI) measured by flow cytometry. *C*omparable results were observed with our HCA assay, and also in this case the HCA assessment of cell death was as sensitive as the flow cytometric PI uptake assessment.

2.3.2 HCA resolves apoptotic and necrotic cell death

The key observation here is that HCA can be implemented to highly resolve the whole process of acute toxicity (illustrated by control nanoparticles). Indeed, one can obtain information on the detailed apoptotic and necrotic mechanism, and subsequent studies have also shown that we are able to identify mechanistic details of cell cycle (relevant for developmental toxicology) and many other “systems”-level toxicity responses. We illustrate the process here to show how much more can be learned than in current simple “life and death” toxicity tests. The outcome of this illustrated the potential of HCA to identify many more subtle forms of even mechanistically interwoven toxicity than all current approaches, and lights the way to a new way of thinking, discussed at the end of the report.

Once it had been established that the nanoparticles to be used were reasonably well mono-dispersed in the complete cell culture medium used for these studies, we proceeded to perform dose–response experiments using a multi-parametric approach that measures changes in cell numbers, nuclear size and intensity, mitochondrial membrane potential, cytosolic calcium, acidification of the lysosomes and plasma membrane integrity in the same samples. The panel of selected cell lines was exposed to increasing concentrations of PS-NH_2, PS-Plain and PS-COOH nanoparticles for 24 and 72 h. After 24-h exposure, all the selected cell types displayed changes in the measured parameters in a dose-dependent fashion; PS-COOH and PS-Plain nanoparticles did not cause any alteration in the cellular markers compared with cells exposed to the vehicle, indicating that they did not elicit any cytotoxic response. However, PS-NH_2 nanoparticles showed dose-dependent alteration of all the measured parameters, resulting in acute toxicity for all the cell lines investigated.

Interestingly, for all the cell lines except RAW 264.7, the decrease in nuclear size and increase in nuclear

intensity, indicative of nuclear condensation, associated with mitochondrial depolarisation (TMRM), increase in lysosomal acidification (LysoTracker Green), calcium deregulation (Fluo-4) and plasma membrane permeabilisation (TOPRO-3). This suggested that the cells underwent apoptotic cell death, consistent with previous results (Xia et al., 2008; Bexiga et al., 2011; Wang et al., 2013), and lysosomal damage; the same parameters indicated nuclear swelling after exposure of PS-NH_2 nanoparticles to RAW 264.7 cells, strengthening the hypothesis that these cells underwent cell death through a different mechanism, potentially necrosis.

A very valuable parameter used in toxicology to describe and normalise the response of cultured cells to different agents with variable degrees of toxicity is the half-maximal inhibitory concentration (IC_{50})/EC_{50} value. The most sensitive parameter for PS-NH_2 nanoparticles (i.e. the parameter with the lowest EC_{50}/IC_{50} value) observed for all cell lines except RAW 264.7 was lysosomal intensity by LysoTracker Green, followed by mitochondrial depolarisation, nuclear condensation, nuclear intensity increase, cytosolic calcium and then loss of plasma membrane integrity. This sequence of EC_{50}/IC_{50} values was consistent with apoptotic cell death, as previously reported (Bexiga et al., 2011; Wang et al., 2013). On the contrary, the EC_{50}/IC_{50} values for the measured parameters in RAW 264.7 cells were very similar, suggesting that in this cell line these events happened simultaneously and led to swelling of the nucleus, rather than its condensation, which is consistent with a necrotic type of cell death. This sequence of EC_{50} confirmed, in agreement with previous observations (Wang et al., 2013), that, after nanoparticles were actively taken up by cells through the endocytic pathways and accumulated in the lysosomes, they activated an apoptotic process that led to depolarisation of the mitochondria with subsequent release of apoptogenic factors and activation of the caspase cascade, finally resulting in nuclear fragmentation and condensation and subsequently plasma membrane rupture (Figure 2.8).

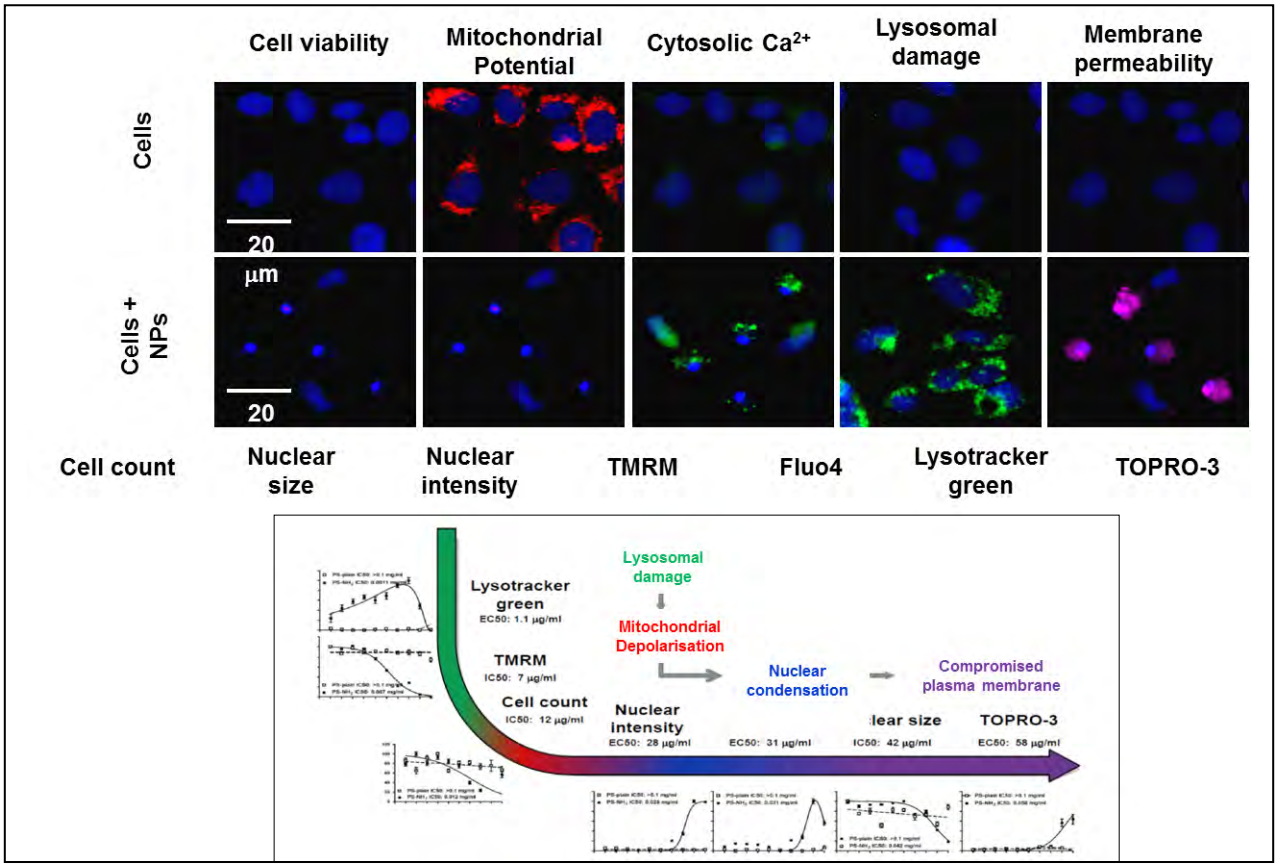

Figure 2.8. Sequences of EC_{50}/IC_{50} thresholds suggest that all cell lines tested except RAW 267.4 execute apoptotic cell death, while RAW 267.4 undergoes a non-regulated form of cell death. EC_{50}/IC_{50} thresholds were calculated for each parameter measured for the selected cell lines exposed to vehicle (ctrl) or increasing doses of PS-Plain, PS-COOH or PS-NH_2 nanoparticles, for 24 h.

2.3.3 Screening of nanoparticle toxicity using HCA

HCA has the potential to analyse several samples in the same experiments; in order to test its potential as a screening tool for nanoparticle toxicity, we exposed astrocytoma 1321n1 and hepatocellular carcinoma HepG2 cells to five different concentrations of nanoparticles, ranging from 0.0001 to 1µg/ml. Thirteen different nanoparticles were used for this screening, after characterisation of their size distribution in the exposure media, as shown in Table 2.1. The two cell types were exposed to all the different nanoparticles at the indicated five doses for 24 and 72 h. HCA analysis was performed for a standard set of parameters, including cell numbers, nuclear morphology/intensity, mitochondrial membrane potential (TMRM), calcium signalling (Fluo-4) and plasma membrane permeabilisation (TOPRO-3).

Of all the nanoparticles tested, only ZnO nanoparticles showed acute toxicity: decrease in nuclear size and increase in nuclear intensity, indicative of nuclear condensation, associated with mitochondrial depolarisation (TMRM), increase in calcium deregulation (Fluo-4) and plasma membrane permeabilisation (TOPRO-3). Silica Ludox Clx showed moderate toxicity, with small changes observed in each of the aforementioned channels, and all the other nanoparticles tested did not cause any change in the parameters analysed (Figure 2.9). These results were consistent with other results reported here and in other publications from the Centre for BioNano Interactions.

Table 2.1. List of nanoparticles assessed in the screening

Particle	Nominal size (nm)
SiO_2	50
SiO_2	200
SiO_2	300
Si-NH_2	50
Si-NH_2	100
Si-NH_2	150
Si-NH_2	200
Si-NH_2	250
Si-Ludox Cl	50
Si-Ludox Clx	50
Cerium dioxide (CeO_2)	70
Zinc oxide (ZnO)	30
Titanium dioxide (TiO_2)	50

2.3.4 Mechanistic studies using HCA

Within this project, we also investigated the potential for extending HCA to assess subtle signalling responses that may have implications for longer-term accumulation.

2.3.4.1 Investigation of lysosomal damage

We have identified that an increase in acidity associated with swelling of the lysosomal compartments is a common feature observed in all cell lines investigated following exposure to PS-NH_2 nanoparticles. Along with our previous findings, this suggests that lysosomal damage is the triggering event that starts the apoptotic cascade. We therefore decided to investigate the mechanisms associated with PS-NH_2 nanoparticle-induced lysosomal damage. It had been already observed many years ago that amphyphylic cationic drugs caused lysosomal damage through accumulation of lipids inside the lysosomes of cells, a phenomenon called lipidosis (Lullman et al., 1978). It was later suggested that a specific class of cationic drugs, aminoglycosides, were able to bind polar lipids and accumulate in the lysosomes, causing the formation of phospholipid-containing myeloid bodies. These bodies would cause the lysosomal membrane to stretch and eventually rupture. More recently, it was also suggested that chloride channels expressed on the lysosomal membranes are central in the regulation of the proton balance inside the lysosomes (Weinert et al., 2010). As these observations were very similar to the swelling and rupturing of lysosomes observed by us previously (Wang et al., 2013), in this study, we employed a LipidTOX™ phospholipidosis and steatosis detection kit for high-content screening (Life Technologies, Carlsbad, CA) to assess the accumulation of neutral and phospholipids in 1321n1 and HepG2 cells following exposure to increasing doses of PS-NH_2 nanoparticles (as in previous experiments) for 24 and 48 h. Exposure to PS-COOH nanoparticles did not cause lipid accumulation. The results suggest that

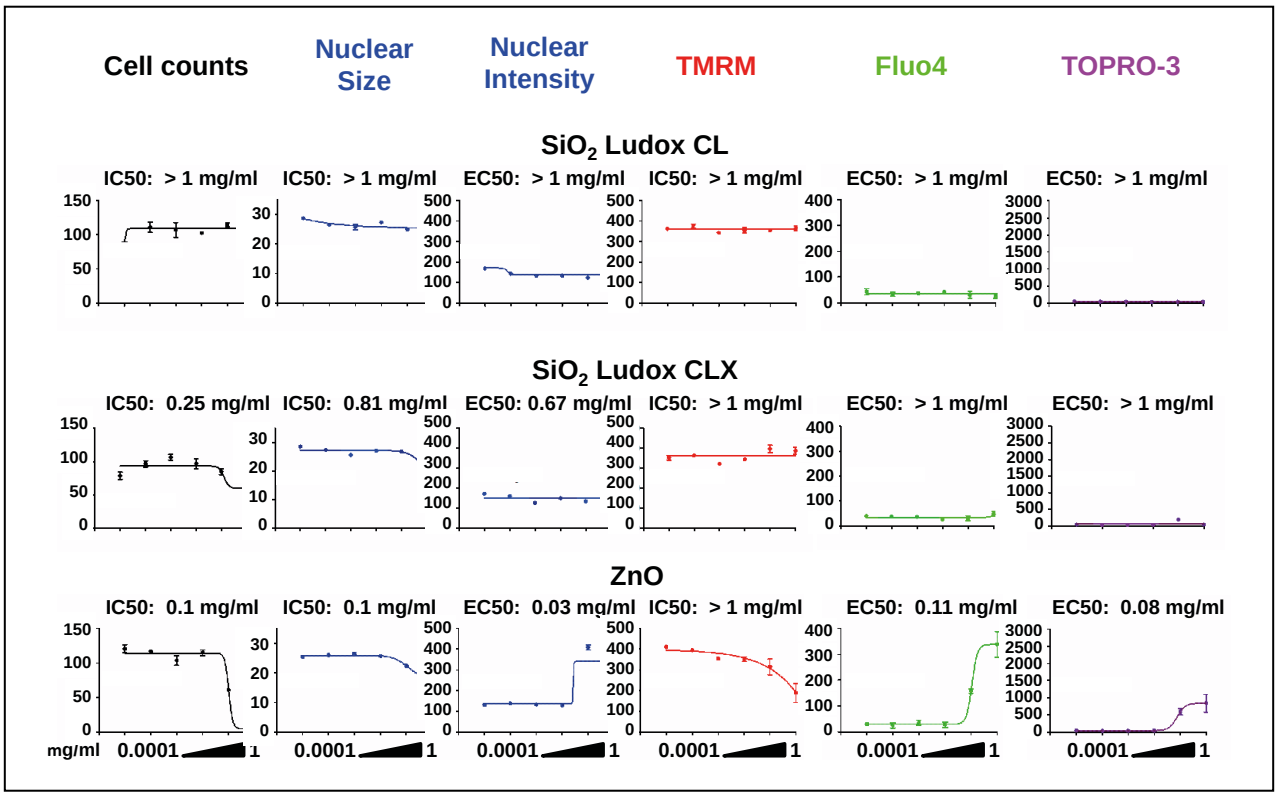

Figure 2.9. Screening of nanoparticle toxicity using HCA. The nanoparticles listed in Table 2.1 were assessed by HCA at the indicated concentrations over 24–72 h. Only ZnO showed an acute toxic response, while silica Ludox Clx showed moderate toxicity and all the other nanoparticles did not affect the cells.

the lysosomal damage caused by PS-NH_2 nanoparticles could be caused by phospholipids trapped on the surface of the nanoparticles that accumulate inside the lysosomes and generate myeloid bodies, which, like aminoglycosides, caused swelling and rupture of the lysosomal membrane. Furthermore, this observation also suggests that LysoTracker Green could be used as a predictive marker for accumulation of phospholipids inside lysosomes caused by cationic nanoparticles (see Figure 2.10).

2.3.4.2 *Caspase-3/-7 activity measured in RAW 264.7, R3/1 and HEK293 cells reveals three distinct responses to the same PS-NH_2 nanoparticles*

HCA illustrated the morphological changes in cells incubated with increasing concentrations of PS-NH_2 nanoparticles coupled with disruption of cellular functions showing cell death via both apoptotic and necrotic mechanisms, depending on the cell line investigated, all of which were confirmed via annexin V and PI co-staining. We first investigated the activation of two effector caspases, caspases-3 and -7, which are downstream of the initiator events of the apoptotic cascade and are common to pathways starting with a stimulus at the level of the plasma membrane, the so-called extrinsic pathway, and those whose stimulus is at the level of the mitochondria, the intrinsic pathway. A chemi-luminenscent assay for caspase-3/-7 cleavage was performed to confirm apoptotic cell death via effector caspase-3/-7 versus necrotic cell death in the three cell lines investigated. R3/1 cells exposed to PS-NH_2 nanoparticles showed an increase in caspase-3/-7 cleavage, confirming that R3/1 cells underwent caspase-mediated apoptosis.

It had been suggested that RAW 264.7 cells follow a necrotic cell death pathway; however, a dose-dependent increase in caspase-3/-7 was observed when they were incubated with PS-NH_2 nanoparticles. HEK293 cells were described as undergoing apoptosis by our HCA and flow cytometry data. However, the caspase-3/-7 activity assay did not reveal any caspase-3/-7 activity, over the exposure times and concentrations measured, that showed clear hallmarks of apoptotic cell death.

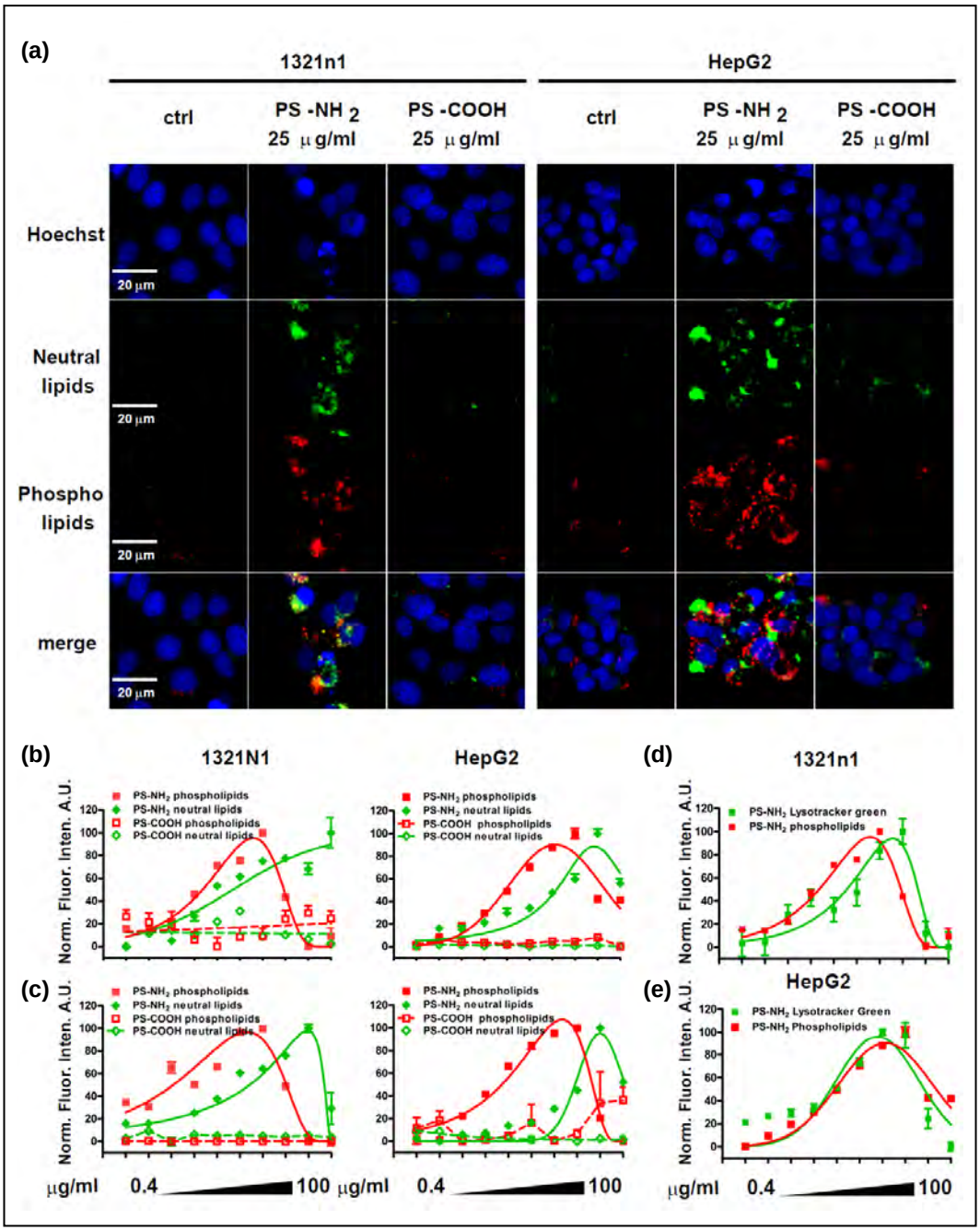

Figure 2.10. PS-NH_2 nanoparticles cause dose-dependent accumulation of phospholipids and neutral lipids in 1321n1 and HepG2 cells. 1321n1 and HepG2 cells were exposed to vehicle (ctrl) or increasing concentrations of PS-COOH or PS-NH_2 nanoparticles for 24 or 48 h and stained with the LipidTOX phospholipidosis and steatosis detection kit (Life Technologies). (a) Representative images of 1321n1 and HepG2 cells exposed to vehicle or 25 µg/ml PS-NH_2 and PS-COOH nanoparticles for 24 h. PS-NH_2 nanoparticles caused intracellular accumulation of phospho- and neutral lipids. Scale bar = 20 µm. (b, c) Graphs showing dose-dependent intracellular accumulation of phospho- and neutral lipids after exposure to nanoparticles for 24 or 48 h. Dose-dependent accumulation was observed in both cell lines after exposure to PS-NH_2 nanoparticles; PS-COOH nanoparticles did not cause lipid accumulation. (d, e) Comparison of LysoTracker Green and phospholipid accumulation after exposure to increasing concentrations of PS-NH_2 nanoparticles for 24 h. Significant overlap can be observed, suggesting that lysosomal swelling is closely correlated with lipid accumulation caused by PS-NH_2 nanoparticles. Data are shown as average ± standard deviation of 45 images acquired from three independent experiments. Data were fitted with a Gaussian curve for visual purposes only.

To further clarify the pathways of apoptosis and necrosis induced in the different cell lines investigated, we subjected protein extracts from cells exposed to PS-NH_2 nanoparticles to immunuoblotting in order to measure the activation of caspase-9, which is activated via the intrinsic pathway of apoptosis. Exposure of R3/1 cells to PS-NH_2 nanoparticles revealed results consistent with activation of the apoptotic pathway discussed previously; RAW 264.7 cells exposed to PS-NH_2 nanoparticles revealed results consistent with the activation of inflammasome-mediated necrosis. Compared with the other cell lines investigated, in the RAW 264.7 cells, we needed to assess caspase activation at shorter exposure times, because extensive protein degradation was observed owing to the rapid cell death process. Interestingly, expression of caspase-9 was absent, which excludes any activation of the apoptotic pathway. The results from the exposure of HEK293 cells to PS-NH_2 suggest that HEK293 cells underwent a caspase-independent type of apoptotic cell death; in addition, exposure to staurosporine, a well-known apoptosis-inducing chemical did not result in activation of caspase-3, suggesting that HEK293 cells do not have a functional caspase cascade pathway.

3 Scientific conclusions

Through this project we have established a paradigm for safety assessment of nanomaterials that involves a series of fundamental steps:

- physico-chemical characterisation of nanomaterials;
- identification of potential artefacts associated with labelling;
- characterisation of the interactions of nanoparticles with the surrounding environment, biological fluids and biological organisms;
- in vitro and in vivo testing for the impact of nanomaterials on human health and on aquatic species.

We have demonstrated that appropriate characterisation of nanomaterials in the relevant biological and environmental fluids used for the study is key prior to safety assessment of nanomaterials. In cells, we observed that fluorescently labelled nanoparticles can contain a significant amount of labile dye which, if not properly assessed, can affect nanoparticle uptake studies. While fluorescence microscopy has been used to provide a very detailed scenario of the intracellular fate of nanoparticles, flow cytometry, a complementary approach also based on fluorescence, allowed quantitative kinetics measurements of nanoparticle uptake. Thus, reproducible and quantitative time-resolved data of nanoparticle uptake into cells have been obtained by using a combination of flow cytometry, fluorescence and electron microscopies. We found that the presence of a layer of biomolecules (corona) on the nanoparticle surface, once in contact with biological fluids, significantly affects cell–nanoparticle interactions. As a consequence of this, the same nanomaterial led to very different biological outcomes when exposed to cells in the presence or absence of such biomolecules in the medium.

Characterisation of the size distribution of nanoparticles in the relevant exposure media is necessary in order to understand the behaviour of the nanoparticle suspension, and its stability over time. Our data have shown that while biomolecules, in particular serum proteins present in the cell culture media, stabilise nanoparticle suspensions, the high salt concentration from environmental media in several cases can destabilise the same nanoparticles and causes them to aggregate. This information is very important in order to understand the eventual differences in the biological impact observed on cells *in vitro* versus in aquatic species.

The nanoparticles sourced within this project included silica and polystyrene nanoparticles, which are currently employed by the food industry as food additives and in packaging materials. Overall, the *in vitro* and *in vivo* studies have revealed that silica nanoparticles did not affect the viability of cells or aquatic species. Only high concentrations of the polystyrene nanoparticles with a positive charge showed acute toxic effects in the organisms investigated.

Finally, we have developed an *in vitro* screening platform based on HCA to characterise the cellular pathways affected by nanomaterials in cells. Following the "3 Rs" of chemical testing – refinement, reduction and replacement – Europe is trying to introduce validated alternative methods to animal testing, whereby *in vitro* testing and the use of simpler organisms (such as zebrafish) are suggested as replacements for testing in rodents. The EU Cosmetics Directive from 2013, which bans the use of animals for the testing of cosmetics products, is the archetypal example of the direction taken by Europe.

Interestingly, we observed that different death pathways were triggered by the same nanoparticles in different cell types: while in most cell types caspase-mediated apoptosis was observed, RAW 264.7 murine macrophages underwent caspase-mediated necrosis and HEK293 hepatocytes executed caspase-independent apoptosis. HCA was also used to screen the effects of several other types of nanoparticles, including silica, zinc oxide, titanium dioxide and cerium dioxide. Therefore, the HCA platform can rapidly provide mechanistic insights on the cellular pathways activated in response to exposure to nanoparticles. Such a platform has great potential for *in vitro* screening of nanomaterials in high-throughput format using complex cell models.

Technical impact summary

The overall pressures implied by the introduction of nanotechnology were discussed in the introduction to this report. The research outlined above leads us to the following conclusions:

1. Although investigations into the impact of nanomaterials in terms of acute toxicity remain important, there should now be much less emphasis on such research. We accept that it would be prudent to continue these research investigations at some level, for particles that may possess novel features (on a case-by-case basis), but in general one now expects that acute toxicity *testing* should be suitably supported as a more applied or industry-oriented programme of testing. We recognise that the use of HCA will make this work effective, rapid and much less expensive.

2. We consider that the major risks of failure in such investigations will arise from poor control of the exposure conditions. Dispersion media are often carried in the species and, if they are unrealistic, may lead to spurious results. It is rarely acceptable in our view to use particles without a dispersing medium. We now recommend a thorough assessment before any such study, in particular matching the exposure medium to the species. The choices, we consider, are now fully resolved at cell level, as evidenced by this report. For example, human serum or fluids should be used to disperse particles for exposure to human cells, taking a species-by-species approach. In the case of environmental exposure of species, we caution that the choice of dispersants may not be fully agreed and may not yet have been resolved satisfactorily in large-scale international programmes. The failure to use any relevant dispersant (e.g. polysaccharides to mimic river water debris) can lead to direct contact between particles and organism (potentially simply sticking to the species) and entirely unrealistic outcomes. Poor dispersions may also lead to spurious outcomes. These exposure conditions must be fully recorded, characterised (e.g. by differential centrifugation) and reported to be reproducible. All of this is distinctive in nanoparticle, rather than chemical, exposure.

3. The appropriate design of a high-content toxicity analysis can lead to far-reaching outcomes. Because of the numbers of endpoints and conditions, one can pick up effects and mechanisms that may prompt interest or concerns beyond naive acute toxicity. Indeed, we would suggest that a widespread HCA study with many endpoints could be a useful prelude before considering exposing any organism and could lead to more focused questions at the organism level.

4. Above all, we conclude that the issue of accumulation of materials, especially if they are not acutely toxic, should be highlighted. This is expressed at cell level by lysosomal accumulation, whereby non-degradable (or slowly degradable) materials, having been previously coated by materials derived from their environment, are irreversibly deposited within lysosomes. The long-term effects of those accumulations should be a high priority for future research. All the materials we have explored have surprisingly long accumulation times. We believe that this can be achieved mechanistically, in combination with *in vivo* studies, and indeed consider that the latter may be less delicate in identifying the processes involved.

5. Furthermore, we recommend that, as a high priority, the degradation products from accumulated, slowly degrading, nanomaterials (identified as having low acute toxicity from short-term exposures) should be explored in research. In particular, methods to measure these aspects should be developed as a priority.

6. Although it is not the topic of the present research, we have recognised from other, related, projects that the shape of nanomaterials may be a topic of interest. In other words, materials of the same size but different shapes may have different outcomes if they reside within cells and organisms for extended periods of time.

In summary, the focus of commercial testing should be acute toxicity. The focus of research should be the role of accumulation (particularly at the cell level) and, in particular, slow degradation, where by-products may

not have had time to have an impact in short-term acute studies. This last question is one of which the research community has essentially no knowledge, and some preliminary studies confirm the need to clarify that as a priority.

References

Allouni, Z.E., Cimpan, M.R., Hol, P.J., Skodvin, T. and Gjerdet, N.R., 2009. Agglomeration and sedimentation of TiO2 nanoparticles in cell culture medium. Colloids and Surfaces. B. Biointerfaces, 68, 83–87.

Anguissola, S., Garry, D., Salvati, A., O'Brien, P.J. and Dawson, K.A., 2014. High content analysis provides mechanistic insights on the pathways of toxicity induced by amine-modified polystyrene nanoparticles. PLOS One, 9, e108025.

Bexiga, M.G., Varela, J.A., Wang, F., Fenaroli, F., Salvati, A., Lynch, I., Simpson, J.C. and Dawson, K.A., 2011. Cationic nanoparticles induce caspase 3-, 7- and 9-mediated cytotoxicity in a human astrocytoma cell line. Nanotoxicology, 5, 557–567.

Casey, A., Herzog, E., Lyng, F.M., Byrne, H.J., Chambers, G. and Davoren, M., 2008. Single walled carbon nanotubes induce indirect cytotoxicity by medium depletion in A549 lung cells. Toxicology Letters, 179, 78–84.

Cedervall, T., Lynch, I., Foy, M., Berggard, T., Donnelly, S.C., Cagney, G., Linse, S. and Dawson, K.A., 2007. Detailed identification of plasma proteins adsorbed on copolymer nanoparticles. Angewandte Chemie International Edition (English), 46, 5754–5756.

Kahru, A. and Dubourguier, H.C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology, 269, 105–119.

Lesniak, A., Campbell, A., Monopoli, M.P., Lynch, I., Salvati, A. and Dawson, K.A., 2010. Serum heat inactivation affects protein corona composition and nanoparticle uptake. Biomaterials, 31, 9511–9518.

Lesniak, A., Fenaroli, F., Monopoli, M.P., Åberg, C., Dawson, K.A. and Salvati, A., 2012. Effects of the presence or absence of a protein corona on silica nanoparticle uptake and impact on cells. ACS Nano, 6, 5845–5857.

Lesniak, A., Salvati, A., Santos-Martinez, M.J., Radomski, M.W., Dawson, K.A. and Åberg, C., 2013. Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. Journal of the American Chemical Society, 2013, 135, 1438–1444.

Lu, J., Liong, M., Sherman, S., Xia, T., Kovochich, M., Nel, A.E., Zink, J.I. and Tamanoi, F., 2007. Mesoporous silica nanoparticles for cancer therapy: energy-dependent cellular uptake and delivery of paclitaxel to cancer cells. NanoBiotechnology, 3, 89–95.

Lullmann, H., Lullmann-Rauch, R. and Wassermann, O., 1978. Lipidosis induced by amphiphilic cationic drugs. Biochemical Pharmacology, 27, 1103–1108.

Lundqvist, M., Stigler, J., Elia, G., Lynch, I., Cedervall, T. and Dawson, K.A., 2008. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proceedings of the National Academy of Sciences USA, 105, 14265–14270.

Mueller, N.C. and Nowack, B., 2008. Exposure modeling of engineered nanoparticles in the environment. Environmental Science and Technology, 42, 4447–4453.

Nemmar, A., Hoylaerts, M.F., Hoet, P.H.M., Vermylen, J. and Nemery, B., 2003. Size effect of intratracheally instilled particles on pulmonary inflammation and vascular thrombosis. Toxicology and Applied Pharmacology, 186, 38–45.

Organisation for Economic Co-operation and Development. OECD Guidelines for the Testing of Chemicals. Available online: http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

Shapero, K., Fenaroli, F., Lynch, I., Cottell, D.C., Salvati, A. and Dawson, K.A., 2011. Time and space resolved uptake study of silica nanoparticles by human cells. Molecular BioSystems, 2011, 7, 371–378.

Tenuta, T., Monopoli, M.P., Kim, J., Salvati, A., Dawson, K.A., Sandin, P. and Lynch, I., 2011. Elution of labile fluorescent dye from nanoparticles during biological use. PLOS One, 6, e25556.

Walczyk, D., Bombelli, F.B., Monopoli, M.P., Lynch, I. and Dawson, K.A., 2010. What the cell "sees" in bionanoscience. Journal of the American Chemical Society, 132, 5761–5768.

Wang, F., Yu, L., Salvati, A. and Dawson, K.A., 2013. The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. Nanomedicine, 9, 1159–1168.

Weinert, S., Jabs, S., Supanchart, C., Schweizer, M., Gimber, N., Richter, M., Rademann, J., Stauber, T., Kornak, U. and Jentsch, T.J., 2010. Lysosomal pathology and osteopetrosis upon loss of H+-driven lysosomal Cl- accumulation. Science, 328, 1401–1403.

Xia, T., Kovochich, M., Liong, M., Zink, J.I. and Nel, A.E., 2008. Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways. ACS Nano, 2, 85–96.

Xing, X., He, X., Peng, J., Wang, K. and Tan, W., 2005. Uptake of silica-coated nanoparticles by HeLa cells. Journal of Nanoscience and Nanotechnology, 5, 1688–1693.

Acronyms and abbreviations

cMEM	Complete minimum essential culture medium
EC_{50}	Half-maximal effective concentration
HCA	High-content analysis
IC_{50}	Half-maximal inhibitory concentration
LAMP1	Lysosomal-associated membrane protein 1
NIR	Near-infrared
OECD	Organisation for Economic Co-operation and Development
PI	Propidium iodide
SF	Serum-free
TEM	Transmission electron microscopy
TMRM	Tetramethylrhodamine methyl ester
v/v	Volume/volume

Appendix 1

Table A1.1. List of commercial nanoparticles used

Manufacturer	Material	Nominal size (nm)
Kisker (Steinfurt, Germany)	Silica yellow–green fluorescent	50
	Silica yellow–green fluorescent	100
	Silica yellow–green fluorescent	300
Polyscience (Niles, IL)	Polystyrene -OSO_3H	50
	Polystyrene -COOH	50
	Polystyrene -OSO_3H yellow–green	50
	Polystyrene -COOH yellow–green	50
	Polystyrene -NH_2	100
Life Technologies (Carlsbad, CA)	Polystyrene -COOH yellow–green	40
	Polystyrene -OSO_3H	100
	Polystyrene -COOH	100
Sigma Aldrich (Gillingham, UK)	Polystyrene -NH_2	50

Table A1.2. Characteristics of the University College Cork-synthesised SiO_2 nanoparticles, including assessment of the elution of the dyes from the particles

Sample name	Actual size (nm)	C, H (%)	Dye (%)	Elution amount after 8 days (ppm)	UV-vis peak (nm)
400-nm SiO_2 @IR-820	290	C = 0.79 H = 1.23	1.215	1.08	348, 370, 759, 825
200-nm SiO_2 @IR-820	240	C = 0.92 H = 1.08	1.415	1.15	347, 370, 761, 835
100-nm SiO_2 @IR-820	100	C = 2.77 H = 1.22	4.26	1.27	348, 367, 753, 839
50-nm SiO_2 @IR-820	50	C = 3.14 H = 1.33	4.83	1.41	347, 370, 760, 838
20-nm SiO_2 @IR-820	20	C = 3.27 H = 1.32	5.03	1.51	347, 370, 757, 814
400-nm SiO_2 @FITC	290	C = 2.41 H = 0.99	3.72	8.19	489
200-nm SiO_2 @FITC	240	C = 1.32 H = 1.33	2.04	4.71	489
100-nm SiO_2 @FITC	100	C = 2.47 H = 1.08	3.815	6.58	488
50-nm SiO_2 @FITC	50	C = 3.65 H = 1.21	5.64	5.47	489
20-nm SiO_2 @FITC	40	C = 4.70 H = 1.21	7.26	4.03	488

Table A1.3. List of the most abundant proteins identified by mass spectrometry on 50-nm silica nanoparticles recovered from cell cultures exposed for 1 h in serum-free conditions

Accession RSp	Name	Molecular weight (Daltons)	SpC SF (human)	SpC cMEM (human)	Cellular component/function
P21333	Filamin-A	280,561.4	137	40	Cytoplasm and cytoskeleton, links actin filaments to membrane glycoproteins
O75369	Filamin-B	277,987.3	125	-	Cytoplasm and cytoskeleton, connects cell membrane constituents to the actin cytoskeleton
P14618	Pyruvate kinase isozymes M1/M2	57,900.17	91	63	Cytoplasm and nucleus, glycolytic enzyme
O43707	Alpha-actinin-4	104,788.5	76	-	Cytoplasm and nucleus, protein transport, regulation of apoptosis
P00352	Retinal dehydrogenase 1	54,826.99	65	-	Cytoplasm, binds free retinal
Q13813	Spectrin alpha chain, brain	284,362.5	65	-	Cytoplasm and cytoskeleton, structural constituent of cytoskeleton
P35579	Myosin-9	226,390.6	56	70	Cell shape, plays a role in cytokinesis
P12814	Alpha-actinin-1	102,992.7	55	-	Cell membrane, cell projection, cytoplasm, cytoskeleton, membrane, focal adhesion assembly, regulation of apoptosis
Q00610	Clathrin heavy chain 1	191,491.7	54	-	Coated pit, cytoplasmic vesicle, membrane, major protein of coated pits and vesicles
Q01082	Spectrin beta-chain, brain 1	274,437.2	53	-	Cell membrane, cytoplasm, cytoskeleton, membrane, actin filament capping
P11413	Glucose-6-phosphate 1-dehydrogenase	59,219.09	46	-	Centrosome, cytosol, internal side of plasma membrane, intracellular membrane-bounded organelle, carbohydrate and glucose metabolism
Q71U36	Tubulin alpha-1a chain	50,103.65	39	19	Major constituent of microtubules, cytoplasm, cytoskeleton
Q9Y490	Talin-1	269,596.3	39	238	Cell membrane, cytoplasm, cytoskeleton
P53396	ATP-citrate synthase	120,762.1	38	-	Cytoplasm, nucleus lipid synthesis, ATP binding, ATP citrate synthase activity

Table A1.3. Continued

Accession RSp	Name	Molecular weight (Daltons)	SpC SF (human)	SpC cMEM (human)	Cellular component/function
P68104	Elongation factor 1-alpha 1	50,109.18	38	30	Cytoplasm and nucleus
P69905	Haemoglobin subunit-alpha	15,247.92	38	34	Oxygen transport from the lung to the various peripheral tissues
P29401	Transketolase	67,834.88	35	-	Cytosol, transferase, involved in energy reserve metabolic processes
P10809	60-kDa heat shock protein, mitochondrial	61,016.47	34	6	Mitochondrion, mitochondrial protein import and marcomolecular assembly
P13639	Elongation factor 2	95,277.08	34	15	Cytoplasm, cytosol and ribonucleoprotein complex, protein biosynthesis, GTP-binding, nucleotide-binding
P07355	Annexin A2	38,579.82	33	1	Basement membrane, extracellular matrix, secreted, positive regulation of vesicle fusion
Q15149	Plectin 3	53,1465.9	32	-	Cell junction, cytoplasm, cytoskeleton, cellular component disassembly involved in apoptosis
O60701	UDP-glucose 6-dehydrogenase	54,989.33	31	13	Cytosol, biosynthesis of glycosaminoglycans
P04083	Annexin A1	38,690	30	-	Cell membrane, cell projection, cilium, cytoplasm; promotes membrane fusion and is involved in exocytosis
P60842	Eukaryotic initiation factor 4A-I	46,124.6	29	3	Cytosol, eukaryotic translation protein biosynthesis
Q14204	Cytoplasmic dynein 1 heavy chain 1	532,071.8	29	9	Cytoplasm, cytoskeleton, dynein, microtubule, transport, microtubule-based movement

Accession RSp, accession number in Uniprot database; cMEM, complete minimum essential culture medium; SF, serum-free; SpC, spectral counts.

AN GHNÍOMHAIREACHT UM CHAOMHNÚ COMHSHAOIL

Tá an Ghníomhaireacht um Chaomhnú Comhshaoil (GCC) freagrach as an gcomhshaol a chaomhnú agus a fheabhsú mar shócmhainn luachmhar do mhuintir na hÉireann. Táimid tiomanta do dhaoine agus don chomhshaol a chosaint ó éifeachtaí díobhálacha na radaíochta agus an truaillithe.

Is féidir obair na Gníomhaireachta a roinnt ina trí phríomhréimse:

Rialú: *Déanaimid córais éifeachtacha rialaithe agus comhlíonta comhshaoil a chur i bhfeidhm chun torthaí maithe comhshaoil a sholáthar agus chun díriú orthu siúd nach gcloíonn leis na córais sin.*

Eolas: *Soláthraímid sonraí, faisnéis agus measúnú comhshaoil atá ar ardchaighdeán, spriocdhírithe agus tráthúil chun bonn eolais a chur faoin gcinnteoireacht ar gach leibhéal.*

Tacaíocht: *Bímid ag saothrú i gcomhar le grúpaí eile chun tacú le comhshaol atá glan, táirgiúil agus cosanta go maith, agus le hiompar a chuirfidh le comhshaol inbhuanaithe.*

Ár bhFreagrachtaí

Ceadúnú

- Déanaimid na gníomhaíochtaí seo a leanas a rialú ionas nach ndéanann siad dochar do shláinte an phobail ná don chomhshaol:
- saoráidí dramhaíola (m.sh. láithreáin líonta talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh. déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- an diantalmhaíocht (m.sh. muca, éanlaith);
- úsáid shrianta agus scaoileadh rialaithe Orgánach Géinmhodhnaithe (OGM);
- foinsí radaíochta ianúcháin (m.sh. trealamh x-gha agus radaiteiripe, foinsí tionsclaíocha);
- áiseanna móra stórála peitril;
- scardadh dramhuisce;
- gníomhaíochtaí dumpála ar farraige.

Forfheidhmiú Náisiúnta i leith Cúrsaí Comhshaoil

- Clár náisiúnta iniúchtaí agus cigireachtaí a dhéanamh gach bliain ar shaoráidí a bhfuil ceadúnas ón nGníomhaireacht acu.
- Maoirseacht a dhéanamh ar fhreagrachtaí cosanta comhshaoil na n-údarás áitiúil.
- Caighdeán an uisce óil, arna sholáthar ag soláthraithe uisce phoiblí, a mhaoirsiú.
- Obair le húdaráis áitiúla agus le gníomhaireachtaí eile chun dul i ngleic le coireanna comhshaoil trí chomhordú a dhéanamh ar líonra forfheidhmiúcháin náisiúnta, trí dhíriú ar chiontóirí, agus trí mhaoirsiú a dhéanamh ar leasúchán.
- Cur i bhfeidhm rialachán ar nós na Rialachán um Dhramhthrealamh Leictreach agus Leictreonach (DTLL), um Shrian ar Shubstaintí Guaiseacha agus na Rialachán um rialú ar shubstaintí a ídíonn an ciseal ózóin.
- An dlí a chur orthu siúd a bhriseann dlí an chomhshaoil agus a dhéanann dochar don chomhshaol.

Bainistíocht Uisce

- Monatóireacht agus tuairisciú a dhéanamh ar cháilíocht aibhneacha, lochanna, uiscí idirchriosacha agus cósta na hÉireann, agus screamhuiscí; leibhéil uisce agus sruthanna aibhneacha a thomhas.
- Comhordú náisiúnta agus maoirsiú a dhéanamh ar an gCreat-Treoir Uisce.
- Monatóireacht agus tuairisciú a dhéanamh ar Cháilíocht an Uisce Snámha.

Monatóireacht, Anailís agus Tuairisciú ar an gComhshaol

- Monatóireacht a dhéanamh ar cháilíocht an aeir agus Treoir an AE maidir le hAer Glan don Eoraip (CAFÉ) a chur chun feidhme.
- Tuairisciú neamhspleách le cabhrú le cinnteoireacht an rialtais náisiúnta agus na n-údarás áitiúil (m.sh. tuairisciú tréimhsiúil ar staid Chomhshaol na hÉireann agus Tuarascálacha ar Tháscairí).

Rialú Astaíochtaí na nGás Ceaptha Teasa in Éirinn

- Fardail agus réamh-mheastacháin na hÉireann maidir le gáis cheaptha teasa a ullmhú.
- An Treoir maidir le Trádáil Astaíochtaí a chur chun feidhme i gcomhair breis agus 100 de na táirgeoirí dé-ocsaíde carbóin is mó in Éirinn

Taighde agus Forbairt Comhshaoil

- Taighde comhshaoil a chistiú chun brúnna a shainaithint, bonn eolais a chur faoi bheartais, agus réitigh a sholáthar i réimsí na haeráide, an uisce agus na hinbhuanaitheachta.

Measúnacht Straitéiseach Timpeallachta

- Measúnacht a dhéanamh ar thionchar pleananna agus clár beartaithe ar an gcomhshaol in Éirinn (m.sh. mórphleananna forbartha).

Cosaint Raideolaíoch

- Monatóireacht a dhéanamh ar leibhéil radaíochta, measúnacht a dhéanamh ar nochtadh mhuintir na hÉireann don radaíocht ianúcháin.
- Cabhrú le pleananna náisiúnta a fhorbairt le haghaidh éigeandálaí ag eascairt as taismí núicléacha.
- Monatóireacht a dhéanamh ar fhorbairtí thar lear a bhaineann le saoráidí núicléacha agus leis an tsábháilteacht raideolaíochta.
- Sainseirbhísí cosanta ar an radaíocht a sholáthar, nó maoirsiú a dhéanamh ar sholáthar na seirbhísí sin.

Treoir, Faisnéis Inrochtana agus Oideachas

- Comhairle agus treoir a chur ar fáil d'earnáil na tionsclaíochta agus don phobal maidir le hábhair a bhaineann le caomhnú an chomhshaoil agus leis an gcosaint raideolaíoch.
- Faisnéis thráthúil ar an gcomhshaol ar a bhfuil fáil éasca a chur ar fáil chun rannpháirtíocht an phobail a spreagadh sa chinnteoireacht i ndáil leis an gcomhshaol (m.sh. Timpeall an Tí, léarscáileanna radóin).
- Comhairle a chur ar fáil don Rialtas maidir le hábhair a bhaineann leis an tsábháilteacht raideolaíoch agus le cúrsaí práinnfhreagartha.
- Plean Náisiúnta Bainistíochta Dramhaíola Guaisí a fhorbairt chun dramhaíl ghuaiseach a chosc agus a bhainistiú.

Múscailt Feasachta agus Athrú Iompraíochta

- Feasacht chomhshaoil níos fearr a ghiniúint agus dul i bhfeidhm ar athrú iompraíochta dearfach trí thacú le gnóthais, le pobail agus le teaghlaigh a bheith níos éifeachtúla ar acmhainní.
- Tástáil le haghaidh radóin a chur chun cinn i dtithe agus in ionaid oibre, agus gníomhartha leasúcháin a spreagadh nuair is gá.

Bainistíocht agus struchtúr na Gníomhaireachta um Chaomhnú Comhshaoil

Tá an ghníomhaíocht á bainistiú ag Bord lánaimseartha, ar a bhfuil Ard-Stiúrthóir agus cúigear Stiúrthóirí. Déantar an obair ar fud cúig cinn d'Oifigí:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig Forfheidhmithe i leith cúrsaí Comhshaoil
- An Oifig um Measúnú Comhshaoil
- An Oifig um Cosaint Raideolaíoch
- An Oifig Cumarsáide agus Seirbhísí Corparáideacha

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag comhaltaí air agus tagann siad le chéile go rialta le plé a dhéanamh ar ábhair imní agus le comhairle a chur ar an mBord.

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Visualisation and quantification of the interaction of fluorescent nanoparticles with ecotoxicologically relevant species

Louise Rocks, Sergio Anguissola and Kenneth A. Dawson

The present programme aimed to clarify some aspects of the nature and consequences of the interaction between engineered nanoparticles and the environment. The team have concluded that most nanomaterials tend not to be acutely toxic, unless the substances from which they are made are also toxic. Those that are acutely toxic can now be readily identified by screening tests.
The research team believe that it is now important to focus effort on other potential issues. In particular, nanoparticles examined for a wide range of sizes and exposure conditions tend to accumulate within cells, and some organisms, with limited (or no) potential to be rapidly cleared from them.

Identifying Pressures

Nanotechnology developments suggest the introduction of many diverse particle types such that a detailed case-by-case analysis of safety will be difficult, without some overarching understanding of the expected effects. Early responses should focus on identifying the key questions to be asked in research and creation of an effective screening safety testing approach that is rapid and inexpensive to apply, requiring limited in vivo study.

Informing Policy

The outcomes of the project were significant in framing the advice and explanations given to the European Commission and the public on the interaction between engineered nanoparticles and the environment, most particularly in pioneering the issues for larger programmes to follow. It began earlier than most other similar studies, and had significant impact on the opinions currently on the website of the Directorate-General for the Environment, and now being widely accepted internationally in the EU, US, OECD and beyond.

Developing Solutions

In this project, an approach for simple high throughput safety assessment of nanomaterials for acute toxicity testing at cell level and simple aquatic species has been established. This method, besides being inexpensive, is also less error prone than previously assessment platforms derived from chemical safety assessments. This satisfies some immediate practical concerns and allows the research agenda to move on to more focused questions.
Acute toxicity testing should be implemented as a more applied or industry oriented programme of testing. A major focus of future research should be the role of nanoparticle accumulation at cell level and, in particular, slow clearance or slow degradation, where by-products may not have had time to have an impact in short-term acute studies.

Comhshaol, Pobal agus Rialtas Áitiúil
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