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Review Article

Engineered nanomaterial risk. Lessons learnt from completed nanotoxicology studies: potential solutions to current and future challenges

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Abstract

PARTICLE_RISK was one of the first multidisciplinary projects funded by the European Commission's Framework Programme that was responsible for evaluating the implications of nanomaterial (NM) exposure on human health. This project was the basis for this review which identifies the challenges that exist within the assessment of NM risk. We have retrospectively reflected on the findings of completed nanotoxicology studies to consider what progress and advances have been made within the risk assessment of NMs, as well as discussing the direction that nanotoxicology research is taking and identifying the limitations and failings of existing research. We have reflected on what commonly encountered challenges exist and explored how these issues may be resolved. In particular, the following is discussed (i) NM selection (ii) NM physico- chemical characterisation; (iii) NM dispersion; (iv) selection of relevant doses and concentrations; (v) identification of relevant models, target sites and endpoints; (vi) development of alternatives to animal testing; and (vii) NM risk assessment. These knowledge gaps are relatively well recognised by the scientific community and recommendations as to how they may be overcome in the future are provided. It is hoped that this will help develop better defined hypothesis driven research in the future that will enable comprehensive risk assessments to be conducted for NMs. Importantly, the nanotoxicology community has responded and adapted to advances in knowledge over recent years to improve the approaches used to assess NM hazard, exposure and risk. It is vital to learn from existing information provided by ongoing or completed studies to avoid unnecessary duplication of effort, and to offer guidance on aspects of the experimental design that should be carefully considered prior to the start of a new study.

Keywords: Nanotoxicology, nanotechnology, nanomaterials, toxicology, hazard, risk

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Introduction

The production and use of engineered nanomateri-als (NMs) is constantly expanding; however, there are still many uncertainties surrounding the potential risks posed by NMs to human health and the environment. As such, investigations that assess NM hazard are essential in order to understand more about the potential detri-mental implications of engineered NMs to human health. Hazard data can then be combined with exposure data to provide information on the risks posed by NMs to allow for the safe, responsible and sustainable development of nanotechnology in the future.

PARTICLE_RISK (2005–2009) was one of the first research projects funded by the European Commission's Framework Programme (FP6) concerned with evaluat-ing the safety of engineered NMs. PARTICLE_RISK was an interdisciplinary and multidisciplinary project that evaluated the potential adverse implications of NM exposure on human health (Table 1). In 2005, research within the discipline of nanotoxicology (concerned with investigating the toxicity of engineered NMs) was in its infancy. However, since that time there has been a rapid expansion in the number of studies concerned with assessing the safety of engineered NMs. This derived, in part, from (i) evidence of NM toxicity within early stud-ies which prompted concern surrounding the potential adverse health effects that may emerge following expo-sure to NMs, and (ii) the anticipated increase in NM use and production and therefore human and environmen-tal exposure (The Royal Society and Royal Academy of Engineering Nanotechnology Report, 2004). Despite an international research effort there is still insufficient data available to conduct the in depth risk assessments required to inform the regulatory decision making pro-cess on the safety of NMs. It is therefore prudent to reflect on what progress has been made since the completion of PARTICLE_RISK and other "early" nanotoxicology studies. This will allow identification of the advances in knowledge that have been made, as well as the limita-tions and failings of these studies in order to determine what lessons can be learnt from their completion. A ret-rospective assessment allows us to take a broader look at the literature in order to evaluate what direction nano-toxicology research is taking and to help guide future testing strategies concerned with assessing NM safety. In this way, we hope to develop more appropriate hypoth-esis directed research in the area of nanotoxicology in the future whose data can be used for risk assessment purposes. This is made especially relevant by the current drive to achieve a harmonised approach with regards to the testing of NM safety, and the greater understanding of what a testing strategy for NMs should encompass.

Identification of commonly encountered challenges within nanotoxicology investigations

The findings from PARTICLE_RISK have formed the basis for this review (Table 1) as it provided us with a starting point to identify the (commonly encountered) limitations of current approaches concerned with the assessment of NM safety. Using this information and relating it to findings from the wider literature we have identified common pitfalls and challenges associated with nanotoxicology research. This is required to overcome the obstacles that still exist within the development of robust approaches to assess NM risk through integration of hazard and exposure data. Of particular interest is determining the most appropriate approach to: (i) select NMs for nanotoxicology investigations, (ii) characterise the physico-chemical properties of NMs, (iii) disperse NMs for hazard investigations, (iv) select NM doses and concentrations for hazard investigations, (v) select relevant models, target sites and endpoints to assess NM safety, (vi) develop alternatives for testing NM safety in animals and, (vii) use the hazard data generated for risk assessment purposes in order to allow the hazard data to be employed in a wider context for use by industry and regulators. We will address each of these issues and offer recommendations and ways forward that could help provide solutions to these existing challenges to address current uncertainties or gaps in knowledge.

Selection of NMs for nanotoxicology investigations

Nanomaterials are a very varied population of materials. Despite the diversity of NMs available the only feature they have in common is their small size (NMs are typically defined as having one dimension <100 nm in diameter).

Therefore, an array of different forms of NMs are available that vary with respect to their physical and chemical characteristics including for example; composition, shape, size, surface area, surface properties, solubility and charge. Only a limited number of NMs that are currently in use or production have been tested within nanotoxicology investigations because of time and financial constraints. Some of these NMs were produced

Table 1. The research findings generated during the PARTICLE_RISK project have been published separately; however, the main findings from the PARTICLE_RISK project are summarized in the table.

Publication	Model used	NMs used and dose/concentration	Dispersion	Conclusion	Limitations
Jacobsen et al., 2007	• FE1 MML mouse epithelial cell line	• 14 nm ufCB (Printex 90) • Single exposure: up to, 100 µg/mL for 24 h • Repeated exposure: 75 µg/mL in 8 exposure rounds (8 × 72 h)	In cell culture medium with serum. Sonication on ice bath.	ufCB at non-cytotoxic levels induces DNA strand breaks and oxidised purines in FE1-MML cells. Prolonged exposure leads to increased cII (1.4-fold) and lacZ (1.23-fold) mutations.	These effects were observed following exposure for a 99% pure carbon particle. However, these results do not necessarily show that all carbonaceous nano particles are mutagenic
Jacobsen et al., 2008	• FE1 MML mouse epithelial cell line	• C60, SWCNT • Up to 200 µg/mL for 24 h	In cell culture medium with serum. Sonication on ice bath	Low genotoxicity of SWCNT and C_{60} compared to ufCB in FE1-MML cells. ROS production was highest for ufCB followed by SWCNT and C_{60}. SWCNT exposure led to cell-cycle G1-arrest.	Characterization revealed ROS production as a likely candidate for the genotoxicity. However, exposures containing antioxidant could verify the direct link
Semmler Behnke et al., 2008	• Wistar-Kyoto rats • Intravenous injection (i.v.) • Intratracheal instillation (i.t.)	• 1.4 nm and 18 nm Au NPs (neutron activated) • 26.5 µg/50 µL (1.4 nm) 2.7 µg/50 µL (18 nm)	Agglomerates were filtered from the 1.4-nm-NP solution prior to exposure. Visual and UV absorption indicated no agglomeration of the 18-nm-NP suspension.	Translocation of NMs across lung and intestinal barriers, as well as the accumulation in secondary target organs is related to the size and charge of NMs. This novel approach to assess NM biodistribution this approach has the advantage that a 100% balance of the biodistribution of the applied NM can be obtained.	This study focussed on gold NPs and there is a need to investigate the biodistribution of a more diverse array of NMs and via different exposure routes (e.g. ingestion). This research has now commenced.
Jacobsen et al., 2009	• C57 and ApoE$^{-/-}$ mice:	• ufCB, 2 nm Au NPs, C60, QDs (positive and negatively charged) and SWCNTs	In 10% BAL Fluid and 0.9% saline. Sonication on ice bath	Lung inflammation, injury and DNA damage. QD elicited by far the greatest response followed by ufCB and SWCNT with C_{60} being least inflammatory, and DNA damaging. Surface area correlated well with inflammatory response for low toxicity particles.	The media caused agglomeration of some particles, and the importance of this is unknown. Comparisons were performed at a single instillation dose of 54 µg. The toxicity detected at QD exposure were likely due to leakage of Cd.
	• Intratracheal instillation	• 18 µg or 54 µg (50 µL administered)			Whilst this study alone cannot be used to confirm the correlation between NM surface area and toxicity, when taken together with findings from the wider literature the importance of surface area to NM toxicity is realised.

(Continued)

Table 1. *(Continued)*.

Publication	Model used	NMs used and dose/concentration	Dispersion	Conclusion	Limitations
Folkmann et al., 2009	• Fisher 344 rats: • Oral gavage	• C60, SWCNT • 0.064 and 0.64 mg/kg body weight	In saline or corn oil. Ultra sound sonication.	Ingestion of SWCNT or C_{60} caused increased levels of 8-oxodG in liver and lung tissue, and was not caused by inhibition of DNA repair. NMs can elicit systemic effects.	It is still unresolved how the exposure of the gut to NMs can result in the oxidation of biomolecules in internal organs. Possibilities include translocated particles and circulation inflammatory molecules. A different dispersant should be considered in the future as corn oil is rich in polyunsaturated fatty acids and was sonicated, which might produce genotoxic compounds
Vesterdal et al., 2009	• ApoE$^{-/-}$ mice at the age of 11–13 or 40–42 weeks: • Intraperitoneal injection	• C_{60} • 0.05 and 0.50. mg/kg body weight	Isotonic saline. Sonication on ice bath.	C60 caused a modest decrease in endothelium-dependent vasorelaxation in aorta segments	The aorta is a conductance vessel and vasomotor dysfunction in other vessels (arterioles, veins) might be affected differently
Vesterdal et al., 2010	• ApoE$^{-/-}$ mice at the age of 11–13 or 48–49 weeks: • Intratracheal instillation	• Printex 90 (ufCB) • 0.05–2.7 or 2 × 0.5 mg/kg body weight	In 10% BAL Fluid and 0.9% saline. Sonication on ice bath	Decreased endothelium-dependent vasorelaxation after two exposures in 11–13 weeks mice. The same exposure did not accelerate the plaque progression in the 48–49 weeks old mice. ICAM-1, VCAM-1, nitrotyrosine unaltered in BCA vessels. Unaltered mRNA levels in the lung tissue of VCAM-1, ICAM-1 and Hmox-1, whereas there was increased MCP-1 expression level	The study was designed to investigate the vasomotor dysfunction of low doses of Printex 90, whereas the dosing regimen might have been too short to promote plaque progression and the mice were relatively old with substantial atherosclerosis at the start of the experiment.
Johnston et al., 2010a	• Human C3A hepatocyte cell line • Primary isolated hepatocyte couplets	• Fluorescent polystyrene beads (20 nm, 200 nm) (Invitrogen) • Concentrations up to 125 μg/mL for up to 1 h	NMs prepared in cell culture medium in the presence or absence of 10% serum	The uptake of NMs by hepatocytes is size dependent, with smaller particles (20 nm) more effectively internalised by cells than their larger counterparts (200 nm). The dispersion protocol used can impact on the behaviour of NMs. Specifically serum is able to enhance particle uptake.	Fluorescent polystyrene beads are useful experimental tools. However, further studies are required to determine the wider applicability of the findings to more diverse forms of NMs
Zuin et al., 2011	• Weight of evidence approach	• C_{60}, SWCNT, ufCB, QD tested during the project	N/A	A weight of evidence approach can be used to investigate the potential of NMs to cause harm to human health, in the absence of sufficient information to conduct a full risk assessment.	Incorporation of other evidence and indicators are needed to improve the procedure and to develop more robust conclusions concerning the NMs hazard.

(Continued)

Table 1. *(Continued)*.

Publication	Model used	NMs used and dose/concentration	Dispersion	Conclusion	Limitations
McGuiness et al., 2011	• Whole blood (human) • Platelets (human) • Erythrocytes (human)	• Unmodified, aminated, and carboxylated polystyrene latex nanobeads (50 nm) • Concentrations up to 260 μg/mL	Used as supplied in most experiments. For some studies NMs were prepared in saline and sonicated for 5 min.	Potency of NM to induce platelet activation is related to NM charge.	There is insufficient toxicokinetic data to predict the fraction of inhaled/deposited NP that become blood borne for specific types of NP. This information is required to allow better interpretation of the data as to whether effects may be seen at plausible exposures
Jacobsen et al., 2011	• FE1 MML, Muta Mouse lung epithelial cell line	• 14 nm ufCB (Printex 90) • Repeated exposure: (75 μg/mL in 8 exposure rounds (8 × 72 h)	In cell culture medium with serum. Sonication on ice bath.	ufCB primarily cause G:C → T:A, G:C → C:G, A:T → T:A mutations in MutaMouse lung epithelial cells. This is in keeping with a genetic fingerprint of high ROS production.	It needs to be verified if all ROS producing particles also causes strand breaks and mutations

A panel of NMs namely single walled carbon nanotubes (SWCNTs), carbon fullerenes (C_{60}), quantum dots (QDs) (positive (+ve) and nega-tively charged (−ve)), ultrafine carbon black (ufCB) and gold were obtained, and characterized (see Zuin et al., 2011) although they toxicity was not assessed in all models investigated. A summary of the experimental approach, and findings of PARTICLE_RISK are provided.
Importantly, no standardized protocols for preparation of NMs were used, and a range of concentrations/doses of particles were tested. However, there was some consistency in the response that was observed across the different models tested, e.g. the greater toxicity of QDs, when compared to other particle types. A small description of the limitations of each study is provided.

in laboratories for research purposes or were obtained from commercial or industrial sources. However, this selected number of NMs may not reflect the diversity of NMs available or on the way to be being introduced onto the market. This is exemplified by the knowledge that one type of NM, such as carbon nanotubes (CNTs), can be available in a wide diversity of forms (Figure 1). Despite the obvious differences in the physico-chemical properties of the CNTs (e.g. morphology) represented in Figure 1, all of these materials would be classified as multi-walled (MW)CNTs, but they are likely to vary with regards to their toxicity. The diversity of NMs available may arise from the production methods used to generate the NMs, or the post-production modifications used to alter the properties of the NMs. For example, CNTs can be produced by a number of different methods (reviewed in Donaldson et al., 2006), and comparative studies have illustrated that CNTs obtained from different sources, and produced by different manufacturing processes can vary with regards to their physico-chemical properties and toxicity (e.g. Lam et al., 2004). Metal catalysts (such as iron or nickel) can be used within the production of CNTs, and these metals can remain within CNTs as an impurity, and the residual catalyst can contribute to the toxicity of CNTs (Shvedova et al., 2003, 2005). Post production modification of CNTs can be used to remove the catalyst metal contaminants but this can also change their physico-chemical properties (e.g. CNTs can become shortened) and their toxicity (Bottini et al. 2006; Wu et al., 2005). Furthermore, NMs can vary from batch to batch from the same supplier and so NM samples can be highly diverse (e.g. in terms of size and morphology of the NMs produced) which can make it difficult to make conclusions regarding the hazards of NMs (Park & Grassian, 2010).

NMs that are anticipated to have wide usage within commercial applications are frequently selected within hazard investigations as these NMs are expected to be produced in high quantities, increasing the potential for human and environmental exposure. However, there is a lack of information from industry regarding NM production levels, and use in consumer products. In an attempt to address this, some national reporting schemes have been implemented which can help identify the extent of NM production and use. The EU chemicals regulation (REACH) is also anticipated to help predict the quantities of NMs that are being produced. In addition, the Woodrow Wilson inventory can be used as a resource to determine which NMs are used in consumer products to the greatest extent. It is recommended that these databases should be used to help prioritise the selection of NMs for nanotoxicology studies.

Those NMs anticipated to pose a hazard to human health due to, for example, existing evidence of toxicity within animal or cell models are also often prioritised within hazard investigations. The Organisation for Economic Co-operation and Development (OECD) have identified a list of “representative NMs” whose safety should be assessed with highest priority (due to concerns regarding their toxicity and anticipated high level of production and use), and the toxicity of these materials is currently under investigation (OECD, 2010). The Institute for Health and Consumer Protection of the European Joint Research Commission (JRC) has

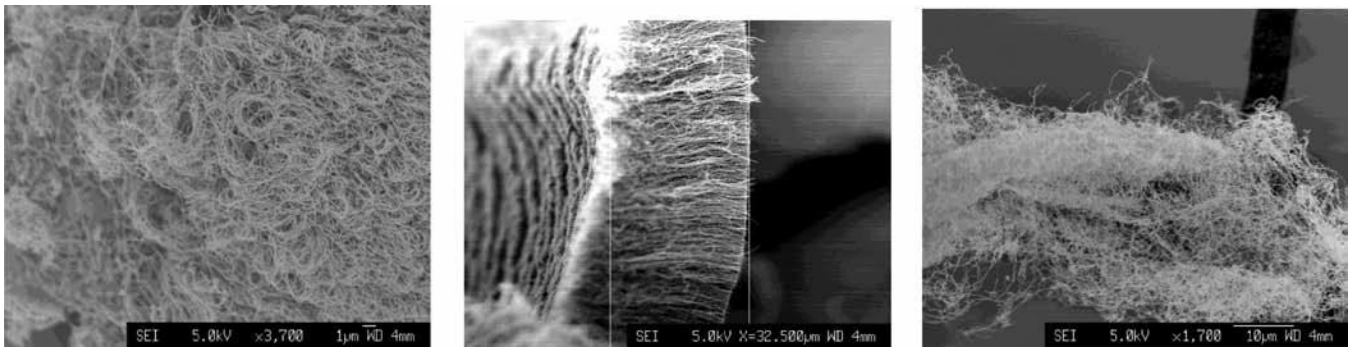

Figure 1. The morphology of different samples of MWCNT, imaged using scanning electron microscopy (SEM). Images courtesy of Matthew Boyles.

established a repository which is regarded as a source of NM "reference materials" that are well characterised and will allow for the reproducibility and reliability testing of NM safety investigations to commence. The use of such NMs by a variety of researchers can allow for the rigorous safety testing of these NMs and enable inter-laboratory comparisons to be conducted. However, these NMs should not be tested in isolation, as they are not reflective of the diversity of NMs under development and use. Instead, it is perhaps more relevant to use them for benchmarking purposes. Therefore, studies in which NM physico-chemical characteristics are systematically altered offer a distinct advantage of studies in which a panel of industrially relevant materials (e.g. OECD) are compared since such panels allow specific hypotheses to be addressed.

It is often the case that a panel of NMs which vary greatly with respect to their physico-chemical characteristics are investigated within hazard studies. These studies often strive to identify the attributes of NMs that are responsible for any observed toxicity and therefore necessitate that the tested NMs are well characterised. This approach has been used with success where relationships between the physico-chemical properties of NMs and their toxic potency have been identified (Cho et al., 2012), as well as demonstrating that NMs can act via different mechanisms of toxicity depending on their physico-chemical characteristics (Cho et al., 2010; McGuinnes et al., 2011; Kermanizadeh et al., 2012). Furthermore, polystyrene beads, which are commercially available in defined size ranges and surface chemistry can be used as experimental tools as they can be systematically modified in order to dissect out the properties of NMs that drive their toxicity (Brown et al., 2001; McGuinnes et al., 2011; Clift et al., 2008; Johnston et al., 2010a). Such information is extremely beneficial within the development of structure activity relationships that could be used to predict the toxicity of NMs based on their physical and chemical properties. In fact, studies have selected NMs to test specific hypotheses relating to the relationship between NM physico-chemical properties and their toxicity. For example, the applicability of robust structure activity relationships, such as the fibre pathogenicity paradigm to NM toxicity has been tested with success, and is reliant on the selection and availability of NMs that have very specific properties (Brown et al., 2007; Poland et al., 2008; Murphy et al., 2011). Within PARTICLE_RISK, quantum dots (QDs) were consistently demonstrated to be very toxic in all models tested (e.g. Jacobsen et al., 2009). Although we have speculated what properties of the QDs were responsible for their toxicity, namely charge, composition and stability, and are confident that the instability of QDs was responsible for the toxicity, further studies would be required to identify with certainty the properties of the QDs that were responsible for mediating the adverse effects observed. However, other studies have demonstrated that the stability and composition of QDs is fundamental to their toxicity (Lovric et al., 2005; Derfus et al., 2004; Hardman, 2006) suggesting that the weaknesses of one study may be addressed by comparison with complementary studies. This knowledge can also be used to inform the experimental design of studies investigating QD toxicity in the future, and in particular it suggests that appropriate controls (e.g. cadmium) should be included within the hazard assessment of QD toxicity and that QD instability should be routinely assessed. Furthermore, this information can influence the selection of QDs within biomedical applications and also to promote their safe design in the future. For example, a wide array of QDs are now available for use and include QDs that have a (zinc sulphide) shell that can improve their stability, as well as non-cadmium based QDs illustrating how the design of QDs can be improved by responding to existing hazard information.

Despite the huge diversity of NMs under use and production, only a small proportion of 'representative' NMs are undergoing safety evaluations. The selection of NMs within hazard investigations is critical, and these studies can extract information regarding the attributes of NMs that confer toxicity. NMs are typically selected for hazard investigations based on: (i) the likelihood of human exposure (i.e. high production and use levels),
(ii) existing evidence of NM toxicity, or (iii) to test specific hypotheses regarding the relationship between NM properties and toxicity. It is recommended that

NMs currently generated by industry, and known to be incorporated within consumer products are made available for testing through collaboration between academia and industry. This type of study is in the minority, but there are successful examples of this type of collaboration (e.g. Park et al., 2008). The need to test explicit hypotheses when evaluating the relationship between NM characteristics (e.g. size, shape, charge etc.) and NM toxicity requires a supply of NMs which are produced with very specific properties. An inability to produce NMs to the required exact specification can obstruct the completion of this type of study; including problems in obtaining NMs with a suitably narrow size distribution, or the need to have a supply of NMs with high purity in sufficient quantities that do no vary greatly batch-to-batch. In the future, it would also be useful to conduct further reviews (e.g. Johnston et al., 2009, 2010b, 2010c, 2010d) or an analysis of NM toxicity relating biological impact to physico-chemical characteristics across an array of studies.

Physico-chemical characterisation of NMs for hazard investigations

A thorough physico-chemical characterisation of NMs in parallel to hazard investigations can help identify what NM attributes are responsible for any observed toxicity. There is debate surrounding the properties of NMs that require assessment within characterisation studies. However, it has been suggested that size, surface area, shape, crystal structure, aggregation/agglomeration, composition, surface chemistry, solubility and charge should be routinely considered (Warheit, 2008). Ideally, an extensive characterisation of NMs would be performed throughout all hazard investigations, but this is not realistic due to financial and time constraints and due to the required access to advanced instrumentation and specialist technical expertise (Stone et al., 2010). Prioritising parameters for assessment and selecting appropriate analytical techniques is not currently achievable. This derives from the fact that there is a very wide range of analytical techniques available to characterise NMs and there is no consensus with regard to which techniques should be applied for each attribute. Furthermore, currently used techniques often have a number of technical and physical limitations, such as their sensitivity (e.g. limit of detection), and each technique is likely to provide different results. This encourages the utilisation of more than one technique to assess each parameter of interest, but this may not always be possible due to time and financial constraints as well as a lack of available equipment or technical expertise (Stone et al., 2010). More than one technique (when available) is often currently applied for the determination of the each parameter of interest (such as NM size). The majority of NM characterisation within early nanotoxicology studies was very limited and conducted in the ‘as produced’ form (i.e. the sample (wet or dry) that is supplied for investigation, which is not dispersed in biological media). However, within toxicological investigations NMs are dispersed in complex biological media (e.g. cell culture medium), and so the NMs interact with biological molecules such as proteins. For example, it is well established that proteins within biological media adsorb onto the surface of NMs to modify NM toxicity, and that the affinity and specificity of protein binding is related to the physico-chemical properties of NMs such as their size, composition and hydrophobicity (Lundqvist et al., 2011). Such interactions (i.e. the formation of NM-protein complexes) are likely to impact on NM properties and toxicity, and so it is essential to understand how the NM preparation procedures can impact on their physico-chemical properties. Methodological advances have facilitated the characterisation of NMs in more complex media, but there are still obstacles that need to be overcome. In order to make substantial progress in this area, it is recommended that more reliable and accurate technologies and methodologies are developed for the detection and characterisation of nanomaterials in complex media.

There is currently a drive to ensure that the physico-chemical characterisation of NMs is conducted within relevant biological media, such as cell culture medium before, during and after hazard investigations. Whilst the importance of this is recognised as it improves understanding of what cells and tissues are exposed to in culture or *in vivo*, it is also necessary to evaluate the properties of NMs within their “as produced” form, as these properties will dictate the behaviour of NMs within different biological media. Furthermore, the adverse health effects elicited by pathogenic fibres and particles (such as asbestos) were known prior to understanding which of the physico-chemical properties of these materials elicited toxicity. Thus, it is possible to identify some of the characteristics responsible for the toxic effects of particles and fibres without information on the how their properties are modified after dispersion in biological media. Specifically, characterisation of these materials in an as-produced form allowed an assessment of what characteristics of NMs drove their pathogenicity within robust structure-activity relationships. Accordingly, there is a need to characterise NMs within their “as produced” form, as well as obtaining information on the NMs within their “as tested” form (before, during and after exposure) to improve an understanding of the cellular response to NMs throughout their life cycle. Of vital importance is maintaining good communication between toxicologists and those conducting the characterisation studies in order to understand the feasibility of conducting a thorough physico-chemical characterisation of NMs alongside hazard investigations. A flexible approach is also required within the physico-chemical characterisation strategy for NMs, as some techniques will be restricted to the analysis of only “dry” or “wet” NM samples. For example, measurements of the BET surface area are restricted to the use of dry (powder) samples. Furthermore, some techniques for characterisation may not be applicable

to all forms of NMs (e.g. DLS is best suited to spherical particles).

Nanomaterials are often obtained from commercial sources for hazard investigations. The physico-chemical characterisation of the NMs allows identification of whether the manufacturer's information on particle physical and chemical properties is accurate. The information provided by NM manufacturers has sometimes been demonstrated to be incomplete or inaccurate, which emphasises the need for independent characterisation (e.g. Park & Grassian, 2010). Importantly, a number of early nanotoxicology studies will not have the extensive characterisation information that is now being asked for. This means that the interpretation of this data can be difficult, making comparisons between different studies challenging. However, the findings of such studies are still useful.

There is a lack of consensus of what NM properties should be routinely characterised and how these should be measured. Recommendations have been made previously with regards to which parameters should be assessed with highest priority (Warheit, 2008), but the requirements for characterisation should be flexible and evolve with time to accommodate new knowledge. We recommend that the specific aims of each study should dictate what level of characterisation is required, rather than arbitrarily conducting rigorous physicochemical characterisation of NM properties during every hazard investigation. For example, hazard investigations do not always require the properties of the NM responsible for the toxicity to be identified but instead focus on the mechanism of toxicity of a NM or their toxic potency. Furthermore, in recent years the mounting pressure (from the nanotoxicology and nanosafety communities) to characterise NMs has resulted in an increase in the amount of characterisation that is conducted; however, the quality of some of this data needs to be considered. There is little benefit in encouraging characterisation of NMs alongside all hazard studies if the data obtained is not interpreted correctly, if inappropriate approaches have been used to investigate the parameter of interest or if the characterisation has not been conducted to a high standard. Collaboration with experts who have access to the required equipment and the knowledge to correctly interpret the data is vital. In addition, more studies whose sole purpose is to systematically evaluate the contribution of NM properties should be conducted in the future, instead of the expectation to achieve this within each individual hazard study.

Dispersion of NMs

NMs have a tendency to agglomerate or aggregate to form larger more complex structures when dispersed in biological media for hazard investigations. A number of approaches may be used to improve the dispersion of NMs including the use of dispersants (such as proteins, detergents and solvents), mechanical and physical processes (such as sonication) and manipulation of ion strength, and pH of the dispersion medium. It is very important to have control of the dispersion characteristics when administering NMs to cells or animals, be it in air or fluid vehicles. Sometimes it is appropriate to aim for monodisperse suspensions of NMs (e.g. when attempting to identify the contribution of a particular NM property to its toxicity), and at other times it is desirable to have a dispersion that is similar to a real-life exposure scenario (which may not be a mono-dispersed preparation of NMs). There is currently insufficient information available on the exposure of humans to NMs or the environment to guide relevant dispersion protocols for toxicological investigations, both *in vitro* and *in vivo*. Before deciding on the dispersion method the aims and purposes of the study need to be considered. The approach taken to prepare NMs for hazard studies is therefore unlikely to be the same for all hazard studies. Instead, a flexible approach may be required that considers the following; the NM under investigation (e.g. its hydrophobicity, its production and use), the likely exposure route, and the potential for translocation from the exposure site. Thus, the aim of the toxicity studies should influence the approach taken, ensuring the dispersion is physiologically or environmentally relevant when possible. Reflecting on the findings of existing hazard experiments is also vital in order to determine whether dispersion procedures are able to influence the toxicity of NMs.

The most appropriate means to disperse NMs is a debatable issue within the discipline of nanotoxicology as the procedures used to disperse NMs may impact on their toxicity. Traditionally, cell culture medium (with or without serum) has been utilised for the preparation of NMs for *in vitro* studies, or saline (with or without protein, such as albumin) for *in vivo* investigations. However, due to the tendency of NMs to agglomerate and aggregate, more diverse and aggressive means of dispersion have been attempted that do not necessarily mimic physiological or environmental conditions. A variety of biological and chemical agents have been used to help disperse NMs and the relevance of their inclusion within NM suspensions, and their ability to modify the toxicity of NMs will be considered. Corn oil has been used to improve the dispersion of NMs (carbon fullerenes (C_{60}) and SWCNTs) prior to exposure via ingestion (Folkmann et al., 2009). The inclusion of corn oil was able to improve the dispersion of the (hydrophobic) NMs such as CNTs and C_{60}. DNA damage within the gut, liver and lungs was investigated, and genotoxicity was observed in the liver and lungs, but there was no significant difference in the level of DNA damage inflicted by the NMs when dispersed in saline or corn oil, suggesting that the improved dispersion of NMs did not impact on their toxicity. However, it was evident that corn oil alone had a tendency to induce DNA damage, which is considered to derive from the production of genotoxic compounds following sonication of the polyunsaturated fatty acids contained in the corn oil (Folkmann et al., 2009). This

study therefore suggests that dispersants and mechanical processes (such as sonication) may influence the dispersion but toxicity of the vehicle, and further studies are required to identify their influence on NM behaviour. Also of relevance is the finding that the toxicity associated with C_{60} has been suggested to derive from the presence of residual solvent (or its derivatives), which have the potential to become intercalated into the lattice structure of C_{60} or are released into the aqueous phase (Isakovic et al., 2006). Careful consideration therefore needs to be given to the inclusion of solvents to disperse NMs for hazard investigations. The improved dispersion of NM suspensions through the inclusion of biologically relevant dispersants has the ability to enhance the toxicity of NMs. Bovine serum albumin (BSA) and dipalmitoylphosphatidylcholine (DPPC, a component of lung surfactant) were able to enhance the dispersion of ufCB, which lead to increased ROS production in cell free conditions, and when macrophage MM6 cells were exposed to ufCB (Foucaud et al., 2007). The inclusion of serum within NM dispersions is able to enhance the uptake of NMs by hepatocytes (Johnston et al., 2010a), which may have implications for normal cell function. Accordingly, substances used to improve the dispersion of NMs are able to impact on their behaviour and toxicity, and the necessity of improving the dispersion of NMs in biological media should be considered within the aims and hypotheses of the study (e.g. does the dispersion mimic real-life exposure scenarios? Is this required?).

The enhanced toxicity of NMs when prepared using different preparation techniques may derive from the improved dispersion of NMs whereby the NMs have a smaller tendency to aggregate or agglomerate. Alternatively, the enhanced toxicity observed may result from the inherent toxicity of the dispersant used. In order to develop the best procedure to disperse NMs, it is vital to consider the aims of the study, but it is likely that a compromise will have to be made between obtaining a "well-dispersed" NM suspension, and ensuring that a physiologically or environmentally relevant NM suspension is generated. Comparative studies have provided insights into the influence of different approaches to disperse CNTs, and the impact on NM toxicity (e.g. Wang et al., 2010). Ideally, the dispersion media should be relevant to environmental or physiological conditions; however, there is currently a lack of information about how NMs behave in different exposure scenarios (e.g. air, soil, water, biological systems), and how their form may change throughout their life cycle (e.g. production, use, disposal and release). Therefore, an attempt should be made to make the NM dispersion as close to real-life conditions as possible and relevant to the aims of the study. Many *in vitro* experiments are conducted using cell culture medium supplemented with serum; however, the composition of the medium could be made more relevant to the *in vivo* situation by considering how the target cell type under investigation may be exposed to the NM *in situ*.

For example, NMs are known to become coated in protein following exposure, and the nature of this protein coating is dictated by the exposure route (Johnston et al., 2012). It is therefore likely that liver derived cells will be exposed to a different form of NM if it is inhaled, when compared to exposure following the injection of NMs directly into the circulation. These differences are primarily due to the protein coating surrounding the NM. This information can be incorporated within the preparation of NMs for hazard studies, for example, NMs can be pre-coated with lung lining fluid (to represent exposure of NMs to the lung), then coated within serum proteins (to represent the transit of NMs in the circulation to the liver) and exposed to liver cells *in vitro* (e.g. Johnston et al., 2012). It is therefore recommended that the approach utilised to suspend NMs within hazard experiments is fully justified by identifying the relevance of the NM preparation procedure to real-life conditions. If a dispersion procedure has been employed to obtain a mono-dispersed suspension of NMs in order to identify the relationship between NM physico-chemical characteristics and biological response this should be clearly communicated, and its relevance to human exposure discussed.

Identifying relevant NM doses and concentrations to use in hazard investigations

To date, the majority of nanotoxicology research has focussed on hazard investigations. However, the simultaneous assessment of NM hazard, alongside exposure assessment studies will allow decisions to be made regarding the risks posed by NMs (Thomas et al., 2009). A greater understanding of the exposure of humans and the environment to NMs is required to inform these risk assessments, and also to direct the design of appropriate hazard investigations, and interpret their findings. This is vital as it is often stated that the doses (exposure of humans or animals e.g. mg/kg) or concentrations (*in vitro* studies e.g. µg/ mL) of NMs used in hazard investigations are too high and not relevant to real life exposures (Thomas et al., 2009). Despite criticisms regarding the relevance of the NM exposures used, the hazard data obtained can still be used within risk assessments, although there are concerns that information on the potential risks of NMs will be based on high dose toxicology if this continues (Thomas et al., 2009). However, there is currently a lack of exposure data available to improve the study design of hazard investigations that allow for the testing of more appropriate concentrations of NMs. Thus, it is challenging to identify physiologically relevant concentrations of NMs to use within hazard investigations, and this lack of information also impedes the interpretation of hazard data.

There are two main approaches that can be followed within hazard investigations. Physiologically relevant concentrations of NMs can be selected; however, a lack of exposure data prevents the utilisation of NM exposures

that are based on real-life exposures. Alternatively, a range of NM concentrations can be used to investigate the dose dependency of NM toxicity. Such studies can be used to dissect out information such as the no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) values that can be used for benchmarking and regulatory purposes. The usefulness of this is exemplified by the determination of an acceptable exposure level for humans to fullerenes based on hazard investigations (Shinohara et al., 2011). Such studies have the advantage that they may actually capture real-life NM exposure levels.

Quantifying exposure to NMs is challenging. This derives, in part, from methodological limitations surrounding the identification and quantification of NMs (Tiede et al., 2008), and a lack of information regarding the production and usage levels of NMs. With regards to exposure assessment, the majority of studies have focussed on workplace exposures, as the greatest potential for exposure is anticipated in this exposure setting (Abbott & Maynard, 2010; Thomas et al., 2009). For example, field studies have indicated that the airborne concentrations of SWCNTs generated during handling were low, at 53 μg/m3, and were predominantly in an agglomerated "clumped" form (Maynard et al., 2004). In addition, workplace exposure to silver (20 ng to 1.18 μg/m^3) and TiO_2 (100 μg to 4.99 mg/m^3) have been quantified (Lee et al., 2011). Occupational studies have focussed on inhalation of NMs, and therefore focussed primarily on airborne concentrations as it is assumed to be the main and greatest route of exposure. However, it is also relevant to consider dermal exposure to NMs. Glove deposits (to investigate the potential dermal exposure to SWCNTs) during handling were estimated at between 0.2 mg and 6 mg per hand (Maynard et al., 2004). Researchers responsible for generating and working with NMs (e.g. to assess their toxicity) may be exposed to NMs in a laboratory setting. Laboratory studies have suggested that on agitation SWCNTs are released into air but that this was a relatively inefficient process and dependent on the level of agitation (Maynard et al., 2004), and that NMs (C_{60} and MWCNTs) can become airborne on handling and (probe) sonication (Johnson et al., 2010). With regards to consumer exposure to NMs, quantitative exposure assessment is currently impeded by a lack of knowledge of the products that contain NMs (Aschberger et al., 2011), and the lack of information surrounding production and usage. However, some studies have looked at the NM content of products which may be used to understand pathways for human exposure and estimate human and environmental exposure to NMs (Benn et al., 2010, 2011; Benn & Westerhoff, 2008). Specifically C_{60} has been detected in four commercial cosmetics ranging from 40 ng/g to 1.1 μg/g, with a single-use quantity of cosmetic (0.5 g) containing up to 0.6 μg of C_{60} (Benn et al., 2011). Silver concentrations within consumer products (such as textiles, and cosmetics) ranged from 1.4 μg/g to 270 mg/g product (Benn et al., 2010). In terms of quantifying environmental exposure, the measurement of NMs within complex environmental matrices is impeded by methodological limitations for the detection and characterisation of NMs in complex media (Tiede et al., 2008). Model based estimates of environmental NM levels have been generated (Mueller & Nowack, 2008; Boxall et al., 2008; Gottschalk et al., 2009). The predicted environmental concentrations varied with regards to the NMs under investigation, and the environmental compartment under consideration. As an example, simulated levels of environmental concentrations range from 0.003 ng/L (C_{60}), to 21 ng/L (TiO_2 NMs) for surface waters, and from 4 ng/L (C_{60}) to 4 μg/L (TiO_2 NMs) for sewage treatment effluents (Gottschalk et al., 2009). Of importance is that these models are based on many assumptions and so the modelled value has a high level of uncertainty (Aschberger et al., 2011).

Exposure assessment studies are essential when evaluating the risks posed by NMs when used in conjunction within hazard information, and can inform the concentrations and doses of NMs used within hazard studies. However, there are few studies that have quantified NM levels in different exposure scenarios (occupational, environmental and consumer), which mainly derives from methodological limitations. More information in this area is therefore urgently required. The life cycle of NMs (from NM production, use and disposal) should also be considered, as the level of exposure, and "form" of the NM is likely to change throughout its life cycle (Abbot & Maynard, 2010; Thomas, 2009). Also of relevance is that the exposure of humans to NMs is likely to be associated with repeated exposures and co-exposures to other NMs or pollutants. There is currently a lack of studies that encompass these issues within hazard investigations, although there are some limited examples of repeated exposures in inhalation studies *in vivo* (e.g. Li et al., 2007) and *in vitro* hazard assessments (e.g. Jacobsen et al., 2008; Møller et al., 2011) which more accurately reflect the exposure of humans to NMs. Further research in these areas is required in the future.

There is debate surrounding the best dose metric to express nanomaterial exposure within hazard investigations. Ordinarily, the mass dose of particles administered to animals or cells is used to quantify exposure (e.g. μg/mL, mg/kg, μg/cm3). However, the importance of particle size (e.g. Ferin et al., 1992; Brown et al., 2001; Gaiser et al., 2009) surface area (e.g. Duffin et al., 2007; Oberdöster, 2000; Jacobsen et al., 2009) and number (Ferin et al., 1992) to particle toxicity is also recognised. Recently Teeguarden et al. (2007) have suggested that *in vitro* cellular dose for adherent cells should relate to the actual amount or particle or rate of particle deposition over time, which is dependent upon multiple factors including particle density, shape, size, agglomeration and the viscosity of the medium. This is a rather complex method of expressing dose which requires further consideration and debate. It is vital

that the dose metric selected is toxicologically relevant (Abbott & Maynard, 2010), so that there should be a relationship between the exposure metric selected and response observed. Therefore, identification of the most appropriate dose metric to utilise needs to be driven by the findings of toxicology investigations (Abbott & Maynard, 2010). Existing studies have provided some clarity regarding the properties of NMs that are influential to their toxicity (such as surface area, size *etc*), but there is currently insufficient information available in this area which promotes the uncertainty surrounding the selection of an appropriate dose metric. In addition conventional monitoring approaches that traditionally quantify exposures on a mass or number basis cannot adequately provide information on the different parameters of NMs (e.g. surface area) due to a lack of suitable equipment (Abbot & Maynard, 2010). Further studies are therefore required to elucidate which approaches are best to measure the desired parameters and relate them to NM toxicity (Thomas et al., 2009).

Exposure assessments have been conducted for NMs, but there has been a large variation in the levels of NMs within occupational, laboratory, environmental and consumer settings, from model or measurement studies. Exposure assessment for NMs has lagged considerably behind hazard studies and as a result very little is known about real-life exposures of humans or other organisms in the environment to NMs. Exposure studies are therefore a real bottleneck for assessing NM risk, and as such novel approaches that enable assessment of NM exposure need to be developed that will allow NM identification, characterisation and quantification within the environment (air, water, soil) and biological specimens. To date, it has been assumed that the monitoring of NM exposure will necessitate the development of more advanced instruments and devices that can quantify the level of NMs in different exposure scenarios and environmental matrices, but relatively little progress has been made in this area due to the difficulties of detecting such small particles, confirming their composition and distinguishing them from other particles in the environment. Innovative approaches to the exposure measurement of humans and the environment to nanomaterials are urgently required.

Due to the many uncertainties surrounding the level of NM exposure it is challenging to recommend suitable concentrations of NMs to utilise in hazard investigations. Instead, it seems appropriate for investigators to justify the use of NM concentrations and relate these to the hypotheses of study. Therefore, when selecting NM concentrations to test within hazard investigations a compromise has to be made between (i) identifying a environmentally or physiologically relevant exposure level, and (ii) investigating the dose dependency of NM responses through the use of a range of doses. Therefore, whilst dose response experiments may not reflect accurately the anticipated level of exposure they do consider a range of doses that may capture exposure levels anticipated within occupational, environmental and consumer setting, and can be used for risk assessment purposes. Importantly, conversions can be made between different dose metrics if sufficient physico-chemical characterisation information is provided by investigators, and this will facilitate making comparisons between different studies. Accordingly, it is suggested that researchers provide the concentrations on a mass, and surface area basis (where possible), but also provide a detailed account of the experimental design elements (e.g. volume of NM suspension administered, number of cells exposed, surface area of exposure site (e.g. culture dish, lungs) that could influence exposure. Therefore, for comparative purposes it would be beneficial for investigators to present exposure dose in terms of mass dose, surface area, where possible. Whilst this information may not be available for "early" nanotoxicology studies, this is recommended for future studies.

One of the central premises of toxicology is that dose is critical to the observed response. Therefore, regardless of the dose metric that is used it is essential that a relationship between dose and toxicological response is demonstrated for NMs. This is not a trivial task as it requires about 10 doses to obtain a reliable fit to a sigmoid dose-response curve; curve fit to a linear dose-response relationship requires about three doses and control, although this is only relevant for mechanistic endpoints related to development of tumours such as DNA lesions and mutations. It is clear that many studies, e.g. those of particle-induced inflammatory responses have far too few doses to obtain meaningful dose-response relationships. It therefore seems more appropriate and a better use of research money to focus on optimal dose-response relationships rather than screening hazards of a panel of MNs with relatively few high-dose exposures. NM toxicity can be related to particle mass, surface area or number; however, to date, the majority of activity in this area has focussed on determining whether a dose metric based on surface area is preferable to one based on mass. This is logical as the surface properties of NMs are fundamental to their toxicity as it is the surface that interacts with cells. Identifying a dose metric that is able to account for the response of cells and organisms to all forms of NMs would be beneficial for the standardisation of testing approaches to assess NM safety, and to allow comparisons to be made between *in vitro* and *in vivo* studies. In addition to particle mass, surface area and number several novel and more elaborate descriptors of NM dose have been suggested, including a model that describes the deposition rate of NMs onto cells *in vitro* over time in order to calculate cellular dose (Teeguarden et al. 2007; Hinderliter et al., 2010). Some other models use a slope analysis (greatest response per unit dose) of the dose response curve to relate NM dose to the biological response (Han et al., 2012; Rushton et al., 2010). It is recommended that alternative expressions of dose are investigated, but these need to be debated more widely before their relevance should be widely accepted.

Identifying relevant models, target sites and toxicology endpoints for NM hazard investigations

Historical drivers for NM hazard investigations

The absence of standardised tests to evaluate the toxicity of NMs meant that initially the most appropriate testing strategy to adopt when determining the safety of NMs relied on knowledge of how ultrafine air pollution particles (equivalent to NMs in terms of size) behaved. The toxicity of ultrafine air pollution particles provided the major basis for concern regarding the potential risks posed by engineered NMs to human health (The Royal Society and Royal Academy of Engineering Nanotechnology Report, 2004), but also the foundations for research concerned with evaluating the safety of NMs and investigating the potential mechanisms by which NMs may exert toxicity.

Interest in evaluating the toxicity of NMs stemmed from a number of findings which demonstrated that size was paramount to particle toxicity, and specifically that as particle size decreases, toxicity generally increases. The size dependency of particle toxicity was extensively investigated in the 1990s and early 2000s using a number of low solubility, low toxicity materials, including titanium dioxide (Ferin et al., 1992), carbon black (Li et al., 1999), and polystyrene (Brown et al., 2001) NMs. At the same time, epidemiological studies found a positive correlation between the level of particulate air pollution and increased morbidity and mortality rates from cardiovascular and pulmonary disease in susceptible individuals that had pre-existing disease (Pope & Dockery, 1999). The ultrafine particle component of particulate air pollution was hypothesised to be principally accountable for eliciting the cardiovascular toxicity associated with exposure (Seaton et al., 1995), with a correlation between ultrafine particulate exposure and adverse health impacts apparent within humans (Peters et al., 1997). Of interest is that the effects of NMs on vascular function and the progression of atherosclerosis (by repeated exposures) are in keeping with studies on air pollution or combustion-derived particles (Møller et al., 2011).

Knowledge of the mechanisms by which ultrafine particles exerted toxicity directed the experimental approaches to consider with highest priority when evaluating the toxicity of NMs within early nanotoxicology studies. Specifically, ultrafine particles were capable of eliciting toxicity by stimulating reactive oxygen species (ROS) production and oxidative stress development, which triggered an inflammatory response via the activation of cell signalling cascades (please refer to Donaldson & Stone, 2003; Oberdorster et al., 2008; Møller et al., 2010 for reviews). Inflammation and oxidative stress are able to promote cell and tissue damage and can culminate in genotoxic, cytotoxic, and fibrotic responses which are associated with disease, including cancer. This knowledge informed the selection of endpoints investigated within the discipline of nanotoxicology. There are a number of commonly used approaches that are used to assess the toxicity of NMs due to evidence of their involvement in mediating the adverse effects of NMs including; NM uptake by cells, inflammatory responses, stimulation of cell signalling pathways, oxidative responses, and genotoxicity. In the main, it is apparent that NMs can elicit toxicity via these processes, which has been observed for a number of NMs including CNTs (Shvedova et al., 2005; Poland et al., 2008; Brown et al., 2007; Johnston et al., 2010d), C_{60} (Sayes et al., 2004; Rouse et al., 2006; Johnston et al., 2010c), and TiO_2 (Ferin et al., 1992; Park et al., 2008; Monteiller et al., 2007; Johnston et al., 2009). Gene microarray and proteomic studies may also provide greater insight into the underlying mechanistic processes responsible for any observed toxicity of NMs (Park et al., 2011), but cannot be routinely considered due to the requirement for access to specialised equipment and their expense. NM interference with toxicology assays is a problem and must be considered within hazard investigations due to the potential for false positive or false negative results to be obtained (Wörle-Knirsch et al., 2006), which promotes the utilisation of two or more independent test systems for each endpoint of interest where possible.

Human exposure to NMs is likely to occur via inhalation, ingestion, injection and dermal absorption in occupational, environmental and consumer settings. Assessing the consequences of pulmonary exposure to NMs is vital and is relevant to all exposure settings. Inhalation studies are urgently required and a limited number of inhalation studies have been conducted (Li et al., 2007). However, intratracheal instillations are more frequently conducted due to the fact that they require less material and are far less costly, but their physiological relevance is questioned. The dose administered via instillation must be carefully considered, as high doses can result in the blockage of bronchi, and death via asphysixation (Warheit et al., 2004). The pulmonary toxicity of ultrafine carbon black (ufCB, 14 nm) has been compared following instillation and inhalation (e.g. Jacobsen et al., 2009; Driscoll et al., 2000), and the response following instillation has been demonstrated to be greater than that observed following inhalation. The enhanced response associated with intratracheal administration is likely to derive from the direct, bolus dose delivered to the alveoli. For inhalation, a possible greater deposition of NMs into the bronchi and bronchioles increases their propensity for clearance. This illustrates that the experimental approach used to assess the pulmonary effects of NMs affects the severity of the response, and thus should be justified.

The use of susceptible models is an important consideration within NM hazard investigations. As observed for pollution particles, there may be members of the population that are predisposed to the toxicity of NMs due to pre-existing (cardiovascular or pulmonary) disease. ApoE$^{-/-}$ mice are hyperlipidemic and prone to develop atherosclerotic lesions similar to those observed in humans (Jacobsen et al., 2009). It has been demonstrated

that the pulmonary inflammatory response elicited by NMs was greatest, and occurred at an earlier time in the ApoE$^{-/-}$ mouse model compared to wild-type (Jacobsen et al., 2009). This highlights that the mice may be primed for a fast response but also an increased sensitivity to the effects induced by NMs, and perhaps insinuating that members of the population eating western diet causing hyperlipidemia may be predisposed to NM toxicity.

Ideally, it would be beneficial to identify biomarkers of exposure and toxicity that can be used to assess the toxicity of NMs. It is recommended that inflammatory and oxidative stress endpoints continue to be routinely considered within hazard investigations for NMs and that they could be used as the basis for a screening strategy for assessing the safety of NMs *in vivo* and *in vitro*. More specifically, it is recommended that *in vitro* cytotoxicity tests could be used, when appropriate (e.g. when assessing the toxicity of NMs with commercial applications), in the first instance for benchmarking purposes and to prioritise which NMs should be investigated within more in depth mechanistic studies. This would also allow for the identification of sub-lethal concentrations of NMs to assess other endpoints of interest such as inflammation, oxidative stress and genotoxicity. However, it is recognised that there are many different NMs in use and development, and it may not be possible to make generalisations regarding the mechanisms underlying the toxicity of NMs, as there is evidence that different NMs elicit toxicity via different mechanisms, and that different cell types respond to the same NMs in different ways. Furthermore, NMs may elicit unique hazards that are not common to all NMs (e.g. mesothelioma-like response induced by CNTs (Poland et al., 2008)). The majority of existing studies have focused on the acute effects of NMs, and therefore there is a need to move towards chronic studies which investigate the long-term impacts of NM exposure.

Biodistribution of NMs following exposure: tracking of NMs following exposure and selection of target sites for hazard investigations

Evaluation of the toxicity of ultrafine pollution particles concentrated on their impact within the respiratory and cardiovascular systems. This derived from the knowledge that exposure to such particles was likely to occur via inhalation, and that the detrimental health impacts arising in association with increased (ultrafine) particulate matter (PM) were most evident in the lungs, and cardiovascular (CV) system of susceptible individuals. The exploitation of NMs within diverse applications means that there are a number of different exposure routes (including inhalation, ingestion, dermal absorption, and injection) associated with the use and development of NMs that must be considered when evaluating their safety. As such, it is necessary to consider what the outcome of NM exposure is at the different exposure sites in the body. Furthermore, assessment of NM toxicity at secondary target sites is required as the localisation of NMs is not restricted to their exposure site. This arises from the distribution of NMs within multiple organs and tissues located at a distance to their portal of entry due to their translocation following exposure (Oberdörster et al., 2005). Biodistribution studies are necessary to direct hazard investigations (e.g. through identification of relevant target sites), but also to interpret the findings of existing studies. NM exposure can occur via inhalation, ingestion or dermal exposure, and it is necessary to consider the transfer of NMs across these biological barriers into blood, and identify their accumulation within secondary target sites.

It is often challenging to detect NMs in biological tissues. The inherent properties of NMs can be used to detect and quantify some NMs following exposure (e.g. near infra red imaging for gold NMs (Cobley et al., 2011) and light fluorescence for carbon based NMs). However, such approaches are not always able to discriminate between NMs themselves and their dissolution products. Consequently, NMs are often modified with radioisotopes, fluorescent tags or chemical doping to enable visualisation and quantification in studies of deposition, absorption, distribution and elimination following exposure in order to investigate NM biodistribution (Oberdörster et al., 2002; Singh et al., 2006). However, the stability of some tags has been questioned as radiolabels may detach from the NM and enter the circulation (Mills et al., 2006; Wiebert et al., 2006; Möller et al., 2006, 2008). An alternative approach used to investigate the biodistribution of NMs is via their neutron activation. This novel approach enables, for the first time, the quantification of the entire NM dose in the animal following exposure, by analyzing each organ and tissue, remaining carcass and total excretion. As such, this approach has the advantage that a 100% balance of the biodistribution of the applied NM can be obtained. This is achieved using gamma-spectroscopy, without any further organ or tissue preparation, to measure the fate of all NMs contained in the entire organism, as well as those excreted from the body at very low NM doses (~10 µg/ animal). All NMs administered can therefore be accounted for in this model. Such studies have demonstrated that the biodistribution pattern of gold NMs (ranging from 1.4 to 18 nm; after neutron activation) following intratracheal instillation into the lungs of rats (Semmler-Behnke et al., 2008), intravenous injection (Semmler-Behnke et al., 2008; Hirn et al., 2011) or ingestion (Schleh et al., 2012) is NM size and charge dependent. Following pulmonary exposure >99% of 18 nm NMs were retained in the lungs (24 h post exposure) and translocation to the circulation was 0.2% (Semmler-Behnke et al., 2008). However, larger fractions of the smaller NMs were able to transfer to the blood and accumulate in secondary target organs and tissues (1.4 nm: 8.5%, 2.5 nm: 3%, 5 nm: 0.4%), and localisation was greatest in the liver and spleen as well as in soft tissue and skeleton (Semmler-Behnke et al., 2008).

This suggests that the ability of NMs to cross the air/blood barrier of the lungs and distribute within the body is a size dependent phenomenon (Semmler-Behnke et al., 2008). Following intravenous exposure, gold NMs also accumulated predominantly within the liver and spleen (Semmler-Behnke et al., 2008). The accumulation of 18 nm Au NMs occurred to a greater extent within these organs than their smaller counterparts, and the 1.4 nm Au NMs had a more widespread distribution including the kidneys, brain, heart (Semmler-Behnke et al., 2008). In addition to the studies described, the preferential accumulation of NMs within the liver and spleen has been repeatedly demonstrated for other NM types, although accumulation within other target sites such as the lungs, blood, kidney, brain, and heart is also observed following intravenous (e.g. de Jong et al., 2008; Sonavane et al., 2008; Sadauskas et al., 2009), ingestion (Schleh et al., 2012) and pulmonary (e.g. Kreyling et al., 2009; Semmler et al., 2004 Oberdörster et al., 2002) exposure. The localisation of NMs in the spleen and liver is likely to be accounted for by their uptake by the Reticulo-Enodthelial system. The absorption of NMs across barriers in the lung and GIT, and their subsequent distribution within the body was demonstrated to be NM size and charge dependent (Schleh et al., 2012). Comparing total translocation after instillation versus gavage the total translocated fraction of negatively charged gold NMs across the air-blood-barrier is significantly higher by about one order of magnitude than through the gut epithelium (Schleh et al., 2012).

Biodistribution studies can help identify potential target sites of NM toxicity. However, the translocation of NMs from their exposure site to secondary targets may not be required in order for them to exhibit toxicity. SWCNT and C_{60} are able to elicit oxidant-mediated damage to DNA (indicated by 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG)) within the lung and liver, but not the colon epithelial cells following ingestion (Folkmann et al., 2008). These studies were therefore important in illustrating that systemic effects can transpire in response to oral NM exposure and that there are differences in the genotoxic potency of particles. The mechanism by which NMs mediate damage at target sites which are at a distance to the exposure site requires further consideration. Effects may be mediated within the lung and liver following oral exposure due to the translocation of the NMs from the gastrointestinal tract (GIT) into the bloodstream and subsequent accumulation within other organs (e.g. Møller et al., 2012). Alternatively, the exposure of the GIT to NMs could stimulate the release of factors that act at distal target sites to elicit damage. It is therefore of interest for future studies to encompass the possibility that systemic effects are evident in response to NM exposure, and to investigate the mechanisms underlying such effects.

Development of animal alternatives

Due to the diversity of NMs available the development of predictive *in vitro* testing systems are an important component of nanotoxicology research, in order to minimise animal use and promote the development of animal alternatives which is necessary from both a financial, time and ethical perspective. This goal was identified some time ago (Maynard et al., 2006), and it is necessary to consider the progress that has been made in this area. The ability of *in vitro* models to predict the *in vivo* response is a debatable issue (Donaldson et al., 2009). As such, we will reflect on whether correlations can be made between *in vitro* and *in vivo* studies to determine if the *in vitro* models that are regularly used to assess NM toxicity are suitable.

It is often argued that *in vitro* models cannot replicate adequately the *in vivo* response to NMs. For example, inflammation is difficult to replicate *in vitro* because it depends on an intact vascular system and a large assortment of cellular and humoural interactions (Cho et al., 2010). Within *in vivo* studies, the response elicited by different NMs is characterised by different patterns of inflammation (in terms of time), resolution, and cellular exudates and thus this variability in inflammatory response and resulting pathology is difficult to predict or detect using *in vitro* models (Cho et al., 2010). The pulmonary toxicity exhibited by NMs (including carbonyl iron, crystalline silica, precipitated amorphous silica, nano-sized zinc oxide, and fine-sized zinc oxide), within an *in vivo* model (administered by intratracheal instillation to rats) was not well correlated to *in vitro* studies (using immortalised rat L2 lung epithelial cells, rat lung alveolar macrophages, and co-cultures of these cells) when cytotoxicity and inflammatory endpoints were compared (Sayes et al., 2007a). Furthermore, the difficulty in interpreting and extrapolating *in vitro* toxicity measurements to *in vivo* effects has been demonstrated for fullerenes (Sayes et al., 2007a). Specifically, the impact of fullerene functionalisation on their toxicity was investigated within *in vitro* (Sayes et al., 2004) and *in vivo* models (Sayes et al., 2007b), and there was little correlation between the findings. However, the *in vitro* models and the *in vivo* models used varied with respect to target organ and endpoints under investigation.

In vitro models are recognised as being useful tools within the investigation of NM toxicity, and in determining their mechanism of action, but their ability to predict the pathogenicity exhibited by NMs is often questioned. However, *in vitro* models have been used with success to identify the physico-chemical properties of NMs that drive their toxicity which is useful within the development of structure activity relationships. For example, a macrophage model has been developed successfully to predict the *in vivo* response (frustrated phagocytosis and asbestos-like pathogenicity in the lungs) to high aspect ratio nanomaterials (Brown et al., 2007; Poland et al., 2008). In addition, *in vitro* models have been useful in demonstrating the relationship between NM surface area and pulmonary asbestos-like toxicity (Donaldson et al., 2008; Jacobsen et al., 2009; Saber et al., 2012). Furthermore, a number of methodological advances have allowed for

the development of more relevant, and sophisticated *in vitro* models. *In vitro* systems used to assess NM safety are often criticised for their lack of complexity. However, the architecture of organs, such as the lung has been encompassed within a triple cell co-culture *in vitro* model using cell lines and/or primary cells that mimics the human alveolar epithelial barrier (Lehmann et al., 2011; Rothen-Rutishauser et al., 2005, 2008). In the future, it is also envisioned that microfluidic systems might also provide a useful screening system for the assessment of NM safety. For example the INLIVETOX FP7 funded project is developing a microfluidic system that incorporates healthy or inflamed GIT, endothelial, and liver components. A challenge has to derive culture and flow conditions that allow the survival and function of the diverse cell types within one system. This has now been achieved and the first nanomaterial exposures are in progress.

When cell lines and primary cells are utilised to assess the hazards posed by NMs *in vitro*, these can be derived from different species. Importantly, cross-species comparisons have been conducted that assess the exposure of humans and the environment, via ingestion (Gaiser et al., 2012). It has been observed that there were commonalities in the response of different species (invertebrates (*Daphnia magna*), fish and human cell lines) to silver and CeO_2 NMs, and therefore that cross-species extrapolations may be possible within NM test development in the future (Gaiser et al., 2012), and this requires further attention. Nanotoxicology hazard investigations should therefore reflect on the contribution of species differences to the observations that are made, as this is an important consideration within risk assessments.

It is not suggested that *in vitro* models are used exclusively to assess the toxicity of NMs, instead it seems more appropriate to view this as a long term goal, and that in the short term the development and validation of more sophisticated *in vitro* models that more accurately reflect *in vivo* conditions should be a priority. Currently, *in vitro* models can help screen and benchmark the toxicity of NMs at different target sites, and be conducted in parallel to *in vivo* studies. Advances on what the best dose metric will help enable comparisons to be made between *in vivo* and *in vitro* studies. Moving forward, it is anticipated that the *in vitro* models being generated can be used to develop high throughput systems to screen NM toxicity, and that ultimately structure activity relationships in conjunction with *in silico* approaches can be used to predict NM safety.

Risk assessment for NMs

Critical to ensuring the safety of NMs is an understanding of the risks they pose to human health. Ordinarily, the risks of materials are assessed through consideration of exposure data and hazard information within risk assessments. Risk assessments for NMs have been attempted using classical regulatory based approaches, but cannot be performed in full due to a lack of exposure and hazard data within the available literature (Aschberger et al., 2010a, 2010b; Christensen et al., 2010, 2011). As a consequence, definite conclusions cannot be reached from these risk assessments that can be used for regulatory decision making (Aschberger et al., 2010b). Specifically, risk assessments for human health require exposure data for various routes of exposure (inhalation, ingestion, dermal absorption) and exposure scenarios, and the establishment of Derived No-Effect Levels (DNELs) that are extrapolated from animal data (to the human situation) using uncertainty factors (Aschberger et al., 2011). Ideally, hazard information would be obtained using standardised methodologies, which are currently not available for NMs.

The challenges and knowledge gaps faced by risk assessors with regards to NMs have been identified previously (Thomas et al., 2009; Aschberger et al., 2011; Savolainen et al., 2010). The main data gaps associated with NM risk assessment include; NM key exposure metrics, dependable exposure scenarios, affordable monitoring technologies, exposure data and models, as well as data on NM translocation and toxicity and associated testing strategies (Savolainen et al., 2010). In addition, the uncertainty in both hazard and exposure assessment is likely to vary with the NM under investigation and its degree of functionalisation (Owen et al., 2009). Several authors (e.g. van Zijverden & Sips, 2009) agree that conventional risk assessment frameworks for chemical substances should be critically evaluated and eventually adapted for application to NMs. Therefore, instead of dwelling on the challenges faced when conducting risk assessments it seems more appropriate to consider what alternative approaches can be considered as interim measures until in depth risk assessments can be conducted for NMs. For example, a number of NM risk tools have been recently proposed (e.g. the Swiss Precautionary Matrix, the Dutch Stoffenmanager Nano, the French Anses system). However, most of these approaches serve as preliminary risk screening tools for industries, and none of the proposed methods can inform regulatory decision making. Minor attention has been paid to the application of novel approaches facilitating the near-term estimation of risks of NMs by integrating the existing data and information. An integral part of the PARTICLE_RISK project was the identification of suitable methodologies for this purpose, until full risk assessments can be conducted, and the Weight of Evidence (WoE) approach was applied (Zuin et al., 2011). WoE is an adaptable approach that allows usage and integration of different types of available data, information, and associated uncertainties as well expert judgment to assess risk and/or hazard (Weed, 2005). It is often used when assessing complicated systems where different data and information need to be integrated. According to the Massachusetts WoE Workgroup, the WoE approach may be defined as “the process by which

measurement endpoints are related to an assessment endpoint to evaluate whether a significant risk of harm is posed to the environment" (1995). The assessment endpoint is the environmental and/or human value that is to be protected, and is usually expressed in terms of a specific receptor (species, habitat, system) and a function or quality of the human health or environment that is to be maintained or protected. The measurement endpoints are all the different pieces of information (called the lines of evidence (LoE)) used to evaluate the assessment endpoint. LoE are a set of information that pertain to an important aspect of the environment or human health (Smith et al., 2002), and they serve to predict hazard or risk. Importantly, several LoE are often associated with a single assessment endpoint (Massachusetts Weight-of-Evidence Working Group, 1995). The WoE approach integrates information provided by multiple LoE, and relates them to a single measure for decision making (Smith et al., 2002). A critical component of a WoE analysis is allocating a weighting to the available information, and this typically considers; the strength of the relationship between each LoE and the assessment endpoint, the quality of the data and experimental design, whether the findings are reproducible (e.g. is there conflicting evidence?) and the nature and severity of the response observed (Massachusetts Weight-of-Evidence Working Group, 1995). Expert judgement is crucial within the appraisal of the value of the available information (i.e. its weighting) (European Chemicals Agency, 2010). The way the WoE is implemented is case-dependent, and so no standard approach currently exists and the approach used will be influenced by the amount of information available and the importance of a decision being made (Burton et al., 2002; European Chemicals Agency, 2010). The WoE approach provides the opportunity to make use of less reliable information by pooling together all available information (European Chemicals Agency, 2010). It is useful in the preliminary hazard ranking of NMs given the scarcity of reliable data on exposure and effects of NMs (Zuin et al., 2011).

In the PARTICLE_RISK project, the WoE approach was applied to ufCB, SWCNT, C_{60} and QDs by integrating and combining physicochemical properties of NM and toxicity data of the NMs, in order to assess their potential hazardousness level for human health (Zuin et al., 2011). One benefit of such an approach has been the assembly of a comprehensive dataset of combined hazard and intrinsic properties of NMs (i.e. size, shape, etc.), to ascertain which of these properties are important for governing potential risk. This is an important aspect because the nature of the data and information gathered, and the way this information is integrated will form the evidence base to enable decision making about risks. Tools like the WoE approach, Multi Criteria Decision Analysis (MCDA) as well expert judgment are suitable approaches for addressing potential risk of NMs which take into account all data gaps and uncertainties concerning NM exposure and effect. This is currently a dynamic area of research and several approaches are still under development. For example, within the FP7-funded project ENPRA a novel approach for human health risk assessment of NMs based on WoE and MCDA is being developed. Its main goal is to quantitatively assess the occupational risks from NMs in order to inform adequate risk management actions and regulatory decisions. Based on the conventional risk assessment paradigm, the approach uses experimental/ estimated exposure and effect data provided by project partners and/or available in the literature that refer to a panel of nanomaterials (including titanium dioxide, zinc oxide, silver nanoparticles and multi-walled carbon nanotubes), to rank and prioritise them for further testing and risk assessment. In ENPRA, the WoE based risk assessment will allow researchers to cluster studied NMs into ordered risk categories in the context of MCDA. It will include a number of steps such as; identifying suitable LoE (e.g. NMs properties, exposure metrics, oxidative stress, DNA damage *etc*.) and stakeholders/ experts relevant to the risk evaluation, assigning a weighting to the evidence, performing the WoE analysis through developed software, and interpreting the results of the model.

It is suggested that the requirements of risk assessment approaches (such as WoE) are used to inform the design of hazard investigations. In the future, other available evidence and indicators, and incorporation of exposure data and information could be included together with new effect evidence to improve the procedure and then to calculate a risk index, to develop more robust conclusions concerning the risk of NM.

Conclusion

The area of nanotoxicology first emerged in the early 2000s (Donaldson et al., 2004) and since this time there has been an exponential increase in the number of hazard and exposure studies concerned with evaluating NM safety. As a result, many advances have been made in improving the design of investigations conducted to evaluate NM toxicity, and in developing a suitable testing strategy to assess NM safety. Importantly, many lessons have been learnt from existing studies and thus the discipline is constantly adapting and evolving in response to new evidence. However, despite this progress there are still many challenges that exist when evaluating NM risk to human health and the environment, and therefore uncertainties surrounding the risks posed by NMs. We have reflected on existing studies, and considered the direction that nanotoxicology research is taking in order to identify challenges that require urgent attention within the exposure and hazard assessment of NMs, and aimed to provide guidance for future research activities to address these issues. It has not always been possible to provide definitive recommendations on the best practical approach to take within the investigation of NM toxicity but guidance has been provided for aspects of

the experimental design that should be considered prior to the start of any hazard investigation for NMs. Multi-disciplinary working is fundamental to nanotoxicology research, and this type of collaboration is essential within the development of innovative solutions to existing challenges.

It is vital that the physico-chemical characteristics that drive NM toxicity are identified to ensure that the safe design of NMs is supported, and the findings can promote the development of legislation and risk management measures to protect against any identified risks of NMs. In conjunction with hazard data, high quality characterisation data is required to support the intelligent and safe design of NMs in the future, and to inform the development of predictive models to screen NM safety. However, this does not mean that every hazard assessment should be accompanied by
a rigorous physico-chemical characterisation of the NM under investigation. Instead, justification for the characterisation approach taken should be explicit for each study (i.e. for what purpose is the characterisation being performed?). Furthermore, the feasibility of conducting the analysis should be considered (i.e. can an extensive characterisation of NMs in biological media be conducted?) and the characterisation should provide high quality data that has been correctly interpreted.

It is suggested that the needs of risk assessments drive exposure assessments and hazard investigations. As with any emerging technology or novel material, the scarcity of exposure and effect data introduces potentially high uncertainty into the characterisation of NMs risk. Many lessons have been learnt with regards to the most suit-able approach required to assess NM safety, and there is a greater understanding of what a testing strategy for NM safety assessment should encompass. However, there are still a number of barriers that obstruct the routine assessment of NM safety using standard approaches. Future priorities will include not only the generation of new exposure and hazard data, but also the applica-tion of suitable approaches and methods to support future decision on NMs risk. We are still some way off having the required information to conduct in depth risk assessments but alternative approaches, such as WoE have been identified as suitable alternatives in the meantime. Whilst the focus of this review was on the potential implications on human health there are many cross-overs that exist with nano eco-toxicology research that should be explored. We are now in a position where we have a greater awareness about the knowledge gaps and research priorities relating to nanotoxicology and this information is informing the development of intelli-gent testing strategies for nanomaterials (e.g. FP7 funded project ITS-NANO; www.itsnano.eu). Moving forward, it is necessary to prioritise the research strategy for nano-toxicology and separate out what is currently feasible from what we ultimately need to know and how we get there.

Declaration of interest

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