HM Government

# Characterising the potential risks posed by engineered nanoparticles

## UK Government research – a progress report

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# 1. Background and Introduction

1 In accordance with the Government's response[1] to the Royal Society and Royal Academy of Engineering report on nanotechnologies[2], a cross-Government group (the Nanotechnology Research Co-ordination Group - NRCG) has been set up to co-ordinate research efforts relating to the potential human health and environmental risks posed by the products of nanotechnologies. This work is aimed at leading to the development of an appropriate framework and measures for controlling any unacceptable risks.

2 The NRCG's first research report, published in November 2005[3], sets out a programme of nineteen research objectives (see Annex 1) to characterise the potential risks posed by engineered nanoscale materials. The report also describes ongoing activities and funding mechanisms to address these objectives. The scope of the report includes the societal issues surrounding the development and use of nanotechnologies.

3 For the purposes of the research programme, engineered nanoscale materials are defined as having been deliberately engineered (i.e. not natural or unintentional by-products of other processes); having two or more dimensions broadly at the nanoscale; and being 'free' within any environmental media at any stage in a product's life-cycle.

4 In order to progress the research objectives set out in the NRCG report, and considering their diverse nature, from metrology, through hazard and risk assessment, to social dimensions, the NRCG has organised them into five interrelated areas, and established five Task Forces with the necessary expertise (see Annex 2 for Task Force membership) to take them forward:

- Task Force 1: Metrology, Characterisation, Standardisation and Reference Materials
- Task Force 2: Exposures – Sources, Pathways, and Technologies
- Task Force 3: Human Health Hazard and Risk Assessment
- Task Force 4: Environmental Hazard and Risk Assessment
- Task Force 5: Social and Economic Dimensions of Nanotechnologies

[1] http://www.dti.gov.uk/science/science-in-govt/st_policy_issues/nanotechnology/page20218.html
[2] www.nanotec.org.uk/finalReport.htm
[3] http://defraweb/environment/nanotech/research/reports/index.htm

5 In formalising this arrangement, the NRCG specifically asked the Task Forces to develop action plans that: (1) drill down into their allocated research objectives; (2) detail priority research areas; (3) provide details of ongoing research projects; and (4) develop proposals to address particular research needs.

6 This report brings these action plans together to provide an overall picture of the Task Forces' progress since November 2005. As well as providing our stakeholders with a general update, its publication is intended to further encourage a co-ordinated and informed approach to addressing this agenda, both within the UK and internationally. While we are seeking to secure appropriate levels of funding to meet some of the objectives, there should be no expectation that the UK should provide the answers to fill all the information gaps. That is why we are working at international level to optimise opportunities for cooperation and collaboration to meet our common goals in nanotechnology research. Particular contacts and working arrangements have been made with the European Commission and EU Member states, the OECD, US institutions such as the Environmental Protection Agency, the National Centre for Toxicology Research, the National Institute for Occupational Safety and Health, the National Science and Technology Council, and the National Institute of Standards and Technology, who all have research programmes in nanotechnology hazard and risk assessment.

7 Structurally, the report is organised into sections that correspond to the action plans of the five Task Forces. For ease of reference, Table 1 summarises the research activities and proposals set out in each of the sections. However, while the development of the plans necessitated close communication and collaboration across the Task Forces, and we have attempted to apply a common format in terms of presentation within this report, each section/action plan should be viewed as the product and responsibility of its respective Task Force. Further, the Task Forces will continue to be responsible for updating the scope and content of their action plans, in line with our changing knowledge.

| Table 1. Summary of Government progress on research objectives | | | |
|---|---|---|---|
| **Project description** | **Active or proposed** | **Est. cost (£)** | **Progress** |
| **RO1 To understand the social and ethical implications of nanotechnologies through a programme of public dialogue and social research** | | | |
| (1) Nanodialogues: examines the practicalities of the concept of 'upstream' public engagement in relation to nanotechnologies. | Active | 240k[4] | Outputs of the project on nanoremediation to be published in Autumn 2006. Project as a whole to report formally in Spring 2007. |
| (2) The Nanotechnology Engagement Group (NEG): multi-stakeholder group charged with mapping out and analysing public engagement activities on nanotechnologies. | Active | 115k | Final report to published in September 2007. |
| (3) Small Talk: project that pulls together the findings of a wide range of activities around the UK that are focused on discussing nanotechnologies with the public and scientists. | Active | 50k | Final project report to be published in Autumn 2006. |
| (4) Environmentally beneficial nanotechnologies: study to determine the nature of and barriers to environmentally beneficial nanotechnologies. | Active | 65k | Project commenced in September 2006, and is due to report formally in March 2007. |
| (5) Expert advice: study to address the role and effectiveness of non-scientific expertise on scientific advisory committees. | Active | 80k | Project commenced in June 2006. An initial set of outputs will be discussed in a Demos pamphlet to be published in December 2006. The project will report formally in March 2007. |
| (6) Multi-level risk governance for nanotechnologies: programme of work to address the adequacy of current risk governance frameworks for nanotechnologies. | Proposed | 1m[5] | Discussions are ongoing to determine partners with which to take forward specific projects. |
| **RO2 To identify the most suitable metrics and associated methods for the measurement and characterisation of nanomaterials** | | | |
| (1) DTI National Measurement System Programmes: Project 3.1 of this programme is developing new measurement methods, and protocols for existing measurement instruments, for the characterisation of micro- and nanoparticles. Particular aspects include surface chemistry and nanomechanics. | Active | 78.6k in 2005/6 | - |

[4] Supported by a grant of £120k from the DTI's Sciencewise programme, with matched funding from other partners.
[5] Matched funding programme.

| Table 1. Summary of Government progress on research objectives | | | |
|---|---|---|---|
| **Project description** | **Active or proposed** | **Est. cost (£)** | **Progress** |
| (2) Method selection for carbon nanotube detection, characterization and monitoring. | Proposed | - | - |
| (3) Carbon nanotube detection and charaterisation. | Proposed | - | - |
| (4) Differentiation between natural and man-made nanoparticles. | Proposed | - | - |
| (5) Low aspect ratio particles: surface chemistry and surface area measurement. | Proposed | - | - |
| **RO3 To develop standardised, well-characterised reference nanoparticles** | | | |
| (1) Research project on prioritisation of nanoparticle reference material requirements (to include two expert workshops). | Proposed | 80k | Contract being let by Defra in Autumn 2006. |
| (2) Development of documentary standards for reference material preparation. | Proposed | - | - |
| **RO4 Understand the properties of nanoparticles in the context of their ignition and explosion potential and assess/develop methods for evaluating this** | | | |
| (1) Investigation of the Fire and Explosion Properties of Nano-particles and the possible quantification methodologies (HSE). | Active | Approx. 300k | - |
| **RO5 Further identification of sources of nanoparticles** | | | |
| (1) Helpdesk - information bulletin service reviewing studies on exposure and potential health effects of nanomaterials relevant to occupational settings. | Active | 52k (Yr1) | The key word lists for the literature searches have been completed. The first bulletin is scheduled for Autumn 2006. |
| (2) An analysis of potential exposures throughout the life-cycle of engineered nanoscale materials (UCL). Study is being completed as part of the NANOSAFE 2 project. | Active | - | - |
| (3) Addressing potential exposure through a life-cycle approach. | Proposed | - | - |
| (4) Project to quantify the amount of exposure within the life cycle of a nanoparticle. | Proposed | - | - |
| (5) Work to differentiate between natural and man made nanoparticles. | Proposed | - | - |
| (6) Identification of further uses and resources. | Proposed | - | - |
| **RO6 Optimisation and development of technologies that enable the measurement of occupational and environmental exposure to nanoparticles via air. Technologies for measurement of nanoparticles via air; RO7 Understanding of fate and behaviour of nanoparticles in air** | | | |
| (1) Nanoparticle Occupational Safety & Health (NOSH) Consortium. | Active | 35k from | Project on going at Du Pont's Experimental Station |

| Table 1. Summary of Government progress on research objectives | | | |
|---|---|---|---|
| **Project description** | **Active or proposed** | **Est. cost (£)** | **Progress** |
| Project will generate nanoparticle aerosols and measure their behaviour; develop a portable measurement device and measure the barrier efficiency of various materials. | | HSE. Overall budget $680,000 | Laboratories and is due for completion in 2007. Progress is good with the reproducible production of $SiO_2$ nanoparticles achieved. Work on respirators proceeds. Papers are in preparation and there will be presentations at the next NIOSH conference. |
| (2) Nanosh: European multi-centre research focused on occupational exposure to nanoparticles and their health effects. Includes university work. | Active | 573k from HSE/ HSL. Overall budget 27m | Preliminary work has started. Plan is to visit Leeds University in October 2006 for initial measurements. Nanosh funding will be used to work on a pitzo-electric means of mass measurement. |
| (3) Nanosafe 2: four main scientific subprojects: (1) measurement of exposure to and characterisation of airborne nanoparticles; (2) potential health effects; (3) procedures for safe production and handling; and (4) standards, regulations and societal implications. | Active | 70k from HSE/ HS Overall budget 8.3m | Will follow the same sampling strategy as Nanosh so results can be compared. Results are starting to come in. HSL will be completing a regulatory review by the end of September 2006 making use of existing UK and EU reviews. |
| (4) Investment Research Programme – Nanochallenge: objectives include the development of the HSL aerosol generation and measurement capabilities – (i) Development of improved methods of collection and characterisation of airborne nanoparticles. The applicability of biological monitoring will also be investigated; (ii) Assessment of dustiness testing for nanomaterials. | Active | Approx 400k | Initial work with carbon black, $TiO_2$ and gassifier fines have been assessed and the results show the dustiness kit doesn't work.<br>A start has been made on assessing collection. This links to the Nanosh project where the Finnish partners are doing some characterisation work. Both groups will follow the same methods. |
| (5) Assessment of the potential use of nanomaterials as food additives or food ingredients in relation to consumer safety and implications for regulatory control. | Active | 40k | Project due for completion January 2007. |
| (6) Assessment of current and projected applications of nanotechnology for food contact materials in relation to consumer safety and regulatory implications. | Active | 68.5k | Project due for completion Spring 2008. |
| (7) Modeling: generation of real exposure data and comparison to predictions of existing environmental and human exposure models. | Proposed | - | - |

| Table 1. Summary of Government progress on research objectives | | | |
|---|---|---|---|
| **Project description** | **Active or proposed** | **Est. cost (£)** | **Progress** |
| Current work on improving human exposure models will be monitored and should finish before work on modeling nanoparticle exposure starts. | | | |
| **RO8 Development of exposure control devices** | | | |
| (1) Investment Research Programme – Nanochallenge. This exposure assessment and control project includes the development of improved methods for the generation of nanoparticle aerosols. | Active | Approx 400k | Initial set up work ongoing. |
| (2) Nanoparticle Occupational Safety & Health Consortium (NOSH). This research has three specific objectives including development of a portable measurement device. | Active | 35k from HSE. Overall budget $680,000 | Meetings with instrument manufacturers show no portable monitoring device available in the short/medium term. |
| (3) Nanosh.: working party 2 will assess the performance of Respiratory Protective Equipment and protective clothing against nanoparticle penetration in real-life conditions. | Active | 573k | Funding agreed. Work starting. |
| (3) Effectiveness of current control measures: a review of control measures currently used for the production, handling and end use of nanoparticles. | Proposed | - | Work will start following a survey, as part of Nanosh, of what control devices are being used. |
| (4) Control Banding project. This links with the COSHH Essentials work and HSE would like to form an international group to develop a Control Banding approach to exposure control. | Proposed | - | A case will be made for the nano control banding work to be included in the Disease Reduction Programme. |
| (5) Assessment of the effectiveness of water filtration techniques against nanoparticles. | Proposed | - | - |
| **RO9 Optimisation, development and application of technologies that enable the measurement of exposure to nanoparticles in soil and water** | | | |
| (1) NERC Aquanet Knowledge Transfer network on natural aquatic colloids. http:/www.gees.bham.ac.uk/research/aquanet/ | Active | 60k | Potential for knowledge and technology transfer from understanding natural aquatic colloids to engineered nanoparticles. |
| (2) Develop a network of contacts in Europe to keep informed of research and field-scale trials and identify opportunities for EC funding of collaborative research or demonstration proposals. | Proposed | - | - |
| (3) A targeted call for measurement technologies for nanomaterials in | Proposed | - | - |

| Table 1. Summary of Government progress on research objectives | | | |
|---|---|---|---|
| **Project description** | **Active or proposed** | **Est. cost (£)** | **Progress** |
| water and soil | | | |
| **RO10 Research to understand the environmental fate, behaviour and interactions of nanoparticles in soils and water** | | | |
| (1) The Environmental Nanoscience Initiative: mechanism to address basic research into fate and behaviour of nanomaterials in the environment (www.nerc.ac.uk/research/programmes/nanoscience). | Active | Approx. 520k | Announcement of funding opportunity October 2006. |
| (2) Current and Predicted Environmental Exposure to Engineered Nanomaterials. | Proposed | Approx. 50k | Defra due to let contract in Autumn 2006. |
| (3) Review of the published literature on the range of nanoparticles developed and used for wastewater remediation. | Proposed | - | - |
| (4) Laboratory study of nanoiron and bimetallic materials currently available for remediation of soil or water. The study will provide a benchmark for decision- making on which nanoparticles are potentially suitable for small-scale releases to provide information on field-scale fate and transport. | Proposed | - | It is intended that this study be funded through a CASE studentship. |
| (5) Field-scale remediation of contaminated groundwater and soil. Comparative assessment of remedial performance using micro- and nano-scale particles, assessment of measurement technologies and investigation of nanoparticle transport. | Proposed | - | - |
| (6) Consideration of the unintentional release of nanoparticles through disposal to landfill or discharge to sewage systems and wastewater treatment plants. Address scale of exposure through disposal routes. | Proposed | - | - |
| (7) Develop an understanding of nanoparticle behaviour and fate in drinking water treatment processes. | Proposed | - | - |
| **RO11 Research to establish a clear understanding of the adsorption of nanoparticles via the lung, skin and gut and their distribution in the body (i.e. Toxicokinetics), identifying potential target organs/tissues for toxicity assessment of nanoparticles in the human body**<br>**RO12 Research to establish a clear understanding of intra and intercellular transport, localisation and toxicity of nanoparticles and their cellular toxicity**<br>**RO13 To establish a clear understanding of whether oxidative stress, inflammatory effects and genotoxicity apply to nanoparticles**<br>**RO14 Research to establish a clear understanding of the deposition, distribution, toxicity, pathogenicity and translocation potential** | | | |

| Table 1. Summary of Government progress on research objectives | | | |
|---|---|---|---|
| **Project description** | **Active or proposed** | **Est. cost (£)** | **Progress** |
| **and pathways for nanoparticles in the airways and lung and their potential impacts on the cardiovascular system and brain.**<br>**RO15 Given the current use of nanoparticles in consumer products there is a need to further our understanding of dermal uptake, penetration and toxicity of nanoparticles**<br>**RO16 To develop testing strategies for human health hazard assessment and assess how fit for purpose current test methods are as applied to nanoparticles** | | | |
| (1) In vitro studies to investigate:<br>• penetration of nanoparticles across physiological barriers (gut, lung, skin, blood-brain, placenta)<br>• penetration of nanoparticles into cells<br>• persistence of nanoparticles in cells and tissues<br>• induction of cellular stress by nanoparticles (oxidative stress and inflammation)<br>• mutagenicity and hence carcinogenicity<br>• damage to cells by nanoparticles (cytotoxicity and apoptosis)<br>• circulatory effects of nanoparticles. | Proposed | - | Task Force 3 commissioned a report from the Health and Safety Laboratory (HSL) on *in vitro* methods for assessing the toxicity of nanoparticles. This was discussed by an *ad hoc* group of experts convened by the Task Force and was edited by them. The final report was compiled by the Chair of Task Force 3 and is provided as Annex 4 to the report. |
| **RO17 Research to establish the uptake, toxicity and effects of nanoparticles on groundwater and soil micro-organisms, flora and fauna, especially in the context of remediation**<br>**RO18 Research to establish the mechanisms of toxicity, toxicokinetics and *in vivo* effects of nanoparticles to key ecological groups (including invertebrates, vertebrates (e.g. fish) and plants). A key aspect of such work should be the facilitating of knowledge transfer from human toxicological studies to inform ecotoxicology** | | | |
| (1) NERC Responsive Mode Funding. | Active | Approx. 750k | 3 recent grants awarded (University of Birmingham, University of Exeter, University of Plymouth). |
| (2) The Environmental Nanoscience Initiative: mechanism to address basic research into fate and behaviour of nanomaterials in the environment. | Active | Approx. 520k | Announcement of funding opportunity October 2006. Launched at international conference held September 2006. |
| (3) International activity monitoring and co-ordination<br>EU Framework Programmes (FP6, FP7); US Environmental Protection Agency NCER Programme. | Active | - | Active engagement with USEPA, EU Framework Programmes, EU Joint Research Centre. Opportunities for research funding under EU FP7 programme. |
| (4) Knowledge transfer and capacity building. | Active | - | 'Environmental Effects of Nanomaterials' – |

| Table 1. Summary of Government progress on research objectives | | | |
|---|---|---|---|
| **Project description** | **Active or proposed** | **Est. cost (£)** | **Progress** |
| | Active | 125k | international scientific conference held September 2006, London.<br>Nanonet – recently NERC funded network on nanoparticles in the aquatic environment |
| **RO19 Define endpoints to be measured in ecotoxicological studies and assess how fit for purpose current standard tests for persistence, bioaccumulation and toxicity are when considering nanoparticles. This should lead to the defining of a suite of standard PBT protocols for use in environmental hazard assessment** | | | |
| (1) Assessment of how fit for purpose current and proposed regulatory ecotoxicological tests are for nanomaterials. | Active | Approx. 25k | Contract to be let by Defra in Autumn 2006. |
| (2) International activity monitoring and co-ordination. SCENIHR Nanomaterials Working Group assessing the Technical Guidance Document (TGD) supporting chemicals regulatory risk assessment. | Active | - | Co-ordinating with European Chemicals Bureau, European Centre for the Validation of Alternative Methods (ECVAM) and OECD.<br>TGD working group due to report early 2007. |

# 2. Task Force 1: Metrology, Characterisation, Standardisation and Reference Materials

## 2.1 Aims of Task Force

8 Task Force 1 is responsible for progressing the research agenda as it relates to metrology, characterisation, standardization, and reference materials. More specifically, its activities are focused on the following research objectives:

RO 2 To identify the most suitable metrics and associated methods for the measurement and characterisation of nanoparticles.

RO 3 To develop standardised, well-characterised reference nanoparticles.

RO 4 To understand the properties of nanoparticles in the context of their ignition and explosion potential, and assess/develop methods for evaluating this.

RO 5 Optimisation, development and application of technologies that enable the measurement of exposure to nanoparticles in soil and water.

## 2.2 Identifying the most suitable metrics and associated methods for the measurement and characterisation of nanoparticles (RO2)

9 Information on the composition and properties of engineered nanoparticles, such as size distribution, shape and particularly surface area, solubility, surface charge and other surface chemical properties, is fundamental to understanding their potential toxicological and ecotoxicological properties. Instruments for surface area measurement, specifically aimed at the toxicology testing community, are beginning to appear commercially, e.g. there are recently introduced particle surface area monitors that give values for the surface area of particles deposited in different regions of the human lung. However, at present, there is little information on the influence of the above nanoparticle characteristics on basic aspects of toxicology and ecotoxicology, mechanisms of action and influence on dose-response relationships and toxicokinetic profiles. Work is also needed to enable characterisation and measurement of nanoparticles in different environmental and biological media, including air, water and soil and a potential wide range of organisms and their tissues and organs.

### 2.2.1 Active research projects

*2.2.1.1 DTI Measurements for Emerging Technologies programme*

10 Project 3.1 of the DTI Measurements for Emerging Technologies programme is developing new measurement methods, and protocols for existing measurement instruments, for the characterisation of micro- and nanoparticles. Particular aspects include surface chemistry and nanomechanics. The project is scheduled to last three years and involves a wide range of UK industrial partners, with the principal work being done at NPL.

### 2.2.2 Research proposals

*2.2.2.1 Method selection for carbon nanotube detection, characterisation and monitoring*

11 There is a particular need for carbon nanotube detection, characterisation and monitoring given the increase in carbon nanotube production, which will find its way into products that will end their lives in the UK. Hence, we are focusing our work in this area.

12 There are no good methods for detecting, characterising and quantifying carbon nanotubes available to the toxicology community, except for Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). These are expensive, slow, have sampling issues and are often extremely matrix-dependent. New methods need to be developed based on the special chemical or spectroscopic properties of nanotubes so that they can be distinguished from carbonaceous “background.” Nanotubes have a few special properties (e.g. very characteristic Raman spectra) that may assist in this, but the underlying metrology of potential techniques needs to be developed to the point where the toxicology community can make use of it. This may involve the development of new instruments, but is more likely to mean new standard protocols for existing instruments.

*2.2.2.2 Carbon nanotube detection and characterisation*

13 Currently only SEM and TEM – with consequent high cost and sampling issues – are available. More rapid methods are needed. Highly structurally specific methods of detection are needed to distinguish nanotube materials against a background of carbon in other forms. Raman and surface-enhanced Raman have promise here, but are not sensitive to the length or aspect ratio of tubes that may be the property of greatest significance to their toxicology. Other methods that may give information on length distribution more directly, such as terahertz spectroscopy, are at an even earlier stage of development.

*2.2.2.3 Differentiation between natural and man made nanoparticles*

14 This is a major issue, especially for nanoparticle producers who must demonstrate that the exposure of their workers to potentially hazardous engineered nanoparticles leads to no significant risk, while relatively large quantities of "ultrafine" products of incomplete combustion (often from petrol and diesel engines) enter through doors and windows. Most instruments for nanoparticle sizing (e.g. by Dynamic Light Scattering) are insensitive to chemical composition. This is a serious limitation for the quantification of engineered nanoparticles, but much less of a problem in traditional applications to quantification of incidental ultrafine particles. Therefore, the engineered nanoparticle community can benefit in only a limited way from the legacy of development of particle sizing for environmental monitoring. New measurement principles are needed.

*2.2.2.4 Low aspect ratio particles: surface chemistry and surface area measurement*

15 For the wide range of low aspect ratio particles, although mechanisms of health and environmental impact are not clear, the physico-chemical properties most likely to be needed as input parameters in future structure activity relationships are:

- surface chemistry
- total surface area
- surface curvature and defect density.

16 Rapid methods of measuring these parameters (e.g. the total surface area of particles in suspension having a specified surface chemistry) are required, initially for toxicological studies, but subsequently for risk assessment based on structure-activity relationships or other models. Where measurement techniques exist, they are not rapid. A scientific scoping study is needed to prioritise potential measurement principles and recommend the most promising methods for development.

### 2.2.3 Monitoring other programmes of work

17 Work on the use and incorporation of nanotube materials into products is led by the USA, Japan and Korea, with some significant work across Europe. Standardisation of measurement and characterisation methods will take place under Working Group 2 of ISO TC229 on Nanotechnologies, and close monitoring of new work items within this working group will be important.

18 UK Research Council funding is substantial in this area and although directed largely at fundamental science and support for innovation, good liaison is needed, especially with nanotechnology Interdisciplinary Research Centres, to make best use of measurement and characterisation expertise.

## 2.3 Developing standardised, well-characterised reference nanoparticles (RO3)

19 Without reliable reference materials, the results of toxicology studies for engineered nanoparticles will be difficult to compare and therefore of limited value in drawing conclusions.

20 Efforts in this area need to address three different types of reference material. Of these, the simplest is the **set of materials for physico-chemical measurement and instrument calibration**. These may comprise, for example, monodisperse particles of different sizes. Although many micrometre-scale particulate size standard materials are available, there are relatively few truly nanoscale reference materials (e.g., the smallest seems to be the US National Institute for Standards and Technology 100nm spherical particulate material). There is also a need for reference materials with the same or similar size distribution, but distinctly different surface chemistry (e.g. aluminium vs. transition metal, metal vs. graphitic). Few studies have compared the performance of particle sizing instruments applied to particles of different chemistry. The likely importance of surface chemistry on particle toxicology has been highlighted.

21 In addition to this set of materials for instrument testing and calibration there is a need for a "library" of materials for toxicology purposes. This library can be further divided into two types. The first is **materials that represent those in high-volume industrial production**. Samples will need to be homogenised and characterised so as to provide a continuous, reliable source to the nanoparticle toxicology community. The quantities of material may need to be significant, especially if the needs of some potential ecotoxicology studies are to be met.

22 The second involves **materials designed to answer specific toxicology questions**. The example here, which may or may not be significant in practice, is the question of nanotube length. By analogy, which may or may not be valid, with asbestos fibre toxicology, short nanotubes, below 1 micrometre in length, may be effectively removed by phagocytosis, whereas longer nanotubes may not. Therefore a set of reference materials of this type may comprise two nanotube materials of 1 and 10 micrometre lengths. (See also Task Force 3 item 4.2.2 for consideration of other nanoparticles as reference materials.)

23 A prioritisation exercise should be carried out on the three categories set out above followed by the development of reference materials to fulfill the most pressing needs.

### 2.3.1 Research proposals

#### *2.3.1.1 Identifying a priority set of reference materials*

24 Defra will let a contract in Autumn 2006 entitled 'Reference Materials for Engineered Nanoparticle Toxicology and Metrology.' The project will seek to provide a list of candidates for inclusion in a set of reference materials to support measurement, toxicology and risk assessment of engineered nanomaterials with particular reference to the UK.

*2.3.1.2 Development of documentary standards for reference material preparation*

25 ISO TC229 on nanotechnologies and/or CEN TC352 on Nanotechnologies could enable the development of documentary standards covering preparation methods that may reduce the need for a centrally-held stock of physical reference material. This may be a particularly viable route to reference materials designed to resolve specific toxicology issues.

### 2.3.2 Monitoring other programmes of work

26 Work on selecting and developing reference materials is going on at a number of centres internationally (see Table 2), and collaboration will be essential to minimise duplication of effort.

| Organisation | Territory | Work in progress |
|---|---|---|
| IRMM (EC Institute for Reference Materials and Measurements) | EU | IRMM have nanoparticle reference materials in development. IRMM are in the process of designing a specific activity within the Reference Materials' projects for the Work Programme of the Joint Research Centre for the 7th Framework Programme (2007-2013) devoted to the development, production and certification of reference materials supporting quality assurance of measurements in nanotechnology/nanosciences. |
| NIST (National Institute for Standards and Technology) | USA | Sub-micrometre standard reference materials (SRMs) have been available commercially from NIST for many years. These are intended for instrument calibration, have little chemical relation to materials of commercial or toxicological interest. The only truly nanoparticle SRM commercially available are NIST's 100nm polystyrene spheres in water suspension (SRM 1963). |
| NIOSH (National Institute for Occupational Safety and Hygiene) | USA | NIST and NIOSH are currently collaborating on the development of a 200nm beryllium oxide reference material. NIOSH have raised awareness of the need for nanoparticle reference materials at recent "Biological and Environmental References Materials" (BERM) conferences, and it is likely that sessions at future conferences in this series will help prioritise needs[6]. |
| Australian National Measurement Institute | Australia | Australia has significant nanoparticle production facilities, collaborating with Oxonica in the UK, for example. The Australian National Measurement Institute has particle sizing (DLS) instruments with which it will characterize materials, but does not yet certify standard reference materials. |

**Table 2. International work in progress on nanoscale material metrology and standardisation**

[6] www.csc.noaa.gov/berm/

## 2.4 Understanding the properties of nanoparticles in the context of their ignition and explosion potential, and assessing/developing methods for evaluating this (RO4)

27 The large surface area of finely-divided nanoparticulate materials means that their explosive potential is difficult to predict on the basis of models developed for larger particles. New models and measurement methods are needed. These are rather different from the models of nanoscale behaviour needed for metrology, characterisation and toxicology, and may involve testing of nanomaterials for bulk fire and explosive properties using modified forms of standard test methods.

### 2.4.1 Active research projects

*2.4.1.1 An Investigation of the Fire and Explosion Properties of Nanoparticles and the Possible Quantification Methodologies*

28 This project, which is funded by the HSE, aims to understand the fire and explosion hazards of selected nano powders. Areas for investigation comprise: explosion properties; ignition properties; accumulation of electrostatic charge; fire properties; ease with which selected powders can be made to form a cloud; and the suitability of standard test methods.

29 The work identified is divided into the work packages that deal with the potential key issues relating to the fire and explosion hazards, in terms of their basic properties as dispersed clouds, layers and when stored in bulk. At this time, the work is focused on the handling of such materials on a small production or laboratory scale.

30 The project will result in guidance on handling the potential explosion and fire hazard properties of nanoparticles, and an article(s) suitable for a scientific peer reviewed journal describing the work completed.

## 2.5 Optimisation, development and application of technologies that enable the measurement of exposure to nanoparticles in soil and water (RO9)

31 Research objective 9 requires methods for measurement of nanoparticles in a specific matrix. In many cases these simply do not exist, and as with research objective 3, a prioritisation exercise will be needed to identify the most important matrices and analytes before committing funds to develop extraction protocols.

### 2.5.1 Active research projects

*2.5.1.1 NERC Network (AQUANET)*

32 There is potential for technology and knowledge transfer from AQUANET, a NERC funded network examining natural aquatic colloids. There are also aspects addressed in the International Life Sciences Institute (ILSI) Research Foundation/Risk Science Institute study[7] on nanoparticle measurement within specific matrices.

### 2.5.2 Research proposals

*2.5.2.1 Develop a network of contacts in Europe*

33 To keep informed of research and field-scale trials and identify opportunities for EC funding of collaborative research or demonstration proposals.

*2.5.2.2 A targeted call for measurement technologies*

34 Given the complexity and diversity of matrices, the underpinning science of quantification of nanomaterials in complex matrices is a priority research need. A call for projects on nanomaterials in water and soil would provide an excellent opportunity.

[7] http://www.particleandfibretoxicology.com/content/pdf/1743-8977-2-8.pdf

# 3. Task Force 2: Exposures – Sources, Pathways and Technologies

## 3.1 Aims of the Task Force

35 Task Force 2 is focused on the sources, pathways and technologies associated with human and environmental exposure to engineered nanoscale materials. Key research objectives are:

RO 5 Further identification of sources of nanoparticles.

RO6 Optimisation and development of technologies that enable the measurement of occupational and environmental exposure to nanoparticles via air.

RO7 Understanding the fate and behaviour of nanoparticles in air.

RO8 Development of exposure control devices.

RO10 Research to understand the environmental fate, behaviour and interaction of nanoparticles in soils and water.

## 3.2 Further identification of sources of nanoparticles (RO5)

36 Both the RS/RAEng report and exposure scoping study identified likely current sources of human and environmental exposure to engineered nanoscale materials. These are:

- occupational exposure in the workplace (human)
- exposure from deliberate environmental releases, e.g. remediation of contaminated ground waters and land (environment and possibly human)
- exposure from 'unintentional' environmental releases, e.g. from fuel additives and in industrial and domestic waste streams (environment and human)
- exposure from consumer products, such as cosmetics (human)
- exposure from medical products, including drugs, treatments and devices (human).

37 However, considerable uncertainties still remain over the sources of engineered nanoscale materials, including unintentional releases into the environment. Moreover, potential exposures need to be considered in terms of a product's life-cycle, which encompasses manufacture, through use, to waste removal or recycling, and highlights links between air, land and water, and between measurement and exposure (see Fig 1).

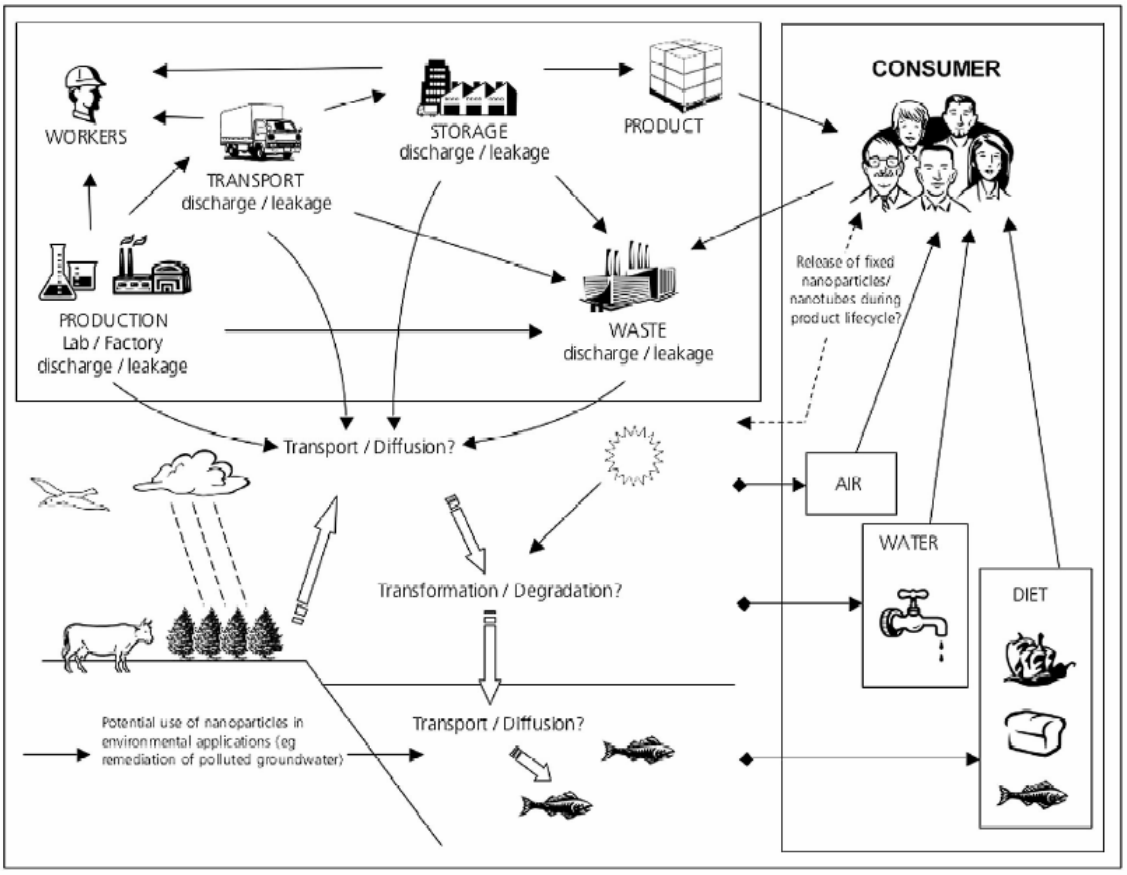


*Figure 1. Some possible exposure routes for nanoparticles and nanotubes based on current and potential future applications. (Adapted from National Institute for Resources and Environment, Japan http://www.nire.go.jp/eco_tec_e/hyouka_e.htm)*

### 3.2.1 Active research projects

*3.2.1.1 Helpdesk proposal*

38 The aim of this work is to provide an information bulletin service reviewing studies on exposure and potential health effects of nanomaterials relevant to the occupational setting. The bulletins produced will be available on the web. HSE is funding the work for year one (£52k). Ongoing funding will be considered following an assessment of costs and benefits.

*3.2.1.2 An analysis of potential exposures throughout the life-cycle of engineered nanoscale materials*

39 Dr Tony Harker of UCL is carrying out an analysis of potential exposures throughout the life-cycle of engineered nanoscale materials. The study is being completed as part of the NANOSAFE2 project (see research proposals for ROs 6&7). One of the materials under consideration is carbon nanotubes in tyres.

### 3.2.2 Research proposals

*3.2.2.1 Addressing potential exposures: a life cycle approach*

40 The Task Force considered that a life cycle overview showing key linkages between components, as conceptually illustrated in Figure 1, is needed. It was agreed that all of the Task Force objectives need to be expressed in this way. Examples suggested for working up were iron used in land remediation, the use of titanium dioxide and carbon nanotubes. The starting point for this work will be to find out if there is any life cycle analysis work already ongoing or completed and whether the approach is practical.

*3.2.2.2 Measurement*

41 There needs to be some quantification of the amount of exposure within the life cycle of an engineered nanoscale material. This calls for detailed study of environmental fate and behaviour through environmental compartments such as sewers, groundwater, surface water and air. The point is that engineered nanoscale materials, on use and disposal, have the potential to migrate between environmental compartments. This will identify which pathways will need to be targeted for exposure and risk management.

*3.2.2.3 Differentiation between natural and man-made nanoparticles*

42 This is a top priority in order to enable meaningful conclusions to be drawn from exposure measurements (see section 2.2.2.1).

*3.2.2.4 Identification of further uses and sources*

43 Other sources of information might be from professional bodies such as The Royal Society of Chemistry, The Institute of Mining and Metals, and The Institute of Physics.

### 3.2.3 Monitoring other programmes of work

44 The active monitoring of work conducted around the globe will require resources. The intention would be to provide a regular quarterly report on any results/actions taken. This would be available to Task Force members, the NRCG and on the web. Sources of information and other programmes of work include:

- OECD working party on nanomaterials which intends to host a database on nanotechnology research including the database from the Project on Emerging Nanotechnologies set up by the Woodrow Wilson Centre[8]

[8] http.www.nanotechproject.org/index.php?id=18

- networks and wider community, including EU Research database, German Federal NanoCare project

- Defra/EA/HSE Landscaping Study: this project has produced a database of companies who are using nanotechnology, and its findings have been published in the journal Occupational Medicine

- information on users/producers from MNT Network

- information on consumer products from the Woodrow Wilson Centre's Inventory of Nano Products

- monitor NANOSCAN, a joint in-house journal scan produced by the Environment Agency for the Nanotechnology Issues Dialogue Group (NIDG).

45 It is possible that the formal monitoring of these sources can be incorporated into the Helpdesk research proposal above.

### 3.3 Optimisation and development of technologies that enable the measurement of occupational and environmental exposure to nanoparticles via air (RO6); and understanding of fate and behaviour of nanoparticles in air (RO7)

46 RO6 links with RO9 (Optimisation, development and application of technologies that enable the measurement of exposure to nanoparticles in soil and water), and RO7 links strongly to RO10 (Research to understand the environmental fate and behaviour and interaction of nanoparticles in soil and water) and the proposed life cycle analysis will illustrate this.

47 At this stage Task Force 2 has placed dermal exposure issues under RO6 and RO7, but they also have strong links to Task Force 3's RO15 (dermal uptake, penetration and toxicity of nanoparticles).

48 The development of protocols and technologies will allow us to develop a more comprehensive understanding of the fate and behaviour of nanoparticles in air (strong link to Task Force 1).

#### 3.3.1 Active research projects

*3.3.1.1 Nanoparticle Occupational Safety & Health (NOSH) Consortium*

49 A consortium of companies, government, academia, and public interest groups, led by DuPont, has formed to sponsor research that will further our understanding of how to best assess and control occupational exposures to

engineered nanoparticles. The total budget for the work is $680k, of which the HSE is contributing £35k.

50 The research has three specific objectives: (1) generate nanoparticle aerosols and measure their behaviour as a function of time; (2) develop a simple, robust, portable device to measure airborne nanoparticles; and (3) measure the barrier efficiency of various materials to nanoparticles.

51 The research will be performed at DuPont's Experimental Station Laboratories, and is due for completion in 2007.

*3.3.1.2 Investment Research Programme – Nanochallenge*

52 The objectives of this programme, which the Health and Safety Laboratory (HSL) is funding to the extent of £400k, include 'the development of the HSL aerosol generation and measurement capabilities.' The exposure assessment and control project includes:

- the development of improved methods of collection and characterisation of airborne nanoparticles. The applicability of biological monitoring will also be investigated;

- the investigation of whether the current method of dustiness testing (used to evaluate dust emissions during handling and transport) is suitable for nanomaterials, and if not, to propose and investigate alternative options for particle dispersal and measurement.

*3.3.1.3 Nanosh*

53 This is a £2.7 million European multi-centre research project that focuses on occupational exposure to nanoparticles and their health effects to provide important information for risk assessment analysis to be carried out on the production and use of nanoparticles in the workplace. The HSE and the HSL have contributed £574k. The two main parts to the project are: (a) exposure assessment in a range of different workplaces where nanoparticles are being produced and/or used; and (b) health effect studies of nanoparticles, including genotoxicity, inflammatory responses and microvascular effects. This work is related to that of TF 3 on *in vitro* testing methods.

54 HSL/HSE leads the work package on exposure assessment with three partners from Germany, the Netherlands, and Poland. The workplan includes assessment of exposure to nanoparticles in a wide range of workplace settings, and assessment of the performance of Respiratory Protective Equipment (RPE) and protective clothing against nanoparticle penetration in real-life conditions. HSL/HSE will concentrate on assessing exposures in the nanotechnology laboratories of a number of UK universities, as well as assessing the performance and suitability of the control measures deployed to

enable guidance to be written on suitable control measures for the processes involved.

55 HSL/HSE will have access to all information generated in the project.

*3.3.1.4 Nanosafe*

56 A consortium of European companies and research laboratories has developed an £8.3 million research programme for the safe production and use of nanomaterials. This was targeted for funding by the EU Framework 6 programme as a large integrated project to last over a 48-month period. The project has 4 main scientific subprojects: (SP1) measurement of exposure to and characterisation of airborne nanoparticles; (SP2) potential health effects of nanoparticles; (SP3) procedures for safe production and handling of nanoparticles; and (SP4) standards, regulations and societal implications of nanoparticles.

57 The main aims of the HSL contribution (£70k) to the project are: (SP1) development of the nanoparticle exposure sampling strategy, including choice of sampler; (SP3) limited measurements of exposure to nanoparticles in the workplaces of UK industrial partners in the project; and (SP4) review of the relevance of current standards for the measurement and control of airborne nanoparticles, including both inhalation exposure and fire and explosion safety.

*3.3.1.5 Assessment on nanoparticles in food*

58 The fundamental approach to exposure estimation for nanoparticles will be the same as for other food chemicals – combining information on food consumption with data on the occurrence of nanomaterials. The latter might be either based on the level of incorporation (for nano-ingredients) or actual measurements (nano-contaminants).

59 The key issues are measurement and characterisation (i.e. speciation, physical differentiation) to confirm whether nanoparticles that are present in the final food are comparable with those used to produce the risk assessment.

60 In order to gather additional information on the food safety and regulatory implications, the Food Standards Agency has commissioned an assessment of new and potential applications of nanotechnology for food contact materials which is due to report in Spring 2008. A similar research project has been commenced in relation to food additives and novel food ingredients. This work is due to be completed in January 2007.

### 3.3.2 Research proposals

*3.3.2.1 Modelling*

61 The first stage will be the generation of real exposure data and then this will be compared to the predictions of existing environmental and human exposure models. There is current work on improving human exposure models and it is recognised that this work should finish before the longer-term goal of work on modeling nanoparticle exposure begins.

### 3.3.3 Monitoring other programmes of work

62 Table 3 sets out the programmes of international work most relevant to addressing RO 6 and RO 7.

| Organisation | Territory | Work in progress |
|---|---|---|
| NIOSH | USA | NIOSH researchers are partnering employers and others in conducting field studies to observe and assess occupational health and safety practices in facilities where nanotechnology processes and applications are used. We will monitor their results via NIOSH publications (e.g. newsletter), through personal contacts and through contact within the Safety of Nanomaterials Interdisciplinary Research Centre (SnIRC). |
| European Commission | EU | Contacts in DTI and information available on EU websites will provide details of the 6th and 7th Framework Programmes and other projects. The new chemicals legislation, REACH, will impact on nanoparticle research. |
| Nanoderm consortium/ industry | EU/ International | Ongoing international work in Dermal Exposure, e.g. Nanoderm (EU project)[9]. This project used a combination of *in vitro* and *in vivo* methods to study the passage of titanium dioxide through human and pig skin cultures. They found that the nanoparticles remained blocked in the upper epidermal layers but penetrate the dermis along the follicular cells. Research is continuing to find out if damaged skin continues to act as a barrier.<br><br>Ongoing work by industry e.g. dermal penetration work funded by cosmetic companies |

**Table 3 International work on exposure to nanomaterials via the air**

### 3.4 Development of exposure control devices (RO8)

63 Given the potential for occupational human exposure and the uncertainties in our knowledge of possible health effects associated with exposure to nanoparticles, exposure control strategies are needed to enable those working in the field to implement the necessary control measures for

[9] http://europa.eu.int/comm/research/index_en.cfm

both aerial and dermal exposure. These should be developed in the form of good practice guidelines for handling and working with nanoparticles.

### 3.4.1 Active research projects

*3.4.1.1 Investment Research Programme – Nanochallenge (see 3.4.1.2)*

*3.4.1.2 Nanoparticle Occupational Safety & Health Consortium (see 3.4.1.1)*

### 3.4.2 Research proposals

*3.4.2.1 Effectiveness of current control measures*

64 This project will be taken forward by HSE and HSL, and will include a review of control measures currently used for the production, handling and end use of nanoparticles. The most common control systems will be identified and have their performance assessed. This will focus on capture or containment of nanoparticles (using systems such as LEV, fume cupboards, laminar flow booths, glove boxes, etc) and include all parts of the system, including leakage through seals and filters. The project will take account of ongoing work in this area and avoid duplication.

*3.4.2.2 Nanosh*

65 As part of the proposal, the laboratories involved in Working Party 2 will assess the performance of Respiratory Protective Equipment and protective clothing against nanoparticle penetration in real-life conditions (see 3.4.1.3 for further details).

*3.4.2.3 Control Banding project*

66 HSE would like to form an international group to develop a Control Banding approach to exposure control. The control Banding project is related to COSHH Essentials work and will start with discussions between HSE and NIOSH.

*3.4.2.4 Water filtration techniques*

67 Assessment of the effectiveness of water filtration techniques against nanoparticles, including efficient removal of nanoparticles from drinking water and wastewater effluent. This will also be important in RO10.

### 3.4.3 Monitoring other programmes of work

68 The active monitoring of work conducted around the globe will require resources. The intention would be to provide a regular quarterly report on any

results/actions taken. This would be available to Task Force members, the NRCG and on the web.

69 The work of Task Force 1 will be important to work in this area.

70 As part of its strategic research programme, NIOSH is currently funding a 1-year project to investigate filter efficiency at the nano-scale. The study goals are to determine:

- if single fibre filtration theory is valid for engineered nanoparticles
- the possible boundaries of the most penetrating particle size range
- the filtration boundaries of nanosized particles in the diffusional capture mechanism range.

71 Initial results were published in October 2005 and the final report is due in late 2006.

72 ICON has commissioned a survey to consider the environmental and human health practices of nanotechnology companies in the United States, including training, types of exposure control (personal protective equipment, respiratory protective equipment, engineering) types of monitoring and disposal.

## 3.5 Research to understand the environmental fate, behavior and interaction of nanoparticles in soils and water (RO10)

73 This research objective links strongly to research objective 9 on 'Technologies for the measurement of nanoparticle exposure in soils and water' which is also being considered by Task Forces 1 and 4.

74 Research is needed to address the fundamental knowledge gaps on the fate and behaviour of nanoparticles in soils and water. This could involve an initial review of existing data followed by laboratory, semi-field and field experiments, and lead to the evaluation/development of exposure assessment models for the terrestrial and aquatic environments.

### 3.5.1 Active research projects

*3.5.1.1 The Environmental Nanosciences Initiative (see 5.4.1.1).*

### 3.5.2 Research proposals

*3.5.2.1 Current and Predicted Environmental Exposure to Engineered Nanomaterials*

75 This project, which is funded by Defra (c £50k) aims to evaluate the current environmental exposure to existing production, uses and disposal (i.e. life-cycle stages) of nanomaterials, and to predict likely future exposures of nanomaterials in development or emerging uses. It will include an attempt to quantify and evaluate the potential distribution of materials between different environmental compartments. It is hoped to let a contract for this project in Autumn 2006.

*3.5.2.2 Review of published literature on the range of nanoparticles developed and used for waste water treatment*

*3.5.2.3 Laboratory study of nanoiron and bimetallic materials currently available for remediation of soil or water*

76 Although a number of nanoremediation products are commercially available, the manufacturers are not UK-based. However, these products may be proposed for use in the UK, currently without the benefit of robust fate and transport data. Such a study will provide a benchmark for government and regulatory decision-making on which nanoparticles are potentially suitable for small-scale releases to provide information on field-scale fate and transport. It is intended that this study will be funded through a CASE studentship/fellowship (see also RO2).

*3.5.2.4 Field-scale remediation of contaminated groundwater and soil*

77 Robust science is needed from controlled field trials to assess the perceived benefits and environmental risks of remediation using nanoparticles. Such work should include a comparative assessment of remedial performance using micro- and nano-scale particles, assessment of measurement technologies and investigation of transport of nanoparticles. This will lead to the development of a predictive fate and transport model and assessment of the impact on microbial communities within the treatment area and down gradient. Collaborative projects with research institution and industry partner associated with controlled field trials are suggested (see also RO9, RO17, RO19).

*3.5.2.5 Consideration of the unintentional release of nanoparticles through disposal to landfill or discharge to sewage systems and wastewater treatment plant.*

78 The life-cycle analysis approach can address the scale of exposure through disposal routes. Particular issues include the mass or flux of nanoparticles unintentionally released on disposal, degradation, partitioning and potential remobilisation of nanoparticles, efficacy of current treatment and disposal systems for managing nanoparticles, and the impact of nanoparticles on biological treatment systems and ecological end-points (see also RO5, RO9, RO17, RO19).

*3.5.2.6 Develop an understanding of nanoparticle behaviour and fate in drinking water treatment processes*

### 3.5.3 Monitoring other programmes of work

79 The active monitoring of work conducted around the globe will require resources. The intention would be to provide a regular quarterly report on any results/actions taken. This would be available to Task Force members, the NRCG and on the web.

80 Network with USEPA on the use of nanoparticles, particularly iron or bimetallic materials, for the remediation of groundwater.

81 Develop a network of contacts in Europe to keep informed of research and field-scale trials on water and soil remediation using nanomaterials and identify opportunities for EC funding of collaborative research or demonstration proposals

# 4. Task Force 3: Human Health Hazard and Risk Assessment

## 4.1 Aims of the Task Force

82 Task Force 3 is responsible for addressing research objectives relating to the toxicology of engineered nanoscale materials. The Task Force is focused on objectives 11-16 in the report "Characterising the Potential Risks Posed by Engineered Nanoparticles: a First Government Research Report" (2005):

RO 11 Research to establish a clear understanding of the adsorption of nanoparticles via the lung, skin and gut and their distribution in the body (i.e. toxicokinetics), identifying potential target organs/tissues for toxicity assessment.

RO 12 Research to establish a clear understanding of inter and intracellular transport and localisation of nanoparticles and their cellular toxicity.

RO 13 To establish a clear understanding of whether oxidative stress, inflammatory effects and genotoxicity apply to nanoparticles.

RO 14 Research to establish a clear understanding of the deposition, distribution, toxicity, pathogenicity and translocation potential and pathways for nanoparticles in the airways and lung and their potential impacts on the cardiovascular system and brain.

RO 15 Given the current use of nanoparticles in consumer products there is a need to further our understanding of dermal uptake, penetration and toxicity in the skin.

RO 16 To develop testing strategies for human health hazard assessment and assess how fit for purpose current test methods are as applied to nanoparticles.

83 Engineered nanoparticles represent a new form of materials which may be presented to the human body and the environment. Exposure may occur during the manufacture of nanoparticles, in the general environment due to its contamination with nanoparticles or deliberately when medicines, cosmetics and other products contain nanoparticles. The range of possible exposure scenarios is vast, as is the range of materials used that can be presented in nano-form. If this is added to the wide range of nano-forms (e.g. particulate, fibres, tubes, simple nanoparticles, complex nanoparticles, and nano-dots) the number of possible combinations of exposure pattern, material presented, and dose becomes almost endless. It is clearly impossible to study every unit of

what might be visualised as a very extensive, multi-dimensional matrix. This matrix is expanding.

84 The problem of assessing the possible toxicological effects of exposure to nanoparticles is likely to be difficult, but it is not hopeless. The principles of toxicology that have been applied successfully to other materials remain valid when nanoparticles are considered. In addition, if the value of determining generic properties of nanoparticles that control their toxicity is accepted, it is likely to be possible to extrapolate from comprehensive studies on well-characterised particles to the possible effects of new materials. It is thus important to discover what controls the toxicity of representative nanoparticles, whether there are unknown factors, and to explore the mechanisms by which these materials exert their effects in the body.

85 This plan sets out some of the principles that should, in the opinion of Task Force 3, be adopted in studying the possible effects of nanoparticles. It is intended that this advice should form an un-prescriptive guide for research workers, and for industrial and regulatory toxicologists involved in assessing the health risks posed by such materials. It may also be useful to those assessing applications for research funding in this fast-growing area. There are, of course, others who have attempted such a task. Detailed reports have been prepared by ILSI, by the US EPA, and jointly by the Committees on Toxicology, Carcinogenicity and Mutagenicity of Chemicals in Food, Consumer Products and the Environment in the UK, and by other bodies. There are no startlingly new suggestions in this plan, and if it differs at all from others, the differences may concern the emphasis placed on certain areas. In preparing the plan, it was noted that work in the general area of nano-toxicology has been spread across a number of disciplines. Work in the pharmaceutical field is clearly advanced, but there has not been sufficient contact between this field and that of respiratory toxicology - closer collaboration is urged. It is clear that the details of the toxicological studies needed on any material presented as nanoparticles will depend on their likely uses or on how they are likely to be encountered.

86 The plan has been divided into three parts:

i. the need for detailed characterisation of particles

ii. the need for *in vitro* studies

iii. the need for *in vivo* studies.

87 This represents, in general, a tiered approach with data on characterisation of nanoparticles being essential prior to any experimental work. *In vitro* to investigate effects in cellular systems/tissues may then be used to provide information on potential mechanisms and to inform the design of *in vivo* studies. However, flexibility is needed, with consideration on a case by case basis, and bearing in mind the purpose of the study, for example, research toxicology or use in hazard assessment to meet regulatory needs.

## 4.2 Research proposals

### 4.2.1 Characterisation of nanoparticles

88 It is clear that there are hundreds of different nanoparticles. These vary in terms of chemical composition and physical dimensions. Even apparently well defined substances also vary in terms of their properties depending on the manufacturing process. In addition, batch to batch variation in properties has been noted. It is thus important that all toxicological work on nanoparticles should include the clearest and most extensive description of the material used. It is hoped that a structure/activity paradigm for nanoparticles toxicity can be developed and that this will allow prediction of toxicity on the basis of physicochemical parameters. This will only be achieved if studies incorporate the measurement of as large an array of particle characteristics as possible. Other groups have documented the range of particle characteristics that could be described and which could contribute to the structure/activity paradigm. These should certainly include information on the composition and dimensions of the particles, on their solubility, their charge, their capacity to produce oxidative stress and their surface properties (see also research objective 2 considered by Task Force 1).

### 4.2.2 Need for a central nanoparticles bank

89 It is unlikely that individual toxicologists will have access to the facilities needed for such extensive characterisation outlined above. It follows that a central bank should be obtained and characterised in as much detail as possible. Toxicologists could then draw samples from the bank with confidence that any two samples of the same materials would be similar. It will be important to study the material in the bank repeatedly: ageing of particles and possible resultant changes in physico-chemical properties will need to be determined. The need for such a bank is very clear to toxicologists: the literature is currently confused as a result of studies being published on poorly characterised material. The bank should begin with a modest range of materials and expand with time (see also research objective 3 considered by Task Force 1).

90 It is hoped that study of a range of nanoparticles will allow inferences to be drawn regarding the key features of these materials that control their toxicity. Thus, it is suggested that the bank should acquire a range of materials that differ in chemical composition, in physical shape and in dimensions. The following materials are suggested for early inclusion (note that for several of these a series of sub-sets should be defined, e.g. carbon black may be obtained in a variety of sizes):

- Titanium dioxide
- Carbon black

- Carbon (C60) fullerenes
- Quantum dots
- Polystyrene particles
- Carbon nano-tubes (single-walled and multi-walled)
- Uncoated superparamagnetic iron oxide nanoparticles (SPION)
- 100 nm PLGA (poly (D,L-lactic co-glycide) acid) nanoparticles.

91 Consideration should also be given to producing samples of nanoparticles that may be generated in the body from material used in implants such as artificial joints.

92 This is only a first attempt at a short list. Advice from experts will be welcome, and the results from work under RO3, section 2.3, will provide greater direction. It will be readily appreciated that if even only 10 materials are identified and that some of these need to be held in a variety of shapes and sizes, the bank would grow quickly. It is appreciated that the costs of maintaining an adequate bank of material might be considerable. Further work is needed on this especially as regards material used as quantum dots where there may be a problem with stability and with C60 fullerenes where acquiring a suitable quantity to provide a useful bank may be difficult. The bank of nanoparticles should be held centrally and material should be made available to research workers without charge.

### 4.2.3 Toxicological studies

93 It is important to stress the need to decide the objectives of toxicological studies before beginning work. This may seem obvious, but there are important differences between studies that are needed for regulatory purposes and those which it would be useful to undertake from a research perspective. It is not our intention to write either a manual for research workers or for regulatory toxicologists but only to stress points that might be important in either field. There seems value in a tiered approach with the use of *in vitro* studies to investigate toxicity in certain cellular systems followed by *in vivo* studies. It should be stressed that studies on human tissue collected post-mortem are also likely to be helpful. Such work is more difficult today than in the past and this is a major problem for research workers in pathology. The Royal College of Pathologists should take a lead in this area and should consider how they might contribute to work on the distribution and fate of nanoparticles in the body.

### 4.2.4 *In vitro* studies

94 *In vitro* studies of the possible toxicological effects of nanoparticles should be undertaken before *in vivo* studies. Current concerns about the use of animals in toxicological testing are making *in vivo* studies more difficult, but only in a few areas have *in vitro* studies been validated for use for regulatory

purposes and such studies are likely to provide only initial data on comparative toxicity of different sized materials. These findings will need to be followed by *in vivo* studies.

95 The Task Force has commissioned a short report on possible *in vitro* approaches to nano-toxicology from the UK Health and Safety Laboratory. This report was considered at a meeting of experts in the field and has been edited in conjunction with these experts. The report is attached as Annex 4 to this report. It should again be stressed that the general principles of toxicology apply to nanoparticles as they do to other materials. Here, a few areas have been selected where useful information could be provided from *in vitro* studies.

96 At the cell/isolated tissue level:

- interactions of nanoparticles with cells and their surface fluids found at the sites of entry of these materials to the body should be studied. Consideration should be given to epidermal, lung and gut epithelial cells and the endothelial cells of capillaries. It is notable that nanoparticles can be generated within the body from surgical implants. In this case uptake by lymphatic capillaries would be likely to be important;

- what happens to the toxicity of nanoparticles after they have crossed surface liquid barriers such as lung lining fluid?

- the interaction of nanoparticles with tissue to tissue barriers in the body and absorption across such barriers, egg gut, skin, lung, blood-brain and placenta;

- what happens to nanoparticles inside cells?

- what happens to cells after that have taken up nanoparticles? Here were are thinking of depletion of cell defences against free radicals and the effects of oxidative stress that may, but by no means must, follow interaction with nanoparticles. Effects of lysosomal functioning may be important as might effects on genetic material. It will be important to know whether individual nanoparticles are mutagenic and thus whether carcinogenicity is likely;

- how do nanoparticles interact with tissues distal to their portal of entry, egg blood, brain and liver? The endpoints studied in such cell systems should depend on an appreciation of the normal specialised function of these tissues. Subtle changes in cell function should be studied: this may be especially important in considering changes in cell functioning in the central nervous system.

97 Almost any aspect of cell biology could be affected by the interaction of nanoparticles with the cell, but whether specific aspects of cell physiology will

be significantly affected will depend on: the inherent toxicity of the nanoparticles for specific aspects of cell physiology; the amount of nanoparticles reaching the cell; and the cell's defence mechanisms. This is not new: it applies widely in toxicology. Also not new are concerns about extrapolating from one cell type to another: from animal to human cells, from immortalised cell lines to normal cells and so on. But these too apply in much toxicological work.

98 There is a need for work involving tissue systems and not only a single type of cell.

### 4.2.5 *In vivo* studies

99 Current concerns about the use of animals in toxicological studies are well recognised but from a regulatory perspective it is likely that the standard methods of *in vivo* toxicology (histopathology, haematology, clinical chemistry, etc.) will prove reliable in detecting the acute and chronic effects of short term and semi-long term exposure to nanoparticles and will be required. If, for example, nanoparticles cause severe damage to hepatic cells the effects would be seen in the clinical chemistry of the blood, in the animal's well being and behaviour and in the histological appearance of the liver at post-mortem. The results from *in vitro* studies may inform decisions as to what particular areas to concentrate upon, or where some investigations in addition to those normally carried out may be appropriate.

100 However, there is always the possibility that an effect that is not detectable by standard methods will occur. Consider, for example, an effect on atherosclerotic plaques. This might not be seen in animals commonly studied: it might be detected only in special strains that have a predisposition to atheroma. This could be a problem: it is known that nanoparticles can affect the rate of development of atherosclerotic plaques in a special strain of rabbit but these would be unlikely to be used in toxicological testing of new nanoparticles. This sort of discovery comes from the research rather than the regulatory side of toxicology. Research is needed to define what nanoparticles *could* do and then regulatory testing is needed to identify levels below which such effects would not be expected to occur. Another example might be the lysosomal storage disease which may have been induced by exposure to non-biodegradable polymers used as plasma expanders during WWII. Detection of such an effect by standard toxicological methods might be unlikely. The implication of this is that new methods may need to be added to the current regulatory methods. The validation of such approaches and acceptance internationally (a requirement for regulatory toxicity guidelines) is likely to prove difficult and expensive.

101 As in the section dealing with *in vitro* methods a few areas have been picked out that the task force felt needed special attention.

102 The route of exposure used should match that likely to be experienced by man. In the case of environmental exposure, the inhalation, oral and dermal routes will be important. In the case of medicinal preparations, these, plus the intravenous and intramuscular routes, might apply. The special case of implanted materials should not be forgotten.

103 As important as an appropriate route of exposure is the form in which the nanoparticles are given. Careful characterisation of the inhaled aerosol will be needed in inhalation studies; in studies involving oral administration the form (solid, with food, liquid etc.) in which the nanoparticles are given will also need to be well characterised. The possibility of alteration of nanoparticle properties by food and secretions of the gut should be considered.

104 Multiple dosing may be needed to replicate the likely pattern of exposure in man.

105 The toxicokinetics of nanoparticles should be studied. This will be difficult without labelled particles. Dispersion patterns in the body, the action of some tissues as sinks or storage sites and the pattern of excretion should all be studied. Dispersion across tissue barriers was mentioned earlier in the *in vitro* section: it may also need to be studied *in vivo*. Dispersion across the blood-brain and placental barriers are potential sources of concern.

106 *In vitro* mutagenicity tests will provide information as to whether there are concerns about the material being a genotoxic carcinogen but *in vivo* studies would be needed to confirm this. Reproductive toxicology will need special attention, particularly if placental transfer is confirmed in specific cases.

107 Consideration should be given to studies employing volunteers. Some such work has already been done in the respiratory toxicology area. Defining the endpoints that should be studied will be important and will depend on the level of invasiveness of the studies, Broncho-alveolar lavage, for example, may be useful for examining intrapulmonary inflammatory responses. Sophisticated electrocardiographic techniques and scanning techniques may also be needed.

### 4.3 Monitoring other programmes of work

108 Monitoring the fast growing field of nanotechnology will require the allocation of financial resources. The key US groups and organisations such as NIOSH and the US EPA are active in this field. Workers at the US National Toxicology Programme at the US National Centre for Toxicology Research and at the US National Institute for Occupational Safety and Health (NIOSH) are planning studies on a range of nanomaterials. It is recommended that the DH Expert Advisory Committees (COT, COC, COM, and COMEAP) keep this subject under review.

## 4.4 Conclusion

109 Investigating the toxicological properties of nanoparticles will require close collaboration between scientists from a range of disciplines. Detailed characterisation of any particles studied is a priority. The establishment of a central bank of particles available to research workers is urged. Both *in vitro* and *in vivo* methods will be needed to examine the toxicological properties of nanoparticles. The methods that will be needed will vary from particle to particle depending on mode of exposure and use. Though this is a new field it is felt that the established principles of good toxicological practice are likely to be applicable.

# 5. Task Force 4: Nanotechnologies Environmental Hazard and Risk Assessment

## 5.1 Aims of the Task Force

110 Building a robust evidence base in the area of nanotechnologies environmental hazard and risk assessment is important for underpinning the development of policy and evaluating appropriate risk management options. Task Force 4 is contributing to this evidence base by addressing the following research objectives:

RO 17 Research to establish the uptake, toxicity and effects of nanoparticles on groundwater and soil microorganisms, animals and plants, especially in the context of remediation.

RO 18 Research to establish the mechanisms of toxicity, toxicokinetics and in vivo effects of nanoparticles to key ecological groups (including invertebrates, vertebrates (e.g. fish) and plants). A key aspect of such work should be the facilitating of knowledge transfer from human toxicological studies to inform ecotoxicology.

RO 19 Define endpoints to be measured in ecotoxicological studies and assess how fit for purpose current standard tests for persistence, bioaccumulation and toxicity are when considering nanoparticles. This should lead to the defining of a suite of standard PBT protocols for use in environmental hazard assessment.

111 In addressing these objectives, the Task Force agreed to:

1. develop and implement an action plan to meet the research objectives 17,18 and 19 outlined in the NRCG research report that relate to environmental risk assessment;
2. identify work areas and associated funding under these research objectives;
3. ensure proposed projects deliver meaningful information for the development of policy;
4. establish links both internally with other task forces and externally (e.g. international initiatives to foster knowledge transfer and minimise duplication);
5. develop mechanisms to foster communication between the science, policy and industrial community in the area of nanotechnologies environmental risk assessment and risk management;
6. develop mechanisms to foster capacity building to meet the research objectives;
7. monitor and report on progress on 1 – 6 above.

## 5.2 Risk assessment: ecotoxicological test methods (RO19)

112 The legislative circumstances under which hazard and risk assessment will be required for a nanomaterial have been reviewed and depend on a number of potential factors including whether it is considered to be a new or existing substance and in certain circumstances production volume. Under current chemicals control legislation the status of a nanomaterial is related to substance identification.

113 The Task Force considered the evaluation of current ecotoxicological methods for hazard assessment as a priority work area.

### 5.2.1 Research proposal

*5.2.1.1 Assessment of how fit for purpose current and proposed regulatory ecological tests are for nanomaterials*

114 The Task Force established the hypothesis that current testing strategies and their associated methods for characterising ecohazard (including bioaccumulation and toxicity) of chemicals are appropriate for nanomaterials, reflecting the fact that in many cases standardised tests assess hazard irrespective of the mode of action of the substance. However, recent work undertaken by the EU Commission's Scientific Committee on Emerging and Newly Identified Health Risks[10] and the ILSI Research Foundation/Risk Science Institute[11] suggest that current test methods may not be fit for purpose and may need to be amended. Defra has therefore commissioned a study to specifically address this. Within this study, those specific aspects of current regulatory test strategies and methods that are *not* fit for purpose for nanomaterials will be identified. The study will make recommendations as to which elements of current testing strategies and methods should be kept, amended or removed and where necessary, reasonable additions made. This will form the scientific basis for empirically testing the hypothesis through investigation.

115 The project will describe the current tests used to assess hazard (specifically ecotoxicity and bioaccumulation) under significant pieces of current and imminent chemicals legislation. It will then review and analyse published and ongoing hazard assessment studies of nanomaterials that have been conducted to date and discuss with researchers their experiences of working with these materials. It will identify which elements of the regulatory tests are likely to be not fit for purpose, giving clear, concise reasons as to why this is the case. This assessment will consider both the hazard endpoints of specific tests and the operational context of protocols (e.g. issues of how the organisms or cells are challenged, delivery and dose measurement of the

[10] http://europa.eu.int/comm/health/ph_risk/committees/04_scenihr/docs/scenihr_o_003.pdf
[11] http://www.particleandfibretoxicology.com/content/pdf/1743-8977-2-8.pdf

substance in question and standardisation, QA/QC issues). Reasonable and pragmatic variants on current test methodologies will then be described, based on these assessments. Finally, a suggested experimental approach for empirically testing the methods and variants will be outlined, forming the basis of the next phase of work in this area.

### 5.2.2 Monitoring other programmes of work

116 In terms of monitoring international work in this area, the European Commission has set up a working group under SCENIHR (The Commission's Scientific Committee on Emerging and Newly Identified Health Risks) to consider the appropriateness of risk assessment strategies and test methods. This group is considering nanomaterials in TGDs (Technical Guidance Documents) that support chemicals legislation (e.g. REACH) and is expected to report in early 2007.

117 It should be noted that hazard assessment is only one component of overall risk assessment and that the outputs of this work area should be considered within an overall assessment of risk that includes exposure and also considers human health risk assessment

## 5.3 Fundamental ecotoxicology and environnmental effects (RO17&18)

118 At present there is very little information on the influence of particle size, shape and number on basic aspects of ecotoxicology, mechanisms of action, influences on dose response relationships (at all levels of biological organisation) and influences on toxicokinetic profiles (adsorption/uptake, distribution, metabolism and excretion). The extent to which fate, behaviour and ecotoxicology of nanoparticles is governed by specific properties, common to some or all nanoparticles, is largely unknown. Such basic research informs the appraisal and development of hazard assessment methods, furthers our understanding of key aspects of hazard and will contribute to our understanding of whether hazard is enhanced in the nano versus bulk form for existing substances. For example, if dose response relationships are not influenced by particle size or number, then current hazard assessments for substances presented in bulk form may be appropriate for nanomaterials.

119 Fundamental questions that underpin this work area were informed by an international two day conference hosted by the Society for Environmental Toxicology and Chemistry/the Society for Experimental Biology, the Society of Chemical Industry, the Environment Agency and Defra in September 2006

'Environmental Effects of Nanoparticles and Nanomaterials'[12] . The following are examples of key questions that will be considered:

- Are dose response relationships (at all levels of biological organisation) influenced by particle size, number or shape?
- Interaction: do nanoparticles influence the fate, behaviour or ecotoxicology of other substances present in the environment (e.g. in the rhizosphere, groundwaters, sediments)?
- Are substances in their nanoform within environmental matrices more persistent, bioaccumulative or toxic when compared to the substance in bulk or dissolved form?
- What factors (e.g. pH, ionic strength, microbiology) influence agglomeration and other aspects of fate and behaviour in the environment? Is agglomeration an important factor mitigating any adverse effects of nanoparticles once in the environment?
- Are the fate, behaviour, interaction and toxicological/ecological effects of nanoparticles governed by specific properties exhibited at the nanoscale (e.g. specific surface properties) that are both measurable and generalisable to certain or all classes of particles?
- Can structure/activity relationships be developed for the behaviour and effects of nanomaterials in the environment?

### 5.3.1 Active research projects

*5.3.1.1 Environmental Nanoscience Initiative*

120 Defra, NERC and the Environment Agency have established the Environmental Nanoscience Initiative[13] (ENI) to address Research Objectives 17 and 18, as well as aspects of environmental fate and behaviour covered by Task Force 2 . NERC has recently awarded approximately £750k of research funds in this area and the ENI will provide approximately £520k of further money to fund small, exploratory grants with a view to providing preliminary data for larger grant applications through responsive mode routes and to encourage capacity building (see below).

[12] www.nerc.ac.uk/funding/thematics/eni/workshops/
[13] http://www.nerc.ac.uk/research/programmes/nanoscience

### 5.3.2 Monitoring other programmes of work

121 Wider international work is also being undertaken in this area, including through, for example, the USEPA's NCER programme[14] and within the EU Framework Programme that is being monitored by the Task Force.

## 5.4 Communication, knowledge transfer and capacity building (RO18)

122 Technical expertise based on working experience in the specific area of nanotechnologies environmental hazard and risk assessment is an area that requires encouragement and development. Developing capacity within the research community in this area is important for the provision of independent advice on environmental hazard and risk assessment to Government.

### 5.4.1 Active research projects

*5.4.1.1 Environmental Nanoscience Initiative*

123 Building capacity is an objective of the Environmental Nanoscience Initiative (see section 5.3.1.1).

*5.4.1.2 NANOMIST and NANOsafeNET*

124 Two existing networks (NANOMIST and NANOsafeNET) are also involved in networking and knowledge transfer.

*5.4.1.3 Nanonet*

125 NERC have recently funded a network on the behaviour of engineered nanoparticles in natural waters which is due to start in 2007.

[14] http://es.epa.gov/ncer/nano/research/index.html

# 6. Taskforce Force 5: Social and Economic Dimensions of Nanotechnologies

## 6.1 Aims of the Task Force

126 The NRCG's first research report, published in November 2005, emphasises the UK Government's commitment to:

RO1 understand the social and ethical implications of nanotechnologies, through a programme of public dialogue and social research.

127 Following the report's publication, a Task Force was convened to consider the implications of work in this wide-ranging area for Government policy on nanotechnologies, and to take forward appropriate research.

128 This chapter details the Task Force's progress since the publication of the Government's first research report in November 2005.

## 6.2 Public engagement (RO 1)

129 The Government's programme of public engagement on nanotechnologies is centred on three projects[15]: Nanodialogues; the Nanotechnology Engagement Group (NEG); and Small Talk.

### 6.2.1 Active research projects

#### *6.2.1.1 Nanodialogues*

130 Nanodialogues[16] is stated to examine the practicalities of the concept of 'upstream' public engagement, which is all about how we can build public value into new technologies and the way they are regulated, before they reach the market. It does this through a series of case studies looking at: the control of nanoparticles to remediate land contamination; the shaping of strategic research directions; the global diffusion of nanotechnologies; and public value and innovation in a corporate environment.

131 The project will report formally in Spring 2007, however, the people's inquiry on nanoremediation has produced an initial set of conclusions, which Defra and the Environment Agency have discussed with the participants. The

[15] http://www.defra.gov.uk/environment/nanotech/research/pdf/nanoparticles-riskreport.pdf
[16] http://www.demos.co.uk/content/SeethroughScience

fieldwork for the project looking at the relationship between nanotechnologies and developing countries has also been completed, and the results will be discussed at a meeting, in Autumn 2006, with the UK Department for International Development.

*6.2.1.2 The Nanotechnology Engagement Group*

132 The NEG[17] aims to bring greater coherence to the increasing number of projects and activities that address the interface between technical and social understandings of the potential risks posed by nanotechnologies. The Group – which is made up of people with expertise and projects in this area – is charged with mapping out and analysing the current practices of public engagement on nanotechnologies. This exercise is intended to inform the Government and others about the conditions under which early public engagement can influence policy and decision-making.

133 The NEG has to date produced an introductory report setting out its working rationale[18]; published a first policy report providing an initial analysis of public engagement exercises on nanotechnologies[19]; and organised a one-day event exploring the tensions and synergies between the needs and expectations of the different parties involved in public engagement on nanotechnologies[20]. The final NEG report will be published in Autumn 2007.

*6.2.1.3 Small Talk*

134 Government is also funding Small Talk, a project that pulls together the findings of a wide range of activities around the UK that are focussed on discussing nanotechnologies with the public and scientists. The project will report formally in October 2006.

### 6.2.2 Monitoring other programmes of work

135 One of the key functions of the Nanotechnologies Engagement Group is to draw together findings from a broad range of public engagement projects on nanotechnologies, both within the UK and internationally, and to relay information on the implications of these activities back to Government.

136 In 2005, UNESCO convened an *ad hoc* group of experts to study and analyse the ethical implications of nanotechnology. The group's work has

[17] http://www.involving.org/index.cfm?fuseaction=main.viewSection&intSectionID=391
[18] http://83.223.102.125/involvenew/mt/archives/blog_37/NEG%20Introductory%20Report.pdf
[19] http://83.223.102.125/involvenew/mt/archives/blog_37/NEG%20Policy%20Report%201%20.pdf
[20] http://83.223.102.125/involvenew/mt/archives/blog_37/Report%20from%20the%20NEG%20workshop%2030%20June%202006.pdf

resulted in a draft "Outline Policy Advice on Nanotechnologies and Ethics,"[21] which suggests four types of actions around: awareness raising; education; research; and policy. The document is now under analysis by COMEST (the UNESCO World Commission on the Ethics of Scientific Knowledge and Technology), and will be discussed in further detail at the 5th Ordinary session of COMEST to be held in Dakar, Senegal, from 5 to 8 December 2006. Prior to this meeting, representatives of the various sciences, policy makers and NGOs involved in nanotechnologies, will meet in Paris to discuss the draft Outline to ensure that COMEST will have adequate materials and proposals to transform it into a COMEST Policy Advice. The chair of the UK Task Force on the Social and Economic Dimensions of Nanotechnologies will participate in this meeting. The Policy Advice will be discussed at the General Conference of UNESCO later in 2007.

### 6.3 Social and economic research (RO1)

137 Important context for the work of the Task Force is provided by *'Nanotechnology: A Report for the ESRC 2006'* by Stephen Wood, Richard Jones and Alison Geldart of Sheffield University. This updates work carried out for the ESRC in 2003, and provides a succinct literature review of the debate surrounding nanotechnology since that time. The report recognises that two broad areas have dominated discussions so far: the possible toxicity of nanoparticles, and issues of public engagement and democratisation of science. The report also draws conclusions as to issues that have yet to be addressed within the nanotechnology debate, grouped around the following main headings:

1. the development of nanotechnology
2. nanotechnology, industry and the economy
3. nanotechnology and internationalisation
4. technology development and society
5. public engagement.

138 There is also stated to be a particular deficit of work on the economic considerations of nanotechnology, especially in relation to understanding the process of technological development.

139 The Task Force has identified four priority research areas. In framing and progressing these, we are mindful of the conclusions of the recent ESRC and NEG policy reports, as well as the focus of ongoing activities, and seek to address some of the gaps in the research literature on the development and control of nanotechnologies.

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21 http://portal.unesco.org/shs/en/file_download.php/9e0a2a81aedc50026ebba0e23d8d1921NanoPolAdvice_Outline_Apr06.pdf

### 6.3.1 Active research projects

*6.3.1.1 Innovation and public value*

140 Defra is taking forward a research project to consider how nanotechnologies can help to address key environmental policy challenges, such as climate change and sustainable energy. The project will also consider potential barriers to the development and realisation of the selected applications, including how Government can help in this respect. The project is expected to report its conclusions by March 2007.

*6.3.1.2 Expert advice*

141 There has been a perceived mishandling of expert advice by Government in several controversial hazard issues (e.g. BSE, GM and Foot and Mouth Disease), and some academics and policy makers now see non-scientific expert advice as a means of emboldening public confidence and legitimacy in the way knowledge and expertise are applied in policy processes. This discussion is particularly important in deciding how we build and use expert advice on the potential risks posed by nanotechnologies. To this end, Defra is taking forward a project to understand how we can best utilise non-scientific expertise on the Advisory Committee for Hazardous Substances, which has responsibility for nanotechnologies. Some of the results of the study will be communicated in a Demos science and society pamphlet, which will be launched in early December 2006, and a more comprehensive set of findings will be published in February 2007.

*6.3.1.3 The future convergence between nanotechnologies and other technologies*

142 The ESRC is planning a workshop on the future convergence between nanotechnologies and other technologies. The workshop will take place during the latter part of 2006 and more information will be provided when it becomes available.

### 6.3.2 Research proposals

*6.3.2.1 Multi-level risk governance*

143 The ESRC is looking to progress a programme of research (with matched funding from others) on the adequacy of current risk governance frameworks for nanotechnologies. The specific research questions that the programme will seek to address include:

- the relationship between private, national, European Union and global resources and their responsibilities in terms of effective governance

- the effective upstreaming of citizen influence in relation to global governance systems

- effective multi-level governance in relation to securing advantages for the range of different private sector stakeholders.

### 6.3.3 Monitoring other programmes of work

144 See section 6.2.2.

## 6.4 Conclusions

145 Government needs to learn from the activities taking place under this programme, as well as those funded by other organisations, to maximise the benefits of any new public dialogue and research initiatives. To ensure that this reflection and learning takes place throughout the duration of the projects, the Task Force chair sits on the steering board of the Nanodialogues project, is a member of the NEG, and is in regular contact with the contractors responsible for Small Talk and the other research projects. It is our intention to publish an initial set of reflections on the role and effectiveness of our public engagement programme before the end of 2006. A final set of reflections will be published in response to the main NEG policy report, which is due in September 2007.

# 7. Next steps

146 This report has summarised Government progress in co-ordinating research to address the potential risks posed by engineered nanoscale materials as well as setting out the research we believe is necessary to gather evidence for an appropriate control structure. Over the next 12 months, the five Task Forces will continue to progress and develop their respective programmes of work, making sure to maximise opportunities for European and international co-ordination. At the end of this period, we will publish a second research report, revisiting the research objectives to which the Task Forces are currently working. We will continue to involve the wider stakeholder and academic community in all areas of our work.

# ANNEX 1
# NRCG Research Objectives (November 2005)

| |
|---|
| **RO1** To understand the social and ethical implications of nanotechnologies through a programme of public dialogue and social research |
| **RO2** To identify the most suitable metrics and associated methods for the measurement and characterisation of nanomaterials |
| **RO3** To develop standardised, well-chacterised reference nanoparticles |
| **RO4** Understand the properties of nanoparticles in the context of their ignition and explosion potential and assess/develop methods for evaluating this. |
| **RO5** Further identification of sources of nanoparticles |
| **RO6** Optimisation and development of technologies that enable the measurement of occupational and environmental exposure to nanoparticles via air.Technologies for measurement of nanoparticles via air |
| **RO7** Understanding of fate and behaviour of nanoparticles in air |
| **RO8** Development of exposure control devices |
| **RO9** Optimisation, development and application of technologies that enable the measurement of exposure to nanoparticles in soil and water. |
| **RO10** Research to understand the environmental fate, behaviour and interactions of nanoparticles in soils and water |
| **RO11** Research to establish a clear understanding of the adsorption of nanoparticles via the lung, skin and gut and their distribution in the body (i.e. Toxicokinetics), identifying potential target organs/tissues for toxicity assessment of nanoparticles in the human body |
| **RO12** Research to establish a clear understanding of intra and intercellular transport, localisation and toxicity of nanoparticles and their cellular toxicity |
| **RO13** To establish a clear understanding of whether oxidative stress, inflammatory effects and genotoxicity apply to nanoparticles |
| **RO14** Research to establish a clear understanding of the deposition, distribution, toxicity, pathogenicity and translocation potential and pathways for nanoparticles in the airways and lung and their potential impacts on the cardiovascular system and brain. |
| **RO15** Given the current use of nanoparticles in consumer products there is a need to further our understanding of dermal uptake, penetration and toxicity of nanoparticles |
| **RO16** To develop testing strategies for human health hazard assessment and assess how fit for purpose current test methods are as applied to nanoparticles |
| **RO17** Research to establish the uptake, toxicity and effects of nanoparticles on groundwater and soil micro-organisms, flora and fauna, especially in the context of remediation |
| **RO18** Research to establish the mechanisms of toxicity, toxicokinetics and in vivo effects of nanoparticles to key ecological groups (including invertebrates, vertebrates (e.g. fish) and plants). A key aspect of such work should be the facilitating of knowledge transfer from human toxicological studies to inform ecotoxicology. |
| **RO19** Define endpoints to be measured in ecotoxicological studies and assess how fit for purpose current standard tests for persistence, bioaccumulation and toxicity are when considering nanoparticles. This should lead to the defining of a suite of standard PBT protocols for use in environmental hazard assessment |

# ANNEX 2

## Membership of the Task Forces

### Task Force 1: Metrology, Characterisation and Standardisation

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Martin Goose (HSE)
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Peter Hatto (Chair of ISO TC229 -nanotechnologies)

### Task Force 2: Exposure – Sources, Pathways and Technologies[22]

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### Task Force 3: Human Health Hazard and Risk Assessment[23]

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Robin Fielder (HPA)
Maureen Meldrum (HSE)
Heike Weber (MRC)
Inga Mills (HPA)
John Garrod (Defra)

[22] N.B. Task Force 2 held a workshop with experts to advise their action plan.
[23] TF 3 had a workshop with experts from the field of particulate toxicology to advise their action plan.

**Task Force 4: Environmental Hazard and Risk Assessment**

Richard Owen (EA, Chair)
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**Task Force 5: Social and Economic Dimensions of Nanotechnologies**

Chris Snary (Defra, Chair)
Anna Marshall (ESRC)
Kieron Stanley (EA)
John Garrod (Defra)

# ANNEX 3

## Acronyms

| | |
|---|---|
| BERM | Biological and Environmental Reference Materials |
| BSI | British Standards Institute |
| CASE | Co-operative Award in Science and Engineering |
| CEN | European Committee for Standardisation |
| CMR | Carcinogenic, mutagenic or toxic to reproduction |
| COC | Committee on Carcinogenicity in Food, Consumer Products and the Environment |
| COM | Committee on Mutagenicity in Food, Consumer Products and the Environment |
| COMEAP | Committee on Medical Effects of Air Pollutants |
| COT | Committee on Toxicity in Food, Consumer Products and the Environment |
| Defra | Department for Environment, Food and Rural Affairs |
| DfID | Department for International Development |
| DH | Department for Health |
| DLS | Dynamic Light Scattering |
| DTI | Department of Trade and Industry |
| EA | Environment Agency |
| EC | European Commission |
| ECVAM | European Centre for the Validation of Alternative Methods |
| ENI | Environmental Nanoscience Initiative |
| EPSRC | Engineering and Physical Sciences Research Council |
| ESRC | Economic and Social Research Council |
| EU | European Union |
| FP7 | EU 7$^{th}$ Research Framework Programme |
| FSA | Food Standards Agency |
| HPA | Health Protection Agency |
| HSE | Health and Safety Executive |
| HSL | Health and Safety Laboratory |
| ICON | International Council on Nanotechnology |
| ILSI | International Life Sciences Institute |
| IOM | Institute of Occupational Medicine |

| | |
|---|---|
| IRMM | Institute for Reference Materials and Measurements |
| ISO | International Standards Organisation |
| LEV | Local Exhaust Ventilation systems |
| MHRA | Medicine and Healthcare Products Regulatory Agency |
| MNT | Micro and nanotechnology network |
| MRC | Medical Research Council |
| NEG | Nanotechnology Engagement Group |
| NERC | Natural Environment Research Council |
| NIDG | Nanotechnology Issue Dialogue Group |
| NIOSH | National Institute for Occupational Safety and Health |
| NIST | National Institute for Standards and Technology |
| NOSH | Nanoparticle Occupational Safety and Health |
| NPL | National Physical Laboratory |
| NRCG | Nanotechnology Research Co-ordination Group |
| OECD | Organisation for Economic Co-operation and Development |
| PAS | Publicly Available Specification |
| PBT | Persistence, Bioaccumulation potential and Toxicity |
| PPE | Personal Protective Equipment |
| RAEng | Royal Academy of Engineering |
| REACH | Registration Evaluation and Authorisation of Chemicals |
| RPE | Respiratory Protective Equipment |
| RS | Royal Society |
| SCENIHR | Scientific Committee on Emerging and Newly Identified Health Risks |
| SEB | Society for Experimental Biology |
| SEM | Scanning Electron Microscope |
| SETAC | Society for Environmental Toxicology and Chemistry |
| SnIRC | Safety of nanomaterials Interdisciplinary Research Centre |
| SPION | Superparamagnetic iron oxide nanoparticles |
| SRM | Standard Reference Material |
| TEM | Transmission Electron Microscope |
| TF | Task Force |
| TGD | Technical Guidance Document |
| UCL | University College London |
| USEPA | United States Environmental Protection Agency |

# ANNEX 4

# Report on *In Vitro* Methods for Assessing the Toxicity of Nanoparticles

Prepared by:
NRCG Task Force 3

August 2006

# Table of Contents

## 1. Preparation of Report

This report was drafted by Drs Rosemary Gibson, Angela Curtis and Gareth Evans of the Health and Safety Laboratory, Harpur Hill, Burton, Derbyshire SK17 9JN. It was discussed by an ad hoc expert group convened to assist Task Force 3. Members of the ad hoc group were allocated sections of the draft which fell into their special areas of expertise and were asked to produce amendments as necessary. The final report was edited by the Chairman of Task Force 3.

## 2. Contributors to the Report

### Drafting Group

| | |
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### Editor

| | |
|---|---|
| Professor R L Maynard | (Health Protection Agency) |

## 3. Summary

Nanotechnology has the potential to revolutionise everything from medicine to clothing and electronics. Indeed many nanomaterials are already on the market. Whilst this technology has enormous potential benefits, there are concerns that the unique properties of nanoparticles (NPs) will also lead to human health problems. Many reviews have recently considered approaches to investigate the toxicology of NPs, and have recognised that preliminary toxicity data can be usefully obtained from *in vitro* studies. In this discussion document, the *in vitro* approaches proposed by Oberdörster and colleagues (2005) in the ILSI review have been considered, and distilled into a set of *in vitro* assays that will allow analysis of the effects of NPs on a range of cellular and tissue endpoints. The important issues of translocation of NPs across physiological barriers and persistence within tissues are also discussed. Summaries of recommendations are provided as boxed inserts in the text. It is important to note that none of the *in vitro* methods discussed here, with the exceptions of mutagenicity studies, can be used alone for risk assessment purposes.

## 4. Introduction

Mankind has long been exposed to airborne particles of a wide variety of sizes (e.g. from combustion), and has developed defence mechanisms to counter their effects. Nanotechnology holds promise of many beneficial applications in medicine, manufacturing, energy efficiency, and environmental remediation. Concerns have, however, been expressed over the potential negative health and environmental impacts of NPs, and their possible long-term side effects. Several reviews have discussed potential human hazard issues concerning the production and application of NPs. The Royal Society and Royal Academy of Engineering Report (2004) identified a number of research areas to be addressed:

- Development of internationally agreed protocols and models for investigating routes of exposure and toxicology, including assessment of different sizes of NPs and different types of coating.

- Fundamental studies of mechanisms of interaction between NPs and cells and their components, particularly in blood vessels, the skin, heart and nervous system.

- Development of protocols for *in vitro* and *in vivo* toxicological analysis of new NPs likely to go into large-scale production.

The report highlighted the knowledge of particle health hazards that has already been gained from studies of quartz, asbestos and air pollution. Whilst the report concluded that it is very unlikely that exposure to engineered NPs could reach levels of air pollution required to cause effects, some conclusions can be drawn from studies of asbestos exposure. The toxicity of asbestos depends on certain characteristics: small fibre diameter allowing penetration deep into the lung, fibre length preventing effective removal by macrophages, low solubility (and hence long persistence), and high surface reactivity that leads to oxidative damage and inflammation. The analogy with carbon nanotubes and fibres means these NPs require careful toxicological assessment, but also leads to the conclusion that some of these characteristics (particularly solubility/persistence, and the capacity to induce oxidative damage and inflammation) should be the focus of *in vitro* toxicological analysis. The report also noted that emphasis should be placed on end-use nanomaterials where the risk of exposure to the particles is greater than those embedded in a larger material (e.g. computer chip).

In its response to the Royal Society and Royal Academy of Engineering Report (2004), the first UK Government report (2005) set out nineteen research objectives of which several can be effectively addressed by *in vitro* toxicology methods:

*Research objective 12:* Research to establish a clear understanding of inter- and intra-cellular transport and localization of NPs and their cellular toxicity.

*Research objective 13:* To establish a clear understanding of whether oxidative stress, inflammatory effects and genotoxicity apply to NPs.

The report recognised that the development of standard methods applied within tiered testing strategies should be a major priority for hazard assessment of NPs, which will also require standardised, well characterised reference materials, against which comparative toxicological data can be benchmarked.

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2005) suggested that current risk assessment methodologies may require modification for application to NPs, since potentially they could cause unique adverse effects, not seen with larger forms of materials. Toxicity of NPs could arise from the characteristics of the chemical itself, its new nanoscale form, or adsorption of other chemicals onto the particles. *In vitro* studies are well suited to provide early indications of whether a chemical of known toxicity in larger forms has adverse health effects when produced in nanoscale form; if the nanoscale form is significantly different in toxicity, this should lead to a more detailed analysis, as if it were a *new* substance. If the nanoscale form has very similar hazard properties *in vitro* to the parent compound, then further testing may not be necessary.

In the UK, the Committees established to give authoritative advice to Government departments/agencies on the toxicity and specifically on the carcinogenicity and mutagenicity of chemicals in food, consumer products and the environment (COT, COC, COM, respectively) have also considered this area and have issued a combined statement. This is available at: http://www.food.gov.uk/science/ouradvisors/toxicity/statements/cotstatements2005/307429

The US EPA (2005) Draft Nanotechnology White Paper highlights the significant lack of knowledge of the environmental, human health, and ecological implications associated with nanotechnology, and points out that the ability of available data on the toxicity of ambient air particles to predict the toxicity of engineered nanomaterials is uncertain; e.g. carbon nanotubes show different dose-responses to graphite when compared using a mass-based dosemetric. Furthermore, NPs may be able to generate both local *and* systemic toxic effects, suggesting that both will need to be considered in toxicological evaluation of these particles. Another significant challenge presented by the complexity and diversity of engineered NPs is whether assessment of one material will be sufficient to assess the toxicity of the same material produced by a different process or with a different coating. The review considers that the guiding principle for all testing will be to establish which NPs are most used or most likely to lead to human exposure, and that these particles should act as representatives for testing and evaluation purposes.

These reviews highlight the urgent need for toxicological testing of existing and emerging NPs, and *in vitro* testing represents a cost-effective route to providing preliminary toxicity information. The ILSI review (Oberdörster *et al*,

2005) suggested the elements of an extensive screening strategy, which would address the physicochemical characteristics of NPs, and investigate their toxicity through *in vitro* and *in vivo* assays. The *in vitro* screening strategy proposed by ILSI formed the starting point for the views set out here. The ILSI review proposes a comprehensive array of *in vitro* cell culture models to investigate the toxicity of diverse nanomaterials in routes of entry and target tissues. The case for a wide range of assays was based upon the need to identify unanticipated target tissue/organ toxicities of NPs. Whilst this comprehensive approach could identify particular pathways of toxicity, it may exceed both the needs and resources of manufacturers, and regulators. Furthermore a large panel of methods risks leading to a lack of clarity regarding the toxic effects of NPs, and does not guarantee that the tests will predict *in vivo* toxicity of NPs.

# 5. *In vitro* Methods for Study of the Toxicology of Nanoparticles

## 5.1 Penetration of NPs across physiological barriers

The body presents a number of barriers to nanoparticles: the external skin, gut and lung epithelia, the internal blood brain barrier and the placenta. The barriers can be broadly considered in three categories: the simple epithelia of the gut, lung and placenta, the very tight, highly developed blood-brain barrier and the stratified epithelium of the skin. Translocation can occur either by paracellular diffusion (between cells) or by transcellular movement through the cells of the barrier. Furthermore, for each NP, passage across the barrier with the potential for subsequent systemic exposure, needs to be distinguished from toxicological effects on the cells or functions of the barrier itself.

### 5.1.1 Gut

Translocation across the intestinal epithelium is often investigated *in vitro* using immortalised cell lines, of which the most popular is the colon adenocarcinoma cell line, Caco-2, isolated from a human primary tumour. These cells can be cultured under standardized conditions (including in the absence of serum), and have been widely used by the pharmaceutical industry to assess drug transport across the gut. They form a highly polarized monolayer *in vitro*, and express many features of the human intestine, including drug and chemical transporters such as p-glycoprotein. More complex *in vitro* models are available which include cells that contain digestive enzymes (Eisenbrand *et al*, 2002), or produce mucin (Nollevaux *et al*, 2006) and Caco-2 cells themselves can be converted into follicle-associated epithelia, which contains specialized M cells that can transport particulate material (des Rieux, 2005); inclusion of these cells may, therefore, be very relevant for evaluation of transport of NPs.

### 5.1.2 Lung Epithelium

The ILSI review proposes that bronchial and alveolar epithelia constitute the major barrier to entry of NPs inhaled into the lung. Transport of NPs across both these epithelia can be evaluated *in vitro*, and there is a wide array of potential cell lines available. Two lines represent good barrier models of bronchial epithelium, namely 16HBE14 and Calu-3 (reviewed by Forbes and Ehrhardt, 2005). 16HBE14 are transformed bronchial epithelial cells, derived from a bronchial adenocarcinoma, and *in vitro*, they form confluent mixed layers of cuboidal ciliated and secretory cells. These cells have been employed for preliminary *in vitro* analysis of translocation of NPs across lung epithelium (Geys *et al*, 2006). For alveolar epithelium, the human cell line

A549 has been used in some studies, but it suffers from the notable disadvantage that it does not form functional tight junctions, and therefore is more permeable than normal lung epithelium. Consequently, Forbes and Ehrhardt (2005) do not recommend any existing cell lines, but propose the use of alveolar type II cells that can be isolated from patients undergoing lung resection surgery, and which when cultured *in vitro*, grow as very flat, extended cells. Aerosol delivery to the surface of the air interface of the cell monolayers is possible by growing the cells on permeable porous membranes, so that the cells can be nourished from medium below, but when the medium is removed from their upper surfaces the cells can be exposed to airborne agents (such as NPs), mimicking inhalation exposure (Bakand *et al*, 2005).

### 5.1.3 Skin

There are a number of *in vitro* methods proposed by ILSI for the skin. Human epidermal keratinocytes (HEK), which are cryopreserved, pooled, human neonatal epidermal keratinocytes, and the immortalized, non-transformed cell line HaCaT, both represent *in vitro* models for skin. These cells are grown as monolayers, and therefore are not appropriate models for evaluation of translocation of NPs across stratified epidermis, which represents a unique barrier system. These models are more appropriately used to examine toxicity in basal epidermal cells (after translocation across the stratum corneum).

To study translocation across the skin, primary isolated human or pig skin can be used, the latter mimicking human skin closely in terms of morphology and permeability. The excised skin is placed, stratum corneum uppermost, in a diffusion cell, in contact with an appropriate receptor fluid, and ensuring a good seal around the tissue. The NPs are applied to the epidermal surface, and the receptor fluid sampled at intervals (automatically in a flow-through diffusion cell), until the end of the experiment, when the dislodgeable dose, the amount associated with the skin sample and the amount in the receptor fluid are analysed. The methods and controls are considered in detail by the OECD in their Test Guideline 428 and their associated guidance document on skin absorption studies (OECD, 2004). These methods have also been reviewed by Bronaugh (2004). These represent potential models for examining translocation of NPs across intact *stratified* skin, but they require some caution in interpretation. The skin is not in contact with a circulatory “sink”, which needs to be taken into account when calculating absorbed dose, and since the skin is not renewing itself as *in vivo*, the levels of NPs in contact with the outer stratum corneum layers might be elevated. Furthermore, the tissue needs to be handled carefully, and its integrity rigorously assessed throughout the experiment. Pre-study controls for skin integrity should be run (and reported) such as measurement of tissue electrical resistance, and trans-epidermal water loss. It is also important to consider carefully from where on the body the skin sample is taken given variations in skin thickness and density of hair follicles.

A more advanced model consists of the isolated, “perfused porcine skin flap model” in which a flap of skin is surgically created and “grown” on the underside of pigs (Riviere *et al*, 1986). This retains microcirculation, but requires a more elaborate, dedicated perfusion chamber.

### 5.1.4 Blood-brain barrier

The blood-brain barrier (BBB) consists of a layer of endothelial cells, with extremely tight junctions between them, leading to a barrier of very low permeability and very high electrical resistance. The barrier is also characterised by highly regulated transport pathways. In addition, glycoproteins are expressed. Many of the transport proteins and enzymes exhibit polarised expression. The two main *in vitro* models of the BBB use freshly isolated or immortalized brain endothelial cells. Primary brain endothelial cells are generally isolated from porcine or bovine brain obtained from abbatoir material, and these form excellent BBB models. There are several brain endothelium-like cell lines, e.g. ECV304 (human) and RBE4 (rat) *In vivo* the function of the BBB is significantly modulated by the close proximity of astrocytes and therefore both primary cells and cell lines should be cultured in the presence of either primary astrocytes or glioma cell lines such as the rat C6 glioma cells (Cecchelli *et al*, 1999). There has been some debate over the validity of the ECV304 cell line as an endothelial cell model, since whilst originally isolated as a spontaneous transformed cell from human umbilical vein endothelial cells, it also has the karyotype of a bladder carcinoma cell line. However, it is useful as a BBB model since glial-ECV304 cell co-culture greatly increases the expression of endothelial phenotype (increased electrical resistance, up-regulated expression of markers) (Abbott, 2002).

### 5.1.5 Placenta

The placenta separates the maternal and fetal circulation and its function is to transport nutrients from mother to fetus and excretory products from the fetus to the mother for removal. The fetus may be more sensitive to toxins than the adult due to rapid development and altered metabolism during gestation and hence the placenta plays a critical role in relation to the health of the newborn child. The placenta is the organ with the greatest variability in terms of morphology, anatomy and function amongst mammalian species. In the human, the placental barrier (PB) is composed of a trophoblast layer (cytotrophoblast and syncytiotrophoblast cells), connective tissue and vascular fetal endothelial cells with the syncytiotrophoblast cells bathed directly in maternal blood. Transport across the placenta is mainly by simple diffusion but selective and active transport systems have been identified on the trophoblast membrane facing the maternal blood. There are a range of *in vitro* systems available to examine placental transfer, including the isolated

human perfused placenta, villus preparations, trophoblast plasma membrane and isolated transporters and receptors (Sastry, 1999). Cultured human placental cells are most suitable for initial studies to determine whether nanoparticles transfer across the PB. It is difficult to generate single cell monolayers using primary trophoblast cultures but three pure trophoblast cell lines (BeWo, JEG and JAr) derived from malignant gestational choriocarcinoma of the fetal placenta are available commercially. The BeWo cell line is stable, easy to maintain by passage, and forms confluent monolayers in a relative short time (4-5 days) when cultured on permeable polycarbonate membranes (Chandorkar *et al*, 1999). The BeWo cell line forms functional polarised tight monolayers, retaining several morphological and biochemical characteristics of human trophoblasts and has been used extensively in many transport studies (Ampasavate *et al*, 2002). A range of transfer and toxicity studies can be carried out using the 2-chamber Transwell® culture system, with the BeWo cells grown on permeable inserts, which is ideally suited for extensive characterisation of the effects of different physico-chemical properties upon nanoparticle transfer and toxicity in the PB. Future work could include modification by co-culture with human imbilical vein endothelial cells (HUVEC) as a move towards generation of a more complex 3D model and comparison with other systems such as the perfused placenta.

*Recommendations:*

1) Translocation of NPs across barriers can be effectively studied *in vitro*. Movement across mucosal cell barriers such as gut and lung epithelium can be effectively studied using simple, polarized monolayers since the movement of the particles is more likely to depend on the properties of the particles themselves rather than inherent properties of these specific barriers. . For assessment of NP movement across stratified cell layers, pig or human skin should be tested in a diffusion cell, as described in the OECD Test Guideline 428. However, evidence to date suggests that NPs do not readily penetrate stratified epithelia

2) Where systemic NP distribution is suspected, translocation across the BBB should also be evaluated, using the simple ECV304 – C6 glioma co-culture model in the first instance.

3) Experiments need to be carefully controlled, and the integrity and function of the barrier demonstrated and reported. For most of the models described, measurement of electrical resistance of the barrier and impermeability to appropriate control agents is straightforward when the cells are grown on permeable membranes (usually required for full barrier function).

4) Once initial information has been obtained on the effects of physico-chemical properties upon transfer of NP across monolayers, future work should include the development of appropriate 3-D models and a comparison with other simple barrier models and more complex models such as the *ex vivo* human perfused placenta.

All these models can be used both for studies of NP translocation as described, but also for analysis of whether NPs alter barrier function, and/or affect physiological transport of other agents across the barriers.

## 5.2 Penetration of NPs into Cells

Understanding the mechanism(s) of particle penetration into cells would be important where NPs are shown to induce cellular reactivity and/or are believed to translocate through cells. Selective transport of many low molecular weight chemicals is mediated by specific systems such as channels, pumps, and transporters. These routes of entry are unlikely to be relevant to the uptake of NPs unless they are prone to degradation to constituent chemicals. The most likely penetration route for NPs is by uptake within membrane vesicles via pinocytosis, potocytosis (cell drinking) or phagocytosis. Alternatively, there may be passive uptake across cell membranes. Phagocytosis usually involves uptake of larger particles, which then enter the lysosomes and may be modified enzymatically. Identification of larger particles following phagocytosis can be quantified using conventional method, eg using fluorescent beads, as well as methods described here.

Pinocytosis is the common route of uptake for small, nano-sized particles such as viruses and macromolecular protein complexes. Uptake by this route can be specific, and activated/mediated by cell surface receptors (specific or non-specific), or non-specific as a consequence of cell drinking (potocytosis). Pinocytosis occurs via at least two mechanisms. The clathrin-dependent pathway (Owen, 2004) involves receptor mediated internalisation of molecules via clathrin-coated pits, and incorporation of the molecules into the endosomal pathway where they may enter lysosomes and the golgi apparatus. In contrast, uptake via caveolae (pits containing the caveolin protein) includes potocytosis (Mineo and Anderson, 2001) by-passes the lysosome and can transport molecules across the cell, into the endoplasmic reticulum, into other endocytic compartments and into the cytoplasm. It should be noted that many nanosized drug, protein and gene delivery systems have been specifically designed to target and internalise into cells via receptor mediated endocytosis leading to trafficking into the endosomal and/or lysosomal compartments of the cell. Clearly, the fate of the particle is important. Particles taken into lysosomes may be enzymatically modified and persist within the cell. Studies of those with lysosomal storage diseases (inborn errors of metabolism) show that accumulation of non-biodegradable materials in lysosome results in pathophysiological changes. Alternatively, transfer of particles across a cell to another tissue compartment may initiate a very different sequence of events. Specific inhibitors of these pathways are available and may identify which are important.

Due to the negatively charged lipids of the plasma membrane, it is generally thought that neutral or cationic particles will be taken up more readily than anionic ones. However, adsorption of NPs to blood proteins or mucosal secretions, eg in the gut or respiratory tract, may significantly modulate uptake

of NPs; e.g. non-specific adsorption of gold NPs to serum proteins leads to their receptor-mediated endocytosis (Chithrani *et al*, 2006). It is possible that the diversity of proteins and other molecules within body fluids may therefore lead to differential uptake of NPs via many different mechanisms. In addition, NPs may display a variety of functional modifications and varied chemistry that will affect particle-cell interactions. It will be important to use well characterised NPs in order to appreciate which mechanisms and features prompt cellular penetration by NPs.

During the development of pharmaceutical nano-sized drug carriers such transport studies have been adopted as routine over the last 30 years. However, there are still difficulties in obtaining quantitative data describing intracellular fate. Techniques that are being used include:

(i) *In vitro* monitoring rates of cellular uptake, exocytosis and transcytosis using radiolabelled and fluorescence-labelled probes. Uptake can be quantified by flow cytometry and visualised by confocal microscopy. The pH and concentration-dependent quenching of fluorophores and also artefacts due to fixation of cells can interfere with these assays.

(ii) *In vitro* use of subcellular fractionation and also confocal microscopy co-localisation techniques can be used to investigate the specific intracellular compartmental localisation of nano-sized particles. This is important to identify potential targets for acute and chronic toxicity.

(iii) Subcellular fractionation can also be used to isolate organelles from cells pre-exposed to nanoparticles and allows organelle function to be measured outside the cell. For example, lysosomes are used to see evidence of enhanced lysosomal membrane leakage following internalisation of polymeric materials.

Once inside the cells, however, the NPs may still be contained in vesicles that enter the endosomal pathway, or they may be found free in the cytoplasm due to passive uptake or caveolar uptake. The mechanism of uptake and route followed by NPs is unclear. To delineate the routes taken, careful localisation of NPs within cells and more specifically within different cellular compartments is required. The particles are so small that differentiation between internalised particles and cellular compounds is likely to be difficult, unless they form aggregates. Detection and identification will depend on methods such as electron microscopy for definitive localisation within cells. New methods, such as scanning ion conductance microscopy may be useful to examine live cell surface interactions with nanoparticles at high resolution, but these techniques are not available for routine use (Gorelik *et al*, 2004). Fluorescence microscopy accompanied by confocal microscopy is an alternative for evaluation of uptake and gross cellular localisation at lower resolution. Cellular fractionation accompanied by NP detection (e.g. by mass spectrometric methods) is another option. All these methods can, however, potentially lead to artefacts during sample preparation or evaluation of results.

*Recommendations:*

1) Successful analysis of penetration of NPs into cells either *in vitro* or *in vivo* will depend on the effective methods for detecting the particles. The current method of choice would be electron microscopy, since it can be applied to many particles without labelling (and potentially altering them). This approach is, however, labour-intensive and requires expensive equipment.

## 5.3 Persistence of NPs in Cells and Tissues

There is a widely accepted link between the persistence of inhaled fibres in tissues such as the lung, and their toxicity. The ILSI review suggests that the well-established protocols for investigating the durability of fibres *in vitro* could be modified for NPs. These assays essentially monitor release of materials from particles incubated in solutions that mimic physiological conditions. In the static dissolution assay, defined amounts of the particles are incubated in sodium oxalate solutions prepared at either pH 4.6 (to mimic the acidic environment inside phagosomes in cells) or at pH 7.0 (to represent the extracellular milieu) for different periods of time (typically 2-4 weeks) (Searl *et al*, 1999). In the continuous flow or dynamic assay, a simulated physiological saline is pumped through a chamber containing a defined amount of the particles for a set period (Searl *et al*, 1999). Quantitative spectroscopic methods or electron microscopy can then be used to quantify dissolution of the particles e.g.inductively coupled plasma atomic emission spectroscopy or flame atomic absorption spectroscopy. Several studies have suggested that dissolution rates of fibres determined using these assays can usefully predict persistence *in vivo* (e.g. Morris *et al*, 1995; Searl *et al*, 1999) and pathogenicity (Hesterberg and Hart, 2001). These assays would also be valuable for analysis of the longevity of NP coatings. However, the approaches for detecting dissolution of the particles might vary with the type of NP, and require optimization.

Analysis of persistence within living cells *in vitro* is more demanding, and the above acellular assays are likely to be more reliable and robust, and the first choice to inform toxicology. Analysis within cells *in vitro* requires quantification of the NPs after different periods of incubation with cells; this is most straightforward for particles that are or can be fluorescently or radioactively labelled. Several effects would have to be taken into account: dilution of particles due to cell division (or non-dividing cells could be used), and modification of the particles by the culture medium (adsorption by proteins etc).

*Recommendations:*
1) The *in vitro* static and dynamic dissolution assays would provide efficient, informative starting points for analysis of NP persistence.

## 5.4 Induction of Cellular Stress by NPs

Cellular stress can be considered as a graded response of the cell to stimuli or insults and it can act as a sensitive indicator of cellular health and viability. Many *in vitro* readouts of cellular stress can inform toxicology if used with appropriate controls and doses of agent that are relevant for actual exposure levels.

### 5.4.1 Oxidative Stress

Reactive oxygen species (ROS) generation has been observed in response to large fibres, ambient particles and a growing number of NPs. A good paradigm for explaining NP toxicity has been proposed by Kelly (2005) and Nel and coauthors (2006); this involves the generation of ROS and the development of oxidative stress. Oxidative stress is, therefore, suggested by the ILSI review authors as an endpoint for multiple tissue types, both portal of entry and target organ, and can be studied in cell-free as well as cellular systems. This suggests that, as a starting point, an indication of oxidative stress could be obtained from experiments with well characterized lung fluids and/or generic cell types, although differences between cell types in terms of their antioxidant defences would dictate downstream responses to the ROS and the overall level of oxidative stress.

There are many ways of quantifying ROS (reviewed by Kelly, 2003; Tarpey *et al*, 2004). These methods will be briefly summarised here and recommendations made for those that are the most robust, reproducible and widely used.

- Depletion of airway fluid low-molecular-weight antioxidants: reduced glutathione (GSH), uric acid and ascorbate. These antioxidants provide the first level of protection against inhaled particles and their depletion can be measured *in vitro* as an estimate of NP oxidative activity or *ex vivo*, following challenge, which gives an estimate of an individual's or patient group's ability to withstand NP oxidant challenge. Airway fluid antioxidant depletion can be accurately and conveniently measured by HPLC (Mudway *et al*, 2001, 2004).

- Low molecular weight antioxidants such as GSH are also present in cells, and the balance of GSH and its oxidized form (GSSG) provides a good indication of the level of oxidative stress. The concentrations of both can be determined either biochemically or by HPLC.

- Lipid peroxidation has been a widely used indicator of free radical formation, frequently measured by detection of thiobarbituric acid (TBA) reactive substances arising from degeneration of lipid peroxidation products (eg, Oberdörster, 2004). Although sensitive, this

method however is not specific since many other biological species react with TBA.

- Colorimetric methods for quantifying ROS generation include measurement of the reduction of ferricytochrome c to ferrocytochrome c, or oxidation (and inactivation) of aconitase by superoxide. The former assay is suggested by Tarpey *et al* (2004) to have "gold standard" status for *in vitro* assay of superoxide; to increase the specificity of the assay the cytochrome c can be acetylated and inhibitors of enzymes, which reoxidise cytochrome c, included.

- Chemiluminescent methods are based on compounds such as lucigenin and coelenterazine, both light-producing chromophores. Lucigenin suffers from the disadvantage that redox cycling can occur, generating further ROS. Coelenterazine avoids this problem, and the intensity of the light emitted is greater than with lucigenin, but its reaction is not specific for ROS, since NOS also increases its luminescence. This problem can be avoided if necessary by using appropriate scavengers.

- Fluorescent approaches are becoming increasingly popular for measuring ROS. One of the most popular reagents is 2'-7'-dichlorofluorescein (DCFH) (eg Brown *et al*, 2001; Hussain *et al*, 2005); the non-fluorescent form, DCFH-DA, is taken up into cells *in vitro* where it is esterified to DCFH and oxidized in the presence of ROS to fluorescent DCF. Although widely used and quite robust, this assay has disadvantages: firstly, the DCFH is not completely "trapped" intracellularly as suggested, but can leak out and increase background signals; DCF can be formed by peroxidases and NOS within the cell regardless of ROS production, and DCF formation is very sensitive to intracellular iron levels, occurring very slowly in the absence of iron.

- Another fluorescent assay for ROS is based on dihydrorhodamine 123 (DHR), which is cell-permeable, acts as a sensitive indicator of mitochrondrial membrane potential, and can be oxidized by ROS to the fluorophore rhodamine 123. Like DCFH, other agents are, however, able to oxidize DHR, and both these assays are, therefore, more suitable for qualitative than quantitative assay of ROS (Tarpey *et al*, 2004).

- Electron spin resonance (ESR) is a technique that directly measures the presence and concentration of oxygen (and other) free radicals. Since most free radicals are very unstable, ESR usually employs exogenous "spin traps" that react with the free radical species, generating more stable adducts with characteristic ESR profiles. The major disadvantages of ESR are cost, lack of specificity of some spin traps and silencing of some adducts by cellular reductants.

- Free radical formation can also be analysed by detection of the hydroxyl radical-specific DNA adduct 8-hydroxydeoxyguanosine (8-

OHdG) using antibodies raised against 8-OHdG in immunocytochemistry or by HPLC.

*Recommendations:*

ROS are short-lived and the body has scavenger systems to deal with them; therefore any assay designed to detect them must be very sensitive (hence expensive) and is subject to artefacts; for this reason the favoured approach is to measure the impact of NP-derived ROS:

1) The biochemical assays for depletion of antioxidants by NPs provide an excellent, cost-effective starting point for assessment of free radical generation. This approach has the physiological advantage in that it measures the body's response to the oxidant challenge.

2) ROS can be measured by ESR, but this requires expensive, dedicated equipment and often additional oxidants such as $H_2O_2$ are needed to generate a signal.

3) Cell-based fluorescence assays are generally more sensitive than colorimetric, and therefore a semi-quantitative analysis of cellular oxidative stress should be obtained using DCFH, with more detailed quantitative analysis of overall oxidative stress from determination of the levels of GSH/GSSG. Appropriate controls should include free radical scavengers (eg, mannitol, DMSO, catalase) to verify the specificity of the measurements.
The ILSI review proposes oxidative stress analysis for many tissue types, but we propose a simpler strategy on the basis that essentially two cell types can give meaningful information on whether a NP is likely to induce oxidative stress.

4) Professional phagocytes such as macrophages (either primary isolated cells, or a cell line such as U937 or THP-1) should be used since they can actively ingest and mount a significant oxidative response to NPs, and may, therefore, represent a "worst-case scenario" for generation of oxidative stress in response to NPs.

5) The second cell type would be one of the models suggested for studying translocation of NPs across barriers such as HaCaT skin cells, since these are not capable of phagocytosis, but can sample their environment by pinocytosis, and therefore are likely to mount a more moderate oxidative stress response, closer to that likely to be produced by other cell types in the body.

### 5.4.2 Inflammation

Inflammation involves intra- and intercellular signalling to coordinate the function of immune and non-immune cells at sites of infection and damage. Neutrophils and monocytes/macrophages of the innate immune system are

important effector cells during acute inflammation. The acute inflammatory response includes capillary vasodilation with exudation of plasma proteins, adhesion of neutrophils to endothelium and chemoattraction of other immune cells into the inflammatory site. There is local activation of leukocytes and release of numerous mediators that lead to elimination of foreign substances by macrophages (phagocytosis) and neutrophils, elimination of cells (apoptosis) and healing of tissue.

Primary cellular targets of inhaled, orally ingested or topically applied NPs will be the epithelium and any resident leukocytes, for example macrophages and neutrophils, and these cells are most likely to trigger an inflammatory response. The interaction of NPs with these cells will critically depend on the composition of secretions, which may modify the characteristics eg, tendency to agglomeration, of the particles. The epithelium and resident leukocytes can then initiate responses by interstitial cells, eg fibroblasts, and those of the cardiovascular system, eg endothelial cells and blood leukocytes. There is evidence that the particles themselves may translocate into the interstitium and circulatory system, supporting the need for investigation of direct NP-interstitial, NP-endothelial and NP-blood leukocyte responses. Other cell types that should be investigated include those from target organs such as liver, heart, nervous system, brain and kidney. Serum and tissue proteins, lipids and other molecules are likely to affect NP-target cell interactions. Clearly, the choice of cell system will depend on the questions asked, relating to the pro-inflammatory potential of the NPs.

The measurement of immune cell functions and the release of inflammatory mediators (eg cytokines and pro-inflammatory eicosanoids) will enable the potential effect of NPs on immune responses to be assessed. This section will focus on the role of specific cell types involved in cellular mediated inflammatory responses. Epithelial cells and macrophages (lung and gut), as well as keratinocytes, are obvious choices. Fibroblasts, endothelial cells, cardiomyotyes, dendritic cells, neurons, glial cells, lymphocytes, spleen cells and hepatocytes are other cells that may be targeted by NPs and in which may it may be important to investigate possible pro-inflammatory effects of NPs. Primary cells can be obtained from animals, but there may be crucial between species differences and initial experiments using animal tissue should, wherever possible, be supported by studies using human sources. However, it may be difficult to obtain sufficient primary human cells for routine testing, and other strategies will be required. For example, it is not possible to generate monocyte-derived macrophages and dendritic cells from human blood in significant numbers. Although there may be between-subject variation, consistent trends will be highlighted. If cell lines are used, they should have been derived from normal tissue, not cancerous tissue, and the cellular mechanisms under investigation should have been proven to match those of the primary cells of interest. Such strategies should also be used when information is required for specific target tissues, as primary human cells from these tissues will be even more scarce.

During infection and irritant responses to toxic chemicals and mineral particles, circulating blood neutrophils and monocytes are rapidly recruited to

the area of insult, adhering to local blood vessels and migrating toward sites of inflammation in response to gradients of chemotactic factors. The priming of neutrophils involves tethering and rolling on the luminal surface of the vascular endothelium. Neutrophil rolling is then arrested by their expression of specific β2 integrins (CD11b and CD18) which complexes with their cognate cell adhesion molecules on the surface of endothelial cells e.g., CD54 (ICAM-1). The most widely accepted method of measuring neutrophil activation status is by flow cytometry and uses specific fluorescent labelled antibodies (eg., to CD11b and CD18 antigens). The priming of circulating monocytes also leads to their differentiation into macrophages with loss of the expression of the cell surface marker CD14 and increased expression of molecules such as CD68 and CD11b (MAC 1a).

The priming of these cells also results in their increased adherence to other cells but also non-specifically to tissue culture dishes. NPs may either enhance or inhibit this process. Adherence assays are a simple cost effective means (Bevilacqua *et al*, 1985) to assess the impact of factors that prime these cell types. Adherence assays are suited to high throughput analysis and automation. The adherent cells are labelled with fluorescent or coloured dyes and the number of cells attached to the surface determined using automated plate reader devices or microscopy. This type of assay should be considered as part of a suite of tests for NPs.

Following the priming of neutrophils and macrophage differentiation, additional stimuli can activate phagocytosis of opsonised organic and inorganic particles, such as of bacteria, apoptotic cells or virally infected cells. The effects of NPs on phagocytosis might be stimulatory or inhibitory. Phagocytosis following/during exposure to NPs can be measured using a variety of probes, but most commonly fluorescent labelled latex beads (not NP-sized; probably about 5 μm), which enable the process to be visualised using a microscope, and quantified using light or confocal microscopy, or low cytometry. Activation of the phagocytic response is also accompanied by the production of free radical burst and release of bactericidal peptides and proteolytic enzymes. There are a wide variety of probes and assays available to measure these processes. The uptake of particles mediated via the Fc receptor can be demonstrated using Fc OxyBURST Green ™ assay (Ryan *et al*, 1990). The Fc OxyBURST Green reagent is bovine serum albumin (BSA) covalently linked to dichlorodihydrofluorescein (H2DCF) and an anti-BSA antibody. When the immune complexes bind to the Fc receptors of the BSA antibody, the non-fluorescent H2DCF molecules are internalised within the phagocytic vesicles and subsequently oxidised to a green-fluorescent product. This process can be visualised using a microscope and quantified using flow cytometry or a fluorescent microplate reader. Limitations of this approach are discussed in section 5.8.1. Thus, it would not be practical to use fluorescent particles as well as a fluorescent endpoint assay. Furthermore, some particles may interfere with the “readout”. Suitable control experiments will be necessary.

Other functional endpoint assays for the activation of inflammatory cells include their release of proteases (serine proteinases and matrix metalloproteinases) and other enzymes involved in free radical production, for example, neutrophil myeloperoxidase (MPO) which is involved in the conversion of hydrogen peroxide to hyperchlorous ions. These methods are suited to high throughput and are cost effective and may bypass some of the limitations of using fluorescence to measure effects. These are simple spectrophometric assays that can rapidly measure cellular activation (eg priming and phagocytosis) under numerous experimental conditions.

Cell signalling pathways and release of mediators that underlie these responses, as well as the responses of other resident cells that trigger inflammation, can also be monitored. The direction of an immune response (e.g., Th1 cytotoxic immunity vs Th2 humoral immunity) is coordinated by specific cytokines produced by immune and non-immune cells, whereas the pattern of chemokine release is indicative of the magnitude and profile of the inflammatory response. All the cellular sources described above can be monitored for release of pro-inflammatory mediators such as cytokines (eg TNF-α, IL-1), chemokines (eg IL-8, MCP-1), nitric oxide, and up-regulation of transcription factors such as nuclear factor kappa B (NFκB) and activator protein 1 (AP-1), known to be important in initiating the inflammatory response. One problem, outlined in section 5.8.1, will be interference by NPs, as they may adsorb the markers of interest and therefore the markers will not be available for assay. Individual cytokines, and chemokines, can be quantified using ELISA although this is labour-intensive and expensive. Recent development of a bead-based multiplex immunoassay system (which uses the same principals as ELISA) and slide antibody arrays, allows multiple proteins such as cytokines, chemokines and proteins involved in cell signalling (eg mitogen activated protein kinases, NFκB, AP-1), to be measured simultaneously in a single sample (Chan *et al*, 2004). These methods considerably reduce the cost and time for completing such an analysis but require the use of dedicated fluorescent bead analysers, or array scanners. Immunoblotting may also be used but this technique is not suitable for high throughput analysis. In general, assays based on immunological detection can be expensive and thus this approach may be more useful for NPs that have demonstrated toxicity and for which further mechanistic information is required. Nitric oxide measurement is a simple spectrophotometric assay, leukotrienes and similar mediators can be measured using sensitive colorimetric, chemiluminescent and fluorescent immunoassays. Again, the caveats listed in section 5.8.1 should be taken into account.

Many of these cellular processes result in the release of intracellular stores of calcium which can be monitored using fluorescent calcium indicator dyes e.g., Fura-2 and Indo-1 (ratio-metric $Ca^{2+}$ indicators). These dyes exhibit different excitation and/or emission wavelengths (Grynkiewicz *et al*, 1985). While Fura-2 is designed for microscopy, Indo-1 is a better choice for flow cytometry. These methods are unlikely to add further information to the phagocytosis assay, but nevertheless may be useful markers of cellular activation. The properties of fluorescent NPs, such as quantum dots, may

overlap with some of the dyes used to quantify specific cellular responses (ef Fura-2) used to measure intracellular calcium, and this must be taken into consideration (see section 5.8.1).

*Recommendations:*

1) To assess cellular responses of phagocytic immune cells to NPs, the use of freshly isolated neutrophils and macrophages, or monocyte-derived macrophages, is recommended, but for high throughput analysis the use of well characterised macrophage cell lines should be considered. Monocyte-derived dendritic cells should be considered for the investigation of the acquired immune response.

2) Measurement of cell surface antigen expression and cell adhesion are the recommended methods to characterise the potential priming effect of NPs on these cells.

3) For measurement of cell activation, recommended methods include assays of phagocytosis and the oxidative burst.

4) To assess the potential of NPs to initiate an inflammatory immune response via target cells, primary cells should be used where possible; failing this, well characterised cells lines derived from normal, not carcinoma, tissue should be used.

5) The measurement of release of inflammatory mediators (e.g. eicosanoids, chemokines, cytokines, nitric oxide) is recommended for the first tier of tests as these demonstrate potential types of inflammatory immune responses and are suited to high throughput analysis.

## 5.5 Mutagenicity/Genotoxicity

This area differs from the other areas where *in vitro* tests are being considered to provide preliminary information on the toxicity of NPs in that there are validated *in vitro* assays that are used to assess the mutagenicity (and hence also potential carcinogenicity) of chemicals. In addition, there are well established strategies for evaluating chemicals for mutagenicity. These are outlined in the guidance published by the independent expert advisory committee established to advise Government departments and agencies on all aspects of the mutagenicity of chemicals, namely the Committee on the Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM). The most recent guidance was published in December 2000; it is available at;
http://www.advisorybodies.doh.gov.uk/com/guidance.pdf

Both in the latest guidelines, and in the earlier version in 1989, the strategy was based on an initial *investigation in vitro* (Stage 1) to assess mutagenic potential using a small number (3 unless exposure was very limited when 2

were acceptable) of well conducted gene mutations and chromosome damage (structural and numerical aberrations ie, clastogenicity and aneugenicity). The three recommended tests are gene mutation in bacteria, chromosome damage in mammalian cells using metaphase analysis (or alternatively the *in vitro* micronucleus test which has the advantage of also readily detecting aneugens), and the mouse lymphoma assay (detects both gene mutations and damage at the chromosome level in mammalian cells). There are internationally recognised OECD guidelines for all these assays except the *in vitro* micronucleus test for which there is a draft OECD guideline under consideration. Provided that these 3 tests are conducted to the current guidelines, they should detect almost all mutagens and the COM believe that there is no justification for screening using *in vivo* tests. Substances negative in these 3 well conducted *in vitro* tests can be regarded for practical purposes as being non-mutagenic.

If positive results are obtained in any one test there is a need for *in vivo* studies in animals to ascertain whether the effects seen *in vitro* could be expressed *in vivo*. Stage 2 of the strategy outlines testing in somatic cells *in vivo* to ascertain whether the chemical should be regarded as an *in vivo* somatic cell mutagen and hence also a potential carcinogen and a possible germ cell mutagen. Negative results from at least 2 appropriately designed assays in different tissues are needed before it can be assumed that activity seen *in vitro* cannot be expressed *in vivo*. Stage 3 involves investigations in germ cells *in vivo* somatic cell mutagens and hence regarded also as potential carcinogens and possible germ cell mutagens, no further testing will be necessary.

It is clearly important to know whether NPs have any mutagenic, and hence also carcinogenic, potential. The recommended strategy using *in vitro* assays for chemicals is considered appropriate. However, there are important additional considerations (these also apply to the *in vitro* assays used in areas other than mutagenicity) regarding the characterisation of the NPs being investigated and the need for measurements in the cell culture system to ensure awareness of any agglomeration/disagglomeration of particles. Thus it is suggested that information from the following 3 well established mutagenicity assays is desirable when assessing the mutagenic potential of NPs.

(1) Assay for gene mutation in bacteris using the *Salmonella typhimurium* assay. The usual 5 strains (covering both base pair and frame shift mutations) should be used, namely TA1535, TA1537 (or TA97), TA1538, TA98 and TA100. In addition data using TA102 would be helpful to identify effects due to reactive oxygen species in view of the sensitivity of this strain to such species. It is recognised that such prokaryotic assays may not reflect some types of cellular interactions that may be important in any NP related genotoxicity, but they do have the advantage of being designed to be particularly sensitive to any inherent DNA activity by the test material (eg little barrier to reaching DNA). Furthermore, this is the most widely used *in vitro* mutagenicity and has proved to be a very sensitive assay for detecting gene

mutation in chemicals. In addition, the assay is complimented by the following 2 mutagenicity assays in mammalian cells.

(2) An assay for clastogeniticy in mammalian cells by metaphase analysis. Either CHO cells or human lymphocytes with mitogen stimulation may be used (these are regarded as giving equivalent results). Some limited information can be obtained also on potential aneugenicity from the incidence of hyperdiploidy, polyploidy and/or modification of the mitotic index; such indications may be followed up by appropriate staining procedures such as fluorescence *in situ* hybridisation or chromosome painting.

(3) An assay for both gene mutations and clastogenicity (various sizes of chromosome deletions) in mammalian cells based on measuring mutations at the thymidine kinase locus in L5178Y cells (the mouse lymphoma assay).

When investigating chemicals in these 3 assays there is a requirement to test both in the presence and absence of an exogenous metabolic activation system such as Arochlor induced rat liver S-9 fraction. It is unlikely that this will be needed for any activation of the well characterised NP being recommended for investigation, and furthermore the S-9 fraction may cause complications due to binding of the NP particles, but some data to confirm this is desirable.

If positive results are obtained with NPs in any of these 3 *in vitro* assays, it will be necessary to investigate *in vivo* in animals before drawing any definite conclusions regarding the significance of these data. The approach outlined in Stage 2 of the COM strategy is, in general, appropriate although with NPs there would be even greater need to consider each compound on a case by case basis, and design further tests on the basis of any knowledge of the distribution of the NP in the body and any tissues of accumulation/toxicity. There are well established assays for investigating mutagenicity in bone marrow eg the *in vivo* micronucleus test (measures clastogenicity and aneugenicity) and for genotoxicity in the liver (unscheduled DNA synthesis, an indirect indicator of DNA damage). If it is appropriate to investigate other tissues a number of approaches are available, although in no cases are there recognised guidelines. These are listed in the COM strategy on investigating the mutagenicity of chemicals noted earlier and include measurement of DNA strand breaks using the COMET assay, measuring DNA adducts using $^{32}$P-postlabelling or measuring point mutations in transgenic animal models.

*Recommendations:*

The list of genotoxicity assays reviewed here is not comprehensive but certain recommendations can be made:
1) Assay for gene mutation in bacteria using the *Salmonella typhimurium* assay should be done.

2) Assay for clastogenicity in mammalian cells using metaphase analysis should be done. Either CHO cells or human lymphocytes with mitogen stimulation should be used.

3) Assay for both gene mutations and clastogenicity (various sizes of chromosome mutations in mammalian cells, based on measuring mutations at the thymidine kinase locus in L517BY cells should be done.

## 5.6 Damage to Cells by NPs

There are a large number of approaches that can be used to investigate cytotoxicity *in vitro*, and many of these are mentioned in the ILSI review. Assays will be considered in turn and recommendations made regarding the most robust and informative.

### 5.6.1 Membrane permeability assays

These assays are based on the fact that the plasma membranes of dead and dying cells become permeable to dyes such as trypan blue or propidium iodide. Trypan blue staining is typically analysed by light microscopy, making it labour-intensive. Propidium iodide (PI) is a fluorescent red, DNA-intercalating dye and uptake by dead cells can be followed either by fluorescence microscopy or flow cytometry.

In contrast the LDH assay, one of the most popular, simple methods for assessing cell death, can readily be done quantitatively and semi-automatically. This assay is based on the release of the cytosolic enzyme lactate dehydrogenase into the culture medium when the plasma membranes of dead and dying cells become permeable. LDH can be detected either colorimetrically or fluorescently.

### 5.6.2 Metabolic assays

There are several indicators of cellular metabolism that are routinely used to assess viability/cytotoxicity *in vitro*. Alamar Blue is a redox indicator that fluoresces when reduced in the medium of proliferating cells. Cell death therefore leads to a quantitative reduction in staining intensity, although strictly this assay measures inhibition of proliferation rather than cell death itself. Neutral red, in contrast, is a vital stain only taken up by live cells; it is used to quantify cell viability and forms the basis for the ECVAM-validated 3T3 phototoxicity test. The MTT assay is based on the ability of mitochondrial dehydrogenases within live cells to reduce tetrazolium salts to coloured formazan products, providing a quantitative indication of viability. However the MTT-generated formazan crystals are insoluble, requiring solubilisation for analysis. Alternative tetrazolium substrates (e.g. XTT, MTS, WTS) have since been developed which form water-soluble products, simplifying and improving the sensitivity of the assay.

Energy failure is also a feature of cell death, and enzyme-based ATP assays have been employed to quantify cytotoxicity. One popular, sensitive assay is based on bioluminescence generated when luciferase catalyses the formation of light from ATP and luciferin.

### 5.6.3 Apoptotis assays

Apoptosis is a controlled form of cellular death that can occur in response to a wide variety of stimuli or insults. Biochemical and morphological changes form the basis for many approaches available for quantifying apoptosis.

- Activation of caspases is critical for cell death, and caspase activity can be quantified in cell extracts using fluorogenic or colorimetric substrates.

- Nuclear changes such as chromatin condensation accompany apoptosis. These can be detected using fluorescent dyes such as the Hoechst 33342, which has a high specificity for DNA (leading to low background fluorescence) and gives rise to more intense staining in apoptotic compared with healthy cells in which the DNA staining is weak and diffuse. DNA fragmentation can be analysed either by gel electrophoresis or labelling of the DNA ends generated in a technique called TUNEL.

- Changes at the mitochondrial level precede the morphological changes of apoptosis and can be used as early markers of cellular death. Dihydrorhodamine (DHR) and its derivative tetramethylrhodamine methyl ester (TMRM) are indicators of mitochondrial membrane potential (MMP), accumulating in the organelles in proportion to MMP (Esposti, 2001); their fluorescence intensity decreases during early apoptosis. Loss of staining therefore provides a sensitive indicator of loss of viability. Release of cytochrome c from mitochondria also accompanies apoptosis and precedes activation of caspases; this endpoint can be followed by fluorescence or cell fractionations, but quantification via these methods is not robust.

- Apoptotic cells are tagged for phagocytosis by exposure of phosphatidyl serine on their plasma membranes, which once labelled with fluorescently-labelled annexin-V can be visualised by flow cytometry or fluorescence microscopy.

*Recommendations:*

For nanoparticle toxicology, the most important consideration is whether the NPs induce cell death, rather than distinguishing the mechanism of death (i.e. apoptosis versus necrosis); since *in vitro*, apoptotic cells progress to secondary necrosis due to the absence of phagocytes, assays that detect necrosis will also detect many late apoptotic cells. Much NP research has so far rightly focussed on simple endpoints such as LDH and MTT assays.

1) We recommend that at least two different assays are used to evaluate NP cytotoxicity: the LDH, neutral red and MTT-like assays are simple and robust; they are quantitative, and can be carried out semi-automatically on spectrophotometric or fluorescent plate readers, allowing efficient, rapid analysis of many samples on cells grown in multi-well plates. The neutral red assay already constitutes part of a validated toxicity test, with a standardized protocol; the LDH and second generation improved MTT-like MTS assays are readily available as commercial kits.

2) It would be possible to apply these techniques to a wide variety of cells, either primary or cultured lines representative of many tissue types. We would suggest that a discrete panel of cell types is used which represent portal of entry and target tissue types: professional phagocytes (e.g. either primary isolated macrophages, or a cell line such as U937 or THP-1), epithelial cells (e.g. HaCaT skin cells) and connective tissue cells (e.g. fibroblast 3T3 cells).

## 5.7 Circulatory effects of NPs

### 5.7.1 Adsorption of NPs to Proteins in the Circulation

The ILSI review notes that the large surface area of NPs could lead to significant adsorption of proteins to particles and changes in NP behaviour. NPs may come into contact with proteins at the point of entry into the body or after crossing into the interstitium or systemic circulation. Opsonization, the process whereby a foreign particle (or organism) becomes coated with opsonin proteins, is the first line of defence of the body against particle invasion (reviewed by Owens and Peppas, 2006). In the blood, opsonization occurs very quickly, often within seconds, and involves interaction with a variety of proteins including complement proteins (C3, C4, C5), immunoglobulins, plasma fibronectin, laminin, collagen or C-reactive protein. Opsonized particles are then recognized by the reticuloendothelial system (RES), which can remove particles from body within seconds (following e.g. intravenous administration). NPs may be phagocytosed either by such “professional” phagocytes i.e. dendritic cells or macrophages, or by other cells. Phagocytes bind to the opsonins and phagocytose the particle, leading where possible, to the degradation of the particle by enzymes or oxidative mechanisms within the cell. If the particles are not biodegradable by this route, they may be cleared by the renal system, if their molecular weight is less than 5 kDa. Particles of radius of more than 200 nm are cleared more slowly than smaller ones by the renal system. Alternatively they may accumulate in the liver or spleen, leading to toxicity if they are not degradable. Interaction between NPs and opsonins will greatly depend on the surface coating of the particle. Binding is mediated by several attractive forces: van der Waal’s, electrostatic, ionic, hydrophobic or hydrophilic depending on the particle and proteins involved. Opsonization of hydrophobic particles generally occurs more quickly than that of hydrophilic particles due to enhanced adsorption of blood serum proteins to their surfaces. Surface

charge also affects the rate of opsonization, neutral particles interacting more slowly than charged. Opsonization is therefore significantly slowed by groups that block electrostatic or hydrophobic interactions, and shielding of the NP with polymer systems such as poly(ethylene) glycol (PEG) can greatly prolong the circulatory life-time of particles. To be effective, such groups should be covalently bound to the NP, since otherwise it is possible for the groups to be displaced by opsonins.

Binding of NPs to proteins in lung lining fluid or blood can be assessed in several ways; including two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Typically, therefore, NPs might be incubated with serum, plasma or other physiological fluid; the particles are then recovered, potentially with adherent proteins, which are then subject to 2D-PAGE.

A more powerful technique for analysis of interactions between proteins and NPs is provided by surface plasmon resonance (SPR), which can be automated within the BIAcore (the most widely used) or similar systems. This optical technique measures the refractive index close to a sensor surface. In the BIAcore, ligands (e.g. proteins from plasma) are immobilized on the sensor surface, and NPs are then injected into the flow cell, and their binding to the proteins on the sensor detected by changes in refractive index. BIAcore has developed the technique such that they have "single pass, multi-spot flow cell systems" that can analyse the interactions between a NP and spots of hundreds of proteins from a physiological fluid on a single "chip" (see http://www.biacore.com/lifesciences/technology/introduction/Flow_cells/index.html).

*Recommendations:*
1) Both 2D-PAGE and SPR technology (eg BIAcore) with multi-spot flow systems are applicable for analysis of interactions between emerging NPs and proteins.

### 5.7.2 Activation of Complement by NPs

The ILSI review suggests that activation of complement by NPs should be studied. The complement system is a proteolytic cascade that complements and amplifies the antibody response to invading pathogens. Complement activation occurs after exposure to fibres such as chrysotile asbestos (e.g. Warheit *et al*, 1985), leading to recruitment of macrophages to lung alveoli, potentially contributing to asbestosis lesions. When activated, the complement cascade leads not only to phagocyte recruitment and destruction of invading organisms, but can also have "bystander effects" (e.g. lysis of nearby cells) if not terminated quickly. Activation of the cascade by NPs could therefore have serious consequences.

Activation of complement can be readily evaluated *in vitro* using a number of methods. The principle of the haemolytic CH50 assay is that the complement cascade is activated when sensitized sheep erythrocytes interact with

complement proteins in human serum, resulting in the lysis of erythrocytes and the release of haemoglobin (Layre *et al*, 2006). Opsonization of NPs reduces the number of complement proteins in serum available to lyse the erythrocytes, leading to a decrease in the CH50 units compared to control i.e. the amount of serum required to lyse 50% of the erythrocytes. Although simple, strictly this method measures opsonization of NPs rather than complement activation. Assays that investigate proteolysis of complement proteins more accurately reflect activation of the cascade by NPs. These methods depend on immunological detection of complement proteins. The simplest method is the "dot blot", in which the sample is spotted onto a filter that is subsequently incubated with an antibody raised against the activated complement protein of interest; detection of antibody binding occurs as in a Western blot (binding of secondary labelled antibody, and then exposure to either a colorimetric or chemiluminescent substrate). This method is more qualitative than quantitative. One of the older methods involves 2D-immunoelectrophoresis (Laurell, 1966), in which the samples containing the complement proteins are electrophoresed through agarose gels impregnated with anti-complement antibodies. The antigen-antibody reaction leads to the formation of precipitation zones in the gels, the sizes of which are proportional to the amount of antigen (complement) present. This method has recently been used for evaluation of complement activation by NPs (Bertholon *et al*, 2006). Enzyme-linked immunosorbent (ELISA) or radioimmuno (RIA) assays can also detect cleavage of complement proteins (e.g. Wagner and Hugli, 1984); many researchers prefer ELISA assays to RIAs, owing to the health and safety issues of handling radioactive material.

*Recommendations:*
Both ELISA and RIA assays for detection of activation of complement are sensitive, robust, reproducible, and quantitative; their specificity depending on the antibodies and conditions employed. They are functional assays, which would give valuable information on the ability of NPs to activate this cascade *in vivo*.

### 5.7.3 Modulation of Blood Coagulation by NPs

Epidemiological evidence suggests a link between inhaled air pollution and adverse cardiovascular effects (Department of Health, 2005). Some NPs have been shown to pass into the systemic circulation rapidly (Nemmar *et al*, 2002), and reports are emerging to suggest that some particles (nano-sized and larger) may modulate blood coagulation even in the absence of pulmonary inflammation.

*Direct effects on platelets or other blood cells*

Platelet aggregation has been demonstrated following exposure to NP (Nemmar *et al*, 2003; Radomski *et al*, 2005). Analysis of platelet activation can be performed in two ways: by following platelet aggregation in a platelet aggregometer after addition of specific agents (eg collagen, ADP) to platelet-

rich plasma, or by isolation of platelets from plasma and analysis of activation-dependent expression of P-selectin on their surface. The latter can be performed readily by flow cytometry. Platelets are obvious targets for studying the potential thrombogenic effect of NP but other blood cells such as monocytes or soluble components of the blood may also be important.

*The endothelium and the fibrinolytic balance*

The endothelial cells control clot formation by modulation of the fibrinolytic balance by production of fibrin-dissolving proteins, such as tPA and their regulatory proteins eg, PAI. Particles are capable of modulating these in ways that favour thrombogenesis (Gilmour *et al*, 2005). The effects of NP on synthesis and release of key proteins involved in coagulation by endothelial cells is an important area for research.

*Clinical tests of time to clotting in blood*

Standardised assays for blood clotting have been in clinical use for many years, and can readily be applied to NPs *in vitro*. Assays fall in two categories: investigation of sequential activation of clotting factors or activation of platelets. Both of these parameters might be affected by NPs. A range of coagulation assays are available, all of which can readily be performed in a simple coagulation analyzer; the most appropriate analyses of the effects of NPs would be:

- *Prothrombin time* measures the status of the extrinsic coagulation cascade in terms of the time taken from addition of e.g. recombinant tissue factor to citrate anti-coagulated plasma to the formation of fibrin threads.
- *Fibrinogen time* measures the rate of clot formation after dilution of the plasma so that the amount of fibrinogen is rate-limiting, and addition of an excess of thrombin.
- *Activated partial thromboplastin time* measures the time to fibrin clot formation when a surface activating agent (e.g. silica) and phospholipids are added to plasma.

*Recommendations:*

1) Platelets and endothelial cells are key players in clotting that have been studied previously for particle effects on thrombogenesis and there are established assays for pro-thrombotic effects in them, eg, platelet aggregometry.

2) Clinical test of time to clotting are low-tech and are more likely to be useful in human subjects than *in vitro*.

## 5.8 Generic Issues

### 5.8.1 Controlling for particle interference

There are a wide variety of endpoints that can be employed in toxicity assessment strategies, as well as several approaches that can be used to measure each endpoint. Many biochemical assays employ the measurement of a coloured or fluorescent product, for example the MTT assay described below generates a blue formazan product that can be quantified by measuring the absorbance at 550 nm. However, a number of nanoparticles are coloured, fluorescent or possess properties that allow them to interfere in such assays. A number of examples of potential interference that should be considered include:

a. The opaque nature of carbon black generates an absorbance reading at 550 nm leading to an overestimation of cell viability using the MMT assay.

b. A study by Worle-Knirsh *et al* (2006) also demonstrates the potential for carbon nanotubes to bind the formazan product generated in the MTT assay, leading to an underestimation of cell viability.

c. $TiO_2$ is used in sunscreen due to its ability to reflect UV light, and since fluorescent dyes are excited by and subsequently emit light that fall within the UV spectrum, it is necessary to ensure that the endpoint measured is a true reflection of the dye and not the particle interference.

d. More obviously, fluorescent nanoparticles such as quantum dots may overlap in terms of excitation and emission wavelength properties with dyes used to quantify specific cellular responses, for example, fura-2 used to measure intracellular calcium.

It is therefore necessary to ensure that such interference is considered during the choice of endpoint, the assay used to assess that endpoint and the subsequent experimental design. In all studies it is essential that the capacity of particles to interfere in the assay is investigated through the use of appropriate control treatments.

A number of endpoints employ the measurement of a cellular product, for example the release of a protein. It is also necessary to ensure that such products can be accurately measured in the presence of nanoparticles. Nanoparticles have a huge surface area to mass ratio with a capacity to bind large quantities of proteins, for example, bovine serum albumin and lung lining fluid proteins (Brown *et al*, 2001). Recent data from several research groups also demonstrate that nanoparticles can bind the protein lactate dehydrogenase leading to underestimation of inflammation. Again, adequate control experiments are required to determine such interactions in order to allow appropriate interpretation of the data generated.

### 5.8.2 Particle preparation

There is currently no agreed standard protocol for the preparation of particles prior to the addition into *in vitro* systems. A number of options are available:

a. Dispersion or sonication in physiological buffer with or without serum
b. Addition of a surfactant to aid dispersion
c. Addition of a solvent to aid dispersion.

The time required to disperse particles via sonication is unclear, and likely to vary with different particles. Sonication may destroy proteins if serum is present and so sonication time needs to be kept to a minimum. There is also no agreement over whether it is best to use a probe sonicator or a waterbath, but generation of a nanoparticle aerosol using the probe should be avoided, as should crossover of particles between samples via contamination of the probe. Waterbaths may not generate sufficient energy to disperse particles, and different locations within the waterbath may be more effective than others. Very little work has been conducted in relation to the effect of surfactants on nanoparticle behaviour, but if such a protocol is chosen it should be relevant to the human body, for example lung surfactants. Finally, a number of ecotoxicology studies have used solvents such as tetrahydrofuran to solubilise C60 followed by rotary evaporation. This has led to much deliberation over the interpretation of the results generated since it is difficult to untangle the effects of the remaining contaminating solvent from that of the particles.

## 6. Conclusions

*In vitro* studies of the possible toxicological effects of nano-particles should be undertaken before *in vivo* studies. We have listed a large number of *in* vitro studies that could usefully be applied to nanoparticles. Those appropriate in a given instance will need to be considered on a case by case basis. We note that current concerns about the use of animals in research are making *in vivo* work more difficult, but recognise that in only a few areas have *in vitro* studies been validated for regulatory purposes. *In vitro* studies are likely to provide initial data on comparative toxicity of different sized materials, with the findings having to be followed up by *in vivo* studies in animals.

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## 8. List of Abbreviations

| | |
|---|---|
| **AP-1** | Activator protein-1 |
| **BBB** | Blood-brain barrier |
| **BSA** | Bovine serum albumin |
| **Caco-2** | Colon adenocarcinoma cell line |
| **CHO** | Chinese hamster ovary |
| **COC** | Committee on the Carcinogenicity of Chemicals in Food, Consumer Products and the Environment |
| **COM** | Committee on the Mutagenicity of Chemicals in Food, Consumer Products and the Environment |
| **COT** | Committee on the Toxicity of Chemicals in Food, Consumer Products and the Environment |
| **DCFH** | 2'7'-Dichlorofluorescein |
| **DCFH-DA** | Non-fluorescent form of DCFH |
| **DHR** | Dihydroryhodamine 123 |
| **DMSO** | Dimethyl sulphoxide |
| **DNA** | Deoxyribonucleic acid |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **ESR** | Electron spin resonance |
| **GSH** | Reduced glutathione |
| **GSSG** | Oxidised form of reduced glutathione (GSH) |
| **H2DCF** | Dichlorodihyrdofluorescein |
| **HaCaT** | Human keratinocyte cell line |
| **16HBE14** | Human bronchial epithelial cell line |
| **HEK** | Human epidermal keratinocytes |
| **HPLC** | High performance liquid chromatography |
| **HUVEC** | Human umbilical vein endothelial cells |
| **LDH** | Lactate dehydrogenase |
| **IL-1** | Interleukin-1 |
| **IL-8** | Interleukin-8 |
| **ILSI** | International Life Sciences Institute |
| **MCP-1** | Monocyte chemoattractant protein-1 |
| **MMP** | Mitochondrial membrane potential |
| **MPO** | Myeloperoxidase |
| **NFκB** | Nuclear factor kappa B |
| **NPs** | Nanoparticles |
| **OECD** | Organisation for Economic Co-operation and Development |
| **8-OHdG** | 8-Hydroxydeoxyguanosine a DNA adduct |
| **2D-PAGE** | Two-dimensional polyacrylamide gel electrophoresis |
| **PB** | Placental barrier |
| **PI** | Propidium iodide |
| **PEG** | Polyethylene glycol |
| **RES** | Reticuloendothelial system |
| **RIA** | Radioimmunoassays |
| **ROS** | Reactive oxygen species |
| **SCENIHR** | Scientific Committee on Emerging and Newly Identified Health Risks |

| | |
|---|---|
| **SPR** | Surface Plasmon resonance |
| **TBA** | Thiobarbituric acid |
| **$TiO_2$** | Titanium dioxide |
| **TMRM** | Tetramethylrhodamine methyl ester |
| **TNF-α** | Tumour necrosis factor-α |
| **US EPA** | United States Environmental Protection Agency |