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Approaches to Develop Alternative Testing Strategies to Inform Human Health Risk Assessment of Nanomaterials

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Abstract

The development of Alternative Testing Strategies (ATS) for hazard assessment of new and emerging materials is high on the agenda of scientists, funders and regulators. The relatively large number of nanomaterials on the market and under development means that an increasing emphasis will be placed on the use of reliable, predictive ATS when assessing their safety. We have provided recommendations as to how ATS development for assessment of nanomaterial hazard may be accelerated. Pre-defined search terms were used to identify the quantity and distribution of peer-reviewed publications for nanomaterial hazard assessment following inhalation, ingestion or dermal absorption. A summary of knowledge gaps relating to nanomaterial hazard is provided to identify future research priorities and areas in which a rich data set might exist to allow ATS identification. Consultation with stakeholders (e.g. academia, industry, regulators) was critical to ensure that current expert opinion was reflected. The gap analysis revealed an abundance of studies which assessed the local and systemic impacts of inhaled particles. Development of ATS for assessment of the dermal toxicity of chemicals is already relatively advanced, and these models should be applied to nanomaterials as relatively few studies have assessed the dermal toxicity of nanomaterials, to date. Limited studies have investigated the local and systemic impacts of ingested nanomaterials. If the recommendations for research prioritisation proposed are adopted it is envisioned that a comprehensive battery of ATS can be developed to support the risk assessment process for nanomaterials. Some alternative models are available for immediate implementation, whilst others require more developmental work to become widely adopted. Case studies are included which can be used to inform the selection of alternative models and endpoints when assessing the pathogenicity of fibres and mode of action of nanomaterial toxicity.

Keywords: nanomaterials, hazard, risk, alternative testing strategy, 3Rs, gap analysis

1.0 INTRODUCTION

The development of Alternative Testing Strategies (ATS) for hazard assessment of new and emerging materials has been high on the agenda of many scientists, funders and regulators for a number of years, largely due to ethical concerns about using animals for toxicity testing. Growing acceptance and implementation of the 3Rs principles (replacement, reduction, and refinement of animal use) within the field of toxicology has led to regulators becoming increasingly receptive to the use of ATS to inform the risk assessment process. For nanotoxicology, the need to develop ATS has become increasingly urgent due to the large number of nanomaterials on the market and under development for a wide range of applications. Such diversity of use leads to the potential for human and environmental exposure via a variety of routes, for which the associated risks need to be understood in order to allow nanotechnology to develop in a safe, responsible and sustainable manner.

To date, assessment of nanomaterial hazard has encompassed evaluation of their local and systemic toxicity using a combination of *in vivo* and *in vitro* models. Over recent years the application of *in silico* models (e.g. quantitative structure activity relationships (QSAR)) to predict nanomaterial hazard has also been explored, however such tools are in the very early stages of development [1, 2]. A variety of different nanomaterials of varied physicochemical properties have been tested using traditional *in vivo* and *in vitro* methods; with silver (Ag), metal oxides (e.g. titanium dioxide (TiO_2)), and carbon nanotubes (CNTs) amongst the most extensively investigated due to their high levels of use and production, and concerns regarding their safety. This has allowed relationships between nanomaterial physicochemical properties and toxicity to be explored. However, the ability to assess the potential risks of this diverse array of nanomaterials using conventional hazard tests is limited by time, budget and ethical considerations regarding the use of animal testing. This is compounded further by the desire to understand how the hazard/risk of each nanomaterial is modified along the value chain (from design to successful use in products) and throughout the life cycle (from pristine nanomaterial to disposed or fragmented/aged product). Thus, increased use of reliable, predictive ATS for the hazard assessment of nanomaterials is essential. Furthermore, where possible the development of

(automated) high throughput (HTP) systems is desirable to enable the testing of a range of nanomaterials and concentrations in fewer experiments.

There are many types of ATS, including simple *in vitro* systems with single cell types, *in vitro* co-cultures, complex 3D *in vitro* models which mimic tissue architecture (e.g. EpiDerm (skin), EpiAirway (lung)), cell-free assays (e.g. enzyme assays), prokaryotic bacteria (e.g. Ames Test), invertebrates (e.g. Drosophila), vertebrates (e.g. zebrafish) and those that incorporate *in silico* modeling. For each model there are also a range of endpoints that can be assessed. Endpoints that have been widely reported to be associated with nanomaterial hazard can be investigated using ATS to screen toxicity but to also obtain a better understanding of the mode of action of nanomaterial toxicity, for example, inflammation, and oxidative stress, and the downstream consequences of these processes (e.g. genotoxicity). In addition model specific endpoints such as Drosophilia reproduction, zebrafish reproduction and development and systems biology approaches (e.g. genomics and proteomics) can be used to further explore the hazard potential of nanomaterials. Using ATS it is often possible to evaluate multiple parameters of interest in one experiment, maximising the amount of information that can be obtained from one study. If used alone each test system may have limited value, however, the breadth of ATS available provides the opportunity to assess several endpoints simultaneously and use batteries of tests (multiple models approach) which together can provide a rich diversity of information. However, assembly of this battery of models and interpretation of the data generated by the different ATS requires an understanding of the limitations of each model, their validation by comparison to animal or human study results (e.g. via *in vitro in vivo* extrapolation; IVIVE), and the use of relevant standards to allow comparability between data sets [3].

There is an expectation that development of ATS along these lines will substantially increase the quantity of hazard information on nanomaterials and their modes of action leading to (1) more effective and rapid screening of nanomaterials; (2) the development of *in silico* quantitative structure-activity relationship (QSAR) models facilitating a 'safer by design' approach to reducing risk to human health; (3) advancement of Adverse Outcome Pathway models and (4) development of a tiered testing strategy for assessment of hazard with a significant reduction, though perhaps not complete elimination in the short term of *in vivo* exposure

and hazard testing to decrease reliance on animal models when demonstrating safety for regulatory purposes.

The following manuscript outlines recommendations for model and endpoint selection and development, and research prioritisation to promote ATS development, which if implemented could focus and speed up efforts to enhance the use of ATS in the risk assessment of nanomaterials. Importantly, the approach described is also likely to be more widely applicable to a diverse range of materials and chemicals.

2.0 RECOMMENDATIONS FOR PRIORITISING ATS DEVELOPMENT

The breadth of ATS available means that not all models can be developed simultaneously and to the same extent. Furthermore, not all ATS will be equally applicable or useful for nanomaterial hazard identification, therefore a need exists to prioritise ATS development. There are several ways in which prioritisation could be considered, which were discussed in the context of human health and environmental risk assessment by the expert Society for Risk Analysis (SRA) workshop held in September 2014 in Washington DC in cooperation with the OECD Working Party on Manufactured Nanomaterials (see Shatkin et al, this volume). For example, a systematic analysis of the current published literature allows identification of gaps in knowledge as well as areas in which there is a wealth of knowledge to draw upon. The literature search should not be restricted to nanomaterials, but include other chemicals to identify what lessons can be learnt from the development of existing ATS, and then applied to nanomaterial hazard assessment. It could be argued that it is a priority to fill the data gaps with regards to nanomaterial hazard, but development and validation of an ATS to represent a hazard assessment for which the hazard is not well understood and is difficult. In contrast, areas which are more data rich provide an opportunity to make decisions for ATS prioritisation on a strong evidence base. The development of ATS is more advanced in some areas than others. For example, due to EU regulatory bans on the use of animal testing for cosmetic safety assessment, ATS model development for investigation of the dermal toxicity of substances is relatively well advanced. In addition, zebrafish are a well established, valuable model when investigating the developmental and reproductive toxicity of substances [4]. ATS for nanomaterials should encourage wider application of such models for the assessment of hazard in relation to human (and environmental) health.

The following strategy to support the use of ATS in the hazard assessment of nanomaterials was discussed:

Step 1. Identify sources of information for the nanomaterial of interest across a variety of models (*in vivo, in vitro, in silico*) via a literature search. Material such as published literature, OECD dataset (including 14 nanomaterials tested via a wide array of methods and laboratories), industrial datasets (e.g. REACH dossiers, EPA submissions) *etc* should be used in order to identify where there is sufficient quantity of data, consistency in study design and measured outcomes and therefore confidence in the hazard information available for a particular type of nanomaterial. In other words, can we identify the low hanging fruit that are ripe for development into the ATS?

Step 2. Divide the data and information available into *in vivo* and *in vitro* data and within the *in vitro* data, list the *in vitro* tests used to assess different endpoints within an ATS, and identify which models reflect or predict the *in vivo* response. This approach will allow identification of a short list of endpoints within an ATS that are worthy of further development. It will also be essential to identify scenarios where there is no correlation between *in vitro* and *in vivo* studies i.e. where ATS may not be appropriate.

Prediction of an *in vivo* result by an *in vitro* assay means one or both of the following: (1) that the results of an *in vitro* assay for several different nanomaterial variants matches the relative order of toxic responses observed for those same nanomaterial variants in an *in vivo* model endpoint; and/or (2) that the intervals of the toxic response observed for those nanomaterials matches the intervals of the toxic response observed in an *in vivo* model. Searches should account for the fact that certain individual *in vitro* assays may not have predictive power on their own, but may serve to improve predictions of *in vivo* responses in combination with results across multiple *in vitro* assays. It should also be noted that *in vitro* assays have the additional benefit that they are useful to probe mechanisms of toxicity that can help to explain *in vivo* observations. This means that it might be useful to broaden this approach to identify additional *in vitro* endpoints that add further detail to understanding the effects of nanomaterial treatments.

Step 3. An assessment of data quality needs to be incorporated into the literature/information sources used to provide the information on which the ATS shortlist is based. This needs to include certainty around

materials tested (i.e. adequate physicochemical characterisation of nanomaterial properties) and certainty around the protocol utilised including consistency with established recommendations or norms and in the data obtained. Reference to relevant human data should be considered where available.

Step 4. Interrogate the usefulness of the short-listed ATS further by assessment of their suitability for a range of nanomaterials. For example some nanomaterials may interfere in particular assays [5] to provide false positive or negative results. This interference may arise due to the surface properties of nanomaterials including catalytic activities [6] and also adsorptive properties [7] [8] such as indicator dyes [9] . They may therefore be used in the separation of selected analytes from complex matrices [10], to transport macromolecules and also for *in vivo* imaging [11] [12]. Nanomaterials may also have optical properties [13] as well as plasmon resonance effects where they absorb light or quench fluorescence in the visible region [14]. It is therefore of no surprise that such surface properties may be operational when chromophores, fluorophores and luminophores are utilised in assay systems to assess a great number of cellular functional disturbances that may be arising from exposure to nanomaterials. As such, discrepancies may therefore arise between these assays depending on the extent of the effect of these surface properties on the chromophores, fluorophores or the luminophores used. Nanomaterials themselves may therefore interfere with these assay systems [15] [16], thus producing inaccurate results.

In addition a critical analysis of the potential to develop each ATS into either a High Throughput Screening (HTS) or a High Content Screening (HCS) approach is needed. When testing the developing ATS, relevant concentration ranges for each nanomaterial need to be identified, based on real-life exposure information, when available. Within the group of ATS taken forward for this interrogation, there will need to be a selection of tools to evaluate the biokinetics of nanomaterials in order to identify potential for uptake, targets for accumulation, toxicity and also clearance due to their biopersistence.

Within the design of each ATS, consideration will need to reflect the likely exposure scenarios (e.g. concentration, route) for each nanomaterial. For example, nanomaterials relevant for exposure via inhalation could be pre-coated in lung lining fluid before introduction into the *in vitro* system to better replicate real-life exposures and mimic the transit of nanomaterials in the body [17].

The development process also needs to include consideration of suitable controls (positive, negative and vehicle), plus controls that are relevant to certain types of nanomaterials (e.g. fibres for high aspect ratio nanomaterials, relevant metal salt solutions for nanomaterials that are prone to dissolution). The preparation of nanomaterials for hazard testing also needs to be carefully considered; there are currently a number of different approaches that can be used to disperse nanomaterials (e.g. sonication (probe or bath), stirring, and inclusion of solvents, proteins or lipids in dispersion medium), and no consensus as to which approach is most appropriate and relevant to real-life exposure conditions.

Once this process is complete a short list of prioritised ATS can then be tested with nanomaterials for which human and animal hazard information is available, in order to test the limitations of each ATS in relation to their power of predictability of the hazard of the tested nanomaterial. The battery of ATS can then be compiled and interrogated further in order to standardise the protocol. At this point, for ATS which overlap in terms of the information they can provide that is suitable for risk assessment, a decision can be made over whether the list could be streamlined further. However, it is often useful to have more than one way to measure the same effect, especially due to the potential for nanomaterials to interfere in some assay types implemented within a particular ATS.

It is unlikely that this approach will identify ATS that are relevant for all routes of exposure, target locations and toxicological endpoints relevant to risk assessment. However, once this battery of ATS is developed this will then allow gaps to be identified in order to focus future ATS development. It is also likely that nanomaterials will be transformed along their life cycle (from pristine nanomaterial to disposed or fragmented/aged/transformed product) and that this should be taken into consideration when performing hazard assessments using ATS.

3.0 THE CURRENT STATUS OF NANOTOXICOLOGY – IDENTIFYING THE LOW HANGING FRUIT

In 2012, the European Project ITS-NANO (http://www.nano.hw.ac.uk/research-projects/itsnano.html) generated a gap analysis of the human hazard literature relating to nanotoxicology. The gap analysis included a systematic search of PubMed (up to December 2011) with pre-defined search terms in order to identify the

quantity and distribution of peer-reviewed publications that are relevant for the hazard assessment of nanomaterials. These results were converted into heat map tables. A heat map is a graphical representation of information where each value within a matrix is represented by a colour. In this instance three colours were used, each representing a range of data within defined limits. The heat maps were initially used to generate a gap analysis of research priorities to be addressed, but for the purposes of the SRA workshop, they provided an opportunity to identify areas in which a rich data set might exist to allow ATS identification for development prioritisation.

This list of identified research priorities was then shared with a wide range of stakeholders (e.g. academia, industry, regulators, NGOs, policy makers) to ensure that they reflected current expert opinion. This was essential as the simple systematic literature search did not include a check of the research quality, only the content, and could not take into account funded research that was recently completed (but not yet published) and research that was still in progress. The gap analysis was then used to generate research prioritisation recommendations, through consultation with these stakeholders, leading to the development of an intelligent testing strategy (ITS) for nanomaterials [18]. The full project report (http://nano.hw.ac.uk/research-projects/itsnano.html) and the publication in Particle and Fibre Toxicology [18] are provided for information. Chapter 3 of the full ITS-NANO report provides the gaps relevant to hazard assessment and suggests strategies to address each gap.

Prior to the SRA workshop, the ITS-NANO gap analysis tables were updated (May 2014) in order to identify the current status of research across different nanotoxicology relevant disciplines, and to analyse areas in which research activity has been most productive over the last 2 years. The workshop in Washington also provided an opportunity to liaise with stakeholders to update this list of research priorities and to provide an expert assessment of the relevance of these priorities, as well as a discussion of how these research gaps might be addressed.

The heat maps were generated from the number of peer reviewed publications identified using the NCBI publications database, PubMed. In order to tailor the searches, a range of specific keywords were identified which represent each area of nanotoxicology interest in relation to hazard assessment for human health, and

was focused on three main routes of exposure; respiratory, ingestion and dermal (Table I). In addition, the searches considered the mechanism of nanomaterial toxicity, the biodistribution of nanomaterials and effects induced at secondary target sites (systemic toxicity), the longevity of the response (acute vs chronic) and the physicochemical properties of nanomaterial that confer toxicity. Both *in vivo* and *in vitro* studies were identified. The publication databases were interrogated using combinations of keywords to form detailed and highly specific search terms. The list of publications identified during each search was examined to identify any duplications, irrelevant publications, or missing relevant publications. During the update (May 2014), the publications were also checked to ensure they had not been included in the previous search conducted during the ITS-NANO project (2012-2014), e.g. if previously detected due to 'early online publication'. The number of publications identified per search was then used to construct updated 'heat maps'.

[TABLE I]

3.1 FINDINGS FROM THE GAP ANALYSIS

Studies focusing on assessment of nanomaterial hazard following the pulmonary route of exposure are most abundant (Tables II and III), when compared to either ingestion (Tables IV and V) or the dermal exposure route (Tables VI and VII). A subset of studies investigated biokinetics following pulmonary exposure, which are also relatively abundant compared to ingestion and dermal exposure studies.

3.1.1 Pulmonary Studies

Pulmonary studies identified during the gap analysis include both local effects in the lung (Table II) as well as systemic effects (Table III), but with relatively more concentrating on systemic effects. In terms of understanding the mode of action of nanomaterial toxicity, studies relating to inflammation dominate the pulmonary studies that identify a local effect in the lungs or in lung cells. In comparison to inflammation, oxidative stress and cytotoxicity studies, investigations of fibrosis, genotoxicity and carcinogenicity are relatively scarce. Note that these data are a combination of both *in vivo* animal models as well as *in vitro* cell culture models. In fact, a range of models were used; for *in vivo* testing rodent (rats and mice) studies are

the most prevalent with administration via intratracheal instillation was most common, with fewer studies administering nanomaterials via inhalation. Such studies typically investigate inflammatory and oxidative stress driven pulmonary responses (e.g. via assessment of inflammatory cell infiltration, cytokine production, antioxidant depletion and histopathological examination). The majority of studies assessed acute effects, following a single administration of nanomaterials. For *in vitro* studies, a plethora of models have been used including; macrophage cell lines (e.g. J774, THP-1), bronchial or alveolar cell lines (e.g. A549, BEAS-2B), primary monocytes/macrophages (derived from the rat lung, or human blood), and co-culture systems (e.g. 3D cell cultures encompassing epithelial cells, macrophages and dendritic cells). Endpoints relating to cytotoxicity, cytokine production, cellular uptake of nanomaterials, oxidative stress (encompassing ROS production or antioxidant depletion) and genotoxicity were most prevalent in *in vitro* studies.

[TABLES II and III]

Similar to the observations for local effects, pulmonary studies considering the modes of action for systemic or distal effects are dominated by inflammation followed by oxidative stress with the biodistribution of nanomaterials following pulmonary exposure rather extensively investigated. In comparison, longer term effects such as fibrosis and carcinogenicity are less abundant for the systemic effects following pulmonary exposure.

[TABLES IV and V]

3.1.2 Ingestion Studies

To date there are very few studies published that have focused on ingestion as a route of exposure for either local or distal impacts of nanomaterials (Tables IV and V). As a consequence there are few studies that identify the mode of action, biokinetics or the relationship between biological impact and nanomaterial physicochemical characteristics following ingestion. *In vitro* models were most prevalent and the models most commonly used to assess the implications of nanomaterial ingestion on the intestinal epithelium included Caco-2 single cell cultures (using undifferentiated or differentiated cells), with more limited studies using co-cultures (e.g. intestinal epithelial and immune cells; M cell model, mucus model) which more closely

mimics the gastrointestinal tract (GIT) epithelium *in vivo*. Studies typically assessed cellular responses related to inflammation, oxidative stress and genotoxicity.

3.1.3 Dermal Studies

For dermal studies, again there are relatively few published studies. Of those that have been published, the majority have looked at the relationship between particle size and either local cytotoxicity, inflammation or oxidative stress. The penetration of limited types of nanomaterials (such as quantum dots) through skin and potential for particle translocation has also been considered using *in vitro* models. Several models have been used including simple *in vitro* cell line mono-cultures (e.g. keratinocytes, fibroblasts), more sophisticated *in vitro* models which replicate skin tissue cell architecture (e.g. EpiDerm), and *ex vivo* tissue models (pig and human). Far fewer *in vivo* studies have been carried out, the majority of which tend to focus on rodent models.

[TABLES VI and VII]

A systematic analysis of the peer reviewed literature, as conducted here, provides an overview of the major areas of research activity. Conversely, this provides an opportunity to identify areas where information is lacking and hence establish research needs for the future. This information will be useful to help researchers and funders prioritise hazard studies in the future. Furthermore, it enables identification of commonly used ATS models (that have been tested for a range of nanomaterials), and where possible, comparison to *in vivo* findings.

4.0 CASE STUDIES

Selection of relevant and sensitive alternative models and endpoints to assess nanomaterial hazard is essential for appropriate interpretation of data. Existing particle and nanotoxicology research can be used to justify and guide model and endpoint selection and to identify the limitations of alternative models.

4.1 Case Study 1: High Aspect Ratio Nanomaterials (HARN) & the fibre pathogenicity paradigm

Identifying the relationship between nanomaterial physicochemical properties and their toxicity is a critical component of hazard studies. This information can be used for a variety of purposes, for example it is required to support the design of appropriate legislation to manage the risks posed by nanomaterials and to encourage 'safety by design' when developing new generations of nanomaterials. High aspect ratio nanomaterials (such as carbon nanotubes (CNTs)) have a structural resemblance to asbestos fibres, causing concern that these materials may induce similar adverse health outcomes to asbestos in the lung (e.g. mesothelioma) following inhalation. Of benefit is that a robust structure activity relationship 'the fibre pathogenicity paradigm' exists for asbestos fibres. More specifically, it is established that long (>5µm), thin (<100nm), biopersistent fibres are likely to be more pathogenic than shorter (<5µm), more agglomerated/entangled fibres (e.g. [19, 20]). This derives from the ability of long, thin biopersistent asbestos fibres to be deposited in the alveolar region of the lung following inhalation, where they interact with alveolar macrophages and mesothelial cells. Long fibres (>5µm) cannot be effectively ingested by macrophages and induce frustrated phagocytosis, stimulating a persistent inflammatory and oxidative response leading to tumour development (for reviews please refer to [19, 21]. The applicability of this paradigm to HARN (e.g. CNTs, silver or nickel nanowires) has been tested using *in vivo* and *in vitro* models. *In vivo* studies have typically exposed mice to CNTs, silver nanowires or nickel nanowires via intraperitoneal [22], pharyngeal aspiration [23, 24] or intrapleural injection [25-27] and assessed inflammation (e.g. differential cell counts, cytokine production, histopathological analysis, granuloma formation), oxidative stress and macrophage uptake of fibres (using microscopy) as indicators of toxicity. Such studies have provided data to support the applicability of the fibre pathogenicity paradigm to HARN. *In vitro* experiments using macrophages (primary, human and animal derived cells or cell lines) further support that fibre length is critical to HARN toxicity and that cytotoxicity, pro-inflammatory cytokine production, cellular uptake and reactive oxygen species production are appropriate, and sensitive markers of the biological response [23, 28, 29].

Thus, based on existing knowledge of fibre toxicity it is possible to recommend the design of a tiered testing strategy which promotes the use of ATS for assessment of HARN hazard. In the first instance, assessment of the physicochemical properties of HARN should be prioritised within the hazard assessment, which can be achieved via the use of microscopy (e.g. scanning electron microscopy (SEM), transmission electron

microscopy (TEM), atomic force microscopy (AFM), light microscopy) to measure fibre length and diameter. If HARN morphology is long (> 5µm) and straight then this indicates that these fibres may be pathogenic. It is also established that iron or nickel contamination can contribute to CNT toxicity [30] and so the purity of samples should also be confirmed via elemental analysis when characterising HARN physicochemical properties. Thus, characterisation of nanomaterial physicochemical properties is a key, early component of hazard studies for fibre-like nanomaterials and can be used to predict their pathogenicity. *In vitro* studies should then be used to assess the response of macrophages to HARN. Macrophage/monocyte cell lines (e.g. J774, RAW 264.7, THP-1) can be used, followed by limited studies using primary human macrophage cells to validate the response exhibited by cell lines, if deemed appropriate (and subject to ethical approval). Endpoints assessed should include i) cellular uptake of HARN by macrophages using microscopy (e.g. light microscopy, TEM, SEM), ii) production of pro-inflammatory cytokines e.g. tumour necrosis factor alpha (TNFα), interleukin (IL)-1β and iii) respiratory burst (production of reactive oxygen species (ROS)). It is advised that 'control' asbestos fibres (e.g. long fibre amosite) are included experiments as a positive control. In order to confirm the findings from *in vitro* experiments it may be appropriate (e.g. to fulfil regulatory requirements) to perform limited, focused *in vivo* (rodent) studies in order to assess fibrosis and mesothelial cell response.

4.2 Case study 2: Mechanism of action of nanomaterial toxicity

A better understanding of the mode of action of nanomaterial toxicity will help support the development of a battery of sensitive, evidence based tests that can be used to screen nanomaterial toxicity using alternative models. Existing information on the mechanism of toxicity of particles and nanomaterials can be used to inform the selection of ATS when testing the toxicity of a nanomaterial of unknown hazard. Prior to the emergence of nanotechnology and hence nanosafety research, most particle toxicology was related to respiratory exposures, including occupational dusts (e.g. asbestos [31], silica [32] and coalmine dust [33]) and air pollution particles (PM_{10}, e.g. [34]). The study of occupational and environmental particles identified oxidative stress and inflammation as key processes in controlling the pathological responses to these particles in humans. For example, both asbestos and PM_{10} have been shown to induce glutathione depletion and ROS production [35, 36] which is indicative of oxidative stress, stimulate activation of the oxidative stress responsive transcription factor nuclear factor kappa B [37, 38], activate Ca^{2+} signalling, and increase

expression and production of pro-inflammatory cytokines such as interleukin-8 and TNFα [39, 40]. The discipline of particle toxicology is well established, and with due regard for the physicochemical and toxicological differences that nano-sized materials have compared to their bulk counterparts, provides an additional source of information on which to base ATS identification and prioritisation. We suggest that this historical information on particle toxicology can be exploited to design hazard studies for nanomaterials, using *in vitro* and *in vivo* models.

As described above, regardless of the target site under investigation in *in vitro* and *in vivo* hazard studies for nanomaterials, assessment of pro-inflammatory and oxidant responses are common. We suggest that hazard studies continue to assess such responses, but also expand the endpoints investigated to enable a better understanding of the cellular and molecular events underlying nanomaterial toxicity. In the first instance, *in vitro* experiments should be conducted, using cell lines that are appropriate to the target site of interest, to probe the cell and molecular events underlying nanomaterial toxicity. Such studies should assess cytotoxicity (e.g. alamar blue, WST-1, neutral red, lactate dehydrogenase (LDH) assays) over a range of nanomaterial concentrations to identify sub-lethal concentrations of nanomaterials to test using more in depth mechanistic studies. When investigating the mechanism of toxicity we suggest that the information obtained from each individual study is maximised. For example, the cells themselves can be used to assess a number of different responses (e.g. cytotoxicity, cellular uptake of nanomaterials, oxidative stress (ROS production, antioxidant depletion), activation of transcription factors, changes in protein levels/activity, changes in gene expression, intracellular Ca^{2+} concentration), whilst the cell supernatant (from the same experiment) can be used to measure indicators of sub-lethal and lethal effects (e.g. cytokine production, release of lactate dehydrogenase respectively). Where possible, highthroughput systems should be used, ideally using automated approaches. Genomics and proteomics analysis may also provide guidance as to which endpoints to consider when screening nanomaterial toxicity. Focused studies using primary cells may be conducted to validate the findings obtained from cell lines. *In vitro* studies will inform the design (e.g. selection of endpoints) of focused *in vivo* (rodent) studies, if deemed appropriate.

5.0 DISCUSSION

The main observations from our gap analysis are that studies investigating the consequences of pulmonary exposure dominate the literature in comparison to ingestion and dermal routes of exposure. For these studies, inflammation and oxidative stress account for the majority of studies investigating the mode of action. These publications include those using inflammation (e.g. neutrophil infiltration, cytokine production) as a biomarker of hazard rather than an investigation of the mode of action. The pulmonary studies include a relatively large number of biokinetic studies as well as the investigation of both local and systemic effects. This suggests that researchers in the field recognise a need to investigate particle translocation and its consequences. This information can also be used to better design *in vitro* studies, whereby knowledge of nanomaterial biodistribution could be used to select relevant target sites to investigate and can be used to inform the preparation of nanomaterials for *in vitro* hazard studies. More specifically studies could suspend nanomaterials in lung lining fluid (LLF) then serum to mimic the transit of nanomaterials from the lung to other sites in the body via the blood (e.g. liver). The literature relevant to pulmonary exposure, including cytotoxicity, inflammation and oxidative stress therefore provides a suitable starting point for identification of suitable ATS to be developed and validated.

In addition, a better understanding of the mode of action of nanomaterial toxicity will lead to the identification of evidence based approaches to screen nanomaterial toxicity in ATS, and also inform the potential consequences of human exposure to nanomaterials. This suggests a need for future research activities to investigate the mode of action, and if possible link to the physicochemical characteristics of nanomaterials. As discussed above, this information can be used to feedback into, and inform and prioritise ATS and endpoint selection for nanomaterial hazard assessment.

Inflammation, oxidative stress and cytotoxicity studies in general relate to acute pulmonary responses in the lung and lung cells, whereas in comparison, the studies requiring longer term, and/or repeated pulmonary exposures (fibrosis, genotoxicity and carcinogenicity) are relatively scarce. This is likely to be driven by the financial, ethical and practical difficulties of conducting longer term studies. Short term inhalation studies (5 day exposures followed by a 28 day investigation) [41-43] have been developed as an alternative to longer

term (e.g. 90 day) studies, in order to minimise the suffering to animals and reduce the cost. However, there is clearly a need to conduct more long term inhalation/pulmonary studies for nanomaterial hazard assessment. To facilitate their utility, ATS also need to be adaptable and facilitate the testing of toxicity of nanomaterials in the long term or following repeated exposures (e.g. [44]).

Research of the consequences of nanomaterial ingestion or dermal exposure has lagged behind those of pulmonary studies. Studies on the impact of nanomaterials on the skin do not generally investigate toxicity/hazard specifically, the majority are investigating the potential for nanomaterial penetration, rather than hazard. This leads to a larger number of publications investigating biokinetics and the potential for uptake into dermal cells and translocation of topically applied nanomaterials to other organs of the body. However, many of the dermal studies investigating penetration have often identified the skin as an effective barrier to nanomaterials (e.g. [45]), possibly reducing the urgency of funders and scientists to investigate this route of exposure. However, skin is exposed purposefully (e.g. sunscreens and cosmetics) and accidentally (e.g. occupational exposure) to nanomaterials, and not all skin types are equally effective, with integrity being affected by disease status, sunburn and age. More research would therefore be useful with compromised skin models [46] and repeat dosing to simulate sunscreen and nanomedicine use. Investigation of the skin irritancy and sensitisation potential of nanomaterials has also been neglected. The OECD have offered guidance on the strategy to assess skin sensitisation and irritation using ATS which should be followed, although the applicability of such test guidelines to nanomaterials requires investigation.

Relatively few studies have evaluated the impact of nanomaterials on the GIT. As observed for the lung and skin, existing studies have focused primarily on assessment of cytotoxicity, inflammatory and oxidative endpoints using *in vitro* models (intestinal epithelial cells). There is some evidence that some nanomaterials are able to cross the intestinal barrier and accumulate in secondary target sites [47], however the applicability of these findings to more diverse forms of nanomaterials needs to be assessed. *In vitro* models have been used primarily to assess the consequences of nanomaterial ingestion for the GIT. However the physiological relevance of the models used needs to be considered and the complex cell architecture, and the low oxygen content of the GIT should be better represented in *in vitro* hazard studies in the future. However, the use of

such complex models is often more time consuming and expensive than more simplistic single cell cultures. Furthermore, the need to disperse nanomaterials in physiological media that considers the transit of nanomaterials through the GIT within *in vitro* studies should be evaluated, to improve physiological relevance. For example acidic conditions of the stomach can modify the physicochemical properties of nanomaterials and therefore their behaviour in the GIT. There has been a lack of *in vivo* studies which have assessed the implications of nanomaterial ingestion for the GIT. However, further research is required in this area as recent studies have observed a disturbance in the diversity and abundance of GIT microflora following nanomaterials ingestion which may lead to adverse health outcomes.

Existing statistical modelling studies connecting nanomaterial characteristics to toxic outcomes, which the development of ATS should accelerate, have either focused exclusively on the results of *in vitro* cytotoxicity studies (e.g. [48-50]) or *in vivo* pulmonary inflammation studies [51]. The success of these analyses suggests that the approach advocated here for the development of ATS is feasible, however in the short-term, the utility to nanomaterial risk assessment of an approach that investigates only on one portion of the *in vitro* – *in vivo* divide is limited. The recommendations outlined here should enable a connection to be developed between these two model approaches, while pointing to the more informative nanotoxicology assays in both realms, and clarifying which nanomaterial characterisation measures are essential.

6.0 CONCLUSION

Finally, the SRA workshop participants generated recommendations to prioritise ATS development for assessment of nanomaterial hazard. In the short term the ATS developed will reflect models and endpoints for which the field of nanotoxicology is already relatively confident (e.g. selection of models and endpoints to assess pulmonary toxicity), and so it will simply move these frequently used and tested methods towards standardised (validated) protocols to be used more directly for risk assessment. However, it is not possible to generate a battery of ATS that can cover all forms of nanomaterials, potential routes of exposure, targets for toxicity or types of toxicological consequence. As demonstrated in the case studies, some ATS are suitable for immediate use within the hazard assessment of nanomaterials, whereas other models require further development to identify whether they are suitable for more widespread use. Therefore, further ATS

development, along with *in vivo* testing and/or human epidemiology will be required to generate a comprehensive battery of ATS able to fully support the risk assessment process.

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TABLES

Table I. Gap analysis: keys words, used in combination to form detailed search-terms used for PubMed database searches.

Nano-relevance (for all searches)	(ultrafine OR nanoparticle OR nanoparticulate OR nanomaterial OR nanotube OR nanotubule OR nanofiber OR nanofibre OR nanowire OR nanowhisker OR nanorod)
Exposure route	
Respiratory	(inhalation OR instillation OR aspiration OR intraperitoneal OR air liquid interface)
Gastrointestinal	Ingestion
Dermal	(dermal OR skin)
Physicochemical properties	
Size	Size
Surface area (SA)	surface area
Charge	surface charge
Aspect ratio	aspect ratio
Solubility	Solubility
Crystallinity	(crystallinity OR crystallinity OR anatase OR rutile)
Composition	Composition
Biological impacts	
Cytotoxicity	(cytotox* OR necrosis OR apoptosis OR cell death OR tryptan blue Or lactate dehydrogenase OR LDH OR MTT OR Sulforhodamine OR WST)
Inflammation	(inflam* OR macrophage OR cytokine OR neutrophil OR PMN OR lymphocyte OR leukocyte OR chemokine OR granuloma OR immune OR phagocytosis)

Oxidative stress	(oxidative stress OR oxidant OR reactive oxygen species OR ROS OR active oxygen species OR radical OR anti oxidant OR glutathione OR lipid peroxidation OR 8-oxoDG OR OHdG)
Fibrosis	(fibro* OR ECM OR TGF beta OR Collagen OR hydroxyproline)
Genotoxicity	(genotox* OR mutation OR comet assay OR micronucleus OR OHDG OR chromosome OR genetic toxicity)
Carcinogenic	(carcinogenic OR carcinogen OR tumour OR mutagenic OR mutagenicity OR carcinogenicity)
Biokinetics	(biokinetics OR toxicokinetics OR biodistribution OR translocation OR uptake OR internalisation OR endocytosis)
Systemic targets	
Lung	(lung OR inhalation OR instillation OR pulmonary OR aerosol OR aspiration OR intraperitoneal)
Liver	(liver OR hepat* OR reticuloendothelial OR kupffer OR bile OR cholestasis)
Spleen/immune	(spleen OR reticuloendothelial OR immune)
Central nervous system (CNS)	(nervous system OR brain OR nerve OR neural OR blood brain barrier OR olfactory OR neuro)
Gastrointestinal (GI) Tract	(gut OR ingestion OR stomach OR intestin* OR peyers OR bowel OR food)
Kidney	(kidney OR renal OR filtration OR glomerulous)
Cardiovascular (CV)	(heart OR endothelium OR blood OR platelets OR clotting OR thrombosis OR nitric oxide OR atherosclerosis OR plaque OR stroke OR infarction)
Reproduction/ development	(sperm OR oocyte OR fetus OR foetus OR reproductive OR developmental OR fertility)
Pleura (retention)	(pleura* OR mesothelium OR stomata)

The following tables identify the total number of publications as of May 2014.

Table II. Publications related to pulmonary exposure, physicochemical properties and biological impact:

Total published as of May 2014

	Biological impact					
PC properties	Cytotoxicity	Inflammation	Oxidative stress	Fibrosis	Genotoxicity	Carcinogenicity
Size	85	206	69	21	9	23
SA	14	49	19	2	4	7
Charge	1	7	2	1	0	1
Aspect ratio	3	8	0	1	0	2
Solubility	9	7	6	1	4	3
Crystallinity	13	24	11	0	2	0
Composition	6	14	8	0	3	2

Key: n = number of publications

n < 15
15 ≤ n ≤ 30
n > 30

Table III. Publications related to pulmonary exposure, systemic effects following pulmonary exposure: Total published as of May 2014

	Biological impact						
Target	Biokinetics	Cytotox	Inflammation	Ox. stress	Fibrosis	Genotox	Carcinogenicity
Lung	170	157	445	158	36	31	29
Liver	51	11	19	17	1	6	2
Spleen/immune	42	7	42	6	1	0	2
CNS	35	8	25	21	1	2	2
GI Tract	3	1	2	0	0	0	0
Kidney	27	11	13	3	1	0	1
CV	42	26	80	46	1	1	1
Repro/dev	3	0	8	5	1	5	2
Pleura (retention)	3	0	12	3	2	1	0

Table IV. Publications related to oral exposure, physicochemical properties and biological impact: Total published as of May 2014

	Biological impact					
PC properties	Cytotoxicity	Inflammation	Oxidative stress	Fibrosis	Genotoxicity	Carcinogenicity
Size	4	3	3	0	2	4
SA	2	0	1	0	0	0
Charge	0	0	0	0	0	0
Aspect ratio	0	1	0	0	1	0
Solubility	0	0	0	0	0	0
Crystallinity	1	0	2	0	0	1
Composition	1	1	1	0	0	1

Table V. Publications related to oral exposure, systemic effects following oral exposure: Total published as of May 2014

	Biological impact						
Target	Biokinetics	Cytotox	Inflammation	Ox. stress	Fibrosis	Genotox	Carcinogenicity
Lung	5	3	2	1	0	0	2
Liver	5	3	0	2	0	1	0
Spleen/immune	1	0	2	1	0	1	1
CNS	3	1	0	1	0	0	0
GI Tract	18	11	5	6	1	4	6
Kidney	3	3	0	0	0	1	0
CV	6	2	1	2	1	1	1
Repro/dev	0	4	0	2	0	1	0
Pleura (retention)	1	2	0	1	0	0	0

Table VI. Publications related to dermal exposure, physicochemical properties and biological impact: Total as of May 2014

	Biological impact					
PC properties	Cytotoxicity	Inflammation	Oxidative stress	Fibrosis	Genotoxicity	Carcinogenicity
Size	36	19	21	6	10	3
SA	3	2	1	0	0	1
Charge	4	2	1	0	1	1
Aspect ratio	1	0	1	0	1	0
Solubility	2	1	1	0	1	1
Crystallinity	5	3	4	1	1	0
Composition	3	1	3	1	1	0

Table VII. Publications related to dermal exposure, systemic effects following dermal exposure: Total as of May 2014

	Biological impact						
Target	Biokinetics	Cytotox	Inflammation	Ox. stress	Fibrosis	Genotox	Carcinogenicity
Lung	3	2	3	0	0	2	2
Liver	7	2	3	2	2	2	1
Spleen/immune	5	1	6	0	0	0	1
CNS	4	2	0	2	2	1	0
GI Tract	3	0	1	0	0	1	0
Kidney	4	0	1	0	1	0	0
CV	7	1	3	0	0	0	0
Repro/dev	1	0	0	0	0	0	0
Pleura (retention)	1	0	0	0	0	0	0