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TITLE

Aligning nanotoxicology with the 3Rs: What is needed to realise the short, medium and long-term opportunities?

RUNNING HEAD

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ABSTRACT

Nanomaterials convey numerous advantages, and the past decade has seen a considerable rise in their development and production for an expanse of applications. While the potential advantages of nanomaterials are clear, concerns over the impact of human and environmental exposure exist. Concerted, science-led efforts are required to understand the effects of nanomaterial exposure and ensure that protection goals are met. There is much on-going discussion regarding how best to assess nanomaterial risk, particularly considering the large number of tests that may be required. A plethora of forms may need to be tested for each nanomaterial, and risk assessed throughout the life cycle, meaning numerous acute and chronic toxicity studies could be required, which is neither practical nor utilises the current evidence-base. Hence, there is scientific, business, ethical and legislative drivers to re-consider the use of animal toxicity tests. An expert Working Group of regulators, academics and industry scientists were gathered by the UK's NC3Rs to discuss: i) opportunities being offered in the short, medium and long-terms to advance nanosafety, ii) how to align these advances with the application of the 3Rs in nanomaterial safety testing, and iii) shifting the focus of risk assessment from current hazard-based approaches towards exposure-driven approaches.

KEY WORDS (max. 6)

3Rs; alternative approaches; nanotoxicology; nanosafety; regulatory testing; *in vitro/in silico*

ABBREVIATIONS

AOP	Adverse outcome pathway
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals

EFSA	European Food Safety Agency
EU	European Union
ITS-NANO	Intelligent Testing Strategy for Engineered Nanomaterials
NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in Research
OECD	Organisation for Economic Cooperation and Development
QSAR	Quantitative Structure Activity Relationship
REACH	Registration, Evaluation, Authorisation & restriction of Chemicals
SCCS	European Scientific Committee on Consumer Safety
STIS	short-term inhalation study/studies
SUN	EU FP7 Project “Sustainable Nanotechnologies”

1. Introduction

Due to their unique physicochemical properties, the potential utility of nanomaterials has been increasingly recognised over recent years. A nanomaterial can be defined as a material which has at least one dimension between 1 and 100 nm in diameter (ISO, 2008). However, there are currently multiple working definitions of a nanomaterial, which means that materials not specifically designed as nanomaterials can in some instances also be classified as “nano”, if for example they contain a fraction in the nano-sized range of >50% of the particle count, as per the EU Recommendation (EC, 2011). There exists a vast array of different nanomaterials and forms that have been placed on the market for numerous applications across a wide range of sectors such as cosmetics, medicine, agriculture, food, textiles, electronics, packaging, and industrial chemicals (e.g. pigments (such as in paints) and construction chemicals; (Nowack, 2015)). Although the many advantages to their use are clear, concerns over their safety remain. In particular it will be useful to consider the following when identifying the potential risks associated with nanomaterials (Stone et al., 2016b):

- What are the potential consequences of nanomaterial exposure for human health and the environment?
- To what degree are humans actually exposed to nanomaterials (i.e., the likelihood that they pose a risk where there is a known hazardous potential)?
- What intrinsic and system-dependent physicochemical properties of nanomaterials confer their toxicity?
- What are the mechanism of actions underlying the toxicity of nanomaterials?
- What are the short and long-term effects of nanomaterial exposure (single, and repeated), and consequences of the bioaccumulation of insoluble and biopersistent nanomaterials?

Data on the hazard potential of nanomaterials is a necessary component of risk assessments (where information from both hazard and exposure assessment are combined to establish safe margins of exposure) and for classification and labelling purposes, to enable registration for marketing and sale.

There are increasing examples, particularly within Europe, where re-evaluations and/or separate evaluations of the safety of different nanoforms are required such as the EU Biocides Regulation (528/2012), the EU Cosmetics Regulation 1223/2009/EC and EU Food Additive Regulation (EC 1333/2008). The European Food Safety Authority (EFSA) has also published Guidance on risk assessment of nanomaterials in food/feed and the European Commission's Scientific Committee on Consumer Safety (SCCS) has released Guidance on risk assessment of nanomaterials in cosmetics. The US FDA has also recently published Guidance for Industry Use of Nanomaterials in Food for Animals (FDA, 2015). Authorisations specifically referring to (nano)materials within size boundaries and/or specific forms may imply that each form of a nanomaterial used in regulated products will have to be tested for safety in its own right under the appropriate regulatory framework, even though some of these materials have been in production and use for many years. This approach could lead to extensive testing of different nanomaterial forms, resulting from for example from modifications to their size, geometry, and/or surface coatings. A desire to understand the behaviour of nanomaterials throughout their life cycle/value chain could also potentially contribute towards an increase in the amount of testing to understand the potential hazards to the consumer and the environment at different stages of the lifecycle. Generally, the toxicity testing of nanomaterials and bulk forms for regulatory purposes has been carried out primarily using a prescriptive list of animal studies which have been traditionally used in the risk assessment of chemicals (e.g. studies conducted in line with OECD Test Guidelines; http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm).

There are however increasing pressures to move away from using traditional toxicity testing where possible (EC, 2014). For example, there are emerging legislative bans on the use of animals in cosmetics testing, and there has been much debate within the field around whether the traditional testing strategies for chemical risk assessment are appropriate for nanomaterials (in a broad sense, and related to the suitability of specific assays)(Nel et al., 2013, Silbergeld et al., 2011, Stone et al., 2016a, Aschberger et al., 2016). For the sustainable development and use of nanomaterials, it is

crucial that the genuine health implications are accurately recognised to ensure that society remains protected from any negative (human health) implications following nanomaterial exposure (Oberdorster et al., 2005). Other particle and fibre types, although not necessarily within the nanoscale, have been shown to cause adverse health effects in humans in the past (for example, asbestos, particulate air pollution and crystalline silica quartz). Thus, questions have been posed regarding whether exposure to nanomaterials could cause similar or more harmful effects, due to their small size and potential distribution patterns in the lung and other organs (Donaldson and Borm, 1998, Donaldson et al., 2010, Stoeger et al., 2006).

An expert Working Group of European regulators, academics and industry scientists led by the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) have identified the potential opportunities being offered in the short, medium and long-term to reduce the reliance on traditionally used animal toxicology tests whilst advancing the science of alternative testing strategies towards the risk assessment of nanomaterials. We also explore what is needed from the nanotoxicology community to ensure these endeavours are translated into genuine gains in the science and practice of nanomaterial safety assessment, and consider these issues in the wider legislative context. It is also important to note that the resulting recommendations may also be widely applicable to other areas of risk assessment that are seeking to move away from the use of animal toxicity tests (Burden et al., 2015).

2. The current landscape: *in vivo* testing strategies within the nanotoxicology field

Within the field, there is an increased desire to replace animal testing with alternative testing strategies when assessing nanomaterial toxicity. However there are a number of reasons why some animal toxicity tests will continue to be necessary in the risk assessment of nanomaterials and other (non-cosmetic) chemicals in the next five to ten years. Firstly, despite extensive research efforts,

there is still a limited understanding of nanomaterial absorption, distribution, stability and persistence in the (human) body (Landsiedel et al., 2012), largely due to technological challenges associated with detection of small quantities of (unlabelled) nanomaterials. Whole organisms continue to be the most scientifically relevant test system as they are capable of capturing effects of nanomaterials after they have been absorbed and distributed (and possibly bio-processed) in the body. Furthermore, standard testing requirements in many regulations demand data from animal experiments, and risk assessors are most experienced, and have most confidence, in interpreting data from animal models. There is also insufficient knowledge of how results generated using non-animal methods compare with data from traditional *in vivo* tests, due to a lack of published studies focused on directly comparing effects seen using alternative models (e.g. *in vitro, in chemico*, invertebrate models) against those observed *in vivo* (e.g. (Snyder-Talkington et al., 2015, Landsiedel et al., 2014b, Krug, 2014)).

The majority of *in vivo* assessments undertaken so far have been intended to assess the effects of inhalation exposure to nanomaterials, as currently the primary populations at risk of exposure to nanomaterials are those working in industry, and thus occupational exposure via inhalation represents a high-priority group (Shatkin and Kim, 2015). Therefore to reflect this exposure route of concern, more pulmonary-orientated research than oral-based studies tends to be performed for nanomaterials (Stone et al., 2016a, Aschberger et al., 2016). Inhalation studies require specialised equipment and are more difficult and expensive to carry out than oral administration studies which are commonly used for other chemicals and products. Hazard assessment of nanomaterials has therefore largely utilised *in vivo* studies carried out using high dose intratracheal instillation, with post-exposure observation periods which are often selected to mimic accumulations resulting from chronic (low dose) exposure. The high doses tested and route of administration employed in these studies are not always relevant to human exposure scenarios, and can result in so-called “overload” of the test system (Morrow, 1988, Oberdorster et al., 2015). To address this, protocols such as short-term *in* vivo inhalation studies (STIS) have been developed and advanced, in order to increase

171 understanding of the effects of inhaled nanomaterials and thus potentially reduce the need for 90-
172 day inhalation studies (OECD Test Guideline 413) (Hahn et al., 2014, Ma-Hock et al., 2009, Landsiedel
173 et al., 2014a). Adoption of the STIS protocol in research studies such as the EU funded Sustainable
174 Nanotechnologies (SUN) project (www.sun-fp7.eu/) has reduced the time, financial and ethical
175 implications associated with testing nanomaterial safety, but have not yet eradicated the need for
176 longer term tests (Gosens et al., 2016).

177 Although the long-term effects of nanomaterial exposure remain a major safety concern, there are
178 few inhalation laboratories equipped to carry out the time consuming and expensive sub-chronic
179 (i.e. 90 day) or chronic (1.5 to 2 year) OECD inhalation tests, and thus there remains limited available
180 animal data on the chronic effects of inhaled nanomaterials, e.g. (Ferin et al., 1992, Pothmann et al.,
181 2015, Kasai et al., 2016). Furthermore, there is uncertainty when extrapolating from short-term *in*
182 *vivo* studies to chronic effects due to limited knowledge regarding nanomaterial biokinetics and
183 accumulation in the human body, and on the progression of short-term effects into adverse, chronic
184 biological impacts.

185 Exposure assessments, which aid in the risk assessment process, are carried out with a focus on the
186 release of nanomaterials over the life cycle of the products and actual aerosol concentrations in the
187 air, with less focus on the determination of the internal body/circulating concentrations that result
188 from such exposure (Pelclova et al., 2017). Furthermore, the patterns of exposure are likely to
189 change over coming years as the industry grows. Although inhalation exposure to nanomaterials
190 currently remains the primary portal of entry largely as a result of occupational exposure, effects on
191 consumers following exposure via oral and dermal routes are becoming more relevant due to the
192 wide array of potential applications possible for nanomaterials (e.g. in cosmetics, food or consumer
193 products), and the increase in nanomaterials on the market. Few data are available as yet on uptake
194 and effects through oral and dermal routes (Stone et al., 2016a), as particulate materials including
195 nanomaterials are typically not often absorbed through intact skin (e.g. see SCCS, 2012). This is a

major challenge in terms of increasing interest in the use of nanomaterials for the transdermal delivery of therapeutics (e.g. reviewed in (Peptu et al., 2015), and the use of specifically-designed nanomaterials for this purpose is a further concern regarding the testing necessary to determine dermal toxicity of nanomaterials. As many nanomaterials intended for dermal application are most likely to be found within cosmetic products, and cosmetics are no longer allowed to be tested on animals in many regions, viable alternatives to models of *in vivo* dermal exposure will be critical in coming years. In fact, the OECD has issued guidance on an integrated approach to testing and assessment (IATA; OECD 2014) which is based on alternative methods that should be employed when assessing the skin irritation and sensitisation potential of chemicals (OECD, 2014b; OECD 2016a; OECD 2016b), and this IATA should be applied to nanomaterials.

The discussion on how to best assess the safety of NMs throughout their life-cycle may trigger the use of large numbers of animals and resources. Furthermore, insufficient knowledge on how stable nanomaterials are during transit within the body and their fate is adding to uncertainty around the utility of data generated in both *in vivo* and *in vitro* studies. Efforts have begun to investigate the stability/degradation of nanomaterials in relevant “body fluid” environments (e.g. (Kagan et al., 2010, Feliu et al., 2016)) and the influence that the formation of nanomaterial–protein complexes (which occurs following nanomaterial exposure, or during their transit in the body) has on the biological response (e.g. (Lundqvist et al., 2011), although there remains a lack of controlled studies which systemically address these questions. The plethora of nanomaterials/forms requiring investigation also means it is impractical to perform *in vivo s*tudies for every single nanomaterial/form. Furthermore, there are general questions being asked in aligned fields such as traditional chemical risk assessment, regarding whether data generated from animal studies really are the most appropriate means of predicting human hazards (Hartung, 2009).

There are also increasing business and legislative drivers towards the re-evaluation of the use of animal toxicity tests; for example risk assessments for the cosmetics/personal care products industry

in Europe (EU Cosmetics Regulation 1223/2009/EC) cannot be carried out in animals. Similar bans are also in place or expected in other geographical regions. This could drastically impact innovation in the development of novel nanomaterial ingredients if suitable alternative methods for gathering safety data are not sought quickly. Other regulations stipulate that animal tests are only carried out as a last resort, e.g. the European chemicals legislation REACH (Registration, Evaluation, Authorisation & restriction of Chemicals), even though animal toxicity tests remain the standard means to fill the information requirements.

3. The vision: aligning the 3Rs with improved safety assessment of nanomaterials

Creating an environment where the use of animals in nanotoxicology is refined, reduced and replaced would help to address societal, business and legislative concerns, and could at the same time could improve the science underlying the safety assessment of nanomaterials. However, a systematic and focused shift towards this vision, and a clearly co-ordinated strategy to enable this will be needed. There is currently an opportunity to create a scientifically-driven paradigm which takes advantage of all the latest scientific and technological developments (Stone et al., 2016b, Hussain et al., 2015) and applies them to promote a "21st century" approach to the risk assessment of nanomaterials. Here we consider the opportunities currently available or under development that within short, medium and long-term timeframes could allow these goals to be achieved.

3.1 Immediately, and in the short term (0-5 years): Reduction and refinement of existing animal models

It is possible to immediately refine (i.e. minimise pain, suffering, distress or lasting harm) and reduce the numbers of animal tests that are currently carried out to assess the safety of nanomaterials. For example, the application of short-term inhalation studies (Landsiedel et al., 2014a), where rats are

exposed to test material aerosols on five consecutive days with 21- or 28-day post-exposure observations could, in the first instance, serve as an early tier test. This would determine whether further sub-chronic and chronic toxicity tests need to be carried out, and in this way would decrease the number of longer term studies. Indeed, as more data from this type of study becomes available it could be used as a screening and grouping tool and hence reduce the need for 90 day *in vivo* studies altogether. It is worth noting that the progression of effects and chronic outcome may not be detected in such a study e.g. those which result from biopersistence. Therefore it is crucial that considerations around the fitness for purpose of short-term studies are made on a case by case basis (as has been previously shown in (Ferin et al., 1992) and (Oberdörster et al., 1990)). There is also potential to combine several endpoints within each animal study, and determine toxicity at both the exposure site (e.g. lungs) and secondary target site (e.g. liver) to maximise the amount of information obtained from each study (e.g. see (Gosens et al., 2015)). Inhalation studies have been carried out which combine organ toxicity, genotoxicity and (albeit limited) biokinetic examinations (Landsiedel et al., 2014a, Cordelli et al., 2017, Maser et al., 2015). Such an approach is frequently applied to academic *in vivo s*tudies, as shown by several previous studies that have assessed a number of biological responses (e.g. inflammation and oxidative stress) in order to better understand the potential mechanisms underlying the adverse biological impact associated with nanomaterials at different target sites (Cockburn et al., 2012, Poland et al., 2008, Shvedova et al., 2005, Labib et al., 2016). Furthermore, European Commission-funded projects frequently perform *in vivo* studies that share tissues between laboratories in order to enable assessment of toxicity at several target sites in one study (e.g. (Kermanizadeh et al., 2016).

Increased incorporation of real-life exposure considerations when designing studies will aid in the application of tiered approaches which can be used to prioritise or waive testing. This could mean that nanomaterials are only tested in long-term animal studies if evidence (from *in vitro* testing) has been gathered first which shows that there is a genuine potential risk. In this way assessments would not only explore hazard potential but would also consider whether a) the nanomaterial is

absorbed, b) long-term exposures are likely, c) sufficient quantities reach the target organ, and d) systemic effects are caused at the doses tested. When appropriate, such an approach could provide justification for exposure-based waiving (an option under REACH guidelines). Such a concern-driven approach based on realistic exposure information is suggested by the EU-funded "Nano-safety cluster" (Oomen et al., 2014), and considerations of exposure are advised under the Scientific Committee on Consumer Safety (SCCS) Guidance on the safety assessment of nanomaterial in cosmetics, and European Food Safety Agency (EFSA) Guidance on the risk assessment of the applications of nanoscience and nanotechnologies in the food and feed chain. There would be great benefit in utilising evidence from clinical data on nanomaterial effects more widely, particularly to aid understanding around likely human exposure levels, and also when evaluating the predictive nature of both animal and non-animal approaches (see Table 1), although it is unclear how much of this information exists or is likely to be generated in this timeframe. Additional information could come from biomonitoring data from occupational settings, as well as initiatives that provide information on the exposure levels to nanomaterials that are possible following contact with, for example, different cosmetics and food products.

The addition of toxicokinetic analyses to short term *in vivo* studies could help with dose setting for subsequent chronic *in vivo* studies, as is the case for chemicals (Creton et al., 2012). Such analyses could be used to determine the relationship between internal exposure and systemic effects. This information is particularly important considering that internal exposure can be influenced by pre-absorption behaviour of the nanomaterial (e.g. agglomeration/aggregation (Pauluhn, 2010)), or the dose selected, as administration of excessively high doses may lead to higher or lower (agglomeration, and thus) exposures (Oberdorster et al., 2015). These effects highlight the importance of ensuring that the doses selected for testing are relevant to levels likely to be encountered by humans and the environment, and to enable cross-species extrapolation. To date, assessment of nanomaterial biodistribution has relied on the use of labelled (e.g. fluorescent, radioactive) nanomaterials (e.g. (Konduru et al., 2014). Fluorescence labels may produce artefacts in

297 biological systems which are not related to the nanomaterial itself (Johnston et al., 2013). Elemental
298 analysis has also been used to detect and quantify nanomaterial (e.g silver) biodistribution, but this
299 approach cannot discriminate between particles or ions. Thus new approaches are required to
300 enable the biodistribution of the diverse array of unlabelled nanomaterials to be performed (for
301 example, the use of Coherent Anti-Stokes Raman Scattering (CARS) microscopy to image particle
302 uptake by cells/tissues; (Johnston et al., 2015).

303 A further area of importance is the current efforts to evaluate, improve and validate current
304 standard *in vitro* test systems for nanomaterial hazard assessment. There is an appreciation that
305 approaches which already have associated OECD Test Guidelines are not always appropriate for
306 nanomaterial testing, and thus there are ongoing activities to address these issues to recommend
307 protocols developed specifically for nanomaterial evaluation (Doak et al., 2012, Pfuhler et al., 2013,
308 OECD, 2014a, Oesch and Landsiedel, 2012, Rasmussen et al., 2016). These efforts will help to redress
309 the problems associated with the relevance and reliability of current *in vitro* assays for
310 nanomaterials, but new test systems may still be required, as it is unlikely that the current models
311 are able to adequately report on all mechanisms leading to adverse effects potentially induced by
312 nanomaterials (Doak et al., 2012, Hirsch et al., 2011). Building knowledge about the mode of action
313 of nanomaterial toxicity (i.e. the cellular and molecular processes driving pathogenicity) will enable
314 informed, evidence based *in vitro* models to be identified, which can be used in the first instance to
315 screen for nanomaterial toxicity and could reduce the number of nanomaterials taken forward for *in*
316 *vivo* testing. There is also scope to apply knowledge of how other non-nano-sized particles and fibres
317 behave, to identify and inform which responses are of most importance and interest when assessing
318 nanomaterial hazard. The OECD has recommended a testing strategy for assessment of skin
319 irritation and sensitisation which uses models of varied complexity, including *in vitro* and *in chemico*
320 test systems (OECD, 2014b), OECD 2016a, OECD 2016b). These protocols have not been widely
321 applied to nanomaterial risk assessment (e.g. for eye irritation testing see (Kolle et al., 2016), but

offer an opportunity to enhance the use of alternative models by the nanotoxicology community and should be more widely used in the future.

3.2 In the medium term (5-10 years): Reduction of animal use through use of existing information, development of more robust, targeted *in vitro* approaches and more predictive computational models

There is scope to leverage existing information to prioritise nanomaterials for testing. One way to achieve this is through grouping, to allow the utilisation of read-across approaches and provide justification for waiving of tests. There is however recognition within the field that the grouping of nanomaterials is complicated and cannot be reliably carried out based on properties such as chemical composition, size or surface coating alone, as the links between these and any adverse biological impacts are complex (Braakhuis et al., 2016). Thus, there has been a need to categorically identify the most appropriate and relevant factors which causally lead to apical endpoints. Currently the most straightforward comparison that can be made is to the bulk counterpart of a nanomaterial, for which there usually exists documented evidence on toxicity and also information on human exposure (Cockburn et al., 2012). So far, a robust structure activity relationship and a good correlation between *in vitro* and *in vivo* studies have been identified for asbestos fibres and carbon nanotubes (Poland et al., 2008, Brown et al., 2007) and work is ongoing to establish such correlations for other types of nanomaterial. Accordingly, existing knowledge on the intrinsic and system-dependent physicochemical properties of nanomaterials which confer toxicity can support evidence based, tiered approaches to testing their pathogenicity. For example in the case of high aspect ratio nanomaterials (HARNs) such as carbon nanotubes (CNTs) fibre length has been correlated to both *in vivo* effects (e.g. inflammation), with increasing fibre length (>5µm) causing greater toxicity (Donaldson et al., 2010). The HARN concept has not yet been adopted for two-dimensional materials, like graphene. This effect has also been observed *in vitro* when macrophages

347 are used as the test system via assessment of the following indicators of toxicity; cytotoxicity,
348 proinflammatory cytokine production, cellular uptake, and ROS production (Kermanizadeh et al.,
349 2013, Brown et al., 2007). Accordingly, for assessing the safety of these types of nanomaterial, the
350 first key step would be identifying fibre length and diameter using (electron) microscopy. It would
351 also be informative to assess the purity of samples through elemental analysis, as iron and nickel
352 contaminants are known to contribute to CNT toxicity (Lam et al., 2004). This would be followed by
353 assessment of *in vitro* macrophage responses (Wiemann et al., 2016) for HARN samples with
354 physicochemical properties of concern (e.g. fibre length, metal content, diameter), followed by
355 targeted *in vivo* testing to confirm *in vitro* findings, and fulfil data requirements (Stone et al., 2016a).

356 Quantitative Structure Activity Relationship (QSAR) models that can be used for prediction of
357 nanomaterial exposure-dose-responseare currently under development for metal-based
358 nanomaterials (Kleandrova et al., 2014, Winkler et al., 2014). There have also been significant efforts
359 in the field focusing on QSAR models and physiologically based pharmaco-kinetics (PBPK) models to
360 predict *in vivo* nanomaterial exposure hazards for human and aquatic organisms developed in FP7
361 European projects including SUN, ENPRA, MARINA and MODENA-COST, designed to provide a basis
362 for in *vitro / in vivo* extrapolations (IVIVE)(Speck-Planche et al., 2015, Puzyn et al., 2011, Winkler et
363 al., 2013, Lin et al., 2016, Carlander et al., 2016, Li et al., 2016). However, whilst such computational
364 models can complement experimental work (Horev-Azaria et al., 2011) they cannot, at this time,
365 replace it and there has been limited success in facilitating IVIVE (Lin et al., 2016). For example, as
366 the extrinsic properties of nanomaterials dynamically change according to the biological
367 environment, correlation of *in vivo/in vitro* test results with their pristine structure and/or intrinsic
368 properties (i.e. the classic (Q)SAR approach) is insufficient. Quantitative Structure-Property
369 Relationships (QSPR) therefore need to be established and also represent an area of increasing focus
370 requiring further development as our understanding of nanomaterial behaviour in complex
371 biological environments improves (Winkler et al., 2013, Hristozov et al., 2014). Thus, at this time

much work will be required to make these models suitable for regulatory risk assessment (Tantra et al., 2015, Winkler et al., 2013).

The enormous diversity of nanomaterials and models (e.g. mammalian cells, rodents, humans, aquatic organisms, terrestrial organisms, plants, bacteria) that must be considered is a barrier to the fast development of QSARs (Kleandrova et al., 2014). As such, high throughput (HTP) automated systems which can be used to fill data gaps are desirable to enable the generation of sufficiently predictive QSAR models. Relating material properties to biological outcomes will also be useful in read-across approaches, and the large body of data recently released from the OECD (www.oecd.org/chemicalsafety/nanosafety/testing-programme-manufactured-nanomaterials.htm) had potential to contribute relevant information on major nanomaterials that could form part of the reference base for improved read-across (Foss Hansen et al., 2016). Recently a decision making framework for the grouping and testing of nanomaterials for human health assessments has been proposed by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Nano Task Force" (DF4nanoGrouping; (Arts et al., 2015, Arts et al., 2016)) which aims to ensure that *in vivo* studies are only performed where there are specific data needs, i.e. when read-across cannot be performed, or when the data supporting read-across is not sufficient. The grouping process proposed considers information such as exposure, the characteristics responsible for the functionality of the nanomaterials (e.g. uptake and system-dependent properties including solubility, agglomeration, dispersibility), and cellular effects (i.e. mechanisms of action), and the link between these factors and apical endpoints. Work is ongoing to build confidence in this strategy (RIVM, JRC, and ECHA, 2016; OECD 2016c); other factors that will benefit from further investigation within a grouping approach include: a) the physicochemical characteristics known to drive biological interactions (including shape and surface area of the nanomaterial); b) the ability of the nanomaterial to enter different cellular compartments (thus allowing for the possibility of a variety of biological responses); and c) the number of nanoparticles interacting with cells. The intention is

397 that a robust grouping approach could be incorporated into regulatory guidance in the medium
398 term, to enable its application at the broader level.

399 Expanding the use of *in vitro* approaches that are specifically targeted towards the fulfilment of data
400 requirements could be possible within this time frame. These would include HTP systems to provide
401 information on nanomaterial physicochemical characteristics, hazards and exposure for use in risk
402 assessment (as envisioned by the ITS-NANO framework (Stone et al., 2014)). This requires a shift
403 towards the use of robust, systematic and comprehensive *in vitro* test platforms that provide an
404 indication of uptake and biological effects of nanomaterials specifically over a range of toxicity
405 endpoints, and consideration of how multiple tests can be integrated to allow for accurate
406 predictions of each endpoint (Clift et al., 2011, Stone et al., 2009, DeLoid et al., 2017). In the
407 medium-term such information will be gained through the application of currently used *in vitro* cell-
408 based test systems (e.g., those applied in chemical toxicity tests and used in the nanotoxicology field
409 currently, as reviewed in (Hartung and Sabbioni, 2011)) or adaptations thereof. In combination with
410 data from high throughput screening this approach will help to build confidence in the use of cell-
411 based systems and will contribute to gaining useable knowledge about the biological reactivity of
412 nanomaterials, as well as a better understanding of their toxicological mechanisms. These platforms
413 may also be used as tools in the early screening of candidate nanomaterials to help ensure that
414 potential to induce toxicity is detected and further understood prior to a substance being
415 administered in animal tests (Clift et al., 2014). The animal tests may then be avoided completely if
416 problematic substances are flagged by these screens, or any necessary animal tests could then be
417 better designed and refined. In addition, innovative technologies which utilise microfluidics, such as
418 "lung-on-a-chip" micro-devices that can accurately replicate specific conditions within the human
419 lung (Huh et al., 2012), and those which could mimic passage of nanomaterials from the gut through
420 blood vessels to the liver (such as (Kim et al., 2016) or that developed in the inlivetox project:
421 http://www.inlivetox.eu/), are becoming available and have potential to contribute useful
422 physiologically relevant information. Concomitant to such progression within cell based *in vitro*

technologies, it should be noted that similar achievements are also being made within computational toxicology, as recently reviewed in (Richarz et al., 2015). As efforts continue in the development of nano-specific *in vitro* tests, it is conceivable that in the medium-term useful models that are currently available may have progressed towards validation.

While efforts in each of these areas are ongoing, it is important that investment continues into refining and reducing the numbers of animals used in the *in vivo* tests that remain mandatory, and from which information will be used to inform the utility of the new/adapted *in vitro* approaches. For example, developing short-term studies for routes other than inhalation (e.g., short term studies for oral administration are being developed as part of the EU-funded project SUN), and improving the technical aspects of STIS, particularly as aerosol generation and characterisation is demanding. Moreover, it is challenging to model actual lung burdens resulting from aerosol inhalation *in vitro*. However, strides have been taken to close this gap, for example in a recent publication where the occupational exposure of an inhalatory dose of carbon nanotubes could be mimicked based upon their physicochemical characteristics (Chortarea et al., 2015).

3.3 In the long term (10 years +): Replacement with accepted non-animal methods

In the long-term many sectors have a desire to move away completely from using animal toxicity tests towards the use of scientifically and regulatory accepted non-animal approaches which bear greater relevance to humans. Like traditional *in vivo* tests, each non-animal method has its own merits and disadvantages, and it is unlikely that one cell-based assay or computational model will ever replace an existing animal test on a 1:1 basis. Thus, the most appropriate methodologies will need to be applied in an integrated assessment and testing strategy (Landsiedel, 2015), which includes weight of evidence considerations. This will negate the use of a predefined test battery even with suitable *in vitro* methods at hand. This will also mean that data packages may need to be

447 designed on a case-by-case basis i.e. in a pragmatic, tiered manner which addresses the necessary
448 data gaps, rather than a conventional "tick-box" approach.

449 Exposure considerations will form an important component of such an integrated approach and
450 could start to be addressed *in vitro* through the incorporation of barrier models, which have
451 potential to allow for investigations into nanomaterial uptake and transport (Bachler et al., 2015,
452 Braakhuis et al., 2015, Endes et al., 2015, Garcia-Garcia et al., 2005, George et al., 2015, Rothen-
453 Rutishauser et al., 2007, Gordon et al., 2015). More complex *in vitro* models will also be important in
454 providing information on barrier penetration and translocation capabilities, such as those which
455 comprise more realistic and physiologically relevant systems than the traditional 2D/monolayer
456 methods. This includes cultures of multiple cell types and growing cells in 3D, which has been
457 demonstrated in the "ready to use" EpiDerm^{TM} system, which more accurately mimics skin (although
458 these types of commercial platforms tend to be expensive) (Wills et al., 2016). Also, the use of
459 human or pig skin explants are used to estimate dermal uptake of nanomaterials (Monteiro-Riviere
460 et al., 2013, Fabian et al., 2016). Three-dimensional tissue models demonstrate functional and
461 metabolic properties that could be considered more representative of the *in vivo* environment, as
462 recently suggested for the identification of eye irritation potential of nanomaterials (Kolle et al.,
463 2016) . Consequently, biological response and outcomes seen in 3D and microfluidics models in
464 relation to toxicity endpoints may be very different to those observed in 2D culture systems, which
465 suggests that they may be more physiologically representative (Chapman et al., 2014, Hu et al.,
466 2010, Clift et al., 2014, Snyder-Talkington et al., 2015, Ucciferri et al., 2014). An emphasis on using
467 human cells and tissues in such models where possible will further increase their relevance in the
468 assessment of human safety.

469 Determining whether the endpoints or biomarkers measured within *in vitro* tests are truly driving
470 the key events that result in adverse effects at an organism level would be facilitated by an increased
471 understanding of mechanisms/modes of action; sufficient acquisition of this type of knowledge

could facilitate in the long-term the subsequent development of adverse outcome pathways (AOPs) specific for nanomaterials. Mapping out the pathways in the systematic manner offered by the AOP paradigm would also help to identify research and data gaps in the toxicity pathways triggered by harmful nanomaterials. This has started to be explored e.g. see (Wang et al., 2015), and under the auspices of the EU's MARINA, NanoSafetyCluster and ITS-NANO (Stone et al., 2014) projects. Application of pathways-based approaches has the potential to improve mechanistic understanding of nanomaterial effects (Nel et al., 2013), and advance the development and implementation of non-animal methods to determine whether substances are likely to cause the key events that result in adverse outcomes. Again, it is crucial that an exposure element is captured in such an activity, a feature not encompassed by the current AOP paradigm. Reliable and advanced *in silico* models, if progressed through the availability of more hazard and physicochemical data generated for example by high throughput systems, could also offer huge benefits to the field in the long-term, and will be key tools for predicting the likelihood of different nanomaterials to induce the key events within toxicity pathways. Large-scale efforts towards such modelling approaches have already been initiated, with projects such as the COST Action TD1204 Modelling Nanomaterial Toxicity (MODENA): http://www.cost.eu/COST_Actions/mpns/Actions/TD1204.

4. Key objectives to achieve the vision

The ultimate aspiration of aligning the 3Rs principles with nanotoxicology is the efficient and reliable risk assessment of nanomaterials through application of a focused, exposure-driven integrated approach which utilises data from animal studies only where it genuinely adds value and concentrates testing on specific scientific questions, feeding back into safe-by-design nanomaterials. Table 1 outlines the expert group's perspective on the key focus areas resulting from the short, medium and long-term goals and the necessary steps to enable this vision, while Figure 1 summarises the major scientific considerations needed in approaching these objectives. It is worth

noting that within the coming years, more information on exposure and effects of nanomaterials will come to light, and this experience should be considered and these steps revised accordingly (Nowack et al., 2016, Sotiriou et al., 2016, Ding et al., 2017).

5. Outlook

This broad level analysis focuses on how the application of non-animal methods could drive advances in the field of nanotoxicology and the potential next steps to achieve this. The proposals have widespread applicability and are relevant across multiple sectors. By prioritising attention on the key focus areas identified in section 4 we recommend that the toxicology community work together to:

- Evaluate and acknowledge the limitations and uncertainties of all *in vivo* and *in vitro* approaches, both traditional and alternative;
- Provide clarity as to which potential effects can be adequately covered in safety assessment and which potential effects require further research;
- Appreciate that there will never be a single system that is suitable for all nanomaterials - different models/frameworks/integrated approaches (some of which are already available) covering different aspects of several nanomaterials, will prove helpful; ultimately a battery of approaches will cover most nanomaterials;
- Design exposure-driven integrated approaches/decision-making frameworks first then seek the methods that provide the appropriate data for this specific purpose.

Achieving the above will rely on:

- Academic scientists to work on systematically addressing the data gaps identified here, and strategically focus and align research;

- Funding bodies, to establish strategic funding calls which have measureable impact and enable the necessary progress within basic research;
- Regulators, to provide guidance on when they can accept non-traditional approaches and data (via case studies, to increase the efficiency of the case-by-case approach that is recommended); and to offer compromise between relying on new approaches and established methods of risk assessment, and adopting non-animal approaches. During the time in which data from both *in vivo* and non-animal tests is being produced, their concurrent consideration will help to maximise understanding of the merits and disadvantages of both approaches;
- Industry, to provide clarity about their needs and requirements, to support the steering of future research efforts.

Finally, the output of these discussions will most likely translate into tangible impacts on the reduction, refinement and replacement of animals with 1) the engagement and support from scientific organisations such as the NC3Rs that is complementary to the efforts of the OECD's Working Party on Nanotechnology, and 2) open, face-to-face discussion and collaboration which incorporates dialogue between all relevant stakeholders (regulators, legislators, funders, industry and academics).

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904 Table 1. Key focus areas resulting from the short, medium and long-term goals and the steps
905 necessary to enable them.

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Key focus areas	**Steps to enable focus areas**
<u>Regulatory framework</u> Framework established to enable implementation of alternative non-animal methods into risk assessment and acceptance, with built-in recognition that it is likely that no single method for hazard assessment or physicochemical data will suffice in isolation	▪ Developing methods to serve specific data requirements of decision-making frameworks. ▪ Validation/standardisation of (alternative) test methods towards their use in hazard and risk assessment. ▪ Increasing regulatory confidence in results from non-traditional methods (via guidelines, training, workshops, dialogue). ▪ Supporting risk assessors to understand the relevance and applicability of *in vitro* data for risk assessment, particularly as there will be a need for extensive resource and expertise to interpret and integrate data from various sources. ▪ Adoption of a rationale to deal with uncertainties and limitations inherent to experimental models (both *in vitro* and *in vivo*). ▪ Ensuring that uncertainty in the results is reflected clearly by risk assessors. ▪ Applying a weight of evidence approach to consider all available evidence from different non-animal methods.

	▪ Engagement of decision makers early on in the process.
Hazard prediction Accurate predictions of toxicity that can be confidently linked to physicochemical properties (not only material properties of the pristine material but also functionality of the nanomaterial, e.g. bio-physical interactions of the nanomaterial with its environment (e.g. body fluids))	▪ Adoption of a dual approach: hypothesis driven studies which test if a particular nanomaterial property impacts on toxicity, and studies which compare the toxicity of panels of nanomaterials. These parallel approaches will aim to identify which properties confer toxicity. ▪ Production and easier access to series of systematically altered nanomaterials (e.g. different nanomaterials of the same material with one characteristic altered to enable hypothesis-driven studies to be performed; although we recognise this could prove challenging). ▪ Standardisation of measurements and methods used for nanomaterial characterisation. ▪ Continuation of data sharing on the characterisation of nanomaterials and hazard information in order to document properties and make connections to adverse outcomes, as is taking place within certain EU projects (via round-robin exercises, etc.). ▪ Pooling existing toxicity and physicochemical data and analysing trends to enable predictions, providing the data is comparable and reliable (i.e. all variables are kept the same).

	▪ Establishment of "reference data" for different endpoints for nanomaterials or other well-known particulate materials (e.g. silica or asbestos) which are deemed "representative" (dependent on the nanomaterial being studied) and the use of appropriate positive controls to relate the effects of the nanoforms in *vitro*/*in vivo*. This involves ensuring that knowledge already in existence in other areas of particle toxicology is utilised to help build knowledge within the discipline of nanotoxicology. ▪ Development of advanced analytical techniques to ascertain levels of exposure.
IVIVE (*in vitro* to *in vivo* extrapolation) Increased understanding of extrapolation between different *in vivo* and *in vitro* models (both *in vivo* vs. *in vitro* and between different *in vitro* models)	▪ Selection of relevant concentrations in *in vitro* models. ▪ Identification of appropriate positive controls/"benchmark" nanomaterials, and comparable studies undertaken using them; this would be useful in potency ranking for hazard identification. ▪ Incorporation of toxicokinetic aspects into tests to enable consistent assurance that nanomaterials are being taken up, and reaching targets and leading to systemic exposure. ▪ Cross-talk between *in vivo* and *in vitro* scientists and a culture shift away from treating each in isolation; this

	has been an aim of projects such as ENPRA in the EU. ▪ Focused efforts to bridge the *in vivo*/*in vitro* divide through targeted investment into developing and better understanding the utility of 3D models, fluidic dynamic models and multi-cellular cultures. ▪ Development of *in vitro* models that allow repeat-dosing to be performed. ▪ Taking into account the utility of other emerging technologies that can provide at least a part of the evidence, such as 'omics'. ▪ Enhanced investigation of mode of action of nanomaterial toxicity.
<u>Validation</u> Consensus reached on how best to validate non-animal approaches: against a) animal or b) human data, considering that human is the species in question, and many *in vitro* approaches utilise cells of human origin	▪ For a), generation of sufficient *in vivo* data, to enable comparisons. This should only be carried out when necessary, in situations where the data are critical and meaningful (i.e. ensuring that exposure and test nanomaterial are well characterised, although considering the multitude of possible nanomaterials and exposure routes, this will be difficult to achieve, but may be aided by grouping approaches). ▪ For b), exploitation of clinical/biomonitoring information (i.e. from the welding/mining/tattooing industries), gathering information from workplaces and environments where nanomaterials are used, and building knowledge of precisely the

	concentrations and constituents of nanomaterials within widely used products such as cosmetics and food additives.
Mode of action/AOPs Adaptation of current standard *in vitro* approaches and improved test item preparation, dosing, and understanding of toxicity mechanisms; followed by utilisation of the mechanistic data they provide to build AOPs	▪ Concerted efforts to target areas where current *in vitro* methods are not adequate (e.g. alveolar absorption), where the entire range of toxicological responses that would be seen *in vivo* are not captured (e.g. lung toxicity), and on better mimicking the realistic exposure situation including consideration around relevant delivery techniques. ▪ Dedicated programmes of work and entering of relevant AOPs into the AOP Wiki.
Publication standards Raised publication standard so that only high quality, relevant and comparable information is generated in *in vitro* studies	▪ Widespread implementation of standardised protocols e.g. which ensure consistency in cell lines used, facilitated by ring trials. ▪ Studies designed with consideration of the scientific question e.g. relevant delivery methods used and toxicologically relevant endpoints assessed, accounting for system dependent material properties, and consideration of *in vitro* effects on a whole organism level e.g. incorporation of components which reflect distal effects caused following local absorption. ▪ Definition and dissemination of scopes and limitations of the tests including open recognition by

	scientists of situations in which *in vitro* tests may not be appropriate e.g. due to temporal and toxicokinetic aspects, and determination of how the predictive capabilities of *in vitro* systems could be utilised in these situations.
<u>QSARs/*in silico* models</u> Necessary characteristics and essential levels of complexity incorporated into computational models	▪ Extensive collaborations between toxicologists, mathematicians and theoretical physicists will produce useable, reliable models. ▪ Expansion of the use of high throughput systems which will enable data gaps to be filled more quickly.

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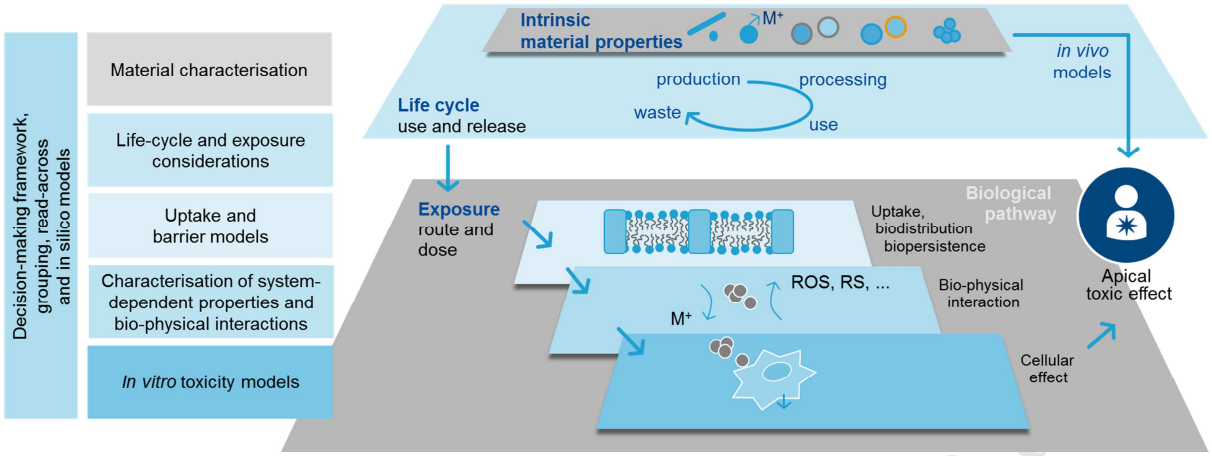
Figure legends

Figure 1. Key scientific considerations to enable realisation of the short, medium and long-term opportunities outlined in section 3. The boxes on the left hand side detail the tools that are necessary towards a) ensuring that intrinsic properties and nanomaterial life cycle are considered in the prioritisation of nanomaterials taken forward into hazard testing, and b) the successful utilisation of non-animal, mechanistic approaches to predict apical toxic effects. Figure adapted from that presented at the second International Congress on Safety of Engineered Nanoparticles and Nanotechnologies (SENN) 2015, Helsinki, Finland by R. Landsiedel.



HIGHLIGHTS

- An expert working group provides a current and forward looking perspective on the 3Rs in nanotoxicology
- Application of non-traditional, alternative methods could improve nanosafety assessment
- There are many short, medium and long-term opportunities to apply the 3Rs within nanotoxicology
- Key focus areas and steps needed to ensure genuine gains are identified.