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

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3 opportunities?

4

5 **RUNNING HEAD**

6 Aligning nanotoxicology with the 3Rs

7

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

48

49 **KEY WORDS (max. 6)**

50 3Rs; alternative approaches; nanotoxicology; nanosafety; regulatory testing; *in vitro/in silico*

51

52 **ABBREVIATIONS**

53 AOP Adverse outcome pathway

54 ECETOC European Centre for Ecotoxicology and Toxicology of Chemicals

57	ITS-NANO	Intelligent Testing Strategy for Engineered Nanomaterials
58	NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in
59		Research
60	OECD	Organisation for Economic Cooperation and Development
61	QSAR	Quantitative Structure Activity Relationship
62	REACH	Registration, Evaluation, Authorisation & restriction of Chemicals
63	SCCS	European Scientific Committee on Consumer Safety
64	STIS	short-term inhalation study/studies
65	SUN	EU FP7 Project “Sustainable Nanotechnologies”
66		
67		
68		
69		

72 increasingly recognised over recent years. A nanomaterial can be defined as a material which has at
73 least one dimension between 1 and 100 nm in diameter (ISO, 2008). However, there are currently
74 multiple working definitions of a nanomaterial, which means that materials not specifically designed
75 as nanomaterials can in some instances also be classified as “nano”, if for example they contain a
76 fraction in the nano-sized range of >50% of the particle count, as per the EU Recommendation (EC,
77 2011). There exists a vast array of different nanomaterials and forms that have been placed on the
78 market for numerous applications across a wide range of sectors such as cosmetics, medicine,
79 agriculture, food, textiles, electronics, packaging, and industrial chemicals (e.g. pigments (such as in
80 paints) and construction chemicals; (Nowack, 2015)). Although the many advantages to their use are
81 clear, concerns over their safety remain. In particular it will be useful to consider the following when
82 identifying the potential risks associated with nanomaterials (Stone et al., 2016b):

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

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

97 (328/2012), the EU Cosmetics Regulation 1223/2009/EC and EU Food Additive Regulation (EC
98 1333/2008). The European Food Safety Authority (EFSA) has also published Guidance on risk
99 assessment of nanomaterials in food/feed and the European Commission's Scientific Committee on
100 Consumer Safety (SCCS) has released Guidance on risk assessment of nanomaterials in cosmetics.
101 The US FDA has also recently published Guidance for Industry Use of Nanomaterials in Food for
102 Animals (FDA, 2015). Authorisations specifically referring to (nano)materials within size boundaries
103 and/or specific forms may imply that each form of a nanomaterial used in regulated products will
104 have to be tested for safety in its own right under the appropriate regulatory framework, even
105 though some of these materials have been in production and use for many years. This approach
106 could lead to extensive testing of different nanomaterial forms, resulting from for example from
107 modifications to their size, geometry, and/or surface coatings. A desire to understand the behaviour
108 of nanomaterials throughout their life cycle/value chain could also potentially contribute towards
109 an increase in the amount of testing to understand the potential hazards to the consumer and the
110 environment at different stages of the lifecycle. Generally, the toxicity testing of nanomaterials and
111 bulk forms for regulatory purposes has been carried out primarily using a prescriptive list of animal
112 studies which have been traditionally used in the risk assessment of chemicals (e.g. studies
113 conducted in line with OECD Test Guidelines;
114 <http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm>).

115 There are however increasing pressures to move away from using traditional toxicity testing where
116 possible (EC, 2014). For example, there are emerging legislative bans on the use of animals in
117 cosmetics testing, and there has been much debate within the field around whether the traditional
118 testing strategies for chemical risk assessment are appropriate for nanomaterials (in a broad sense,
119 and related to the suitability of specific assays)(Nel et al., 2013, Silbergeld et al., 2011, Stone et al.,
120 2016a, Aschberger et al., 2016). For the sustainable development and use of nanomaterials, it is

123 (Oberdorster et al., 2005). Other particle and fibre types, although not necessarily within the
124 nanoscale, have been shown to cause adverse health effects in humans in the past (for example,
125 asbestos, particulate air pollution and crystalline silica quartz). Thus, questions have been posed
126 regarding whether exposure to nanomaterials could cause similar or more harmful effects, due to
127 their small size and potential distribution patterns in the lung and other organs (Donaldson and
128 Borm, 1998, Donaldson et al., 2010, Stoeger et al., 2006).

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

139

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

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

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

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

173 et al., 2014a). Adoption of the STS 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

198 nanomaterials for this purpose is a further concern regarding the testing necessary to determine
199 dermal toxicity of nanomaterials. As many nanomaterials intended for dermal application are most
200 likely to be found within cosmetic products, and cosmetics are no longer allowed to be tested on
201 animals in many regions, viable alternatives to models of *in vivo* dermal exposure will be critical in
202 coming years. In fact, the OECD has issued guidance on an integrated approach to testing and
203 assessment (IATA; OECD 2014) which is based on alternative methods that should be employed
204 when assessing the skin irritation and sensitisation potential of chemicals (OECD, 2014b; OECD
205 2016a; OECD 2016b), and this IATA should be applied to nanomaterials.

206 The discussion on how to best assess the safety of NMs throughout their life-cycle may trigger the
207 use of large numbers of animals and resources. Furthermore, insufficient knowledge on how stable
208 nanomaterials are during transit within the body and their fate is adding to uncertainty around the
209 utility of data generated in both *in vivo* and *in vitro* studies. Efforts have begun to investigate the
210 stability/degradation of nanomaterials in relevant “body fluid” environments (e.g. (Kagan et al.,
211 2010, Feliu et al., 2016)) and the influence that the formation of nanomaterial–protein complexes
212 (which occurs following nanomaterial exposure, or during their transit in the body) has on the
213 biological response (e.g. (Lundqvist et al., 2011), although there remains a lack of controlled studies
214 which systemically address these questions. The plethora of nanomaterials/forms requiring
215 investigation also means it is impractical to perform *in vivo* studies for every single
216 nanomaterial/form. Furthermore, there are general questions being asked in aligned fields such as
217 traditional chemical risk assessment, regarding whether data generated from animal studies really
218 are the most appropriate means of predicting human hazards (Hartung, 2009).

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

223 in the development of novel nanomaterial ingredients if suitable alternative methods for gathering
224 safety data are not sought quickly. Other regulations stipulate that animal tests are only carried out
225 as a last resort, e.g. the European chemicals legislation REACH (Registration, Evaluation,
226 Authorisation & restriction of Chemicals), even though animal toxicity tests remain the standard
227 means to fill the information requirements.

228

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

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

239

240 **3.1 Immediately, and in the short term (0-5 years): Reduction and refinement of existing** 241 **animal models**

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

247 further sub-chronic and chronic toxicity tests need to be carried out, and in this way would decrease
248 the number of longer term studies. Indeed, as more data from this type of study becomes available
249 it could be used as a screening and grouping tool and hence reduce the need for 90 day *in vivo*
250 studies altogether. It is worth noting that the progression of effects and chronic outcome may not be
251 detected in such a study e.g. those which result from biopersistence. Therefore it is crucial that
252 considerations around the fitness for purpose of short-term studies are made on a case by case basis
253 (as has been previously shown in (Ferin et al., 1992) and (Oberdörster et al., 1990)). There is also
254 potential to combine several endpoints within each animal study, and determine toxicity at both the
255 exposure site (e.g. lungs) and secondary target site (e.g. liver) to maximise the amount of
256 information obtained from each study (e.g. see (Gosens et al., 2015)). Inhalation studies have been
257 carried out which combine organ toxicity, genotoxicity and (albeit limited) biokinetic examinations
258 (Landsiedel et al., 2014a, Cordelli et al., 2017, Maser et al., 2015). Such an approach is frequently
259 applied to academic *in vivo* studies, as shown by several previous studies that have assessed a
260 number of biological responses (e.g. inflammation and oxidative stress) in order to better
261 understand the potential mechanisms underlying the adverse biological impact associated with
262 nanomaterials at different target sites (Cockburn et al., 2012, Poland et al., 2008, Shvedova et al.,
263 2005, Labib et al., 2016). Furthermore, European Commission-funded projects frequently perform *in*
264 *vivo* studies that share tissues between laboratories in order to enable assessment of toxicity at
265 several target sites in one study (e.g. (Kermanizadeh et al., 2016)).

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

273 justification for exposure-based waiving (an option under REACH guidelines). Such a concern-driven
274 approach based on realistic exposure information is suggested by the EU-funded “Nano-safety
275 cluster” (Oomen et al., 2014), and considerations of exposure are advised under the Scientific
276 Committee on Consumer Safety (SCCS) Guidance on the safety assessment of nanomaterial in
277 cosmetics, and European Food Safety Agency (EFSA) Guidance on the risk assessment of the
278 applications of nanoscience and nanotechnologies in the food and feed chain. There would be great
279 benefit in utilising evidence from clinical data on nanomaterial effects more widely, particularly to
280 aid understanding around likely human exposure levels, and also when evaluating the predictive
281 nature of both animal and non-animal approaches (see Table 1), although it is unclear how much of
282 this information exists or is likely to be generated in this timeframe. Additional information could
283 come from biomonitoring data from occupational settings, as well as initiatives that provide
284 information on the exposure levels to nanomaterials that are possible following contact with, for
285 example, different cosmetics and food products.

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

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

325 **3.2 In the medium term (5-10 years): Reduction of animal use through use of existing**
326 **information, development of more robust, targeted *in vitro* approaches and more**
327 **predictive computational models**

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

349 2015, 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-response are 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 pharmacokinetics (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

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

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*

425 development of nano-specific *in vitro* tests, it is conceivable that in the medium-term useful models
426 that are currently available may have progressed towards validation.

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

437

438 **3.3 In the long term (10 years +): Replacement with accepted non-animal methods**

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

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™ 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

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

488

489 **4. Key objectives to achieve the vision**

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

500

501 **5. Outlook**

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

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

517

518 Achieving the above will rely on:

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

523 regulators, to provide guidance on when they can accept non-traditional approaches and
524 data (via case studies, to increase the efficiency of the case-by-case approach that is
525 recommended); and to offer compromise between relying on new approaches and
526 established methods of risk assessment, and adopting non-animal approaches. During the
527 time in which data from both *in vivo* and non-animal tests is being produced, their
528 concurrent consideration will help to maximise understanding of the merits and
529 disadvantages of both approaches;

- 530 ■ Industry, to provide clarity about their needs and requirements, to support the steering of
531 future research efforts.

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

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Key focus areas	Steps to enable focus areas
<p data-bbox="186 186 462 215"><u>Regulatory framework</u></p> <p data-bbox="186 329 592 862">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</p>	<ul style="list-style-type: none"> <li data-bbox="673 186 1364 291">▪ Developing methods to serve specific data requirements of decision-making frameworks. <li data-bbox="673 329 1364 500">▪ Validation/standardisation of (alternative) test methods towards their use in hazard and risk assessment. <li data-bbox="673 538 1364 710">▪ Increasing regulatory confidence in results from non-traditional methods (via guidelines, training, workshops, dialogue). <li data-bbox="673 748 1364 1071">▪ Supporting risk assessors to understand the relevance and applicability of <i>in vitro</i> data for risk assessment, particularly as there will be a need for extensive resource and expertise to interpret and integrate data from various sources. <li data-bbox="673 1110 1364 1281">▪ Adoption of a rationale to deal with uncertainties and limitations inherent to experimental models (both <i>in vitro</i> and <i>in vivo</i>). <li data-bbox="673 1319 1364 1433">▪ Ensuring that uncertainty in the results is reflected clearly by risk assessors. <li data-bbox="673 1471 1364 1643">▪ Applying a weight of evidence approach to consider all available evidence from different non-animal methods.

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).

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

	<p>through targeted investment into developing and better understanding the utility of 3D models, fluidic dynamic models and multi-cellular cultures.</p> <ul style="list-style-type: none"> ▪ Development of <i>in vitro</i> 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.
<p><u>Validation</u></p> <p>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 <i>in vitro</i> approaches utilise cells of human origin</p>	<ul style="list-style-type: none"> ▪ For a), generation of sufficient <i>in vivo</i> 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

<p><u>Mode of action/AOPs</u></p> <p>Adaptation of current standard <i>in vitro</i> approaches and improved test item preparation, dosing, and understanding of toxicity mechanisms; followed by utilisation of the mechanistic data they provide to build AOPs</p>	<p>food additives.</p> <ul style="list-style-type: none"> ▪ Concerted efforts to target areas where current <i>in vitro</i> methods are not adequate (e.g. alveolar absorption), where the entire range of toxicological responses that would be seen <i>in vivo</i> 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.
<p><u>Publication standards</u></p> <p>Raised publication standard so that only high quality, relevant and comparable information is generated in <i>in vitro</i> studies</p>	<ul style="list-style-type: none"> ▪ 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 <i>in vitro</i> 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

	<p>aspects, and determination of how the predictive capabilities of <i>in vitro</i> systems could be utilised in these situations.</p>
<p><u>QSARs/<i>in silico</i> models</u></p> <p>Necessary characteristics and essential levels of complexity incorporated into computational models</p>	<ul style="list-style-type: none"> ▪ 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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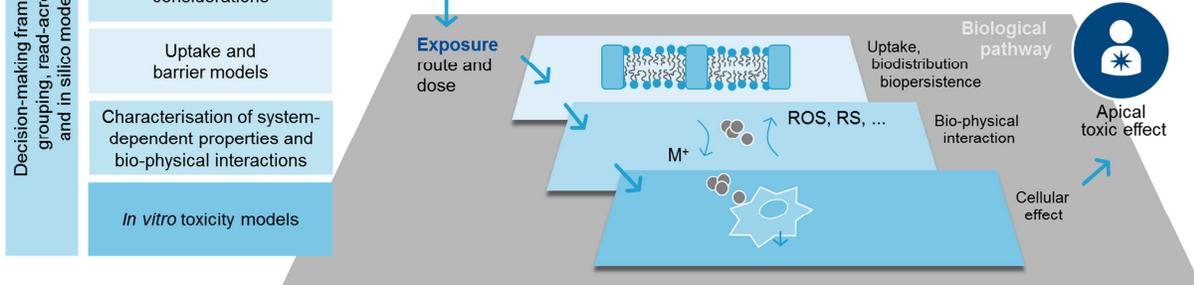
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911 opportunities outlined in section 3. The boxes on the left hand side detail the tools that are
912 necessary towards a) ensuring that intrinsic properties and nanomaterial life cycle are considered in
913 the prioritisation of nanomaterials taken forward into hazard testing, and b) the successful
914 utilisation of non-animal, mechanistic approaches to predict apical toxic effects. Figure adapted from
915 that presented at the second International Congress on Safety of Engineered Nanoparticles and
916 Nanotechnologies (SENN) 2015, Helsinki, Finland by R. Landsiedel.

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- 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.

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