An Integrated Approach to Testing and Assessment to support grouping and read-across of nanomaterials following inhalation exposure

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Short title

IATA for grouping of nanomaterials

Key words

IATA, grouping, read-across, nanomaterials, inhalation exposure, testing strategy, case study

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18 ## Abstract

19 Here we describe the generation of hypotheses for grouping nanoforms (NFs) following inhalation
20 exposure and the tailored Integrated Approach to Testing and Assessment (IATA) with which each
21 specific hypothesis can be tested. This is part of a state-of-the-art framework to support the
22 hypothesis-driven grouping and read-across of NFs, as developed by the EU-funded Horizon 2020
23 project GRACIOUS. Respirable NFs, depending on their physicochemical properties, may either
24 dissolve in lung lining fluid, or in acidic lysosomal fluid after uptake by cells. Alternatively, NFs may
25 also persist in particulate form. Dissolution in the lung is therefore a decisive factor for the
26 toxicokinetics of NFs. This has led to the development of four hypotheses broadly grouping NFs as
27 instantaneous, quickly, gradually and very slowly dissolving NFs. For instantaneously dissolving NFs,
28 hazard information can be derived by read-across from the ions. For quickly dissolving particles, as
29 accumulation of particles is not expected, ion toxicity will drive the toxic profile. However, the
30 particle aspect influences the location of the ion release. For gradually dissolving and very slowly
31 dissolving NFs, particle-driven toxicity is of concern. These NFs may be grouped by their reactivity
32 and inflammation potency. The hypotheses are substantiated by a tailored IATA which describes the
33 minimum information and laboratory assessments of NFs under investigation required to justify
34 grouping. The GRACIOUS hypotheses and tailored IATA for respiratory toxicity of inhaled NFs can be
35 used to support decision making regarding Safe(r)-by-Design product development or adoption of
36 precautionary measures to mitigate potential risks. It can also be used to support read-across of
37 adverse effects such as pulmonary inflammation and subsequent downstream effects like lung
38 fibrosis and lung tumor formation after long-term exposure.

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41 ## Abbreviations

Line	Abbreviation	Definition
42	ALI	air-liquid interface
43	AOP	Adverse Outcome Pathway
44	$DCFH_2$-DA	dichlorodihydrofluorescin diacetate
45	DN	decision node
46	EPR	Electron Paramagnetic Resonance
47	ER	endoplasmic reticulum
48	ESR	Eletron Spin Resonance
49	FRAS	Ferric Reduction Ability of Serum
50	GTTC	Genetic Toxicology Technical Committee
51	H-I-G	Hypothesis for Inhaled NFs that Gradually dissolve
52	H-I-I	Hypothesis for Inhaled NFs that Instantaneously dissolve
53	H-I-Q	Hypothesis for Inhaled NFs that Quickly dissolve
54	H-I-S	Hypothesis for Inhaled NFs that very Slowly dissolve
55	HSP	heat shock protein
56	IATA	Integrated Approach to Testing and Assessment
57	IT	intratracheal instillation
58	JRC	Joint Research Centre
59	KE	key event
60	LLF	lung lining fluid
61	MoA	mechanism of action
62	NF	nanoform
63	NM	nanomaterial
64	OECD	Organization for Economic Co-operation and Development
65	8-OHdG	8-hydroxy-2-deoxyguanosine
66	PC	physicochemical

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67 PLF phagolysosomal fluid

68 POD point of departure

69 ROS reactive oxygen species

70 SbD Safe(r)-by-Design

71 SOP standard operating procedure

72 STIS short-term inhalation study

73 ## 1. Introduction

74 Manufacturing and functionalizing of materials at the nanoscale leads to an array of nanoforms (NFs)
75 of each nanomaterial (NM), that may vary in physicochemical (PC) properties such as chemical
76 composition, size, morphology and surface characteristics. The definitions of a NM and a NF as given
77 by the European Commission are shown in the Supplementary materials (Table S1). Apart from
78 expected benefits, modification of NFs may also pose a hazard to human health to a greater or lesser
79 extent than the unmodified NF. Risk assessment requires comprehensive physicochemical
80 characterization as well as sufficient exposure and hazard data for each NF, but testing every unique
81 NF for their potential adverse effects would demand substantial resources including large numbers
82 of animals.

83 Grouping and read-across are evolving into important tools in the safety assessment of chemical
84 substances, including NFs. Formation of a group requires the properties of the grouped substances
85 to be similar or follow a consistent trend. For chemical substances grouping is typically based on
86 evidence of similar chemical structures, common functional groups, common precursors, or likely
87 common breakdown products (REACH, Annex XI, 1.5 and OECD guidance) [1]. Read-across allows
88 prediction of specific fate and hazard endpoints for one or more substances (target material(s)) in a
89 group, by using data for the same endpoint from another substance in the same group for which
90 more information is available (source material) [2]. This approach can be used to fill data gaps where
91 hazard data is lacking thereby minimizing the need to perform additional *in vivo* studies for each
92 group member. Grouping of NMs typically involves the grouping of different NFs of one chemical
93 substance or the grouping of a nano- and a non-nanoform(s) of one chemical substance. It requires
94 similarity in physicochemical parameters with known relevance for human and environmental
95 hazard. Key intrinsic material characteristics as highlighted in the ECHA guidance for grouping NMs
96 (Appendix R.6-1) include chemical composition, impurities and functionalization in addition to
97 particle size, shape and surface area [3]. System-dependent properties governed by the

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surroundings in which the NF is placed (e.g. dissolution rate in biological media, surface reactivity and dispersibility) should also be considered to support grouping [4].

In recent years, several scientific approaches for grouping and read-across of NFs have been developed [5-9]. The EU-funded Horizon 2020 project, GRACIOUS has taken these approaches a step further by developing a state-of-the-art framework to support the hypothesis-driven grouping of NFs and streamline the risk assessment process [10]. Read-across between NFs of the same group can be utilized as an efficient and effective tool to obtain toxicological information and fill data gaps without resorting to animal testing of individual NFs for hazard assessment, including for a regulatory setting. Within the GRACIOUS Framework a number of 'pre-defined' grouping hypotheses have been generated, based on clear toxicokinetic pathways or mechanisms of action (MoA). These allow the user to quickly recognize a potential hazard which may be applicable to the NF(s) under investigation [10].

GRACIOUS has also developed tailored Integrated Approaches to Testing and Assessment (IATA) which gather evidence in order to justify (or reject) grouping of a target NF and a source material. The IATA sets out a tiered testing strategy, which reflects the different information needed and levels of uncertainty acceptable for different grouping purposes. Here we propose a number of purposes for which the use of the inhalation IATA will be appropriate:

1. Grouping to guide and support the development of materials and NFs that are Safe(r) by Design (SbD).
2. Grouping to promote the adoption of precautionary measures for materials for which limited hazard data is available.
3. Facilitating the generation of a read-across argument for filling in a data gap to comply with regulations.

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The substantiation of a grouping decision is underpinned by the demonstration of similarity between group members, which helps the user to assess whether a target NF is sufficiently similar to a source material to allow grouping and to assume the target NF will induce similar toxicity compared to the source material. For SbD, for the adoption of precautionary measures, and for screening if regulatory read-across could be possible, a qualitative similarity assessment based on expert judgement is sufficient. For regulatory read-across quantitative mathematical similarity assessment is necessary to compare the NF to the source material. Here we describe the generation of GRACIOUS 'pre-defined' hypotheses for grouping NFs where inhalation exposure is a primary concern, and the tailored IATA to test each specific hypothesis. The use of the IATA, including qualitative similarity assessment, will be demonstrated using benchmark materials.

2. Grouping Hypotheses

Within the GRACIOUS framework, the user is first asked for basic information to identify the NFs under consideration and their potential uses in order to identify the most appropriate hypotheses to test [10]. In addition, the basic information gathers information needed in order to tailor the outputs of the grouping and read-across exercise to the purpose of grouping. According to the GRACIOUS Framework, the basic information therefore requires the user to identify: the purpose of grouping, basic physicochemical characteristics and the use/exposure scenarios.

Four hypotheses have been generated for grouping NFs with predicted similar fate and a subsequent assessment of similar hazard following the inhalation route of exposure (see Table 1). The hypotheses include both acute and repeated exposure.

148 Table 1: GRACIOUS 'pre-defined' hypotheses for inhalation exposure to NFs.

Short title	Hypothesis
Instantaneously dissolving NFs (H-I-**I**)	Respirable NFs with an instantaneous dissolution rate: Following inhalation exposure, the toxicity is driven by and is therefore similar to those of the constituent ions or molecules.
Quickly dissolving NFs (H-I-**Q**)	Respirable NFs with a quick dissolution rate: Following inhalation exposure both NFs and constituent ions or molecules may contribute to toxicity, but there is no concern for accumulation. Toxicity (also) depends on the location of the ionic or molecular release.
Gradually dissolving NFs (H-I-**G**)	Respirable NFs with a gradual dissolution rate: Following inhalation exposure both NFs and constituent ions or molecules may contribute to toxicity and there is some concern for accumulation. Toxicity (also) depends on the location of the ionic or molecular release.
Very **s**lowly dissolving NFs (H-I-**S**)	Respirable NFs with a very slow dissolution rate: Following inhalation exposure, toxicity is driven by the NFs and accumulation of NFs in the lungs can lead to long-term toxicity.

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151 2.1 Dissolution as a critical descriptor

152 Information on the use and most relevant exposure route of the NF is gathered as part of the basic
153 information at the start of the GRACIOUS framework and guides the user whether inhalation
154 exposure is expected [10]. Each of the four inhalation hypotheses are shortlisted within the
155 GRACIOUS Framework when the aerosolized NFs under investigation are within the respirable range
156 (< 4.2 μm) [11]. Upon deposition in the respiratory tract, NFs first come into contact with mucus in
157 the upper respiratory tract and lung lining fluid (pH7.4) in the deeper lung, respectively. Depending
158 on the PC properties of the specific NFs, they may either dissolve in mucous and lung lining fluid, or
159 in acidic phagolysosomal fluid (pH 4.5) after uptake by cells, or persist within the lung, interstitium or
160 lung-associated lymph nodes for an extended period of time. Deposited particles within the upper
161 respiratory tract and tracheobronchial tree are cleared by different mechanism including mucociliary
162 transport within the first hours [12]. Our grouping hypotheses are concerned with the fate and
163 potential hazard posed by NFs which reach the distal regions of the lung, where accumulation may

164 occur leading to chronic adverse effects in the local tissue as this context is considered the primary
165 concern following inhalation exposure of NFs.
166
167 There are several approaches for grouping and read-across, which identify dissolution under
168 simulated physiological conditions as a crucial criterion for grouping and subsequent read-across
169 between NFs [5, 13-18]. Oberdörster and Kuhlbusch describe in their recent review that "because
170 the *in vivo* dissolution rates of engineered nanomaterials can differ widely, it is too simplistic to
171 group ENM just into soluble and poorly soluble materials" [19]. There are currently no scientifically
172 sound cut-off thresholds to define groups according to dissolution rate as the transition from very
173 slow to quick dissolution rate is continuous. However, here the following pragmatic thresholds are
174 suggested to facilitate the preliminary grouping of NFs into broad categories:

175 1. **Instantaneously dissolving NFs**: threshold of $t_{1/2} < 10$ minutes in lung lining fluid (H-I-I).
176 2. **Quickly dissolving NFs:** threshold of $t_{1/2} < 48$ hours in lung lining or lysosomal fluid (H-I-Q).
177 3. **Gradually dissolving NFs:** threshold of $t_{1/2} > 48$ hours and < 60 days in lung lining or lysosomal
178 fluid (H-I-G).
179 4. **Very slowly dissolving NFs:** threshold of $t_{1/2} > 60$ days in lysosomal fluid (H-I-S).
180
181 In this article, we refer to instantaneously, quickly, gradually and very slowly dissolving NFs to
182 describe the dissolution rate by which NFs release ions/molecules/atoms and thereby alter their
183 (physical) state or entity.
184
185 The pragmatic thresholds are set to reflect the impact of dissolution within the biologically relevant
186 timeframe for cell interaction and cellular clearance from the lungs e.g., 'instantaneous' dissolution
187 within 10 minutes suggests NFs do not persist long enough to be phagocytosed by alveolar
188 macrophages or translocate through the epithelial barrier, therefore particle-triggered hazard is
189 negligible. Alternatively, a longer half-life in lung lining fluid indicates the potential for particle-cell

interactions and uptake of NFs into the lysosomal compartment of the resident pulmonary cells which may ultimately trigger particle-related toxicity. The grouping hypotheses, H-I-Q, H-I-G and H-I-S, address a number of biological outcomes which may result from differing half-lives within the lung lining fluid and/or acidic environment of the lysosome.

Quick dissolution (defined as a half-life of < 48 hours in lysosomal fluid) reflects a timeframe whereby NFs may be taken up by cells, in particular alveolar macrophages, but dissolve rapidly to constituent ions within the acidic environment of the lysosome [20]. This mechanism directly delivers potentially toxic ions to the intracellular environment, which may lead to specific toxic effects such as cell death or activation of pro-inflammatory pathways [21, 22]. Accumulation of particles is not likely due to their quick dissolution and so direct toxicity driven by ions will be most relevant.

Gradual dissolution considers both the particles and NFs which may persist in particulate form for some time but gradually degrade in either in the lung lining fluid or in the acidic lysosomal environment to their constitute components indicating a slow release of ions over time [23]. If exposure exceeds the dissolution and clearance rates of the particle components, NFs may potentially accumulate within the lungs [23, 24]. Thus, toxicity may be driven by both ion and particle effects and may incorporate both direct effects due to toxic ion release or highly reactive particle surface, as well as chronic effects due to slow release of ions over time. Therefore, for quickly and gradually dissolving NFs, the IATA considers both the dissolved and the particulate fraction of the NFs under investigation.

Very slow dissolution is defined by a threshold half-life > 60 days in lysosomal fluid, derived from the extensive literature on the biopersistence of poorly soluble particles in the rat lung [25-27]. Biopersistent NFs will remain as particles in the pulmonary environment over an extended period of

216 time and may accumulate in cells and tissue. Toxicity will be dictated by physical interactions
217 between the NFs and cells, such as through excessive build-up of NFs [25, 28] or specific NF
218 reactivity [29].
219
220 According to Geiser and Kreyling (2010), about 90 % of very small particles deposited in the alveolar
221 region are cleared by alveolar macrophages, which are subsequently eliminated via mucociliary
222 clearance. Other particle clearance pathways from the lung are via the interstitium and lymphatic
223 system, through re-appearance of particles from the interstitium onto the epithelial surface and via
224 translocation to the blood (potentially leading to accumulation in secondary target organs) [27, 30,
225 31]. On repeated exposure to biopersistent NFs the clearance mechanisms can be overwhelmed,
226 leading to NF accumulation and chronic inflammation, that might ultimately lead to fibrosis and/or
227 cancer [25]. Therefore the targeted testing for these NFs differs significantly from those particles
228 that instantaneously dissolve, by focusing on particle-triggered toxicity, including biopersistence,
229 potential accumulation and long-term effects.
230

231 2.2 Biological reactivity as a critical descriptor

232 The mechanism of particle induced toxicity is not yet fully understood. The previously described
233 concept of impaired clearance does not explain the different inflammatory potencies of different
234 NFs. Current research shows that a range of intrinsic factors like shape, size, coating, composition,
235 crystallinity, impurities [15, 32-34], and extrinsic factors such as pH, temperature, ionic strength and
236 protein binding may modulate the surface reactivity of NFs. Thus, surface reactivity was considered
237 as an essential parameter for building and justifying a grouping strategy for very slow, gradual and
238 quick dissolution NFs.
239
240 Several approaches to grouping and read-across acknowledge surface reactivity, such as reactive
241 oxygen species (ROS) production as a key parameter [5, 8, 9]. The imbalance between ROS

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242 generation and ROS scavenging leads to elevated ROS levels within cells, non-selective oxidation of
243 biomolecules [35, 36] and oxidative stress associated with endpoints such as cytotoxicity,
244 genotoxicity or inflammation [37-41]. The induction of oxidative stress (via ROS induction and
245 inflammation) is thought to play an essential role in the mechanism behind nanomaterial toxicity
246 [42, 43 , 44, 45 , 46 , 47 , 48 , 49].
247
248 For grouping and read-across it is insufficient to assign NFs into either a 'not reactive' or 'reactive'
249 category as the level of ROS production by NFs can differ greatly. It is therefore essential to take the
250 potency of NFs into account to substantiate a read-across argument.
251

252 2.3 Inflammatory potential as critical descriptor

253 A key effect of NFs after inhalation is their ability to induce pulmonary inflammation [25, 50-53].
254 Inflammation is considered an important mechanism of action by which NFs may cause toxicity [54].
255 It is related to various adverse outcomes that have been associated with NF exposure, including
256 pulmonary fibrosis and cancer [26, 55]. Inflammation is indicated *in vivo* mainly by an increase in
257 neutrophils and pro-inflammatory cytokines in the bronchoalveolar lavage fluid or via
258 histopathological examination. In *in vitro* lung models, inflammation is generally indicated by the
259 induction of pro-inflammatory cytokines [15, 56].
260
261 Inflammation is a complex process involving many cell types, chemokines and cytokines. Also,
262 depending on the exposure concentration and duration, inflammation can resolve over time. For NF
263 exposure, the main concern is that repeated exposure might lead to chronic inflammation that does
264 not resolve. Given the complexity of inflammation, it is not sufficient to categorize NFs into
265 'inflammogenic' or 'non-inflammogenic'. Similar to reactivity, the potency of the target and source
266 NFs in terms of inflammation potential should be compared to assess their similarity to allow
267 grouping and the subsequent building of a read-across argument.

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269 3. Integrated Approaches to Testing and Assessment

270 The GRACIOUS IATA is structured in a decision tree format which logically follows the fate of the NFs
271 from the initial inhalation exposure to deposition along the respiratory tract and the subsequent
272 potential for interactions with resident pulmonary cells which may lead to toxicity and disease
273 pathogenesis. The decision tree uses a series of decision nodes (DNs) to generate the information
274 needed for critical descriptors in order to selectively distinguish NFs which may be grouped
275 according to the specific inhalation grouping hypotheses (Figure 1).

276

277 The hypothesis for instantaneously dissolving NFs (H-I-I), can be used to perform read-across to the
278 molecular form. If the hypothesis is rejected because the NF does not meet the threshold of $t_{1/2} < 10$
279 minutes in lung lining fluid, then other hypotheses can be considered in which the location of the ion
280 release will affect the toxicity (Figure 1).

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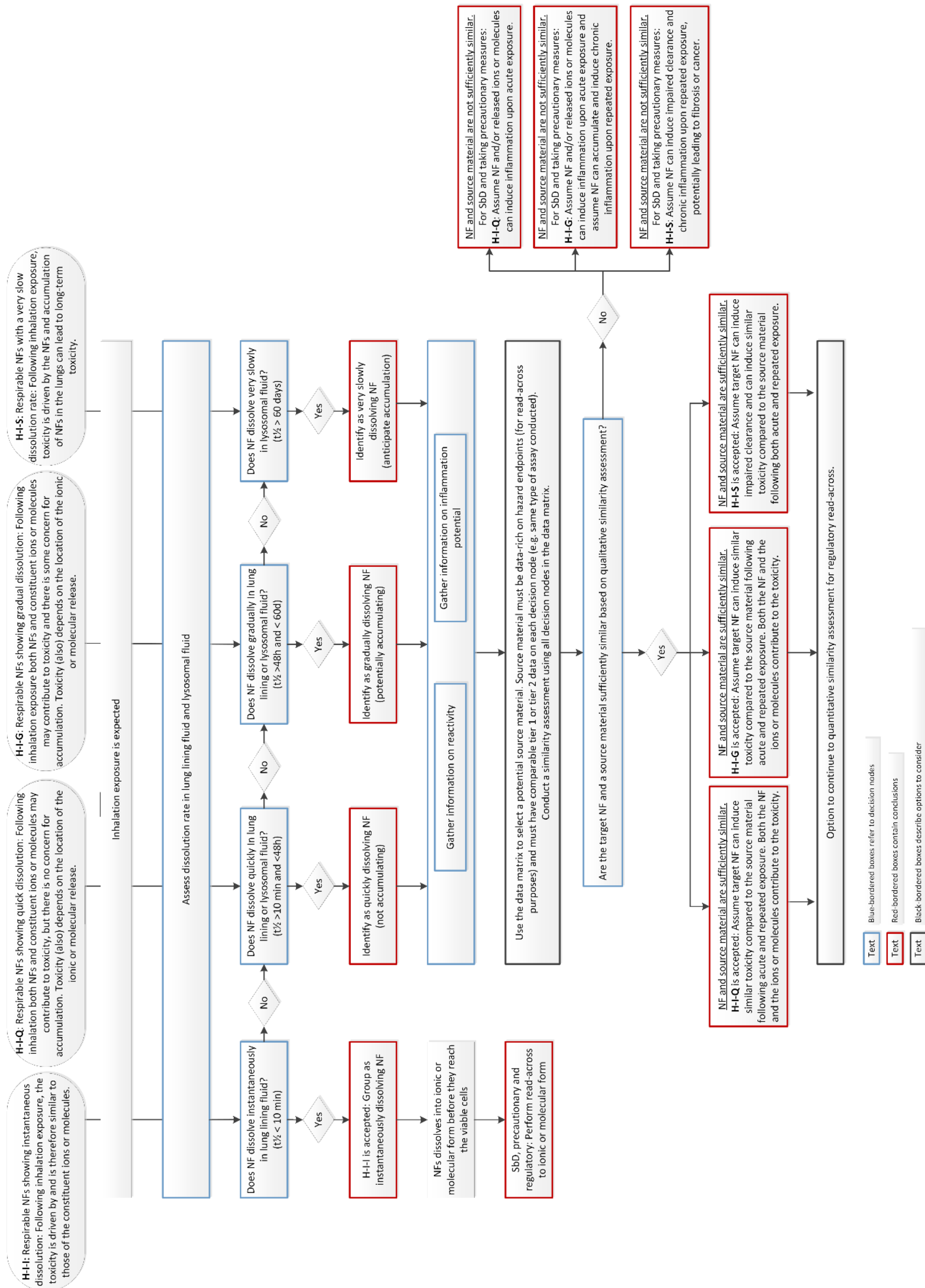

284 *Figure 1. IATA decision tree for assessing whether a NF belongs to the group of instantaneously*

285 *dissolving, quickly dissolving, gradually dissolving or very slowly dissolving NFs, including directions*

286 *on the implications of grouping and subsequent options for read-across.*

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288 For NFs that quickly dissolve ($t_{1/2}$ < 48 hours) there is no concern for particle accumulation and
289 toxicity can mainly be attributed to the ions. However, the particle aspect influences the location of
290 the ion release. A benchmark material that fits into this hypothesis is ZnO (JRCNM01100a, formerly
291 known as NM-110). For ZnO NFs, the particles can be taken up by cells leading to intracellular ion
292 release [57] referred to as the Trojan horse effect [21, 22]. This leads to different effects compared
293 to exposure to zinc salts [58-61].

294

295 For gradually dissolving NFs ($t_{1/2}$ > 48 hours and < 60 days in lysosomal fluid), both the particle and
296 the ions contribute to the toxicity, and the location of ion release affects toxicity. As the dissolution
297 rate is not quick, particle accumulation cannot be discounted for upon repeated exposure. A
298 benchmark material that falls into this hypothesis is synthetic amorphous silica, SiO_2 (JRCNM02000a,
299 formerly known as NM-200) [24]. This material has a half-time of 3.6-4.5 days in lung lining fluid and
300 29-35 days in phagolysosomal fluid and has been shown to induce inflammation after intratracheal
301 instillation [62]. As toxicokinetics are important in this hypothesis, comparison to a source material
302 of similar chemical composition to the NF(s) under investigation is needed for read-across.

303

304 Very slowly dissolving NFs ($t_{1/2}$ > 60 days) are of concern as they can accumulate and may induce
305 long-term effects upon repeated exposure. Benchmark materials that fit into this hypothesis are
306 CeO_2 (JRCNM02102a, formerly known as NM-212), DQ12 quartz silica, and TiO_2 (JRCNM01005a,
307 formerly known as NM-105). The dissolution rate of these materials is very slow and they are known
308 to induce long-term effects in rats upon repeated exposure. CeO_2 JRCNM02102a induced chronic
309 inflammation and fibrosis after 90 days inhalation exposure [63]. DQ12 quartz induced chronic
310 inflammation and fibrosis after 90 days inhalation exposure [64] and cancer after chronic 2-year
311 inhalation exposure [65]. TiO_2 JRCNM01005a induced chronic inflammation and cancer after chronic
312 2-year exposure [26, 28]. These long-term effects are related to impaired clearance in rats at high

313 exposure concentrations caused by extensive accumulation of the particles. Intensive discussions are
314 ongoing about the human relevance of these effects. From a risk assessment point of view, the
315 pulmonary toxicity needs to be considered relevant for human hazard assessment [25].
316

317 ## 3.1 Tiered testing

318 Each DN of the IATA is linked to a tiered testing strategy, which provides practical guidance on how
319 to efficiently assess the target NF (Figure 2). The testing strategy is tiered to enable the burden of
320 data gathering and testing to be tailored to the purpose of grouping, with higher tiers reflecting the
321 greater information requirements to support a grouping decision with higher levels of confidence
322 [10]. The choice of tier reflects the initial purpose for grouping, the associated level of uncertainty
323 considered acceptable for the user's needs and sometimes the suitability of the recommended
324 methods for the NF under investigation. Lower tier testing may facilitate rapid and cost-effective
325 SbD decision making on whether to continue with a product development, despite the relatively
326 high level of uncertainty with this grouping decision. On the other hand grouping and read-across for
327 regulatory purposes may require a higher degree of scientific justification based on higher tier
328 testing. When available, standardized methods (standard operating procedures (SOP) such as OECD
329 TG or ISO protocols) are recommended for inclusion in the tiered testing strategy.
330

331 The tiered testing strategy provided in Figure 2 addresses particle induced hazard in the lung and is
332 relevant to the IATA for hypotheses H-I-Q, H-I-G and H-I-S. It is less relevant to H-I-I, since this
333 hypothesis is addressed by assessing the hazard of the constituent ions or molecules. The outcome
334 of the tiered testing strategies provides the required information needed by the DNs of the IATA to
335 identify which hypothesis is most appropriate for grouping the NFs under investigation. The
336 following sections provide a more detailed description of each DN.
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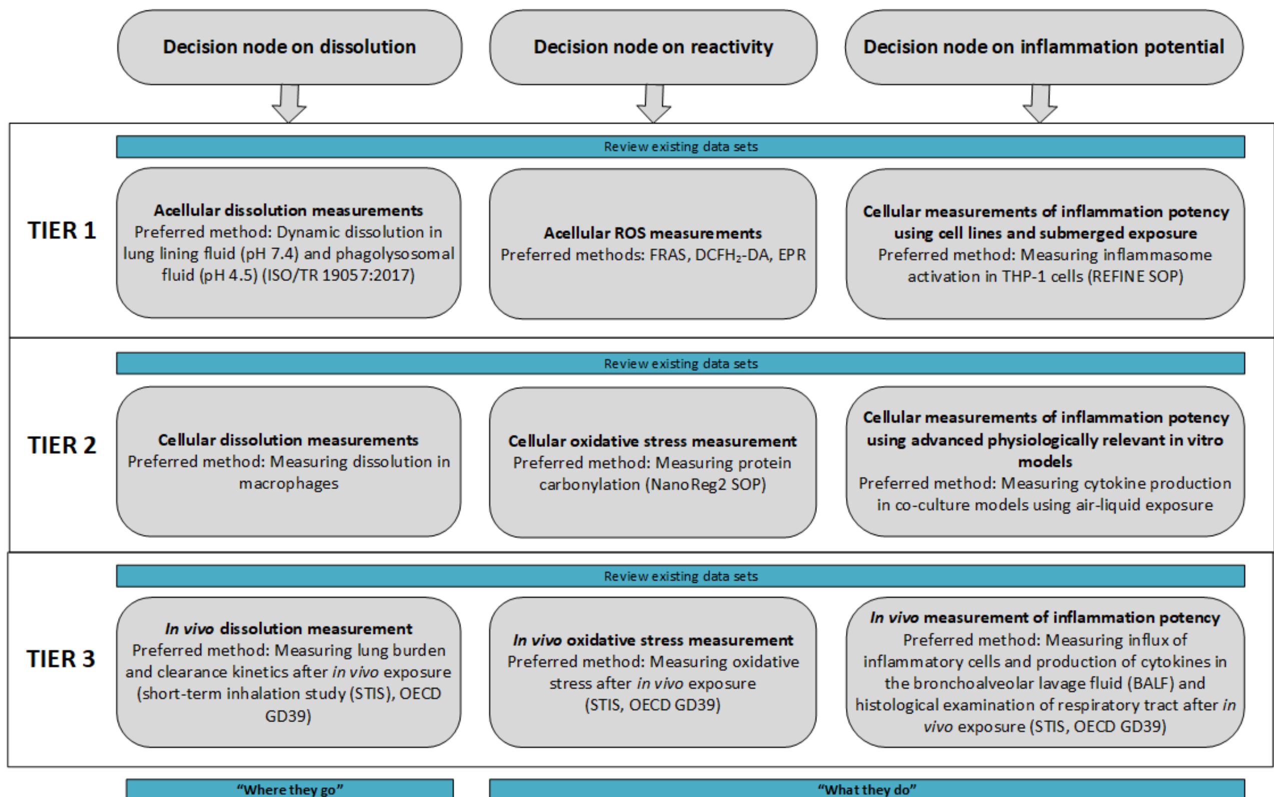

338

339 *Figure 2. Tiered testing for each decision node in the IATA for hypotheses H-I-Q, H-I-G, and H-I-S.*

340

341 ## 3.2 Dissolution decision node

342 The first DN in the IATAs is on the dissolution rate of the NF. Tier 1 testing for this DN includes
343 assessment of the NFs dissolution in simulated lung lining fluid at pH 7.4 (LLF) and phagolysosomal
344 fluid at pH 4.5 (PLF) under static or dynamic conditions. Tier 2 testing for the dissolution DN includes
345 measurement of durability in cellular systems such as macrophages. Tier 3 consists of *in vivo*
346 measurement of lung burden and clearance kinetics.

347

348 Considering that living organisms are dynamic systems, static solubility tests do not reflect the *in*
349 *vivo* turnover of the respective physiological media. Testing NF dissolution in an acellular continuous
350 flow system is considered the preferred method in Tier 1, as the results of the continuous flow
351 system are consistent with data from short-term *in vivo* studies [66]. Standardized ISO protocols for
352 these flow-through or flow-by systems that mimic the non-equilibrium physiological conditions are

353 available (ISO/TR 19057:2017). Simulant media need to be sufficiently complex to offer oxidative,
354 reductive and pH-driven dissolution pathways [67]. For inhalation exposure, both LLF and PLF are
355 relevant media [68].
356
357 Tier 2 examines durability in cellular systems, which take into account a number of dynamic and
358 physiologically relevant environments and pathways to NF degradation [69-71]. As cellular models to
359 assess durability are not yet well standardized, there is currently no SOP available, however, studies
360 have shown incubation with macrophages to be at least as predictive of biodurability as acellular
361 assays for NFs [66] and useful to clarify the specific mechanism of particle degradation [72]. As such,
362 progression to Tier 2 is envisioned to be only used in some cases where a more physiologically
363 relevant cellular system is required to better understand mechanism.
364
365 The determination of biopersistence of NFs requires long-term *in vivo* assays and therefore is not
366 required for initial grouping. Depending on the purpose of grouping, Tier 3 testing may be required
367 to confirm whether acellular *in vitro* durability corresponds with an accumulation of NFs in tissues.
368 For this a short-term inhalation study (STIS) can be used with a 5-day exposure period and a
369 recovery time of e.g. 28 days for very slowly dissolving NFs. The updated OECD test guidelines for
370 inhalation exposure now recommend lung burdens and clearance rate to be included as recorded
371 endpoints [73, 74]. To support grouping of NFs at Tier 3 the IATA requires clearance rate to be
372 included as an endpoint, to provide evidence of similarity in biopersistence. This information can be
373 used in case available for the source material.
374
375 Application of the tiered testing strategy to assess dissolution allows the NFs to be placed into one of
376 four groups: instantaneously dissolving, quickly dissolving, gradually dissolving and very slowly
377 dissolving.
378

379 ### 3.3 Reactivity decision node

380 For the reactivity DN, Tier 1 assessment relies on acellular measures of ROS generation, Tier 2
381 includes measurement of ROS/oxidative stress in cells and Tier 3 includes *in vivo* measurement of
382 oxidative stress.
383
384 A panel of several acellular tests considered appropriate as a starting point to assess reactivity are
385 included at Tier 1. They include Ferric Reduction Ability of Serum (FRAS), Electron Paramagnetic
386 Resonance (EPR) and Dichlorodihydrofluorescin diacetate assay (DCFH$_2$-DA). The FRAS assay uses
387 antioxidant components in human serum as reporter molecules, providing an indirect read-out of
388 ROS generation. The assay has been demonstrated to be suitable for testing both metal-containing
389 NFs and carbonaceous materials [75]. EPR spectroscopy, which is also called Electron Spin
390 Resonance (ESR), measures the transition between electron spin states of paramagnetic molecules,
391 and can be used to study species with at least one unpaired electron. Using different spin probes,
392 spin traps, different types of ROS species can be quantified. EPR has the least interference with
393 hydrophobic and colored substances, however, carbonaceous materials can interfere with the assay.
394 DCFH$_2$-DA assay can be used in Tier 1 to assess acellular ROS production. This assay has been widely
395 used to assess the ROS production of particles and NFs [76]. DCFH$_2$-DA assay is suitable for testing of
396 carbonaceous materials [75].
397
398 Different approaches to Tier 1 assessment of surface reactivity may be taken dependent on the
399 purpose of grouping. For example, for SbD purposes where the aim may be to compare similarity of
400 surface reactivity across NFs of different chemical composition or NFs with the same core and a
401 different coating, a combination of assays would be recommended for a broader assessment of
402 reactivity. Conversely, for grouping NFs for regulatory purposes, such as the development of a read-
403 across argument, comparison of surface reactivity of different NFs or non-NFs via a combination of

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404 assays might add unnecessary complexity. Therefore, a single assay that is sensitive to the
405 substance-specific reactivity should be selected [75].
406
407 Tier 2 involves cellular assessment of oxidative stress as a biological consequence of NF reactivity.
408 More work is required to confirm the most appropriate tests to be incorporated into this tier.
409 Currently assays such as cellular $DCFH_2$-DA assay, protein carbonylation, Nrf2 antioxidant response
410 pathway, Endoplasmic Reticulum (ER) stress, Heat Shock Protein (HSP) activation, glutathione
411 depletion and lipid peroxidation are recommended for inclusion. Measuring protein carbonylation in
412 cells has been shown to give a similar ranking of NFs compared to adverse reactions (such as
413 inflammation) after short-term *in vivo* inhalation studies (STIS) [77]. Measuring glutathione
414 depletion showed a correlation between *in vitro* and *in vivo* exposure for amorphous silica
415 nanoparticles [78]. The disadvantage of measuring glutathione is that is easily is reduced during
416 sample preparation making it difficult to assess the reduced and the oxidized form. An alternative
417 method could be the use of antioxidants to assess whether specific endpoints (e.g. cytokine
418 production) are oxidant mediated.
419
420 If Tier 3 *in vivo* studies are required to enable a grouping decision or to facilitate a read-across
421 argument, measuring glutathione depletion and lipid peroxidation after short-term inhalation can be
422 considered. In addition, endpoints such as oxidative DNA damage (by measuring 8-hydroxy-2-
423 deoxyguanosine (8-OHdG)) may be included in the histopathological assessment of tissue to provide
424 evidence of oxidative stress *in vivo* [39].
425
426 For NFs that are considered either gradually or quickly dissolving based on their dissolution rate, the
427 relative contribution of the ion and particle components to the toxicity observed during hazard
428 testing will need to be determined [79]. Also the potential for the particle and ion to interact to
429 enhance toxicity should be considered [76].

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431 ### 3.3 Inflammatory potential decision node

432 The next step for grouping according to the IATA is to assess the potential of the NFs to elicit an
433 inflammatory response compared to the source material. Endpoints for assessing the lung
434 inflammatory potential should be informed by adverse outcome pathways (AOPs) relevant for
435 pulmonary disease, to ensure the information gathered is targeted and can be interpreted in terms
436 of disease relevance. Therefore the Tier 1 and Tier 2 *in vitro* assays could be selected based on the
437 measurable Key Events (KE) outlined in the AOP [55]. Inflammatory potential can be tested in tiers
438 from simple *in vitro* assays using cell-lines and acute endpoints (Tier 1), to more complex and
439 physiologically-relevant *in vitro* models incorporating multiple cell types and using air-liquid
440 interface (ALI) exposure (Tier 2). If necessary, Tier 3 recommends *in vivo* hazard assessment using a
441 short-term inhalation study (STIS).
442
443 Starting at Tier 1, we recommend simple *in vitro* screening assays following well established
444 protocols. The preferred assay measures inflammasome activation in the human monocyte cell-line
445 THP-1 (SOP from REFINE (Vandebriel et al. submitted 2021)). NLRP3 inflammasome activation is an
446 important step in the immune response to NFs [80], as it contributes to pulmonary diseases
447 including asthma, COPD, fibrosis and cancer [80-82]. Inflammasome activation appears to regulate
448 the balance between tissue repair and inflammation after inhalation of NFs [83] and is therefore key
449 in understanding the inflammation potential of NFs. Several NFs have been shown to activate the
450 NLRP3 inflammasome, including Ag, CeO_2, CNTs, polystyrene, TiO_2 and SiO_2 [84]. Another suitable
451 Tier 1 *in vitro* assay that can be used to assess macrophage activation and inflammatory potential of
452 NFs is based on rat alveolar macrophages (NR8383). According to two recent publications, NR8383
453 assay outcome showed reasonable predictivity to *in vivo* STIS for more than twenty NFs including
454 AlOOH, $BaSO_4$, different CeO_2, Fe_2O_3, TiO_2, different nano ZrO_2, and ZnO, different amorphous SiO_2
455 and graphite nanoplatelets, and two nanosized organic pigments [85, 86].

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456

457 Submerged exposure can greatly alter particle characteristics compared to the airborne state.
458 Therefore, at Tier 2 we recommend using an air-liquid interface (ALI) exposure to mimic inhalation
459 exposure more closely [87]. Several researchers have shown that using ALI exposure improves the
460 predictive value of *in vitro* systems [88-92]. Another way of enhancing predictivity is to better mimic
461 physiological relevance of the *in vitro* model by using co-cultures or tissue models cultured from
462 primary cells. The downside of these more complex models is that these methods have not been
463 validated or standardized and are undergoing constant optimizations to allow better predictions
464 [87]. SOPs and publications [91] [93] from the H2020 project PATROLS (https://www.patrols-
465 h2020.eu), provide useful information towards improved standardization of these methods.
466

467 As inflammation is a complex process, Tier 3 short-term inhalation studies (STIS) [94] might be
468 required to substantiate a read-across argument. If a target NF and a source material show similar
469 potency in a short-term study, this can be used to substantiate a read-across argument for the
470 hazards after repeated exposure from the source to the target NF. We recommend that if *in vivo*
471 studies are considered, inclusion of Tier 3 measurements for all DN (dissolution and reactivity) are
472 combined within one study to avoid additional *in vivo* testing for the other DN. STIS should be
473 performed following recommendation of OECD Guidance Document 39 [95]. Nose-only is the most
474 preferred exposure mode [96]. In case that a study according to OECD test guidelines [73, 74] is
475 required later on for regulatory purposes, the STIS data can assist the scientist to appropriately
476 design their regulatory study.
477

478 ## 4. Demonstration of IATAs

479 Based on the information gathered on each DN, similarity can be assessed between the target NF
480 and the source material. Depending on the purpose, this similarity assessment can be qualitative or
481 quantitative.

482 - Qualitative: Use the IATAs to gather the evidence required to assess whether NFs are
483 sufficiently similar to be grouped. Qualitative similarity assessment may be based on
484 information from a variety of assays deemed appropriate to answer the IATA, justified by
485 expert opinion. Qualitative similarity assessment based on expert judgement can help Safe-
486 by-Design, and is the first step for regulatory read-across. Based on such qualitative
487 similarity precautionary measures can be taken in workplace.
488 - Quantitative: Based on the outcome of the qualitative similarity assessment, perform a
489 detailed quantitative similarity assessment employing mathematically derived limits of
490 similarity between group members within each individual assay of a DN to support read-
491 across to fill a data gap.

492

493 The IATA as presented here directs the collection of the minimum relevant evidence needed to
494 conduct similarity assessment to confirm the proposed substances/NFs can be grouped, and to
495 subsequently support any read-across arguments relevant to the hypothesis. Below, we focus on
496 qualitative assessment of the similarity between NFs.

497

498 ### 4.1 Selection of source materials

499 To form a preliminary group, a source material first needs to be selected against which the NF under
500 investigation is compared. There are several considerations for selecting a source material (or
501 materials), which depends on the purpose of grouping. For SbD and for adopting precautionary
502 measures, less detailed information on similarity is needed. In this case, the target NF can be
503 compared to a data-rich benchmark material such as the reference materials from the JRC

504 repository. These benchmark materials can also serve as positive and negative controls to indicate
505 the maximum and minimum responses in an assay. To be considered acceptable for regulatory read-
506 across a high level of similarity is needed to justify filling a data gap using information from a source
507 material. In this case, the source material should be of similar chemical composition. For example,
508 NFs that differ in morphology or coating can be compared, or the target NF can be compared to its
509 bulk non-nano counterpart.
510

511 4.1 Benchmark material for the IATA on very slowly dissolving NFs: CeO_2

512 Cerium dioxide (CeO_2) NFs are widely distributed as they are used as polishing materials, absorbents,
513 exhaust catalysts, conductors and electrode materials. CeO_2 NFs are known to have a very slow
514 dissolution rate. As a case study, we selected two well-characterized reference materials,
515 JRCNM02102a (formerly known as NM-212) and JRCNM02101a (formerly known as NM-211),
516 supplied by the Joint Research Centre (JRC). Both NFs of CeO_2 are uncoated and produced by
517 precipitation, however these NFs of CeO_2 differ in size and morphology. Table 2 shows some key
518 characteristics of JRCNM02102a and JRCNM02101a reported by the JRC [97]. JRCNM02102a in
519 particular has been studied extensively, including long-term inhalation studies; such studies are
520 lacking for JRCNM02101a. JRCNM02102a is known to have a half-life > 60 days [98]. Results from 90-
521 day inhalation studies show that JRCNM02102a can accumulate in the lungs upon subchronic
522 exposure leading to chronic inflammation and fibrosis, therefore as JRCNM02102a is considered a
523 very slowly dissolving NF that can induce long-term effects. H-I-S was selected as the most
524 appropriate pre-defined hypothesis for potentially grouping different NFs of CeO_2.
525
526
527
528
529

530 Table 2. Particle characteristics of JRC materials JRCNM02102a and JRCNM02101a.

	Primary particle size	**Specific surface area**	**Morphology from TEM image**
NM-211	<10 nm up to 20 nm	27.8 ± 1.5 m^2/g	Spherical with regular morphology
NM-212	<10 nm up to 100 nm	64.9 ± 4.1 m^2/g	Polyhedral with irregular morphology and non-homogenous size distribution

531

532 The aim of the case study exercise was to assess whether the IATA can be used to support the
533 grouping of JRCNM02102a and JRCNM02101a on the basis of a common fate and hazard potential,
534 despite certain dissimilarities between the NFs as highlighted in Table 2. The potential IATA
535 outcomes for this case study are outlined in Box 1. Following IATA for the hypothesis on very slowly
536 dissolving NFs, data was gathered to address each DN (Table 3 and 4).

537

Box 1: Potential IATA outcomes

- Accept grouping hypothesis and use outcome for SbD of new NFs.
- Accept grouping hypothesis and use to design precautionary measures by assuming target NF will cause similar long-term effects compared to the source NF.
- Accept grouping hypothesis and then progress to building a read-across argument (the final similarity may still be unacceptable).
- Reject grouping hypothesis because the NFs dissolve at different rates with may lead to different toxicokinetics (and therefore different bioaccumulation and long-term effects)
- Reject the grouping hypothesis because one appears much more reactive (more potent) than the other or produces ROS/oxidative stress due to a different mechanism of action.
- Reject the grouping hypothesis because one appears much more inflammogenic than the other (more potent).

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539 Table 3. Data matrix for JRCNM02102a as a benchmark material for very slowly dissolving NFs.

	NM212		
Tier	**Dissolution**	**Reactivity**	**Inflammation potential**
1	Flask dialysis < 1 µg/L [97]. Static in PLF < 0.001 Wt % (recrystallizing) [98, 99]. Dynamic in PLF: <0.28 ng/cm²/h [99]. Half-time > 365 days (Wohlleben et al. 2021 in prep)	FRAS: 16.7 sBOD at 1000 m²/L.	Inflammasome activation: at 10 – 30 µg/cm² [100]. Submerged exposure: Increased TNF-α in NR 8383 at 22.5 µg/ml [85].
2		ALI exposure: No oxidative stress observed up to 3 µg/cm² [101]; Submerged in co-culture: No oxidative stress up to 10 µg/m² [101].	ALI exposure: No release of cytokines up to 5 µg/cm² [100]; ALI exposure: Increased IL-6 and IL-1β at 1-3 µg/cm² [101]; Submerged in co-culture: Increased IL-1β, IL-6, IL-8 and TNF-α at 10 µg/cm² [101].
3	5d and 28d study: T ½ 40 days at 0.5 mg/m³, T ½ > 200 days at >5 mg/m³ [98]. Instillation: T ½ ~ 140 days at 1 mg/kg bw [102]. 28d study: No significant reduction of CeO_2 content in lung and extrapulmonary organs at 48h and 72h after exposure to 20 mg/m³ [103]. 90d study: impaired clearance at 3 mg/m³ [104]. 2-year study: T ½ 86, 114, 164 and 200 days at 0.1, 0.3, 1.0 and 3.0 mg/m³ [105]	28d study: Oxidative stress (8-OH-dG) not demonstrated at 20 mg/m³ [106]. 90d study: Increased expression of oxidative stress-related genes at 3 mg/m³ [107], increased 8-OH-dG at 3 mg/m³ [62].	5d study: increased neutrophils in lavage fluid at 0.5 mg/m³[98], 28d study: granulomatous inflammation at 5 and 25 mg/m³ [98]; increased neutrophils at 2.5 mg/m³ [106]. 90d study: neutrophilic infiltration and granulomatous inflammation at 3 mg/m³, progression to fibrosis [104].
Evaluation	**Very slowly dissolving *in vitro*; accumulation and very slow clearance *in vivo.***	**No oxidative stress *in vitro* in cells; ambiguous results *in vivo.***	**Induction of cytokines *in vitro* and inflammation *in vivo.***

540

541

542 Table 4. Data matrix for JRCNM02101a to test the IATA for very slowly dissolving NFs.

	NM211		
Tier	**Dissolution**	**Reactivity**	**Inflammation potential**
1	Flask dialysis < 1 µg/L. Static in PLF < 0.001 Wt % (recrystallizing)[98]. Dynamic in PLF: <0.73 $ng/cm^2/h$ [99]. Half-time > 365 days (Wohlleben et al. 2021 in prep)	FRAS:13 sBOD at 1000 m^2/L.	Submerged: Increased TNF-α in NR 8383 at 22.5 µg/ml [85].
2			
3	5d and 28d study: High lung burden 3 weeks after exposure to 25 mg/m^3 [98, 108]. 28d study: No significant reduction of CeO2 content in lung and extrapulmonary organs 48 h and 72 h after exposure to 10 mg/m^3 [103].	28d study: Oxidative stress (8-OH-dG) not demonstrated at 10 mg/m^3 [106].	5d study: increased neutrophils in lavage fluid at 0.5 mg/m^3[98]. 28d study: increased neutrophils at 1.2 mg/m^3 [106].
Evaluation	**Very slowly dissolving *in vitro*; accumulation and very slow clearance *in vivo.***	**Little information available. No oxidative stress observed *in vivo.***	**Induction of cytokines *in vitro* and inflammation *in vivo.***

543

544

545 Following the DN in the IATA, we can perform a qualitative similarity assessment to compare the two
546 CeO_2 NFs (table 5). From the available data it is clear that both NFs are very slowly dissolving and
547 have the potential to accumulate in lung tissues following inhalation exposure. This might lead to
548 long-term effects upon repeated exposure. A limited number of studies were identified reporting on
549 the reactivity of JRCNM02102a and JRCNM02101a, however from this data set neither NFs appears
550 to intrinsically produce high levels of ROS or induce significant oxidative stress *in vitro* or in short-
551 term *in vivo* studies. JRCNM02102a exposure however resulted in increased expression of oxidative
552 stress-related genes and increased 8-OH-dG after 90-day inhalation [62]. Both JRCNM02102a and
553 JRCNM02101a were shown to induce pro-inflammatory responses in simple *in vitro* assays which
554 was reflected in the development of acute and persistent inflammation *in vivo* after short-term
555 inhalation exposure [98, 106]. Therefore the hypothesis that both JRCNM02102a and JRCNM02101a

556 can be grouped as slowly dissolving NF with the potential to cause long-term toxicity in the lung can
557 be accepted.
558
559 Table 5. Comparison of JRCNM02102a and JRCNM02101a based on the IATA following the
560 hypothesis for very slowly dissolving NFs.

IATA DN	JRCNM02102a	JRCNM02101a
Dissolution	Very slowly dissolving *in vitro*; accumulation and very slow clearance *in vivo.*	Very slowly dissolving *in vitro*; accumulation and very slow clearance *in vivo.*
Reactivity	Little information available. No oxidative stress observed *in vivo* after 28d exposure, while oxidative stress was observed *in vivo* after 90d exposure.	Little information available. No oxidative stress observed *in vivo* after 28d exposure.
Inflammation	Induction of cytokines *in vitro*; inflammation *in vivo* (5d and 28d exposure).	Induction of cytokines *in vitro*; inflammation *in vivo* (5d and 28d exposure).
IATA OUTCOME	Accept hypothesis: Following chronic inhalation exposure, accumulation of NFs in the lungs can lead to long-term toxicity. Form Group, for SbD and for adopting precautionary measures: Assume NM211 can cause similar toxicity compared to NM212 upon long-term exposure.	

561
562
563 For the purpose of SbD or for adopting precautionary measures, the acceptance of the grouping
564 hypothesis supports the prediction that JRCNM02101a can induce impaired clearance and
565 granulomatous inflammation that can progress to fibrosis as reported for JRCNM02102a after 90
566 days inhalation exposure.
567

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568 ## 4.2 Benchmark material for the IATA on quickly dissolving NFs: ZnO

569 Zinc Oxide NFs (ZnO) was chosen as a case study material to exemplify the substantiation of the pre-

570 defined hypothesis, H-I-Q. We collected data relevant to each DN for a single specific ZnO NF,

571 JRCNM01100a (formerly known as NM-110).

572

573 Table 6: Data matrix for ZnO JRCNM01100a as a reference material for quickly dissolving NFs.

Tier	Dissolution	Reactivity	Inflammation potential
1	Static system: <0.05% dissolution in LLF, >90% dissolution in PLF [109]. Static system: 67% dissolution in PLF [99]. Dynamic system: K_{diss}: 204 ng/cm^2/h in PLF Complete dissolution confirmed after 7 days by TEM [99].	FRAS assay: intermediate reactivity [109].	Submerged: ncreased production of TNF-α and IL-8 in THP-1 [110]. Submerged: Increased IL-8 in Human hepatoblastoma C3A cells: [111]. Submerged: Increased IL-8 and MCP-1 in dHL-60 neutrophil cell [112]. Submerged: Increased levels of TNF-α production in HMDM [113].
2	Cellular: 51% dissolution after 24h in NR8383 macrophages [99]. Cellular: complete dissolution after 24h in THP-1 [110].	Cellular, submerged: Dose dependent decrease in reduced GSH and total glutathione antioxidant in human hepatoblastoma C3A cells [114]. p47^{phox} NADPH oxidase-mediated ROS formation in RAW 264.7 [115]. DCFH$_2$-DA cellular: ROS release in 16HBE cells [113]. Cellular, submerged: Upregulation of heat-shock proteins genes (HSP) at 4h in THP-1 [116].	Submerged: Modifications of genes involved in inflammation, apoptosis and mitochondrial dysregulation at 4h in THP-1 [116]. Submerged: Severe tissue destruction at 10–1000 µg/mL at 24h in rat precision-cut lung slices [117]. Molecular responses of A549 cells measured by multiple 'omics' platforms at 24h: metallothionein induction, depletion of antioxidants, repressed DNA repair, induction of apoptosis. Responses to NM110 similar to Zn^{2+} ions, suggesting that the mode of action is mediated by dissolved metal ions rather than by the physical NF [118].
3	Intratracheal instillation (IT): No ZnO NM agglomerates observed inside the BAL macrophages after 24h [119].		IT in mouse: Increased total number, IL-6, LDH and protein in lavage fluid at 64 and 128 µg/mouse [119]. IT in mouse: Increased acute-phase response at 11, 33 and 100 mg/kg bw [120].
Evaluation	**Quick dissolution in the low pH acellular assays. Evidence of quick dissolution within cells after uptake.**	**Reactive in acellular assays and cellular assays.**	**Induced pro-inflammatory signaling in vitro. Acute resolving inflammation in vivo.**

	No accumulation in vivo.		Toxicity is driven by intracellular release of toxic ions rather than particle-driven.

574

575

576 Evidence of dissolution rate is sufficient to identify JRCNM01100a as quickly dissolving, and the
577 reactivity and inflammatory data suggest toxicity is driven by the intracellular release of toxic ions
578 rather than the NF itself. Data from studies conducted on other forms of ZnO NFs further support
579 the conclusion that ZnO NFs can be considered quickly dissolving NFs with minimal potential for
580 accumulation. Accordingly, hazard results from the intracellular dissolution of ZnO NFs to toxic ions
581 have been demonstrated by both *in vitro* and *in vivo* models [57, 59]. Grouping via H-I-Q will
582 therefore allow the similarity assessment between NFs to be framed by the likely relevant
583 mechanism of action driving the potential hazard. Available *in vivo* data for JRCNM01100a consists
584 of 2 intratracheal instillation (IT) studies. IT studies have major shortcomings as for example it is very
585 difficult with IT to get a material spread evenly among the lung lobes and all material could end up in
586 a single lobe and by-pass the upper respiratory tract. In addition, usually unrealistically high
587 exposure doses are being used for IT leading to a bolus effect regardless of the toxicity of NFs [96].
588 Therefore, IT is not considered a physiological route of exposure. However, NFs with high toxicity
589 have been shown to induce persistent inflammation, while NFs with low toxicity induced only
590 transient inflammation after IT. IT could be useful for screening for hazard of NFs [96].

591

5. Discussion

Here, we present a range of inhalation grouping hypotheses which are evidence based, employing knowledge from a wide range of published data. In addition, we present a novel tailored IATA supported by a tiered testing strategy to provide the evidence needed to support, reject or refine these grouping hypotheses. Each hypothesis takes into consideration the physicochemical characteristics of the NFs (what they are), the route of exposure and toxicokinetics (where they go) and their hazard (what they do). For the physicochemical characteristics, dissolution rate was found to be an efficient mechanism by which to group NFs, as this determines their biopersistence and their fate and behavior. Coupling the biopersistence with assessment of the hazard in terms of surface reactivity and pro-inflammatory potential allows further refinement of the group.

Thresholds were provided for the dissolution rate based on biologically relevant timeframes for cell interaction and cellular clearance from the lungs. Clearly particles which dissolve instantaneously ($t_{1/2}$ < 10 minutes) in lung lining fluid, will not persist for sufficient time to induce particle mediated effects. For this reason hypothesis H-I-I supports the argument to read-across from the ionic or molecular form of the same substance to a NF. In contrast, particles which are very slow to dissolve ($t_{1/2}$ > 60 days) may induce particle mediated toxicity and bioaccumulate (H-I-S) [25-27], with the potential to cause longer-term hazards. For the particles that have intermediary dissolution, the toxicity could be driven by particles and/or dissolution products. The rate of release of dissolution products will influence the rate at which these products are released in the cell and so their toxic potential, as well as the duration of particle persistence in the cell and so any biological effects imparted by the residual particles. We therefore set two thresholds, one for gradual dissolution with a half-life of greater than 48 hours in lysosomal fluid (H-I-G) for which accumulation cannot be discounted for, and one for quick dissolution with a half-life of less than 48 hours in lysosomal fluid (H-I-Q). However, these values are not strictly fixed. Values close to the thresholds can be supported by use of a similarity assessment.

618

619 The remaining wording of each hypotheses is less well prescribed, in order to allow flexibility.
620 Instead, the evidence generated by use of the IATA provides the more precise details required to
621 define a group, and can be tailored to support read-across for a specific hazard endpoint e.g.
622 repeated dose toxicity following inhalation exposure. For example, the hypothesis for particles
623 which dissolve quickly could be used to group particles with very low reactivity, or alternatively to
624 group particles with relatively high reactivity. For regulatory purposes, the need to provide
625 thresholds for such descriptors is prevented by incorporation of robust and quantitative methods of
626 assessing similarity (White paper reference).

627

628 The IATA includes DN on reactivity and inflammation potential for assessing similarity between the
629 target NF and a source material. Surface reactivity and inflammation potential are included as both
630 are considered key toxicity parameters for NFs after inhalation exposure [5, 8, 9, 25, 50-53]. They are
631 both associated with pathological outcomes; oxidative stress is associated with genotoxicity and
632 inflammation [37-41], and inflammation with pulmonary fibrosis and cancer [26, 55]. A key toxicity
633 parameter that is currently not included in the IATA is genotoxic potential. The main reason for not
634 including this here is that current *in vitro* assays for testing genotoxic potential need modifications
635 before they can be used to test NFs [121]. Experts from the Genetic Toxicology Technical Committee
636 (GTTC) critically reviewed published data on genotoxicity assessment of NFs and found large
637 variation in tests and systems used for *in vitro* assays. They concluded these results cannot be
638 interpreted and first modifications of the current *in vitro* assays is needed [121]. In addition, the
639 experts of GTTC conclude that it appears that genotoxicity by NFsis mainly induced via a secondary
640 effect (such as via oxidative stress and/or chronic inflammation) and not via direct DNA interaction.
641 Based on the recommendations by GTTC, a testing strategy for assessing the genotoxic potential of a
642 NF is being developed. The here presented IATAs to support grouping and read-across of

643 nanomaterials following inhalation exposure can then be updated accordingly with a DN on
644 genotoxicity.
645
646 Also, systemic toxicity in secondary organs and local toxicity within the upper respiratory tract are
647 not specified in the IATA. As stated before, respirable particles are the focus of our IATA. However,
648 the particles sizes of NFs usually cover a range and they will deposited within the entire respiratory
649 tract depending on the aerodynamic size distribution. At the deposited site, e.g. nasal cavity or
650 larynx, NFs may cause local toxicity. For instantaneously, quickly and gradually dissolving NFs, the
651 local toxicity of the released ions can be assessed by read-across to the ionic or molecular form.
652 Potential particle-triggered toxicity at the upper respiratory tract may be assessed by particle
653 surface-reactivity and inflammation potency. Finally, local toxicity to the upper respiratory tract can
654 be assessed in Tier 3 STIS. Thus, this point is covered by the IATA.
655
656 Systemic toxicity can occur in the case of translocation of the NFs or their ions to the blood. For
657 instantaneously, quickly and gradually dissolving NFs, the released ions might translocate to the
658 blood. For these NFs, read-across to the ionic or molecular form can be performed for assessing
659 systemic toxicity. For very slowly dissolving NFs, translocation of the particles depends strongly on
660 their physical-chemical properties and the region of deposition. NFs deposited in the upper
661 respiratory tract will be cleared via mucocilliary transport and are subsequently swallowed and
662 cleared via the gastro-intestinal tract. NFs that deposit in the alveoli might translocate to the blood.
663 The translocation and systemic toxicity in secondary organs can only be assessed in *in vivo* inhalation
664 studies. Because the existing data of repeated dose inhalation studies with very slowly dissolving NFs
665 did not give indication for any systemic toxicity in secondary organs, and there were no established
666 Tier 1 and Tier 2 tests available, we decided not to include systemic toxicity in our IATA.
667

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668 IATAs have been proposed by the OECD for streamlining of information gathering and testing for
669 hazard assessment of chemicals. In the context of GRACIOUS, we have used them to streamline the
670 evidence identification and generation to test specific grouping hypotheses. We assessed the
671 suitability of the hypotheses and the IATA through application of case studies. The case studies
672 included CeO_2 JRCNM02102a, CeO_2 JRCNM02101a and ZnO JRCNM01100a, for which much data is
673 available and which we propose as benchmark materials. Such benchmark materials will be useful
674 for comparison to the NF of concern or for identifying the range of biological relevance (maximal or
675 minimal biological response) for a particular descriptor. The data identified via the IATA was
676 gathered into a matrix, providing insight into data gaps for these benchmark materials. The IATA
677 starts with a DN addressing dissolution in order to identify the most relevant of the hypotheses. The
678 CeO_2 NFs have a very slow dissolution rate, relevant to H-I-S, while ZnO exhibits quick dissolution,
679 relevant to H-I-Q. Regarding dissolution, sufficient data was available for Tier 1 to assess the relevant
680 thresholds, but little data is available on Tier 2 assays (dissolution in cells). Such Tier 2 methods are
681 quite laborious and do not provide much added value compared to the dynamic acellular dissolution
682 assay. We therefore suggest that in many instances the Tier 1 assays are sufficient for assessment of
683 dissolution in relation to grouping. A Tier 2 assessment of dissolution may be more relevant to the
684 hypotheses where gradual or quick dissolution intracellularly is relevant (H-I-Q and H-I-G).
685
686 For the other DNs, sufficient data was observed for Tier 1 assays and for Tier 3 *in vivo* studies, while
687 there is limited data available for Tier 2 assays. The proposed Tier 2 assays are generally of higher
688 complexity than Tier 1 assays, plus they are relatively innovative and therefore lack standardization.
689 During application of the IATA during grouping, Tier 3 *in vivo* data might be lacking for some NFs.
690 Tier 2 data could therefore be needed to provide the data required for a similarity assessment
691 between NFs within the group. Innovative Tier 2 assays such as co-cultures, primary cells and/or
692 exposure at the air-liquid interface, may be more predictive due to a higher physiological relevance,
693 or by allowing identification of the mechanism triggering toxicity. For example, some ALI models

Internal

694 show a better correlation to *in vivo* data than submerged models [88-92]. The disadvantage of more
695 complex models, at this time, is that optimizations are ongoing and therefore standardization is
696 currently lacking. For grouping purposes, it would be ideal to have an assay that is simple and
697 predictive at the same time. Tier 2 assays will require optimization to deliver this need. In future as
698 Tier 2 assays are validated and evidence builds to demonstrate such assays are suitably and reliably
699 predictive of hazard, the waiving of Tier 3 in vivo testing may be justified reducing the reliance on
700 animal testing for NM hazard.
701
702 As described above, once the data is collected into a matrix a qualitative or quantitative similarity
703 assessment can be conducted. Qualitative approaches can be used to inform the SbD of NFs, or for
704 adopting precautionary measures. For regulatory read-across, a quantitative similarity assessment
705 between group members is needed. For the purpose of read-across to fill a data gap for regulatory
706 hazard assessment, such as extrapolation of the 90-day inhalation study point of departure (POD)
707 from JRCNM02102a to JRCNM02101a, a read-across argument will need to be built. This will require
708 a quantitative similarity assessment to compare the potencies of the target (JRCNM02101a) and the
709 source NF (JRCNM02102a), based on the available data gathered using the IATAs. Several methods
710 of quantitative similarity assessment have been generated and will form the basis of a White Paper
711 and a further 12 publications to be published in NanoImpact (to be submitted by June 2021). A full
712 description of these methods is therefore beyond the scope of this paper.
713
714 The grouping approach and IATAs presented here will be integrated in the overall GRACIOUS
715 framework [10]. The GRACIOUS framework will guide the user through the different steps to
716 hypothesis selection and subsequent IATA testing to allow grouping [5-9]. The GRACIOUS framework
717 will be available as a guidance document and also as a software "blueprint" tool (to be published
718 Sept 2021). Linked to NM databases (e.g. eNanoMapper), the open-access blueprint will facilitate
719 the rapid identification of potential group members or potential source materials and provide a

720 user-friendly interface to facilitate the use of the IATA to support grouping and subsequent read-
721 across.
722
723 Grouping approaches are necessary to perform risk assessment based on limited data. The
724 GRACIOUS approach presented here provides an intuitive way to group NFs based on hypotheses
725 and using an IATA that guides the user to an outcome. We believe this approach is a step forward to
726 streamline hazard assessment of NFs and hope it will be expanded in the future to allow growth of
727 safe nanotechnology.
728

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734

735 ## Author Disclosure Statement

736 No competing financial interests exist.
737

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