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ARTICLE

Integrated approaches to testing and assessment for grouping nanomaterials following dermal exposure

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ABSTRACT

Exposure to different nanoforms (NFs) *via* the dermal route is expected in occupational and consumer settings and thus it is important to assess their dermal toxicity and the contribution of dermal exposure to systemic bioavailability. We have formulated four grouping hypotheses for dermal toxicity endpoints which allow NFs to be grouped to streamline and facilitate risk assessment. The grouping hypotheses are developed based on insight into how physicochemical properties of NFs (i.e. composition, dissolution kinetics, size, and flexibility) influence their fate and hazard following dermal exposure. Each hypothesis is accompanied by a tailored Integrated Approach to Testing and Assessment (IATA) that is structured as a decision tree and tiered testing strategies (TTS) for each relevant question (at decision nodes) that indicate what information is needed to guide the user to accept or reject the grouping hypothesis. To develop these hypotheses and IATAs, we gathered and analyzed existing information on skin irritation, skin sensitization, and dermal penetration of NFs from the published literature and performed experimental work to generate data on NF dissolution in sweat simulant fluids. We investigated the dissolution of zinc oxide and silicon dioxide NFs in different artificial sweat fluids, demonstrating the importance of using physiologically relevant conditions for dermal exposure. All existing and generated data informed the formulation of the grouping hypotheses, the IATAs, and the design of the TTS. It is expected that the presented IATAs will accelerate the NF risk assessment for dermal toxicity via the application of read-across.

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Artificial sweat fluids; dissolution; read across; nanoform

1. Introduction

Skin exposure to nanomaterials (NMs) or nanoforms (NFs) may occur through the intentional use of consumer products (e.g. cosmetics, sunscreens, and wound dressing) and therapeutic applications (e.g. drug delivery) or unintentionally, including occupational settings, where NFs are manufactured, used or handled (e.g. aerosol, dust and paste formulations) (Gautam, Singh, and Vijayaraghavan 2011; Shepard and Brenner 2014; Mohajerani et al. 2019). The definitions of an NM and an NF are shown in the Supporting Information (Table SI1). The term NF is preferred in this paper for its regulatory relevance in the European Union (EU).

Skin irritation and sensitization data are required as part of the core data set to support safety assessment of substances under most regulatory frameworks for chemicals in the EU, including the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) (European Commission (EC) 1907/2006), the Cosmetic Product Regulation (CPR) (EC 1223/2009), or the Biocidal Product Regulation (BPR) (European Commission (EC) 528/2012). For instance, the CPR prohibits the testing of cosmetic products and cosmetic ingredients on animals when the substances are

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exclusively registered for cosmetic uses. On the other side, within other regulatory frameworks (e.g. REACH), a stepwise approach is considered to minimize the need for animal testing. The Organization for Economic Cooperation and Development (OECD) recommended that *in chemico* and *in vitro* approaches for skin irritation (OECD TG 439 2021), corrosion (OECD TG 430 2004; OECD TG 431 2014, OECD TG 435 2015), and sensitization (OECD TG 442C 2018; OECD TG 442D 2018; OECD TG 442E 2018) are prioritized over *in vivo* studies (e.g. OECD TG 404 2002; OECD TG 406 2021; OECD TG 429 2010; OECD TG 442A 2018; OECD TG 442B 2018). However, a standalone *in vitro*, *in chemico,* and *in silico* test is generally not sufficient to predict dermal toxicity, but instead, it is a battery of tests. For this reason, to assess whether a substance causes skin sensitization the so called "defined approach" (DA) is proposed (OECD TG 497 2021). This consists of a combination of *in chemico, in silico,* and *in vitro* methods which provide information on the key events of the skin sensitization adverse outcome pathway (AOP) (OECD TG 168 2012) and result in data interpretation using a fixed data interpretation procedure (DIP) (e.g. a mathematical, rule-based model).

Moreover, regulatory and ethical demands have driven also the development of Next Generation Risk Assessment (NGRA) for skin sensitization to replace animal models. Especially substances used exclusively as cosmetic ingredients benefit from this new approach. The applicability of the NGRA framework was demonstrated by using the case study of geraniol (a face cream ingredient) (SCCS/1628/21).

In silico or grouping and read-across approaches (see section Grouping and read-across – specific considerations for nanoforms within European regulatory frameworks) can further reduce experimental testing and could be valid alternatives to *in vivo* studies when *in chemico/in vitro* methods are not applicable for the substance, or the results are not adequate for classification and risk assessment (Annex VII of the REACH Regulation).

Although dermal penetration is not considered as being part of key events of the skin sensitization AOP (OECD TG 168 2012) is a prerequisite for a substance to cause skin sensitization (ECHA 2017a). Moreover, its contribution to systemic bioavailability is a highly relevant parameter in both the CPR, the BPR, and in other assessments conducted by the Scientific Committee on Consumer Safety (SCCS). For instance, in the SCCS/1618 (2021), focused on the safety of NFs in cosmetics, information on dermal penetration is considered one of the key recommendations for safety assessment of NFs intended for use in cosmetics as the application of NFs in cosmetics is generally based on the industry assumption information that there is no dermal penetration. In this work, we have focused on meeting the requirements for regulators in the EU when investigating the toxicity of NFs following dermal exposure, but the issues raised are relevant globally.

1.1. Grouping and read-across - specific considerations for nanoforms within European regulatory frameworks

A chemical substance can exist in many different NFs (European_Commission (EC) 275 2011). In the EU, since January 2020, NFs of substances need to be registered under REACH (European Commission (EC) 1881 2018). Registration can be done for a single NF or sets of similar NFs, where the latter case means a special form of grouping with all the NFs being within clearly defined and narrow boundaries such that all the hazard, exposure, and risk assessments can be performed jointly (European Commission (EC) 1881 2018). The grouping has evolved as an important tool in the hazard assessment of NFs as it minimizes the need for experimental testing, with NFs being grouped based on similarities in their physicochemical (PC) properties, fate, and/or hazard. Once a group has been established, read-across may be performed, e.g. to fill in data gaps for one toxicological endpoint.

Read-across is an approach used to predict endpoint-specific information (results of an experimental study) of a 'target' substance (i.e. for which data is lacking) by using data from another similar substance (the 'source material'). The generic guidance on read-across established that structural similarity is a prerequisite for any grouping and read-across approach (OECD 2014; ECHA 2017b).

For substances, according to the OECD guidance (OECD 2014), the rationale underpinning grouping may be based on similarity due to:

- common functional group(s)
- common constituents or chemical classes

- similar carbon range numbers
- common AOP or mode of action
- likelihood of common precursors and/or breakdown products via physical or biological processes that result in structurally similar chemicals
- incremental and constant change across the category.

The generic ECHA guidance for all substances (ECHA 2017b) mostly follows these same principles. The ECHA also issued specific guidance for NFs (ECHA 2019). In this case, structural similarity (based on size, surface area, etc.) alone is not sufficient to justify read-across, and similarity by functional properties (e.g. dissolution, reactivity, etc.) needs to be demonstrated (ECHA 2019).

Under the framework of the CPR (EC 1223/2009), the use of read-across is considered in the characterization of the toxicological profile of substances. Although no specific guidance has been issued to address NF dermal exposure, the SCCS recently issued Scientific Advice that includes criteria for prioritization of risk potential of NFs in cosmetics (SCCS/1618 2021). Indeed, SCCS has identified some NF PC properties (e.g. size, chemical nature, surface modifications/coating, and persistence to dissolution) and exposure conditions (e.g. frequency and the amounts used and potential for systemic exposure and accumulation in the body) that could raise safety concerns on NFs when used in cosmetic products. Under the BPR (European Commission (EC) 528/2012), read-across is also considered, but no specific guidance addressing NFs has been issued so far.

1.2. The GRACIOUS framework and the dermal grouping hypotheses generation

Grouping decisions need to be hypothesis-driven and evidence-based (ECHA 2019). Identification of the PC properties that can be mechanistically linked to a hazard endpoint is the initial requirement for the generation of a scientifically robust hypothesis for grouping to support read-across for this endpoint. The EU Horizon 2020 funded project GRACIOUS has generated a Framework to support the generation and testing of grouping hypotheses to facilitate read-across for NFs (Stone et al. 2020).

Grouping hypotheses have been formulated following an in-depth systematic survey of the published and gray literature. For human health, these hypotheses consider exposure via different routes, namely inhalation (Murphy et al. 2021; Braakhuis et al. 2021), oral (Di Cristo et al. 2021), and dermal exposure. For the environment, these hypotheses consider release into different compartments including water, sediment, and soil (Stone et al. 2020). Each of the GRACIOUS grouping hypotheses is accompanied by a tailored Integrated Approach to Testing and Assessment (IATA) (OECD 2018) which specifies what information is required to accept or reject the grouping hypothesis and to frame the similarity assessment between the target NF(s) and the source material(s). The similarity assessment is required to ensure the NFs are sufficiently similar to be grouped; this assessment could be qualitative (based on expert judgment) or quantitative (mathematically derived limits of similarity) depending on the purpose of the grouping (Jeliazkova et al. 2021).

The IATAs are structured as decision trees that are formed from a series of decision nodes (DNs) that ask for specific information on relevant grouping criteria (such as physicochemical parameters, fate, and hazard biomarkers). Questions posed in each DN of an IATA can be answered with information obtained from the existing literature/data, based on expert judgment, or by newly generated data, guided by a tiered testing strategy (TTS). IATAs have already been generated for respirable high aspect ratio NFs (Murphy et al. 2021), for non-fibrous respirable NFs (Braakhuis et al. 2021), and ingested NFs (Di Cristo et al. 2021).

In addition to supporting regulatory dossier generation, the GRACIOUS Framework also allows application of grouping and read-across for several other purposes, including the adoption of precautionary risk management measures, the design of more efficient hazard testing, and for safe(r) by design (SbD) decision-making.

In this manuscript, we summarize the rationale and evidence from existing data on skin irritation, skin sensitization, and the dermal penetration of NFs that underpin the development of the dermal grouping hypotheses, IATAs, and the design of the TTS. Experimental work reported here on NF dissolution in artificial sweat further informed the design

of the IATAs and the TTS for the dermal grouping hypotheses.

2. Evidence required to substantiate the human dermal grouping hypotheses, the IATA formulation, and design of the TTS

To ensure the grouping hypotheses were evidence-based, a targeted literature search on existing information on the properties of NFs which influence their fate and hazard following dermal exposure was conducted. Information on *in vivo/in vitro* NF skin irritation, skin sensitization, skin penetration, and lastly *in vitro* dissolution of NFs in sweat simulant fluid was collected using online resources such as PubMed and Google scholar databases. Publications from 2007 to 2021 were screened. When available, studies evaluating commercial formulations (NFs embedded in products) were taken into account. The following sections summarize the main information identified and utilized.

2.1. Skin irritation

The limited existing data available on skin irritation of NFs has been summarized in Table SI2. Most of the existing studies suggest that there is no NF-specific induction of skin irritation. However, the diversity of materials tested in skin irritation studies is still limited. Knowledge of the chemical composition of NFs can be used to identify NFs that may exhibit irritant effects and more specifically NFs that contain substance(s) classified under CLP as skin irritants or sensitizers or that degrade to the same ionic or molecular form as a substance classified as skin irritant or sensitizer. Such irritant effects are then dependent on NF dissolution, and the ability to identify the contributions of the ionic and particle fractions of the NF to skin irritation. Due to the lack of available experimental evidence on skin-irritating NFs, it is currently not possible to formulate grouping hypotheses on skin irritation based on NF PC properties other than chemical composition and dissolution rate.

2.2. Skin sensitization

The limited data available on skin sensitization of NFs has been summarized in Table SI3. The majority of NFs tested so far have been concluded to be non-sensitizers. Exceptions relate to nickel and silver NFs, which induced sensitization in studies that used a nonstandard modification of the local lymph node assay (LLNA) test involving co-administration of lipopolysaccharide (LPS) (Hirai et al. 2016). Some silver NFs were also identified as skin sensitizers in studies that do not involve the LPS co-administration (Kim et al. 2013; Zelga et al. 2016), but in other studies they were negative (SCCS/1596 2018). The discrepancies between studies are likely to be due to differences in the silver NFs PC properties that were tested, and the experimental design employed in the studies.

As described for skin irritation, the chemical composition of NFs is important and NFs of substances that exhibit sensitizing effects in their ionic or bulk forms would need special attention. In addition, NF dissolution should be assessed to identify the contribution of the ionic and particle fractions to toxicity (Peijnenburg et al. 2020). To cause sensitization, NFs would need to reach viable layers of skin, so the assessment of skin penetration of the NF is important. It is possible that NFs (or their dissolution products) could interact with proteins, potentially changing the conformation of those proteins to promote skin sensitization (Grundström and Borrebaeck 2019).

Therefore, the assessment of skin penetration could proceed with the assessment of skin sensitization, or at least should be assessed in parallel to skin sensitization to better understand the outcome of dermal exposure.

2.3. Skin penetration

At the moment limited information is available on dermal toxicity of NFs in relevant formulations in occupational and consumer real-life exposure scenarios (e.g. NFs industrially used pastes or NFs incorporated in consumer products). This hampers the direct applicability of some of the IATAs that we are presenting. However, NF migration and dissolution studies in exposure-relevant formulations/products, with emphasis on potential changes in agglomeration/aggregation state and surface properties, would allow this gap to be filled.

Some reviews on the dermal penetration of NFs, are available (Poland et al. 2007; Lohani et al. 2014; Marquart et al. 2020), including our recent review

which has an emphasis on quantitative parameters (Gimeno-Benito et al. 2021). Lohani et al. (2014) reported that NF penetration was restricted to the uppermost layers of the stratum corneum. Poland et al. (2007) concluded that, despite many conflicting results, absorption of particles in the nano range through the skin is possible, although to a very low degree. Marquart et al. (2020) found indications for a decrease in dermal penetration with increasing particle sizes, agglomeration, and positive surface charges. In our recent review, Gimeno-Benito et al. (2021), we also concluded that a small percentage of the applied NF dose penetrated the skin surface and reached deeper skin layers (even for systemic toxicity studies), and we proposed a worst-case dermal penetration value of 1% for NFs. Since quantitative studies were often based on elemental analysis, such penetration values might partly be due to NF dissolution.

Skin penetration is also linked to NF flexibility. For instance, NFs that act as nanocarriers can be specifically modified to change their morphological conformation when in contact with the cell membrane leading to a higher penetration by the particle itself (Teixeira et al. 2010; Rastogi, Anand, and Koul 2009; Fang et al. 2008).

The datasets included in the review by Gimeno-Benito et al. (2021) covered a considerable span of surface properties including hydrophobic and hydrophilic, with surface charge including positive, negative, or neutral. The need to include NFs varying in such PC characteristics highlights the challenge of deriving relevant boundaries for scientifically valid grouping hypotheses from a limited but diverse data set. Most NFs included were considered spherical with particle sizes ranging from 2.1 nm to submicron sizes, although in several studies nanorods were also included (reported aspect ratios up to 4:1). Due to concern that the penetration of NFs increases with decreasing particle size, together with a limited number of studies with particles below 5 nm, the derived worst-case penetration estimate applied to NFs equal or larger than 5 nm.

2.4. Dermal dissolution of nanoforms

Dissolution is widely recognized as a key determinant of NF biokinetics, influencing bioavailability, transformation, biopersistence, and therefore toxicity (Utembe et al. 2015). Dissolution is strongly dependent on media parameters such as pH, ionic strength, counter-ions, and complexing agents (Utembe et al. 2015 and Innes et al. 2021). In the design of the inhalation (Braakhuis et al. 2021) and oral (Di Cristo et al. 2021) grouping IATAs, dissolution was the first key step of each IATA to determine whether the exposures involved intact NFs and/or dissolved ions/molecules, as well as the location of potential dissolution and predict the biopersistence of the NF. For the lung, the relevant fluids for dissolution were the lung lining fluid (LSF) and the phagolysosomal fluid (PSF), and for the oral IATA the relevant fluids were the oro-gastrointestinal (OGI) fluids and again the PSF.

Similarly, in the context of dermal toxicity, the biological fluids of the skin determine the extent of dissolution and ions release from the NF and could be used to justify grouping and read-across for dermal penetration and to explain the eventually observed dermal toxicity. It is expected that the fraction of NFs that penetrate viable layers of the skin would be taken up by immune cells (e.g. macrophages and Langerhans cells). Therefore, NF dissolution in lysosomes is also likely to be relevant in terms of predicting NF accumulation in the skin.

Although little work has been done on assessing the dissolution of NFs in artificial sweat, different methodological approaches have been used proposed (Table SI4). The main message of these works (Li, Chen, and Jiang 2007; Qian et al. 2021; Windler et al. 2012; von Goetz et al. 2013; Peloquin, Baumann, and Luxton 2020; Hedberg et al. 2021; Hui et al. 2019) is that NF dissolution in sweat is dependent on the NF chemistry and environmental conditions (e.g. pH). In the case of NFs present in formulations or products such as textiles, their composition will affect their migration, agglomeration/aggregation state, eventual release, and dissolution. Some of the studies in Table SI4 actually evaluated the dissolution of NFs incorporated in textiles or skin products. Different standardized artificial sweats vary in their composition (Table SI5), especially for the absence (e.g. International Organization for Standardization (ISO) 3160-2 2015, European Standard (EN) 1811 2011) or presence of amino acids such as histidine (ISO 105-04), a representative amino acid of the complex mixture of

proteins commonly present in sweat (Murphy et al. 2019; Harvey, LeBouf, and Stefaniak 2010). Histidine is a well-known complexing agent for bivalent metal ions, with decreasing specificity for cobalt, nickel, and zinc, respectively (Zhou et al. 2013; Glover, Bury, and Hogstrand 2003; McDonald and Phillips 1963). Binding is stronger at basic pH, but ineffective at acidic pH (Zhou et al. 2013), therefore, the presence of amino acids, such as histidine, may impact the dissolution rate of metal-containing NFs. Existing data, therefore, suggests that the release of mono- or bi-valent metal ions from NFs can be expected in artificial sweat in diverse conditions simulating the acidic or neutral physiological pH (Sabella et al. 2014).

Existing studies on NF dissolution in biological media have also varied for whether the test system employed to assess NF dissolution uses dynamic (flow-through, tangential flow, following the ISO_19057:2017) or static conditions.

The dynamic continuous flow systems allow constant replenishment of test fluid and removal of dissolved ions, preventing local saturation points, and therefore aids NF dissolution. In contrast, dissolution is likely to be slower under static test conditions as this method would encourage local saturation points, along with a potential variation in pH due to released solutes, or precipitation and/or nucleation (Alexander et al. 1994; Christensen et al. 1994; Bohner and Lemaitre 2009). Dynamic systems are not without their complications such as the ability of NFs to leak through the filtration systems, resulting in an overestimation of ion release (Utembe et al. 2015). Components of more complex fluids, such as proteins or lipids, may result in the obstruction and eventual rupture of membrane pores used in dynamic systems (Ansoborlo et al. 1999). In addition, the reproducibility of dynamic systems may be hampered by the large volumes of fluid, which are particularly labor-intensive (Farrugia 2002). Moreover, it is not trivial to select a flow rate that most accurately represents a physiological scenario.

On the other hand, static systems involve the exposure of known masses of the particles to a fixed volume of simulated fluid. This method could lead to saturation phenomena of the exposure media leading to the inhibition of dissolution when equilibrium is reached. However, they can still be employed with some caution, such as use with low concentrations and short exposure times to minimize saturation (Utembe et al. 2015).

Limited studies have compared these methods for assessing NF dissolution and there are conflicting outcomes as to which approach (static vs dynamic) is most suitable. For example, the low dissolution rate of gold NFs was found to be similar in a static and dynamic system, when assessed in a cell culture medium and a simulated lung fluid (Breitner et al. 2018). However, the low dissolution rate of $BaSO_4$ NFs was shown to increase in a dynamic system compared to a static system when using a lysosome-simulant fluid, with this increased dissolution correlating well with *in vitro* dissolution and clearance *in vivo* (Keller et al. 2020). No comparative studies have used simulated sweat.

3. Results-dermal grouping hypotheses

Four grouping hypotheses that consider the fate, as well as local and systemic toxicity of NFs following dermal exposure, have been generated (Table 1) based upon the evidence gathered during the literature review detailed above. The grouping hypotheses we have developed for the dermal route of exposure are focused on grouping NFs based on their chemical composition (H-D-1),

Table 1. Human health grouping hypotheses for NFs developed for the dermal route of exposure (H-D-).

Human dermal hypotheses	
H-D-1	NFs with constituent substance(s) or degradation products classified for dermal irritation or sensitization: Dermal exposure to the NFs will result in comparable dermal irritation or sensitization depending on NF dissolution rate.
H-D-2	NFs with an instantaneous dissolution: Following dermal exposure, instantaneously dissolving NFs will dissolve into their molecular or ionic form and will cause similar toxicity as substances instantaneously releasing, dissolving and/or transforming into the same ionic or molecular forms.
H-D-3	NFs that are not biopersistent: Dermal exposure to NFs will not lead to accumulation of NFs or subsequent systemic toxicity.
H-D-4	NFs that are larger than 5 nm and which are not flexible: Following dermal exposure, NFs will result in limited or no dermal absorption and no dermal or systemic toxicity.

dissolution kinetics (H-D-1,2,3), size, and flexibility (H-D-4) as these properties relate to specific fate and hazard outcomes.

4. Results- IATAs to support grouping hypotheses and read-across

For each grouping hypothesis, a tailored IATA has been generated, that is a decision tree diagram that guides the users on the information needed to allow the grouping hypothesis to be accepted or rejected.

4.1. H-D-1: NFs with constituent substance(s) or degradation products classified for dermal irritation or sensitization: Dermal exposure to the NFs will result in comparable dermal irritation or sensitization depending on NF dissolution rate

This hypothesis suggests that NFs with constituent substance(s) or degradation products already classified for skin irritation or sensitization could also be considered as skin irritants or skin sensitizers depending on their dissolution rate (Figure 1). The main purpose of this hypothesis is to conclude whether the presence of chemical components with CLP classifications for dermal irritation/sensitization (European Commission (EC) 1272/2008) should lead to the same CLP classification for the NF. When a NF is part of a product or formulation, the aging (e.g. agglomeration, dissolution, change in surface properties) and release of the NF from that system needs to be assessed by the producers according to regulators (Fytianos, Rahdar, and Kyzas 2020; SCCS/1611 2019), simulating relevant use/exposure conditions. Subsequently, the remaining questions of the H-D-1 IATA can be considered. The same approach could be applied to the other developed IATAs, extending the applicability of the dermal IATAs to NFs as part of products. The DNs of the IATA for this grouping hypothesis request gathering information on chemical composition, including impurities, as well as dissolution to support read-across of hazard data between the CLP, classified chemical

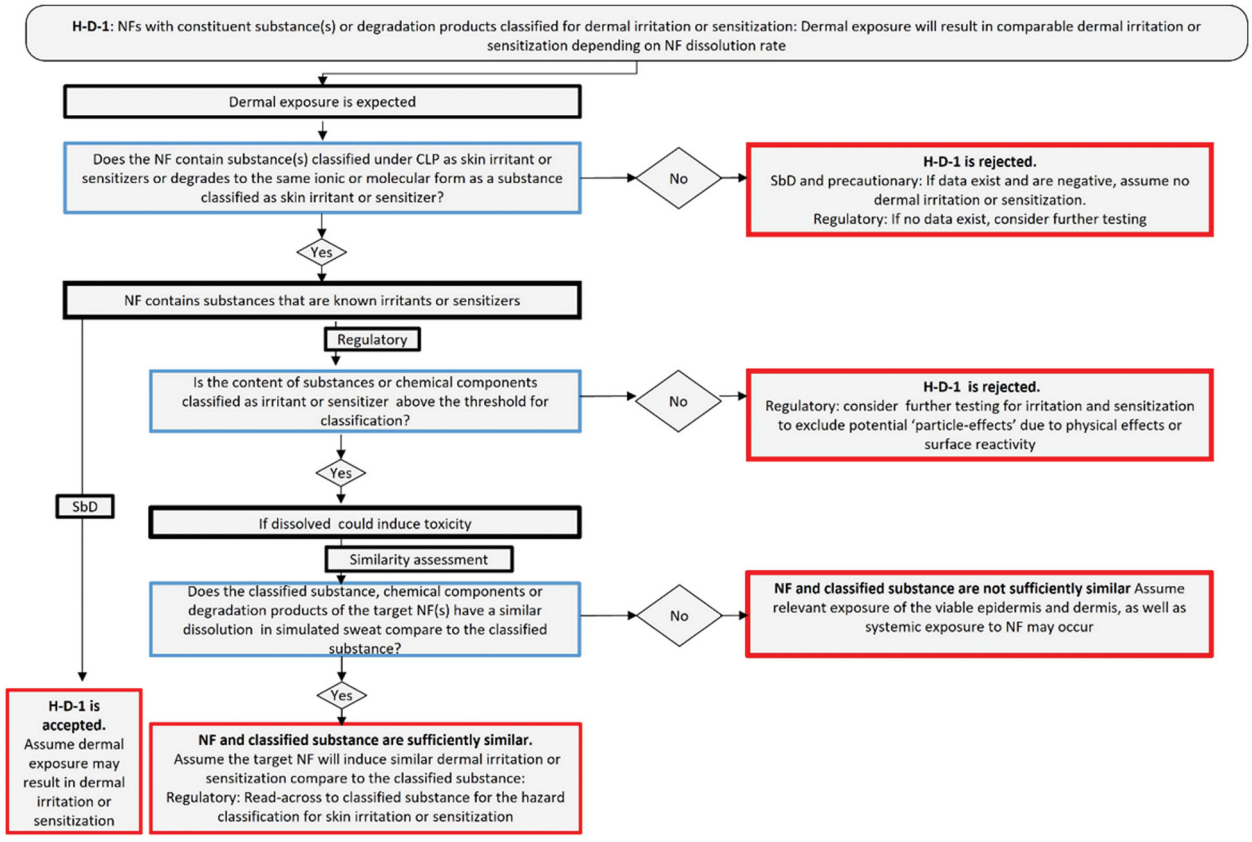

Figure 1. IATA for H-D-1. Blue bordered boxes are decision nodes, red bordered boxes are hypothesis conclusions, black bordered boxes describe options to consider.

components, and the NF. The first DN asks whether the NF contains substance(s) classified under CLP as a skin irritant or sensitizer. If existing information on chemical composition data is not available via databases or the peer-reviewed literature or is not sufficient, the IATA suggests relevant testing via the TTS (see section Results-DNs and their associated TTS). For regulatory purposes, the user is prompted to continue in the IATA and provide further evidence on the amount and bioavailability of the classified substance present as or in the NF. For SbD and precautionary purposes, the assumption that dermal exposure may result in dermal irritation or sensitization based on the chemical composition alone may be sufficient to support grouping and decision making according to common hazard outcomes.

In the second DN, the composition of the classified substances/degradation products in/from the NF is compared with the threshold limit for the classification of mixtures (following the principles outlined by the CLP regulation). If the irritant/sensitizer substance(s) is present at a low level (i.e. below the threshold) the NF would not be classified as a skin irritant or sensitizer.

In contrast, if the content of these classified substances/degradation products in/from the NF is above the classification threshold, then the user proceeds to the next DN which addresses the dissolution of such irritant or sensitizer substances, by looking at the amount of dissolved fraction over a relevant time frame. Here, a similarity assessment for regulatory purposes is required to compare the classified substance and the target NF(s) for the dissolution rate. Indeed, the dissolution kinetics of the NF can provide insights on the presence and role of NFs and/or constituent ions for dermal adsorption and penetration if measured in time frameworks relevant for skin physiology (see more experimental information on dissolution in section Results-DNs and their associated TTS).

Residence times of chemicals in the skin usually vary from 8 to 24 hours, which also allow removal by desquamation and skin washing. In the case of particles, these may accumulate in hair follicles leading to longer residence times. For example, on *in vitro* porcine skin (an appropriate model for human tissue) particles of 320 nm were detected in hair follicles up to 10 days after exposure (Lademann et al. 2007). The fraction penetrating the skin in its particulate form (in this timeframe) is estimated to be minor (<1% for most NFs) (Gimeno-Benito et al. 2021), so could be neglected in terms of contribution to the release of irritants/sensitizers. These data altogether suggest that 10 days is a conservative but relevant residence time for NFs in the skin.

The estimated percentage of dissolution over this time frame (10 days), could be informative of the fraction of irritant/sensitizer substance released, and therefore whether it would or would not exceed the proposed threshold for classification. The generic concentration limits for the classification of mixtures for skin sensitizers and skin irritants are 0.1% and 1% (European Commission (EC) 1272/2008) respectively. If no specific concentration limit applies for the substance of interest, such levels could be used as thresholds. For example, if the % dissolution of skin sensitizing substance(s) over 10 days (versus total NF mass) is below the 0.1% threshold, that NF would not require CLP classification as a skin sensitizer/irritant.

The outcome for this IATA will therefore help predict skin sensitization/irritation endpoints from a classified substance to target NFs and thus support CLP classification. If the release rate of CLP classified components from a target NF is shown to be the same or lower than the dissolution rates of a classified substance, it is considered acceptable to read-across negative hazard data from the source to the target. Indeed, if toxicological data showing negative hazard data for skin sensitization/irritation endpoints exist for the classified substance then a similar conclusion of negligible hazard potential can be assumed for the target NFs, once that particle specific effects for the same endpoint are excluded.

On the other hand, a low hazard potential cannot be assumed to apply to a target NF with a higher rate of dissolution of CLP classified constituents. For this grouping hypothesis the higher the dissolution rate, the higher is the concern regarding potential hazard, thus only dissolution data in relevant simulated fluids (sweat or the intended formulation) should be acceptable. Dissolution data from other physiological media should not be regarded as valid approximations.

4.2. H-D-2: NFs with an instantaneous dissolution: following dermal exposure, instantaneously dissolving NFs will dissolve into their molecular or ionic form and will cause similar toxicity as substances instantaneously releasing, dissolving and/or transforming into the same ionic or molecular forms

Our second grouping hypothesis suggests that following dermal exposure, NFs with an instantaneous dissolution in sweat fluids will dissolve into their molecular or ionic form before they reach the viable layers of the skin (Figure 2). The main DN in the IATA is focused on the dissolution in simulated sweat (see section Results-DNs and their associated TTS for details). If this half-life is below 1 hour the hypothesis is accepted and read-across to the dissolution products is possible. On the contrary, if the half-life value is above 1 hour the user should reject the hypothesis and should consider that exposure of viable layers of the skin to particles is possible and that the NF may exhibit toxicity via particle and/or ion mediated effects (see H-D-1, H-D-3, and H-D-4). The 1-hour timeframe is a threshold derived from the analysis of existing studies on skin penetration summarized in the review of Gimeno-Benito et al. (2021). From this analysis, it appears that skin penetration of NFs is a rather slow process. From a regulatory perspective, only EFSA has defined a relevant cutoff value for the dissolution rate of nanomaterials in the conditions of the gastrointestinal tract (EFSA Scientific Committee 2021). In other areas, regulators, such as the ECHA, do not define any cutoffs about nanoparticle dissolution (ECHA 2021) but we believe that the proposed cutoff, as physiologically relevant, could be a good proposal to suggest in regulatory contexts. In that sense, this cutoff provides a starting point for discussion with regulators where publications on the background of the cutoff are a key requirement. The same comment applies to the other cutoff proposed in the H-D-3 IATA (see section H-D-3: NFs that are not biopersistent: Dermal exposure to NFs will not lead to accumulation of NFs or subsequent systemic toxicity). This IATA does not include any other DNs but drives the user decision depending on the purpose of grouping. For SbD and precautionary measures, no nano-specific risk assessment is needed while for regulatory purposes read-across to non-nanomaterial forms with the same soluble constituent ionic or molecular forms can be performed. Here, the user can assume the same CLP classification and dermal Derived No-Effect Levels (DNELs) for the NF as for the source material.

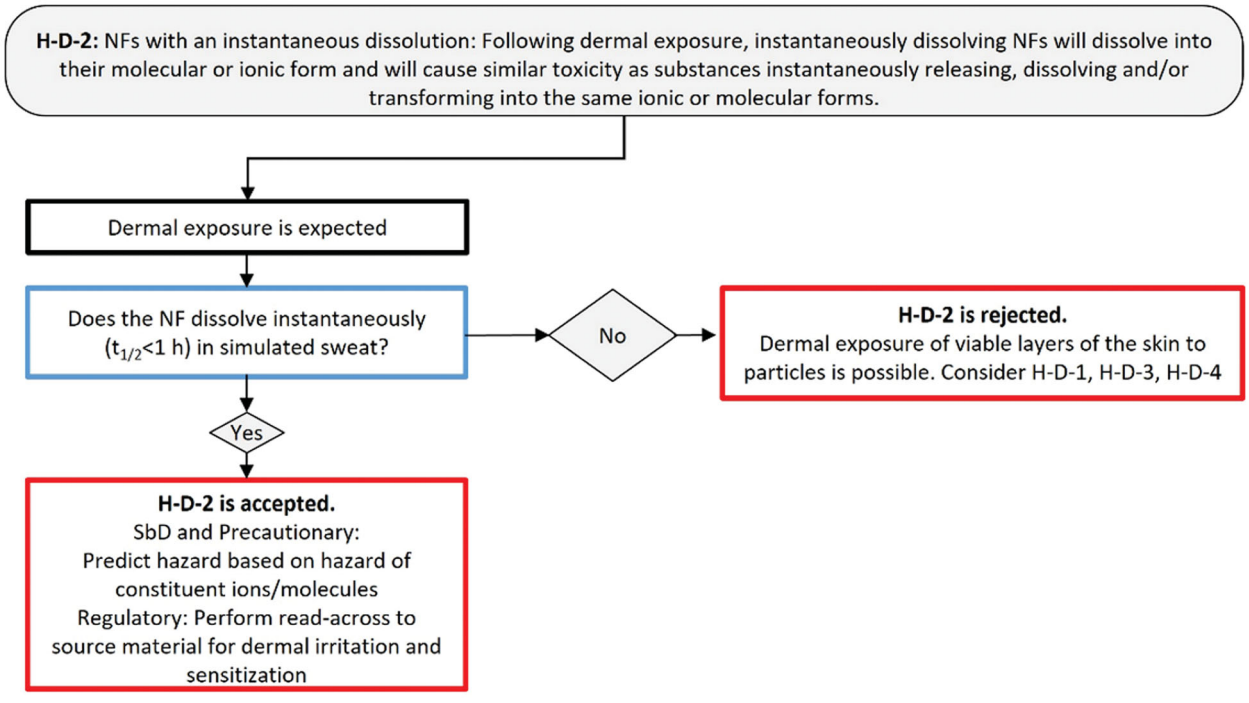

Figure 2. IATA for H-D-2. Blue bordered boxes are decision nodes, red bordered boxes are hypothesis conclusions, black bordered boxes describe options to consider.

4.3. H-D-3: NFs that are not biopersistent: Dermal exposure to NFs will not lead to accumulation of NFs or subsequent systemic toxicity

NFs which do not dissolve instantaneously in simulated sweat may potentially be absorbed across the skin barrier and come into contact with the interstitial fluid of the viable dermal layers (neutral pH) or PSF (low pH) after uptake by resident cells (e.g. macrophages and Langerhans cells). If skin penetration/absorption occurs, the measurement of dissolution in relevant biological compartments (i.e. PSF) can therefore be relevant to predict biodurability, intracellular accumulation, local and possibly systemic toxicity. This grouping hypothesis states that NFs that quickly dissolve in PSF fluid will not lead to accumulation or subsequent particle-specific systemic toxicity (Figure 3).

The main purpose of this hypothesis is to exclude systemic toxicity associated with the translocation of NFs into the blood following dermal exposure. Therefore, the main DN in this IATA is the measurement of NF dissolution in simulated PSF as this fluid mimics the acid phagolysosomal environment of residence macrophages, the cells responsible for particle clearance from tissues (see section Results-DNs and their associated TTS for details) (Koltermann-Jülly et al. 2018). If the dissolution half-life is below 48 hours, it is concluded that there are no concerns about the systemic accumulation of NFs as they dissolve rapidly to constituent ions within the acidic environment of the lysosome (Braakhuis et al. 2021). This threshold reflects a timeframe whereby NFs may be taken up by cells (e.g. macrophages), but they will dissolve rapidly to ions within the acidic environment of the lysosome (Keller et al. 2020) by delivering potentially toxic ions to the intracellular environment (Naasz, Altenburger, and Kühnel 2018; Hsiao et al. 2015). Therefore, the dissolution half-life below 48 h does not exclude per se any concerns. Indeed, it only excludes the eventual potential of the NF to create bioaccumulation according to the current data (Braakhuis et al. 2021). The possibility of systemic hazard linked to other toxicity mechanisms is thus not excluded. For regulatory purposes, the user should perform a similarity assessment for dermal toxicity based on comparison to source materials which may include non-biopersistent NFs and/or constituent ions or molecules, to support read-across. This process helps the user to assess whether a target NF is sufficiently similar to the source material to allow grouping and to assume the target NF will induce similar toxic outcomes compared to the source material. Therefore, this IATA can enable read-across to be performed on dermal toxicity endpoints for NFs of the same substance. To proceed with such similarity assessment the user should consider some NF key features highlighted in the IATA DNs (size, chemical composition, hydrophobicity, and reactivity) for deriving similar toxicity outcomes between the target NF(s) and the source material (see section Results- IATAs to support grouping hypotheses and read-across).

For the DN which addresses the size of the NF, the user should also consider agglomeration. Large/strong agglomerates are expected to limit dermal penetration to a greater extent than smaller or more labile agglomerates, and this is therefore a key parameter to consider when comparing two NFs. Moreover, agglomeration would generally be higher in a media with higher ionic strength, as in the case of sweat (Barreto et al. 2015; Truong et al. 2012; French et al. 2009). Here, the source material requires a smaller aggregated/agglomerated size than the target material to support read-across.

Aggregated size (also in the case of stable agglomerates) is likely to be a stronger determinant of dermal penetration than constituent particle size since disaggregation in the skin is unlikely and should be evaluated using the same test method and media for all NFs under comparison. However, there is no direct experimental evidence of this, since aggregated/agglomerated size is typically not evaluated in NF dermal penetration studies.

The subsequent DN to consider for the similarity assessment is focused on NF chemical composition. Chemical composition should, in principle, not influence dermal penetration, beyond changes due to hydrophobicity or reactivity that are considered in an independent DN. An exception could be when additives, surface treatments, or impurities are known to be dermal penetration enhancers (Lane 2013; Williams and Barry 2012). However, as the IATA is mainly used for read-across for dermal toxicity endpoints such as sensitivity/irritation, then similarity in chemical composition becomes more relevant. To support read-across with the lowest

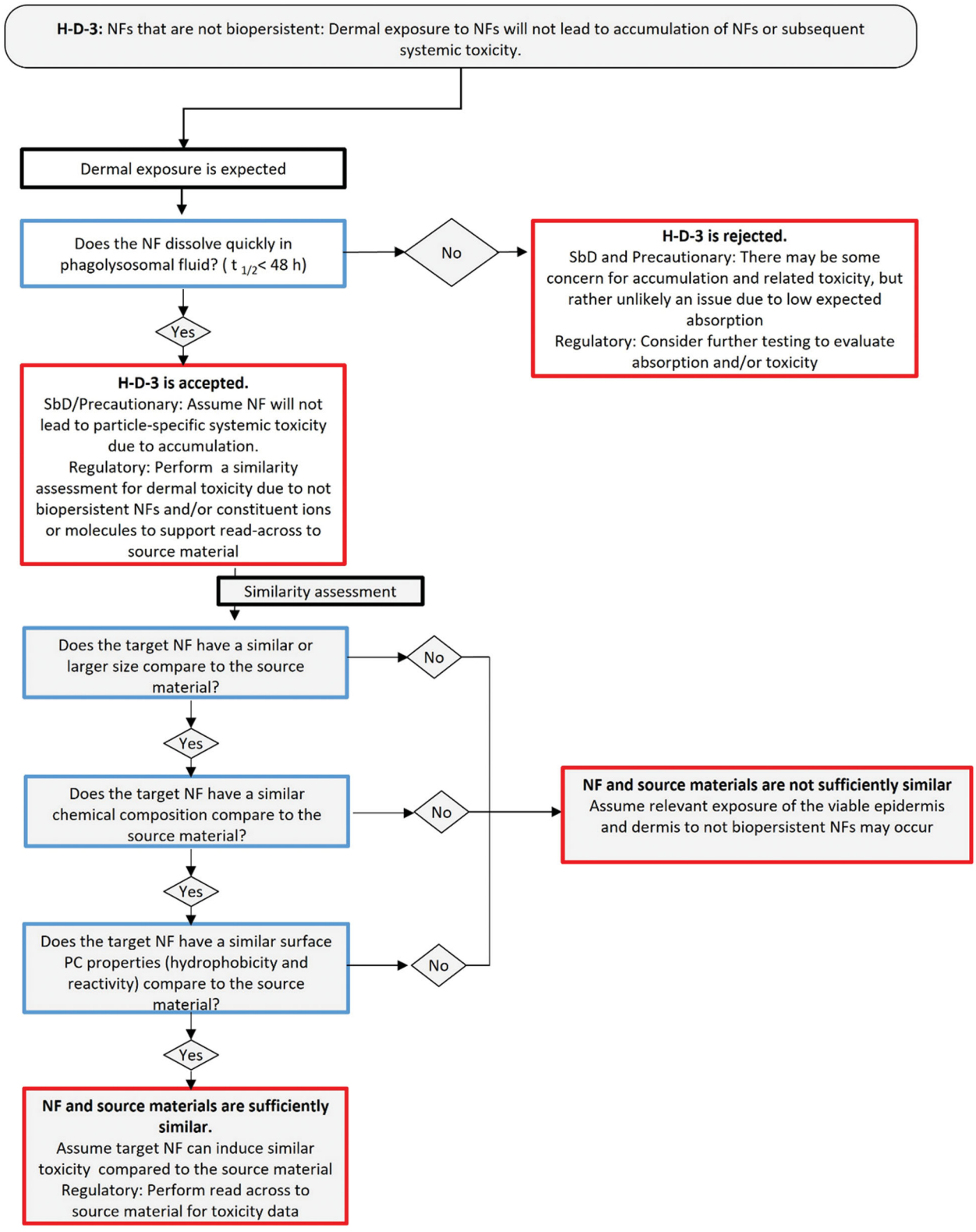

Figure 3. IATA for H-D-3. Blue bordered boxes are decision nodes, red bordered boxes are hypothesis conclusions, black bordered boxes describe options to consider.

level of uncertainty would require NFs to have identical chemical constituents (core and surface) and also similar relative content of impurities and additives. Currently, due to the limited number of studies reported, it is not possible to predict the impact of different surface treatments or chemical

compositions on NF fate and hazard after dermal exposure. However, as the number of datasets increases, it might be possible to also justify read-across, in terms of absorption or particle-specific effects, between NFs with different surface treatments or even chemical composition, as long as they are similar in the remaining parameters covered by the DNs of this IATA.

Lastly, the IATA contains a specific DN on two distinct PC surface properties of an NF, such as the hydrophobicity and reactivity, that can influence the dermal penetration. In relation to hydrophobicity, the lipidic nature of the stratus corneum is a very effective barrier for hydrophilic substances, whereas the viable epidermis is an effective barrier for highly lipophilic compounds (ECHA 2017a). Substances with moderate hydrophobicity have generally been considered to penetrate the skin easier than those with more extreme (hydrophilic or hydrophobic) values, with optimal values around log P of 2 to 3 (Kasting et al. 2019). Indeed, hydrophilic substances are known to preferably penetrate through skin appendages, e.g. hair follicles and sebaceous glands (Kasting et al. 2019), a route that might also be relevant for NFs. The ECHA guidance reduces the default absorption factor for substances from 100% to 10% when their log P is outside the range [-1, 4]. Log P values cannot be calculated for NF, as they are based on octanol-water distribution at equilibrium. Instead, surface contact angle to water can be used as a descriptor of NF hydrophobicity, with $\theta < 90^{\circ}$ considered hydrophilic and $\theta > 90^{\circ}$ considered hydrophobic. Further data is needed to establish which range of surface contact angles could be considered sufficiently similar to support read-across of NFs.

Surface reactivity is expected to be the main determinant of the toxicity of NFs (ECHA 2019). Indeed, highly reactive NFs might induce irritation reactions. For instance, NFs with photocatalytic properties (e.g. titanium dioxide and zinc oxide NFs) are considered highly reactive (Sanders et al. 2012, Wang et al. 2013) as such PC properties increase the production of reactive oxygen species (ROS).

Moreover, although a specific sensitizing response to NFs is rather unlikely, they could behave as co-adjuvants, exacerbating the sensitizing responses to other sensitizers. Read-across toward source NFs with higher reactivity/toxicity in any of these assays than the target NFs should be possible and generally conservative so that if a similarity threshold would still be deemed necessary, this could be rather wide. Otherwise, differences in equivalent effect doses for two NFs in any of these studies should be rather narrow, possibly aligned to the typical reproducibility of these assays.

4.4. H-D-4: NFs that are larger than 5 nm and which are not flexible: following dermal exposure, NFs will result in limited or no dermal absorption and no dermal or systemic toxicity

This hypothesis considers particle translocation across the dermal barrier (Figure 4) and the purpose of this IATA is to group NFs to enable read-across for dermal penetration or toxicity endpoints, in terms of particle-specific effects. Due to the focus on particle translocation, this hypothesis does not apply to NFs that undergo instantaneous dissolution. The first DN in this IATA gathers information on dissolution in sweat and thereby allows exclusion of instantaneously dissolving NFs for which particle-specific effects are not of concern and to identify NFs with longer dissolution half-times for which particle effects are of higher concern.

The second DN addresses NF flexibility. Some NFs are flexible or can change their morphological conformation. It has been demonstrated that such property enhances particle penetration through the skin (Teixeira et al. 2010; Rastogi, Anand, and Koul 2009; Fang et al. 2008). For instance, flexible nanomaterials, such as ethosomes and polymeric nanoparticles favor drug penetration in comparison to nanoparticles that do not allow for modifications to their matrix softness and traditional liposomes, respectively. However, Dianzani et al. (2014), highlighted that there are controversies regarding the ideal flexibility of nanoparticles to optimize skin penetration for nanoparticles used for topical drug delivery. Therefore, NFs that are flexible or can change their morphological conformation are outside the limits of the applicability of the H-D-4 IATA. Information about NF flexibility may be known to the user, as it may be related to its function. Otherwise, it can be deduced, qualitatively, from TEM microscopy analysis (see section Results-DNs and their associated TTS for details) or evaluated by a quantitative method such as that

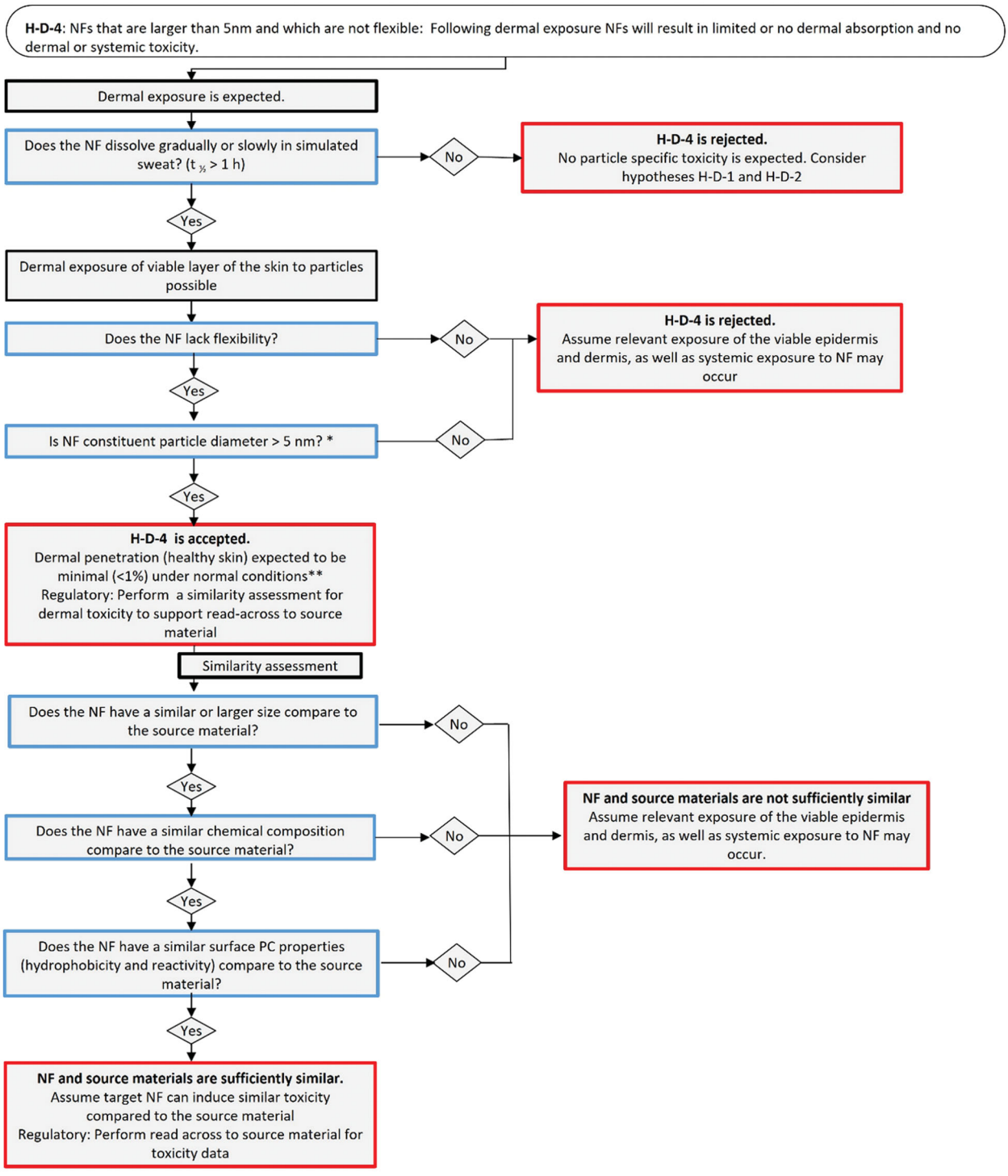

Figure 4. IATA for hypothesis H-D-4. Blue bordered boxes are decision nodes, red bordered boxes are hypothesis conclusions, black bordered boxes describe options to consider. * for NFs > 5nm the users can proceed to the similarity assessment only if there is an appropriate source material (i.e. smaller than target, for which robust data are available for comparison). **'Under normal conditions' e.g. excluding formulations/coatings intended to increase penetration, and occlusion conditions.

described in Song et al. (2011). However, at this point, this property is only qualitatively considered in the IATA, and this decision node is intended to be addressed as a yes/no decision, even when the IATA is used to compare nanoforms. For NFs lacking flexibility, it is widely hypothesized that increasing size decreases dermal absorption (Bos and Meinardi 2000).

The threshold of 5 nm (the third DN) that we propose for this IATA is considered a threshold

above which dermal penetration is limited, with an estimated default dermal penetration value of no more than 1% of the applied dose (Gimeno-Benito et al. 2021).

If the target NF(s) meet the criteria of the initial DNs related to dissolution, flexibility, and size, a preliminary group can be formed which suggests that dermal penetration of these group members will be limited. On the contrary, if grouping is made for regulatory read-across, a similarity assessment, the same described in section H-D-3: NFs that are not biopersistent: Dermal exposure to NFs will not lead to accumulation of NFs or subsequent systemic toxicity for not biopersistence NF, should be performed. Here, information about size, chemical composition, and surface properties should help the user in understanding if the target NF elicits similar toxicity compared to the source material. Evidence of sufficient similarity could mitigate concerns regarding potential systemic hazards posed by target NFs. If target and source NFs are below the 5 nm threshold a similarity assessment can still be conducted according to the IATA but read-across can only be supported if the source material is smaller than the target and the limits of similarity are narrow.

5. Results-DNs and their associated TTS

Here a brief introduction of the TTS, its usefulness in general, and how it serves the dermal IATAs for different purposes is given. To make a grouping decision, information needs to be gathered for each DN of the IATA. Accordingly, we provide guidance on which methods and analytical considerations should be used to collect the information required to address the question that is posed in each DN for an effective grouping. The methods proposed in the TTS are very versatile and can be applied to "pristine/as produced" NFs as well as to NFs incorporated in consumer products.

The TTS we have developed (Figure 5) starts with a recommendation to review the existing (published) data which may be used to address the DN information requirements. If additional data needs to be generated, we propose to first use simple acellular *in vitro* assays covered by Tier 1. To make the results suitable for harmonization, the use of standardized methods is suggested (e.g. ISO or OECD guidelines), when available. However, when standardized methods are lacking, standard operating procedures (SOPs) developed at the level of large-scale EU projects are suggested. If grouping and read-across are not possible using the Tier 1 tests then "substance-specific" testing could be directly performed for the target hazard endpoint (i.e. irritation, sensitization, and dermal toxicity) according to the OECD recommendation (OECD 2014) by using *in vitro* cellular-based assays at Tier 2 or *in vivo* tests at Tier 3. In the following sections, the Tier 1 assays associated with each DN of dermal IATAs are described.

5.1. Chemical composition analysis

NF chemical composition can be analyzed using a range of techniques, including X-ray spectroscopy, analytical electron microscopy, and the inductively coupled plasma mass spectrometry (ICP-MS) (Cheong et al. 2017). For example, for the metal-based NFs, we suggest the ICP-MS analysis (International Organization for Standardization (ISO) 80004-6 2021) as this technique can provide high sensitivity and multi-element determination, allowing the characterization of lower-diameter metallic NFs (Galazzi et al. 2020).

5.2. Dissolution

5.2.1. Dissolution in sweat fluids

NF dissolution in sweat is crucial for many of the IATAs reported (H-D-1, H-D-2, and H-D-4). The dissolution kinetics are measured according to International Organization for Standardization (ISO) 19057 (2017) and expressed as half-life values ($t_{1/2}$). Dissolution should be assessed in physiologically relevant simulated sweat fluid using a static or dynamic set-up depending on both media composition and model system requirements according to the information collected by the literature (Table SI4). Different recipes to prepare simulated sweat are available (Table SI5). We recommend the use of simulant sweat fluids that include representative amino acids of human sweat and to apply both a basic and an acidic pH, i.e. pH 5 and pH 8. Importantly, when assessing the similarity of NFs within the resultant group, the same method for assessing dissolution must be used for all NFs under

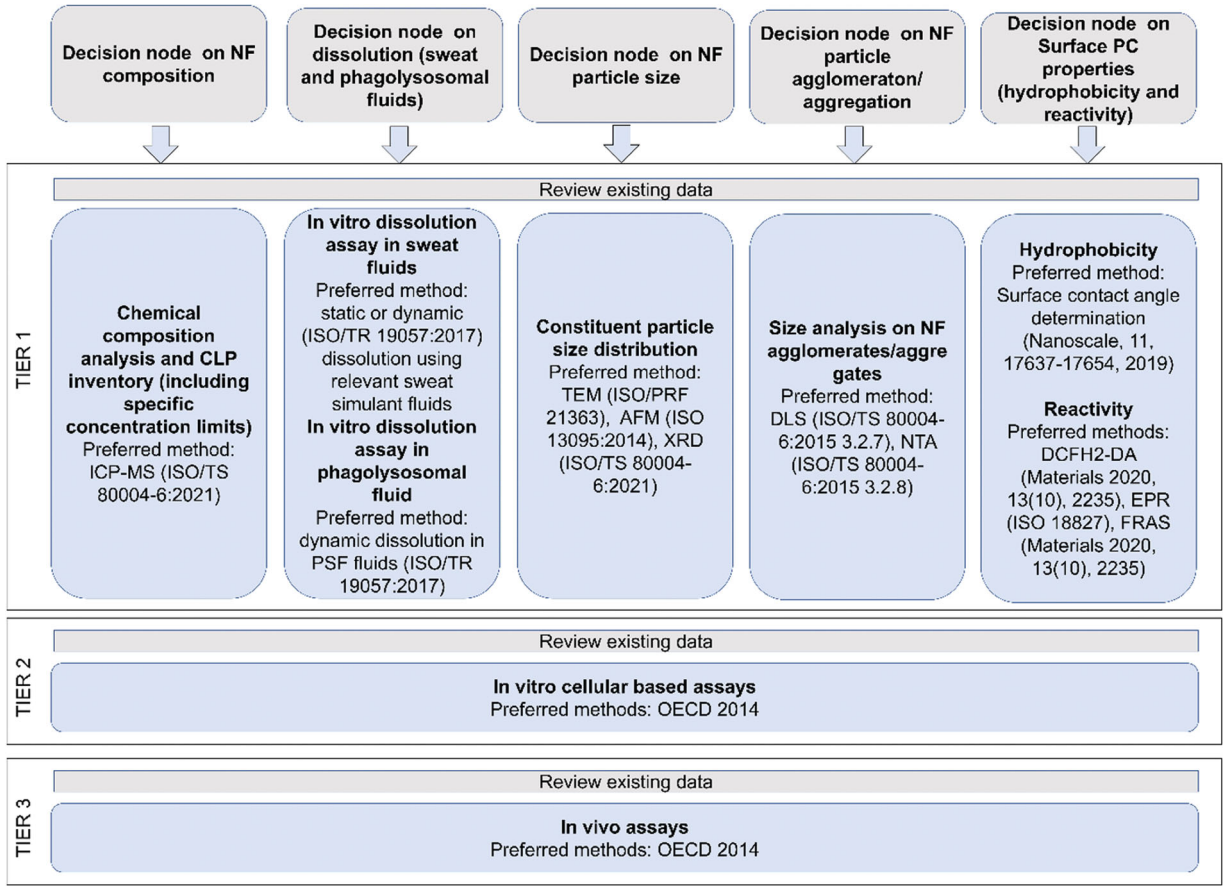

Figure 5. TTS developed for each DN of the IATAs for hypotheses H-D-1, H-D-2, H-D-3 and H-D-4. The TTS provides specific acellular *in vitro* methods to use to satisfy each DN of the dermal IATAs in Tier 1 and more general cellular *in vitro* and *in vivo* methods to evaluate the specific hazard endpoints (i.e. dermal irritation, sensitization and toxicity) at Tier 2 and Tier 3, respectively.

investigation to allow comparison. Whilst simulated sweat is the preferred test media to assess NF dissolution, if dissolution $t_{1/2}$ in water or other physiological media already exists, such data could be considered to make a grouping decision.

5.2.1.1. Role of the molecular composition of the artificial sweat in dissolution rate: experimental evidence. Addressing the significant gap in data relating to the dissolution of NFs in artificial sweat we performed experimental work to inform the design of the TTS for assessing NF dissolution following dermal exposure (Figure 6). First, we identified source materials to test. We selected data-rich benchmark materials from the JRC repository, namely zinc oxide (ZnO) (JRCNM62101a020084) and silicon dioxide (SiO_2) (JRCNM02000a990128) NFs that were tested in different sweat simulations. As sweat is found at moderately acidic to neutral pH levels (Bandodkar et al. 2013), we assessed NF dissolution at pH 5.5 and pH 8. Furthermore, for simulant sweat, we compared the dissolution rate of the zinc NFs in the presence and absence of histidine to better understand how the composition of the media influenced NF dissolution at pH conditions (pH 8.0) corresponding to the histidine maximum activity in terms of ion binding. The methodology used to assess NF dissolution is described in the supporting information. Briefly, using a static set up, as a low flux in human skin is normally expected, NFs were incubated in ISO 105-E04 simulated sweat solutions (Table SI4) at a pH of 5.5 or 8.0 for 10 minutes at 37 °C (for further details, see Supplementary Material) and dissolution was determined (Figure 6). Figure 6(A) reports the $t_{1/2}$ values of ZnO and SiO_2 NFs calculated according to Keller et al. (2020). The identified $t_{1/2}$ showed that, regardless of the pH, ZnO dissolves more quickly compared to SiO_2 (Figure 6A). These results are in contrast to other studies which established that pH greatly affects the dissolution of zinc oxide NFs, as reported for example in Król, Mizerna, and Bożym

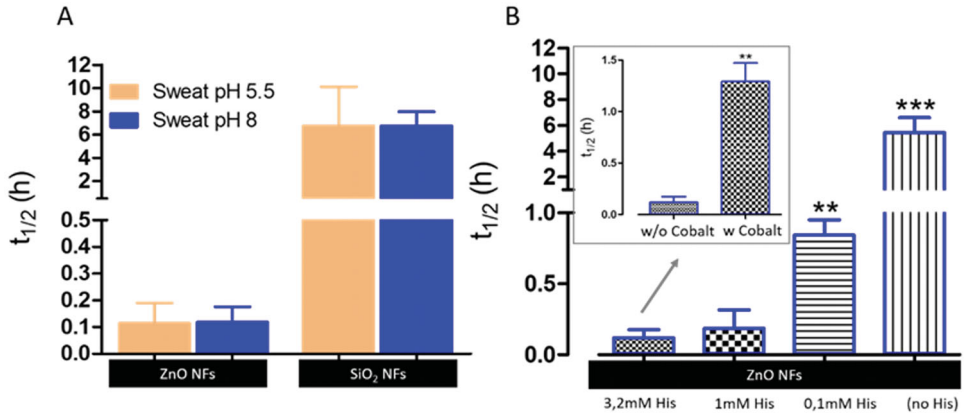

Figure 6. (A) Half-life value ($t_{1/2}$) of zinc oxide (ZnO) (JRCNM62101a020084) and silicon dioxide (SiO_2) (JRCNM02000a990128) NFs in simulant sweat fluids at pH 5.5 and 8.0 ($n = 3$). (B) Half-life values ($t_{1/2}$) of ZnO NF at different concentrations of histidine in ISO sweat fluid pH 8.0. $p < 0.01$ and $p < 0.001$ *vs.* complete ISO sweat ($n = 3$). Inset: Half-life values of ZnO NFs in ISO sweat pH 8.0 with 3.2 mM histidine pre-incubated with or without 1.6 mM Co^{2+}. $p < 0$, 01 *vs.* ISO sweat.

(2020). To evaluate the role of histidine in promoting NF dissolution, International Organization for Standardization (ISO) 105-E04 (2013) simulated sweat at pH 8.0 was incubated with ZnO NFs in the presence of increasing concentrations of histidine (0, 0.1, 1, and 3.2 mM, the latter corresponding to the working concentration suggested by the ISO). Histidine had a negligible role in the dissolution of ZnO at acidic sweat pH, however, it accelerated the dissolution of ZnO NFs at the higher pH. It is hypothesized that this is due to the sequestration of zinc ions through the active coordination binding sites in histidine. As shown in Figure 6(B), $t_{1/2}$ values of ZnO progressively increased with decreasing histidine concentrations. To further confirm the direct action of histidine, cobalt ions (known as the most effective agonist for the histidine coordination binding sites (Watters and Wilkins 1974; Louie and Meade 1998), were added to ISO 105-04 simulant sweat to block the coordination binding sites of histidine. Figure 6(B) shows that, when the histidine binding sites are blocked, $t_{1/2}$ values of ZnO increase. This data further confirms that histidine plays a direct role in enhancing the dissolution of ZnO by sequestration of Zn^{2+} ions. No additional studies were conducted with silica NFs, but it is also known that solutions with basic amino acids, such as histidine, could enhance the dissolution rate of silica (Kawano, Hatta, and Hwang 2009; Kawano and Obokata 2007; Ehrlich et al. 2010). Indeed, histidine can interact more strongly with the negatively charged surface of amorphous silica than other non-basic amino acids. Such binding is due to histidine dissociation, thus forming cationic species. The results from our study indicate the importance of using physiologically relevant simulant fluids, informed by the exposure scenario of interest, to assess NF dissolution. Based on the results for ZnO NFs, it can be concluded that, for NFs with a high dissolution in relevant simulant sweat fluids (with histidine), grouping with the corresponding dissolved ions can be supported for similar fate and hazard. This concept forms the basis of the developed grouping hypothesis for the dermal route of exposure related to instantaneously dissolving NF in sweat (H-D-2, Figure 2). Further hypotheses have been proposed to group NFs for which a slower dissolution rate presents the opportunity for the NFs to remain in particulate form following dermal exposure, and potentially be absorbed in their particle form across the skin barrier (H-D-1 and H-D-4).

As described previously, dissolution can differ between physiological media. Indeed, we investigated the possibility of predicting dissolution of NFs in artificial sweat based on dissolution data generated in neutral pH simulated LSF and low pH PSF in a dynamic set-up. This could allow extensive dissolution testing to be minimized in the future as existing data may be used as a surrogate for estimating dissolution in different biological media. Available studies suggested strong pH dependency, where ZnO NFs dissolve quicker in acidic PSF fluid than SiO_2 NFs, whereas SiO_2 NFs dissolved faster in basic LSF fluids (Table SI6) (Wohlleben et al. 2019; Keller et al. 2020; Keller et al. 2021). As summarized

in Table SI6, the results in ISO 105-04 simulant sweat for both ZnO and SiO_2 NFs showed remarkably quicker dissolution than in LSF (pH 7.4) and PSF (pH 4.5). As metal ions are likely to be sequestrated by histidine and indeed a higher dissolution rate was observed for ZnO and SiO_2, NFs in sweat simulant as compared to lung fluids, it is assumed that the outcome of dissolution studies in water or using physiological fluids other than sweat would generally be conservative (keeping the threshold at <1 h), but acceptable. Once more data becomes available on the comparative dissolution of NFs in sweat versus other commonly available physiological media, more guidance could be provided on the most relevant media to select.

5.2.2. Dissolution in phagolysosomal fluid

Testing NF phagolysosomal dissolution in a dynamic condition is considered the preferred method as the results are consistent with data from *in vivo* studies (Koltermann-Jülly et al. 2018). However, data on a static dissolution test would also be acceptable (and conservative) to address this DN. Therefore, both static and dynamic methods may be used. Also in this case, if the IATA was being used to assess the similarity between NFs, the same dissolution fluid, and the same method should be applied. Read-across of hazard data between a source NF with a similar or higher dissolution $t_{1/2}$ in PSF fluids than the target NF could be used to mitigate concerns of bioaccumulation and associated systemic toxicity after dermal exposure to the target NF. PSF is the preferred test media to address this DN, for which a standard recipe is accessible from the International Organization for Standardization (ISO) 19057 (2017). If dissolution $t_{1/2}$ in water or other physiological media already exists, such data could be considered. For most NFs, dissolution in simulated PSF fluid, mostly due to its acidic pH, would be expected to be higher than that in water or LSF as recently reviewed by Innes et al. (2021). Nevertheless, for some particles (i.e. silica NFs) we have observed the opposite (Table SI6). Therefore, some material-specific cases may require additional testing. The outcome of dissolution studies in water or using physiological fluids other than PSF fluids would generally be conservative and acceptable to address this DN (considering the same threshold). An exception would be NFs of substances that have been shown to dissolve quicker at neutral than at acidic pH. Within the GRACIOUS project, we have assessed the dissolution of several NFs and only the silica NFs show a higher dissolution in LSF fluids than in PSF fluids (Keller et al. 2021 and Table SI6). Given the fact that the use of dissolution data in other fluids would generally be conservative, in some scenarios, the user may still want to refine the assessment using dissolution testing in PSF fluid. Available information on the relative dissolution of NFs of the same substance in different media in the literature should be carefully reviewed and may help in concluding whether such additional testing would or not be worthwhile.

5.3. Particle size

The assessment of the NFs constituent particle size can be carried out by using transmission electron microscopy (TEM) following International Organization for Standardization (ISO) 21363 (2020), atomic force microscopy (AFM) following International Organization for Standardization (ISO) 13095 (2014), or X-Ray Diffraction (XRD) following International Organization for Standardization (ISO) 80004-6 (2021).

5.4. Particle agglomeration/aggregation

To measure the aggregate/agglomeration status of the NFs the suggested methods are dynamic light scattering (DLS) or Nanoparticle Tracking Analysis (NTA) following the International Organization for Standardization (ISO) 80004-6 (2021). However, agglomeration will change depending on the media in which this is evaluated. At this moment, to establish a default dermal penetration value, the use of the constituent particle size is considered conservative and sufficient.

5.5. Surface PC properties

5.5.1. Hydrophobicity

As no standardized methods are available to test the hydrophobicity, we suggest the surface contact angle determination method that can be found in Wohlleben et al. (2019).

5.5.2. Reactivity

Intrinsic NF reactivity could be addressed by acellular reactivity assays, such as the 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) following the method and SOP described in Boyles et al. (2022) and Peijnenburg et al. (2020), the electron Paramagnetic Resonance (EPR) following the International Organization for Standardization (ISO) 18827 (2017), and the ferric reducing ability of serum assay (FRAS) following Peijnenburg et al. (2020). If phototoxic NFs are considered, the reactivity assay should be performed under UV irradiation. The use of a combination of assays for regulatory implications is recommended.

6. Conclusions

Available data on skin irritation, skin sensitization, dermal penetration of NFs, as well as experimental data on NF dissolution in sweat simulant fluids was implemented to formulate grouping hypotheses and IATAs that can be used to support read-across for dermal toxicity endpoints. Although these IATAs focused mostly on direct exposure to "pristine/as produced" NFs, migration and dissolution studies can be used to extend their applicability to NFs when embedded in product formulations to account for real-world exposure scenarios. Such IATAs can be thus considered a proof-of-concept to accelerate the NF risk assessment for dermal toxicity suggesting scientific evidence-driven grouping for NFs.

The generated grouping hypotheses, accompanied by tailored IATAs, focus on i) the dissolution of NFs and release of ions or substances that can induce skin irritation or sensitization according to CLP classification criteria; ii) the dissolution of NFs where a generation of a non-nanoform form is expected before reaching viable layers of the skin, thus allowing read-across from other NFs or non-NFs of the same substance; iii) the lack of biopersistence of NFs which allows waiving concerns on systemic accumulation and potential long-term toxicity of some NFs that dissolve quickly in simulant lysosomal fluids; and iv) limited dermal penetration, with the possibility to use a worst-case estimate for dermal penetration. The cutoffs reported in the dermal IATAs are of course not recognized by any regulator bodies. However, their values fit and are aligned with the works conducted by Gimeno-Benito et al. (2021) and Braakhuis et al. (2021). Both the presented cutoffs are physiologically relevant, as they consider whether constituent ions or molecules, particles, or both contribute to toxicity and allow for grouping NFs according to their biopersistence. Running OECD working groups (e.g. OECD project on Determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biological media) are producing data on dissolution in different biological environments (including some relevant for dermal exposure) and dissolution rates for a range of nanomaterials will be compared. Moreover, comparable dissolution assays (as those suggested by the current paper) are used in the regulatory contexts and the template for data analysis is in common with the GRACIOUS project. This hopefully will allow in the future an initial step of validation of the proposed cutoffs and corresponding test assays for a regulatory context.

Dissolution is a parameter of high relevance in the dermal fate and hazard of NFs. Paradoxically, there has been very little work done on the dissolution of NFs in sweat simulant fluid, which is considered the most relevant fluid for this exposure route. To address this limitation and better include these suggestions in the recommended TTS of the dermal IATAs, we conducted some experimental work to identify what media should be prioritized when assessing NF dissolution following dermal exposure. We concluded that the extrapolation of dissolution data obtained in other physiological media is challenging and, that ideally, the user should select a physiologically relevant simulant fluid that is relevant to dermal exposure. Here, the simulant sweat fluid with a physiologically relevant composition (i.e. presence of amino acids component) and both low and neutral pH (i.e. 5.5 and 8) is recommended.

The boundaries suggested in the IATAs for each grouping hypothesis, both to establish groups or support read-across, are based on limited available data. Such boundaries should be refined once more data becomes available, with a more detailed characterization of the tested NFs. Indeed, as per ECHA requirement, a grouping hypothesis needs to be evidence-based, (even if limited), therefore the IATAs can be then adapted as soon as novel studies in the field will come. This will also facilitate the identification of key NF properties associated with

adverse effects and will allow the development of additional IATAs to support the read-across of dermal toxicity endpoints for NFs.

Disclosure statement

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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