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Refinement of the selection of physicochemical properties for grouping and read-across of nanoforms

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ABSTRACT

Before placing a new nanoform (NF) on the market, its potential adverse effects must be evaluated. This may *e.g.* be done *via* hazard and risk assessment. Grouping and read-across of NFs is a possible strategy to reduce resource consumption, maximising the use of existing data for assessment of NFs. The GRACIOUS project provides a framework in which possible grouping and read-across for NFs is mainly based on an evaluation of their similarity. The impact of NFs on human health and the environment depends strongly on the concentration of the NF and its physicochemical properties, such as chemical composition, size distribution, shape, *etc.* Hence, knowledge of the most relevant physicochemical properties is essential information for comparing similarity.

The presented work aims to refine existing proposals for sets of descriptors (descriptor array) that are needed to describe distinct NFs of a material to identify the most relevant ones for grouping and read-across. The selection criteria for refining this descriptor array are explained and demonstrated. Relevant protocols and methods are proposed for each physicochemical property. The required and achievable measurement accuracies of the refined descriptor array are reviewed, as this information is necessary for similarity assessment of NFs based on individual physicochemical properties.

1. Refining the selection of nanoforms' physicochemical proprieties relevant for grouping and read-across

REACH, Regulation (EC) No 1907/2006 on Registration, Evaluation, Authorisation and Restriction of Chemicals, is the overarching chemicals legislation in the EU and lays down information requirements for substances to be placed on the market in the EU. The European Chemicals Agency, ECHA, oversees the implementation of REACH. Some of the annexes of REACH were amended in 2018 (Commission Regulation (EU) 2018/1881, 2018) to include a definition of nanoform (NF), which is the term used in REACH for nanomaterial, and specify information requirements for NFs.

The data requirements for REACH registration can be fulfilled in several ways, *e.g.* by testing, using literature data or by 'grouping and read-across'. The latter approach is well established for chemicals (not being NFs) and both OECD and ECHA have published several guidance documents (ECHA, 2008; ECHA, 2012a; ECHA, 2013; ECHA, 2017a; ECHA, 2021a; ECHA, 2021b; ECHA, 2016; OECD, 2014) addressing grouping and read-across for chemicals and specifically also for nanomaterials/nanoforms; the EU approach builds on the OECD approach. The OECD defines grouping as the general approach for assessing more than one chemical at the same time. More specifically, the OECD

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explains that "***A chemical category** is a group of chemicals whose physicochemical and human health and/or ecotoxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern, usually as a result of structural similarity*.", which enables "*In the **read-across approach**, endpoint information for one chemical (the source chemical) is used to predict the same endpoint for another chemical (the target chemical), which is considered to be "similar" in some way (usually on the basis of structural similarity or on the basis of the same mode or mechanisms of action)*". The principles of these concepts in the EU legislation are the same. However, for grouping different NFs of the same substance, consideration of the molecular structural similarities alone is not sufficient to serve as a justification (REACH Annex XI). Mech et al. (Mech et al., 2019) provide an overview of the possibilities of applying grouping and read-across to nanomaterials in EU chemicals legislation. For REACH, the current ECHA guidance focusses on grouping of NFs within the same substance registration. Jeliazkova et al. (NanoImpact VSI) give an overview of the terminology used in GRACIOUS including the terms 'grouping' and 'read-across'.

The EU (European Union) Horizon 2020 project GRACIOUS (Grouping, Read-Across, Characterisation and classification framework for regulatory risk assessment of manufactured nanomaterials and safer design of nano-enabled products) has developed a framework for grouping and read-across of nanoforms, and physicochemical properties of nanoforms are considered basic information to enter that Framework (Stone et al., 2020).

The GRACIOUS Framework considers the following key intrinsic and extrinsic NF physicochemical properties affecting the biological behaviour, and environmental fate and transformation (speciation, dissolution, *etc.*) of NFs:

- Composition, including impurities
- Particle size
- Shape
- Crystallinity
- Chemical nature of the surface
- Specific surface area
- Density
- Surface charge
- Surface hydrophobicity
- Water solubility, including dissolution rate
- Dustiness
- Dispersibility
- Biological reactivity

These properties are in line with those requested for NFs by REACH and further detailed in recommendations by ECHA (Comandella et al., 2020) and are key physicochemical properties that may be possibly relevant for grouping and read-across of nanoforms. The properties composition, crystallinity, particle size, shape, chemical nature of the surface (surface chemistry), and specific surface area are considered to be priorities because they are essential to uniquely identify a NF in line with the additional registration information requirements for NFs under REACH (Commission Regulation (EU) 2018/1881, 2018).

The intrinsic properties particle size and particle size distribution (included in the determination of the "constituent particle size") and specific surface area are related. However, whereas it is possible to calculate the specific surface area when the size distribution and shape of the particles are known, the opposite is possible only under very restrictive assumptions for the particle size and shape distribution. Therefore, it is recommended to determine both the particle size distribution and the specific surface area, as also required by REACH.

The properties listed above include those used in grouping and read-across for NFs for various purposes, including the regulatory requirement to address the information requirements laid down in the amended Annexes to REACH (Commission Regulation (EU) 2018/1881, 2018). The OECD Working Party on Manufactured Nanomaterials' (WPMN) testing and assessment programme, suggested that more properties of both intrinsic and extrinsic nature (Rasmussen et al., 2018) are relevant, but this program was not focused on grouping purposes. The refined list presented above is generally in line with the DF4nanoGrouping approach (Arts et al., 2015), but it should be noted that DF4nanoGrouping does not use all the above information to come to grouping decisions and that the DF4nanoGrouping focuses on potential effects upon inhalation, *i.e.* human health hazard assessment. For instance, surface charge and hydrophobicity are not used by DF4nanoGrouping, which instead uses the "functional assay" strategy of measuring agglomeration and reactivity directly.

Additional physicochemical characteristics are included in the detailed steps of the GRACIOUS Framework, but they are only needed when a specific Integrated Approach for Testing and Assessment (IATA) of a grouping hypothesis requires them (Murphy et al., 2021; Stone et al., 2020). Under REACH, grouping and read-across may be used to generate information to fill data gaps on intrinsic properties (REACH, Article 13) of substances, and as release and exposure are not intrinsic properties the ECHA guidance on grouping and read-across does not include properties related to emission and release of and exposure to NFs. The expansion of the DF4nanoGrouping approach from occupational health to include as well grouping for consumer aspects and environmental purposes in nanoGRAVUR (Nanostructured materials - Grouping for occupational health and consumer and environmental protection and risk mitigation; 2018, (Wohlleben et al., 2019)) uses additional extrinsic properties such as resilience of the NEP (nano-enabled product).

In this paper, we have analysed the IATAs proposed in GRACIOUS to identify which of the physicochemical properties proposed in the GRACIOUS Framework are actually used in these IATAs and how frequently they are used. This has been compared to the REACH regulatory information requirements and the DF4nanoGrouping data requirements. Based on this analysis, the list of properties can be refined.

2. Methods, descriptors and technology readiness level of the selected properties for grouping and read-across

An understanding whether grouping of different NFs is possible, an understanding of the available methods, most suitable descriptors and the readiness of these methods (including their level of standardization) to be implemented in a standardized way (to allow for data interpretation between different studies) is essential to understand the suitability of descriptors for similarity assessment of NFs for the purpose of grouping and read-across. The similarity of NFs in this context is based on individual NF properties. As discussed in detail elsewhere (Jeliazkova et al., 2021; NanoImpact Virtual special issue (VSI)), to substantiate grouping for regulatory purposes a pairwise, property-by-property evaluation of the similarity is recommended. This can be done using various, well-established algorithms, as shown elsewhere (Jeliazkova et al., 2021 NanoImpact VSI). Similarity assessment also requires understanding of the achievable accuracy of the available methods, because it must be demonstrated that measured differences between NFs are greater than the achievable accuracy of the method (Cross et al., 2021; NanoImpact VSI). This is needed to have confidence that the measured differences are indeed real.

This section provides an overview of the methods, descriptors and technology readiness level to investigate the selected properties as well as a detailed discussion of essential resources for protocols associated with each of the physicochemical properties of relevance identified in the GRACIOUS basic information step (Stone et al., 2020).

2.1. Summary of suitable methods for each of the selected properties

The methods and descriptors most suitable for obtaining information on each of the selected physicochemical properties, also useful for grouping purposes, are listed in Table 1, as well as links to ongoing

Table 1
The GRACIOUS draft selection of methods, descriptors and representative test materials for the physicochemical properties of a NF.

Property	Method*	Descriptors* [metric]	Representative test materials tested*	More input expected from past and/or ongoing projects*
Constituent particle shape triggers HARN hypothesis	SEM or TEM NanoDefine methodology (Mech et al., 2020), consistent with REACH nanoforms	Aspect ratio [unitless]	$BaSO_4$ IRMM387 (NM-220), $BaSO_4$ IRMM381, $CaCO_3$ IRMM384, alumina-coated TiO_2 IRMM388. HARN: NM-402, NM-400	ND
Constituent particle size distribution	SEM or TEM for confirmation NanoDefine methodology, consistent with REACH nanoforms. DLS, CLS, FFF or ES-DMA for screening	Median of minimum external dimension [nm]	$BaSO_4$ IRMM387 (NM-220), $BaSO_4$ IRMM381, $CaCO_3$ IRMM384, alumina-coated TiO_2 IRMM388. HARN: NM-402, NM-400	ND
Composition incl. iImpurities and additives	Identify composition by XRF, ICP-OES, ICP-MS or XRD (applicable for inorganic materials) HPLC (for organics)	Impurity >1% (consistent DF4nanoGrouping and ECHA footnote to CLP (Regulation (EC) No 1272/2008, 2008)	Not relevant. Cut-offs given by CLP and Swiss reporting scheme for NFs	ACENano Analytical and Characterisation Excellence
Chemical nature of the surface **surface chemistry**	XPS, TGA-MS/IR, MALDI-TOF-MS, solvent extraction combined with GC–MS and/or LC-MS (or combinations of all mentioned techniques)	Informative for GRACIOUS IATA and methods. Probably not in itself a criterion for similarity of (nano)forms, instead several sur-face-induced biological inter-actions are measured directly	ND	ACEnano
Specific surface area	BET, VSSA by gas adsorption ISO 9277, 2010; Hackley and Stefaniak, 2013; OECD Guidance 318	Informative for GRACIOUS IATA and methods. Probably not in itself a criterion for similarity of (nano)forms, instead several surface-induced biological interactions are measured directly	ND	Gov4Nano Implementation of Risk Governance: meeting the needs of nanotechnology
Surface charge	Zeta-potential with pH titration or potentiometric titration for NF Charge density	Informative for GRACIOUS IATA and methods. Probably not in itself a criterion for similarity of (nano)forms, instead several surface-induced biological interactions are measured directly	ND	ND
Surface hydrophobicity	Potentially: sessile drop, water contact angle (Xiao and Wiesner, 2012); OECD TG under development (based on: Desmet et al., 2017; Valsesia et al., 2018)	Informative for GRACIOUS IATA and methods. Probably not in itself a criterion for similarity of (nano)forms, instead several surface induced biological interactions are measured directly	ND	ACEnano; Gov4Nano; OECD
Density	He-pycnometry	Informative for GRACIOUS IATA, probably not in itself a criterion for similarity of (nano)forms	ND	ND
Physicochemical hazards not specific for NF: human toxicity, ecotoxicity	Not specific for NF GHS Globally Harmonized System of Classification and Labelling of Chemicals and CLP (Regulation (EC) No 1272/2008, 2008)	H-phrases: informative for GRACIOUS IATA, probably not in itself a criterion for similarity of (nano)forms	Not relevant	ND
Rigidity for HARN IATA	No validated method yet established	Modulus of elasticity [MPa] (for MWCNT: diameter [nm])	NM-400 (non-rigid)/ NM-401 (rigid)	ND
Solubility in aqueous medium quickly soluble	OECD TG draft: in 5 mM $NaHCO_3$, pH 7 at 10 mg/L, 24 h	% dissolved	CuO (OECD): soluble (given in TG draft)	OECD
Solubility: Ion-releasing triggers the solute IATA	OECD TG draft: incubate 100 mg/L in relevant medium, measure ions	Dissolved ions [mg/l]	CuO (OECD): ion releasing with >0.1 mg/L Cu^{2+}	OECD
Dissolution kinetics in relevant media	Flow-through dissolution + ICP-MS as requested and implemented by Oberdörster and Kuhlbusch, 2018	Pulmonary: k [ng/cm^2/h] (Oberdörster and Kuhlbusch, 2018)	$BaSO_4$ NM-220, CeO_2 NM-212, ZnO NM-110 (uncoated) or NM-111 (coated)	PATROLS (Physiologically Anchored Tools for Realistic nanomateriaL hazard assessment), ACEnano
Transformation of the NF	TEM, optional SAD, XPS (Koltermann-Jülly et al., 2018)	Qualitative or NanoDefiner (Brüngel et al., 2019) image analysis	ND	PATROLS, ACEnano
Dispersibility approximated by Homo-agglomeration	Human health perspective (DF4nanoGrouping): Agglomeration in serum-containing medium + DLS or AUC Environmental perspective: OECD TG 318 in relevant medium (instead of 3*3 Ca*NOM media = 54 measurements)	Follow up to read-across similarity by DLS/VCM, AUC.	TiO_2 NM-105, Ag NM-300 (OECD, 2017c)	ACEnano
Dispersibility approximated by	Possibility after (Geitner et al., 2017)	Attachment efficiency (α)	ND	ACEnano, nanoFASE (Nanomaterial Fate and Speciation in the

(continued on next page)

Table 1 (*continued*)

Property	Method*	Descriptors* [metric]	Representative test materials tested*	More input expected from past and/or ongoing projects*
affinity and thus hetero-agglomeration				Environment), OECD
Biological Reactivity	ESR cell free in water (ecotoxicity), ESR cell-free +FRAS (on human serum) (human toxicity)	ESR: relative to negative representative material FRAS: relative to LoD and positive representative material	$BaSO_4$ (neg), Mn_2O_3 (pos)	ACEnano, PATROLS ISO TS18827

* The abbreviation "ND" stand for "Not determined.". The other abbreviations are listed elsewhere in this paper.

projects which are expected to further optimise and develop the methodology. For a number of these properties and methods Rasmussen et al. (Rasmussen et al., 2018) indicate their availability, for example, OECD test guidelines and/or ISO (International Organization for Standardization) standards.

2.2. Relevant sources of protocols for intrinsic and extrinsic physicochemical properties

There are numerous sources of information relevant to NF physicochemical characterisation, including the OECD WPMN, EU regulatory guidance (ECHA, 2019, 2021b), reviews (especially those from ProSafe (Promoting the Implementation of Safe by Design)) of methods (Rasmussen et al., 2018; Stefaniak et al., 2013; Steinhäuser and Sayre, 2017); of properties used in frameworks (Oomen et al., 2018) and overviews from projects (Jantunen et al., 2017). These documents outline proposals for the basic information relevant for the basic starting information to enter the Framework.

Importantly, the ECHA practical guide on "How to report read-across and categories" (ECHA, 2012b) does not specify methods, but refers to guidance R7.1 for "advice on some of the parameters" and refers to ECETOC (DF4nanoGrouping) for a "supplementary information table that includes available analytical methods for parameters relevant for read-across and grouping of nanoforms" (ECHA, 2021a). In R7.1 (ECHA, 2021a), many alternative methods are provided for each property, whereas DF4nanoGrouping selects one specific method per property. It should be noted that also other regions than Europe recognise the possible difference between different nanoforms and that the testing itself may influence the results obtain. Hence, *e.g.* the US-EPA recommends to differentiate discrete forms "using the same test medium and method […] as even minor changes […] can result in large differences in the measured results" (US-EPA, 2017).

During the GRACIOUS project we have explored several alternative methods to identify the method and metric with highest relevance for grouping and read-across purposes.

The following sources of information are perceived as essential for physicochemical methods, because they focus on grouping and read-across with a selection of methods:

- DF4nanoGrouping, Table SI_2 (intrinsic) and SI_4 (extrinsic) (Arts et al., 2015), which are specifically mentioned in the ECHA grouping guidance (ECHA, 2017b).
- ProSafe review of methods, Table 1 (Steinhäuser and Sayre, 2017) that serve grouping purposes and provide up to four alternative methods for each property.
- ECHA guidance R7.1 (ECHA, 2021a) as a supporting reference.
- ISO/TR 13014:2012 which is a technical report that introduces the terminology (descriptor, measurand, *etc*). The techniques that were prioritized for GRACIOUS are selected from the ISO technical report. However, if the prioritized techniques are not applicable to certain types of material, alternatives may be found in the ISO 13014 tables.

There are numerous alternative sources of physicochemical methods for different purposes, as reviewed extensively in the key sources listed above. GRACIOUS is aware of important parallel developments, including OECD TGs. Recently Rasmussen et al. (2018) assessed the physicochemical measurement methods applied in the OECD testing and assessment programme and identified methods that could be useful for the testing of nanoforms. Methods relevant to GRACIOUS have been summarised in Table 1.

The nanoGRAVUR project (Wohlleben et al., 2019) filled several method gaps identified by ProSafe (Steinhäuser and Sayre, 2017) and improved the methods originally proposed by DF4nanoGrouping (Arts et al., 2015) such as method updates for "Water solubility, including dissolution rate". Quickly soluble materials may be identified early in the process, *e.g.* by using the Health Canada static method (Avramescu et al., 2017), which is very similar to the "screening method" in the OECD draft TG on solubility. At a later stage, dynamic dissolution testing may be required (Bove et al., 2017), such as the flow-cell geometry selected by WHO/IARC for fibre biodissolution ranking (IARC, 2002), which then also requires the dissolution rate metric in $ng/cm^2/h$ as recommended by Oberdörster and Kuhlbusch (Oberdörster and Kuhlbusch, 2018).

These approaches are all based on the availability data also at the property description level. In order to ensure that such relevant data would be collected in a systematic way, for each property GRACIOUS had as a task to check whether a NANoREG data logging and database template already exists (Totaro et al., 2017). Following this check, some additional templates were developed in GRACIOUS (Gottardo et al., 2019).

For the majority of properties, no certified reference materials currently exist, and even only a limited number of representative test materials (Roebben et al., 2013) are available. Without such materials, which allow *e.g.* confirming the outcomes of measurements of properties of other unknown materials, the uncertainty associated to the measurement results is significantly higher. The importance of representative materials for grouping was already stressed by NIOSH (NIOSH, 2013).

3. Towards the refining of descriptor and parameter arrays to contribute to a framework guidance document

Here we outline the basis for refining the descriptors and parameters, which contributes to the GRACIOUS Framework Guidance Document (Guidance on the GRACIOUS Framework, 2021). To enable the grouping and read-across based on the hypotheses developed in GRACIOUS, a list of important NF physicochemical properties was proposed and considered, see Table 2. The physicochemical properties of interest were selected based on their relevance in identifying NFs and how strongly they influence the NFs stability/reactivity. This selection is in accordance with REACH and reflected in guidance by ECHA, OECD recommendations, and outcomes from previous EU projects (*e.g.* MARINA, NANoREG), the German NanoGRAVUR project, and the DF4nanoGrouping approach developed by ECETOC. A comprehensive list of physicochemical properties of potential high relevance for NFs was thus proposed, see Table 2. The physicochemical properties are in line with the GRACIOUS nomenclature based upon definitions in GUIDEnano knowledgebase extended and refined with new insights from GRACIOUS

Table 2
The selection of properties that are considered relevant for hypotheses based on intended use, release and exposure. The form and rate of release after incorporation into a solid matrix may be assessed by these properties.

Physicochemical property	Scale type	Minimum relevant range*	Maximum relevant range*	Critical values that a group should not cover unless with justification*	For GROUPING PURPOSE: Width of a band/ Floating or fixed bands*	Required accuracy*
Aspect ratio	LOG	<0.01	>1000	3 (shape category of ECHA guidance)	x10/Floating around the group median, or range of x10 below the source NF if the read-across source is part of the NF group.	x2
Particle size	LOG	<10 [nm]	1000 [nm] for NFs, and > 1000 nm for non-NF during R-X	100 nm (only NFs can be grouped, not the non-nano-form) 5 nm (biokinetics and translocation change for smaller particles)	X3/Floating around the group median	10% of upper band limit
Surface area	LIN	<20 [m^2/g]	>180 [m^2/g]	ND	TBD/Floating around the group median	TBD
Surface charge	LIN	<−30 [mV]	>+30 [mV]	ND	20 mV/Fixed	5 mV
Surface hydrophobicity	LIN	<20 [°]	>90 [°]	ND	TBD/TBD	10°
Density	LIN	<1	>10	ND	TBD/TBD	TBD
Dustiness	LOG	<10 [mg/kg]	>10,000 [mg/kg]	ND	x10/Floating around the group median	x2
Chemical composition	LOG	TBD	TBD	TBD	TBD	TBD
Water solubility	LOG	<0.001 [mg/L]	>1000 [mg/L]	ND	x10/Floating around the group median	x2
Dissolution rate	LOG	<0.1 [ng/cm^2/h]	>100 [ng/cm^2/h]	ND	x10/Floating around the group median	x2
Dispersibility (attachment efficiency)	LOG	$<10^{-5}$	$>8 \times 10^{-1}$	ND	x10/Floating around the group median	x2
Biological reactivity	DCFH, EPR: LIN FRAS: LOG	(assay-dependent descriptors and values)	(assay-dependent descriptors and values)	ND	DCFH, EPR: 30% of positive control FRAS: x10	DCFH, EPR: 10% of positive control FRAS: x2

* The abbreviation"LOG" stand for "Logarithmic", "LIN" for "linear", "ND" for "not determined" and "TBD" for "to be determined". x2, x3 and x10 stand for a width band of 2, 3 and 10 fold, respectively.

deliverables and reporting templates developed during the GRACIOUS project (Gottardo et al., 2019).

The scope is to further reduce the number of elements in the descriptor array and include only the parameters that have an optimal ratio of significance (for grouping and read-across) to accessibility and cost in order to generate a final set of properties and methods for inclusion in the Framework Guidance Document. The next section describes the criteria used to facilitate the selection of the most significant and relevant properties.

3.1. Criteria for selecting the most significant and relevant properties

Each of the listed physicochemical properties (see Tables 1 and 2) was selected because of its importance in determining the basic starting information and so is deemed to be a suitable candidate to enable possible grouping and read-across of NFs. It is important to point out the key role of previous guidance, projects, legislation and approaches in addition to an extensive key bibliography review (*e.g.* Arts et al., 2015; Oomen et al., 2015; Oomen et al., 2018; Rasmussen et al., 2018; Stefaniak et al., 2013; Steinhäuser and Sayre, 2017) to propose a first set of properties.

The further selection of the most significant/relevant properties for read-across purpose will be based on the following main criteria:

i) Requirements from IATAs
ii) Relevance to IATAs
iii) Outcomes of case studies
iv) Method development status (technology readiness level; TRL)
v) Estimated cost

For each of these criteria the level of importance (*e.g.,* low, medium and high) will be proposed.

3.1.1. Requirements from IATAs

Firstly, the frequency at which a given physicochemical property occurs in the different IATAs developed in GRACIOUS, as well as in the decision trees from the basic information stage, is of interest. Thus, the number of times a physicochemical property was cited in the IATAs concerning the hypotheses dealing with both human health (HH) and the environment (ENV) is counted, see Table S1. In addition, physicochemical properties cited in the contextual information and release/exposure IATAs are also considered. Such information is the starting point (exposure scenario and compartment of release of the NFs) for the different IATAs developed for both the HH and ENV compartments. The fact that a physicochemical property may be directly or indirectly mentioned in the IATAs is also represented in Table S1, *e.g.* specific surface area is implicitly needed for the determination of the dissolution rate as the dissolution rate is expressed in units of ng/cm^2/h, thus if the dissolution rate is mentioned in a IATA, the specific surface area is indirectly needed.

3.1.2. Relevance to IATAs

In addition to the frequency of appearance of these physicochemical properties of interest, their relevance within the IATAs is also a criterion used to refine the parameter array list. To qualitatively classify relevance, the positioning of physicochemical properties within the IATA will be assessed to evaluate their relevance. For example, physicochemical properties used as a pre-screening criterion in an IATA will be regarded as less relevant than if the property serves as a cut-off value that justifies read-across and grouping.

3.1.3. Outcomes of case studies

The outcome of the future case studies will indicate whether the level of frequency, importance and relevance that are proposed for each physicochemical property and defined by them being required in the

IATA are appropriate, and thus will successfully lead to a justified grouping decision within the IATAs. If the physicochemical properties thought to be important (due to high relevance and frequency in a number of IATAs) prove to be less critical in justifying a grouping decision when the IATAs are tested using case studies, the parameter array could be refined. Also, outcomes of the case studies will inform refinement of the IATAs themselves which change the level of importance of some physicochemical properties through reduced, or higher frequency and changed relevance of a property across the IATAs.

3.1.4. Method development status

The status of the method development is also taken into consideration with a simplified three-level TRL assessment: low, medium, high.

3.1.5. Cost

Finally, the last criterion to refine the property selection is the estimated cost to obtain information to assess the properties. It mainly comprises materials, instruments, staff and time, and cost for sample preparation and sample analysis.

4. A worked example evaluating the “class level” of physicochemical properties: chemical composition

The following example demonstrates the approach to evaluate the significance and relevance of the physicochemical property “Chemical composition (including impurities)”.

The NF chemical composition was mentioned in 5 of the 8 environmental IATAs and 7 of the 12 human health IATAs developed during GRACIOUS (Table S1). For the environmental IATAs the “chemical composition” property was mentioned thrice directly (mainly toxicity of the ions released during dissolution processes and/or particulate toxicity of the NF) and twice indirectly by referring to the attachment efficiency. The chemical composition is mentioned in 2 of the 14 IATAs dealing with exposure and NF release compartments (Exposure). The IATAs including chemical composition are mentioned below. The name given to the IATAs is the one from the GRACIOUS project. “E” stands for Environment and “H” for Human health. For the IATAs dealing with the environment, “G” stands for aquatic compartment, “S” for soil compartment and “SW” for soil-water (sediment) compartment. For the IATAs dealing with the human health, “I” stands for inhalation, “O” for oral and “D” for dermal. For the exposure IATAs “Oc” stand for occupational setting, “R” for environmental release and “HE” for human exposure.

4.1. Direct references to three Environmental IATAs

- E-G-1: NFs in the aqueous environment: Following aqueous exposure dissolution rate and attachment efficiency (derived from dispersion stability) are the main driving forces that determine NF fate in aqueous environments, and are sufficient as input in fate modelling of NFs. Lethal and sub-lethal toxicity to representative aquatic species is driven by the fate and toxicity characteristics in aqueous environments of either NF particles or solutes or both.
- E-G-2: NF with a very slow dissolution rate in environmentally relevant media: Biopersistence potential is likely which triggers (long-term) hazard concerns.
- E-S-1: NF with a very slow dissolution rate and high affinity with the solid soil phase: Persistence in soil is likely which triggers (long-term) hazard concerns.

4.1.1. Indirect reference to Environmental IATA

- E-WS-1: NF in sediment environment: For NFs in benthic systems lethal and sub-lethal toxicity to representative benthic species is driven by their dissolution
- E-S-1: NF with a very slow dissolution rate and low affinity with the solid soil phase: Following soil exposure NF mobility in soil follows ground water flows. NFs in this group can cause acute lethal and sub-lethal toxicity to representative soil species.

4.2. Direct references to seven Human Health IATAs

- H-I-3: 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.
- H-I-5: Respirable, partially dissolving NFs: The hazard of the particle will be based on both ion/molecular toxicity and particle toxicity and on the location of the molecular or ionic release.
- H-O-2: NFs with a quick dissolution: Following oral exposure both NFs and constituent ions or molecules may contribute to local inflammation in the OGI tract, but there is no concern for NF accumulation
- H-O-3: NFs showing gradual dissolution: Following oral exposure both NFs and constituent ions or molecules may lead to local inflammation in the GIT. Partial dissolution
- H-D-1: NFs with an instantaneous dissolution: Following dermal exposure NFs will dissolve into their molecular or ionic form before they reach the viable layers of the skin and will cause similar toxicity as substances quickly releasing, dissolving and/or transforming into the same ionic or molecular.
- H-D-2: NFs with constituent substance(s) or degradation products classified for dermal irritation or sensitization: Dermal exposure to the NFs may result in dermal irritation or sensitization.
- H-D-4: NFs that are not flexible and have a constituent particle size larger than 5 nm: Following dermal exposure NFs will result in limited or no dermal absorption.

4.3. Direct references to Exposure IATA

- Oc-R-outdoor-1: NF transformation through incineration
- Oc-HE-D-1: Textile wearing

The IATAs’ requirements regarding the “chemical composition” property is thus classified as “High”. Indeed, a property was arbitrarily considered of “High” importance in terms of being required if it was cited at least in 10 of the 34 total IATAs (“Medium”: 4–10/34; “Low”: <4/34).

In addition, the relevance in the IATAs of this property is “High” as it has direct implications for toxicity assessment, and possibilities for and outcomes of grouping and of read-across.

The analytical methods developed to access the chemical composition of NFs (*e.g.* ICP-MS/OES) are well established techniques for element quantification and the method development level is thus also considered to be “High”.

The cost to determine the chemical composition are estimated as “High” as, in addition to relatively high cost of analysis, the material also needs to be digested, which may require expertise and time for adequate sample preparation.

4.4. Evaluation of all physicochemical properties

Following the approach above, the need for, and relevance and significance of each physicochemical property previously identified, but also the method development status and estimated cost, are evaluated with each IATA and the results are presented in Table S1.

5. Concepts of achievable and required accuracy

To simplify the import of literature data as well as to reduce the effort and cost in determining the physicochemical properties necessary for a robust NF grouping and read-across, the levels of required precision and resolution are introduced. This concept is based on "achievable accuracy" and "required accuracy". The achievable accuracy for the determination of a given physicochemical property is limited by several factors, including an appropriate and complete specification of the measurand, the sample preparation, the method of measurement, and the measurement procedure. Approximations and assumptions incorporated in the measurement methods and procedures can be refined in order to lower the level of uncertainty (Baratto, 2008; GUM, 1993). To derive the required level of accuracy, we estimated for each NF physicochemical property the categorization boundaries (*e.g.* the different value classes to categorize NFs) which include 1) the scale type (*e.g.* linear or logarithmic scale) which follows from the relevant range, 2) the minimum and maximum biologically relevant range for a given physicochemical propriety when it is relevant for the physicochemical property and, 3) the band width and a recommendation whether the bands should be fixed or floating.

The acceptable width of similarity for individual properties is a function of the scale type, the critical range of values that are biologically or environmentally relevant and the achievable accuracy of the method. To validate acceptable limits of similarity for grouping, calibration using case studies against *in vivo* outcomes is required. The acceptable limits of similarity will depend on the purpose of the grouping and the hypothesis that is addressed. The widths proposed in Table 2 concern hypotheses based on intended use, release and exposure. For other grouping purposes, the width of these bands may be narrowed or widened on the basis of scientific justification.

In practice, the required specification of the measurement is dictated by the required accuracy of the measurement. The measurand should be defined with sufficient completeness with respect to the required accuracy so that for all practical purposes associated with the measurement its value is unique (GUM, 1993). An in-depth discussion on required and achievable accuracy in the context of similarity assessment of NFs is provided in (Cross et al., 2021, NanoImpact VSI).

An example of the "required accuracy" is presented in this section for the determination of the NF surface charge (*e.g.* zeta potential value). The zeta potential refers to the electrical potential at the shear plane and may be used to estimate the charge density by converting the experimental zeta potential value to the effective charge density of colloids which is usually determined by potentiometric titration. For a NF the required accuracy may only need to be sufficiently accurate ($+/-$ 5 mV) to permit the classification of the NF in different classes (*e.g.* $> +30$ mV or < -30 mV: stable particles; [+30 to −30] mV: unstable particles, Fig. 1). The two classes were suggested based on the common stability domains of NFs considering only electrostatic stabilization processes and

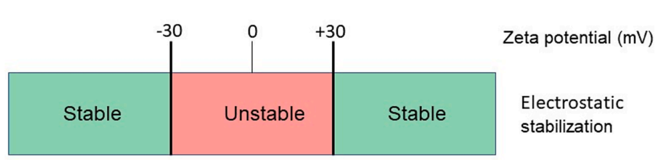

Fig. 1. The different boundaries of zeta (ζ) potential values to permit classification/characterisation of the NFs based on their electrostatic stability.

the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Patel and Agrawal, 2011).

Where possible, the required level of accuracy for all of the investigated NF physicochemical properties is presented in Table 2. The required accuracy is suggested (or will have to be determined (TBD)), for each of the physicochemical properties of interest for read-across and grouping purpose.

It has to be stressed that floating bands may be advocated to increase the usefulness for the purpose of comparing the provisional NF group members. For the specific parameter that varies most within the group (as compared to the span from min to max relevant range), we propose a factor of appropriate heterogeneity across the group, in the context of grouping hypotheses based on intended use, release and exposure.

However, for the purpose of Safe by Design (SbD), a categorization with fixed bands might be useful. As highlighted, the boundaries of these fixed bands may have a biological relevance. A key study (Meesters et al., 2019) investigating the influence of physicochemical properties on the fate and potential exposure of NFs in the environment, was used as a reference for the proposition of the different categories presented in Table 2, in particular the minimum and maximum relevant ranges for dissolution rate and dispersibility.

In some cases (*e.g.* the shape and size distribution of constituent particles), a critical value (or critical values) may exist and has been proposed based either on biological relevance (*e.g.* critical size for NF being a size smaller than 5 nm (in diameter) owing to biokinetic and translocation changes) or due to its wide acceptance by the scientific expert community. Although grouping based on biological similarity may be easier if groups remain entirely below or entirely above such a critical value (*e.g.* an aspect ratio of 3 is used by the ECHA guidance (ECHA, 2019) to differentiate elongated ($\geq$3) from spheroidal ($<$3) NFs) it is however not the case for all materials of interest. Grouping of spheroidal and elongated NFs may be justified, if no NF fulfils HARN criteria from a size, aspect ratio and biopersistence point of view. In addition, the type of scale (linear *vs* logarithmic) that should be adopted for some of the physicochemical properties (*e.g.* aspect ratio, *etc.*) is not always obvious and will mainly result from the wideness of the proposed value range (Janer et al., 2021).

6. Summary

In the GRACIOUS Framework for grouping and read-across of nanoforms physicochemical properties of nanoforms are considered basic information to enter that Framework, and each nanoform is characterised by a set of descriptors (descriptor array) needed to distinguish is as a distinct NFs of a material. This paper presents an analysis of the IATAs proposed in GRACIOUS to understand which of the intrinsic and extrinsic physicochemical properties proposed in the GRACIOUS Framework are actually used in these IATAs and how frequently they are used. The physicochemical properties were also compared to the REACH regulatory information requirements and the DF4nanoGrouping data requirements. The physicochemical properties of interest were selected based on their relevance in identifying NFs and how strongly they influence the NFs stability/reactivity. This analysis provides a basis for refining the descriptor array used in the GRACIOUS Framework to include only the parameters that have an optimal ratio of significance (for grouping and read-across) to accessibility and cost in order to generate a final set of properties and methods for inclusion in the Framework Guidance Document.

For each physicochemical property, the paper provides an overview of the measurement methods, descriptors and technology readiness level to investigate the property as well as a detailed discussion of essential resources for protocols associated with each of the identified physicochemical properties.

The criteria used to facilitate the selection of the most significant and relevant properties is also described and are based on i) requirements from IATAs, *i.e.* the frequency at which a given physicochemical property occurs, directly or indirectly, in the different IATAs and in the decision trees from the basic information stage ii) relevance to IATAs, and to qualitatively classify relevance, the positioning of each physicochemical property within the IATAs was assessed; for example, physicochemical properties used as a pre-screening criterion in an IATA will be regarded as less relevant than if the property serves as a cut-off value that justifies read-across and grouping; iii) outcomes of future case studies, which will indicate whether the level of frequency, importance and relevance that are proposed for each physicochemical property in the IATA are appropriate; iv) method development status (technology readiness level; TRL) and v) estimated cost, mainly comprising materials, instruments, staff and time, and cost for sample preparation and sample analysis. For each of these criteria the level of importance (*e.g.* low, medium and high) will be proposed.

The required level of accuracy needed for NF physicochemical properties was proposed as an additional criterion as *e.g.* this would easy the use of data from literature. The required level accuracy has categorization boundaries associated, mainly based on scale type (linear *vs* logarithmic) and suggested minimum/maximum ranges, to allow NF grouping and read-across based on similarity assessment. The outcome of this work will be part of the basis for the GRACIOUS (and future projects) framework design and refinement for grouping and read-across of NFs.

Abbreviations

ACEnano	EU H2020 project 'Analytical and Characterisation Excellence in nanomaterial risk assessment: A tiered approach'
AUC	Analytical Ultracentrifugation
BET	Brunauer-Emmett-Teller method
CLP	Regulation (EC) No 1272/2008 on classification, labelling and packaging of substances and mixtures
CLS	Centrifugal liquid sedimentation
DF4nanoGrouping	A decision-making framework for the grouping and testing of nanomaterials
DLS	Dynamic light scattering
DLVO	Derjaguin–Landau–Verwey–Overbeek
DCFH	Dichlorodfluorescein
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECHA	European Chemicals Agency
ENV	environment
EPR	Electron paramagnetic resonance
ES-DMA	Electrospray-Differential Mobility Analysis
ESR	electron spin resonance
EU	European Union
FFF	Field flow fractionation
FP	Framework Programme
FRAS	Ferric reduction ability of serum
GC	Gas chromatography
GOV4NANO	EU H2020 project 'Implementation of Risk Governance: meeting the needs of nanotechnology'
GRACIOUS	EU H2020 project 'Grouping, Read-Across, CharacterIsation and classificatiOn framework for regUlatory risk assessment of manufactured nanomaterials and Safer design of nano-enabled products'
GUIDEnano	EU FP7 project 'Assessment and mitigation of nano-enabled product risks on human and environmental health: Development of new strategies and creation of a digital guidance tool for nanotech industries'
H2020	Horizon 2020
HARN	High Aspect Ratio Nanoparticles
HH	human health
IARC	International Agency for Research on Cancer
IATA	Integrated Approaches to Testing and Assessment
ICP	Inductively coupled plasma
IRMM	Institute for Reference Materials and Measurements (Part of the European Commission's Joint Research Centre, which has since been re-organised, so IRMM does not exist under that name anymore)
ISO	International Organization for Standardization
LC	Liquide chromatography
LIN	Linear
LOG	Logarithmic
MALDI	Matrix Assisted Laser Desorption/Ionization
MARINA	EU FP7 project 'Managing Risks of Nanoparticles'
MS	mass spectrometry
MWCNT	Multi-walled carbon nanotubes
NANOREG	EU FP7 project 'A common European approach to the regulatory testing of nanomaterials'
NanoDefine	EU FP7 project 'Development of an integrated approach based on validated and standardized methods to support the implementation of the EC recommendation for a definition of nanomaterial'
NanoGRAVUR	German national project 'Nanostructured materials - Grouping for occupational health and consumer and environmental protection and risk mitigation'
ND	not determined
NEP	nano-enabled product
NF	nanoform
NIOSH	National Institute for Occupational Safety and Health (USA)
NOM	Natural organic matter
OECD	Organization for Economic Co-operation and Development
OES	Optical emission spectrophotometry
ProSafe	EU H2020 project 'Promoting the Implementation of Safe by Design'
REACH	Regulation (EC) No 1907/2006 on Registration, Evaluation, Authorisation and Restriction of Chemicals
SAD	System Analysis and Design
SbD	Safe by Design
SEM	Scanning electron microscopy
SSA	specific surface area
TBD	To be determined
TEM	transmission electron microscopy
TG	OECD Test Guideline
TGA	Thermogravimetric analysis
TRL	technology readiness level
VCM	Volumetric centrifugation method
VSI	Virtual special issue
VSSA	Volume specific surface area
WHO	World Health Organization
WPMN	(OECD's) Working Party on Manufactured Nanomaterials
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction
XRF	X-ray fluorescence

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Author statement

All authors contribute to the development and to the writing of the manuscript.

Declaration of Competing Interest

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

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Appendix A. Supplementary data

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