Materials Today 90 (2025) 68–85

Contents lists available at ScienceDirect

Materials Today

journal homepage: www.elsevier.com/locate/mattod

Minimising data needs to support the safer design of multicomponent nanomaterials – Application of grouping

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ARTICLE INFO

Keywords:
Grouping
Similarity assessment
Nanomaterials
Safe and sustainable by-design
IATA

ABSTRACT

There is an ongoing demand to develop options to reduce hazard testing of substances and materials on a case-by-case basis. Grouping approaches offer a way to share or re-use safety-related information between similar substances, providing insights that can inform the Safe and Sustainable by-Design (SSbD)[2] of new materials.

Here, an existing grouping hypothesis template for single-component nanomaterials (NMs)[3] is expanded to facilitate systematic consideration of grouping for multicomponent nanomaterials (MCNMs)[4] relevant to SSbD. Modifications to the template include additional information on a) the complexity of physical and chemical composition; b) the emerging properties driving the MCNM functionality; c) the potential for MCNM components to transform with different rates, leading to complex exposure scenarios; d) prioritisation and simplification of grouping decisions related to material properties (what they are), fate/toxicokinetics (where they go) and the hazard mechanisms (what they do).

Existing information and data are used to formulate a matrix of sub-hypotheses that individually relate one (or more) indicators of 'what they are' to a single indicator of either 'where they go' or 'what they do'. The resultant sub-hypotheses are easier to assess than the all-encompassing over-arching hypothesis required for regulatory application of grouping. The estimated level of impact of each indicator is used to prioritise the sub-hypothesis assessment. Accepting or rejecting each prioritised sub-hypothesis is facilitated by the application of tiered testing strategies promoting the use of relevant existing data, new approach methodologies and machine learning-based models. A case study of SiO_2@ZnO MCNM is provided to demonstrate the template's usefulness in an SSbD context.

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[2] SSbD: Safe and Sustainable by-Design.
[3] NMs: Nanomaterials.
[4] MCNMs: Multicomponent Nanomaterials.

https://doi.org/10.1016/j.mattod.2025.08.024
Received 16 January 2025; Received in revised form 22 July 2025; Accepted 24 August 2025
Available online 6 September 2025

Introduction

The development and use of engineered nanomaterials (NMs) has expanded, particularly over the last 20 years [1]. Over this time, the complexity of these materials has increased to improve functionality, resulting in innovative advanced materials (AdMa) including multi-component nanomaterials (MCNMs; Fig. 1) [2]. Related terms to MCNMs found in the literature include second generation nanoparticles [3,4], composite (nano)particles [5], hybrid nanomaterials [6,7], nanohybrids [8], nanocomposites [9,10], multifunctional nanomaterials [11], multicomponent nanoparticles [12], multimaterial systems [13], multicomponent nanostructures [14] or smart multifunctional nanoparticles [15]. Although the term does not have a formal and harmonized definition, this paper will consider MCNMs to consist of at least two chemically distinct components, with at least one of them, or the combined construct, being at the nanoscale (1–100 nm).

The OECD's description of AdMa [16] identifies that intentional combination of different components is conducted to obtain new or enhanced properties that drive functionality (Supplementary File 1 -Table S1). Those properties may also indicate differences in release, fate/toxicokinetics (where they go), and (eco)toxicity (what they do). However, how information on the new or enhanced properties of MCNMs or MCNM-enabled products could be used to identify or predict the hazard has not yet fully explored. For example, reactive properties (e.g., redox reactivity) could be used to predict oxidative stress in biological systems potentially leading to outcomes like inflammation, fibrosis and/or even tumour formation. Since functionality related information is usually known early in the MCNM design process and sometimes even actively manipulated, it can be exploited from the very early phases for safety considerations.

The European Commission has developed a Safe and Sustainable-by-Design (SSbD) framework that aims to support consideration of chemical or material risk as well as sustainability [17–19]. This framework takes into consideration the entire life cycle of chemicals or materials, from early innovation stages through to the end of life. By considering SSbD from early innovation stages the likelihood that materials fail at the market entry stage, due to safety or sustainability issues, is hopefully reduced. SSbD implementation (with respect to safety) includes both the use of less hazardous compositions and strategies to reduce release and exposure. Importantly, functionality is weighed alongside the identified environmental health and safety concerns. The SSbD process often compares several variations of an innovative material, as well as one or more "conventional" materials, which can be used as a benchmark [20]. Such safety comparisons can be facilitated by grouping. For regulatory purposes, hazard grouping of NMs requires identification of similarities including key descriptors [21] related to physicochemical characteristics (what they are), fate and behaviour/toxicokinetics (where they go) and (eco)toxicity (what they do). If the similarities are sufficiently justified, existing safety-related information can be read-across from source substances (with data) to target substances (lacking data), thereby reducing the need to generate new hazard data (especially from studies that use animals). Whilst in regulations read-across is hazard endpoint specific, under SSbD its use is up to the users.

To support the hazard-grouping of NMs the European GRACIOUS project built upon previous efforts [22–24], to form a state-of-the-art grouping and read across framework [25]. The GRACIOUS framework includes a template to support the generation of a grouping hypothesis [26] (Fig. 2), that predicts whether NMs with similar characteristics, in the same exposure context, will cause similar hazard outcomes.

While extensive evidence is needed for regulatory purposes to demonstrate similarity for grouped substances, we propose that a less stringent approach can be taken for SSbD. For example, grouping for SSbD can rely more heavily on New Approach Methodologies (NAMs) [27–29] rather than animals (often used for regulatory purposes), as recommended in the European Commission SSbD framework [18,19]. The range of NAMs available are increasingly diverse, such as high throughput screening [30,31] and *in vitro* assays or *in silico* tools. Decision trees known as Integrated Approaches to Testing and Assessment (IATAs) provide a structure to identify and prioritise relevant NAMs. The use of existing of new data generated by these NAMS is needed by innovators to understand which compositional and structural features of the MCNM might minimise hazard whilst maintaining functionality. In this way, the chance of generating products that would fail in the later stages of development due to safety concerns could be reduced.

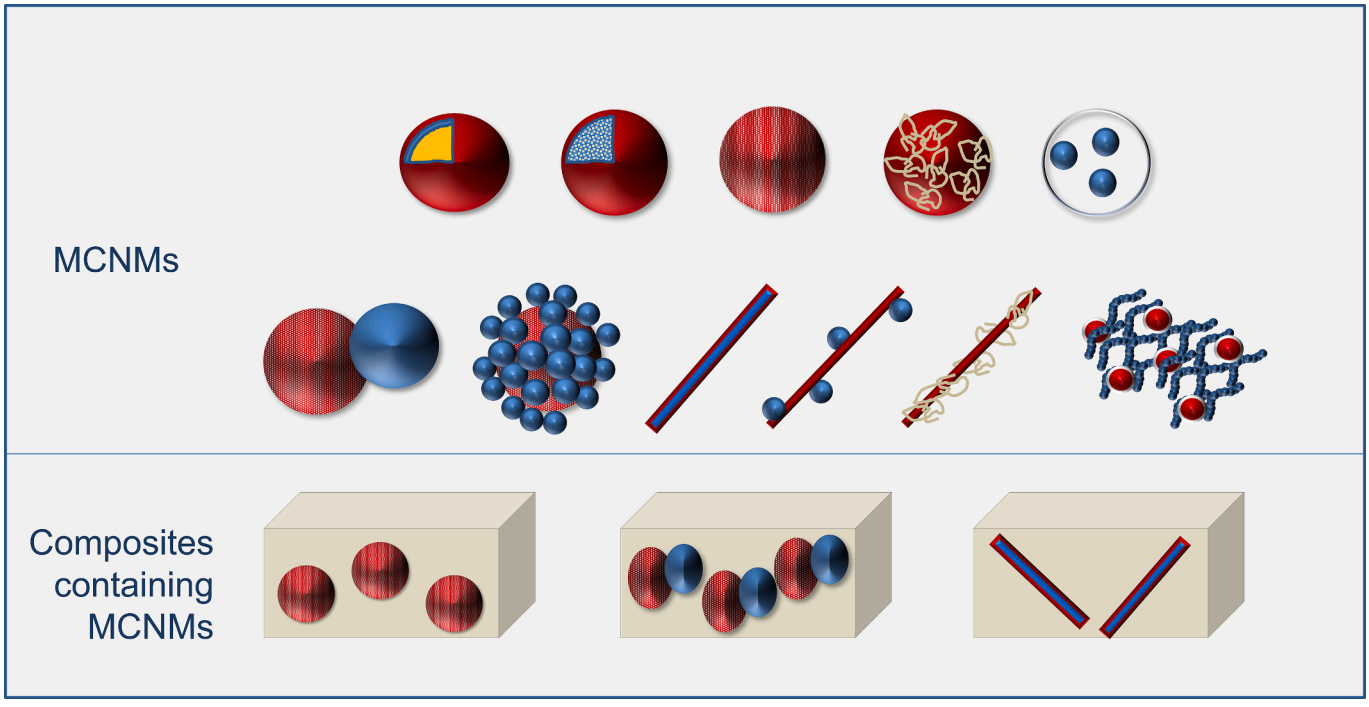

Fig. 1. MCNM diversity. MCNMs can be considered as derived purposefully from industrial engineering processes for specific applications, as well as incidentally released from composites during their production, use, recycling or disposal.

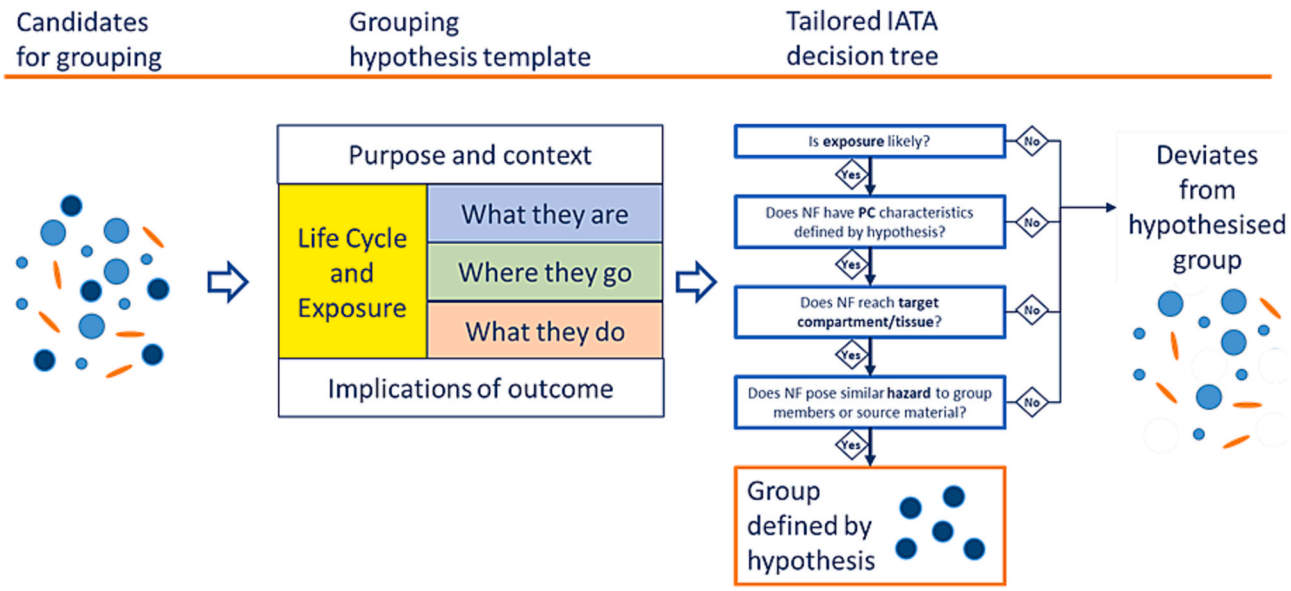

Fig. 2. Basis of NM grouping. The diagram shows how the hypothesis and associated IATAs are used to group nanoforms according to the GRACIOUS framework [32]. NF: nanoform; PC: physicochemical characteristics.

For MCNMs, grouping is potentially more challenging than for mono-constituent NMs and classic "non-nano" substances, due to the complexity of composition, the specific spatial relationship between components and their potential for different transformations, enhanced properties, and potential interactions in biological/environmental media.

This paper, which is the result of collaboration between experts from the European projects SUNSHINE, HARMLESS and DIAGONAL, shows the adaptation of the GRACIOUS grouping framework to support the SSbD of MCNMs. Through a case study (SiO_2@ZnO MCNM) we demonstrate how the adapted grouping hypothesis template systematically guides and organizes the information gathering for group members. The choice of case study was based on (i) the existing availability of data for the individual components within the MCNM, (ii) the industrial relevance of this product currently under development for inclusion in cement or mortars to remove pollutants from the air, and (iii) the evidence of enhanced photocatalytic functionality due to the MCNM structure.

Methods

The adaptation of the GRACIOUS framework aims to address emerging needs in the field of nanomaterials: 1) the possibility of extending the application of the "grouping" concept to more complex materials, such as MCNMs, beyond conventional engineered NMs; 2) the opportunity of applying a clear methodology for the generation of tailored IATA addressing the hypothesis generated through the framework; 3) to reduce data requirements in order to support early innovation stages rather than regulatory applications of grouping.

Through a first workshop involving experts from different European projects the issues driving the complexity of grouping for MCNMs were described. In order to support SSbD rather than regulatory applications, the original template structure [26] was edited to enhance flexibility, limit the information required and reduce the complexity of physicochemical transformations driven by the MCNMs. The edits included adaptation of the template to allow the generation of a range of relatively simple grouping sub-hypotheses aiding the prediction of the potential impact of the 'what they are' (column) parameters on the 'where they go' and 'what they do' (row) parameters. The revised template was designed to allow SSbD users to focus on a limited number of decision nodes, which can be selected based on users' needs (e.g., prioritisation of the component impacts, methods, or cost considerations).

The new template was shared during an online workshop in January 2023, which involved participants from SUNSHINE, HARMLESS and DIAGONAL projects. Via a series of draft texts, meetings and use of a case study described in the next sections, the hypothesis template was iteratively refined.

Results and discussion

The complexity of MCNMs affects safety and grouping

Several reasons were identified to explain why the grouping of MCNMs is more complex than for conventional engineered NMs:

(i) *Enhanced properties:* The enhanced properties of MCNMs (Supplementary File 1 - Table S1) may influence functional properties, fate, hazard and therefore risk. They could therefore be used to inform grouping hypothesis formulation. A detailed overview of the enhanced properties, their potential impact on release, and the size of that impact, along with a qualitative level of confidence of this assessment can be found in Swart et al [33]. Within SSbD approaches, the modification of functional properties of an MCNM could be used to modulate hazard and/or exposure profiles, and hence risk mitigation. However, this modification must be balanced against the need to preserve the material's functionality.

(ii) *Mixture effects:* The components of a MCNM may act independently from each other or in combination. The first scenario assumes either identical mechanism of toxicity and same target site, or different mode-of-action (MoA) and different biological target site [34,35]. When components sharing the same MoA interact, mixture effects such as synergism, potentiation or antagonism are likely to occur. Therefore, knowledge of the MoA of each component could influence grouping hypothesis formulation and inform SSbD strategies to manipulate hazard and/or exposure. The correct definition of the MoA impacts the identification of the Adverse Outcome Pathway (AOP). A more detailed definition of additive effect, synergism, potentiation and antagonism is provided in the supplementary information file (Supplementary File 2 - Fig. S1).

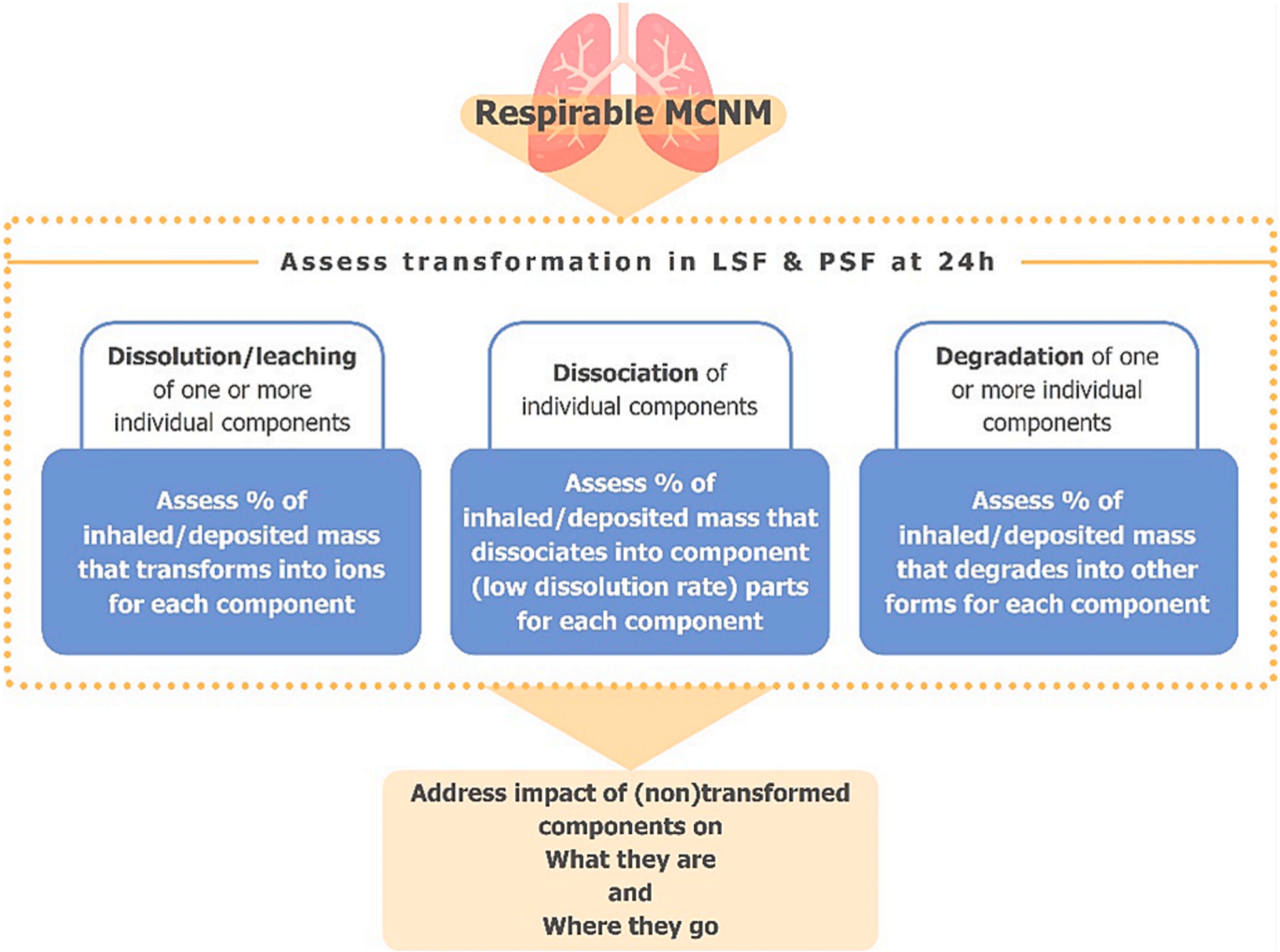

Fig. 3. Example of MCNM transformation upon inhalation. Considerations required for inhaled MCNM undergoing transformation in extracellular and intracellular fluids (lung simulant fluid (LSF) and phagolysosomal simulant fluid (PSF). Similar scenarios are relevant for ingestion (oro-gastrointestinal fluid and PSF) and skin (sweat and PSF).

(iii) *Transformation of Components:* Different components of an MCNM may undergo transformations at different rates. In the context of NMs, dissolution rate has been identified as a key transformation influencing grouping hypotheses, as this parameter allows the users to identify whether exposure (and as consequence the potential toxicity) is to ions/molecules alone, particles alone or a mixture of the two [21,36–38]. For MCNMs dissolution rates may be similar (congruent) for all components, leading to particle shrinking, or different (incongruent), leading to their leaching [39] with or without an overall decrease in MCNM size or structure. During dissolution, some released ions may be more reactive than others, leading to toxicity associated with this substance or even the formation of new species [34,35] i.e., by complexation with bioavailable anions or cations). Alternatively, a shell/core or embedded structures can shield more soluble components, preventing or slowing their dissolution.

Other transformations such as dissociation of components (e.g., detachment of a nanoparticle from the surface of a core particle), degradation of one or more components (e.g., enzymatic degradation of an organic coating), and other processes leading to a breakdown of the molecular structure (generating smaller, less functional, molecules and materials) need to be considered. Both dissociation and degradation processes could occur under different conditions (e.g., extracellular fluid, (phago)lysosomal fluid, and other environmentally relevant conditions) (Figs. 3, 4). Such transformations could therefore lead to a different fate and toxicity of the intact MCNM and its individual components, as compared to the single free components.

These potential complexities in the behaviour of MCNMs after release and exposure mean that the template presented makes particular consideration of transformation. As an example, Fig. 3 describes the options that should be considered when examining toxicity to the lung following inhalation. The first consideration is the assessment of whether the MCNM or components of the MCNM dissolve, dissociate or degrade in biological fluid at the point of entry into the body or in an environmental compartment. If the answer is 'yes', the users consider the nature of the transformation and whether it is complete, partial or unchanged at a designated time point (e.g., 24 h after deposition into lung lining fluid (LLF)). For MCNMs with partial or unchanged transformation in the initial compartment, the same considerations are required for subsequent compartments (e.g., intracellular). In case the material transforms, an understanding of the rate of transformation is also required to assess similarity of MCNMs.

Given the complexity of these considerations, we recommend as a first step the use of decision trees to identify the questions that need to be answered; the second step would be identifying the method best designed to answer such questions. Fig. 4 shows a decision tree focussed on dissolution, but similar decision trees could also be developed for

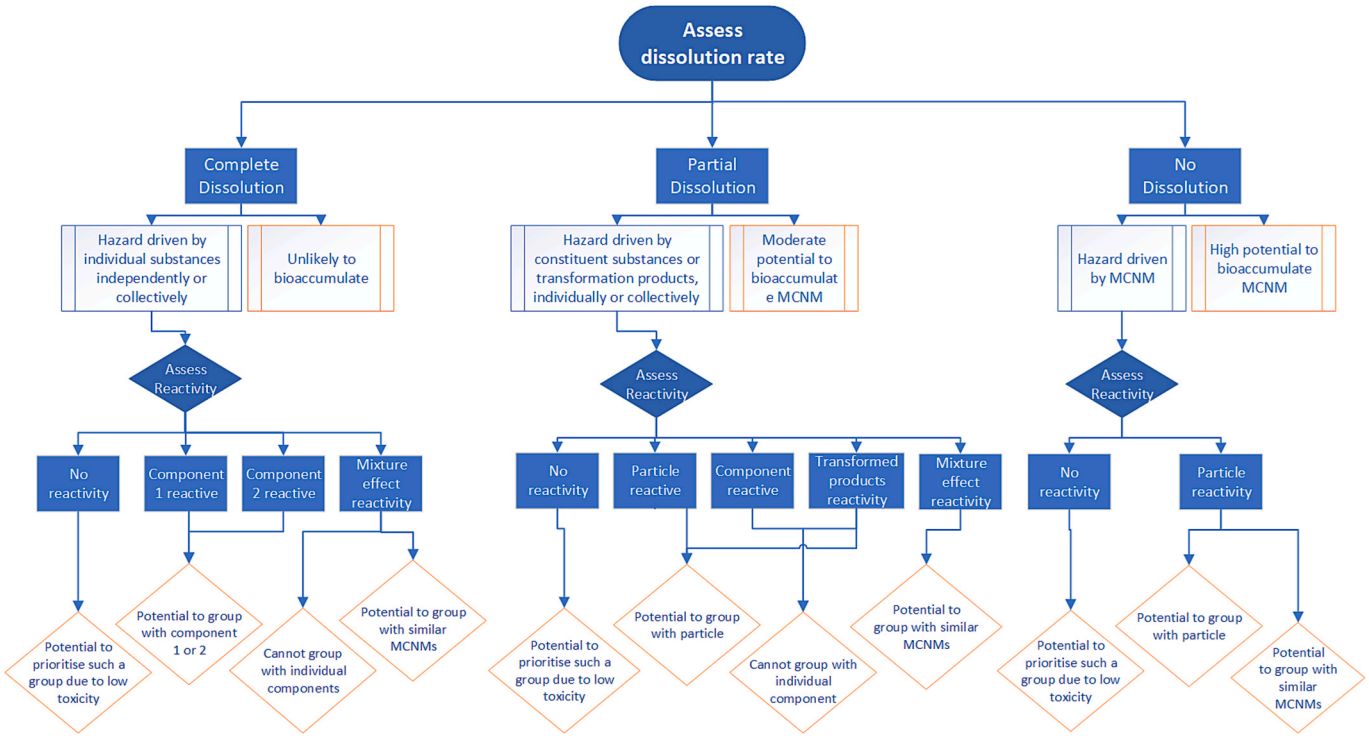

Fig. 4. Dissolution decision tree. Dissolution rate [21,36] can be used to inform hazard assessment. Since MCNMs can include substances in different proportions and with different dissolution rates, the users will need to take this into account. "Prioritisation" is intended for further stages of product development; whether doubts about safety of the MCNM still exist the decision three invites the users to group the MCNM with other substances to help in the prediction of its potential hazard. The figure is applicable to fluids relevant to the route of exposure for humans or to an environmental compartment. Similar diagrams could also be drawn for other transformations such as dissociation or degradation rates of components.

other types of transformations. For assessing dissolution, established methods are available and relevant to a range of body fluids [36,39]. If dissolution is complete and the hazards of each component are known, then the leachable mass % [39] can be used to assess the absolute hazard of each released component, allowing the users to identify whether the actual hazard is the sum of each individual released component (additive) or whether mixture effects may occur. If, however, dissolution is only partial, uptake of the remaining MCNM components into the cell is assumed, so that the users consider whether any of the hazard is particle/MCNM driven. Going back to the example reported in Fig. 3, if there is no transformation of the MCNM in extracellular fluids, then the users assume cellular interaction which may include uptake of the complete MCNM into cells. Then the users are prompted to consider the surface reactivity of the particle as well as the potential for transformation inside cells via (phago)lysosomal fluid. If there is transformation, the users are again prompted to consider whether the transformation is complete or not, and therefore whether the intracellular exposure is to the constituent ions/molecules or a mixture with particles. Again, the users are prompted to relate the released ions/molecules to their quantity in the original MCNM and to their known hazards.

Along with such transformations, it is also worth considering the potential to bioaccumulate. While Fig. 4 indicates where particle forms of MCNMs may persist and therefore may bioaccumulate, it is worth noting that this process might be true also for those components that transform into non-particulate forms.

Grouping of MCNMs – template overview

A brief overview of the content of the updated MCNM grouping template is provided in Fig. 5. The following text outlines the content of each section of the template.

Section 1 includes:

(i) The MCNM(s) of interest, along with their intended use and required functionality.
(ii) Potential benchmark substances spanning MCNMs, individual components, and constituent substances (see Section 1).
(iii) The purpose of grouping (for SSbD or Regulatory purposes).
(iv) The proposed group members, including information from (i) and (ii).
(v) Information on production, use and other relevant life cycle stages.
(vi) Prediction of exposure hotspots based on (i) and (v).

Section 2 addresses:

(vii) The context based upon Section 1, and identifies the priority environmental compartment(s), and for humans, the route of exposure.
(viii) Hazard endpoints of relevance to the context.
(ix) MoA or AOP information available for the MCNM, its components or constituent substances.

Section 3 details (as a matrix):

(x) Physicochemical properties (what they are) of the MCNMs and benchmarks.
(xi) Enhanced properties of the MCNM.

Section 4 addresses:

(xii) The relationship between 'what they are' (including enhanced properties) with 'where they go'. This leads to the formation of a series of grouping sub-hypotheses and consideration of whether the impact of each physicochemical or enhanced properties on 'where they go' is minor or major.

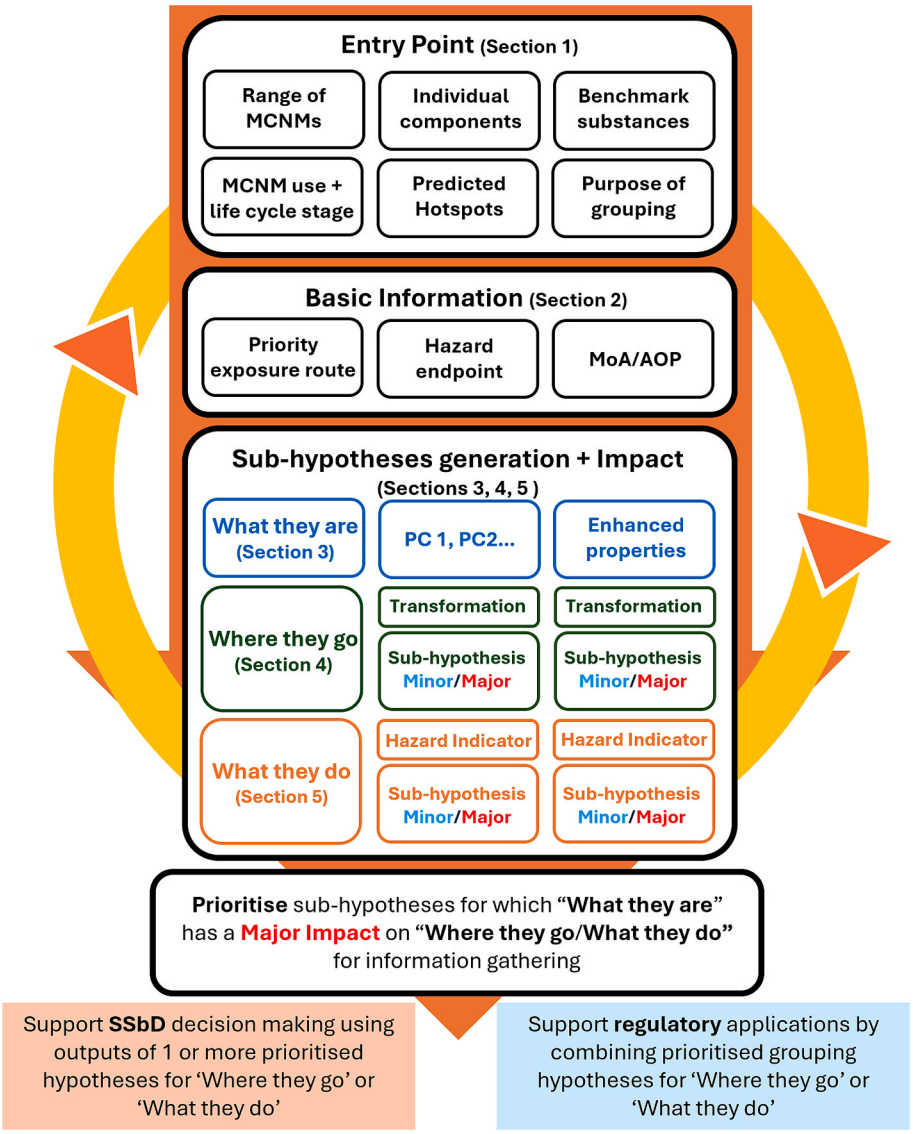

Fig. 5. MCNM grouping template overview. The scheme shows how to support information gathering to enable the SSbD of MCNMs. The arrows show the iterative approach.

(xiii) The required information for assessing each sub-hypothesis and the methods (in the form of a tiered testing strategy) that could be used to generate the information, if it is not already available.

Section 5 focuses on:

(xiv) The relationship between 'what they are' and 'enhanced properties' with 'what they do'. This again leads to the formation of a series of grouping sub-hypotheses and consideration of whether the impact of each physicochemical or enhanced properties on 'what they do' is minor or major.

(xv) The identification of information required to assess each sub-hypothesis and the methods (in the form of a tiered testing strategy) that could be used to generate the information, if it is not available.

The complexity of MCNMs is considered at an early stage of the grouping process. The use of a series of sub-hypotheses allows the identification of relevant existing information, and if needed the specific pertinent comparative fate and hazard investigations to be prioritised depending on the predicted impact to inform SSbD. These information building blocks are laid out in the template as individual cells within a table. We have termed these cells 'decision nodes' which are equivalent to the decision nodes of the GRACIOUS IATAs used for grouping of NMs [21,26,36]. However, in this context the process is simplified as there is no need to structure the decision nodes in a decision tree format or to complete them in a specific order.

The users initially input all available data into the relevant parts of the different sections (Fig. 6), before assessing whether sufficient information is available to support SSbD decision making or returning to prioritised sections to fill information gaps. As input to the template proceeds, additional useful information may become apparent (e.g., the need for an additional benchmark material). Whilst the process of completing each section is therefore likely to be iterative, a SSbD decision can be made without completion of all sections.

A more detailed explanation of each template section is provided in the main text below, followed by a practical demonstration of how to use the template for a selected case study (text boxes). The template filled in with the specific information of the case study is provided as supplementary information.

Section 1			
• **Description of MCNM, intended use and functionality:**			
• **Purpose of grouping:**			
• **Proposed Group members:**			
• **Similarity assessment for relevant decision nodes** (which comparisons are needed?):			
• **Production, use and other relevant life cycle stages:**			
• **Predicted hotspots:**			
Section 2			
• **Priority exposure route:**			
• **Hazard endpoint:**			
• **Mode of action/AOP:**	Description + Flow diagram (whether available)		
Section 3			
	Physicochemical identity - 'What they are'		
	Chemical intrinsic hazard 1, 2....	**PC characteristic 1, 2 ...**	**Enhanced properties**
MCNM 1, 2 ...			
Component NM/substance 1, 2			
Benchmark 1, 2			
Section 4			
Predicted Impact of 'What they are' on 'Where they go': Minor or major			
Deposition in target organ or environment	Impact of *Chemical intrinsic hazard* on deposition Minor/Major/ No impact	Impact of *PC 1,2...* on deposition Minor/Major/ No impact	Impact of *Enhanced properties* on deposition Minor/Major/ No impact (e.g., enhanced component A/B reactivity may impact deposition due to attractive/repulsive electrostatic charges).
Transformation in relevant fluid 1, 2 ...	Impact of *Chemical intrinsic hazard* on trasformation in relevant fluid 1,2... Minor/Major/ No impact (e.g., component A/B rapidly/slowly/does not dissolve in different pH)	Impact of *PC 1,2..* . on trasformation in relevant fluid 1,2... Minor/Major/ No impact	Impact of *Enhanced properties* on tranformation Minor/Major/ No impact (e.g., enhanced component A/B reactivity may impact transformation via modified interaction between NM and media).
Mixture effects	Impact of *Chemical intrinsic hazard* on Mixture effect Minor/Major/ No impact	Impact of *PC 1,2...* on Mixture effect Minor/Major/ No impact (e.g., size of component A/B affects dissolution rate of component A/B thus A:B ratio thus achievent of threshold concentration inducing synergism)	Impact of *Enhanced properties* on tranformation Minor/Major/ No impact (e.g., enhanced component A/B reactivity may impact).
Grouping hypothesis	MCNM can be grouped with component A and/or component B according to similarity in transformation of common chemical constituents (or NA)	MCNM can be grouped with component A and/or component B according to similarity in PC 1 (or NA)	E.g. MCNM can be grouped with component A and/or component B according to similarity deposition/transformation predictable from enhanced properties
IATA Decision Node and Similarity Assessment	Assess transformation of MCNM + individual components. Dissolution in relevant fluid 1 should/should not be assessed (or NA)	MCNM can be grouped with component A and/or component B according to similarity in PC 1 (or NA)	E.g. Assess Enhanced properties of MCNM + individual components
Section 5			
Impact of 'What they are' on 'What they do'			
Hazard indicator 1, 2 ...	Impact of *Chemical intrinsic hazard* on hazard indicator 1,2... Major/Minor/ No impact	Impact of *PC 1,2...* on hazard indicator 1,2... Major/Minor/ No impact	Impact of *Enhanced properties* on hazard indicator 1,2... Major/Minor/ No impact
Grouping hypothesis	MCNM can be grouped with [other MCNM, individual component NM and/or benchmark material] according to similarity in hazard outcomes of common chemical constituents (or NA)	MCNM can be grouped with [other MCNM, individual component NM and/or benchmark material] according to similarity in hazard indicator 1 outcomes predicted from PC 1 (or NA)	E.g. MCNM can be grouped with [other MCNM, individual component NM and/or benchmark material] according to similarity in hazard outcomes predicted from MCNM enhanced properties, functionalisation/chemistry (or NA)
IATA Decision Node and Similarity Assessment	Assess / Do not assess Hazard indicator 1, 2 ...	Assess / Do not assess Hazard indicator 1, 2 ...	E.g. Assess / Do not assess Hazard indicator 1, 2 ...

Fig. 6. MCNM working template overview. The basic information defining the case study and the interconnections between physicochemical identity (Section 3, light blue), fate (Section 4, green) and hazard (Section 5, orange) are displayed. Indication of minor/major/noimpact is expert based by the users. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Section 1 – entry point

Section 1 of the template sets out the background information needed for MCNM grouping (Box 1; Supplementary File 3 - Table S2). Users are first asked to input a 'Description of MCNM, intended use and functionality'. The description includes chemical composition, indication of the relative proportion of each component and the structure of the components relative to each other (examples are provided in Fig. 1). The intended use informs likely release and exposure scenarios. Information on functionality is needed to inform considerations of enhanced properties that might influence 'where they go' and/or 'what they do'. At this stage additional physicochemical characteristics are not required.

Next the users define the 'Purpose of grouping' to focus the level of evidence required to justify grouping. A regulatory risk assessment will require stronger evidence of similarity than grouping for SSbD purposes.

The term 'Proposed Group Members' refers to the materials/substances being grouped. This could include an MCNM along with one or more of its individual components, other MCNMs, or MCNM-containing nano-enabled products (NEPs). If a company has a range of MCNMs to consider for development, the putative group might include all candidate MCNMs, with grouping used to inform their prioritisation for use or development. In some groups, single component materials may be included, that should match the form (e.g., size, shape) and composition of the components of the MCNM. However, this is not always possible since some components would not exist in isolation (e.g., the shell of a core shell structure). In such a case, a pragmatic approach could be used to identify alternatives.

At the initial stage of the process, users must also identify benchmark materials or substances with known properties, physicochemical characteristics and/or hazards. Benchmarks may also include other MCNMs, if available. Benchmarks allow for the identification of the biologically relevant range of a specific hazard test, ensuring the relevance of any data for the MCNM. Benchmarks may also inform the measurable range that can be detected by the experimental method used (which might be different to the biologically relevant range) [40,41]. Furthermore, for those benchmarks that are highly toxic, the users may wish to establish that the MCNMs are "not similar" to these substances, and *vice versa* for those benchmarks of low toxicity. It is worth noting that benchmarks are not positive or negative controls. Instead, they are intended to be used across all the data sets/studies in the grouping exercise, rather than in a single data set/study. Positive and negative controls could also be included for each study to demonstrate that the experimental system is working effectively.

The 'Similarity assessment' section identifies the logical comparisons to be made between putative group members and allows the users to exclude comparisons not relevant to the purpose to simplify the decision making. Such logical comparisons might include pairwise comparison between an MCNM and each individual component of the MCNM, a pairwise comparison between different MCNMs or multifactorial comparison across a panel of substances (including for example a control benchmark material of known hazard). A comparison between different components might also provide information on their relative hazards.

The 'Production, use and other relevant life cycle stages' adds to the information under 'Description of MCNM, intended use and functionality'. Production methods are important as they may impact hazard and functionality (e.g., precipitated silica, fumed silica and mined clays). Steps in the production, use, and predicted life cycle can be used to identify potential exposure hotspots. The 'predicted exposure hotspots' are then prioritised to identify the exposure routes and contexts or populations of most concern. Different exposure routes (e.g., inhalation of aerosolised substances or dermal exposure), or differing contexts (e.g., human health or release into the environment), will require separate

Box 1
Case study: Entry Point (Template Section 1).

The case study here described consists of an MCNM (conventionally abbreviated as SiO_2@ZnO; Supplementary File 3 - Table S2) synthesized from commercial mesoporous SiO_2 NM powder (average particle diameter of 20 nm, 95.5 % purity, NanoAmor, TX, USA) and $ZnAc_2{\cdot}2H_2O$ (purity 99.5 %, ITW Reagents S.r.l.). Briefly, the zinc acetate dihydrate was deposited on the SiO_2 NM core in a 1:2 ratio, through a calcination/hand grinding process to form the desired core–shell structure with ZnO NM as the outer shell [42]. The MCNM SiO_2@ZnO was provided by the Andalusian Innovation Centre for Sustainable Solution (CIAC). The material is proposed by the manufacturer for use in the construction industry, where it is mixed with a mortar cement and applied to the exterior surfaces of buildings with the purpose of improving human health through ZnO photocatalytic decontamination of hazardous oxides of nitrogen (NOx) gases to form NO_3^- [43]. The presence of the SiO_2 component in the MCNM improves the ZnO performance in terms of reactivity [44], stability, and dispersibility on the building materials (e.g., mortar). There are several reasons for the enhanced surface reactivity. The MCNM exhibits a decreased energy band gap which drives enhanced photocatalytic activity to form free radicals in water [45]. The SiO_2 further enhances this process by increasing the surface area and providing active sites, which improve ROS generation and reduce electron-hole recombination [45–47].

Here we aim to test whether the specific MCNM SiO_2@ZnO can be grouped with either mesoporous SiO_2 or ZnO NMs, through the understanding of similarities in their fate and hazard potential. The early identification of the fundamental drivers of toxicity will then help the re- design of a safer MCNM.

The assessment of the peer reviewed literature revealed existing hazard data for the constituent components (summarised below), but not for the MCNM. The similarity assessments will therefore be a pairwise comparison between the SiO_2@ZnO MCNM and each of the individual components, i.e. the mesoporous SiO_2 (identical to that found in the MCNM) and a ZnO NM for relevant fate and hazard endpoints, identified in Section 5. The ZnO and SiO_2 NMs were provided by CIAC. The SiO_2 NM was identical to the version in the MCNM. The ZnO NM was manufactured using the same reagents and processes as for the ZnO in the MCNM. However, the exact form of ZnO in the MCNM could not be replicated in the absence of the SiO_2 NM, and so the resultant ZnO NM is only a surrogate rather than an exact comparison.

Mechanical mixing of MCNM into the mortar has been identified as a potential exposure hotspot which may result in inhalation exposure of the aerosolised MCNM. Analysis of the life cycle of the MCNM, indicated that once incorporated and covalently bonded into the cement, it no longer exists as a discrete MCNM. Therefore, release of the intact MCNM from the concrete during use of end-of-life is unlikely. Whether weathered SiO_2@ZnO nanomaterials are released during end-of-life is uncertain, however previous analyses performed by applying EN 12457-3:2004 [48] and ISO 2812:2018 [49] identified no or negligible release of Zn or Si from the cement [42], confirming the stability of MCNM incorporation. This suggests that exposure post incorporation into the mortar is not relevant to consider at least at an early innovation stage.

While other exposures during the manufacturing process might be expected (e.g. dermal exposure during mixing into the mortar), the consideration of multiple exposure scenarios is not necessary at the early innovation stages. Instead, it is more prudent to focus on the highest likely exposure route, or the exposure route associated with the greatest hazard. Assessment of the literature for the components of the multicomponent nanomaterial indicates that inhalation is a route associated with hazard. At a later innovation stage, the user could consider more or all exposure routes.

templates to be completed to provide relevant assessment of hazard and similarity between substances specific to the context. Frequently the MCNM considered for SSbD within the same case study are likely to have similar uses and life cycles, which will limit the potential exposure scenarios to be considered.

Section 2 – hazard and mode of action

Section 2 (Box 2; Supplementary File 4 - Table S3) of the template focuses on using the gathered information to define a series of grouping sub-hypotheses. These sub-hypotheses allow the identification of individual decision nodes that tailor the information needs to support SSbD of MCNM(s) under evaluation.

The first step is for the users to define the context – whether related to human health or environmental compartments – based on identified exposure and release hotspots of concern.

Next, the users define the relevant hazard endpoint(s) of most concern for the exposure context identified. The hazard endpoint could be that required in regulations, but for SSbD they could be more flexible. Any MoA or AOP information is useful to identify the descriptors [21] that feed into decision node design required to gather the evidence needed to demonstrate similarity between group members. It is worth noting that this approach builds upon known toxicity, rather than novel toxicity (not yet anticipated). For early innovation stages with limited resources, it is more efficient to focus on known toxicity than to investigate a wide range of new toxicities that might be relevant for standard risk assessment.

Section 3 – determining 'What they are'

Section 3 (Box 3; Supplementary File 5 - Table S4) considers the question 'what they are' and identifies the need to consider:

- The different physical and chemical components of the MCNM.
- Interaction between components via mixture effects that may influence hazard.
- Enhanced properties that may induce additional or novel hazards.

In case of a core–shell structure, details of the outer layer/shell composition, strength of the bonds between components, thickness, and/or the presence of additional surface decorators (e.g., metals, functional groups, if any) will be required in addition to the standard parameters needed for NM characterisation. Within an SSbD context more flexibility is accepted. Thus, parameters may be measured or derived from computer modelling (e.g., quantum mechanics), to provide quantitative descriptions of various aspects, including size, shape, surface properties, charge and electronic distribution [78,79]. This becomes particularly significant in the context of newly designed MCNMs because theoretically calculated parameters allow the users to understand 'what they are' even before synthesis. This information also allows the suitability of the benchmark materials to be assessed. The template can be expanded to add further physicochemical characteristics as required.

Section 4 – considering 'Where they go' including transformations

Section 4 (Box 4; Supplementary File 6 - Table S5) considers 'where they go', which is informed by the use and exposure scenarios of section 1, and the 'what they are' information in Section 3. The headings for each column of Section 3 also act as the column headings in Section 4 to

Box 2
Case study: Basic information (Template **Section 2**)

The completion of Section 2 for the SiO_2@ZnO MCNM case study highlighted the potential for human exposure by inhalation (Supplementary File 4 - Table S3) as confirmed by a questionnaire completed by the CIAC Foundation [42].

Upon deposition in the respiratory tract, inhaled NMs contact mucous in the upper respiratory tract and neutral pH lung lining fluid (LLF) in the deeper lung [54]. Deposition in the upper respiratory tract is likely to be associated with effective clearance via the mucociliary escalator, unless dissolution is immediate. NM dissolution may occur in mucous and LLF or, in acidic (phago)lysosomal fluid if taken up by macrophages or epithelial cells. If the MCNM or any of its components do not dissolve they will persist within the lung *interstitium*, or lung-associated lymph nodes for an extended time. Following inhalation of pathogenic particles of low solubility (e.g., crystalline silica), adverse outcomes such as chronic lung inflammation [55], fibrosis [56], and carcinogenicity [57,58], are possible due to a continued exposure, and so these endpoints are important to consider for inhaled MCNMs that do not dissolve completely. With respect to hazard, crystalline SiO_2 NM (CAS n° 7631–86-9) in the form of quartz or cristobalite is toxic and classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans [59,60].

Most notifiers to the EU Classification and Labelling database have classified amorphous silica under CLP as not hazardous [61] but it does not have a harmonized classification as decided by regulators. However, a substance evaluation under the European Union regulation Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) Regulation N° 1907/2006 [62] authored by The Netherlands raises concerns that the substance may present adverse effects sufficient for classification [63]. The report concludes that there is sufficient evidence to justify reconsideration of classification and labelling for repeated dose toxicity (STOT RE) in the lung/respiratory tract for SiO_2 NM powders. The European Chemicals Agency (ECHA), in consultation with other evaluating member states, will evaluate the new available evidence and therefore may update this classification. In other *in vivo* studies, spherical and mesoporous silica were similar in their ability to increase airway hyper-responsiveness in mice treated with the antigen ovalbumin [64]. A follow-up study by Han et al [65] at a higher concentration and for a prolonged period (10 mg/kg, 3 times weekly for 2 weeks), also found airway hyper-responsiveness to the allergen ovalbumin. Although these particles appear to have adjuvant effects, there is no indication in the literature that they are respiratory sensitizers. According to the literature, various forms of silica NMs, including mesoporous, can dissolve in LLF [66], but it is likely to be sufficiently slow that the particulate form will persist long enough (hours) to be taken up into cells and/or be translocated to lung-associated lymph nodes [54].

Therefore, the hazard endpoints of inflammation, fibrosis and carcinogenicity should be considered to influence SSbD considerations.

The study by Braun et al. (2016) [66] also indicates the dissolution of mesoporous SiO_2 NM is slower at lower pH values. This would suggest that if taken up into cells, dissolution in (phago)lysosomal fluid will be slower, allowing the particles to persist, further justifying their potential to induce the hazard endpoints identified. ZnO (CAS no.: 1314–13-2) is classified by the harmonised classification and labelling system approved by the EU as may damage fertility or the unborn child, is harmful if swallowed, is harmful if inhaled and may cause damage to organs through prolonged or repeated exposure. However, some of the registrants suggest these toxicities are caused by impurities or additives. Some of the lung evidence comes from ZnO in welding fumes, which is associated with pulmonary toxicity in welders, with high doses resulting in metal fume fever [67].

The mechanism of toxicity in humans is documented to involve lung inflammation [68]. Using animal models, a single exposure to ZnO NM has been demonstrated to induce pulmonary inflammation (neutrophil influx) and lung damage (protein in BAL) at doses of 64 and 128 μg in C57BL/6N mice within 24 h following intratracheal instillation [69]. In another study with longer exposures via inhalation (e.g., at 3 weeks, 3.5 mg/m^3, 4 days per week) [70] an increase of macrophages in BAL fluid and a moderate increase in IL-12 (p40) and MIP-1α were observed, but no other inflammatory or toxic responses were detected. In contrast, Jacobsen et al [71] demonstrated a dose-dependent (25–100 μg) pulmonary cytotoxicity and mortality in mice, which was attributed to dissolution and therefore exposure to Zn^{2+} ions. Doses below 6 μg caused acute toxicity including damage to the lung epithelial barrier, as well as oxidative stress. Any mice that survived the higher exposure dose exhibited lung fibrosis. For the NM110 form of ZnO, pulmonary exposure has been shown to induce strong inflammatory and acute phase responses in mice and humans, which is linked to dissolution of ZnO in lysosomes [72]. Since ZnO NM has been demonstrated to induce toxicity both to humans, animal models and in the environment [73], consideration of its transformation will be essential. Dissolution studies of ZnO NM indicate quick dissolution in artificial lysosomal fluid [74], but agglomeration in artificial interstitial fluid [70]. Combining the available MoA for mesoporous SiO_2 NM and ZnO NM, a putative MoA for SiO_2@ZnO MCNMs has been developed (Fig. 7). The MoA incorporates the cascade of biological changes resulting from interactions between the MCNM and biological target (what they do). This MoA can be assessed by the GRACIOUS Inhalation IATA [21,75] and GRACIOUS genotoxicity tiered testing strategy [76]. The decision nodes (and IATA) can be extended to include more targeted hazard assessment which can be guided by the Lung fibrosis AOP173 [56] and Lung carcinogenicity AOP303 (under development) [77] to demonstrate mechanistic similarity.

relate ‘what they are’ and enhanced properties to considerations of ‘where they go’.

Considerations of ‘where they go’ may include MCNM deposition (e.g., for inhalation) transformation (e.g., dissolution, dissociation, and degradation) in relevant environmental liquids, extracellular body fluids (e.g., lung lining fluid, oro-gastro-intestinal fluid, sweat) and inside the cell (phago)lysosome (Figs. 3 and 4). Transformation determines the fate of both the MCNM and its single components, with different components potentially having different transformations; this information is needed for further understanding of hazards. Similarity in transformation may be used to demonstrate possible similarity in the mechanism underlying the toxicity or bioactivity of the MCNM. Differences identified in overall bioactivity, or the underlying mechanism of toxicity can then be used to improve safety such as identifying the need to modify composition/structure to reduce hazard or exposure. Section 4 is also an opportunity to consider whether interactions between the different components of the MCNM might lead to mixture effects (whether observed) that influence ‘where they go’.

The result of the above exercise is to generate sub-hypotheses linking the effect of a specific physicochemical characteristic (including enhanced properties) to the fate or toxicokinetics of an MCNM.

For each interaction, the users should indicate whether the effect is expected to be minor (evidence of no, or little association identified via literature) or major (some evidence of association identified via literature search). More expert users could further stratify the major impact according to the nature (*in vitro* vs *in vivo*), quality (e.g., Klimisch score

Box 3
Case study: 'What they are' (Template Section 3)

Section 3 highlighted the relatively large size of the benchmark ZnO provided by CIAC, compared to the form within the MCNM. For this reason, a smaller benchmark of NM110 ZnO from JRC [80] was also included due to the abundance of information on its physicochemical characteristics and toxicology. However, the physicochemical characterisation of the MCNM quickly established the lack of similarity in physical form and further assessment of transformation processes highlighted significantly higher dissolution rate. For this reason, an additional control of soluble $ZnCl_2$ was added to the case study to simulate complete zinc dissolution. While such data might not ultimately lead to grouping of the MCNM with these representations of the constituents, they are still useful to inform on the potential MoA and the hazard indicators that are relevant to assess. The dissolution information from the MCNM therefore provided the information to feedback into the choice of putative group members, allowing an iterative approach to the group membership. This iterative approach means that the number of members in a group may fluctuate throughout the process as candidates are either identified via expert judgement by the users as "not similar" to each other, or further information is gained on the characteristics of the MCNM that warrant addition of new candidate members.

[81]) and completeness [82] of evidence available. Such an approach will be useful at later innovation stages (when progressing towards regulation).

The parameters likely to induce major impacts on 'where they go' can therefore be prioritized for grouping hypothesis generation and for identifying the parameters to be measured (or predicted).

The next step is to test one or more of the prioritised sub-hypotheses based on the extent of the predicted impact. There is no specific order in which the "flagged" sub-hypotheses (and decision nodes) need to be tested; in addition to the predicted relevance (i.e., major impact), the choice can be influenced by availability of data and relevant methods to generate missing data. To support this process, the template prompts the users to identify the information required to test the sub-hypotheses. The cells in the working MCNM grouping template (Fig. 6) that correspond to these information requirements are the decision nodes. It is worth noting that the same decision node may appear several times within the matrix, so that the same data can be used for assessing several sub-hypotheses. For each decision node we recommend structuring suitable methods into a tiered testing strategy [21,25,26,36,75,82,83]. While, several tiered testing strategy structures have been proposed, they have in common the use of more simple approaches in earlier tiers, progressing to more complex methods in later tiers. For example, in the project GRACIOUS Tier 1 represents simple assays (e.g., *in silico, in chemico* (cell free), *in vitro* with a single cell type), Tier 2 represents more complex *in vitro* systems (e.g. multiple cell type 3D tissue culture models), and Tier 3 uses animals, typically rodents. However, SSbD being the purpose of this exercise, the use of animal studies is discouraged. For all three tiers the presence of existing data is identified before promoting the generation of new data. Similarly, in a tiered testing strategy for ecotoxicology testing, Tier 1 represents *in silico, in chemico* or acute invertebrate testing, Tier 2 includes longer term exposures of invertebrates, while Tier 3 could use multiple species to represent a mesocosm. Other examples of tiered testing strategies have also been published, for example via the European Commission's SSbD framework [18,19] in which tier 0 refers to toxicological concerns based on chemistry/structure, tier 1 refers to *in silico* tools, tier 2 *in vitro* tools and tier 3 *in vivo* studies. It is important to note that the *in silico* tools available for chemicals are not directly applicable to nanomaterials or MCNMs. *In silico* tools for physiological based kinetic models to predict distribution in the body do exist for NMs and can be incorporated into IATAs [84]. In contrast, the *in silico* tools available for prediction of NM or MCNM hazard are not yet widely applicable, although some are available in the literature [85–87].

While it is not essential that testing methods are standardised (e.g., OECD, ISO), the better established and validated the method, the more reliable the data and subsequent decisions will be. In addition, the use of standardised methods will allow a more efficient transition into regulatory applications of grouping. However, the inclusion of NAMs like PBK models into IATAs to predict toxicokinetics of advanced materials might help in streamlining their acceptance also for regulatory purposes [84].

The resulting data can be compiled into a data matrix to allow assessment of similarity. Demonstrating similarity in the specific physicochemical or enhanced properties between the test panel members under investigation will support grouping substances for a specific decision node, i.e., if size is assumed to have a direct impact on deposition in the lung, demonstrating similarity between the size of a panel of MCNMs will support the sub-hypothesis that each MNCM will deposit in the same region of the lung.

The assessment of similarity will be more accurate if identical methodology has been used to generate the data for the substances compared.

Section 5 – assessing 'What they do'

In order to simplify the evaluation of the expected impacts of the MCNM physicochemical properties on 'what they do', so called "hazard indicators" have been identified considering the most common parameters of reactivity (e.g. ability to generate reactive oxygen species), cytotoxicity, inflammatory potential and genotoxicity [21,36]. However, the users can add or delete different indicators that are relevant to a specific MoA. The ability of each physicochemical characteristic to influence each hazard indicator is assessed and the extent of impact either predicted or based on existing data. The parameters likely to induce major impacts on 'what they do' can therefore be prioritised for grouping sub-hypotheses generation and for identifying the parameters to be measured (or predicted), again by applying tiered testing strategies [21,25,36–38,75]. As for Section 4 ('where they go'), MCNM's transformation also plays a major role in influencing 'what they do'. Therefore, the role of dissolution/leaching, dissociation and degradation requires consideration to attribute hazard to the relevant components (Fig. 3). The format of the table allows the systematic identification of individual sub-hypotheses that relate key physicochemical and enhanced properties to the hazard indicator, so that the users can consider one or more factors to feed into the SSbD decision making process.

Assessing similarity

Similarity assessment is required to determine whether the putative group members can be effectively grouped to justify the sharing or reuse of data. The similarity should be assessed for the different decision nodes. Similarity can be assessed both qualitatively, and by applying quantitative algorithms [95,96]. Qualitative methods, such as expert judgement or use of thresholds, are obviously less stringent than quantitative methods, however in an SSbD context it is up to the users to decide whether qualitative methods are sufficient for their needs.

For the quantitative similarity assessment, the users can compare two group members at a time (pairwise) for each measured parameter to

Box 4
. Case study: 'Where they go' (Template Section 4).

In Section 4 of the SiO_2@ZnO MCNM case study (Supplementary File 6 - Table S5), the physicochemical characteristics and the enhanced properties listed in Section 3 are replicated at the top of the matrix to remind the users about the impact of the substance characteristics on their fate.

The particle sizes suggest that aerosolised particles could be inhaled [88–90].

The predicted MoA indicates contact with the lung lining fluid and or (phago)lysosomal (Fig. 3 and Fig. 4). Mesoporous silica has been shown to dissolve in the neutral pH of the lung lining fluid [66], so the dissolution rate of SiO_2 within the MCNM and the one of the individual component mesoporous SiO_2 NM was compared. The structural transformation of the MCNM post incubation with lung lining fluid could also be assessed. For example, microscopic analysis of the SiO_2 NM post-incubation in the lung simulant fluid shows that SiO_2 NM undergoes partial dissolution [91], requiring the users to consider both dissolved and undissolved forms.

For the ZnO NMs, dissolution in LSF is very slow [91], suggesting that the undissolved form is particularly relevant in this compartment. For the SiO_2 and ZnO particles that do not completely dissolve, we need to consider uptake of particles into cells (see below).

If transformation includes dissociation, then consideration of the fate of different components needs to be addressed. Currently, dissociation of an MCNM into the individual components is technically challenging to quantify; therefore, such a decision node could only be addressed by qualitative microscopy.

Persistent components that could be taken up into cells need to be considered for dissolution in (phago)lysosomal fluid. ZnO NM dissolves rapidly in the low pH, while SiO_2 NM dissolution is very slow [91].

Applying the considerations described above to this specific case study, the following sub-hypotheses were formulated:

- SiO_2@ZnO could be grouped with SiO_2 NM and/or ZnO NM according to similarity in transformation of common chemical constituents.
- SiO_2@ZnO could be grouped with SiO_2 NM and/or ZnO NM according to similarity in shape.
- SiO_2@ZnO could be grouped with SiO_2 NM and/or ZnO NM according to similarity in deposition predictable from size.
- SiO_2@ZnO could be grouped with SiO_2 NM and/or ZnO NM according to similarity in transformation predictable from size.
- SiO_2@ZnO could be grouped with SiO_2 NM and/or ZnO NM according to similarity in deposition predictable from surface area.
- SiO_2@ZnO could be grouped with SiO_2 NM and/or ZnO NM according to similarity in deposition predictable from enhanced functionality, but only if mixture effects/interactions do not drive enhanced properties.
- SiO_2@ZnO can be grouped with SiO_2 NM and/or ZnO NM according to similarity in transformation (dissolution rate or % leachable mass) predictable from enhanced properties, but only if interactions between components do not drive them.

The users do not need to assess all sub-hypotheses. For example, the sub-hypotheses related to size and surface area effects on deposition or transformation could be combined for spherical particles without porosity and with a relatively smooth surface.

However, while size is likely to influence 'where they go', surface area may have less impact.

Subsequent rows indicate the parameters that can be measured to assess each hypothesis. For the SiO_2@ZnO case study, dissolution leading to % leachable mass of ions is clearly important as this provides information on the release of zinc and silicon ions to contribute to the MoA.

Size is useful to confirm similarity in deposition pattern in the lung and potential for transformation [88–90] In addition, enhanced properties could be checked in relation to the potential for impacts on 'where they go'.

Based on information gathered in Section 3 we can say that 1) the MCNM and the mesoporous SiO_2 NM overlap in size range, but the SiO_2 NM has a much larger surface area (Supplementary File 5 – Table S4); 2) Surface analysis of MCNM structure (TEM-EDX) indicates that the surface pores of the mesoporous SiO_2 are filled with ZnO, resulting in a reduced surface area; 3) the ZnO surrogate NM from CIAC is 10 times larger than the MCNM, while 4) the surface area is much smaller as expected for a smooth surface; 5) the ZnO NM110 is about 2 times larger than the MCNM, which again results in a smaller surface area. Together, this information indicates potential lack of relevance of the use of ZnO NM110 as a benchmark.

Moreover, dissolution analysis of CIAC materials revealed that Zn dissolution rate from SiO_2@ZnO MCNM was much higher than for the surrogate ZnO NM or ZnO NM110, suggesting the need of including a representative soluble form of Zn, like $ZnCl_2$, as potential benchmark.

make a matrix of comparisons or conduct a multidimensional comparison between all group members for all measured parameters simultaneously [95]. Currently, pairwise methods are favoured for regulatory applications as the outcomes are more robust than the multidimensional comparisons [95]. For SSbD approaches, the pairwise methods will be easy to apply to individual data sets associated with individual sub-hypotheses and their associated decision nodes. It will, in future, be possible to automate such processes computationally, reducing the burden on the users to understand and conduct the analysis.

Recently, two quantitative pairwise methods were evaluated and assessed for their applicability to MCNMs, with a particular focus on incorporating intrinsic and extrinsic properties of single-component NMs as well as the enhanced properties of the MCNMs. The first method by Zabeo et al. [97] is based upon Ordered Weighted Average allowing for grouping of NMs without being affected by the addition of new candidates to the dataset. The method was then improved by using Asymmetric Sigmoid based normalisation which allows for a clearer differentiation between values close to the biologically relevant threshold [98].

Additionally, the Bayes factor similarity method [96] has been extended to describe similarities using diverse experimental functional, physicochemical data properties and toxicological endpoints. However, in the extended version of the methodology local groups of similar MCNMs per data source inform the decision for a final grouping of the integrated data [99].

Furthermore, when assessing similarity, one could also consider the relative importance of the physicochemical properties used for grouping. For instance, properties like dissolution and degradation,

Box 5
. Case study: 'What they do' (Template Section 5).

In Section 5 of the SiO_2@ZnO case study (Box 5; Supplementary File 7 - Table S6), the physicochemical characteristics and enhanced properties listed in Section 3 are replicated at the top of the matrix to remind the users about the impact of the substance characteristics on the potential hazard.

First the extent of the predicted impact of physicochemical characteristics on hazard was stated to help in the sub-hypothesis generation and prioritization. Complete dissolution of ZnO NM is associated with significant hazard [91].

Previous studies [92,93] indicated how the redox cycling of divalent Zn^{2+} cations could generate reactive oxygen species, a key step in driving all identified hazard indicators. For the specific case study, composition, structure, size and surface area have all been identified to have a major impact on the hazard indicators of surface reactivity, cytotoxicity, inflammation and genotoxicity. Thus, additional physicochemical characterization was required to verify how the two components were linked within the MCNM. This revealed that ZnO penetrates the SiO_2, reducing its porosity as further supported by the reduced surface area (see Supplementary File 5 – Table S4). However, the presence of ZnO on the surface does not change the overall shape or size of the MCNM, as the ZnO coating appears very thin and mainly present in the SiO_2 pores.

Since the ZnO coating is incomplete we concluded that it would not prevent the access to the SiO_2 surface by any biological fluids or cells. This finding, together with the considerations listed in Box 2, Box 3 and Box 4 (e.g., SiO_2 NM partly dissolves at neutral pH while ZnO NM does not, ZnO NM dissolves at low pH while SiO_2 NM does not), can be used to support grouping to inform SSbD. The enhanced properties and mixture effects were also predicted to have a major impact on all the included indicators of hazard. This is because the mechanism of driving the enhanced properties is the exact mechanism proposed for driving the hazard indicators listed above in this section. Examples of sub-hypotheses considered for this case study (including those indicating potential for mixture effects) are provided below:

- SiO_2@ZnO −MCNM can be grouped with other MCNMs, individual components SiO_2 NM and/or ZnO NM and/or benchmark material according to similarity in hazard outcomes predicted from common chemical composition.
- SiO_2@ZnO −MCNM can be grouped with other MCNMs, individual components SiO_2 NM and/or ZnO NM and/or benchmark material according to similarity in interactions between components leading to mixture effects.
- SiO_2@ZnO −MCNM can be grouped with SiO_2 NM and/or ZnO NM according to similarity in shape.
- SiO_2@ZnO −MCNM can be grouped with other SiO_2@ZnO MCNMs for which the shell prevents availability of core material thereby preventing mixture effects. This hypothesis is relevant if the group is expanded to include additional MCNMs.
- SiO_2@ZnO −MCNM can be grouped with other SiO_2@ZnO MCNMs, individual component NM and/or benchmark material according to similarity in hazard outcomes predicted from size.
- SiO_2@ZnO −MCNM can be grouped with other SiO_2@ZnO MCNMs, individual component NM and/or benchmark material according to similarity in hazard outcomes predicted from surface area.
- SiO_2@ZNO −MCNM can be grouped with other SiO_2@ZnO MCNMs, individual component NM and/or benchmark material according to similarity in hazard outcomes predicted from MCNM enhanced properties.

Sub-hypotheses (Supplementary File 7 - Table S6 red text) were assessed by measuring the hazard indicators of cytotoxicity, inflammatory potential or genotoxicity. These parameters were measured because the assays were cheap, quick, available and less prone to interference by the particles than for other endpoints. However, the user could choose to substitute these endpoints for others such as surface reactivity. Tiered testing strategies are available to test these parameters [21,76]. This means, from a SSbD point of view that the information gathered can be relatively focused. Importantly, due to the overlap in the mechanism of enhanced functionality and hazard induction, if progressing to later innovation stages it would also be advisable to assess surface reactivity and related impact on cells. Any of the indicators above could be used for this, although they could be supplemented with an assessment of oxidative stress (e.g., glutathione depletion) [94], which is a more specific and direct measure of such a mechanism of toxicity. By considering how physicochemical identity impacts fate and hazard, the parameters to be included in a similarity assessment are readily identified.

In the case of SiO_2@ZnO the IATA decision nodes should address any or all the following: deposition, dissolution in biological media, cytotoxicity, inflammatory potential, genotoxicity and surface reactivity.

which are major factors in assessing hazard, should be assigned higher weights, whereas characteristics such as MCNM shape should be assigned a lower weight. The assignment of weights to sub-hypotheses could be accomplished using, for example, decision tree learning algorithms. As discussed in previous papers [95], the quality of data will be important in determining the accuracy of the similarity analysis. The considerations made on the specific case study are described in Box 6.

Making a decision to progress with innovation (or not)

The implications of safety grouping to SSbD will vary according to whether:

(i) The MCNM group members are all found to be **similar.** The outcome will be **dependent on the level of hazard** (which can be gauged via comparison to the benchmark materials).
   a. If the hazard is relatively high, and exposure is likely, the users can utilise the information identified by the grouping activity to identify strategies to reduce hazard and/or exposure. For example, if one substance is found to drive the toxicity, substitution for a less toxic component could be considered, providing that the MCNM retains its desired functionality.
   b. In contrast, if the hazard is relatively low, the users can exploit this information for all current and future group members when making decisions about application and design and focussing on functionality (and sustainability) if the changes remain within the boundaries of the group.

(ii) The MCNM group members are **not found to be similar.** Several options can be considered:
   a. Remove group members from the extremes/borders of the group, thereby decreasing the group size.

Table 1
Comparison of the MCNM grouping template to support SSbD with the regulatory frameworks for supporting nanomaterial grouping and read across. The final column highlights the innovations associated with the SUNSHINE template.

Grouping and Read Across methodology	ECHA/REACH	GRACIOUS framework	SUNSHINE template
Reference	[22,24,62]	[25,26]	This manuscript
Substances supported for grouping and read-across	Simple, single substance nanomaterials.	Simple, single substance nanomaterials.	Complex multicomponent nanomaterials consisting of more than one substance.
Overall description	General framework to highlight considerations suitable for nanomaterial grouping and read across.	Detailed framework (with user guidance and a software blueprint) to support stakeholders to conduct grouping and read across of nanomaterials in line with REACH.	Template that identifies the different components, enhanced properties, and mixture effects that could impact on grouping decisions.
Purpose	Regulatory applications of grouping and read across.	Predominantly regulatory applications of grouping and read across.	Predominantly SSbD applications of grouping, using a simplified process to speed up early innovation.
Grouping hypothesis generated	Describes hypothesis needs, including physicochemical properties and hazard impact.	Template to support development of a comprehensive hypothesis that includes physical chemical properties, exposure route and hazard impact.	Template to support generation of a series of sub-hypothesis that focus on individual physicochemical properties related to route of exposure and hazard impact. Includes considerations of complexity and enhanced functionality from early innovation stages.
Grouping hypothesis testing	The evidence gathered should address all elements of the mode of action.	The evidence gathered should address all elements of the mode of action.	Not all information is needed. E.g., there is no need to address all elements of the mode of action. Can focus on the most relevant, readily available or cheaply acquired data.
Grouping hypothesis testing progression	If a hypothesis is rejected, then grouping and read across cannot be conducted.	If a hypothesis is rejected, then the framework supports iterative adaptations to generate an improved hypothesis.	The user can either cease development at an early innovation stage to save resources and time, or they can progressively address more decision nodes as confidence in safety increases.
Grouping hypothesis testing – Methods	Suggest that NAMs can be used to generate evidence of similarity.	Provides exposure route specific IATAs (decision tree structure), including tier testing strategies for each individual decision node.	Template includes a matrix of individual decision nodes that relate to each sub hypothesis. No need to generate a complex decision tree. Tiered testing strategies can be used for the decision nodes addressed.
Grouping hypothesis testing – Completeness and reliability of data	Requires complete data sets of high quality, generated via standardised methods.	Requires complete data sets of high quality, generated via standardised methods (where possible).	Decision making at an early innovation stage can be made with incomplete data sets using non-standard methods. Not every single parameter needs to be assessed.
Grouping hypothesis testing – method variability	Must use an identical protocol for all data acquisition used within a single similarity assessment.	Must use an identical protocol used for all data acquisition within a single similarity assessment.	Decision making at an early innovation stage can use data generated with different methods.
Similarity assessment	REACH states that similarity should be assessed but does not stipulate the methods.	Several methods are provided to quantitatively assess similarity [95].	Can be assessed by expert judgement at early innovation stages. Can use quantitative methods as development progresses.
Read-across	General method provided to highlight considerations suitable for read across.	Detailed methodology provided with options to iteratively improve read across hypothesis.	Not always essential to conduct read across at early innovation stages. If conducted more likely to be expert opinion than via a quantitative method.
Approach disadvantages	Time consuming, requires lots of high-quality data and therefore potentially expensive. Relatively difficult to achieve regulatory acceptance.	Time consuming, requires lots of high-quality data and therefore potentially expensive.	False positives or negatives may be more frequent than the other approaches. Progress to regulatory applications requires use of data of higher quality and completeness.
Approach advantages	Generates high quality data to support decision making and reduce animal use for regulatory purposes.	Detailed support to generate high quality data to support decision making and reduce animal use for regulatory purposes. Aims to increase potential for regulatory acceptance.	Offers the ability to extend grouping to more complex materials. Allows the user to identify the most pertinent information to inform early decision making. Relatively quick and cheap, providing agile support to product development. Can be increased in detail as innovation proceeds.

b. Divide the putative group into 2 or more groups, allowing the users to reject the most hazardous group.
c. Reject the grouping sub-hypotheses, leading to either reformulation of the hypotheses or assessment on a case-by-case basis.
d. If an obvious ranking occurs between the group members, this information can be utilised to prioritise the MCNMs of lowest hazard but with effective functionality, or to identify characteristics that can be applied to other similar MCNMs to improve their hazard potential.

For a putative **group of MCNMs plus representative individual components**:

(iii) If the group members are found to be similar it is likely that the hazard is driven by one or more of the components, without interactions or influence by the enhanced properties.
(iv) If the MCNMs and the components are not found to be similar, then either:
   a. The components have the same MoA but different reactivity
   b. The components do not have the same MoA when compared to the MCNM
   c. Mixture effects could be driving the toxicity of the MCNM, and other similar MCNMs may exhibit similar mixture effects; or
   d. Enhanced properties could be driving the toxicity of the MCNM, and other similar MCNMs may exhibit comparable impacts driven by the enhanced properties

For these last two points it is possible that the enhanced properties are also driven by mixture effects, meaning that these last two points could be identical. In addition to assessing similarity of the group members, the outcome of using the template may allow prediction of their relative hazards (e.g., ranking), allowing prioritisation of a specific MCNM or smaller group of MCNM for further development. An MCNM grouping hypothesis therefore does not necessarily support the formation of a group of MCNMs that are predicted to pose the same fate/hazard (which would allow data to be data re-use or read-across between group members), but rather indicates where the similarity between MCNM and/or individual components exists that can inform the design and use of a safer MCNM [99]. The users can then apply the information on the similarity of a particular parameter to make a prediction of potential fate/hazard outcomes and therefore determine where existing data for another substance/NM/MCNM could be used to inform SSbD. Any changes to the MCNM design triggered by the grouping approach would need to assess the potential impact on the enhanced functionality of the MCNM and its usefulness in the proposed product. Considerations about SSbD decision-making for the specific case study are reported in Box 7.

One potential limitation of this approach is that the template does not encourage the identification of novel toxicities associated with the enhanced properties, as these may be difficult and expensive to determine at the innovation stages. For such cases, we would suggest that a generalised IATA (e.g., addressing cytotoxicity, oxidative stress, inflammation, genotoxicity) could be used relevant to a specific route of exposure. A more extensive hazard assessment would be required potentially to identify novel hazards induced by enhanced properties. We suggest that at early innovation stages such information is not necessary, but as knowledge expands to understand such novel effects, they can in future be included during innovation. However, if this information is available, it could also be easily inserted into the template matrix. Such information will be essential if progressing to a regulatory grouping application.

Conclusion

This work describes the application of an MCNM hypothesis template that structures the gathering of relevant information for similarity assessment between MCNMs and their components. Such an approach could also be used to compare a range of MCNMs in order to use SSbD to prioritise their development. Further case studies have been conducted [100] with manuscripts in preparation [101].

This template allows the process of grouping to be transparently described and to effectively inform SSbD approaches for MCNMs. The resultant MCNM grouping template is therefore structured to ensure a systematic approach for the identification of a range of highly relevant, high impact and focused grouping sub-hypotheses, as well as allowing the users to prioritise the use of pre-existing data and non-animal alternative methods. The use of a table or matrix format within the template removes the necessity to generate a hierarchical decision tree. The hierarchical decision trees usually used in IATAs are complex, difficult to generate and time consuming to validate. The decision tree structure is essential for regulatory applications where detail is required to support evidence gathering in a strategic manner and therefore regulatory relevant decision-making. In contrast, the table format suggested here is easily generated and is more suitable for SSbD, where prioritisation can be driven by user needs or information availability. It is worth noting that some decision nodes might be common and applicable to multiple hypotheses, thus the assessment of such a decision

Box 6
. Case study: using the similarity assessment to inform SSbD.

It is unlikely the SiO_2@ZnO will be "similar to" SiO_2 NM or ZnO NM in every IATA decision node, nor do they need to be. For example, SiO_2@ZnO and ZnO NM are different in size 20nm vs < 200 nm) [42] which would prevent similarity in lung deposition. This dissimilarity in size does not prevent certain decision nodes being employed to indicate where a SSbD modification could minimise hazard, e.g., if cytotoxicity to SiO_2@ZnO is shown to be "similar to" ZnO NM, then ZnO NM could be substituted for a less hazardous NM or substance, if this does not adversely affect the overall MCNM functionality. If such a substitution is not possible then the safe production and use of the MCNM may require limits of exposure and the adoption of occupational hygiene measures currently recommended for handling hazardous materials such as ZnO.

Two similarity assessment methods were applied to dissolution data for SiO_2 NM, ZnO NM and SiO_2@ZnO across a range of toxicological and ecotoxicological media [99]. Interestingly, for the considered time frames, similarity between the SiO_2@ZnO MCNM and its single component SiO_2 NM was observed in most of the biological media, whereas ZnO dissolution rate was found to be dissimilar from the other two group candidates [99].

Similarity in the dissolution rate of a substance from an MCNM and the dissolution rate of the individual substance or NM may allow re-use of hazard data for hazard endpoints driven by the release of the component ions.

If the MCNM dissolves to release toxic ions the users may consider altering the MCNM composition to reduce the hazard based on this decision node alone.

What can we then conclude from the SiO_2@ZnO case study and similarity assessment?

- In terms of 'what they are', key parameters to measure for a qualitative assessment of similarity are composition, size, surface area, surface coating (morphology and composition), surface reactivity.
- Surface reactivity was proposed as an indicator of enhanced functionality and could be useful to assess similarity.
- In terms of 'where they go', the key parameters to measure for assessment of similarity are size and transformation.
- In terms of 'what they do', the key parameters to measure (hazard indicators) were cytotoxicity, inflammatory potential and genotoxicity. For each parameter, potential for mixture effects should be assessed provided that previous sections highlighted the simultaneous presence of multiple components.
- Single component (surrogate) ZnO NM and representative ZnO NM110 could not be grouped with the MCNM and so were not suitable sources to allow re-use of hazard data. The high release of Zn ions from the MCNM (20% from MCNM against~10% from ZnO in RPMI medium supplemented with 10 % FBS and 1 % Penicillin/Streptomycin – Supplementary File 8 – Fig. S2) suggests that $ZnCl_2$ might be a better benchmark. The hazard information (Section 3 and/or Section 5) for $ZnCl_2$ could therefore be re-used for the MCNM.
- The mesoporous SiO_2 NM individual component could potentially be grouped with the MCNM, and therefore the hazard information (Section 3 and/or Section 5) for SiO_2 NM could be re-used for the MCNM.

Box 7
. Case study: helping decision-making.

With respect to the SiO_2@ZnO case study, if the hazards of mesoporous SiO_2 NM and $ZnCl_2$ are considered suitable for the application (subject to available exposure and risk mitigation measures), then at the early innovation stages enough information exists in the case study provided to continue to the next stage of development.

In fact, cytotoxicity of the (MC)NMs was assessed in THP-1 cells which indicated that the MCNM was more "similar to" the cytotoxicity and pro-inflammatory responses induced by ZnO NM rather than SiO_2 when compared at the same mass exposure concentration, probably due to the release of Zn ions. There was a slight potentiation of the response compared to ZnO alone suggesting 1) a minor interaction between NM components which may impact the bioactivity of the ZnO when in MCNM form [42], or 2) the higher release of Zn ions from MCNM rather than ZnO NM when compared at the same mass exposure concentration (Supplementary File 8 – Fig. S2) impacts the biological effect. Therefore, at early innovation stages the hazard of the SiO_2@ZnO MCNM can be assumed to be comparable to $ZnCl_2$ for acute effects (via expert judgement). Based on 'where they go' (dissolution in lysosomal fluid), the hazard can be assumed to be comparable to SiO_2 for (sub)chronic effects as this component will remain after dissolution of the ZnO component and does only poorly dissolve in lysosomal conditions.

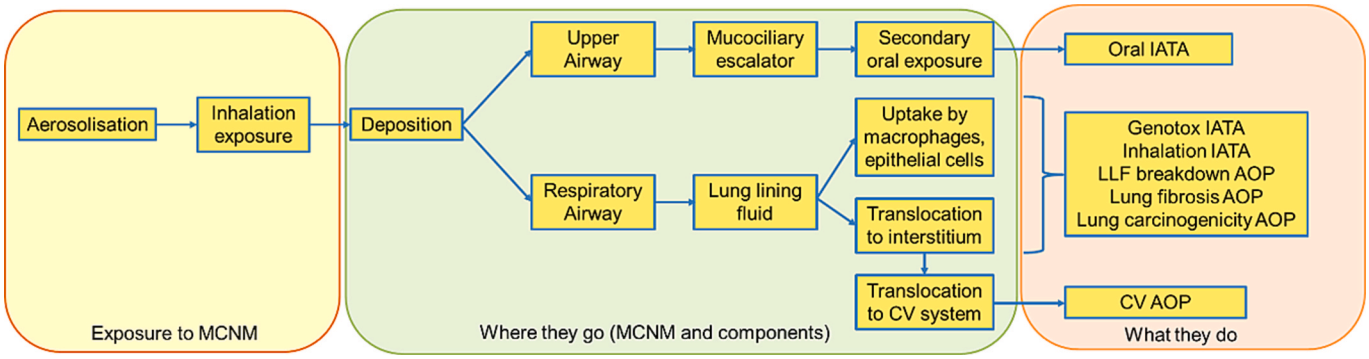

Fig. 7. MoA for inhalation exposure to MCNMs. The MoA diagram focuses on local fate, but translocation into the cardiovascular system (CV) and other organs as well as the oral exposure route could also be considered. The MoA has been generated considering both AOP 302 (LLF breakdown AOP) [50,51] and AOP 237 (CV AOP) [52,53] and it does not prescribe the need for translocation to induce an acute phase response.

node can provide confidence across a range of hypotheses. The flexibility of this tool allows to generate sub-hypotheses that are aligned to the stage of product development. For example, early in the process, more gross assumptions, hypotheses and result processing can be used to rapidly screen out candidates from development and to get an overview of the factors driving toxicity. In line with the EC's SSbD Framework, the template provided here firstly promotes the use of NAMs structured via tiered testing strategies and facilitates decision making at early innovation stages even in the absence of a complete or robust data sets. Later on, in product development where the changes to the product will be more subtle, a more quantitative approach to optimise toxicity and similarity assessment could be used. For example, this could include the application of standardised methods to test all materials within the group. In addition, the further along the innovation path users progress, the greater the number of decision nodes should be considered. The proposed grouping template thus offers a valuable tool not only to identify opportunities to re-use existing data to support the SSbD of MCNMs, but also to support decision-making during the early stages of innovation, particularly when relying on non-animal methods. Table 1 describes the main features of the SUNSHINE template compared to the existing methodologies.

However, while the resultant template is complex, it is designed to be incorporated into software (e.g. a decision support system) so that the users are supported through the provision of the information in steps, reducing their need to understand the complexity. Software can also include the algorithms and machine learning functionality to support similarity assessment in a quantitative manner. Such approaches have already been used in GRACIOUS [102] and SUNSHINE [103] projects.

Finally, while this study has focused on human health effects, a similar approach could be used for grouping in relation to environmental hazards.

CRediT authorship contribution statement

Vicki Stone: Writing – original draft, Supervision, Methodology, Funding acquisition. **Elisa Moschini:** Conceptualization, Formal analysis, Data curation, Writing – original draft, Methodology. **Fiona Murphy:** Conceptualization, Formal analysis, Data curation, Writing – review & editing, Methodology. **Neil Hunt:** Writing – review & editing. **Magda Blosi:** Writing – review & editing, Funding acquisition. **Danail Hristozov:** Funding acquisition. **Helinor Johnston:** Writing – original draft, Methodology. **Finlay Stenton:** Formal analysis, Data curation. **Alicja Mikolajczyk:** Writing – review & editing, Funding acquisition. **Agnes G. Oomen:** Funding acquisition, Writing – review & editing. **Otmar Schmid:** Writing – review & editing, Funding acquisition. **Georgia Tsiliki:** Writing – review & editing, Funding acquisition. **Andrea Brunelli:** Formal analysis, Data curation. **Elena Badetti:** Formal analysis, Data curation. **Ulla Vogel:** Writing – review & editing, Funding acquisition. **Agnieszka Gajewicz-Skrętna:** Writing – review & editing, Formal analysis. **Wendel Wohlleben:** Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work has received funding from the European Union's

HORIZON 2020 Research & Innovation Programme under grant agreement no. 952924 (SUNSHINE), no. 953183 (HARMLESS) and no. 953152 (DIAGONAL).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mattod.2025.08.024.

Data availability

The authors confirm that the data supporting this work is available within the electronic supplementary information and/or Sunshine project database https://www.sunshine.greendecision.eu/database.

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