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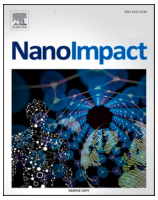

Next Generation Risk Assessment approaches for advanced nanomaterials: Current status and future perspectives

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ABSTRACT

This manuscript discusses the challenges of applying New Approach Methodologies (NAMs) for safe by design and regulatory risk assessment of advanced nanomaterials (AdNMs). The authors propose a framework for Next Generation Risk Assessment of AdNMs involving NAMs that is aligned to the conventional risk assessment paradigm. This framework is exposure-driven, endpoint-specific, makes best use of pre-existing information, and can be implemented in tiers of increasing specificity and complexity of the adopted NAMs. The tiered structure of the approach, which effectively combines the use of existing data with targeted testing will allow safety to be assessed cost-effectively and as far as possible with even more limited use of vertebrates. The regulatory

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Regulatory readiness
Grouping
In vitro approaches
In vitro-in vivo extrapolation (IVIVE)
In silico approaches
In chemico approaches

readiness of state-of-the-art emerging NAMs is assessed in terms of Transparency, Reliability, Accessibility, Applicability, Relevance and Completeness, and their appropriateness for AdNMs is discussed in relation to each step of the risk assessment paradigm along with providing perspectives for future developments in the respective scientific and regulatory areas.

1. Introduction

Engineered nanomaterials have been produced for over 20 years and are used in almost every industrial sector. Looking back in time, certain pigments were industrially produced in nanoform in the last 100 years, but especially in the past two decades the degree of engineering of targeted structures for specific functionalities has improved, and over the time, the complexity of such materials has increased. The most recent innovations are referred to as advanced materials. The term advanced materials does not have a regulatory definition yet, but the Organisation for Economic Co-operation and Development (OECD) agreed on a working description: 'advanced materials are understood as materials that are rationally designed to have new or enhanced properties, and/or targeted or enhanced structural features with the objective to achieve specific or improved functional performance' (OECD, 2022a). Not all advanced materials are nanomaterials (European Commission, 2022), but this manuscript is focusing specifically on the advanced nanomaterials (AdNMs). These, in many cases, are nanocomposites formed by two or more functional components (*e.g.*, nanoparticles, nanocrystals, organic molecules, internal structures) conjugated by strong molecular bonds, or by a nanomaterial with a unique chemical composition modified by hard or soft coatings (Banin et al., 2014; Wohlleben et al., 2017). Some of the most widely used AdNMs are (combinations of) carbonaceous (*e.g.*, fullerenes, carbon nanotubes, graphene) or metallic (metal or metal oxide) nanoparticles with or without organic components (*e.g.*, polymers, macromolecules, and enzymes). Other AdNMs rely on nanostructures such as nanofiltration or ultrafiltration composite membranes, internally porous insulation materials or concrete systems that have grown into multistructural and multicomponent materials by a reactive process (Wohlleben et al., 2017; Stark et al., 2015).

The AdNMs enable technologies that effectively support innovation in a broad range of industrial sectors such as construction, structural and functional materials, food, healthcare, energy, cosmetics, and electronics; however, due to their more sophisticated nature and potentially more dynamic interactions with biological and ecological systems it has been challenging to assess their environmental, health and safety (EHS) risks (Mech et al., 2022; Gottardo et al., 2021). EHS assessment of AdNMs is complicated by substantial data gaps for these emerging materials and the lack of standardised tools to address their complex properties and interactions. To overcome these challenges, substantial resources have been invested into the development of methods that facilitate the assessment of AdNMs. This includes over a decade of EU research projects, standardisation, and harmonisation activities (*e.g.*, OECD's Working Party on Manufactured Nanomaterials (WPMN)), which have attempted to establish future-proof testing methods that can generate reproducible results in a cost-efficient manner, and as far as possible minimizing the use of experimental vertebrates.

Risk assessment is a central theme in the regulation of chemicals. It is defined as 'a process intended to calculate or estimate the risk to a given target organism, system or (sub) population, including the identification of attendant uncertainties, following exposure to a particular agent, taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system' (OECD, 2003). Risk assessment can be in relation to human health or the environment, and broadly consists of hazard assessment, exposure assessment and risk characterization, including uncertainty analysis (Nielsen et al., 2007). The hazard assessment mostly relies on data from animal experiments, which has increasingly come under pressure. Society is questioning the overall performance, sustainability, continued relevance, and the ethics of this system, demanding a change in the testing approaches (Schmeisser et al., 2023). This has led to incremental refinement of the regulatory system due to the policy ambitions to *replace* animal testing, *reduce* the use of vertebrate animals and *refine* the tests (3R principles) (Moné et al., 2020). The most obvious change is the ban of *in vivo* testing on cosmetics in 2013 (*European Parliament and of the Council, Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on Cosmetic Products*, 2013). These efforts have opened a new frontier of research and regulatory advancement into the Next Generation Risk Assessment (NGRA) paradigm.

The first reference to NGRA dates back to 2010, when the US EPA initiated the 'NexGen' program with the aim of developing a new paradigm for the next generation of risk science based on the application of emerging molecular, systems biology and computational methods (Cote et al., 2012). The International Cooperation on Cosmetics Regulation (ICCR) first explored the possibility to apply such a paradigm in real life through an integrated strategy for risk assessment of cosmetics ingredients (Dent et al., 2018) which has already been tested in hundreds of substances. NGRA can be defined as an exposure-driven risk assessment approach that integrates New Approach Methodologies (NAMs). NAMs are defined as 'any technology, methodology, approach, or combination of approaches that can provide information on risk assessment without the use of vertebrate animal studies' (ECHA, 2016). It is important to emphasize that in the acronym 'new' refers to alternatives to the conventional animal-based approach to risk assessment and not to the degree of technological novelty of a NAM. These alternative approaches include grouping, *in vitro*, *ex vivo*, *in chemico* and *in silico* methods (*e.g.*, advanced *in vitro* assays such as 3D organoids, quantitative structure–activity relationships (QSARs), high-throughput screening bioassays, omics, microphysiological systems, machine learning models and artificial intelligence). The goal of NAMs is to increase the speed and decrease the cost of testing, while avoiding the use of animals when generating the data needed for the regulatory approval of new substances such as AdNMs. In addition to promising faster and more efficient toxicity testing, NAMs have the potential to fundamentally transform the current regulatory landscape by allowing more human-relevant decision making based on both hazard and exposure assessments (Schmeisser et al., 2023). In fact, in contrast to the widespread belief, the regulatory accepted animal models are not without limitations, and the results obtained from them may not always accurately predict the human situation (Van Norman, 2019). However, the expectations that NAMs would fit into the established risk assessment framework by addressing existing regulatory endpoints without the use of experimental animals is to some extent misleading. NAMs often provide information of a different kind, which can be as informative and potentially even more relevant for risk assessment than the *in vivo* models (Schmeisser et al., 2023). The main challenge for NAMs is their acceptance and routine applicability in regulatory decision making, which has traditionally relied on data from animal experiments. Therefore, it is crucial to ensure the regulatory readiness of these emerging methods (Bal-Price et al., 2018). The regulatory acceptance of NAMs is important also for industries: the application of such alternative approaches can help to reduce R&D and regulatory compliance costs and reduce the time required for new materials/products to reach the market. This can also allow industrial companies to better align to the 3R principles.

The quality of NAMs can be assessed by their (1) compatibility with regulatory frameworks (*i.e.*, they enable the assessment of an endpoint of regulatory relevance with equal or higher sensitivity and efficiency) and (2) usefulness and usability by the industries complying with regulations and regulatory requirements. This is not trivial and will require that NAMs are Transparent, Reliable, Accessible, Applicable and Complete (*cf.*, TRAAC framework) (Shandilya et al., 2023).

There are a few NAMs that have already reached the level of readiness for application in a regulatory context. For example, traditionally used animal tests for chemically-induced effects such as eye irritation or skin sensitization have been successfully replaced by NAMs (Caloni et al., 2022). Furthermore, various regulations accept well justified grouping and read-across approaches, where data gaps are filled with existing information from similar substances and materials (*e.g.*, ECHA's Read Across Assessment Framework (RAAF)) (ECHA, 2017). ECHA and OECD provide additional guidance for grouping and read-across approaches of nanomaterials (OECD, 2014; ECHA, 2019) and the EU Horizon 2020 projects GRACIOUS, HARMLESS, SUNSHINE and POTENTIAL have provided scientific support (Stone et al., 2020).

A recent ECHA report identified eight NAMs specifically developed for nanomaterials (Jagiello et al., 2022), some of which referred to ISO Standards (ISO, 2019; ISO, 2018; ISO, 2016) and one to the European Food Safety Authority (EFSA) Guidance on risk assessment of nanomaterials (EFSA, 2018). There may also be other NAMs developed for chemicals that could be adapted for testing nanoforms (OECD, 2022b). On this front, EU projects such as PATROLS, Gov4nano, RiskGone, the Graphene Flagship and NanoHarmony have contributed to the development and adaptation of NAMs to AdNMs. A recent review proposed a toolbox of more than fifty NAMs, the majority of which were concluded to be potentially applicable for the risk assessment of nanomaterials (Nymark et al., 2020). However, progressing further to the application of such NAMs in regulatory risk assessment for more complex toxicological endpoints is not easy or straightforward.

This is why for those mainly systemic endpoints risk assessment still largely depends on animal studies. In living organisms many physiological processes interact with each other; the toxicokinetic behavior of the substance determines its internal concentration in the different tissues in time, and consequently, the internal exposure. Simulating the internal exposure in NAMs is a major challenge. In contrast to organisms, NAMs often focus on one or a limited number of specific aspects of exposure, toxicokinetic behavior or hazard. It is therefore often necessary that for more complex toxicokinetic and toxicological endpoints a battery of NAMs is applied. The US Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products (ICCVAM, 2018) emphasizes the need of defining the context of using NAMs and applying flexible and fit-for-purpose validation approaches. Indeed, it is critical to clarify how each NAM (in a battery) relates to the endpoint and how exactly the output of the NAMs can be used in a regulatory decision.

Combining NAMs for the assessment of complex toxicological endpoints of regulatory relevance requires a structured framework. The need to establish such an integrated NGRA framework that is aligned to the conventional regulatory risk assessment paradigm is the central concept of this perspective paper. We propose a framework that is exposure-driven, endpoint-specific, makes best use of any pre-existing information, and can be implemented in tiers of increasing specificity and complexity of the adopted NAMs. The tiered structure of the approach, which effectively combines the use of existing data with targeted testing will allow safety to be assessed cost-effectively and as far as possible without the use of experimental animals. The approach involves assessment of regulatory readiness of the adopted NAMs, which is demonstrated in specific examples. The relevance of the NAMs for the AdNMs are discussed in relation to the hazard and exposure assessment steps of the risk assessment paradigm along with providing the perspectives of the authors for future developments of alternative methods in these areas.

2. NGRA framework for AdNMs

In this manuscript we propose a conceptual NGRA framework for AdNMs, which is based on the latest scientific and regulatory knowledge. This approach is exposure-driven and employs Integrated Approaches to Testing and Assessment (IATA) and NAMs to enable use of existing information and targeted generation of new data as far as possible without the use of experimental animals. The proposed approach is designed to be flexible and efficient (Bos et al., 2015). It is flexible in the sense that it is able to address different assessment goals depending on the assessor's needs, considering all data already available as a starting point, and selecting the most appropriate tools targeting the data gaps. It is efficient as it aligns with the collection of the data needed for the risk assessment according to the assessor's goals and enables targeted testing instead of fulfilling predefined data requirements. Efficiency is also achieved through enabling the possibility to read-across existing information from a source material to a target material based on grouping. These features of the proposed approach result in optimal balance between compiling the best quality current data and the testing efforts required to perform robust regulatory risk assessment.

The proposed NGRA framework is exposure-driven, meaning that risk assessment starts with the identification of the most relevant exposure scenarios along the life cycle of an AdNM. This may for instance relate to scenarios with the highest potential of exposure (worst case scenario) or to a specific life cycle stage of concern, depending on the assessment goals. This exposure-driven approach is essential for the subsequent optimization of hazard testing as the identified exposure scenarios are related to specific exposure routes or environmental compartments, which narrows down the need for hazard testing to a limited set of (eco)toxicity endpoints. Similarly, the identified life cycle stages of concern would point to the forms of the materials (*e.g.*, pristine, released, weathered/aged) that are most relevant to include in the testing program.

Once the relevant exposure scenarios are identified, all available information for each one of them should be evaluated. The relevant data encompasses both intrinsic and extrinsic physicochemical characteristics as well as release, biodistribution, environmental fate, exposure, and hazard aspects. This initial 'pre-assessment' would help to evaluate if (some of) these scenarios may give rise to a concern, *i.e.* that it cannot be ruled out that a human health and/or ecological risk may be present. If risk cannot be ruled out, the type of information that best serves the risk assessment should be determined, the gaps in the existing dataset should be assessed and guidance should be provided on how to obtain any missing information. Data gaps should initially be filled in a straightforward and relatively simple way with easy-to-handle tools with the possibility to use more sophisticated methods, as needed, further down the risk assessment process. To achieve this, we propose a tiered approach implemented by the application of IATA. IATA are a non-standard approach proposed by OECD (OECD, 2022c), which can be instrumental for combining experimental and/or *in silico* modelling NAMs to generate EHS data more cost-efficiently and without the use of animal experiments.

Our proposal for a tiered NGRA framework using IATA is rooted in the results of many previous developments going back to 2012. The EU project ITS-NANO was the first to generate a research strategy that aimed at informing development of future IATA for engineered nanomaterials (Stone et al., 2013). In 2014 a summary of nano-specific IATA was published as an outcome of discussions of the NanoSafety Cluster Group 10 (Byrne et al., 2014; Oomen et al., 2014), which presented a vision for concern-driven integrated approaches for the (eco)toxicological testing and assessment of nanomaterials. This study presented for the first time a tiered approach in which material properties, exposure, biokinetics and hazard data are integrated for the purpose of accelerating the risk assessment process and reducing testing costs and the use of experimental vertebrates. This tiered approach was fundamental to the risk assessment methodologies for nano and biomaterials developed

in the follow up EU FP7 MARINA (Bos et al., 2015) and BIORIMA (Giubilato et al., 2020) projects. The tiered approach was also embraced by the Horizon 2020 GRACIOUS project, which developed 39 tiered IATA that support hypothesis-driven grouping of nanoforms (Stone et al., 2020; Braakhuis et al., 2021; Murphy et al., 2023; Murphy et al., 2022). The adaptation/development of tiered IATA for multi-component AdNMs is also a major objective of the more recent EU Horizon 2020 SUNSHINE and HARMLESS projects. The projects above were all led by the co-authors of this manuscript.

Learning from the outcomes of these key projects, we advocate that in order to acquire the data needed for NGRA in a strategic and efficient manner, it is logical to apply a concern-driven approach composed of two main stages and incorporating all steps of the conventional risk assessment for chemicals. The stages are (1) Problem formulation and (2) Risk assessment (including exposure andhazard assessment as well as risk characterization) (*cf.* Fig. 1). To optimise testing, the proposed approach relies as far as possible on current information, but since in most cases the AdNMs are novel emerging materials for which the existing data may be very limited, new exposure and hazard data will need to be generated. The goal is to do this by implementing tiered IATA with the higher tiers increasing in terms of the specificity and complexity of the adopted NAMs.

2.1. Stage 1: Problem formulation

The main goal of the Problem formulation stage is to set the scope of the risk assessment by defining the information requirements as well as outline the strategy to collect the required data in a flexible and efficient way. The proposed NGRA approach is exposure-driven so the Problem formulation stage is structured in the following two steps: (1) exposure scenario identification and (2) pre-assessment of available data (i.e., on release, human biodistribution, environmental fate, human and environmental hazard) for the identified exposure scenario(s).

To properly identify relevant exposure scenarios along the life cycle of AdNMs (from synthesis to end of life), it is important to confirm that a potential release may occur. For each relevant exposure scenario, at minimum, the dataset must identify and define the life cycle stage (*e.g.*, use phase), the nanoforms (*e.g.*, powdered graphene), routes of exposure (*e.g.*, inhalation) and biological target (*e.g.*, lungs). The information collected should be carefully analyzed to prioritize hotspots of concern for further assessment in Stage 2, which is Risk assessment. Additional information such as release/exposure, but also biodistribution, environmental fate, human and environmental toxicity, as far as relevant for the specific scenario, must be included. The results of such an initial exposure scenario identification and the subsequent analysis of the available data in the pre-assessment step would be a green flag

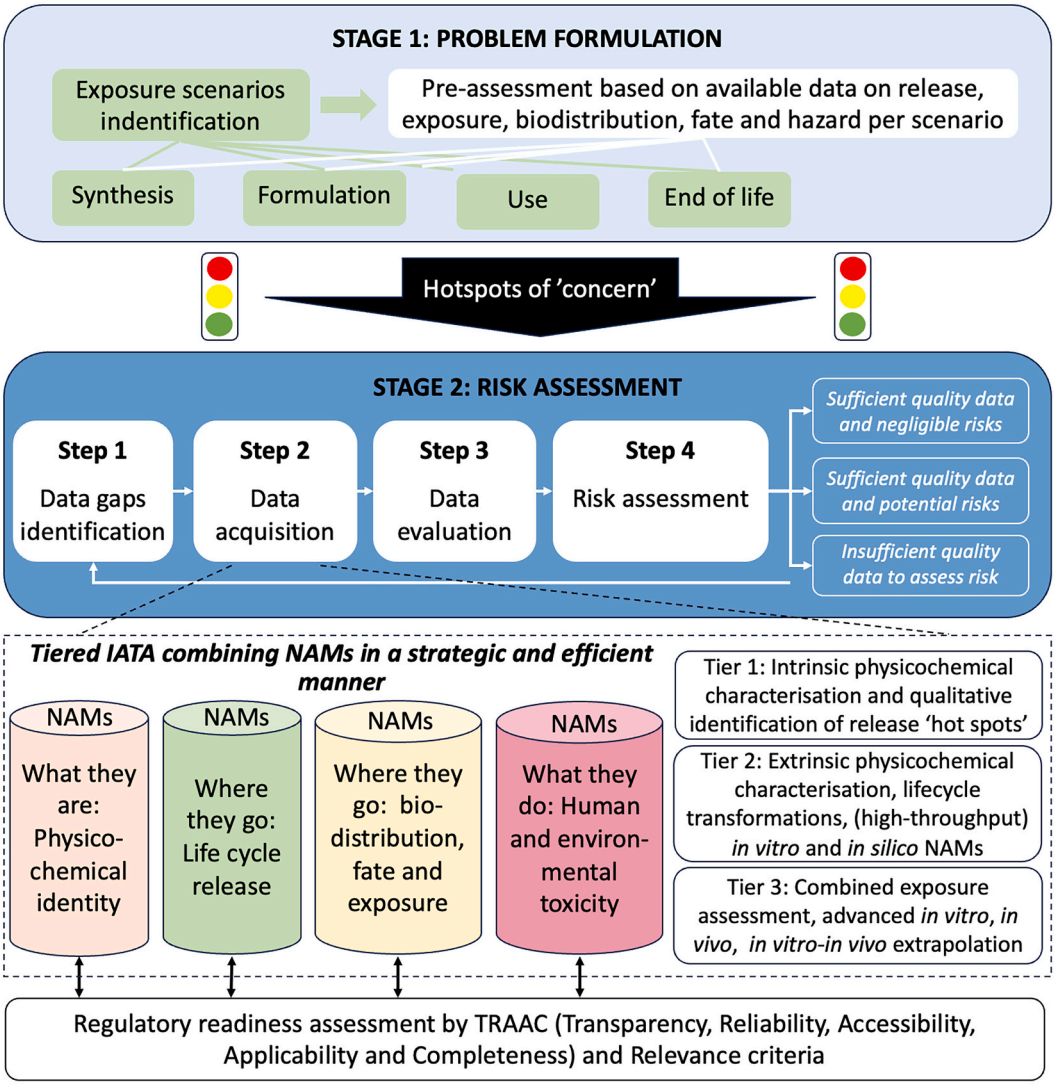

Fig. 1. Conceptual framework for exposure-driven NGRA of AdNMs.

(meaning, no concern), a yellow flag (insufficient information available) or a red flag (potential concern). Concerns should be described as detailed as possible based on the available information. In addition to identifying areas of concern to address in the risk assessment by additional testing and assessment, the conclusions of this early pre-assessment could also be useful for product or material development as they could lead to 'go'/ 'no go' decisions in the early stages of innovation. In some cases, such screening results could eliminate the need for further testing by identifying negligible exposure potential or hazard potency very early in the assessment process.

2.2. Stage 2: Risk assessment

The Risk assessment of AdNMs should be implemented as an iterative process consisting of four steps: (1) Data gaps identification, (2) Data acquisition, (3) Data evaluation, and (4) Risk characterization. Step 1 identifies the most important gaps in the data needed for the risk assessment. Step 2 consists of read-across of existing information and/or targeted generation of new data to fill the data gaps. This is broadly exposure and hazard related information, or more specifically, physicochemical identity ('what they are'), life cycle release, biodistribution, fate and exposure ('where they go') and human and environmental toxicity ('what they do') data. The data is acquired following a tiered IATA composed of NAMs, including approaches for grouping and read-across to make use of existing information. In this step, the regulatory readiness of the selected NAMs is evaluated, if not previously known, using a set of established criteria (*cf.* 3.3.1) to ensure that the produced datasets will be considered/accepted by the regulators. Step 3 involves evaluating the acquired data in terms of established quality criteria such as completeness, relevance, reliability, adequacy (Klimisch et al., 1997; Basei et al., 2022; Kase et al., 2016). If the completeness of the available information is insufficient to perform risk assessment that is acceptable to regulators, then the assessor is iteratively directed back to Step 2 to collect additional information. In Step 4, it is decided if the available data is sufficient to perform a risk assessment. If the answer is 'yes', exposure and hazard assessment are performed, risk characterization is completed, and no iteration is needed. Risk assessment should account for the potential of combined exposure *via* multiple exposure scenarios (*e.g.*, a worker exposed during the production stage can also be exposed as a consumer or consumers may also encounter the material *via* the environment). However, it should be considered that due to the more complex identity of the AdNMs, they may exhibit changing physicochemical properties in different life cycle stages or exposure media which may complicate combining exposure and risk across scenarios, thus requiring the generation of additional data. Moreover, a clear assessment of uncertainty and variability will be necessary prior to making any conclusion. There are three main conclusions that can be drawn from the risk assessment: (1) the available data is insufficient to draw a final conclusion on potential risks, (2) the available data is sufficient, and no risks are expected, (3) the available data is sufficient, and risks are possible. If the conclusion is (1), then the assessor is iteratively directed back to Step 1 'Data gaps identification' and the process starts over again until all uncertainties become acceptable from the risk assessor's point of view in line with the regulatory requirements. For conclusion (2), the iterative process ends. In case of conclusion (3), assigning risk management measures will be necessary, including Safe by Design (SbD) strategies, or the target exposure scenario(s) should be avoided. In case of conclusions (2) and (3), a sustainability assessment (enviromental and socioeconomic) may be added as further evaluation steps, which would then extend the proposed NGRA approach in line with the European Commission's Safe and Sustainable by Design SSbD framework (Caldeira et al., 2022). The integrated assessment of safety and sustainability to support the implementation of the EC SSbD framework is however out of the scope of this manuscript.

Step 2 of the NGRA approach involves the use of existing information (by read-across) and/or the generation of new exposure and hazard related data to fill data gaps identified in Step 1. The data acquisition will be supported by IATA with tiers of increasing specificity and complexity of the adopted NAMs (Tiers 1 to 3). Only the highest Tier 3 involves targeted *in vivo* testing if the lower tiers are not able to generate data that is acceptable by the regulators, or if the involved NAMs or the quality/completeness of the datasets are not compliant with the regulatory principles for a confident conclusion on risks. The targeted *in vivo* results are useful also for selecting realistic concentrations for *in vitro* tests and for validation of their outcomes. They are necessary also for the development of certain computational methods: *e.g.*, *in vivo* kinetic studies can inform physiologically based pharmacokinetic (PBPK) models.

Tier 1 will identify intrinsic physicochemical properties (*e.g.*, composition, size, shape) relevant for early hazard screening with a focus on novel properties that determine the advanced nature of AdNMs. Tier 1 also involves qualitative pathway analysis (based on insights from material flow analysis (MFA)) which allows identification of release 'hot spots' based on limited information on the AdNMs' released forms (*e.g.*, nanoforms, dissolved ions). For human hazard assessment, Tier 1 involves acute *in vitro* assays with cell lines, *in chemico* (cell free) tests and/or *in silico* (QSAR) modelling. For environmental hazard assessment, Tier 1 includes acute endpoints in single representative test species or cell lines as well as QSAR modelling.

Tier 2 will focus on a characterization of the extrinsic physicochemical properties that influence the environmental fate, biodistribution and the human and ecological toxicity of the AdNMs (*e.g.*, dissolution, agglomeration/aggregation, surface charge, reactivity, generation of reactive oxygen species, hydrophobicity/hydrophilicity, partitioning coefficients). Tests should be performed to determine the transformations of the materials during their life cycle focusing on release, human uptake and translocation, environmental fate and exposure, and to establish any relationships between those. To enable assessment of these aspects, Tier 2 includes probabilistic MFA models (Sun et al., 2014; Gottschalk et al., 2009; Mueller and Nowack, 2008; Gottschalk et al., 2010; Sieber et al., 2020; Mennekes and Nowack, 2023; Ivanović et al., 2022) to make it possible to (1) anticipate alterations of AdNMs during all life cycle stages prior to their release, and (2) quantify the releases of AdNMs to the environment. Based on these emission estimates, the fate as well as the distribution, concentration, and accumulation of the released materials in the environment can be modelled by multimedia fate models similar to SimpleBox4Nano (Meesters et al., 2014). In addition, Physiological based pharmacokinetic (PBPK) models should be applied for *in silico* prediction of biodistribution, uptake and transport, including cellular localisation, and to support *in vitro-in vivo* extrapolations (*cf.* 3.3.3). Tier 2 will prioritize simple *in chemico* (for *e.g.*, assessing dissolution in relevant biological medium) or cellular assays using simple or more complex methods such as co-cultures. It also involves cost-efficient methods to evaluate if the materials are Persistent, Bioaccumulative and Toxic (PBT)/very Persistent and very Bio-accumulative (vPvB), and to assess their acute and chronic toxicity and endocrine disruption potential.

Tier 3 involves a more complex and ambitious approach for combined exposure assessment. Specifically, a mechanistic multiscale approach should combine the Tier 2 models, connecting the MFA and the fate models with probabilistic models for uptake and biodistribution. The result will be a comprehensive exposure assessment approach that allows to cover the full life cycle of the AdNMs, from the release to their final fate. Tier 3 also involves advanced NAMs including (1) innovative *in vitro-in vivo* extrapolation techniques that enable the extrapolation of *in vitro* concentrations to human exposure doses, (2) advanced 3D *in vitro* models including multiple cell types organised in a physiologically relevant structure (*e.g.*, reconstituted epithelial tissues made of primary human cells) and/or exposure relevant (*e.g.*, air liquid interface), allowing acute and/or longer-term exposures under realistic conditions, (3) long-term environmental endpoints and multiple species interacting within microcosm tests, and (4) targeted sub-chronic and chronic *in vivo*

experiments to fill outstanding data gaps and to validate the outcomes of the *in vitro* experiments. Moreover, Tier 3 should combine and adapt *in chemico*, *in silico*, and *in vitro* models to determine uptake and bioaccumulation, and to identify potential PBT/vPvB AdNMs. Last but not least, all these data could then be used as the starting point to establish the cornerstones – *i.e.* so-called characterization factors for the toxicity assessment of releases of AdNMs along the life cycle – in order to enable subsequent evaluation of the environmental sustainability of these substances (Salieri et al., 2020).

To operationalize the tiered approach described above, a substantial progress in NAMs development is required for the adaption of the existing approaches to the more complex identity and interactions of the AdNMs. Moreover, a key challenge to overcome is the interpretation of the results of the NAMs in risk assessment.

The following paragraphs describe the shared perspective of the co-authors of this manuscript on the ongoing and future developments in NAMs that can support a successful implementation of the proposed NGRA framework for AdNMs.

3. Emerging methods to support NGRA of AdNMs

3.1. Grouping and read across

Grouping as a basis for read-across of existing information between similar substances is a key approach to optimise and target testing efforts while decreasing the need for animal experiments (Oomen et al., 2015). ECHA and OECD provide detailed guidance for grouping and read-across for nanomaterials (OECD, 2014; ECHA, 2019). However, using grouping in a regulatory dossier has not been extensively demonstrated, especially when nanoforms of the chemical substances are involved, due to a lack of clarity on the evidence required (Jeliazkova et al., 2022).

The EU research project GRACIOUS developed a state-of-the-art framework to support grouping of nanoforms (Stone et al., 2020). The GRACIOUS framework was built upon the work of previous EU research projects (*e.g.*, NANoREG, ITS-NANO, MARINA), the ECETOC's Nano Task Force (DF4NanoGrouping) (Arts et al., 2015), and the ECHA's Partner Expert Group (Clausen et al., 2021). The framework supports hypothesis-driven grouping which is based not only on intrinsic physicochemical properties and (eco)toxicological effects, but also on extrinsic (system-dependent) descriptors of exposure, toxicokinetics and environmental fate.

The GRACIOUS framework implements an grouping hypothesis template (Murphy et al., 2023) which supports the assessor to include consideration of the physicochemical properties (what they are), their fate or toxicokinetics (where they go), associated with a specific use and life cycle stage, and the associated hazards (what they do). Application of the template has resulted in generation of grouping hypotheses relevant to human hazard related to inhalation (Braakhuis et al., 2021; Murphy et al., 2023), ingestion (Di Cristo et al., 2021) and dermal (Di Cristo et al., 2022) routes of exposure. In addition, environmental hazard grouping hypotheses are available for aquatic and sediment environments (Murphy et al., 2023; Cross et al., 2022). The template allows the hypothesis to be structured to relate similar physicochemical characteristics, in the same exposure context, to a resultant similar hazard due to the same Mode of Action (MoA).

Without the use of an animal study, it is unlikely that one single experimental model or test could be used to test such a grouping hypothesis. To guide the user to identify a suitable battery of tests a series of IATA were designed. The IATA are formulated as decision trees that consist of a series of questions (known as decision nodes) that allow identification of key information requirements. Each decision node is supported by a tiered testing strategy composed of state-of-the-art and (where available) standardised methods for data generation. Each decision node is addressed sequentially, and the data is compiled into a matrix covering all tested materials/substances and decision nodes. The data for each decision node can then be assessed for similarity. If similarity is demonstrated for the decision node, grouping is supported; however, if the data lacks similarity, the decision node is rejected, the IATA is exited, and grouping is not applicable.

According to regulatory guidance, expert judgement can be used to assess similarity, but to make the process less subjective, a range of quantitative tools can be applied (Jeliazkova et al., 2022). Such tools calculate how pairs of nanoforms, or group members are related or close to each other. A comparable approach is often used in clustering (*e.g.*, hierarchical clustering) and in classification (*e.g.*, K-Nearest Neighbours). The data is often assessed *via* distance metrics, such as the Euclidean distance, which measure the dissimilarity between two data points (Jeliazkova et al., 2022). Innovative approaches such as correlation metrics (*e.g.*, Pearson's correlation measure), and likelihood-based approaches (Tsiliki et al., 2022; Zabeo et al., 2022) have also been employed to measure similarity between nanoforms, including to AdNMs.

To enable grouping and read-across of advanced materials, the GRACIOUS framework has been extended for multi-component nanomaterials by the EU projects SUNSHINE and HARMLESS and also for a broader range of AdNMs in the EU project POTENTIAL. The modifications address the complexity of composition (what they are) with a special focus on enhanced properties. These projects have concluded that future application of grouping to AdNMs should consider the interaction between different components in a composite material, and the potential for the components to transform (dissociate, disintegrate or dissolve) with different kinetics in different physiological and environmental media along the life cycle, leading to complex exposure scenarios and potentially to mixture (synergistic, antagonistic, additive) effects.

3.2. Exposure assessment

Exposure assessment provides an evaluation of the extent to which humans and the environment are exposed over the entire life cycle of chemical substances such as the AdNMs or the products enabled by them. However, the conventional approach to exposure measurements often involves procedures, which are overly costly and time consuming to apply on a case-by-case basis. The current situation can be improved by application of NAMs such as screening-level lab experiments as well as *in silico* models to assess release, biodistribution, environmental fate, human and environmental exposure (Wambaugh et al., 2019). The application of such NAMs holds promise to decrease the cost and increase the speed of safety assessments, but also to overcome some of the current technical limitations of detection and quantification systems that are still not adapted for the field measurement and monitoring of nanomaterials. On the other hand, drawing conclusions on risks based solely on the NAMs can be complicated due to the transformations of the AdNMs during their life cycles. The complexity added by the identity and the interactions of these new materials can make model generalizations difficult (or impossible). In such situations the exposure assessments will need to be driven (or rely entirely) on experimental methods (*e.g.*, using aerosol sampling instruments and other devices) applied on a case-by-case basis.

Therefore, the state-of-the-art necessitates a 'fit-for-purpose' use of NAMs in combination with exposure measurements. The latter, however, can be considered rather to be an 'enabler' to fill in the data and knowledge gaps, which can render the development and use of *in silico* NAMs more efficient. If the *in silico* exposure NAMs are routinely used within qualitative and quantitative software-based risk assessment tools (*e.g.*, NanoSafer, Stoffenmanager nano, LICARA Nanoscan, SUNDS), there is a huge potential for these tools to learn from such 'case studies' and become more accurate. This is needed as most of these approaches are currently based on conservative assumptions due to the lack of evidence. For instance, many risk assessment tools consider the dustiness of the nano-scaled powders to be the highest, thus overestimating their risk of exposure *via* inhalation despite the fact that recent data has

suggested that this is not always true (Jiménez Garavito et al., 2023).

In addition, the typical tiered approach in exposure assessment (Hollander et al., 2011) describes the stepwise hierarchy of going from a lower tier (qualitative) modelling with few data requirements, but higher uncertainty in the model output, towards higher tiered approaches in which the uncertainty is reduced to derive a more accurate quantitative estimate of exposure, but with more demanding input requirements. As shown in Nymark et al. (2020) (Nymark et al., 2020), the existing exposure NAMs fit well within the same tiered approach of the exposure assessment and align with the increasing technology readiness level within the innovation value chain with more complex NAMs at the later stages.

To facilitate the *in silico* exposure modelling in the proposed NGRA framework, data gaps can be filled by using NAMs that are most suitable for specific exposure scenarios. There should be a focus on standardisation of the exposure scenarios, which supports the harmonisation of exposure scenario description with conditions of use. The first step will be to create a map of the data gaps *versus* relevant NAMs. Such NAMs include *e.g.* (1) methods for dustiness and solubility testing (to better predict the release of AdNM components or degradation by-products), (2) improved generalized inhalation exposure models (incorporating aerosol dynamics), (3) consumer and dermal exposure models, (4) release screening experiments, (5) environmental fate and human biodistribution models, (5) read-across of exposure measurement data (based on grouping of exposure scenarios) to make optimal use of existing information, and (6) make better use of exposure databases to underpin and validate exposure assessment models.

The above needs to be done for the entire life cycle of the AdNM, so not exclusively focusing on the manufacturing or production, but also including occupational, consumer and environmental exposures during the use phase of the nano-enabled product as well as the end of life stage (*e.g.*, recycling, incineration, landfilling). This can be greatly facilitated by the combination of MFA with the NAMs for release, environmental fate, and exposure. This can include not only models but also screening experimental tests that address the transformations of the materials in relevant physiological and environmental media (Meesters et al., 2014; Guo et al., 2020; Cao et al., 2021). These methods can be further combined with biodistribution (PBPK) models to assess the concentrations of the (transformed) materials in different organs (internal exposure), which is essential information to inform more realistic *in vitro* studies as well as *in vitro-in vivo* extrapolations (*cf.* 3.3.3).

To fully enable the proposed NGRA framework the field should progress towards a multiscale modelling approach for combined exposure assessment. Such a mechanistic approach can integrate all relevant exposure scenarios, combining MFA and environmental fate models with (probabilistic) models for uptake and biodistribution. The result of applying this approach will be a comprehensive exposure assessment that covers the full life cycle of an AdNM and accounts for all possible sources of exposure for different targets (*i.e.*, workers, consumers, and the environment).

3.3. Hazard assessment

3.3.1. NAMs vs regulatory endpoints

The EU Chemicals Strategy for Sustainability identified the need to *'innovate safety testing and chemical risk assessment to reduce dependency on animal testing while improving the quality, efficiency, and speed of chemical hazard and risk assessments'* (European Commission, 2020). REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), which is the main regulatory framework covering nanomaterials in Europe, states that it should *'promote the development of alternative methods for the assessment of hazards of substances'*. Besides *'for human toxicity, information shall be generated whenever possible by means other than vertebrate animal tests, through the use of alternative methods, for example, in vitro methods or qualitative or QSAR models or from information from structurally related substances (grouping or read-across)'*. Following the REACH amendments from 2016 and 2017, which included NAMs to address regulatory endpoints, a substantial increase in the use of alternative approaches such as read-across, eye irritation/damage and skin irritation/corrosion tests, has been observed in chemical dossiers (Patlewicz et al., 2024). The ban on *in vivo* experimentation for cosmetics and cosmetic ingredients developed or imported in the European Union (2013) further paved the way for the development of alternative testing, mainly focused on the dermal route. Similarly, EFSA has supported the implementation of testing strategies including NAMs in its risk assessment guidance (EFSA, 2018; More et al., 2021).

Regulators are increasingly using data produced through NAMs, at least to cover data gaps by grouping and read-across, although grouping of nanoforms has not been extensively demonstrated in regulatory dossiers. Similarly, even if several NAMs have now been developed and accepted at OECD level to cover for regulatory relevant end points addressing skin sensitisation, skin irritation and corrosion, eye irritation and corrosion, mutagenicity, genotoxicity and phototoxicity (Table 1 and Table 2), those still need to be adapted to nanomaterials (Table 3). Currently only three ISO standards have been validated for nanomaterials covering photocatalytic activity of nanoparticles for NADH oxidation (ISO 20814) (ISO, 2019), the MTS assay (ISO 19007) (ISO, 2018) and the use of CM-H_2DCF-DA assay for the evaluation of nanoparticle-induced intracellular reactive oxygen species (ISO 19006) (ISO, 2016).

As previously discussed, individual *in vitro* methods are unlikely to cover for an *in vivo* test outcome, however those NAMs may be combined together to inform on the hazard potential of an AdNM. One success story is represented by skin sensitisation, where a well-developed Adverse Outcome Pathway (AOP) exists, and the first three Key Events can be assessed by a combination of *in chemico* and *in vitro* methodologies organised in a testing approach (OECD TG 497). On this front, a recent combined effort between the OECD and the EU Gov4nano project, led by Switzerland, produced the OECD document entitled '*Study Report on the Applicability of the Key Event-based TG 442D for in vitro sensitization testing of nanomaterials'*, representing the first steps into the adaptations of the skin sensitisation strategy to nanomaterials (OECD, 2022b). Other regulatory relevant end points such as skin/eye irritation and corrosion, for which NAMs are already available, may be directly used with solid nanomaterials and adaptations are not expected, since dispersions are not required as the *ex-vivo* models are directly exposed to the substances.

Genotoxicity represents another regulatory relevant end point that is pertinent for NAM development and adaptations, since it is addressed by a combination of *in vitro* and *in vivo* assays (with the exception of cosmetics). Shortcomings regarding nanomaterials have already been identified, those represent the study of nanomaterial cellular uptake, exposure durations and staging with respect to other assay components (*e.g.*, cytochalasin B), or the use of the S9 metabolic fraction (Doak et al., 2023; Elespuru et al., 2018; Doak et al., 2012). Past and present research projects including national and EU initiatives (*e.g.*, PATROLS, RiskGONE) have made good progress on adaptation of different assays such as the micronucleus assay, mammalian gene mutation test, and comet assay, and on the development of NAMs based on advanced *in vitro* models of lung, liver, skin *etc.* with simultaneous assessment by both micronucleus and comet assays (Doak et al., 2022). Furthermore, a new OECD Guidance Document has been released, detailing the adaptations required for the *in vitro* micronucleus assay for genotoxicity testing of nanomaterials (OECD, 2022d). Thus, current efforts suggest that further adaptation of relevant assays to address *in vitro* genotoxicity regarding nanomaterials will be achieved in the foreseeable future.

The main challenge for the emerging NAMs is to ensure their compatibility with regulatory frameworks (regulatory readiness) as well as their usefulness and usability by the industries complying with the regulations and the regulators themselves. This requires to ensure the Transparency, Reliability, Accessibility, Applicability and Completeness (TRAAC) of the methods (Shandilya et al., 2023). *Transparency* refers to clear communication about the methods, their strengths, and limitations

Table 1
OECD Test Guidelines used to assess toxicity through *in vitro* NAMs under the OECD Testing Programme (OECD, 2018).

		ZnO	MWCNTs	Au	Fullerenes	SWCNTs	SiO_2	CeO_2	Ag	TiO_2
OECD Test Guideline										
Dermal absorption	428	X								
Skin corrosion	431	X	X							
Eye Irritation	437	X								
Genotoxicity	471	X	X	X	X	X	X		X	
	473	X	X		X	X	X		X	
	476	X	X				X			X
	482						X			
	487						X			X

Table 2
Cytotoxicity and genotoxicity assays used the in OECD Testing Programme (OECD, 2018) *NRU: Neutral Red Uptake, **AB: Alamar Blue; ***CFE: Colony Forming Efficacy, ****DSB: Double Strands Break.

Assay	ZnO	MWCNTs	Au	Dendrimers	SWCNTs	Fullerenes	SiO_2	CeO_2	TiO_2
Cytotoxicity									
ATP CellTiter Glow			X						
NRU*	X			X					X
LDH		X	X	X	X	X	X	X	X
MTT				X	X	X	X	X	X
XTT			X						
Cell Impedance			X						X
Trypan Blue						X			
BrdU						X			
AB**						X			
WST-1						X		X	
Cell counts						X			X
CFE***								X	
Genotoxicity									
Comet assay					X		X		X
DSB****					X				

(*e.g.*, boundary of use). *Reliability* measures the quality, correctness, and consistency of output. It entails that information about the uncertainty and variability of the test should be available, and the quality of the test can be assessed. This includes that different positive and negative controls should be in place, as well as reference substances/materials. Moreover, it should be clear how these controls would perform to assess the quality of a test. *Accessibility* refers to the findability and usability of the methods. *Applicability* means that the applicability domain of the methods is clearly communicated. *Completeness* measures how comprehensively the methods align to the relevant regulatory frameworks (*e.g.*, REACH) and cover their nano-specific requirements. In addition to the TRAAC criteria, we propose the *Relevance* of the NAM for risk assessment as another essential criterion for regulatory readiness. It measures how clear and well justified is how the data of a NAM can be adequately interpreted in risk assessment. Each of the five TRAAC pillars consists of several assessment criteria, which are illustrated in Fig. 2 and detailed information on each of them is provided in the Supplemental Information. To illustrate the application of these pillars/criteria to assess the regulatory readiness and the usefulness/usability of alternative approaches we applied those to a set of key emerging NAMs carefully selected to represent different levels of regulatory maturity. These NAMs and a summary of the obtained results are presented in Table 4, while the detailed results of the assessment are presented in the Supplemental Information. The assessments of the NAMs were performed by authors of this manuscript who are the developers and/or experts in the specific methods. They were based on a thorough study of the related literature and were verified in expert meetings, which took place in the period October 2023 – April 2024.

The TRAAC framework was originally developed for exposure/risk assessment and decision support tools. This is the first time this framework was applied to NAMs and the results are very encouraging. In the experience of the authors who performed the assessments its criteria were relatively easy and straightforward to implement, and the obtained outcomes provide a meaningful assessment of regulatory readiness and applicability for both experimental and *in silico* NAMs. Some criteria, however, were not directly applicable and need to be revised for NAMs. For instance, as far as cell models are concerned, the criterion describing 'collaborative effort' was not applicable and may be already covered by 'acceptance at OECD level' and 'peer review publications'. Time and cost efficiency may need to be reformulated to be compared with current *in vivo* benchmarks. Socio-economic aspects may not be directly relevant for the assessment of safety assessment tools. In addition, 'inclusion of a license' is not always necessary and very often the intention is that a NAM is open access with no restrictions; this criterion therefore needs to be revised to take this into consideration. The criterion 'advice for improving the output' is not really applicable for tests that have a hazard endpoint as they are not designed for providing advice. The majority of the Completeness criteria focused on characterization of physicochemical properties and while the expectation is that this should be carried out as part of any hazard assessment, it is not included specifically in any *in vitro* model standard operating procedure (SOP). This has made the evaluation of Completeness impossible when the NAM is assessed in isolation of the study context in which it is applied, which resulted in the outcome 'not applicable'.

3.3.2. *In vitro models*

In view of the progressing number of new AdNMs being produced and their increasing diversity and complexity, conventional animal testing strategies have reached their limits of capacity and are challenged by ethical accountability. As a consequence, the demand for smart *in vitro* based testing techniques is increasing. Advanced models hold promise to reproduce *in vivo* responses, mimicking organotypic cellular constitutions and cell circuits. On the other hand, conventional *in vitro* models are likely more appropriate for high throughput and high

Table 3
Current activities on-going at the OECD Working Party of Manufactured Nanomaterials (WPMN) on the development and adaptation of Testing Guidelines and Guidance for nanomaterials regarding physico-chemical and toxicological end points using NAMs.

Title	Status
Test Guideline on particle size and size distribution of manufactured nanomaterials	Test Guideline 125 (Completed 2023)
Guidance on determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biological media	Expected 2025
Guidance on identification and quantification of the surface chemistry and coatings on nano- and microscale materials	Expected 2025
Test Guidance on determination of surface hydrophobicity of manufactured nanomaterials	Test Guideline 126 (Completed 2023)
Test Guideline on determination of the dustiness of manufactured nanomaterials	Expected 2025
Guidance on the determination of concentrations of nanoparticles in biological samples for (eco) toxicity studies	Expected 2024
Test Guideline on dissolution rate of nanomaterials in aquatic environment	Expected 2024
Guidance on assessing the apparent accumulation potential for nanomaterials	Expected 2025
Guidance on environmental abiotic transformation of nanomaterials	Expected 2025
Study Report and preliminary guidance on the Adaptation of *In Vitro* Mammalian Cell Based Genotoxicity TGs for Testing of MNs	OECD Publication No. 359 (Completed 2022)
Applicability of the key event-based TG 442D for *in vitro* skin sensitisation testing of nanomaterials	OECD Publication No. 382 (Completed 2023))
Integrated *in vitro* approach for intestinal fate of orally ingested nanomaterials	Expected 2025
Validation of the *In Vitro* Micronucleus assay for Engineered Nanomaterials	Ongoing – no date
Test Guideline on particle size and size distribution of manufactured nanomaterials	Test Guideline 125 (Completed 2023)
Guidance on determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biological media	Expected 2025
Guidance on identification and quantification of the surface chemistry and coatings on nano- and microscale materials	Expected 2025
Test Guidance on determination of surface hydrophobicity of manufactured nanomaterials	Test Guideline 126 (Completed 2023)
Test Guideline on determination of the dustiness of manufactured nanomaterials	Expected 2025
Guidance on the determination of concentrations of nanoparticles in biological samples for (eco) toxicity studies	Expected 2024
Test Guideline on dissolution rate of nanomaterials in aquatic environment	Expected 2024
Guidance on assessing the apparent accumulation potential for nanomaterials	Expected 2025
Guidance on environmental abiotic transformation of nanomaterials	Expected 2025
Study Report and preliminary guidance on the Adaptation of *In Vitro* Mammalian Cell Based Genotoxicity TGs for Testing of MNs	OECD Publication No. 359 (Completed 2022)
Applicability of the key event-based TG 442D for *in vitro* skin sensitisation testing of nanomaterials	OECD Publication No. 382 (Completed 2023))
Integrated *in vitro* approach for intestinal fate of orally ingested nanomaterials	Expected 2025
Validation of the *In Vitro* Micronucleus assay for Engineered Nanomaterials	Ongoing – no date

content testing with potential real-time transmission of results (Kokot et al., 2020; Toprani et al., 2021; Watson et al., 2014; Pyrgiotakis et al., 2018; Yang et al., 2020). Finally, application of these advanced methods needs to be as comprehensive and predictive in identifying materials of concerns, as were conventional animal studies.

Scientists have developed a range of more physiologically realistic models, establishing organotypic or even organoid culture systems to mimic organ complexity, for example lung organoids (Kastlmeier et al., 2022) and air liquid interface models (Braakhuis et al., 2023) (Lacroix et al., 2018) for pulmonary hazard assessment of inhaled materials. Several advanced *in vitro* models representing the human lung, liver and gastro-intestinal tract were also developed (Doak et al., 2022). The predictive power of these sophisticated models needs to be confirmed on a case-by-case basis, so it is challenging to apply them in high throughput assays that allow to test a large number of materials at comparable conditions. Accordingly, comparable simple cell-based assays, using immortalized cell lines with all their disadvantages, find their applications for *in vitro* high-throughput and high-content screening assays, allowing dose-response assessments and modelling, as well as analyses of biological processes and toxicity pathways, kinetics and dose extrapolation (Nymark et al., 2020). In contrast to throughput, organotypic models hold promise because of their capacity for mechanistic studies helping to better understand the interactions of AdNMs with specific biological structures in addition to their potential to support the 3R principles. Here, the importance of the selection of the realistic dose and the exposure conditions with relevant endpoints deserves particular notice, as most mechanistic studies require high doses and simplified exposure conditions to generate statistical significance with low replicates or repeats. However, to ensure the obtainment of relevant and realistic outputs, advanced *in vitro* models should be combined with appropriate exposure systems that allow for the estimation of the effective delivered dose. A lot of progress has been achieved so far in this direction (Doak et al., 2022) but additional efforts are still required to develop high throughput exposure systems especially for the delivery of dry materials.

3.3.3. *In vitro-in vivo extrapolation (IVIVE) methods*

Currently, regulatory accepted risk assessment of AdNMs is strongly relying on animal (*in vivo*) experiments and relatively little weight of evidence is given to results from cell-based *in vitro* studies. The allometric scaling of *in vivo* point of departure (PoD) hazard data (*e.g.*, lower confidence limit of the benchmark dose (BMDL) or lowest observed adverse effect level (LOAEL)) to human hazard combined with human exposure scenarios provides the basis for risk management *via* definition of 'safe' exposure limits. With improved *in vitro* technologies, there is an increasing interest in reliable methods for *in vitro-in vivo* extrapolation (IVIVE) of BMDLs/LOAELs. However, there is currently no comprehensive IVIVE method suitable for all *in vitro* and *in vivo* models and all types of AdNMs (examples of AdNMs are given in a systematic way inWohlleben et al., 2024). In this section we highlight two key elements for reliable IVIVE, namely the relevance of internal dose and the role of integrative IVIVE modelling. These elements will be elucidated for inhaled nanomaterials, which have been associated with adverse health outcomes including persistent pulmonary inflammation, lung cancer and fibrosis as well as cardiovascular effects (Peters et al., 2011; Halappanavar et al., 2020). Despite this focus on inhaled nanomaterials, many aspects of pulmonary IVIVE are also relevant for AdNMs and for organs other than the lung.

Historically, mass has been the most widely used dose metric for particle-induced toxicity. However, for poorly soluble or bio-persistent nanomaterials the biological interactions occurring at the surface of these materials are strong determinants of the material-specific hazard profile. This is corroborated by a large body of evidence indicating that surface area - not mass, volume or number – is the toxicologically most relevant dose metric, which eliminates many size-dependent effects observed with mass or number as dose metric (Schmid and Stoeger, 2016; Maynard and Kuempel, 2005; Cosnier et al., 2021). This paves the way for surface reactivity-based hazard classification.

IVIVE is often hampered by using exposure concentration as surrogate for the toxicologically relevant tissue-delivered dose. Surface area has also been observed as a suitable dose metric for size-dependent toxicological effects in cell-based *in vitro* assays (McLean et al., 2023). However, such effects have often been obfuscated by the widespread use of particle concentration exposure rather than cell-delivered (internal)

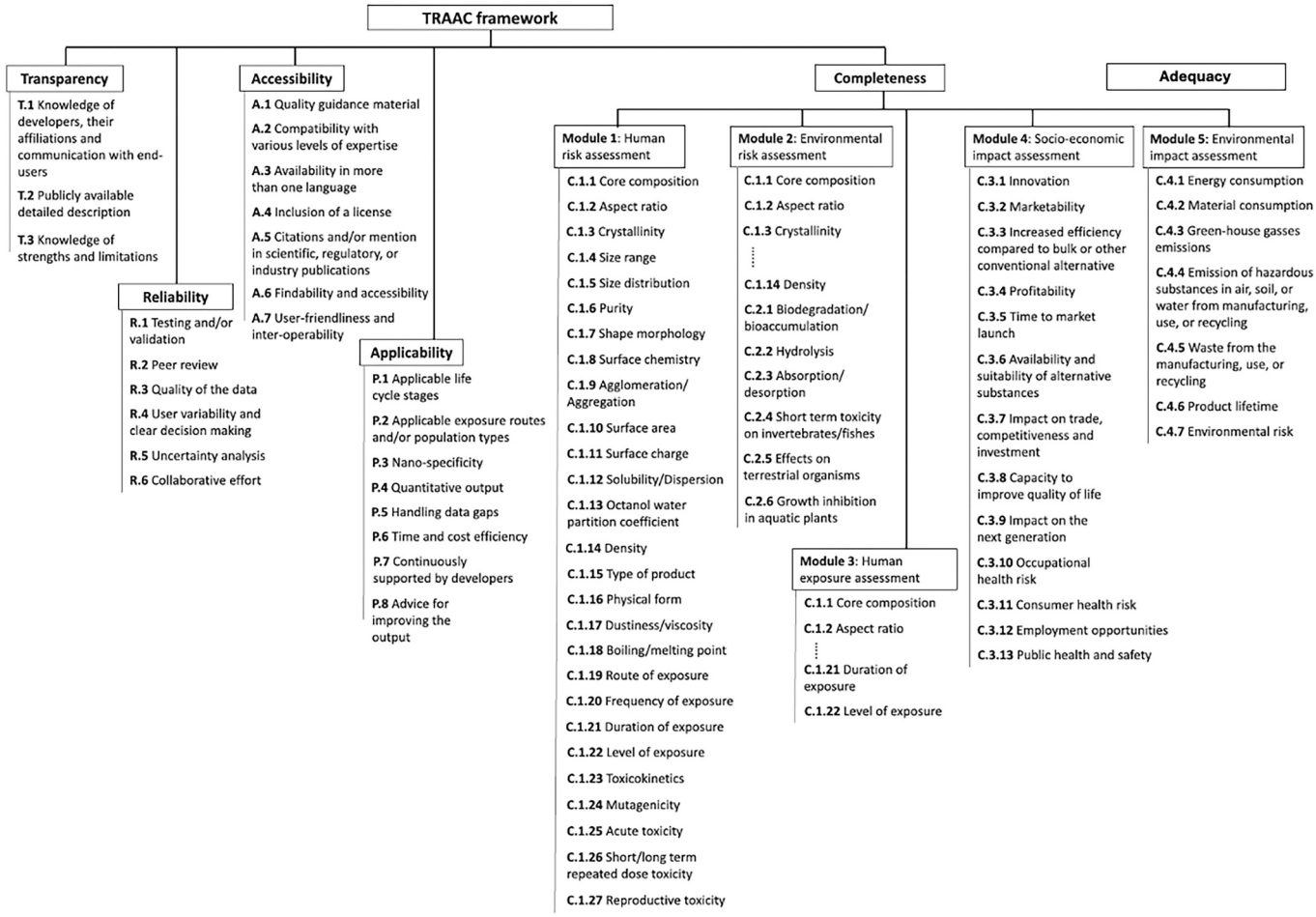

Fig. 2. Overview of the TRAAC framework: five pillars of the framework, i.e. Transparency, Reliability, Accessibility, Applicability and Completeness, and their respective constituting criteria plus an additional criterion Relevance (for risk assessment) to be assessed by expert judgement. Five modules within the Completeness pillar are also shown with the parameters/criteria for the completeness evaluation within each module (After Shandilya et al. (2023) (Shandilya et al., 2023)).

dose (*e.g.*, mass or surface area) in toxicological *in vitro* studies (Krug, 2014; Romeo et al., 2022). For alignment of *in vitro* and *in vivo* dosimetry, not exposure levels but tissue-normalized (internal) dose (*i.e.*, nanomaterial dose per surface area (or mass) of exposed tissue) is the prerequisite for empirical determination of *in vitro – in vivo* dose conversion factors (McLean et al., 2023; Schmid and Cassee, 2017). Internal dose is often determined with either analytical techniques (inductively coupled plasma mass spectrometry (ICP-MS), absorbance spectroscopy) or computational dosimetry models (*e.g.*, Multiple-Path Particle Dosimetry Model (MPPD) or *In vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model) which have been developed for spherical particles but can be adapted to non-spherical particles using the effective density concept (Schmid and Stoeger, 2016; Lizonova et al., 2024; Schmid et al., 2007).

As an example of a promising integrative modelling approach to IVIVE of hazard assessment we present the so-called Predictive Toxicogenomics Space (PTGS), which was constructed based on big data analysis, bioinformatics, and early artificial intelligence concepts (Kohonen et al., 2017). The first demonstration of IVIVE applicability of PTGS dealt with predicting the risk of drug-induced liver injury from drug molecules. Toxicogenomics assessments in diverse liver cell-based *in vitro* models, including monolayer and organotypic cultures, relative PTGS and toxicogenomics reference datasets from large-scale toxicogenomics databases (TG-GATEs) and Connectivity maps constituted the initial analysis to generate scores for PTGS activation. Thereafter, from coupling of the scoring results to limited PBPK modelling data, the PTGS-derived results were able to predict risk of drug-induced liver injury with higher accuracy than standard non-omics-based *in vitro* models' data. Modelling applying overall the 14 components that constitute the PTGS additionally were shown to inform on toxic MoA and to serve for grouping of chemicals and drug molecules. Follow up work within the advanced (nano)materials safety evaluation area, most notably within the EU-funded NanoSolveIT and HARMLESS projects, the PTGS concept has undergone evaluation for broadening its applicability. Such efforts have been built on diverse initial toxicity and dose-response evaluations of materials by low, medium, or high-throughput screening analysis and benchmark dosing to derive PoD data with cell culture models. Under a tiered approach evaluation concept, PTGS assessment of LOAEL is subsequently applied to deepen the MoA evaluation considering gene ontologies generally, but also from assessment of Key Events within AOPs. Under testing, PTGS toxicity scoring might be especially useful to assess hazard of AdNMs based on several constituents where transformation, leaching, *etc.* cause situations where mixture toxicity could be envisioned to occur. Overall, the PTGS concept promises to be a useful modelling tool for deepened hazard characterization, where numerous means of reverse translation from the *in vivo* to the *in vitro* situation *via* PoD/LOAEL calculations and AOP-coupled MoA assessments might be especially informative.

In general, the development of NAMs predictive of human health effects requires reliable methods for IVIVE. This hinges on the availability of a large body of dosimetrically and toxicologically accurate human health data, which will have to be supplemented by *in vivo* data for a more detailed mechanistic understanding of the underlying MoA.

Table 4
Regulatory readiness and usefulness/usability of emerging NAMs assessed in terms of Transparency, Reliability, Accessibility, Applicability and Completeness by applying the TRAAC framework as well as in terms of Relevance (for risk assessment) by expert judgement. Each of these six pillars consists of several criteria, characterized by scores (on a scale of 0.1- unfulfilled, 0.5- partially fulfilled and 1-completely fulfilled) and weights (on a scale of 1 to 5) pre-defined in (Shandilya et al., 2023). The details of the scores assigned to each criterion are reported in the Supplemental Information, while the final results of integrating the criteria scores into an index for each pillar (by weighted average) are summarized in this table. The final indices for the pillars are on a continuous scale from 0 (unfulfilled) to 1(completely fulfilled). The assessments were performed by authors who are the developers and/or experts in the specific NAMs.

Type of NAM	Approach	Intended purpose	Trans-parency	Relia-bility	Accessibility	Relevance	Applicability	Completeness	References
HARMLESS High-Content Analysis (HCA) *in vitro* tox profiling for toxicity endpoints	Safety assessment based on read-across (internal exposure)	Grouping based safety assessment across similar materials	0.49	0.48	0.3	0.5	0.16	1.0	N.a.
In vitro alveolar barrier (ALIsens)	*In vitro* data related to a barrier/organ specific mode of action	Prediction of the respiratory sensitization potential of chemicals and nanomaterial	1.0	0.74	0.6	0.5	0.56	Not applicable	(Chary et al., 2019) WO2018/122219A1: *In vitro* alveolar model for prediction of respiratory sensitization (OECD, 2022e)
MucilAir	Reconstituted primary human airway epithelial *in vitro* model from healthy and unhealthy individuals	Prediction of acute and chronic lung toxicity. Multiple end points, disease models	0.83	0.74	0.88	0.5	0.8	Not applicable	EURL ECVAM TM2014–03 (EU): Transepithelial permeability assessment using an *in vitro* cell model of the human airway epithelium.
HepG2 Liver spheroid models	*In vitro* hazard characterization data; considers metabolic activation	Hazard characterization and assessment (genotoxicity, cytotoxicity, inflammatory response)	0.83	0.81	0.82	0.5	0.78	Not applicable	(Conway et al., 2020; Llewellyn et al., 2020; Llewellyn et al., 2021; Llewellyn et al., 2022)
Human quadruple cell primary healthy hepatic spheroid model	Physiological *in vitro* model composed of hepatocytes, the resident macrophages (Kupffer cells), sinusoidal endothelial cells and stellate cells	Acute and chronic hazard assessment (cytotoxicity, inflammation, metabolic activity, albumin secretion, histopathology)	0.79	0.92	0.8	0.5	0.84	0.3	(Kermanizadeh et al., 2022; Kermanizadeh et al., 2019; Kermanizadeh et al., 2014)
Human quadruple cell primary disease hepatic spheroid model	Pathophysiological *in vitro* model composed of hepatocytes, Kupffer cells, sinusoidal endothelial cells and stellate cells - can replicate mild liver disease (steatosis) and more severe hepatic condition (metabolic dysfunction associated steatohepatitis)	Acute and chronic hazard assessment (cytotoxicity, inflammation, metabolic activity, albumin secretion, histopathology)	0.63	0.92	0.33	0.5	0.68	0.3	(Kermanizadeh et al., 2022)

3.3.4. *In chemico* approaches

In chemico NAMs concern the risk screening by measurement of properties that are known to relate to exposure or to hazard. Such properties are often 'extrinsic' in the sense that one measures the interaction or behavior of the AdNMs under certain well-controlled conditions. *In chemico* NAMs are by definition abiotic, and do not use living cells, as opposed to *in vitro* NAMs. To support hazard screening, *in chemico* NAMs are typically derived from Key Initiating Events of an AOP. A well-known example is the measurement of surface reactivity. Several assays can measure the radical generation and each assay has different applicability domains, and different sensitivities to specific mechanisms of radical generation. Another example is given by the measurement of the dissolution rates and halftimes under simulated physiological conditions. Both reactivity and dissolution are recommended by ECHA for the justification of grouping of nanomaterials and are used to support registration of sets of similar nanoforms. However, AdNMs can induce assay interferences, *e.g.*, adaptations to reactivity assay protocols were required to make the assay that was originally

developed for simple metal-oxide nanomaterials applicable to innovative graphene-based materials. Hence, for AdNMs additional quality control is recommended to detect and remove interferences. Additionally, if the AdNM is multicomponent, the potential transformation of chemical and/or physical structure under physiological conditions must be assessed by suitable *in chemico* NAMs, which will likely derive from the simpler dissolution tests developed for conventional nanomaterials. The detection of the potential mixture of pristine AdNMs, transformed AdNMs or newly formed species, and dissolved components can be a challenging analytical task, but provides guidance to a targeted application of further NAMs on the observed structures. It also provides important feedback to grouping approaches, which may have to reconsider the most suitable source materials, which may include each component of the AdNM, and the transformation products. If the multicomponent AdNM includes polymer components, new NAMs need to be considered that were not relevant for the mostly metal-based conventional nanomaterials. The polymer component, if exceeding 0.1%, would need to be assessed for its solubility, solidity, and biodegradability to assess the AdNM against the criteria of the ECHA restriction of intentionally produced polymer microparticles (commonly known as microplastics).

To support exposure screening, the *in chemico* NAM has to simulate the stress that the AdNM (or the final nano-enabled product incorporating the AdNM) will experience during the life cycle, primarily derived from the intended use. The most commonly required *in chemico* NAM for exposure screening address dustiness, which provides, in its standardised form, the respirable dustiness index as a measure of the propensity of a powder to generate dust when agitated. However, beyond the gravimetric evaluation of the filter from the cyclone, that same filter can also be evaluated by more advanced analytics for the potential demixing of a multicomponent AdNM. Specifically, mixed pigments may be evaluated for the content of titanium dioxide (TiO_2) in the released dust by ICP-MS analysis, or for the content of crystalline silica (quartz, a carcinogen) by X-Ray Diffraction (XRD) analysis. In analogy, the selective detection of the individual components of an AdNM, or of products that incorporate AdNMs, is an obvious adaptation of established life cycle tests that screen for releases, such as the highly standardised simulation of food contact, or weathering tests that assess environmental releases. Previously, selective detection was important to differentiate the leaching of impurities from the migration of nanoparticles, but it can as well address the different components of an AdNM.

3.3.5. *In silico models*

The practical application of Machine Learning (ML) algorithms for prediction, grouping and read-across, has been increasingly interesting to researchers as an effort to improve human relevance. Overall, *in silico* ML models are considered to be cost and time efficient alternatives to traditional testing methods by utilizing the data collected from nano-specific measured properties and experimental conditions. For that reason, they can be considered as NAMs that gain more attention as the volume of good-quality nano-specific data is constantly increasing. *In silico* NAMs have been employed under various scenarios, having as a final goal to produce a model that primarily fits the data well but also is accepted by regulators. Overall, their aim is to gain an insight into features effecting toxicity and predicting possible adverse effects, however with respect to their purpose of use, they can be divided into three main categories: predictive QSARs, similarity assessment methods for grouping and read-across models. Among the three *in silico* methods, QSARs are dominant approaches in toxicological research. QSARs are NAMs using different statistical methods able to capture complex relationships between chemical structures and high tier toxicological endpoints. Nowadays, traditional QSARs have matured to include ML algorithms (QSAR-like ML-based models) which are particularly useful when high numbers of datapoints are available to train the model, for instance when omics data are available. Support vector machines, random forest, nearest neighbour models, and artificial intelligence based network algorithms, are some of the models mostly used (Allen et al., 2022; Zhao et al., 2020; Mayr et al., 2016). Specifically, emerging ML algorithms based on statistical criteria (minimum variance, Akaike Information Criterion (AIC)) and modern dimensionality reduction techniques (nonlinear/independent Component Analysis, matrix factorization, deep learning autoencoders) are powerful tools that can provide better results than classic Principal Component Analysis, when applied to nonlinearly correlated properties. Typically, the chemical space from which a model is derived should be clearly defined in order for the prediction to be considered reliable (Gadaleta et al., 2020). New substances with unknown toxicity but known properties can then be provided into the model to 'estimate' their toxicity, assuming that they are included in the applicability domain space of the model (Ciallella et al., 2021). Although, ML models are used mainly to predict nano (eco)-toxicological outcomes, they could also be important for suggesting improvements to both the design and the strategic use of *in vitro* test methods, and to provide data needed for the correct application of these testing methods, such as prediction of volatility and solubility of chemicals needed to perform some *in vitro* assays.

Grouping and read-across models are desired by industry, but still not well accepted by regulators in the field of nanomaterials. Nevertheless, ECHA published that read-across is used in one of every four cases (25%) in REACH dossiers (ECHA, 2020). Read-across is not a NAM *per se*, but a weight of evidence argument which may be supported by the use of NAMs in the area of similarity assessment. ML models can substantially support similarity assessments, challenging the expert opinion key requirement of an analogue approach. Unsupervised techniques, such as clustering methods can identify patterns in unclassified or unlabeled data, *e.g.*, in relation to organism grouping. Reinforcement learning techniques, including those based on neural networks can support MoA based similarity assessment and grouping using omics data. *In silico* methods are also employed for filling data gaps for toxicological and other endpoints (Myatt et al., 2018) in the prediction of complex toxicities and adverse effects, *e.g.*, repeated dose toxicity. In particular, ML models can generate essential understanding of key inter-component and nano-bio/eco interactions and MoA (such as AOPs) of the AdNMs as a basis for similarity assessment, grouping and read-across.

4. Discussion

The AdNMs of a particular chemical composition can consist of various nanoforms, which may exist as particles, in multi-component structures, different surface coatings as well as aggregates and agglomerates with characteristics that may dynamically change over time along the life cycles of the materials. This complexity is further increased by the differing rates of degradation and toxicities of the separate and interacting components, and their potentially more complex interactions with biological and environmental systems. These components may interact with each other, with other nanomaterials and/or chemicals, leading to additive, synergistic or antagonistic effects (mixture toxicity). It is obviously not feasible to test each transformation of these components/nanoforms by using animal studies to assess their effects for risk assessment purposes. Therefore, a more flexible and adaptive NGRA framework that employs alternative methods for targeted testing and assessment is proposed in this manuscript. This framework implements an exposure-driven tiered approach, starting with identification of hotspots of safety concerns in relevant exposure scenarios, complemented by NAM-based biological activity screening across a broad toxicological space that informs intelligent testing to generate data for regulatory decision making. The iterative structure of the approach allows for a high degree of flexibility to generate the data needed for the specific purpose (SbD vs. regulatory risk assessment) by means of targeted testing and assessment. The approach also allows to iteratively go back to previous steps to refine the data if it is considered

insufficient in terms of quantity or quality for a robust risk characterization conclusion. However, two issues remain to be solved in this regard: guidance is needed on minimum data requirements as well as decision thresholds for the required quantity (*i.e.*, completeness) and quality (*e.g.*, relevance, reliability, adequacy, statistical significance) of the data as per the criteria defined by Klimisch et al. (Klimisch et al., 1997) and further elaborated by others (Basei et al., 2022; Kase et al., 2016). It should be noted however that some regulatory scientists argue that adequacy should not be part the definition of data quality since in the REACH context adequacy is synonymous with regulatory data acceptability, while the acceptability of the data is dependent not only on its quality, but also on the regulatory requirements. Some toxicologists would also exclude relevance, since this is also highly dependent on the regulatory context of use. In any case, for the above criteria the process of assessing data quality is more complicated for data derived from non-standardised NAMs as compared to animal data generated according to internationally accepted standards. Nevertheless, the mere amount of data generated from such alternative methods makes this information important to consider in regulatory decision making. The reporting of data generated by NAMs is therefore highly desirable but guidance on the interpretation of such data is needed before alternative approaches can be confidently applied in risk assessment.

The proposed framework is exposure-driven: Starting the risk assessment with identification of relevant exposure scenarios is key for developing targeted testing strategies as this provides essential initial information on relevant exposure routes, related endpoints, test media, and relevant forms of the materials (pristine vs. weathered/aged). It should be noted however that especially for new AdNMs it may sometimes be impossible to foresee the exposure scenarios for all possible applications. Moreover, performing a risk assessment for every possible exposure scenario will be challenging. Therefore, guidance should be developed on how to prioritize critical exposure scenarios as well as scenarios that can serve as 'worst case' benchmark for others. This can be supported by exposure-based grouping to enable read-across of information between scenarios. The concept of combined exposure considering all possible sources is key, but it should be acknowledged that combining different scenarios for integrated exposure assessment will be complicated by the complex/dynamic physicochemical transformations that the AdNMs can undergo along their life cycles in different exposure media. This is a challenge which could be addressed by future advancements in exposure NAMs.

The proposed framework is considered to adequately address the issues involved in the gathering and evaluation of data for the risk assessment of emerging materials where the available datasets are often fragmented. This is done by thoroughly assessing the available and newly generated data for completeness and quality based on established criteria. In addition, to ensure the regulatory acceptance of the acquired datasets the regulatory reediness of the tools is also assessed using criteria from the recently published TRAAC (Transparency, Reliability, Accessibility, Applicability and Completeness) framework (Shandilya et al., 2023). The latter is of high importance as many NAMs have not yet reached the level of maturity allowing their outcomes to be accepted for regulatory decision making. The results of applying the TRAAC framework showed that its criteria are generally broad enough to be applicable to both experimental and *in silico* NAMs. The completeness criterion, however, was very challenging to apply as it refers to the entire set of regulatory information requirements which can never be accomplished by a single NAM. In three out of six performed TRAAC assessments this criterion was considered not applicable at all (*cf.* Table 4). Therefore, it is clear that the scope of the completeness criterion needs to be narrowed down to the completeness of information to fulfill only the specific information requirement(s) covered by the particular NAM. In addition, the TRAAC framework defines reliability as 'quality, correctness, and consistency of output'. In the context of experimental NAMs, especially *in vitro* methods, reliability also relates to reproducibility; therefore, we suggest including reproducibility in the definition of reliability when the framework is applied to experimental NAMs. In general, the results of applying the TRAAC framework to NAMs are encouraging as it turned out to be generally applicable, but as with any such methodology without detailed guidance different assessors can easily reach discordant conclusions; therefore, explicit guidance for NAMs will be necessary. Moreover, the inclusion of pre-defined weights is not appropriate as the weighting is highly context-dependent, and therefore we recommend leaving the assignment of weights up to the assessor.

The proposed framework involves the integration of NAMs in IATA, which can have a substantial impact on optimising the cost of testing and replacing animal experiments. The IATA are decision trees that can lead the assessor through implementing a battery of NAMs to generate data on specific risk-related endpoints. To target the testing, it should be based on a tiered approach with the specificity and complexity of the adopted NAMs increasing in the higher tiers. Basing such IATAs on AOPs is particularly promising (Tollefsen et al., 2014) as exemplified by the development of the OECD guideline 497 for skin sensitization (OECD, Guideline No. 497, 2024), which has been accepted by regulators. It should be noted that if the adopted NAMs have not reached the level of maturity required for the regulatory acceptance of their results, such alternative approaches can be applied in combination with established *in vivo* methods in the highest tier. This can in many cases ensure better relevance for the risk assessment process, while achieving the goals to optimise testing and reduce animal experiments. IATA can also be applied for identification of the key information required to accept, reject of refine the grouping hypothesis (Stone et al., 2020; Braakhuis et al., 2021; Murphy et al., 2023; Di Cristo et al., 2021). This can support read across for data gap filling prior to engaging in testing and assessment. Therefore, the combination of IATA and NAMs can provide a powerful methodology for acquiring the data needed for risk assessment in a more efficient and strategic manner.

There has been substantial progress made in recent years and the direction towards the increased adoption of NAMs in regulatory practice has been set. These methods have obvious benefits (*e.g.*, for local toxicity and skin sensitization well-established NAMs already fully replace their conventional *in vivo* counterparts (Leist et al., 2012)), but there are major challenges that have prevented their successful application for other essential endpoints. These challenges are rooted in the way legislation implements the current animal-centric risk assessment paradigm (Schmeisser et al., 2023). The REACH regulation specifies that alternative methods can be applied but they need to be '*adequate for the purpose of classification and labelling and/or risk assessment*' and should allow the risk assessor to perform quantitative hazard characterization (*i.e.*, derive a reliable 'Point of Departure' such as a BMDL or a LOAEL). In other words, these methods should provide a robust alternative to the specific animal test designs set out in the REACH Standard Information Requirements. This can be quite challenging as the fitness for purpose of NAMs in relation to a specific context of use is often unclear. This challenge is most evident for complex endpoints such as repeated-dose, carcinogenicity, or reproductive toxicity as to provide an alternative to the conventional animal studies the assessor will need to apply a battery of NAMs, each addressing a Key Event in a complex network of AOPs. The assessor will also have to demonstrate how the results of such a battery approach are adequate for Classification and Labelling. This is however practically impossible as the current Classification, Labelling and Packaging (CLP) complex regulatory endpoints (repeated-dose, carcinogenicity, or reproductive toxicity) are tailored to data either obtained in humans or from *in vivo* animal experiments (United Nations, 2003) (*European Commission, Regulation (EC) No* 1272/2008 on Classification, Labelling and Packaging of Substances and Mixtures, 2008).

It is therefore clear that the legislative frameworks (*e.g.*, REACH, CLP) and the related regulatory guidance need to be adapted to NAMs. The guidance is essential for the broader application of such alternative methods as it provides clarity and regulatory predictability for the stakeholders involved. The lack of regulatory guidance is one of the

main reasons for the hesitancy of the risk assessors from industry to apply NAMs, which is an issue not only for nanomaterials but also in the broader realm of chemicals (Sewell et al., 2024). Indeed, data produced by using single NAMs is currently not perceived by the regulatory community as sufficient to conclude on most endpoints. Therefore, despite the fact that it is a time-consuming and tedious process, regulatory guidance will need to be revised to also include battery approaches (Krewski et al., 2009).

The tiered structure of the proposed NGRA framework implies sequential NAM battery design, first screening broadly and qualitatively for local or systemic effects before engaging in targeted further testing. However, the focus on the apical systemic toxicity endpoints in the classical animal tests can be an obstacle to the implementation such an approach in the current regulatory practice (Schmeisser et al., 2023). The majority of NAMs provide results at the molecular, genomic, transcriptomic, proteomic, or cellular level, which can be indicators of apical effects at the organism level, but they cannot provide direct evidence of such effects. The focus on mechanistic information on early Molecular Initiating Events or Key Events of an AOP that may or may not result in an apical adverse outcome comes with a level of uncertainty that many regulators feel uncomfortable with (Schmeisser et al., 2023). Therefore, to establish trust in the predictive reliability of such NAMs sufficient *in vivo* validation will be required with the animal study including mechanistic information related to the Molecular Initiating Events or Key Events. However, to fully reproduce a (sub)chronic repeated dose toxicity test a complex battery of different NAMs would be needed. This can add significant additional complexity compared to the classical animal tests and is an issue not only for nanomaterials but also for chemicals (Sewell et al., 2024). Nevertheless, testing insoluble or poorly soluble materials using NAMs can be technically more challenging that testing soluble chemicals. These factors can contribute to cases in which a risk assessment of AdNMs carried out by the application of NAMs may not be less costly than one performed *via* conventional *in vivo* methods (Schmeisser et al., 2023; Sewell et al., 2024). These alternative approaches, however, if designed properly have the potential to be more informative and potentially even more relevant to humans that the *in vivo* experiments. It should be acknowledged in this context that NAMs provide a different type of information than animal studies. In a NGRA framework the apical endpoints need to be redefined and, in some cases, even abandoned (Schmeisser et al., 2023) in favor of an approach that is driven by the analysis of biological mechanisms and toxicological MoA relevant to humans. To ensure this, it is essential to demonstrate that a given NAM is not only robust and reproducible but also that it is biologically relevant and fit for its intended purpose (ICCVAM, 2018).

To achieve its impact the NGRA paradigm should be implemented in a concerted effort from all stakeholders. This will be challenging as safety assessment approaches vary according to the stakeholders involved and due to differences in the underlying regulatory frameworks (*e.g.*, chemicals, food, cosmetics). Therefore, dialogue and continuous collaboration among the regulators, industries, methods developers, and the standardisation community is needed to achieve the level of harmonisation needed to enable this long anticipated change of paradigm.

5. Conclusions

This manuscript proposes a flexible and adaptive NGRA framework that implements an exposure-driven tiered strategy, which iteratively applies NAMs for targeted testing and assessment of AdNMs. This approach includes continuous monitoring of the completeness and quality of the data delivered from these alternative approaches. It enables the integration of the NAMs in IATA, which can help to acquire the information needed for risk assessment in a more efficient and strategic manner, thereby having substantial impact on optimising the cost of testing and on replacing animal experiments. Despite the obvious benefits of applying NAMs for the safety assessment of AdNMs, there are still major challenges to overcome, which are rooted in the way legislation implements the current animal-centric risk assessment paradigm. Therefore, the legislative frameworks (*e.g.*, REACH) and the related regulatory guidance need to be adapted to NAMs. Moreover, dialogue and continuous collaboration among all stakeholders (policy makers, regulators, industries, researchers, the standardisation community) is required to promote a change in the current mindset from risk assessments based on apical effects towards a more mechanistic predictive risk analysis based on NAMs without losing sight of their *in vivo* anchorage.

CRediT authorship contribution statement

Danail Hristozov: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Elena Badetti:** Writing – review & editing. **Paolo Bigini:** Writing – review & editing. **Andrea Brunelli:** Writing – review & editing. **Susan Dekkers:** Writing – review & editing. **Luisa Diomede:** Writing – review & editing. **Shareen H. Doak:** Writing – review & editing. **Wouter Fransman:** Writing – review & editing, Writing – original draft, Conceptualization. **Agnieszka Gajewicz-Skretna:** Writing – review & editing. **Elisa Giubilato:** Writing – review & editing. **Laura Gómez-Cuadrado:** Writing – review & editing. **Roland Grafström:** Writing – review & editing. **Arno C. Gutleb:** Writing – review & editing. **Sabina Halappanavar:** Writing – review & editing. **Roland Hischier:** Writing – review & editing. **Neil Hunt:** Writing – review & editing. **Alberto Katsumiti:** Writing – review & editing, Conceptualization. **Ali Kermanizadeh:** Writing – review & editing. **Antonio Marcomini:** Writing – review & editing. **Elisa Moschini:** Writing – review & editing. **Agnes Oomen:** Writing – review & editing. **Lisa Pizzol:** Writing – review & editing. **Carlos Rumbo:** Writing – review & editing. **Otmar Schmid:** Writing – review & editing, Writing – original draft, Conceptualization. **Neeraj Shandilya:** Writing – review & editing, Methodology. **Vicki Stone:** Writing – review & editing, Writing – original draft. **Stella Stoycheva:** Writing – review & editing. **Tobias Stoeger:** Writing – review & editing, Writing – original draft, Conceptualization. **Blanca Suarez Merino:** Writing – review & editing, Writing – original draft. **Lang Tran:** Writing – review & editing. **Georgia Tsiliki:** Writing – review & editing, Writing – original draft. **Ulla Birgitte Vogel:** Writing – review & editing. **Wendel Wohlleben:** Writing – review & editing, Writing – original draft, Conceptualization. **Alex Zabeo:** Writing – review & editing.

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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