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Review

Systems toxicology to advance human and environmental hazard assessment: A roadmap for advanced materials

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ABSTRACT

Ideally, a Systems Toxicology (ST) approach is aimed at by (eco)toxicologists, i.e. a multidisciplinary area incorporating classical toxicological concepts with omics technologies, and the understanding of this through computational data sciences, chemistry, mathematics, and physics modelling. As outlined in several public reports (e.g. from ECHA-European Chemical Agency and EFSA-European Food Safety Authority), the way forward in the coming years in Europe is to integrate New Approach Methodologies (NAMs) (including omics technologies) into hazard and hence risk assessment (RA). Adverse Outcome Pathways (AOPs) describe a sequence of events in response to stress, from the molecular initiating event until an adverse outcome, which is relevant to RA or regulatory decision-making. AOPs are one of the facilitators to integrate mechanistic data into RA, but it is urgent to increase the inclusion of the vast mechanistic knowledge available, especially for the RA of novel smart and advanced materials (AdMa) with multifunctional characteristics. There are still many challenges to the routine usage of NAMs, e.g. omics-based information. Here, we summarise the current state of the art of ST, the benefits of human and environmental health cross knowledge and the available methods and output. The importance of this area has been highlighted for many years but is even more pressing in the context of AdMa. Furthermore, we outline the challenges and suggest recommendations for future implementation.

Contents

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Nomenclature

AdMa	Advanced Materials.
AI	Artificial Intelligence.
AOPs	Adverse Outcome Pathways.
ATAC-seq	Assay for Transposase Accessible Chromatin sequencing.
EC	Effect Concentration.
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals.
ECHA	European Chemicals Agency.
EFSA	European Food Safety Authority.
EHS	Environmental Health and Safety.
ENMs	Engineered Nanomaterials.
ERA	Environmental Risk Assessment.
FAIR	Findable, Accessible, Interoperable and Re-usable.
GD	Green Deal.
GSEA	Gene Set Enrichment Analysis.
HC	High Content.
HTP	High Throughput.
iPCS	induced Pluripotent Stem Cells.
ISO	International Organization for Standardization.
ITS	Intelligent Testing Strategies.
KBRM	Knowledge-Based Risk Management.
KE	Key Events.
LC	Lethal Concentration.
MoA	Mode of Action.
MIAME	Minimum Information About a Microarray Experiment.
MIE	Molecular Initiating Event.
ML	Machine Learning.
NAMs	New Approach Methodologies.
NMs	Nanomaterials.
NOEC	No Observed Effect Concentration.
OECD	Organisation for Economic Co-operation and Development.
OoC	Organ on a Chip.
PoD	Point of Departure.
RA	Risk Assessment.
REACH	Registration, Evaluation and Authorization of Chemicals.
RNAseq	RNA sequencing.
SSbD	Safe and Sustainable by Design.
SB	Systems Biology.
scRNA-seq	single cell transcriptomics.
SPOT	Sample Preparation from multi-Omics Technologies.
ST	Systems Toxicology.
TDTK	Toxico-Dynamics-Toxico-Kinetics.

Introduction

Systems biology / Systems toxicology

Systems biology (SB) is a holistic approach to understanding complex biological systems, utilizing various high throughput and high content methods delivering a wealth of information. Therefore, mathematical and computational models for the interpretation of large datasets are needed [1]. Omics technologies, such as transcriptomics, proteomics and metabolomics, allow to interrogate a significant fraction of biomolecules of a particular type in an untargeted manner and, hence, play a central role in the field of systems biology. These technologies are particularly effective to comprehensively and quantitatively evaluate the molecular consequences of effectors under study and are the engines for the generation of large datasets at multiple levels of biological organization [2]. Although omics techniques have contributed extensively to medicine, drug development and biotechnology, these methods are not yet routinely applied in toxicology – systems toxicology (ST) – and towards regulatory hazard assessment. A series of workshops and reports have been conducted to elaborate on the promises and challenges of omics techniques in chemical risk assessment [3–8]. The usefulness of omics technologies has clearly been identified within the different tiers of regulatory hazard identification and assessment, e.g. the classification of substances and definition of similarity, the elucidation of their modes of action, the identification of species-specific effects and the demonstration of human health relevance [9]. Single-omics techniques facilitate the identification of

biomarkers of a particular type but are limited when it comes to gaining a systematic understanding of biological mechanisms-of-action (MoA) or adverse outcome pathways (AOP). The integration of multiple omics layers is therefore considered the way forward to allow for a comprehensive coverage of molecular responses and to achieve a holistic biological understanding of how chemical exposure impacts living organisms [10–14]. When applied to toxicology, multilayer omics techniques support the clarification of the chemicals MoA [15,16], with the investigation of dose-dependent alterations being of particular interest. These could also inform on the point of departure (PoD) or dose at which significant key events (KE) of molecular alteration occur, and on possible AOPs [17–19]. Multilayer omics have been used to discover predictive biomarkers of toxicity, both in vivo and in vitro [20], which facilitates the development of alternative models to animal experimentation, following the 3 R principles and recommendations.

Systems biology, with its aim and ability to decipher molecular changes quantitatively and functionally, will therefore provide a central starting point for establishing a holistic ST approach. In line with the above, ST is described as a multidisciplinary concept incorporating classical toxicological concepts and tools with omics technologies and understanding them through computational data sciences, chemistry, mathematics and physics modelling [21]. Further, through integrating in vivo and in vitro toxicological data, the ST approach aims at understanding how adverse effects of toxicants are measured, assessed and understood in a biological context [22]. Understanding the MoA in a biological context allows for a transfer of knowledge across. Due to clear shortcomings and limitations of traditional toxicological approaches, i.e. they are mainly black box type testing [23], a paradigm shift towards ST is the best way forward to offer an acceptable understanding and related protection of human and environmental health from materials released to the surroundings [21].

Human vs Environmental SB/ST

Omics technologies can provide an in-depth picture of the molecular level, raising questions re. how those findings can be translated to the organism, community and the ecosystem level, and, in terms of ecotoxicology, how those results can be extrapolated between species [24,25]. In fact, in reality AOPs (including their quantitative versions) should be regarded as AOP network as common key events are shared. It is well known that response mechanisms, especially responses to stress, are among the most conserved across species. Hence, despite the variety of existing animal species, studies on the mechanisms of response to stress can rely on surrogate species, and knowledge can be transferred (see Fig. 1 for a schematic representation). Thus holistic omics approaches facilitate not only the building of AOPs in their quantitative form and/or in AOP networks, as part of SB/ST.

Human

Available methods (cell, tissue, organ, organism) overview & knowledge output. As stated above, the integration of omics studies with existing in vivo and in vitro testing strategies would foster a comprehensive and in-depth systems toxicology approach. Over the past decades, several molecular high throughput and high content technologies have been established to provide primarily mechanistic information and may therefore help to identify the pathways of toxicity.

Until now, transcriptomics has attracted most of the efforts devoted to the implementation of omics techniques within risk assessment. A main reason for this preference is that microarray platforms and sequencing techniques allow for an extremely high coverage, i.e. up to 95% of genes can be detected in mammals [26]. However, transcriptomics cannot stand alone, as this information corresponds to an indirect relationship between gene expression and phenotype or adverse outcome of a potential toxicant. Proteomics emerge as a very promising diversification, as they also account for translational and even post-translational regulatory effects on the protein levels, i.e. closer to the phenotype. Untargeted proteomics allow for the detection of up to 10,000 proteins, whereas targeted methods may be more limited but allows for more specific insights in sub-proteomes. A third omics level is metabolomics, which represent the entirety of small molecules (< 1500 Da) within a specific cell, organ or organism [27]. Metabolomics are even more heterogenic than the aforementioned omics because they include different levels [13]. They are also typically smallest with respect to their feature size. Of course, the outcome is highly dependent on the specific method being used. An example of a multiomics study, e.g. Quirós et al. [28] analyzing mitochondrial stress upon chemical exposure, showed the detection of 15174 genes (Ion Torrent sequencing approach), 8269 proteins (TMT labeling approach) and 1021 metabolites (iFunnel Q-TOF).

Further interesting approaches involve phosphoproteomics and epigenomics. The first covers the reversible, covalent phosphorylation of proteins that is particularly relevant for intracellular signaling [29], while the latter focuses on the various modifications of DNA and the proteins orchestrating their three-dimensional structure.

The recently established single cell omics techniques, e.g., single cell transcriptomics (scRNA-seq) [30], enable the capturing of complex profiles within singular cells constituting a certain tissue. There are for example various specific scRNA-seq methods [31], but the overarching goal is acquiring the transcriptome of each cell in this tissue, and thus presenting cellular heterogeneity by distinguishing gene expression profiles between different cells, cell types, and even between the same cell type in different cellular states [32]. The difference of increased granularity is highly valuable and has been already successfully applied in cancer research and drug and biomarker discovery (see for example the review of [33] and [34]. Rather recently, scRNA-seq has also been applied in a toxicological setting, where it was used for characterization of zebrafish testes upon TCDD exposure [35]. ATAC-seq (Assay for Transposase Accessible Chromatin sequencing) is another omics technique working at single cell resolution and is designed to measure chromatin accessibility [36]. It has been used in diverse applications, such as the general assessment of the comprehensive accessible chromatin landscapes in various cell types in different tissues and organs [37,38], cancer research to identify tissue-specific chromatin activity of regulatory regions in tumours [39,40], or embryonic development, which is known to feature active chromatin reprogramming [41].

Due to the virtually infinite number of variants for ENMs, NAMs is particularly relevant for nanotoxicology. Omics are important to inform on the mode-of-action of ENMs, which is helpful in the context of grouping and read-across, one of the most commonly used alternative approach for filling data gaps relevant for REACH (Registration, Evaluation and Authorization of Chemicals). The major drawback is the lack of data for well-selected material classes, in such a way that they are useful for grouping and/or read-across.

Different xenobiotics utilize different entrance routes, target different organ systems and, thus, trigger different primary molecular responses. Additionally, a single compound is able to trigger different responses in different organs: direct thyroid toxins such as Propylthiouracil (PTU), for example, will specifically affect the thyroid system through the initiation of hypothyroidism [42]. Other organs, e.g. the liver, will show an unspecific clearance-related response (increased oxidative phosphorylation, biotransformation of xenobiotics, etc.), but might also show severe responses such as hepatotoxicity [43,44]. Various organisations have proposed novel methods, for example EFSA (European Food Safety Authority) outlined several distinct but interacting scientific areas i.e. development

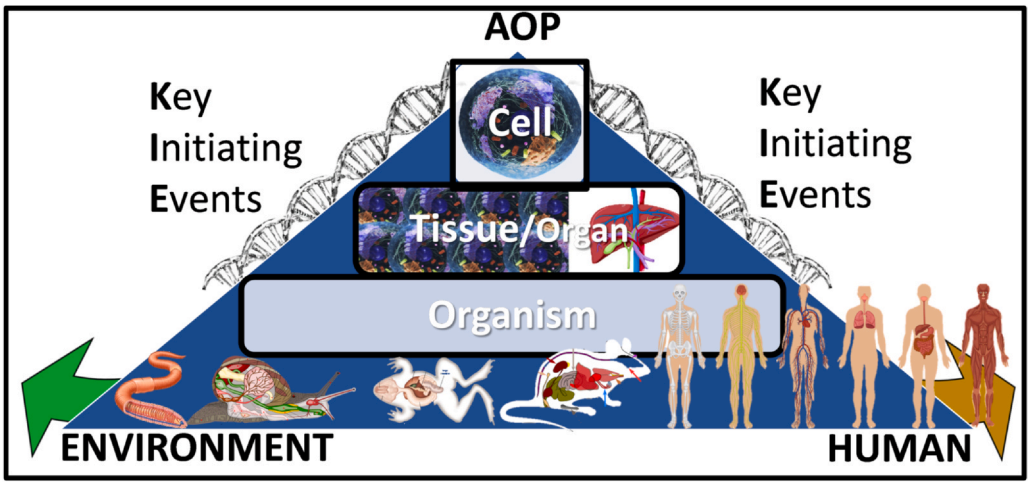

Fig. 1. Representational schematic of the aspects common to various species, namely cell, tissue and organ, up to the non-common and varied forms of life at the organism level. AOPs (Adverse Outcome Pathways) have a series of common events at the cell level – genotypic – up to tissue/organ and become more differentiated at the specific organism level – phenotypical – where human and environmental species diverge.

of additional AOPs/AOP networks, advanced cell culture models including Organ on a chip (OoC) (e.g. based on induced pluripotent stem cells, iPCS), toxicokinetic assessment with a focus on physiological based kinetic modelling (PBK), exposome, human susceptibility, data integration and new concepts in human risk assessment [7]. As to the OoC, microfluidic culture devices represent one of the recently established methods in the search for in vitro human microphysiological systems that can recapitulate organ-level and even multi-organ functions [45].

There are different systems that e.g. recreate organ-level structures or model organ physiology, and by coupling two or more chips, multi-organ systems can be created that mimic whole-body physiology as well as drug distribution and disposition [45].

Another promising approach is to use organoids, which are three-dimensional cell structures that could be derived from many mammalian tissues, both normal and diseased tissue, from adult stem cells and from pluripotent stem cells. Those organoids closely resemble the anatomy and function of the organ of origin and have been shown to mimic complex microenvironments and physiological functions [46–48]. In the field of toxicology, where there are various traditional models, organ-on-chip systems and human organoids are expected to blaze new paths in future research by overcoming current limitations, such as reduction of animal testing and species-to-species extrapolation [49–51].

While omics data (especially multi-omics) derived from multiple organs for the same exposure are still scarce for ENMs, evidence suggests distinct (transcript)omics signatures in different biological systems [52], complicating the extrapolation of the response observed in one organ to other organ types. At the same time, identifying commonalities could guide the development of NAMs based on data-driven grouping of exposures, predictive models and targeted testing strategies. Interestingly, a deeper dive beyond the transcriptomics, and towards the regulatory layer, could reveal commonalities spanning the tree of life, while also showing high levels of specificity to ENM exposures [52]. This, on the other hand, suggests that the commonly applied transcriptomic analyses could be supported by investigation of the regulatory mechanisms behind specific responses. Such mechanisms should obviously be studied in relevant species. For human health studies the experiments are carried out on vertebrate models, however these models are often based on surrogate species e.g. *Mus musculus* (mouse), *Danio rerio* (zebra fish), etc., where metabolic inter-species differences have to be taken into account when attempting to identify universal responses also for humans.

Test designs (concentrations (dose-response, ECx, NOEC, etc.), exposure time, etc.). Experimental design is a critical step that sets boundaries to the data under generation. When investigating the MoA following an exposure to a material, there are some factors to be considered, including the selection of appropriate species, test system setup, exposure doses (general and at target site), and exposure duration, as extensively reviewed, e.g. [7,13,53]. While the selection of these crucial conditions varies based on the goal of the study, there are some specific considerations for omics data. The cytotoxic effects of an exposure in an in vitro system can mask other molecular signals, complicating their assessment. Hence, it is advised to generate omics data with sublethal doses. Similarly, the selection of the doses should be guided by the final aim of the experiment. If an analysis of the dose-response is of interest, the selected doses should be suitable for the modelling while also considering the appropriate selection of models and parameters for omics data [54]. Furthermore, considerations of the time of exposure should be made according to the molecular level of interest as well as the purpose of the experiment. For instance, changes in transcription could be assessed after a few hours of exposure, while epigenetic signals, such as DNA methylation or histone modifications, need a longer time to be established. Similarly, a one-time point setup provides a snapshot into the MoA, while a time-course setup informs on the dynamics of the response and adaptation, guiding the development of potential AOPs. These considerations hold true for studies relevant for both human and environmental health risk.

Environment

Available methods (cell, tissue, organ, organism) overview & knowledge output. To implement a systems toxicology approach for environmental species, e.g. for invertebrates, there are a variety of species for which standard test-guidelines have been standardized, assessing the phenotypical effects. For phenotypic effects, survival and reproduction are among the most common endpoints covered by OECD tests, e.g. [55–59]. These test systems and endpoints are good starting points and represent a basis for linking additional effect levels in order to get a better cover. The various levels of organization, from molecular, cellular and tissues to organ levels, are far less ubiquitous or established than the above mentioned standardized guidelines for phenotypic effects [60]. However, not all standard species have optimized omics methods/techniques implemented or readily available – hence, this is a challenge and a gap where further efforts are required. There are, nevertheless, few key examples where substantial progress has been made that can be

used and further explored, e.g. *Enchytraeus crypticus* [61,62], *Folsomia candida* [63], *Daphnia* sp. [64,65], *Danio rerio* [66], for which the species' genomes have been sequenced and high-throughput tools are available, e.g. transcriptomics. *Enchytraeus crypticus* is an example that progressed significantly towards a multiple level systems toxicology option, besides the currently sequenced genome [61]. It is a species with one of the most complete sets of tools, including transcriptomics[67], metabolomics[68], proteomics [69], epigenetics[70,71], oxidative stress biomarkers[72], histology and immunohistochemistry[73]. Further, there has been a focus on testing NMs and, hence, in this case it also represents a collection of various NMs data, namely for Cu[68,69,74,75–78], Ag [72,73,79,80–82], Ni[83,84], Au[85,86], WCCo[87–89], TiO_2[90,91], ZrO_2[92] or MWCNT[93,94].

When data covers most of the layers, the potential for interpretation is prominent, the proteomics can anchor if the significantly different gene expression is related, the metabolomics can evidence cues and associations, and the observed phenotypic effect can be linked, e.g. to the phenotypic Effect Concentration (EC), e.g. EC10 or EC50 level. Hence, such alternative test methods are highly supported for inclusion in hazard assessment and to meet regulatory preparedness, and will provide information of MoA, KE and AOPs [23]. The present test guidelines are, in many cases, one-point measures of effects, i.e. observing the phenotypic effect at one given time point. However, combining the multi-level omics with further in-depth test designs provides a further advantage and added knowledge to the impacts and mechanisms, e.g. full life cycle[95], OECD extensions[87], multigeneration[77] and multispecies tests [96] can ramp up the interpretation of observed effects and support an approach that is far more ecologically relevant and provide sustainable solutions. Again, these more detailed and advanced tests are available for much fewer species and less data is available.

In terms of ST, human health related studies are more advanced than environmental studies in certain areas, where there is a longer track of experience and implementation, e.g. for medical and pharmaceutical purposes. Nevertheless, there are many lessons that can be integrated both ways across human and environment. While human health studies need surrogate species (e.g. mouse) or extrapolation from in vitro studies, for the environment, multiple relevant species can be tested as full organisms (in vivo) and as in vitro, which allows for actual experimental variety and options that cannot be performed in humans. Because MoA of stressors are some of the most conserved among taxa, cross-species extrapolation provides a highly relevant source of knowledge. Hence, the greatest advantages merge both sources of knowledge and progress in the environmental area and will also benefit human health studies.

For the environment, the *Enchytraeus* species provides an example of how omics and NAMs (New Approach Methodologies) can be linked and provide direct information to the hazards assessment. In a series of papers, it has been shown how alternative test designs combined with omics information at the transcript, proteome and the metabolome levels can provide extensive understanding of the hazard, when these measures are directly related to phenotypic endpoints, e.g. EC10 for reproductive output. The omics information provided AOP relevant insight, linked to the population level effect and the AOPs supported an understanding of other life stages impacts (see [23]).

Test designs (concentrations (dose-response, ECx, NOEC, etc.), exposure time, etc.). Test designs at the standard organism level tests (OECD, ISO) commonly target a full dose response modelling and, hence, an ECx (Effect Concentration) approach is recommended (more doses can balance less replication). A NOEC (No Observed Effect Concentration) approach, fewer test concentrations and more replicates, has significant caveats, as it does not aim at capturing the ECx, but is also an option in the guidelines. Standard OECD guidelines mostly require 1, but sometimes 2, exposure times, e.g. in the OECD for *Eisenia fetida*[97] survival is assessed at day 28 and reproductive output at day 56. The sampling times are in principle adjusted to the species life cycle duration for the target endpoints, although they are rather generic. Extending the test to a full life cycle test type will introduce additional sampling times, e.g. for *Enchytraeus crypticus* [98] it is recommended to sample at 1–13 days (hatching), 13–18 days (growth), 22–25 days (maturity status, survival) and 46 days (reproduction). This kind of testing informs on crucial life stages important for population dynamics and on where key physiological, e.g. endocrine, changes are taking place. It would be highly beneficial to identify how, and which omics are useful at such life stages.

To link to molecular and phenotypic levels, omics test designs should select organism sublethal concentrations (< LC50) for exposure and typically select few ECx, e.g. reproduction EC10, −20, −50, −80. In terms of exposure time, 1–3 dates are selected. Typically for transcriptomics, responses happen immediately after exposure, for proteomics, shortly after and, hence, exposure duration is expected to range between 2 and 3 days to 14–21 days. Naturally, all of these settings can be adjusted to the study aim. If the main question is when a certain mechanism is triggered, then a dedicated time exploration design may be pursued, where the investment is in many sampling times and less concentrations. A full set of many concentrations and many sampling times is not feasible for various reasons (e.g. resources).

Advanced materials (AdMa) including ENM

The materials developed are becoming ever more advanced, from chemicals over nanomaterials to advanced materials (AdMa) or smart materials, the latter two in some cases having a new or enhanced functionality and/or multiple components [99]. Keeping pace with such technological developments is very challenging for hazard assessment oriented regulation, especially if only (previous) black-box testing is available [23]. However, these AdMa bring opportunities for optimizing function while reducing collateral damage as they have new or improved functionalities. In many cases, such functionalities are created at the nanoscale. The importance of these materials is also highlighted in various strategy papers from different strong global economies. For example, the European Green Deal (GD), which is the European Union's new growth strategy [100], outlines the urgency of becoming a sustainable climate neutral and circular economy by 2050 and highlights that the development of AdMa is critical to reaching some of the transitions and goals [101]. AdMa are central to the design of innovative technologies and products, ranging from systems engineering to energy harvesting and energy storage to biomedicine [102].

Timely anticipation of potential human and environmental health related negative effects are, of course, needed. If not obvious from a logical viewpoint, it is clear from the present climate and biodiversity crisis that we need to act much earlier than previously. This requires that regulation keeps pace with innovation. This is generally described by the term "regulatory preparedness", which requires a trustful information exchange between regulators and innovators, supported by various tools for horizon scanning. At the same time, there is the constant need to assess the available tools for hazard, exposure and risk assessment for their fitness. Test methods need to be updated or newly developed and most importantly standardized (e.g. as OECD test guidelines, guidance documents). Finally, existing regulatory frameworks have to be assessed to identify needs for amendment [103]. It is not clear how to deal with the huge complexity of AdMa. For instance multi-component materials bear several challenges but enable new functionalities [104,105]. Such materials include e.g. artificially-architectured materials designed to have material properties beyond those of the

individual components and active materials that are at the boundary between materials and devices. A recent report commissioned by the German Environment Agency identified eight clusters of advanced materials, ranging from (DNA-based) biopolymers to advanced alloys comprising two or more constituents [106]. As these components are often structurally highly ordered and internally synergizing, classifying these materials as simple "mixtures" (as commonly done for cocktails of conventional chemicals) will not suffice. Furthermore, it is unclear whether a new functionality also entails new kind of risks for human and/or environmental.

Challenges for SB/ST of AdMa

The challenges for SB/ST of AdMa are to some extent overlapping with general challenges for SB/ST also to e.g. chemical. Hence, to overcome these challenges for AdMa there must be a continuously con-currently and integrative development in the areas of chemicals and of materials, including the advanced forms.

Availability of methods and standardization

The lack of standard protocols for data generation for analysis and the subsequent lack of verifiable reproducibility are the main obstacles to overcome when considering omics techniques as a validated application in the regulatory process [9]. The absence of best practices for transforming omics results into an easy-to-use framework for regulatory purposes has also been identified as a focal point [6,107]. However, as a starting point, the OECD published reporting frameworks for transcriptomics and metabolomics in regulatory toxicology,[1] and a first analysis framework for the specific application of microarrays and RNA-seq for regulatory agencies has just recently been developed [108].

There are a variety of different approaches for an integrative multi-omics data analysis, for reviews see [109–111]. In general, the methods can be characterised as (i) conceptual integration, meaning that different omics layers are analysed separately and combined later on to infer a comprehensive conclusion, and (ii) statistical integration, which aims at identifying statistical associations spanning different omics-layers and samples. However, each applied method will lead to a large quantity of findings on the molecular level in terms of significantly altered genes, proteins or metabolites. To clearly identify and understand molecular responses and to translate those findings to higher levels of organization, mechanistic insights must be inferred. This is a crucial step in single as well as multi-omics data analysis and is quite frequently accomplished through gene set and pathway enrichment analysis. Therein, previous results from differential gene, protein and/or metabolite analysis are used to identify molecular gene sets or pathways that are up- or down-regulated upon specific conditions, such as chemical exposure. Several dozen single-omics enrichment methods (for a review see [112]) and a few multi-omics pathway enrichment tools are already available, e.g., Paintomics [113] or MutliGSEA [114].

Transcriptomics, proteomics and metabolomics generally capture fast and transient events, which may or may not predict long-term adaptation, depending on the organisms' surplus resources. Hence, it is important to get information of key pathways that lead to long term effects, this, however, requires extensive testing and knowledge transfer. On the other hand, epigenetic signals occurring at the chromatin level are slower to establish, but remain measurable in the biological system even long after the exposure has been removed [115]. Scala et al. [115] have implemented a network-based multi-omics model integrating genome-wide DNA methylation, mRNA and miRNA transcriptomics in order to characterise the MoA of a collection of 10 carbon nanomaterials [115]. By following both the patterns of transcriptional adaptation and cytokine secretion, Kinaret et al. [116] described specific abilities of different types of carbon nanotubes to polarise macrophages into M1 or M2. More recently, multi-omics data have also been used to characterise the transcriptional and epigenetic MoA of multi-walled carbon nanotubes on a model of human macrophages in vitro [117].

The great potential of the vast amount of publicly available omics data can outweigh the lack of uniformity of the data so far, provided there is a clear protocol for data analysis. At first glance, it might seem daunting to approach the countless data resources from individual omics experiments given the lack of format and annotation uniformity. However, this metadata would become of large value if appropriate storage (databases) and harmonization procedures can be assigned to it. For example, TG-Gates is a toxicogenomics database that stores gene expression data from rat liver and kidney samples (in vivo) and rat and human hepatocytes (in vitro) of 170 drugs (https://toxico.nibiohn.go.jp/english/index.html).

In the absence of broad, robust, and uniform reference data sets, collection of public data is the common solution for modelling approaches that require large amounts of data [118]. At the same time, the curation and harmonization of public data is a massive undertaking that requires careful evaluation of each data set, often accompanied with insufficient metadata. This, however, is not a sustainable solution, as the amount of data keeps rising exponentially, nor does it always result in a full capture of nanospecific metadata and hence this causes an insufficient interoperability [119]. Instead, robust standards and guidelines for data generation and reporting are needed to generate data that would be FAIR (findable, accessible, interoperable and re-usable) from the start. There are currently vast community-defined suggestions for FAIR data in the context of AdMa [119–121]. While these guidelines and suggestions promote complete reporting that eases data reuse and interoperability, they do not ensure high data quality that arises from experimental design and execution [122]. For omics data it is obviously important that this is tightly linked to already established reporting ways, e.g. as required when data are published in journals (MIAME). Tackling this issue requires the establishment of optimized standard operating procedures as well as careful reporting and justification of the experimental design and its execution.

For the environment, a clear challenge for ST implementation is the lack of harmonization of available methods for the sub-cellular level studies. The standardization of guidelines by organizations like OECD or ISO promotes not only repeatability around the world, but also the building of a data bank – standardized methods are well accepted and performed much more often than non-standardized ones [123]. Further, there is a need to establish quality criteria to ensure minimum acceptable ranges within the scientific community based on larger samples and not the individual labs. Hence, among the challenges of implementing a ST are the lack of recognized method standardization and quality criteria. Nevertheless, the myriad of available alternatives (e.g. peer-reviewed published studies) are an opportunity to build on, some of the methods are actually very consolidated and could be ready for a standardization process.

Data completeness (studies where all levels of organization are included)

Eco-/Toxicologic data sets that cover molecular responses as well as cellular and even more complex organizational levels are extremely rare. When focusing on omics data sets alone, there are only a handful of data sets that cover multiple omics layers within, e.g. [28]. If the necessity of paired samples can or should be neglected, studies on different omics layers, but with an identical study design, can be combined. However, omics data sets are distributed over

[1] https://www.oecd.org/chemicalsafety/testing/omics.htm

several repositories, and typically there are no links between corresponding data sets [13]. Furthermore, the annotation with meaningful metadata is still a major concern and hampers the identification of potential case study data sets. However, efforts to identify suitable omics data sets for data integration efforts have been made by searching publicly available omics repositories in a general fashion [124] or with a chemical-driven focus [125].

Similarly, for environmental studies, one of the major hurdles comes from the completeness of the data set for a particular stressor. As mentioned, there are also fewer species where all tools are ready to implement. Hence, very few case studies have a full data set available, i.e. multi-omics plus multi-organism endpoints (one example includes the dataset built for *E. crypticus* exposed to CuONM [68,69,74,75–78]).

Required resources

Which omics layers are necessary to sufficiently detect pathway perturbation at the molecular level is not easily answered or universal. It was, however, recommended to select those layers that maximize the coverage for the pathways in focus. From a current point of view, it is beneficial to include both transcriptomics and proteomics layer. This might be contradicting at first sight, since they provide comparable insights on a molecular level, however, their integration mutually improved the detection of response pathways [13]. Metabolomics provide a totally different point of view in terms of molecules and response time scales that are targeted. Phosphoproteomics might be an additional layer that provides valuable information with reference to signaling interrogations.

There is a large effort underlying the wide testing coverage; if one considers that for a certain NM a full data set should be obtained, this means that a minimum coverage should include the screening of omics (transcript-, metabol-, proteomics) and organism endpoints (e.g. for the environment the hatching success, growth, survival, reproduction, multigeneration). This represents a high resource demand, both in terms of time and finances and requires a high level of expertise to perform and explore all data results.

A potential turning point is the establishment of methods to derive multiple omics data sets from a common sample like SPOT (sample preparation from multi-omics technologies), which helps to alleviate financial demands and the amount of needed resources through the combined extraction of transcriptome, metabolome and proteome [126].

Exposure times vs level of organization

Omics refer to several layers of events and provide information on features that can either refer to transcriptomics, metabolomics or proteomics. Each omics layer covers different time scales, with metabolomics showing the widest range, starting from seconds after exposure to multiple hours or even days. Which exact time points to choose for aligning multiple omics layers in a multi-omics data integration is also highly uncertain and a serious issue, since it depends on many factors such as the exposure scenario, the response that should be measured, which omics layer to include and the number of timepoints that can be addressed. It is well known that most observed adverse outcomes are the result of a cascade of events, e.g. a certain gene regulates a protein synthesis, and this causes reduced reproduction. The test design will aim to capture the window of events, i.e. for genes shortly after exposure, for organisms later and for proteins in between, generically speaking. As outlined in the AOPs guideline from OECD[127], a key initiating event (KIE) and a series of follow up events occur not only at different biological levels (cell, tissue, organ, etc.), but also in a time sequence (early, later). Although all of this is consensual, there are some technical aspects to consider - time influences toxicity. Hence, and because most exposure tests run under static conditions, the exposure may change (be reduced) throughout the test duration. This means that there is awareness of the implications different exposure times may have, especially if the material has a fast half-life. This is not a new challenge, TDTK (Toxico-Dynamics-Toxico-Kinetics) approaches aim to tackle some of these challenges.

Integration of knowledge & data analysis

There have been several publications in recent years that discuss the utilization of multiple omics layers in a toxicological setting [14,128,129]. The first, and probably most important, requirement is the consideration of a multi-omics compatible study design right from the beginning. Crucial aspects like sampling strategies, sampling points and required omics layer have a direct influence on the applicable integration method. Any future adaption significantly hampers the power of the data integration approach and should be avoided [13].

The selection of which respective omics layers to use is not only a financially, but also a toxicologically motivated one. As previously discussed, each different omics layer adds distinct levels of information. Another important aspect deals with the measurement strategy in each omics layer: targeted approaches tend to be more accurate, but might yield only a small snapshot of results, whereas untargeted approaches can produce tens of thousands of data points and offer the possibility of detecting previously unknown findings, such as the detection of new splice variants in RNA-seq data. When using highly unbalanced data sets in statistical integration methods, specific findings in small data sets tend to get buried by waves of information originating from a large omics data set. This makes a rigorous filtering for each omics layer right at the beginning inevitable. Further aspects embrace proper data normalization [130] and preparation prior to the integration procedure [131]. Large datasets are the common nominator in such a field and progress towards AI (Artificial Intelligence) methods employing for instance machine learning techniques are obvious [132] although currently still used seldom.

Model vs non-Model organisms

The advent of high-throughput techniques blazed the way for gaining molecular insights with an unprecedented resolution clearly facilitating their ever-widening expansion into other fields such as ecosystem research and ecotoxicology. Especially in those fields, studies are oftentimes conducted on non-model organisms. The definition of model and non-model organisms is subject dependent, but generally speaking, non-model organisms are poorly studied or not at all [133]. The lack of a 'ground truth', such as genome annotation or a reference proteome, substantially exacerbates reliable and meaningful data analysis, since gained results cannot be compared to their original state, thus hampering the full potential of the omics experiment. To overcome those obstacles, huge efforts in terms of an adaptation of the experimental setting as well as the subsequent data analysis must be made. The precise technical challenges are highly dependent on the specific technique to be applied. For transcriptomics studies, meaningful biological insights are commonly derived from lists of genes found to be differentially expressed. Such an analysis depends crucially on the availability of accurate gene and transcriptome annotations, but the reliable inference of functionally relevant information is a major challenge in ecological and evolutionary genomics research [134]. Two different approaches have usually been applied: a *de novo* assembly from sequenced reads alone, e.g. [135], and a guided assembly by utilizing a reference annotation from a (closely) related species, see e.g. [136]. Both methods bear some uncertainties with reference to the genomic divergence and accuracy of the reference genome and the

amount and quality of a *de novo* transcriptome assembly [137]. A third option, direct mapping of sequencing reads to a related genome reference, is becoming more and more popular through the ever-widening availability of genomes and the increasing robustness of mapping tools. Similar challenges must be faced in proteomics. The main hurdle, according to [138], to open up the field of untargeted proteomics for an unprecedented use in non-model organisms is an accurately annotated search space to find the measured peptides. They propose to either (i) apply additional effort to assemble a *de novo* genome or (ii) use RNA-seq to generate predicted protein sequences. Such eminent drawbacks are far less prominent in metabolomics, where the major challenge is the detection and identification of novel compounds, so-called 'unknown unknowns' [139].

Advanced materials

To understand the toxicology of AdMa and to assess the specific challenges that come with the distinct physicochemical properties of AdMa, it is important to first evaluate what aspects of the materials that can be linked to risk, that is linked via the exposure and hazard part. It is also critical to understand whether the generated wealth of knowledge on the toxicity of ENMs can be used for all AdMa. It is, however, well recognised that AdMa may not be covered by the definitions of nanomaterials and pose different characterisation and testing challenges [104]. In general terms, four key aspects can be identified, as further substantiated below:

Toxicity testing of AdMa

Increasing innovation in nanotechnology enables the development of AdMa capable of performing specified tasks, so-called smart NMs [104,140]. Some of these AdMa are designed to undergo changes in their physicochemical properties in response to a specific stimulus. Examples of such AdMa include light-driven molecular motors and smart nano-pesticides. For example, it is now possible to design nano-pesticides that minimize biocidal leaching. Thus, they prevent bioaccumulation in non-targeted organisms, which is a drawback of traditional pesticides [141]. The nano-pesticides can be designed to target specific tissues in plants and remain passive or be activated by stimuli such as pH or a specific enzyme [142–144], leading to e.g. triggered and controlled cargo release in the target organisms. In all cases, the key question is what drives the interactions of AdMa with cells and biomolecules in organisms, and what are the toxicological consequences – this presents many challenges.

There are few medical-oriented toxicological studies in which the effect of activated smart AdMa has been investigated: such tests are still uncommon [145,146]. The question is whether and to what extent the response to a stimulus must be considered in toxicological studies. Risk assessment based on the passive form of smart AdMa is insufficient to assess their risk [104]. For example, smart AdMa with different sizes and shapes may respond similarly to a stimulus, or some fractions of a smart AdMa may unintentionally respond to an uncontrolled stimulus to induce toxicity in non-target organisms. The controlled functionality of smart AdMa therefore adds another level of complexity to the toxicological studies of AdMa. Further, some AdMa may have intentional targets, which become un-intentional in other organisms or may, indeed, interact differently within other organisms. Compared to present day test guidelines, which mainly focus on phenotypic effect, multi-omics testing shows great promise.

Dynamics of the toxicity profile: impact of the complex nature of AdMa on the fate and toxicity profile

It has been shown that fate of ENMs influences their entry pathways in organisms and their toxicity. In line with this, considerable effort has been put in understanding the fate and subsequent toxicity of stable and of soluble ENMs and to differentiate between the toxicity of particulate and ionic forms of ENMs [147]. These issues are the same for AdMa. For an AdMa composed of quickly dissolving and stable ENM components, linking the fate to biological and ecological effects might be complicated. The key issue is: What will cause the effects? One key issue is whether enhanced toxicity occurs compared to the toxicity of the constituents, which means $1 + 1 > 2$ (synergistic effect), or no toxicity takes place, which means $1 + 1 < 2$ (antagonistic effect) in the case of multi-elements AdMa. An illustrative example in this respect is provided by [148], investigating the toxicity of a nanostructure composed of ZnO with Ag ENMs on its surface to *Daphnia magna*. The toxicity of ZnO and Ag ENMs as single components along with their nanostructure (ZnO/Ag) were tested. The authors concluded that neither the toxicity of a mixture of ZnO and Ag nor the toxicity of ZnO/Ag nanostructure can be predicted based on the toxicity of their components. The toxicity of the nanostructure, however, was higher than predicted based on the toxicity of the individual components. This example clearly illustrates that it is complicated to link the observed toxicity of an AdMa to any of the properties of its components. Detailed fate and effect assessment of newly generated AdMa seems needed as experimental data, let alone predictive fate and effect models, are lacking.

Influential factors

One of the main advantages of AdMa is the possibility to design them with different physicochemical properties, such as size, shape, aspect ratio, hydrophobicity, functionality, etc. Systematic studies have been performed to test the influence of ENM physicochemical properties on their toxicity [149]. The findings have confirmed that chemical composition is not the only factor influencing the toxicity of ENMs, but other physicochemical properties can play important roles as well. This will also be true for other AdMa, where the same materials with different organisational form may induce diverse toxic responses. For example, the multi-elemental and functional properties change their uptake pathways and interactions with cells and, subsequently, their toxicity to organisms.

Comprehensive characterization of AdMa, therefore, is required to obtain information on material characteristics such as size distributions, surface charge, shape, surface area, impurities, etc. in AdMa and, in particular, in relation to intended functionality. This information might not be sufficient for investigating the toxicity of some AdMa that are multi-constituents or for different ENMs in one nanocomposite and/or composite materials that can be activated upon stimulation inside or outside an organism. It should also be considered how to test multifunctional AdMa that are triggered by internal or external stimuli. The question could be whether such triggers should be included in the test design. Although improving, many toxicological studies still do not report a detailed characterization of tested AdMa even for single element ENMs, partly due to the limitations in analytical capability and availability. This, again, illustrates that the omics approach combined with priory structural and chemicals should increase the knowledge potential.

Protein coronas and toxicokinetics of AdMa

It is well known that when ENMs enter the body of an organism, the surfaces of the particles are rapidly covered by biomolecules, such as proteins, forming the so-called "protein corona" [150]. The protein corona consists of proteins, which absorb to an ENM for a few minutes up to several hours, where low-affinity high-abundance proteins may initially adsorb to the surface of NMs and are replaced over time by lower abundant proteins with higher affinity [150]. The formation of a protein corona is dictated by the physicochemical properties of ENMs, such as size, aspect ratio, surface charge and chemical composition.

For AdMa, the formation and evolution of a protein corona is also controlled by the physicochemical properties of the particles. Sorption of biomolecules on the surface of AdMa confers a new biological identity to the materials, which influences the biological fate and biodistribution of the particles in various organs, tissues and cells in organisms. The generated data on the biological fate of protein coated ENMs can help to understand the biodistribution of AdMa, and this will be supported by an omics-based hazard approach. For example, it is known that adsorption of proteins facilitates the recognition and uptake of particles by immune cells, which are involved in the uptake and metabolism of foreign particulates [151,152]. This knowledge can also be applied to AdMa. However, some physichochemical properties of AdMa, such as the multi-elemental composition and implementation of switchable properties in some AdMa, which imparts dynamic properties to the ENMs, will add another dimension to the biological fate of AdMa, consequently complicating the prediction of their biodistribution and hazard. Clearly, more studies are still needed to understand the elimination pathway of AdMa from different model organisms with toxicological purposes.

Recommendations

Test setup

Human health (Toxicology) & environment (Ecotoxicology)

To produce good-quality data that provides meaningful insight into the MoA of exposures, a multi-omics design should be accounted for from the beginning. Although this requires major investments in terms of cost and time, the value of such unified data sets cannot be matched by integrating data from various sources and experimental designs. At the same time, compromises are often inevitable. The high cost of omics data generation together with precious exposure materials and models are typically a limiting factor for the setup. Hence, it is of utmost importance to define the purpose of the experiment ahead of time to understand the best approach for the question at hand. This builds the foundation and defines limits for the future use of the data. The selection of the exposure model, dosage, timing and the omics layers to be screened are fully dependent on the goal of the analysis and as a result may vary greatly. For instance, assessing the transcriptome together with methylome may provide insight into gene regulation or long-term adaptation, but does not tell whether the changes correspond to the protein levels. Similar considerations account for other omics techniques applied. Different methods gain different insights with quite distinct feature sizes. While a comprehensive coverage of biomolecules in one omics layer may be desirable for pathway detection, it may be debilitating for a multi-omics integration with considerably smaller omics layers. It is therefore worth considering whether a more targeted analysis would be sufficient instead. Otherwise, a rigorous filtering prior to the integration is key. The decision as to which omics methods should be used is also related to the organism that is assessed. For example, for non-model organisms, targeted approaches, wherever possible, clearly facilitate the subsequent data analysis workflow due to its inherent ‘ground-truth’.

However, each choice made during the test setup, from the experimental design to analysis and modelling, should be carefully justified and reported in the context of the research question.

In the transcriptomics layer, a significant reduction in financial cost, complexity in sample preparation, and subsequent bioinformatic analysis is achievable through the application of the TempO-Seq method [153] which comes at the cost of significantly reduced comprehensiveness. This platform allows for targeted transcriptomic analysis to perform high-throughput gene expression analysis on a sentinel gene set S1500 + [154]. In a comparison of 43 chemicals falling into five distinct modes-of-actions, TempO-Seq and established approaches for measuring the genome-wide transcriptome showed consistent results [155].

Detailed reporting of the metadata is fundamental. A comprehensive reporting framework (RF) is available from OECD [67], where guidance is provided on the experimental design, technical measurements, data analysis, etc. Guidelines are available for metabolomics [156] and transcriptomics [123], but have been harmonized wherever possible to facilitate that other omics layers can follow most of these recommendations. This also supports reproducibility and replicability as well as the reuse and integration of data, which, in turn, helps to build confidence towards omics-based evidence in general.

The actual data analysis and, in terms of multi-omics, integration of methods that comply with the demands of regulatory agencies are still in their infancy. There is a discrepancy between current multi-omics study applications, starting from the actual study design, including the decision of which omics layers to use, the data integration method to be applied and, most prominently, how those results can be transferred/transformed into an appropriate format that can be assessed by authorities. Following an ECETOC workshop on regulatory acceptance for the application of omics data in 2016, it has been suggested that the ever-evolving nature of omics techniques and analysis workflows are best dealt with by applying extensive reporting framework on data generation, processing, and analysis, rather than using overly prescriptive and inflexible protocols for the collection and analysis [9157,158]. Therefore, to ensure reproducibility and interoperability, this will be an inevitable step forward. However, a ’reproducibility crisis’ has recently been diagnosed [159], criticizing the degree of reproducibility in biomedical research [160]. Particularly in regulatory aspects, reproducibility becomes a sheer necessity [161]. Of course, this holds true for all aspects along the regulatory decision-making process and is equivalently important when omics data analysis is brought into the equation. The analysis of high throughput sequencing data is a complex multi-step process. Most of those steps can be achieved by a competing set of publicly available tools for which dozens of parameters can be fine-tuned. Therefore, this landscape of options and opportunities paves the way for critical reproducibility and consistency issues. To cope with the demands of reproducibility during the data analysis, several precautions should be taken: workflow management systems, e.g. uap [162], enable researchers to keep track of the consistency and integrity of the analysis. Furthermore, working environments containing libraries and tools needed for the analysis should also be reproducible. The widely used open-source package management system conda[2] provides basic and easy-to-use functionalities for this task. Containerization of the entire analysis workflow, together with all requirements in terms of software, dependencies, libraries, etc., are currently the best solution to achieve an individually tailored, reproducible and transportable data analysis. Frameworks like Docker [163] (or Singularity [164]), can be installed on any machine and, hence, ensure identical results on different devices.

A suite of “best practices” can be followed to obtain the most and best information. For example, in the context of an AOP framework in ecotoxicology, a tiered approach is recommended. A kind of reversed engineering is planned with the phenotypical adverse outcome being assessed first. The importance of this relates partly to the fact that the knowledge of the dose-response and effect concentrations (ECx) are necessary to select sub-lethal doses for the molecular studies. On the other hand, the aim is to associate and link the mechanisms to effects of regulatory concern [127], i.e. a phenotypical adverse outcome, e.g. reproduction. We here propose the following (see Fig. 2) and in this order:

[2] https://docs.conda.io/en/latest

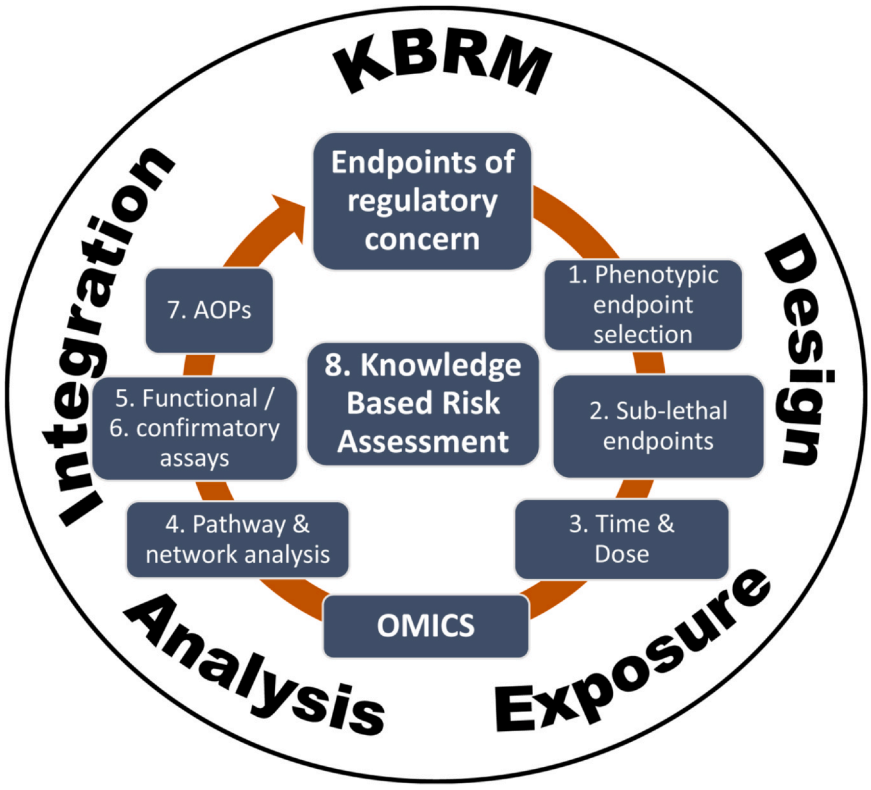

Fig. 2. Schematic representation for the strategy to achieve a knowledge-based risk assessment, including omics, and via a stepwise process: 1) Design, 2) Exposure, 3) Analysis and 4) Integration of data, towards a knowledge-based risk management (KBRM).

1. Assess phenotypic parameters of regulatory concern (survival, growth, reproduction, etc.).
2. A) Select phenotypic chronic endpoint, e.g. reproduction (lethal effect levels often surpass the window of interest for mechanisms/regulation). B) Select sub-lethal ECx or a range, e.g. EC10/20 & EC50, preferably have a dose-response.
3. Consider the relevant exposure times (t) for the omics: $t_{transcriptomics} < t_{proteomics}$. A previous dedicated exploration of exposure duration study is recommended to obtain a confidence interval.
4. Use data analysis best practices, including pathway and network analysis.
5. Use the obtained results to generate hypotheses and move to ITS (Intelligent Testing Strategies) that can target functional studies and potentiate confirmation.
6. Assess relevant functional cellular endpoints based on previous information.
7. Translate the various layers onto an AOP framework.
8. Contribute to Knowledge Based Environmental Risk Assessment (KB-ERA), i.e. integrate mechanistic information in RA.

Exposure route prioritization. To date, omics data derived from ENM exposures is highly biased towards the respiratory system [118]. Of all possible uptake routes into the human body, inhalation is of crucial interest for risk assessment of ENMs because this is the most critical entry point [165]. AdMa materials may also have other uptake routes, intentionally or unintentionally. Traditional toxicology relies on in vivo studies to this end. Currently, the risk assessment of NMs implies case-by-case testing, which would require too much time and major financial costs, besides a high demand for test animals. The rapid increase in new-marketed AdMa pushes the possibilities of this procedure to the limits. Typically, hundreds of animals are used over several years to evaluate each material variation. Thus, large investments are needed to ensure the safety of these new materials. Current regulatory approaches are overwhelmed by the rapid development of new generations of ENMs. Hence, a way forward is to understand the mechanisms-of-action, as this will allow to transfer knowledge from one material to another.

Omics, and eventually multi-omics techniques, will be an essential toolkit to derive modes- and mechanisms-of-action that could be further used in grouping approaches to facilitate risk assessment [166]. The European Food Safety Authority recently published a guidance document for grouping chemicals based on available information, such as MoA aiming at human risk assessment [167].

FAIR complying data

It is important that research in the omics field is performed following the standards towards FAIR (findable, accessible, interoperable and re-usable) data. It enables ground-breaking steps in the nanosafety domain, specifically in the areas of hazard and risk assessment and development of AOPs, among others. Although the global nanosafety community is in many places in an advanced stage in terms of FAIR data, omics datasets need to follow clear rules for their sustainable implementation, improving FAIRness and data reuse. These activities within the nanosafety community aim at supporting researchers in the nano-EHS field to commit in sharing data according to the FAIR principles, while profiting from the data reuse.

Concretely, it is fundamental that resulting raw data from omics experiments is uploaded into repositories (e.g. the PRIDE Archive for proteomics datasets), while the meta-data is clearly described using the templates [168] provided within the eNanoMapper [169,170] database.

Risk assessment of AdMa

Classification

It is well recognised, also by the regulators, that the current risk assessment does not adequately cover the AdMa [104,171,172]. An important step to be taken before developing and implementing non-animal alternative test methods is the classification of advanced materials for toxicological and risk assessment purposes. This classification is a key requirement for any testing strategies in order to enable optimal use of data generated by non-animal testing alternatives by, for instance, grouping of AdMa and read-across of data from similar materials. After all, given the large number of AdMa, individual toxicity testing is not feasible. Developing a strategy to classify AdMa into different groups based on their physicochemical properties that are likely to involve similar toxicology pathways could be a potential solution to efficiently initiate and implement non-animal testing approaches. The key issue here is basically to categorize AdMa based on their physicochemical properties. Such an initial classification was, for instance, developed by the German Environment Agency [103] and could be modified to consider the possible toxicological pathways of an AdMa. Chemical composition, functionality and the volume-specific surface area can be proposed as the main particle properties for clustering AdMa. The benefits of such a classification are to: (1) enable differentiation between AdMa based on their properties that might induce specific hazards, (2) provide measurable criteria that can be integrated into toxicological concepts, (3) provide a framework to categorize the increasing number of AdMa into few identifiable classes, where AdMa within each class potentially induce toxicity through the same pathway and thereby facilitating evaluations, and (4) facilitate a faster pathway to identify the hazard of new AdMa.

Hazard assessment

Recent recommendations for the testing of a group of advanced nanobiomaterials (NBM) [173] envisage three main lines of action for hazard assessment: 1) Mode of action: a tiered approach testing prioritizing the most affected species, 2) Long-term exposure testing: based on standard test guidelines, but increasing exposure duration and increasing sampling detail, and 3) New approach methodologies (NAM): beyond standard testing, exploring the mechanisms via alternative testing, e.g. omics.

Bringing the three main lines of action for hazard assessment of AdMa (as depicted above) one step further into the current risk assessment procedures, the obvious question pops up of whether current toxicological guidelines can cover advanced materials. The Organization for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO) have for many years performed extensive work on developing toxicological test guidelines. These test guidelines support the harmonization across different labs and support the generation of reliable data. Such data are essential as the basics of current risk assessment procedures. The test guidelines are traditionally developed for soluble chemicals and not specifically for particulate materials. This challenge has already been faced for NMs, and in this respect the question was raised regarding the adequacy of these test guidelines for the purpose of assessment of the hazards of NMs. Recently, OECD Guidance [174] was developed, which addresses aquatic and sediment testing of NMs. Considerable progress has been made through the consensus process in the development of Guidance Document 317. Nevertheless, some challenges still need to be resolved, and the evolution in advanced materials science to generate different materials might further challenge the adequateness and fit-for-purpose of documents such as GD 317. As such, considering nano-specific properties in the future version of GD 317 might not be the only requirement anymore, but one should also evaluate the requirement to consider e.g. the response of smart NMs to a stimulus or their multifunctional ability. For example, the current technical guidance disregard the particles, ions and other components that can be released from multi-element advanced materials or disregard the dynamic changes in the structure or functionality of smart advanced materials.

Integration of ST

Many of the challenges related to risk assessment of AdMa can be supported by an ST approach using non animal test methods, both in respect to current ENM and future AdMa. For example, an ST approach can inform on the biological mode of action of a material, e.g. by identifying key event or AOPs, which will allow for a better understanding of the hazards and how to mitigate such hazards. Such information can be utilised to develop better and more sustainable materials, i.e. safe and sustainable by design (SSbD). For the environment, a clear example of how to enhance the environmental risk assessment via combining alternative test strategies for a standard test species with comprehensive omics evaluation can be directly seen in e.g. [90,91]. These partly deal with safer by design (by doping) and partly to understand the biological action.

Understanding biological action of AdMa also allow for a transfer of knowledge across various ENMs and AdMa, similarly to the classification (see above) and read-across approach that now is recommended based on physicochemical properties [171], and the related [175,176]. Understanding MoA will particularly be valuable to gain information across species, since many fundamental stress mechanisms are highly conserved. For the environmental compartment, this should be linked to the extensive ecological knowledge available (e.g. through the European Biodiversity Atlas, https://www.eea.europa.eu) to provide informed and sustainable decisions. For human health, such information should be linked to the extensive human biological knowledge (e.g. through the Human Cell Atlas, https://www.humancellatlas.org/) to provide informed and safe decisions.

AOPs

A promising alternative approach in risk assessment is the concept of the Adverse Outcome Pathway (AOP). It gathers all mechanistic knowledge of a substance at different biological levels, resulting in a robust framework to contribute to regulatory decision processes [177,178]. AOPs aim to describe the substance mode of action as a series of key events [179,180], which is fundamental in the development of alternative test strategies. System biology and omics technologies are valuable methods to evaluate the key events preceding the adverse outcome.

While omics data has been used to support the generation of AOPs [181–184], further integration of these concepts present great potential for the development of NAMs. A recent study by Saarimäki et al. [185] showed how molecular annotation of KEs/AOPs supports chemical safety assessment and biomarker discovery and successfully identifies relevant adverse outcomes of chemicals, both in vivo and in vitro. This further guides the interpretation of omics-based (in vitro) evidence and helps to translate it to robust NAMs while bringing it a step closer to regulatory acceptance. Although these approaches depend on the robustness and coverage of established AOPs, the systematic links between molecular alterations and KEs/AOPs can also support the generation of novel AOPs.

At first such information may be considered only additional information to the risk assessment, however, by developing consistent and agreed frameworks for data collection, processing, interpretation, storage and curation ways to integrate the different omic layers in a reliable and repeatable way will allow for a direct implementation in the regulatory assessment [3186]. Also, for the NAMs, they may initially be considered additional information, but as we become better at integrating this into an ecological context, this may be directly implemented in the regulatory assessment

[186]. In line with this, both System Biology Approaches and NAM have been recommended and supported by regulatory authorities, e.g. ECHA (European Chemicals Agency) [187] and EFSA [3186,188]. The use of omics in RA has been thoroughly considered, and its importance in reducing vertebrate animal testing is well recognized as well as its potential in reducing in the cost per chemical. A validation step to anchor the phenotypic effects to omics is essential prior to full implementation. As outlined above, it is clear that NAMs provide information that allows for a reduction in animal testing, but NAMs that consider the general biology of the tested organisms/population, e.g. various life stages or multigenerational aspects, will seriously enhance the knowledge and understanding of the hazard and related risk.

Risk assessors need data from recognized sources and in a format that can facilitate their interpretation and introduction onto RA. The AOP "describes a sequence of events commencing with initial interaction(s) of a stressor with a biomolecule within an organism that causes a perturbation in its biology (i.e., molecular initiating event, MIE), which can progress through a dependent series of intermediate key events (KEs) and culminate in an adverse outcome (AO) considered relevant to risk assessment or regulatory decision-making" [189]. AOPs represent one of the tools and methods of integrating mechanistic data onto RA.

A roadmap

Timeframes for the development and implementation of topics such as chemical modelling [190] and generation of omics data [191] have been presented previously, and thorough consideration of the benefits for risk assessment have been included, e.g. [3]. Here, we outline some main highlights expected for the next 5–10 years (see Fig. 3) based on current progress.

5 years

As outlined in several public reports, e.g. from ECHA and EFSA in Europe [186], the way forward in the coming years is to integrate the NAMs and the omics technologies, first as supporting evidence and later as a full part of the risk assessment. Key issues here are the general agreement and acceptance of the tool, which should be consistent, reliable and relevant. To mitigate this a system toxicology database that integrate the omics data would be critical to actually apply the knowledge to use, e.g., for development of AOPs that are simple and implementable for regulatory decision making. Further, very large datasets are often seen as an associated burden due to the complexity and the multiple possible ways to analyse these. However, machine learning techniques will become much more integrated in the process of complex data, if these ML techniques are automated and formalised to the required task, they will greatly relief this burden. In line with this, the establishment of bioinformatic workflows on high-dimensional (multi-)omics data to derive lower dimensional and interpretable representations such as signatures or pathways should receive continuous attention. The validation of sentinel gene sets is expected, and these can be used for high-throughput transcriptomics screening in a toxicology setting, targeting regulatory application. This will also decrease the time consumption, costs, and interpretability. These findings will be linked to AOPs. However, a focus point should also be linking these approaches to a broader biological/ecological context and knowledge. For example, extensive species biological and ecological biodiversity knowledge exists outside the roam of risk assessment related research, this can identify empty spots that need to be prioritized in the next years. Such knowledge integration can provide definite input to make the assessment more sustainable. Initiatives for this are already on the way, but much more research should be performed in this area – there should, in fact, be flagship programmes across various regions. The assessment of longer-term exposure of materials and AdMa, e.g. full life cycle, multigenerational, is subject to several initiatives towards the standardization and regulatory inclusion. Many environmentally relevant species will become omics models and have available and optimized methods.

10 years

As with 'classical' chemicals, where regulatory agencies like EFSA are currently developing approaches for assessing the combined exposure to multiple contaminants, risk assessment of mixtures containing multiple ENMs, of AdMa and/or of compounds with low molecular weight will be highly challenging to assess given the sheer complexity. Detailed guidance documents and assessment strategies will be required and made available to consider those combinations in the regulatory process. Strategies on grouping and read-across of materials for the cumulative assessment of groups of materials will be routinely established and used, gaining experience on their merits and warranting optimal use of the scarcely available data.

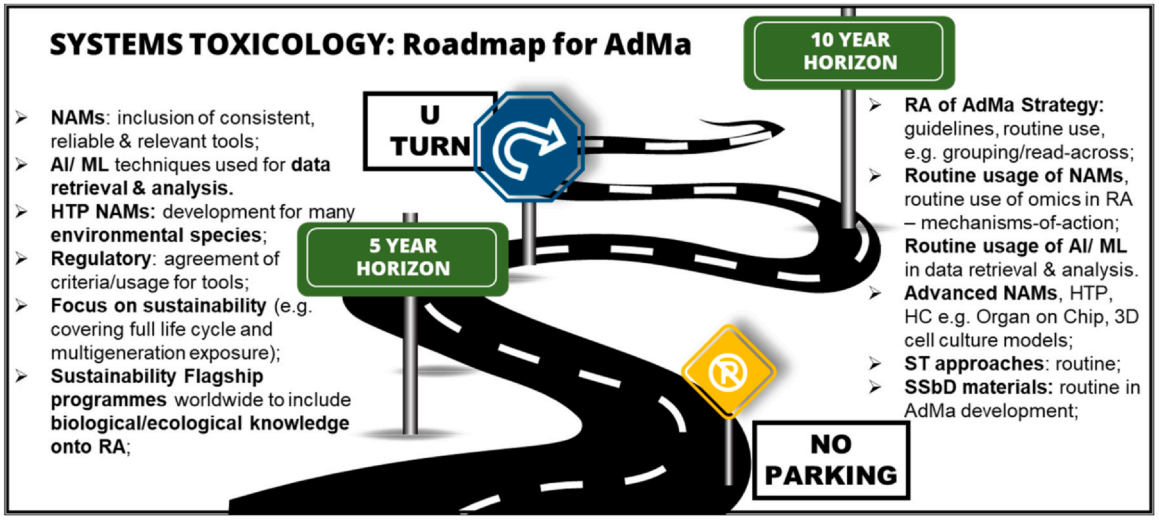

Fig. 3. Roadmap for Advanced Materials (AdMa). Timeframe for the development of systems toxicology (ST) and implementation in Risk Assessment (RA). NAMs: New Approach Methodologies; AI: Artificial Intelligence; ML: Machine Learning; HTP: High-Throughput; HC: High Content; SSbD: Safe and Sustainable by Design.

Producers and innovators will reach a high level of capacity for SSbD novel AdMa. This, together with the established advanced data analysis techniques (machine learning, artificial intelligence), will also promote the more routine use of omics in the RA, both for individual AdMa across materials.

The establishment of new test systems to increasingly switch from (multi-)omics analysis of sentinel species to alternative approaches like Organ on a Chip and 3D-based cell culture models will help to cope with the 3 R requirements. The need for across-species extrapolation and its inherent source of errors or misinterpretations is limited. Along with the establishment of 3D-based cell culture models test structure, the way forward is multiple cell-types and multi-organ settings, using single-cell omics to accommodate cell-type-specific effects and inhomogeneous exposure. For sustainability, the understanding (and assessment) of long-term and chronic exposure of materials and AdMa is a very important aspect. Improved NAMs and predictive models are available to monitor those effects and to identify the materials of highest concern and groups/organisms of specific concerns.

CRediT authorship contribution statement

M.J.B. Amorim: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **W. Peijnenburg:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **D. Greco:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **L.A. Saarimäki:** Writing – original draft, Writing – review & editing. **V.I. Dumit:** Writing – original draft, Writing – review & editing. **A. Bahl:** Writing – review & editing. **A. Haase:** Funding acquisition, Writing – review & editing. **L. Tran:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **J. Hackermüller:** Funding acquisition, Writing – original draft, Writing – review & editing. **S. Canzler:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing. **J.J. Scott-Fordsmand:** Conceptualization, Funding acquisition, Resources, Writing – original draft, Writing – review & editing

Data Availability

Data will be made available on request.

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.

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