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Opportunities and Challenges for Integrating New In Vitro Methodologies in Hazard Testing and Risk Assessment

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Nanomaterials are defined as materials with at least one dimension of 100 nm or less. Their small size confers unique properties that may alter the toxicity profile when compared to larger forms of the same material, requiring additional considerations for safety assessment. There has been a rise in the development of nanomaterials for many applications, and although traditional approaches for toxicity testing may address some of the new toxicity concerns, many may not be directly applicable to nanomaterials and new tools or approaches may need to be developed. Since nanomaterials can exist in many different forms, each of which may cause different adverse biological effects, reliance on traditional in vivo models for safety assessment will simply not be feasible or sustainable, given the volume of materials that may need to be tested. It is essential to consider and develop new in vitro methods that can be applied for hazard identification and risk assessment. Many challenges are associated with using alternative approaches to ensure they are as robust and reliable as traditional in vivo approaches, but by overcoming these issues and adopting new testing strategies there are opportunities to improve safety assessments and reduce the reliance on animal-based toxicity testing strategies.

1. Introduction

Prior to registering and marketing any new pharmaceutical, cosmetic, or (agro)chemical product, including those containing nano-scale components, manufacturers must, by law, generate safety data. These are assessed by regulatory agencies to determine the potential hazards to human health and/or the environment. The data is then incorporated into a risk assessment, which considers the hazards associated with the likely exposure to these substances. Safety testing requirements vary depending on sector, product type, and geographical region/country. Tests performed on living animals (i.e., in vivo) have traditionally been regarded as the "gold standard" for deducing the hazardous effects of any manufactured and/or accidentally produced component (including any intermediate products produced as part of the manufacturing process as well as the toxicity of any metabolites that may be produced). However, industries are increasingly moving away from using animal models in safety testing, particularly toxicity testing for scientific, business, and ethical reasons. From a scientific perspective, in vitro testing/models can help unpick the mechanistic information as to how a substance may exert adverse biological effects, which although an in vivo model may identify, do not generally give information on how this may occur. There will always be the issue of cross-species extrapolation with using in vivo models; using in vitro models with human cells may overcome some of these issues, although these are associated with other limitations which will need to be considered.[1] Furthermore, animal models often do not accurately predict adverse reactions in humans or environmental responses. When selecting a model, it is important that the advantages and limitations of both the in vivo and the in vitro methods are acknowledged and properly understood, including ethical considerations. Significant harm and distress is inflicted onto the test animals during preclinical safety testing and large numbers of animals are used (e.g., in 2017 alone over 850 000 animals were used in regulatory toxicity and safety testing in the European Union [EU]).[2] These ethical and scientific concerns have led to an increasing desire to apply the 3Rs principles; replacement, reduction, and refinement of animals in research (**Table 1**).[3] Finally, in practical terms, animal studies can be technically demanding, laborious, and expensive, especially when considering long-term exposure studies. However, for some technologies this may equally apply to alternative methods (e.g., organo-typic cell culture and advanced physiologically based approaches/fluid-flow systems) particularly in terms of any initial set-up, training, and equipment costs. By applying the 3Rs to the regulatory requirements of safety testing for nanomaterials, in vivo testing can be minimized in favor of robust

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Table 1. The standard and contemporary definitions of the 3Rs (www.nc3rs.org.uk/the-3rs).

	Standard	Contemporary
Replacement	Methods which avoid or replace the use of animals	Accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals
Reduction	Methods which minimize the number of animals used per experiment	Appropriately designed and analyzed animal experiments that are robust and reproducible, and truly add to the knowledge base
Refinement	Methods which minimize the number of animals used per experiment	Advancing research into animal welfare by exploiting the latest in vivo technologies and by improving understanding of the impact of welfare on scientific outcomes

and predictive in vitro methodologies which do not affect the rigor of scientific safety tests.

Manufactured nanomaterials can be defined as a natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm.[4] Their unique physical properties have led to a notable rise in their development and production within a vast array of different applications. This includes the use of different nanoforms of the same material which are known to elicit significantly different adverse biological effects and consequently each form of a nanomaterial will need to undergo safety assessment under the appropriate regulatory framework.[5] The sheer number of different nanomaterials/nanoforms that may be marketed in coming years means it is not feasible or sustainable to use in vivo approaches to test the hazard posed to human health by both developed and proposed nanoforms.[6] It is therefore an area where using alternative methods to the traditional in vivo tests needs to be given priority, acknowledging that there are additional or different considerations when applying new approaches in the safety assessment of nanomaterials compared with traditional chemicals.

2. Opportunities to Utilize Alternative Approaches in Nanomaterial Safety Assessment

In response to the drivers to reduce the reliance on in vivo toxicity testing strategies, legislation has been implemented to compel a shift away from the traditional animal-based approach towards hazard assessment. For example, from 2013 the EU banned the marketing of cosmetics containing ingredients that have been tested on animals.[7] As the EU accounts for a significant percentage of the marketbase for cosmetics, this has effectively led to a worldwide ban on animal safety testing for cosmetics, and other regions have since followed suit with similar legislation.[8] This has given an enormous incentive for the development and innovation of non-animal approaches, including in vitro assays which use human or animal tissues, organs, or cells. These approaches can be quicker and cheaper to conduct than in vivo tests, while providing "human-relevant" mechanistic insights, which could potentially make predictions of toxicity (or "adverse outcomes") more accurate.[9,10] There are some examples of in vitro approaches already being used in regulatory safety testing. For example, in 2017 the EU's regulation concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)[11] information requirements were amended to make non-animal testing methods the default for skin corrosion/irritation (e.g., using the Corrositex in vitro membrane barrier test method; organisation for economic cooperation and development (OECD) test guideline (TG) 435, and the Reconstructed Human Epidermis method/EpiDerm model; OECD TG 439), serious eye damage/eye irritation (e.g., the Bovine Corneal Opacity and Permeability Test Method; OECD TG 437), and skin sensitization (e.g., Direct Peptide Reactivity Assay; OECD TG 442C). The latest statistics show that the use of non-animal tests have tripled for skin corrosion/irritation, quadrupled for serious eye damage/eye irritation, and increased more than 20-fold for skin sensitization under REACH for the period 2017–2019.[12] With these advances of in vitro methodologies made in the cosmetics sector and used in regulatory safety testing it is anticipated that confidence will be given to other sectors to move away from the traditional in vivo models. Current practice, however, is that in vitro toxicity assays are often used internally within companies to prioritize substances for further development and inform later in vivo testing strategies, which can significantly reduce the number of animals used for mandatory safety testing.

It should be noted that while many alternative approaches do not use animals directly, animal derived products such as fetal bovine serum may still be used. However, there is increasing interest for these assays to also be free from animal-derived products to improve human relevance and reproducibility, and to reduce the use of animals.[13] There are a number of activities in progress to help facilitate this, including the fetal calf serum-free database (RRI:SCR-018769) which has been set up to provide information on animal free media for culture and an NC3Rs CRACK IT Challenge (https://www.nc3rs.org.uk/crackit/animal-free-vitro) which aims to replace animal-derived products in OECD TGs.

3. Advanced In Vitro Systems Could Be Used to Increase the Physiological Relevance of New Methodologies

As new methodologies have emerged over the past decade, advanced in vitro approaches beyond simple single-cell systems (i.e., monocultures) have increased. In an effort to more closely mimick the in vivo microenvironment and better relate results to whole organism biology, multicellular models currently under development (but not yet formally validated for regulatory safety testing) include, for example, the lower lung, the liver, the gastro-intestinal tract, and the brain (i.e., cerebral organoids or mini-brains).[14–17] Sophisticated approaches include varying the geometry of cell cultures (e.g., spheroids), incorporating dynamic movement (e.g., breathing motion

within alveolar cell models, the use of air–liquid interfaces), and fluid dynamics (e.g., replicating the circulatory system). Despite this, it remains difficult to compare in vitro data to in vivo outcomes, for example, due to differences in substance concentrations applied, how these substances interact with the cellular systems, and "real-life" internal organism exposure levels. Mathematical/computational models are being developed to inform in vitro to in vivo extrapolation (IVIVE) and the interpretation of in vitro data. Currently, there is an active shift toward using these approaches with the plethora of existing in vivo data sets. One example of such research is through the EU H2020 project "Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment" (PATROLS; https://www.patrols-h2020.eu), where advanced in vitro models of the human lung, gastro-intestinal tract, and liver are being tested to predict engineered nanomaterial hazard using concentrations determined from historical in vivo data sets via IVIVE approaches. These include physiologically based pharmacokinetic models, routinely used to predict systemic or organ/tissue exposure in humans, particularly for oral routes of administration.[18] This information can also help to ensure relevant concentration/dose setting in both in vitro and in vivo studies through "reverse dosimetry."[19] However, demonstrating that models are fit for purpose can be difficult for industry sectors such as agrochemicals and industrial chemicals where human data are not readily available to validate the predictions. Furthermore, human exposure routes are likely to be different for substances released into the environment compared with pharmaceuticals, and there are limited models available that consider non-oral exposure (e.g., dermal, inhalation). A key challenge remains in building confidence in the utility of such models and reducing the uncertainty associated with them, before they are accepted for practical/regulatory use.[18] Efforts are being made to overcome this, particularly in the area of substance screening and prioritization, such as through the US environmental protection agency (EPA) ExpoCast program.[20] It is imperative though that increased cross-sector collaboration and regulatory engagement is conducted in order to further establish and overcome the barriers toward wider uptake of such in silico approaches.

4. Pathways-Based Approaches Will Support the Shift toward Non-Animal Safety Assessment

Within the safety assessment field there has been increased focus to better understand how exogenous substances exert their toxic effects. This has led to the formulation of the adverse outcome pathway (AOP) concept and the development of a web-based platform—the AOP Knowledgebase (https://aopkb.oecd.org/) to bring together as much information as possible on how adverse effects are elicited via mechanistic (toxicology) pathways. The idea being that rather than looking only at which adverse effects occur in a whole organism following substance exposure (as is the case with in vivo approaches), methods are used to examine whether, at a cellular level, the substance of interest causes critical (or "key") events within biochemical pathways that are known to result in adverse outcomes (e.g., fibrosis, cancer) in organisms or populations.[21–23] If such toxicity pathways are well understood, the likelihood of a substance causing an adverse outcome at the organism level can be predicted using non-animal methods developed to detect effects on the known key events. This approach also has the advantage of identifying key mechanisms of action for follow-up studies. For AOPs to be useful for decision-making they need to be quantitative, so that the threshold required for a pathway to progress from key event to the next is understood.[24] Although AOPs originally focused on traditional chemicals, attention has turned more recently to applying the concept to the safety assessment of nanomaterials. In theory, since AOPs are chemically agnostic, that is they describe and monitor the consequence of a chemical system interaction independent of the physicochemical properties of the toxicant, the same AOPs will be applicable to traditional chemicals as for nanomaterials, if the same molecular initiating event is triggered. However, due to their size and physicochemical properties there are notable differences in how nanomaterials can access and interact with biological systems and there are a number of examples of AOPs that are relevant to nanomaterials.[23] The project "Advancing Adverse Outcome Pathway Development for Nanomaterial Risk Assessment and Categorization" under the auspices of the OECD's Working Party on Manufactured Nanomaterials (WPMN) is underway to examine how the nanotoxicology literature can be used to identify the key events relevant for manufactured nanomaterials. The aim of this project is to develop testing strategies around the measurable biological events which incorporate in vitro approaches to inform regulatory decisions regarding nanomaterial safety.[23] Barriers to the uptake of AOP-driven approaches in a regulatory setting, aside from data gaps, include the length of time and effort needed for an AOP to be developed and officially endorsed by the OECD; this requires provision of dedicated resource. An example of this is through the EU H2020 project "SmartNanoTox," which is developing AOPs for nanomaterial induced respiratory toxicity (http://www.smartnanotox.eu/). Furthermore, without better understanding the effect of size-associated properties of nanomaterials on AOPs, it will not be possible to define applicability domains for these approaches.[23,25]

5. The Short and Long-Term Prospects for Integrating New In Vitro Methodologies in Hazard Testing and Risk Assessment

In the short-term, there is certainly scope for the wider use of in vitro assays for screening and prioritization of nanomaterials. These could be new in vitro assays that have been specifically developed for testing nanomaterials or assays already validated for the toxicity testing of traditional chemicals with modifications where appropriate. An example where modifications are needed is in the assessment of genotoxicity, such as the micronucleus assay (OECD TG 487), as the traditional approach can impact the interaction of the nanomaterials with the cellular system, which can block nanomaterial uptake and therefore prevent proper assessment of the nanomaterial in

question. Modifications will ensure that relevant bio–physical interactions of the nanomaterial with its environment (e.g., uptake and dispersion, and interaction with body fluids) are replicated, which can be highly dependent on the physicochemical properties of the nanoform being tested.[6] High throughput screening assays could be particularly useful in this space as financial, time, and ethical reasons-related safety testing of each unique nanomaterial for the potential adverse effects is virtually impossible. Data from adapted/nano-specific in vitro assays, in combination with existing in vivo data and the use of computational models, will also help to identify links between structural, biological, or physicochemical properties of nanomaterials and their toxic effects. Increased knowledge gained using in vitro or modeling techniques on the release, dispersion, and uptake of nanomaterials will help to better understand likely levels of whole organism exposure.[26] This will aid in a) better determining the most relevant route of administration for animal studies for industries where they are still mandatory; b) informing decisions to waive animal studies; and c) ensuring that nanomaterials are tested at doses and in models that are reflective of likely human exposures.[6]

In the longer-term, the goal will be the replacement of animal toxicity studies with more predictive, human-relevant methods. This is a particularly pressing need for the cosmetics industry, which can no longer fall back on traditional in vivo approaches. This will be supported by the application of integrated approaches (**Table 2**) which in part utilize human cells and 3D cell models, including those being developed under large-scale projects such as PATROLS (www.patrols-h2020.eu). In vitro systems capable of mimicking key physiological processes such as nanomaterial penetration and metabolism will also be essential. Computational approaches will also be key in reducing the need to test exposure to and toxicity on a case by case basis in the assessment of nanomaterials. The GRACIOUS project, (www.h2020gracious.eu) is developing a framework for the grouping and read-across of nanomaterials enabling a shift away from the case-by-case risk assessment paradigm. Consequently, this will improve the efficiency of risk analysis and decision making for the safer design of nanomaterials.[27,28]

6. Overcoming the Challenges Associated with Using Alternative Approaches

Although the benefits of rethinking the current testing paradigm are clear, a complete shift toward replacing the in vivo tests that have been used for many years, and which the industry and regulators are very familiar with, does pose significant challenges. Challenges which still require much research and discussion in order to realize the full replacement of in vivo testing strategies with relevant, efficient, and effective alternative systems. To date there have been no new cosmetic ingredients, including nanomaterials, marketed since the animal testing ban despite publication of Guidance on the Safety Assessment of Nanomaterials in Cosmetics by the EU's Scientific Committee on Consumer Safety—highlighting the complexity of moving away from traditional animal-based safety testing.[2] Table 2 highlights two of the key challenge areas and efforts being made to overcome them. This includes efforts by international collaborations such as the Malta Initiative. The Malta Initiative (https://www.nanosafetycluster.eu/international-cooperation/the-malta-initiative/), which is comprised of EU member states, the European Commission, European Chemicals Agency (ECHA), and industry, is working to adapt existing or develop new OECD TGs to ensure nano-specific considerations are incorporated to fulfill regulatory requirements.

One of the key issues highlighted in Table 2 is the time and cost associated with formal validation processes, including those led by validation bodies such as the European Centre for the Validation of Alternative Methods (ECVAM), and approval of methods as per OECD test guidelines.[32] This means that often, by the time an approach has been approved or formally validated the science may have moved on and the method may no longer be state of the art. Going forward, it is therefore critical to streamline approaches to expedite these processes, and formal validation may not always be appropriate. Instead, the use of performance standards may be used to facilitate the validation of similar test methods for the same endpoint.

7. Conclusion

There are ethical, scientific, and practical reasons for moving the safety assessment field away from traditional animal-based approaches used to assess the hazard of traditional chemicals and nanomaterials. The EU's decision to ban animal testing for cosmetics is an example of a unique motive to advance the development of in vitro approaches for safety testing, while at the same time improving the science underlying decision-making. There is great potential to improve the accuracy of human and environmental risk assessment of new products, including through the use of sophisticated human-relevant models based on human cells or 3D human tissues. Pathways-based approaches offer a framework to support the prioritization of non-animal assay development and application—which can be nanomaterial-specific, where necessary. There remain barriers to overcome in satisfying regulators that these approaches will meet their requirements. These include the need to rethink formal validation processes in the interest of timely adoption of new methods and their maximum exploitation; this may be overcome by the creation of faster approaches to assure robustness and reliability of methods such as the adoption of performance-based test guidelines as outlined in Table 2. Another key challenge includes the need to develop integrated strategies and weight of evidence (WoE) approaches to support the combination of multiple lines of evidence and data that will be needed to fully replace current animal tests. Guidance on applying WoE approaches and a more flexible approach to regulatory requirements will be needed for this to occur. The vision being that in vitro and other non-animal (e.g., in silico) approaches can be swiftly proven to be robust and reproducible and form critical components of integrated approaches which are used in place of animal tests in (nano) toxicology testing strategies across all sectors, with wide acceptance across regulatory frameworks.

Table 2. The issues and how to overcome them during the use and validation of alternative approaches.

	The issues	Overcoming the challenges
Accelerating validation processes and adoption in practice	• Lengthy and costly "validation" processes still required to ensure new approaches are robust and reproducible. • Usually involve conduct of large-scale ring trials to ensure intra- and inter-laboratory reliability, and assessments to ensure that the methodology is fit for purpose. • Significant time lag between the development of new in vitro approaches and their acceptance by regulators in place of animal experiments or alongside limited in vivo data sets. • For skin corrosivity (one of the simplest adverse effects to model in vitro), it took almost 20 years for the OECD to adopt a validated test guideline and nearly 30 years for an integrated assessment approach to be developed and put into use. • It took >20 years to formulate the OECD TG for the in vitro micronucleus test, due to the need for consensus across the board for new OECD TGs. • By the time test guidelines have been validated and adopted, science and technology may move on. • A lack of robust nano standards (e.g., positive/negative controls) for toxicity (although some nanomaterials, e.g., carbon black and titanium dioxide are also currently used in this context, due to the wealth of knowledge/data sets concerning these nanomaterial types). • Validated in vitro assays to predict the lengthiest and most severe animal tests (to assess for example acute systemic toxicity, repeat dose toxicity, carcinogenicity, and developmental and reproductive toxicity) currently do not exist.[29] • Animal models may not be the most appropriate measure to benchmark new methods against, as they are not 100% accurate.	• New and existing in vitro models must continue to be standardized, so that the data generated are comparable and of the highest quality, and that studies are robust, reproducible, relevant, and fit for purpose. • Critical that a more streamlined approach to demonstrate robustness and utility becomes available, to expedite the process of ensuring regulatory relevance and adoption in practice. • The OECD has begun developing "Performance-Based Test Guidelines" (PBTGs; e.g., OECD 2015)[30] which set out performance standards to facilitate the development and validation of similar test methods for the same hazard endpoint. New, similar test methods can be added in a timely fashion to the PBTG after review and agreement that the performance standards are met—rather than individual assays having to undergo full validation processes.
The need to integrate and consistently interpret new types of data	• Individual in vitro assays will not replace current animal tests on a one-to-one basis and the integration of data from multiple approaches will be required. • Integrated Approaches to Testing and Assessment (IATAs) encourages the development and use of batteries of complementary tests to satisfy regulatory concerns. • The successful use of integrated approaches relies on: a) incorporation of the necessary levels of biological and chemical complexity which also encompass exposure considerations; and b) regulators having confidence in making safety decisions based on types and combinations of data they are not currently familiar with, often by applying WoE considerations, that is, the integration of multiple lines of evidence to determine their relative support for possible answers to a scientific question.[31] • IATA-style approaches can vary from those that are flexible and judgment-based, through to defined approaches which consist of a fixed data interpretation procedure with a defined set of information sources (e.g., those currently under development by the OECD for skin sensitization). • In addition to in vitro approaches, IATAs will need to incorporate other non-animal approaches including grouping/read-across and in silico methods, which may need to be nano-specific/modified for nano-scale application.	• Genotoxicity tests have traditionally been conducted on DNA damage and mutation induction in isolation. The field is now moving toward integrating multiple endpoints for more holistic safety assessment, including the potential for carcinogenesis. These approaches use both flow-based systems and image analysis platforms to assess DNA damage in conjunction with cell cycle perturbation, activation of key signaling pathways (e.g., p53), and changes to cell bioenergetics and oxidative stress induction.[22] • AOPs play a key role in IATA development but this is dependent on continued development of well-characterized AOPs, which can be a complex and lengthy process requiring collaborative effort within the scientific community.[21] • AOPs need to incorporate thresholds—that is, the quantitative relationships—required for progress between the key events in the pathways which ultimately result in toxicity. • Single linear AOPs should not be considered—rather "networks" need be built that link shared/overlapping key events between different toxicity pathways.[23,24] • Suitable and clear guidance on applying WoE to decision-making will mean in theory firmly fixed or predefined test batteries will no longer be required and there will be greater flexibility in terms of the methods and data that can be utilized. WoE approaches can also include data from the open or "grey" literature and guidance is needed to guide quality assessments and interpretation of such data. • There will need to be sufficient flexibility within the regulatory frameworks and a culture shift away from the current prescriptive nature of safety assessment. • Training tools to aid in the interpretation of data from new models will be vital to aid in their uptake in the regulatory community.

Table 2. Continued.

The issues	Overcoming the challenges
	• Ongoing activities include the GRACIOUS project (www.h2020gracious.eu), GOV4NANO (https://www.gov4nano.eu), PATROLS (www.patrols-h2020.eu), NANOINFORMATIX (https://www.nanoinformatix.eu), RISKGONE (www.riskgone.eu), SmartNano Tox (http://www.smartnanotox.eu/), NANOSOLVIET (www.nanosolveit.eu), and the Malta Initiative (https://www.nanosafetycluster.eu/international-cooperation/the-malta-initiative/). Nanotechnologies, Advanced Materials, Biotechnology, and Advanced Manufacturing and Processing (NMPB) (https://ec.europa.eu/programmes/horizon2020/en/h2020-section/nanotechnologies-advanced-materials-advanced-manufacturing-and-processing-and) 13, 14, 15, and 34 projects. Activities within the OECD Working Party on Manufactured Nanomaterials (WNT) (Working Group of National Co-ordinators of the TGs programme) and WPMN groups. • To streamline the process of risk assessment through grouping of nanomaterials.

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Conflict of Interest

The authors declare no conflict of interest.

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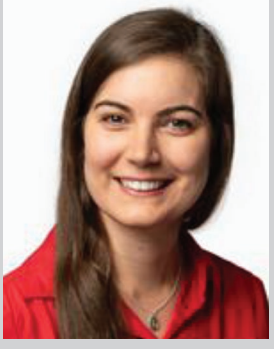

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