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The Road to Achieving the European Commission's Chemicals Strategy for Nanomaterial Sustainability—A PATROLS Perspective on New Approach Methodologies

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The European Green Deal outlines ambitions to build a more sustainable, climate neutral, and circular economy by 2050. To achieve this, the European Commission has published the *Chemicals Strategy for Sustainability: Towards a Toxic-Free Environment*, which provides targets for innovation to better protect human and environmental health, including challenges posed by hazardous chemicals and animal testing. The European project PATROLS (Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment) has addressed multiple aspects of the *Chemicals Strategy for Sustainability* by establishing a battery of new approach methodologies, including physiologically anchored human and environmental hazard assessment tools to evaluate the safety of engineered nanomaterials. PATROLS has delivered and improved innovative tools to support regulatory decision-making processes. These tools also support the need for reducing regulated vertebrate animal testing; when used at an early stage of the innovation pipeline, the PATROLS tools facilitate the safe and sustainable development of new nano-enabled products before they reach the market.

1. Introduction

To reach the target of building a more sustainable climate neutral and circular economy by 2050, the European Commission published its ambitious agenda for chemicals regulations in the European Green Deal in 2020.[1] The European Green Deal is a strategy that aims to overcome the challenges of climate change and environmental degradation, transforming the EU into a resource-efficient and competitive economy. To help achieve a reduction in chemical pollution and exposures to hazardous chemicals at levels that are harmful to human health and to the environment, the European Green Deal encompasses the *Chemicals Strategy for Sustainability: Towards a Toxic-Free Environment*. This strategy starts by

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acknowledging that the European Union has one of the most comprehensive regulatory frameworks for chemicals, supported by a strong scientific knowledge base. Using this foundation, clear targets for innovation are designed to "rapidly and effectively" protect human and environmental health, by strategically responding to challenges posed by hazardous chemicals, minimizing the use of, and where possible substituting chemicals of concern.[2] The *Chemicals Strategy for Sustainability* is also supported by a parallel European Union agenda for sustainable growth: the Commission's *Circular Economy Action Plan*, adopted in March 2020.[3] This highlights targets for sustainable growth, while reducing pressure on natural resources and thus, has overarching synergies in relation to the safe and sustainable design of products across their life cycle.

The *Chemicals Strategy for Sustainability* includes an array of dedicated actions to support its targets, spanning a range of topics that have been considered as key barriers to enhancing the innovation in production and use of chemicals that are safe and sustainable by design. Many of these actions focus on establishing a paradigm change in our approach to chemical safety testing and risk assessment. For example, next-generation new approach methodologies (NAMs) are required to promote the reduction and replacement of animal testing, with EU legislation now in place to strongly encourage the reduction and replacement of animal testing (Directive on the protection of animals used for scientific purposes (2010/63); the Regulation on cosmetic products (1223/2009); Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (2007/2006); and Classification, Labelling and Packaging act (CLP) (1272/2008)). In addition to these recommended changes in REACH and CLP legislation, EU policies are also being introduced regarding endocrine disruptors, as well as combinatorial effects of chemicals and engineered nanomaterials (NMs) as example areas of concern where traditional risk assessment with heavy reliance on in vivo testing is coming toward an end. However, uptake of NAMs requires minimizing the current uncertainty in regulatory application of non-animal approaches to hazard characterization. It will be important to foster innovations in in vitro and in silico models that are predictive and representative of in vivo responses to implement non-animal testing, while minimizing uncertainty and continuing to support new developments in the chemicals industry.

Another aspect highlighted by the *Chemicals Strategy for Sustainability* involves the need to minimize and substitute the use of chemicals that, at one or more points in their life cycle, can have chronic adverse effects toward human health and the environment. Currently, testing for chronic impacts requires repeated and extended exposure studies involving large numbers of animals. Thus, to address this action and simultaneously reduce, refine, and replace animal testing, it will be crucial to establish robust and widely accepted in vitro and in silico testing approaches that are strongly linked to adverse health effects in humans.

While the new (eco)toxicology paradigm is pushing forward with an agenda to reduce, refine, and replace testing in animals, it is important to note that regulatory applications must generate safety assessment based on well standardized and validated methods, such as those published by the Organisation for Economic Cooperation and Development (OECD) and the International Organisation for Standardisation (ISO). Although data derived from non-validated methods that do not have an OECD test guideline (TG) are considered in a risk assessment, they are not as heavily weighted. Thus, it is expected that newly developed hazard characterization approaches are standardized in accordance with international protocols to promote their use within international regulatory frameworks, through the mutual acceptance of data (MAD).[4] This is a significant barrier toward the move away from animal testing because typically, validation of new methods and development of an internationally accepted OECD TG is measured against data generated using animal models, which in most cases, are scarce or unavailable. In addition, validation of methods and establishment of OECD TG can take more than 10 years. Therefore, encouraging early adoption of NAMs by risk assessors, prior to establishing OECD TGs, will be critical to achieving the targets set out by the *Chemicals Strategy*. One way to facilitate this is to make use of relevant data that are currently being generated using NAMs that have associated standard operating procedures (SOPs) and evidence of inter-laboratory transferability and high reproducibility. Such data are actively being generated in academic and industrial laboratories, but often, this data are not captured for regulatory purposes as it does not find its way into regulatory dossiers. Thus, a concerted effort to establish good working practices that readily allow access to appropriate data will be of great support for regulatory risk assessment approaches in the future. This is particularly true for emerging advanced and multi-component materials where hazard characterization data may be generated in parallel with the design phases as a result of safe- and sustainable-by-design approaches.

To complement the development of NAMs, adverse outcome pathways (AOPs) are a useful concept making use of biological mechanistic pathways to predict human and environmental hazard outcomes following not only acute but also prolonged and/or repeated exposure scenarios. The main components of the AOP include the MIE (molecular initiating event), intermediate KEs (key events) progressing through the cellular, tissue, and organ level, culminating in an adverse outcome (AO; **Figure 1**). The KEs in the AOP are sequential and, thus, are causal. The relationship between the two KEs, that is, the level of change in an upstream KE required to initiate the downstream KE, is described as key event relationship (KER) and is depicted by an arrow placed between the two KEs. Hence, AOPs are toxicity road maps that allow systematic organization of complex, multivariate, and heterogeneous experimental data in a simplified and modular format, helping to identify knowledge gaps. The AOP concept has therefore recently been used in human toxicology and has been endorsed as suitable for risk assessment by the OECD. Several NM-relevant AOPs have also been described.[5–7] Consequently, the mechanistic evidence

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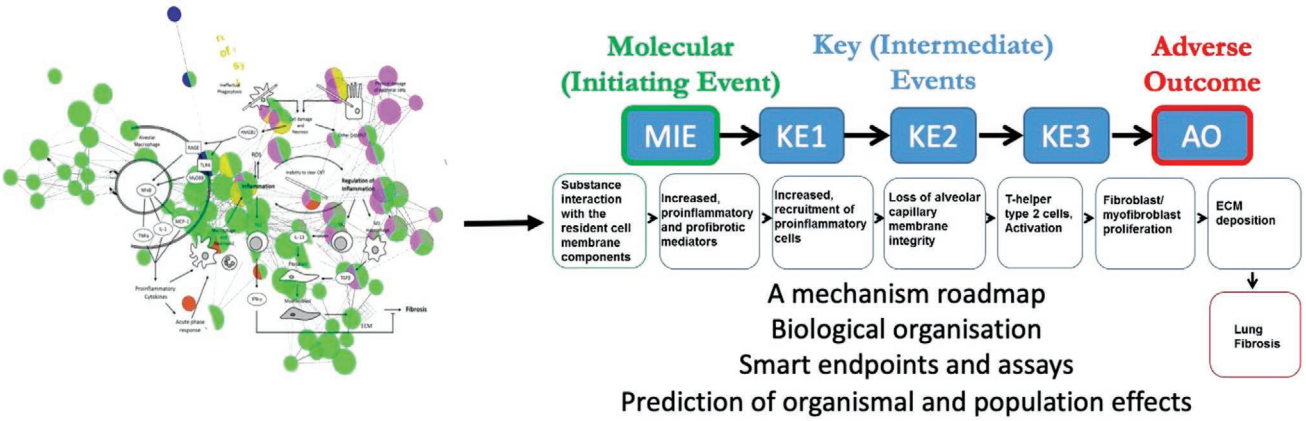

Figure 1. AOPs permit easy visualization of complex disease phenomenon, highlighting the main biological players and events for further research. On the left is a multi-layered and multi-node biological network (colors indicate different biological events and associated molecular players), showing the complexity of the lung fibrotic disease process. Presented on the right is a single, linear mechanism of lung fibrosis derived from the complex network of mechanisms in an AOP framework, depicting only the most essential events or key events involved in the fibrotic disease process. The specific key events can serve as focus points for the development of new approach methodology development. MIE, molecular initiating event; KE, key event; AO, adverse outcome.

identified in the context of AOPs is guiding the identification, design, and development of NAMs that target defined KEs of the pathway, permitting the strategic design and development of experimentally informed testing tools and strategies for the focused generation of "fit-for-purpose" data.[5,8]

2. Achievements in the PATROLS Project Supporting the Chemicals Strategy

Over the last 10–15 years, there has been an impressive expansion in our understanding of NM safety as significant investment has been made to advance our knowledge in this field. This is exemplified by the NanoSafety Cluster projects (https://www.nanosafetycluster.eu/) funded through the European Commission. This collection of projects addresses the safety of materials and technologies enabled by the use of nanoforms, and encompasses an array of aligned topics spanning nanomaterial toxicology, ecotoxicology, exposure assessment, mechanisms of interaction, risk assessment, and standardization of methods developed. To date, most of the human and environmental hazard assessment studies conducted on NMs have focused on acute, and in some cases, high-dose exposures. However, it has been uncertain how these results extrapolate to realistic low dose, repeated and chronic exposures, which present a significant challenge in the risk assessment of NMs. This problem has been compounded by the limited predictive power of current in vitro hazard identification tests most often owing to the lack of consideration for ENM-specific and physiological parameters. For example, simple in vitro monocultures poorly mimic realistic exposure conditions; they lack anatomical and physiological complexity and do not accurately model responses to long-term exposures. Extensive in vivo testing to address these knowledge gaps is not sustainable as chronic exposures are expensive, time consuming, and have ethical concerns. Thus, the project "PATROLS" (Physiologically Anchored Tools for Realistic nanOmateriaL hazard aSsessment (www.patrols-h2020.eu)), was funded by the European Commission to focus on these specific concerns and has subsequently become ideally positioned to support several aspects of the *Chemicals Strategy*. The project was aimed at establishing and standardizing a battery of innovative, next-generation physiologically anchored hazard assessment tools that more accurately predict adverse effects in human and environmental systems caused by long-term, low-dose engineered NM exposure. The project aimed to provide a suite of methods to support regulatory decision-making processes, and it was unique, as it covered and connected all aspects of nanosafety testing spanning in vitro, in vivo, and in silico approaches.

Examining human health risk assessment, for example, requires the building blocks for implementation of an alternative non-animal approach depicted in **Figure 2**. Traditionally, in chemical risk assessment, epidemiological or animal toxicological data are used to derive a no-observed-adverse-effect level or lower confidence limit of a benchmark dose as point of departure. This facilitates derivation of human health guidance values such as a reference dose, derived no-effect level (DNEL), or acceptable daily intake, or calculation of risk estimates for non-threshold effects such as most carcinogenic effects. Within Figure 2, the building blocks inside the large arrow have been proposed as the main pathway toward non-animal alternative methods by Romeo et al. based on insights from the EU project NanoRIGO.[9] Most of these identified building blocks were part of the scope within PATROLS. The following sections describe some of these methodological advances that facilitate reduction in animal testing and simultaneously assist in discriminating NM with a potential for chronic adverse outcome effects.

2.1. Complex Human Lung, Liver, and Intestinal Culture Models for Long-Term NM Exposure

PATROLS developed a range of realistic and reliable in vitro 3D tissue models of the human lung, intestine, and liver for NM safety assessment. The models were specifically designed to support exposure scenarios that were more relevant to the

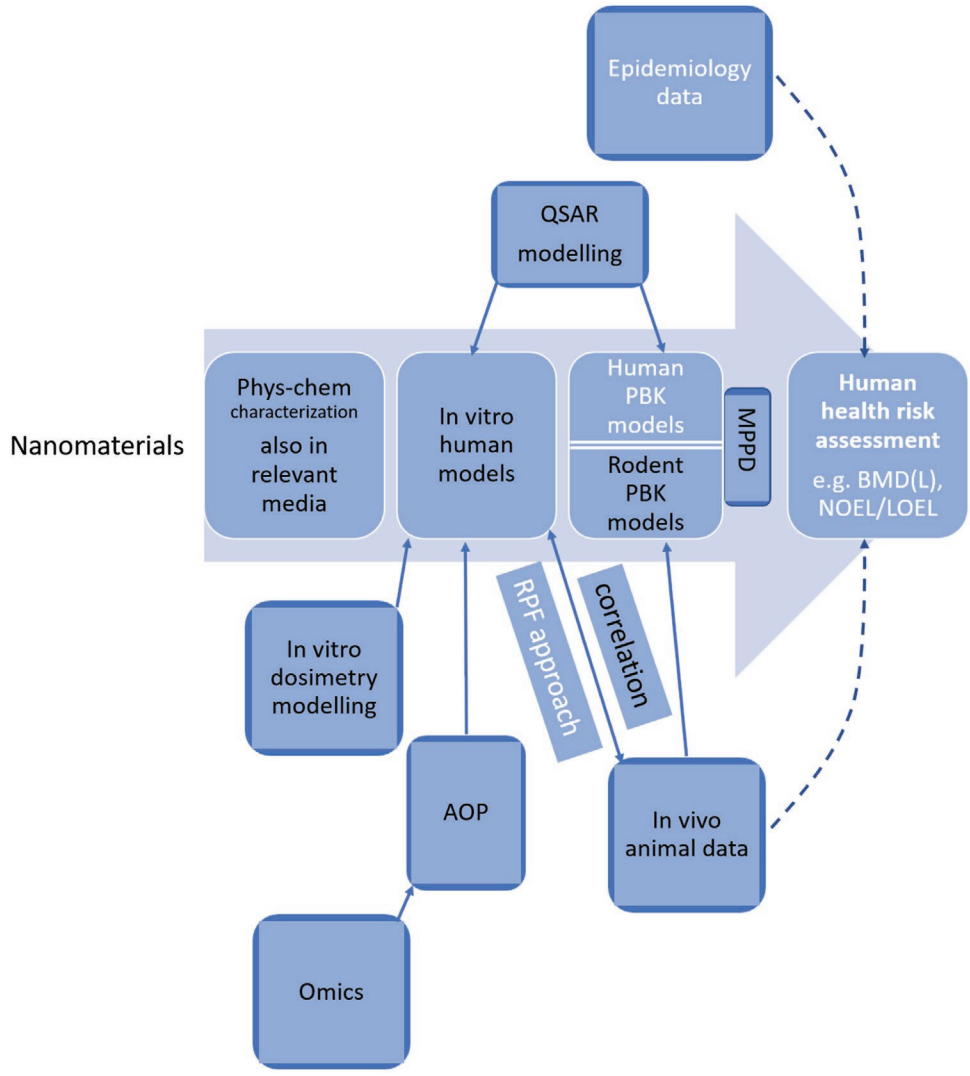

Figure 2. Graphic representation of the building blocks for an alternative methods approach in human health risk assessment of NM. The classical approach uses human epidemiology data and animal toxicological data for the risk assessment. The building blocks inside the large arrow depict the pathway for the alternatives for the assessment as proposed by Romeo et al. 2021.[9] Blocks in black font, such as physico-chemical characterization, quantitative structure activity relationship (QSAR) models, omics approaches, and adverse outcome pathways (AOPs) are elements where the PATROLS project has contributed to their further development. Blocks in white font, such as the relative potency factor (RPF) approach of human physiologically based kinetic (PBK) models, contain essential components for human health risk assessment, but were outside the scope of PATROLS.

longer term and repeated exposure scenarios experienced by humans. In addition, the in vitro testing systems developed under PATROLS more realistically mimicked human physiology, in terms of mode of exposure and biological response, closing the in vitro—in vivo divide in hazard characterization, and thereby reducing the need for animal testing.

From an inhalation toxicology perspective, it is now possible to use alternative, in vitro approaches as tools for understanding both short-term and longer-term (multiple days up to several weeks) impacts of aerosolized substances (e.g., particles, fibers, and chemicals) toward lung cell models mimicking the airways and gas exchange regions of the lung. A suite of specific experimental protocols has been created that encompass more physiologically representative human lung cell cultures in which cells are exposed at the air–liquid interface (static, or under fluid/dynamic flow and/or breathing patterns) combined with realistic exposure strategies (either wet or dry aerosol) that can be implemented for any form of aerosol exposure (i.e., particles, fibers, or chemicals) using in vivo extrapolated exposure concentrations. It is important to add that recommended NM doses in vitro are calculated based on data derived from animal experiments, that is, rat or mouse models, or use information on potential NM exposure in the occupational setting combined with predicted lung surface area concentrations.[10]

The advanced in vitro lung models developed are based upon co-culture systems using permeable trans-well membrane inserts allowing the confluent growth of an epithelial layer complemented with immune cells (e.g., macrophages), interstitial cells (e.g., fibroblasts), or additional barrier cell types (e.g., endothelial cells). An air–liquid interface is included,

enabling a similar spatial arrangement of cells as observed in vivo (human). The advanced in vitro lung models can be combined with numerous state-of-the-art analytical and microscopic techniques to determine the impact of aerosol exposures upon cell structure and function.[11–13] Furthermore, in addition to standard hazard endpoints, these advanced in vitro lung cell culture systems can be utilized for: i) high-throughput (HTP) approaches and ii) to predict the human in vivo response via AOP-driven transcriptomics-based analyses.

For the human liver and intestine, 3D models have been generated consisting of multiple cells orientated in a manner that allows (patho)physiologically relevant cell-to-cell interactions to occur. These models can be utilized to evaluate a range of toxicological and functional endpoints following acute, long-term, and repeated exposures. The 3D liver models developed within PATROLS consist of a primary hepatocyte-based microtissue and a cell line–derived spheroid. The primary liver microtissue models contain hepatocytes, Kupffer cells, endothelial cells, and stellate cells, and can be cultured for up to 21 days.[14] The cell line–based model is derived from HepG2 human hepatocyte cells and was developed to expand the evaluation of toxicological endpoints to include genotoxicity, which requires a larger population of dividing cells.[15,16] Both liver models have been well characterized and demonstrate improved liver-like functionalities than simple monolayer cultured cells, substantially enhancing their patho-physiological relevance.[16,17]

The in vitro model for the human intestine developed in PATROLS is a triple cell co-culture that includes a mucus-producing goblet-like cell line, together with a differentiated epithelial and immuno-competent macrophage-like cell line.[18] The presence of mucus as an additional physical barrier is important to consider in the hazard assessment of NM, as studies have shown that the mucus can entrap administered particles, preventing them from reaching the epithelium.[19,20] Thus, the absence of mucus in in vitro systems might lead to a significant overestimation of cell–particle interactions.[21] The human intestinal model also includes an optional artificial digestion protocol to mimic the influence of stomach passage, whereby ingested NM subsequently interact with the strongly acidic gastric juice and the alkaline intestinal fluid.[18,22] Furthermore, a second intestinal triple culture model has been developed that incorporates M-cells (specialized epithelial cells in close association with Peyers patches), also with the capacity for mucous formation.[23]

Although models discussed above are representative of healthy human tissues (equivalent to testing substances in healthy animals), it is important to note that the toxicological response in vulnerable populations with a pre-existing disease may be different. Current chemical risk assessment does not take into consideration the impact of exposure to an exogenous agent when pre-existing disease may be present in the exposed individual. An ability to calculate such comparisons using in vitro models provides a significant advantage to understanding wider population effects and therefore provides significant added value for extrapolation. Thus, within PATROLS, applied cell culture models representing a range of common human disease states were also developed. These have included an in vitro inflamed lung cell model; in vitro models representing benign fatty liver (steatosis), pre-fibrotic non-alcoholic steatohepatitis (NASH), liver inflammation, and liver fibrosis; as well as a model of the inflamed intestine.[18,24] Interestingly, these diseased models are more susceptible to NM than the equivalent healthy models and are more sensitive to the toxicological insult.[25]

All the in vitro models developed within PATROLS readily facilitate the evaluation of standard hazard endpoints, including cytotoxicity, genotoxicity, and (pro-)inflammatory and (pro-)fibrotic responses, and have been further expanded to inform the design of HTP screening approaches. However, expanding the application of the lung, intestine, and liver models to wider hazard prediction endpoints linked to known AOPs was also considered and implemented. For example, one advanced lung cell co-culture of epithelial and macrophage cells, cultured at the air–liquid interface has been assessed for its predictive nature for the titanium dioxide (TiO_2) in vivo response based upon AOP 173 (www.aopwiki.org/aops/173, inflammation-mediated lung fibrosis) using PCR array technology, and further examined for a more global understanding using a transcriptomic approach. This approach has also been applied toward evaluating the predictivity of liver HepG2 spheroids for hepatocarcinogenicity following NM exposure.[26]

For all in vitro models described in this section, detailed SOPs have been developed, which are open access, several of which have been published with accompanying instruction videos to enable new laboratories to learn and implement the project-created experimental methods (https://www.patrols-h2020.eu/publications/sops/index.php).[11,16,27] Inter-laboratory trials have been conducted with several of these SOPs, demonstrating their transferability and reproducibility across two to three laboratories, to facilitate greater uptake across multiple stakeholder communities for future toxicology assessment(s).[28] Thus, the catalogue of in vitro methods generated by PATROLS provide a strong non-animal suite of methods to evaluate the safety of NM, which can also be expanded to chemicals and can be applied to evaluate materials across their life cycle, linking with many of the objectives highlighted within the *Chemical's Strategy*.

2.2. Advanced Algae, Daphnia, and Zebrafish Larvae Testing for Long-Term NM Exposure

From an ecotoxicology perspective, we now have a significantly improved understanding of the longer-term (multiple days up to several weeks) impacts of NM exposure, across different trophic levels, and have also developed short-term early warning assays for chronic effects.

For algae, the so-called LEVITATT (LED vertical illumination table for algal toxicity tests) test setup has been developed.[29] This utilizes LED illumination from below the vessel containing the algae to allow a homogenous light distribution and temperature control while minimizing intra-sample shading. The setup optimizes the full sample volume for biomass quantification and at the same time ensures sufficient influx of CO_2 to support exponential growth of the algae. Additionally, the material of the test containers can be tailored to minimize adsorption and volatilization. These features tackle the challenges that are inherent to NMs and other non-soluble

chemicals and limit the light interference challenges that inherently exist. The experimental protocols have been published as a NAM for chronic exposures of algae, and an inter-laboratory trial has been executed across multiple partners. This demonstrated transferability, reproducibility, and robustness of the NAM, thereby facilitating uptake across multiple stakeholder communities for future toxicology assessment(s).

For daphnids, an advanced multi-generation test was developed by integrating the specific recommendations for ecotoxicological testing of NM as defined in OECD Guidance Document 317, into a long-term *Daphnia magna* reproduction test (OECD Test Guideline 211).[30] Because fate and dynamics of NMs over long-term exposures are important, the feasibility of assessing multi-generational effects in the first generation of offspring derived from exposed *D. magna*, while maintaining test conditions in accordance with regulatory test guidelines and guidance documents, was evaluated.

The zebrafish embryo is an excellent model organism for systematic toxicological testing of chemicals under the European REACH initiative as they allow identification of target pathways. Zebrafish offer a mapped and annotated transcriptome together with a rich repertoire of genetic, molecular, and cellular manipulation tools.[31] Within PATROLS, a transgenic fish line for early signaling of NM exposure was developed, facilitating the study of early markers of effect with a fluorescence reporter system.[32,33] This novel testing approach enabled the analysis of oxidative stress, a key mechanism by which NM induces cellular damage, leading to chronic effects of NMs on sensory systems, including olfaction, responses in neuromasts, and ion regulatory systems, and later leading to gill damage. Furthermore, Brinkmann et al. (2020) developed a protocol for a germ-free zebrafish larvae test, with clear steps to sterilize embryos of zebrafish, and to allow inoculation of microbes that the larvae encounter, a so-called gnotobiotic technique.[34] It is known that microbiota reside in and on animals, interacting closely with their hosts, modulating all immune responses and energy metabolism.[35] By combining gnotobiotic techniques with acute toxicity tests, Brinkmann et al. showed that host-associated microbiota protect zebrafish larvae against particle-specific toxic effects of silver nanoparticles.[34]

All these experimental model systems developed in PATROLS allowed the screening for lifespan and population-relevant adverse responses following NM exposure. For algae, this included the commonly reported apical endpoints, like growth expressed as biomass, but also included early warning biomarkers like chlorophyll content and types of pigmentation. For daphnia, the fecundity and reproduction numbers were measured as well as biomarker responses at the genetic level related to oxidative stress and reactive oxygen species formation, while for zebrafish larvae, apical development endpoints were recorded alongside biomarker responses at the genetic level and related to oxidative stress and inflammation. These enhanced in vitro ecotoxicological models can be combined with numerous state-of-the-art analytical and microscopic techniques to determine the impact of exogenous agents upon adverse responses, ranging from early marker responses (cell and physiological biomarkers), to impacts of species responses (morphological, physiological and apical endpoints) and population endpoints (fecundity and reproduction).

2.3. Methods for Extrinsic Physicochemical Characterization and Dosimetry

A key aspect of a tiered testing strategy for NM risk assessment involves selection of fit-for-purpose physicochemical characterization endpoints of relevance to hazard testing systems and exposure scenarios. Additionally, standardized or validated methods that meet the regulatory standards are required. In PATROLS, the strategies to characterize physicochemical properties involved consolidation of tests and methodologies to effectively define dose (what is delivered to in vitro and in vivo models), and characterization of the distribution, transformation, and amount of material potentially delivered to a biological target (**Figure 3**). This approach enabled identification of new physicochemical properties that explained the fate and adverse effects observed in the in vitro 3D tissue models following NM exposure,[22,36] and thus, can be used within the safe- and sustainable-by-design of NMs, in support of the *Chemicals Strategy for Sustainability*.

Furthermore, a suite of in silico tools was developed, spanning both hazard prediction models and models to understand NM dosimetry within in vitro and in vivo mammalian and ecological testing systems. Apart from ALI-based lung cell systems, the calculation of the delivered dose is often not considered in studies that deal with corresponding in vitro hazard testing systems due to difficulties in achieving supporting measurements in parallel. This was addressed in PATROLS by testing system-dependent properties in biologically relevant media, under highly controlled conditions, and applying this data in the development of in vitro dosimetry models. This allowed identification of the most suitable input parameters and measurement methods to improve dosimetry models further. One example was the in vitro particokinetics model, which provided an understanding of NM diffusion, sedimentation, and dissolution in cell culture medium and cellular uptake.[38] This model allows enhanced interpretation of the actual NM dose reaching the cells in a submerged in vitro environment. Furthermore, a graphical user interface for the in vitro 1D distorted grid dosimetry model was established, which makes it more user-friendly to calculate the deposited particle dose at different time-points.[39]

Similarly for ecological testing systems, the actual bioavailable fraction to which species are exposed (with a multitude of different exposure routes) is often not considered due to difficulties in achieving supporting measurements in parallel. PATROLS addressed this limitation by explicitly accounting for adsorption and absorption of NMs to organisms for which new analytical measuring techniques like single particle, single cell inductively coupled plasma-mass-spectrometry (ICP-MS), and advanced imaging techniques have been used. Collecting this type of data allows a greater mechanistic understanding of processes at the interface of exposure–biota.

2.4. In Silico Hazard Prediction

Another challenge faced by the field involves advancing the use of in silico modeling for hazard prediction of NM (eco)toxicological effects. Thus, to address this, PATROLS developed a range of mathematical models of in vitro and in vivo systems to

Figure 3. The PATROLS physicochemical testing strategy detailing the endpoint for evaluation, the corresponding methods applied, and the corresponding results and outputs. A) PATROLS dissolution multi-method platform;[22,37] B) PATROLS pro-oxidative multi-method platform.

predict the chronic effects from NM exposure, coupled to scaling of exposure-dose-responses between these two systems. For the lung system, a novel transcriptomic-based and AOP-informed nano-QSAR (quantitative structure–activity relationship) model has been developed.[40] This approach makes use of 1) an AOP established for lung fibrosis (www.aopwiki.org/aops/173) to

rationalize and select the upstream KEs; 2) perturbations in transcriptomics pathways (as opposed to traditionally used expression changes in single or multiple gene targets) as endpoints targeting the selected upstream KE; and 3) carbon nanotubes as model NMs that cause lung fibrosis in experimental rodent models, to identify their specific structural features underlying the fibrosis mechanism. This tool is being expanded to support the application of the AOP-anchored QSAR modeling scheme for predicting pulmonary pathology induced by nine nanoTiO_2 forms following inhalation by female adults C57BL/6 mice. Additionally, quasi-nano-QSAR models have been developed to provide predictions of the sensitivity of *Daphnia magna* to a variety of metal-based NM exposures.[41] Ultimately, this mathematical estimation has the prospect of leading to a user-friendly model allowing the calculation of a full dose-response curve when only a single effect concentration is reported. Thus, the model facilitates assessment of NM safety and is instrumental in efficiently prioritizing, ranking and grouping NMs.

For the rat system, a PBPK model to estimate delivered dose of NM in vivo has been implemented within PATROLS. The model comprises inhalation, oral, and intravenous routes of exposure. It includes several target organs and tissues: liver, tracheo-bronchial lung region, lung parenchyma, brain, spleen, and kidney. In PATROLS, the system has been based on the model from Li et al. as a starting point.[42] Subsequently, PBPK model parameters were estimated based on cerium dioxide (CeO_2) NM-212 and TiO_2 data.[43,44] In this analysis, probability distributions of critical model parameters have been inferred by fitting the model to the data (determining the likelihood of the model) and using Markov–Chain Monte Carlo sampling to construct the posterior probability distributions of the model parameters. These probability distributions of model parameters have been used as a priori estimates (so-called "priors") of the model parameters in the analysis of the distribution studies conducted. The model can support the identification of both chemicals and NMs with a potential for inducing chronic adverse health effects by evaluating their potential to accumulate in certain organs.

3. Lessons Learned and Recommendations

The development and application of innovative testing strategies will underpin the goals within the *Chemicals Strategy* that are focused on "reduce(ing) dependency on animal testing," but also to "improve the quality, efficiency, and speed of chemical hazard and risk assessments." However, to achieve this, a more dynamic approach is required to increase the acceptability of NAMs in a regulatory setting at an earlier stage of development (prior to OECD Test Guidelines being established), to promote their transition into use. While the work in the PATROLS project has focused on the development of NAMs tailored toward NM testing, they are also more broadly applicable to evaluating chemicals. However, for NAMs and the data that are derived by them to be accepted in a regulatory risk assessment framework, several factors need to be taken into consideration:

- Reproducibility: The transferability of protocols for NAMs and their reproducibility should be established and demonstrated within inter-laboratory trials. To achieve reproducibility in different labs, protocols must be harmonized and standardized, and it has been shown that the more complex the methods are, more extensive staff training is needed. This is not easy, as every lab has its own preferred protocols and consumables suppliers. To achieve standardization and harmonization of in vitro procedures, the listing of all materials used, coupled to detailed specification of every single step in a protocol are required. Providing this level of detail allows the protocols to be easily taken up by nearly all contributors within inter-laboratory trials. Additionally, it is important to sufficiently train personnel involved in inter-laboratory comparisons, preferably in-person, as this is essential to support reproducibility, particularly where the NAM SOPs are complex or specialized equipment is required. Albeit that space is often a limiting factor in the length of a manuscript to be published in a (peer reviewed) journal, we promote that people publish their method with as much detail as possible, using the opportunities for online supplements.
- Predictiveness: While highly complex cell models and/or dosing regimen may better represent the in vivo situation and usually result in the test systems being more predictive, this is not always the situation. For example, extended and repeated exposures are possible with the improved lung and liver models. Although these complex multi-cellular test systems exhibited greater sensitivity than standard monolayer cultures, and differences in toxicological endpoints were noted between acute and prolonged exposures to NM, the use of repeated exposure scenarios were not significantly different in terms of biological effect, as compared to a single exposure applied for the same duration.[12,36] However, the use of the primary EpiAlveolar model and repeated exposures over 3 weeks to low-concentration NM aerosols resulted in a robust and predictive outcome regarding inflammatory and fibrotic responses.[45] The use of the air–liquid interface lung cell models that are exposed to aerosolized materials either in a repeated manner, or over a prolonged time, may pave the way forward to mimic exposure and biological complexity and to reach regulatory acceptance of tests and test outcomes. Few adverse outcomes can at present be accurately predicted by using a combination of in vitro tests supplemented with computational models. Yet, this will be the way forward to reduce the use of experimental animals.
- Representativeness: In vivo exposure data are considered the gold standard against which new in vitro methods should be directly compared. However, there is an increasing appreciation that in vivo data also suffer limitations as they are not always reproducible in different species, nor are they representative of human responses due to significant physiological differences between humans and other species.[46–48] Nonetheless, in PATROLS, adverse effects observed in experimental animals (rats and mice) have been linked to effects that were induced in human cell–based models. To what extent the in vitro models predict human health effects remains to be investigated as such human exposure and effect data are lacking. However, this information will be of importance moving forward, especially if effects noted in

animals may also not always be representative of what occurs in humans with a similar exposure scenario.
- Realistic: For solid and complex materials such as NM and advanced materials, biological effect doses must be assessed in in vitro models to allow extrapolation. This requires careful characterization of the test material, as the dose may have to be modeled rather than being measured. Sample preparation of NM and advanced materials may significantly affect the biological responses and toxic potency. Thus, standardized sample preparation protocols are recommended and should be included in the research report.
- Validation: There are currently challenges around validation of NAMs for nanosafety due to the limited amount of comparable in vivo data available. In the absence of NM in vivo data, within the PATROLS method development pipeline, well-understood chemical controls were included, enabling the tests to be evaluated against standard in vitro approaches, which supported the demonstration of improved performance. The costs associated with validation are prohibitive; thus, large-scale international funding opportunities focused on requirements to support NAM assay validation, including large-scale ring trials, which is necessary to attain regulatory acceptance, are of great importance. Given the absence of such public financial support, AOP approaches may be useful to increase confidence in the NAMs and that new methods address the appropriate endpoints.

To date, many of the nanosafety approaches under development have focused upon simple, often single-component NM. The European Commission's *Chemicals Strategy for Sustainability*, however, points to the importance of novel or advanced materials as innovation that is needed to reach targets on a sustainable climate, neutral, and circular economy. Innovations in material manufacture are leading to more complex arrangements consisting of multi-component nanomaterials and mixtures, which differ substantially from the traditional NM. Thus, to enhance the applicability domain of some tools developed for NM in PATROLS, they have been transferred for implementation within new European projects (e.g., HARMLESS and SUNSHINE) to assess the hazard of this new generation of advanced materials, thus, future-proofing the NAMs to ensure their applicability to new emerging technologies that will drive the initiatives underlying the European Green Deal.

The PATROLS project has generated over 50 SOPs spanning a range of in vitro and in silico NAMs to support both human health and environmental hazard assessment of NM. Interlaboratory trials were embedded within the project to demonstrate the transferability and reproducibility of several SOPs. While these methods have not been fully validated, the time needed for validation is substantially longer than the target timelines in the *Chemical Strategy for Sustainability*. Hence, in the meantime, it is important to note that NAMs, such as those developed in the PATROLS project, can be applied in a tiered testing strategy to reduce the need for in vivo evaluation by providing meaningful weight of evidence data. Furthermore, these methods will be important at an early stage of the innovation process (e.g., for screening purposes) within integrated approaches to support waiving and therefore avoiding the need for in vivo tests.

4. Conclusion

The work performed in PATROLS has advanced the state-of-the-art in NM human and environmental hazard assessment through the provision of improved and innovative tools that (for human hazard) could support the reduction of animal testing by applying the approaches at an early stage of the product development pipeline within a safe-and sustainable-by-design strategy. The project has tailored these novel testing systems to improve our understanding of the consequences of long-term NM exposure in both humans and environmental species, spanning:

1) Methods to characterize extrinsic NM properties in realistic, complex biological matrices;
2) In silico tools to enable extrapolation of in vitro findings to effects observed in rodents by dosimetry and exposure assessment modeling (e.g., (Q)SARs, PBPK);
3) Innovative, heterotypic in vitro models of the human lung, intestine, and liver that more closely mimic human physiology and include more realistic exposure strategies;
4) Predictive ecotoxicity tests relevant to a range of species along the food chain; and cross-species and read-across models.

Thus, the outcomes from the PATROLS project have addressed multiple aspects of the *Chemicals Strategy for Sustainability* to minimize the requirement for in vivo assessments by providing next-generation in vitro testing approaches for human hazard assessment. PATROLS also provided a set of tools to support the understanding of the hazard outcomes both for human health and the environment, all of which facilitates the safe and sustainable development of new products before they reach the market (https://www.patrols-h2020.eu/publications/sops/index.php).

Future acceptance and the transition to use of NAMs in regulatory frameworks will be critical in realizing the goals of the *Chemicals Strategy for Sustainability*. To achieve this, open dialogue between method developers and end-users, including those in industry, contract research organizations, and the regulatory risk assessment community, will be crucial to build a wider understanding of the context in which the NAMs can be applied, and their strengths and limitations. Empowering individuals in the risk assessment field by raising awareness and visibility of these testing approaches will help to build end-user confidence. This in turn will encourage the incorporation of NAMs in regulatory evaluation strategies, particularly as part of WoE considerations, thereby accelerating their uptake and use.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

ecotoxicity, in silico models, in vitro 3D models, nanomaterials, nanosafety, physico-chemical characterization

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