MACRAMÉ

Informed Recommendations on the Needs for Test Guideline Developments and the Standardization of Test Methods

Draft

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I. Introduction

These *Draft Informed Recommendations on the Needs for Test Guideline Developments and the Standardization of Test Methods* were initially developed within the EU-funded project MACRAMÉ.

The MACRAMÉ project is fully aligned with the EU ambitions to secure the safety and sustainability of new chemicals, materials, products and processes in order to strive for zero pollution and toxic-free environments. MACRAMÉ concentrates on the development of methodologies that are applicable to Advanced Materials (AdMas) and AdMa-enabled commercial products. This is achieved through development and demonstration of novel methodologies or adaption of existing methods, and by advancing their harmonisation & standardisation on three MACRAMÉ Material Families of inhalable carbon-based AdMas of various morphologies and dimensions. These materials include graphene-related 2D materials (GR2M), carbon nanofibres (CNFs, including carbon nanotubes (CNTs)), and poly lactic-co-glycolic acid (nano)particles (PLGA).

During the method development for safety testing of carbon-based AdMas and AdMa-enabled products, MACRAMÉ gains valuable scientific and technical experiences. These form the basis for these *Draft Informed Recommendations on the Needs for Test Guideline Developments and the Standardization of Test Methods*. The MACRAMÉ project also identified needs and gaps towards regulatory and policy requirements for AdMas, captured in the Needs Assessment Report on Regulatory & Policy Frameworks. Needs and challenges mainly lie in addressing the whole life cycle of (products with) AdMas. This requires combined imaging and analytical approaches to quantify transformed AdMas. In addition, this needs life cycle thinking in material design and developing sector-specific methodologies and tools for Safe-and-Sustainable-by-Design (SSbD), along with incentives to encourage their use. Input on highly required validated and standardised test methods was gathered by MACRAMÉ during multiple events, including the 1st Risk Assessors Summit [1], online Harmonisation and Standardisation Workshops [2] and online Webinars [3].

Hence, the draft informed recommendations outlined in this document are based on the expertise of a wide range of experts and the experiences gained during the MACRAMÉ project. These draft informed recommendations will be disseminated amongst stakeholders to gather feedback in multiple ways, i.e. through an online survey, discussions during the Joint Regulatory Risk Assessors Summit, and a subsequent webinar. Each chapter is dedicated to one recommendation. It is composed of the recommendation itself, a short description of the issue, a brief overview of the state-of-the-art and an information about the envisioned outcome and the required action that are required to take towards this envisioned outcome. Included is a selection of relevant references given at the end of each recommendation.

Method development and standardisation applicable for nanomaterials and other AdMas is highly needed due to their specific challenges in safety testing. Chemical risk assessment is based on testing of chemicals due to substance specific effects. AdMas possess further complexity due to their morphological properties on top of their chemical identity. It is well known that particles come with their own hazard profiles, e.g. due to their biopersistence or specific (fibrous) shape [4]. When cell-based toxicity testing comes into play, AdMa need to be tested administered in their particulate form to identify such a specific particulate hazard profile. Current in vitro test methods might not be applicable for testing of particles or fibres, or might not (sufficiently) cover morphology or transformation

aspects. Furthermore, specific AdMas come with additional challenges towards physico-chemical characterisation and analysis, e.g. identification of multi-component materials or carbon-based materials in test systems or tissues. There have been some advances in the last 10 years in adapting methods, which allow for the testing of (simple) nanoparticles (e.g. in EU projects Gov4Nano [5], NanoHarmony [6]), and on how best to test GR2M (e.g. in the Graphene Flagship [7]). Testing of AdMas can build on these advances and experiences in the nanomaterial field, although the higher complexity of AdMas may pose further challenges.

The overarching goal of the MACRAMÉ Informed Recommendations is to show the specific need for test method development and standardisation of AdMas. This should enable regulatory testing of AdMas that is acceptable and manageable by industry, regulators and other stakeholders. With the list of draft recommendations provided in this document we also aim to address different stakeholders:

- inform and encourage method developers to fill the identified gaps.
- inform and encourage policy makers and funding agencies to fund support the necessary developments.
- support OECD, ISO, CEN and other standardisation and validation bodies and encourage to put emphasis on this topic in the future.

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1. MACRAMÉ. 1st Risk Assessor Summit. Available at https://macrame-project.eu/press-and-events/#RA-Summit1.
2. MACRAMÉ. Harmonisation and Standardisation Workshops. Available at https://macrame-project.eu/press-and-events/#HS-Workshop2.
3. MACRAMÉ. Webinars. Available at https://macrame-project.eu/press-and-events/#MACRAME-Webinars.
4. Rittinghausen, S. *et al.* The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. *Particle and fibre toxicology* **11,** 59 (2014); 10.1186/s12989-014-0059-z.
5. EU Project. Gov4Nano. Available at https://cordis.europa.eu/project/id/814401.
6. EU Project. NanoHarmony. Available at https://cordis.europa.eu/project/id/885931.
7. Graphene Flagship. Available at https://graphene-flagship.eu/.

1. Physico-chemical Characterisation of Advanced Materials

Recommendation

Develop and establish a guidance on a minimum set of physico-chemical characterisation parameters to be determined for safety testing and profound risk assessment for different categories of advanced materials.

Issue

Proper physico-chemical characterization provides the detailed and accurate information needed to assess, identify and manage risks effectively, potentially in grouping approaches. However, complete physico-chemical characterisation of a material can be very extensive and time-consuming. The question arises, which minimum physico-chemical characterization parameters are required for risk assessment of specific categories of AdMas and their intended use.

State-of-the-art and resources to be considered

Physico-chemical characterization reveals the physical properties, composition and microscopic structure of a material. This material characterization helps to identify the intrinsic properties of a material, such as shape, size, flammability, and reactivity. Understanding these properties is critical for recognizing potential hazards associated with the material. A category of AdMa for which an identification of essential physico-chemical characterisation parameters was performed is the graphene family of materials (GR2M). For GR2M, Wick et al. [1] identified three essential physico-chemical properties: number of layers, C/O ratio and average lateral dimensions. The Graphene Council expanded this set to characterize GR2M regardless of production method and raw materials used in its Graphene Classification Framework (GCF) [2]. The GCF lists the 19 key graphene material characteristics that should be reported in the technical data sheet for commercial application. Of these 19, nine are considered the minimum set of detail necessary to provide a one-sentence shorthand description (syntax) for any type of form of GR2M. These are layer count, lateral size, particle shape, oxidation, functionalisation, form, solvent (if dispersed), production method, and raw material. The GCF is now being developed into an official ISO standard [2]. Next to the minimum set of relevant characteristics and the syntax, this standard is set to comprise methods of testing of each characteristic, a range of the expected measurement values for benchmarking, and to define a standardized technical data sheet. These methods were at least in part applicable to even more complex AdMas, which contain GR2M embedded in a variety of organic matrices as investigated in MACRAMÉ. A report by ECHA also stresses the need for thorough characterization of each type of graphene and 2D material including their production and fate but proposes minimal requirements with chemical composition, structure, lateral size, and number of layers as the only mandatory characteristics. However, other important characteristics have also been reported demonstrating the lack of agreement in the scientific and regulatory communities even for this well-investigated AdMa class. For other and especially emerging AdMas, such lists are not existing at all.

For other AdMas, no such evaluation scheme has been conducted, although the European REACH Regulation aims to characterise nanoforms of substances by a minimum of particle size distribution, shape, volume specific surface area and surface chemistry [3]. Nevertheless, given the broad scope of AdMas as described by the OECD [4], defining a universal set of requirements for all categories of AdMas

is likely improbable. The lack of (certified) reference materials for AdMas further complicates the issue. It might, therefore, be more practical to establish requirements tailored to specific categories of materials. Yet, as shown by the large variety within the realm of 2D materials, defining specific categories remains very challenging. Similar challenges exist for multi-component materials (see recommendation 3).

In-depth characterization of AdMas could focus on critical properties such as size, shape, chemistry, physical attributes, stability, and oxidation state. The OECD TG 124 [5] and OECD TG 125 [6], along with related OECD/ISO documents, for example provide comprehensive methods for determining the volume-specific surface area of manufactured nanomaterials and the nanoparticle size and size distribution (1-1000 nm), respectively. Using an appropriate testing medium during dispersion and characterization is key to obtaining accurate and meaningful results not only for characterization but also for the application of toxicological in vitro methods.

Output / product(s) envisioned and action(s) required

The envisioned product is a guidance to identify the minimal morphological information needed for physico-chemical characterisation of AdMas. To effectively achieve this, it is imperative to properly categorise AdMas. In addition, this will depend on the purpose of the assessment, a decision tree to guide users appears needed to determine the minimal characterization requirements that are needed (based on material type, use, purpose and exposure).

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2. Graphene Council. Graphene Classification Framework (GCF) https://www.thegraphenecouncil.org/page/GCF. Available at https://www.thegraphenecouncil.org/page/GCF.
3. EU Commission. Commission Regulation (EU) 2018/1881 of 3 December 2018 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annexes I, III,VI, VII, VIII, IX, X, XI, and XII to address nanoforms of substances, http://data.europa.eu/eli/reg/2018/1881/oj (2018).
4. OECD. Advanced Materials: Working Description. OECD Publishing, 2023, https://doi.org/10.1787/4b5ba38d-en.
5. OECD. Test No. 124: Determination of the Volume Specific Surface Area of Manufactured Nanomaterials. OECD Publishing, 2022, https://doi.org/10.1787/abb72f8f-en.
6. OECD. Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials. OECD Publishing, 2023, https://doi.org/10.1787/af5f9bda-en.

2. Detection and Quantification of Advanced Materials in Biological Matrices

Recommendation

Develop and establish a guidance for the detection and quantification of advanced materials and especially carbon-based materials in biological matrices (e.g. test media, biological matrices).

Issue

The lack of regulatory guidance on the detection and quantification of AdMas upon contact with biological matrices causes great uncertainty. This makes it impossible to fully apply regulation and guidelines, which would require detection and/or quantification of materials for classification purposes. This is further worsened for carbon-based materials, for which identification is at least severely hindered especially if a material with carbonaceous composition is to be detected within biological matrices.

State-of-the-art and resources to be considered

Many of the qualitative and quantitative techniques that are used for the detection and quantification of materials and AdMas are based on the different physico-chemical properties of the materials, as compared to the biological background. For example, Secondary Ion Mass Spectrometry (SIMS) is a powerful technique that allows for the analysis of surfaces and therefore may be applied to solid samples. SIMS enables the identification of elemental composition, isotopic ratios, and molecular structures with high spatial precision. There are also some SIMS techniques that make use of lower energy and allow the detection of small molecules. These can be useful as some AdMas are made of polymers, which could be detected using e.g. Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS). However, SIMS requires for the sample to contain some element that can be distinguished from the biological background. Thus, the detection of AdMa composed only of elements common in biological samples (e.g. C, N, O) is difficult with this technique. Similar issues hamper the use of other methods. In the past, nano-SIMS was successfully used for the detection of CNTs embedded within a biological matrix, but only be using traces of non-carbon catalyser elements used in the production of the CNTs.

When it comes to detection and quantification of AdMas in biological fluids and in cell culture media, there are other types of limitations that need to be considered. In principle, liquid samples are subjected to the same limitations described above for solid samples. However, strategies aiming at isolation and separation of the dispersed materials can be deployed. In this case, it is important that the isolation method achieves separation of the AdMas in a quantitative and reproducible way, without altering the physico-chemical properties of the material in a way that would hinder further characterization. It is also important that proper blanks and controls are included within the experimental protocol. Dynamic Light Scattering (DLS) and Nanoparticles Tracking Analysis (NTA) are among the most available and used techniques for the detection of particle size distribution and quantification of nanomaterials and AdMas in aqueous media. They are relatively accessible and simple to use and provide data on the hydrodynamic diameter of particles. Therefore, they may suffer some severe limitations because they assume that the shape of the AdMas is spherical, thus results with AdMas that are non-spherical are not reliable.

There is a large interference with other components present in the cell culture media. The methods work best with ultrapure water, but this is not representative of the particle behaviour in cell culture media. Furthermore, the dispersion to be analysed with dynamic light scattering (DLS) or particle tracking analysis NTA needs to be "sufficiently stable", which is an arbitrary definition. Dispersion stability depends, along with other parameters, on the nature of the AdMa, on temperature, pH, ionic strength, concentration and presence of proteins (e.g. serum). These parameters will dictate the behaviour of the material in dispersion and determine aggregation and sedimentation.

To detect, visualize and identify particles in cells and tissue-like cell cultures, numerous methods have been developed during the last decade. Darkfield Microscopy (DFM), Hyperspectral Imaging (HSI), Confocal Raman Microscopy (CRM), Laser Scanning Microscopy (LSM), Infrared Microscopy (IRM) or ToF-SIMS are all useful methods, which not only detect but, within limits, can also identify materials. in cells and tissues [1–3]. This is increasingly relevant for AdMas containing mixed and/or carbonaceous materials, e.g. GR2M or CNT [4]. Here, CRM or IRM may help identify materials in questions and could even quantify the number of inclusions, serving as a semi-quantitative approach. Analytical methods such as laser ablation induced coupled plasma mass spectroscopy (LA-ICP-MS) can quantify at least metal-based AdMas in single cells. Nevertheless, the quantitative analysis of AdMas in cells and tissues is still an issue and further development is needed to cover the whole spectrum of materials.

Output / product(s) envisioned and action(s) required

The available microscopic techniques that allow for the detection and quantification of AdMas within biological matrices now need to be combined in a reasonable and most versatile way for which a guiding document should be developed. This is particularly needed in the case of carbon-based materials, which are difficult to detect and quantify with currently available approaches. Whenever these are available, e.g. microscopy techniques based on confocal Raman spectroscopy and/or HyperSpectral imaging, maybe employed but there still is an urgent need of generation of an adequate guidance. This needs to link to the respective SOPs of the methods. This links also to the recommendation 10 in which the detection and quantification of AdMas and their transformed versions in released fragments is outlined.

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1. Veith, L. *et al.* Detection of ZrO_2 Nanoparticles in Lung Tissue Sections by Time-of-Flight Secondary Ion Mass Spectrometry and Ion Beam Microscopy. *Nanomaterials (Basel, Switzerland)* **8** (2018); 10.3390/nano8010044.

2. Vennemann, A., Alessandrini, F. & Wiemann, M. Differential Effects of Surface-Functionalized Zirconium Oxide Nanoparticles on Alveolar Macrophages, Rat Lung, and a Mouse Allergy Model. *Nanomaterials (Basel, Switzerland)* **7** (2017); 10.3390/nano7090280.

3. Seiffert, S. B. *et al.* LA-ICP-MS and Immunohistochemical Staining with Lanthanide-Labeled Antibodies to Study the Uptake of CeO2 Nanoparticles by Macrophages in Tissue Sections. *Chemical research in toxicology* **35,** 981–991 (2022); 10.1021/acs.chemrestox.1c00433.

4. Chaloupková, Z., Žárská, L., Belza, J. & Poláková, K. Label-free detection and mapping of graphene oxide in single HeLa cells based on MCR-Raman spectroscopy. *Analytical methods : advancing methods and applications* **15,** 5582–5588 (2023); 10.1039/d3ay01122d.

3. Testing of Multi-Component Advanced Materials

Recommendation

Develop and establish a guidance on how to perform risk assessment for multi-component advanced materials.

Issue

Multi-component AdMas may pose unintended risks due to a synergistic effect of their individual components. This raises questions on the regulatory preparedness for such AdMas, as they may induce mixture effects that may not be adequately addressed in regulatory frameworks, if such AdMas are not identified as (special) mixtures. The lack of regulatory clarity for multi-component AdMas leads to uncertainties for manufacturers and their investments in these materials.

State-of-the-art and resources to be considered

For multi-component nanomaterials (MCNMs) the issue was recently identified that current regulations (e.g. REACH) may not fully be prepared for such materials [1]. As a result it may not be straightforward what the regulatory requirements for MCNMs would be, partly based on unclarity on the term that does not have an (inter)nationally agreed description or definition. For AdMas the issue of regulatory preparedness may even be bigger, as these more often tend to be composed of multiple components. As a result, different terms carrying an equivalent meaning are found in the literature (nanocarriers, nanocomposites, hybrid materials, etc). However, the REACH definition of a substance includes mono and multi-constituent substances, which may translate into multi-component nanomaterials being classified as a mixture. The current approach is then to individually test each component, which may overlook potential hazardous synergistic effects, although applying a standardised mixture assessment factor (i.e. adding an additional safety factor to address for this uncertainty) may address this to some extent. Similar issues are known for chemicals (e.g. OECD Guidance Document 23 [2]), but the complex nature of advanced multi-component materials may give rise to additional issue.

More detailed assessments of release and bioavailability of the different components of multi-component AdMas, however, may provide more clarity on the potential of mixture toxicity. As a first step to address mixture toxicity, the development of a guidance to perform dissolution of multi-component nanomaterials in biological relevant media (pulmonary fluid and gastrointestinal tract) is currently underway. This effort is working on three SOPs and represents the first step to address toxicity of multi-component nanomaterials [3]. Further testing may then be performed on the leachates of the multi-component nanomaterials, that may provide further insights in potential synergistic/antagonistic effects.

Output / product(s) envisioned and action(s) required

Guidance is needed on how to enable the adequate testing of multi-component AdMas. This may include an integrated approach for testing and assessment (IATA) for an effective assessment.

For regulations clarity on the terminology is essential to provide clarity on the regulatory requirements for multi-component AdMas.

1. Hunt, N. *et al.* Regulatory preparedness for multicomponent nanomaterials: Current state, gaps and challenges of REACH. *NanoImpact* **37,** 100538 (2025); 10.1016/j.impact.2024.100538.

2. OECD. Guidance Document 23 on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Publishing, 2019, https://doi.org/10.1787/0ed2f88e-en.

3. Di Cristo, L. *et al.* Critical aspects in dissolution testing of nanomaterials in the oro-gastrointestinal tract: the relevance of juice composition for hazard identification and grouping. *Nanoscale advances* **6,** 798–815 (2024); 10.1039/d3na00588g.

4. Predicting Toxic Potential of Fibres Based on Physico-Chemical Properties

Recommendation

Develop a guidance for the determination of toxic potential of fibres based on physico-chemical properties i.e. critical dimensions, biopersistence and rigidity. This needs to be backed-up by determining and establishing relevant threshold values and developing regulatory accepted test methods.

Issue

It is well known that inhalation of naturally occurring mineral fibres such as asbestos can lead to the development of lung diseases and even incurable mesothelioma. The same is true for manufactured (advanced) fibres if they have specific properties, i.e. specific dimensions, biopersistence and rigidity. Establishing relevant threshold values for these properties is pivotal for SSbD as well as for regulatory testing.

State-of-the-art and resources to be considered

Fibres have a fibre specific toxicity if have specific dimensions, are biopersistence and are rigid [1]. Determining these properties can help determine if a specific fibre material can lead to lung diseases or mesothelioma or not, without requiring extensive (toxicity) testing. The critical dimensions according to WHO counting rules are set to a width of less than 3 µm, a length greater than 5 µm, and a minimum aspect ratio of 3:1. The critical dimensions can be either fulfilled by single fibres or fibre-shaped bundles (agglomerates, aggregates). Standardised and harmonised methods for the size determination of fibres are available (e.g. OECD TG 125 [2]).

The biopersistence is a combination of non-clearance (linked to the length and rigidity of the fibres) and the biodurability defined by a low solubility / dissolution rate in relevant biological fluids. Standardised and harmonised methods for the determination of solubility and dissolution rate are available or under development (OECD TG 105 [3], OECD TGP project 1.5 [4]).

The rigidity is linked to the failure of the macrophages to bend and therefore incorporate the fibre leading to frustrated phagocytosis [5]. The diameter of a fibre (bundle) may provide a proxy for rigidity, but harmonised/standardised methods are lacking for this property.

Output / product(s) envisioned and action(s) required

To support material developers in testing if their fibres are critical, a Guidance Document leading through the determination of critical dimensions, biopersistence and rigidity is needed. To support this development the following activities are required.

Critical dimension: The critical values for the fibre width and length are well established and standardised measurement procedures are established (e.g. OECD TG 125 [2]). A challenge is the measurement of fibre bundles. Development of sample preparation and measurement procedures is needed.

Biopersistence: An *in vivo* half-life threshold that induces no fibrosis or tumours need to be determined and correlated to solubility/ dissolution rates [6]. Based on this threshold values for solubility/ dissolution rates need to be established and linked to standardised measurement protocols, that also specify the relevant test parameters (fluids, pH, temperature,…).

Rigidity: A threshold value for critical rigidity, inducing frustrated phagocytosis need to be determined and established. For the determination of the threshold value the macrophage assay is a valuable instrument, that needs to be further developed and standardised. Additional methods for the determination of the rigidity need to be developed, those can be in vitro methods (e.g. macrophage assay) as well as physico-chemical testing approaches (e.g. using electromechanical resonance or micromanipulation).

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1. Rittinghausen, S. *et al.* The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. *Particle and fibre toxicology* **11,** 59 (2014); 10.1186/s12989-014-0059-z.
2. OECD. Test No. 125: Nanomaterial Particle Size and Size Distribution of Nanomaterials. OECD Publishing, 2023, https://doi.org/10.1787/af5f9bda-en.
3. OECD. Test No. 105: Water Solubility, 1995, https://doi.org/10.1787/9789264069589-en.
4. OECD. Guidelines for the Testing of Chemicals. Available at https://www.oecd.org/en/topics/sub-issues/testing-of-chemicals/test-guidelines.html.
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6. Madl, A. K. & O'Neill, H. C. Fiber biodurability and biopersistence: historical toxicological perspective of synthetic vitreous fibers (SVFs), the long fiber paradigm, and implications for advanced materials. *Critical reviews in toxicology* **52,** 811–866 (2022); 10.1080/10408444.2022.2154636.

5. Sample Preparation for Safety Testing of Advanced Materials

Recommendation

Develop and establish a guidance on sample preparation for safety testing of advanced materials specified for respective categories of advanced materials, specific test method, biological system, and/or endpoint.

Issue

AdMas need to be tested in their particulate form. This raises challenges for sample preparation. Generally, the aim is to test the 'as produced' material, while the material generally needs some preparation steps to allow a proper dosing in the test system, potentially changing the properties of the material. To further complicate the issue, sample preparation may differ depending on the tested endpoints. Physico-chemical characterisation of the 'as produced' material, for example, may need different sample preparation from that for toxicity testing.

State-of-the-art and resources to be considered

While some AdMas may have their own (additional) challenges, for nanomaterials issues and potential solutions have been identified and published by OECD [1]. This document is currently in revision and will change considerably (publication expected Q3/Q4 2025). While some of the potential solutions identified may be applicable to AdMas as well, the updated document remains focused on nanomaterials.

The often complex multi-component nature of AdMas challenges their physico-chemical characterisation. The measurement equipment for physico-chemical parameters may require steps that may already change (some of) the physico-chemical parameters of the material (e.g. certain electron microscopy techniques require the material to be in a vacuum). For in vitro toxicity testing with cell models most challenges are related to the fact that many tests require exposure in an aqueous medium. This generally then requires ways to disperse the (particulate) materials. Depending on the test system and particle size, dispersion stability can be the result of an interaction between the dispersed substance and the specific (aqueous) medium used. For facilitate an easy data interpretation, it is important to ensure stability under exposure conditions. However, other methods may benefit from the fact that micron-sized particles finally settle, e.g. onto bronchial cell culture models, or are ingested by macrophages after deposition. In any case the dispersion may be designed in such a way that it mimics the expected exposure situation. For environmental aqueous media guidance is available for difficult test chemicals [2], but this does not sufficiently cover difficulties with particles. Therefore, for nanomaterials an OECD TG 318 is available has been developed that provides methodology to disperse nanomaterials in aqueous media as well as methodology to determine the stability of such dispersions [3]. These methodologies can be adapted to AdMas. For media for human health testing, similar methodologies may work, but these methodologies may need to be scrutinised further for the differences in aqueous media. The specific behaviour of multi-component materials may show further challenges in sample preparation (see recommendation 3).

Within the MACRAMÉ project SOPs are being developed for filtering respirable particles from aerosols to allow further testing. This also includes methods for sampling relevant subfractions, the different

steps of detaching particles for filters, and (potential) necessary fractionation for further testing. These steps may also influence the material itself, i.e. the particles in the air may differ from particle samples detached from filters and/or fractionated and should be carefully considered in the light of the applicability of the intended testing system (e.g. lung model).

Output / product(s) envisioned and action(s) required

The guidance on sample preparation for safety testing of AdMas should specify the required steps for the respective categories of advanced material, specific test, biological system, and/or endpoint.

In order to develop such a guidance, SOPs need to be developed for the adequate sample preparation in the media needed for the different safety testing methods required for AdMas. The guidance should link to existing OECD documents and ISO standards and highlight the special needs for the appropriate sample preparation of AdMas.

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1. OECD. Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials. OECD Publishing, 2012, https://doi.org/10.1787/ed430e1d-en.
2. OECD. Guidance Document 23 on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Publishing, 2019, https://doi.org/10.1787/0ed2f88e-en.
3. OECD. Test No. 318: Dispersion Stability of Nanomaterials in Simulated Environmental Media. OECD Publishing, 2017, https://doi.org/10.1787/9789264284142-en.

6. Controlling and Describing the Dosimetry of Advanced Materials

Recommendation

Develop and establish a guidance on controlling and measurement of the dosimetry for adequate and accurate in vitro and in vivo testing for human and environmental toxicity of advanced materials with realistic and relevant particle doses.

Issue

Meaningful in vitro experiments with lung cell models to assess the hazard potential of AdMas require the sound description of relevant doses. Administered dose, delivered dose and, if possible, also cellular doses (potentially in different cell types) need to be described using state-of-the art analytical methods (see [1] for definition).

State-of-the-art and resources to be considered

Unlike well-defined particles, many AdMas exhibit complex morphologies and/or large size distributions. They are also subject to chemical alterations during their life span. Researchers need to carefully consider this complexity before selecting most relevant in vitro experiments mirroring possible routes of exposure. The exposure situation, and the possible uptake of AdMa particles into cells should be qualitatively described using appropriate microscopic imaging methods suitable to detect the particles in question. Also for lung cell models, which were a major topic in MACRAME, the influence of barrier structures such as mucus or lung surfactant as found in some evolved bronchial or lung models should be taken into account. Additionally, 3-dimensional cultures exist that mimic target tissue structures such as the lung parenchyma and these may require further evolved quantitative methods to describe particle uptake by different cell types (e.g. epithelial plus phagocytic cells). Wherever possible, quantitative uptake data should be made available.

Dosimetry can affect the interpretation of hazard effects. In any test system, the administered dose of an AdMa should range from the no-effect level up to a reasonable worst-case scenario, whereby the delivered dose reaching the cells or test organism is most decisive and should be determined.

In case of inhalation exposure, aerosol application is also an option and different model systems for the upper (bronchial) and lower airways (lung parenchyma) may be used to investigate inhalable or respirable particles size, respectively. When AdMas are to be tested for inhalation toxicity in vitro, inhalable or respirable particulates should be made available to competent cell cultures, e.g. as particle suspensions or as dry powder aerosols. The still most common and regulatory relevant dose metric is mass, although specific surface area may be more relevant for nanomaterials. A reasonable dosing of AdMas to assess a potential hazard may be derived from the lung burden typically found in inhalation studies (e.g., according to OECD TG 413 [2]).

Dosimetry is also an issue in case of ecotoxicity experiments, especially when prolonged exposure periods are used. Similar to cell culture testing it needs to be considered that AdMas stability may be challenging in ecotoxicity exposure media (such as freshwater, marine water, or cell culture medium). Hence, accurately assessing the delivered dose (i.e., the actual exposure dose received by cells or organisms) is essential to avoid both overestimation and underestimation of assay outcomes.

Therefore, it is recommended to quantify the delivered dose throughout the exposure period (e.g., at 24, 48, or 72 hours).

In MACRAMÉ, methods for detecting graphene in cells were developed (MACRAMÉ Deliverable in preparation).

Output / product(s) envisioned and action(s) required

A better guidance on a dosimetry oriented towards a realistic particle dose and distribution in human and animal tissues is needed as it will increase the predictability and acceptance of upcoming in vitro NAMs. The quantitative description of delivered and cellular dose is one major key especially for single and repeated dose applications, which become possible with suited cell culture models (LIT). The further development of quantitative high-resolution methods based on a combination of microscopic, analytical, and (mass) spectroscopic methods is expected at least in part to needed to cover the growing complexity of AdMa analyses (see recommendation 2).

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1. Schmid, O. & Cassee, F. R. On the pivotal role of dose for particle toxicology and risk assessment: exposure is a poor surrogate for delivered dose. *Particle and fibre toxicology* **14,** 52 (2017); 10.1186/s12989-017-0233-1.

2. OECD. Test No. 413: Subchronic Inhalation Toxicity: 90-day Study. OECD Publishing, 2018, https://doi.org/10.1787/9789264070806-en.

7. Testing and Assessment Strategies for Profound Risk Assessment of Advanced Materials Depending on Identified Exposure Points

Recommendation

Develop an Integrated Approach to Testing and Assessment (IATA) for advanced materials that guides risk assessors towards indispensable tests on an advanced material and/or advanced material containing product, depending on and consider the relevant identified exposure points as well as taking into account the advanced material's and/or product's associated fate and mobility.

Issue

The large number of different AdMas being produced represents a real challenge to ensure all are safe and fulfil regulatory obligations. Furthermore, assessment of their sustainability requires that the life cycle of AdMas and/or AdMa-containing products are taken into account. This requires a more effective approach to testing as it will be impossible to test each and every material and each step in their life cycle.

State-of-the-art and resources to be considered

For nanomaterials the issue was already identified that it will be impossible to test each and every nanoform for safety assessment. To help tackle this issue, the GRACIOUS project developed different IATAs representing different exposure routes to guide and implement testing in a meaningful and cost-effective way. An IATA will provide a stepwise approach to testing, which is cost effective while eventually addressing regulatory endpoints. It allows to initially group nanoforms based on physico-chemical properties (e.g. dissolution), which will inform a stepwise testing strategy towards the more complex (regulatory) endpoints [1]. It can help focus the assessment, e.g. by first using test systems that mimic the various human biological barriers to allow uptake predictions and identifying most relevant exposure routes. For environmental endpoints fate testing can help identify the environmental compartment of most concern. Life cycle assessment can further inform the IATAs by identifying relevant exposure points in the life cycle, as such identifying the starting point of an IATA. Based on a generalised approach [2], the GRACIOUS project developed 17 IATAs to address human hazard and 23 IATAs to address environmental hazards [3]. These can serve as starting points for the developments of IATAs for AdMas. The IATAs represent different exposure scenarios covering inhalation, ingestion, dermal, aqueous, soil, sediment and air. Information needs are generally overlapping and followed a similar approach. The HARMLESS project has developed tools to incorporate such approaches in safe-and-sustainable-by-design [4]. Test method developments in MACRAMÉ can inform decisions in IATAs and contribute to their further development for AdMas.

Output / product(s) envisioned and action(s) required

The envisioned outcome is one IATA or a series of endpoint specific IATAs for AdMas that guides risk assessors effectively towards indispensable tests on an AdMa and/or AdMa-containing products. The IATA(s) will take into account the relevant identified exposure point and subsequent fate and mobility of the (transformed) AdMa assessed.

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8. Identify Releases of (transformed) Advanced Materials along their Life Cycle

Recommendation

Develop and establish a guidance on a best approach identify where (transformed) advanced materials are released along their life cycle to inform a more dedicated risk assessment.

Issue

For sustainability assessment a closer look to the entire life cycle of a (product containing) AdMas is essential. Such an assessment may already inform design issues related to the first steps in manufacturing an AdMa. Furthermore, identifying critical steps in the life cycle where (transformed) AdMas are release will inform and shape the risk assessment for (product containing) AdMas. Guidance is needed on an effective material flow analysis approach that can effectively identify such critical steps.

State-of-the-art and resources to be considered

To determine if a new product is a more sustainable alternative or not, an analysis of its entire life cycle is required. An improvement e.g. in the use of mineral resources during the manufacturing phase for example might be overshadowed by an increase in the human toxicity during the end-of-life of the product. The (energy and) materials requirements along the entire life cycle of the product will depend on the different processes applied in each phase of the life cycle, the machinery used, and the chemical properties of the materials. Moreover, during the different phases of the life cycle, there will be losses of materials and/or chemicals into water, air and/or soil. The impact of these losses will depend on the properties of the materials, the substances that compose them, and the route of exposure to this new availability of the materials and chemicals.

Therefore, it is important to define the material flows that might take place at the life cycle phases of a new product and incorporate these into a testing concept [1]. A material flow analysis can be performed to represent these flows, and mainly (dynamic) probabilistic models can be used [2,3]. Such an analysis will help identify the different forms and materials released as well as the environmental compartments where they are released [4,5]. Their environmental fate will provide further insights on where and how to obtain the data needed for the risk assessment. To apply a material flow analysis it is essential to first clearly define the system boundary and scope of the study. This will then inform the exact approach for the material flow analysis, e.g. a static probabilistic (not considering stocks) or a dynamic and probabilistic model (considering the dynamic of the system and stocks of the material in specific compartments along the life cycle).

Output / product(s) envisioned and action(s) required

Guidance is envisioned on a best approach to identify transformation and release steps for (products containing) advanced materials along their life cycle. This should include guidance on Material Flow Analysis studies as a pre-experimental approach to pinpoint form-specific releases of the material under investigation. This would then inform on simplified released mass or concentration of the

specific-released form, which would then guide towards the (environmentally) relevant (eco)toxicity experiments to be used in (environmental) risk assessment.

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9. Influence of Physical Transformations in the Environment on Risk Profiles of Advanced Materials

Recommendation

Develop and establish a guidance on how to test the transformations of the physical form of advanced materials occurring in the environment.

Issue

To what extent transformation of advanced (nano)materials and/or their constituent parts alters the exposure and hazard profile of the material is an important question in risk assessment. It is currently not always clear what the most relevant transformation processes are, and (standardised) methodologies may not always be available to determine/quantify these processes.

State-of-the-art and resources to be considered

Test methods exist to determine abiotic and biotic transformations of chemicals (e.g. OECD TG 301 [1], OECD TG 302B [2], OECD TG 307 [3], OECD TG 308 [4], OECD TG 309 [5]). These mainly aim to capture (bio)transformation processes that transform organic chemicals into different chemicals, ultimately often resulting in carbon dioxide and water. For particulate advanced (nano)materials different environmental transformation become important beyond the chemical transformations that are currently the focus of standardised test methods. Not only different chemicals may be formed, but it may also lead to physical change, e.g. decrease in particle size or loss of coating. To further complicate the issue other transformation processes may play a role as well, and these may occur under natural conditions, e.g. through weathering or exposure to UV radiation. As the physical characteristics may also influence the environmental fate and toxicity of particulate materials, it is crucial to monitor such transformations in test systems to assess environmental fate and toxicity.

Physical transformations are dynamic processes influenced not only by the intrinsic physicochemical properties of advanced (nano)materials, such as size, composition, surface area, surface charge, and morphology, but also by external factors like temperature, pH, ionic strength, and the presence of inorganic and organic constituents in the surrounding medium. A crucial first step in identifying the most relevant physical transformations of advanced (nano)materials is, therefore, to define the environmental compartment where these transformations may occur. Physical transformations can vary significantly between soil, freshwater, and marine environments.

Apart from processes like dissolution or (hetero)agglomeration (OECD GD 318 [6]) also sulphidation in anoxic conditions (in soil/sediments) may lead to changes in physical properties (e.g. decrease or increase of particle size, or loss of coatings) [7]. Furthermore, biotic transformations may lead to physical changes in advanced (nano)materials, e.g. when a protein corona is formed [8], or when bacteria use only a specific coating as a carbon source.

For abiotic transformations of nanomaterials a Guidance Document is under development in OECD (TGP Project 3.16 [9]), but for biotic transformations further work is still needed, also for nanomaterials, to identify to what extent current biodegradation test methods are applicable for advanced (nano)materials [10].

The OECD "Physical-Chemical Decision Framework to Inform Decisions for Risk Assessment of Manufactured Nanomaterials" [11] may provide a starting point, but to fully address the transformation issue, further detailed stepwise approaches (e.g. integrated approaches for testing and assessment (IATAs)) may need to be developed.

Output / product(s) envisioned and action(s) required

As a first step a scoping review could be performed to identify the most important transformation processes that may occur in each of the different environmental compartments in which advanced (nano)materials may end up and that influence physical changes that are relevant for risk profiles of these materials.

Subsequently guidance documents and/or harmonised/standardised test methods (ISO/OECD) to reproduce these scenarios and evaluate the transformation of advanced (nano)materials under controlled conditions.

Test methods may include modelling tools to predict physical transformation of advanced (nano)materials in different environmental compartments under defined environmental conditions (i.e. pH, salinity, temperature) [12,13]. Integrated approaches for testing and assessment (IATAs) and any related guidance should also take these aspects into account.

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10. Standardised Release Testing of Advanced Materials along their Life Cycle

Recommendation

Develop standardised release tests adequately mimicking relevant exposure scenarios. This enables a qualitative and quantitative analysis of (potential) release from advanced materials and from advanced materials-containing products along their life cycle. This should include detection and characterisation methods for released (fragments of) materials.

Issue

Standardised release tests adequately mimicking relevant exposure scenarios are needed for a comparable qualitative and qualitative analysis of fragments (potentially) released from AdMa-containing products along their life cycle. Thorough analysis of the released fragments suitable to the material properties of AdMas should be performed subsequently to the release test. At the moment not enough standards are available to cover the testing of AdMas along their life cycle.

State-of-the-art and resources to be considered

Exposure models are important tools for (regulatory) exposure assessment. Many of these models require release test data as input [1,2]. Release of AdMas along their life cycle is also becoming more important for sustainability assessment and for safety-by-design approaches.

Within exposure scenarios, several activities could lead to a release of (fragments containing) nanomaterials or other AdMas. These released materials need to be adequately analysed in order to identify whether these contain the respective AdMa, a transformed version of the AdMa, the matrix material, transformed matrix material or mixtures of those [3]. For exposure assessment as well as safety testing, profound data about the number of released fragments and about their composition and morphology is required [4,5].

So far, the implementation of releases of AdMas is a challenge, especially morphology- and component-specific release information. The determination of both characteristics of released fragments requires adequate detection and analytical methods also considering the complex nature of fragments being composed of matrix material, AdMas or transformed versions of both. In early design phases with low amounts of material available and not yet scaled-up production processes in place, release tests could provide the required information on release and potential exposure.

Few of the available standardised release tests are validated for nanomaterials but none are validated nor standardised for other AdMas yet. For some activity process no release test is established at all. An OECD WPMN Guidance informing about available release tests for nanomaterials is currently developed.

Besides the release test per se, methods for the collection, detection, quantification and characterisation of the released fragments need to be tailored to adequately assess AdMas and their transformed versions (see also recommendation 8). Especially for multi-component materials and for carbon-based AdMas the detection and characterisation methods are yet to be evaluated for their applicability.

In MACRAMÉ for some of the processes in the life cycle methodologies are explored for use in release testing of AdMas. The Taber Abrader was used for testing the release of GR2M from epoxy matrices. Cryo-milling was used to simulate recycling processes at the end-of-life of CNT-containing products and incineration was performed to mimic waste treatment at the end-of-life.

Output / product(s) envisioned and action(s) required

The required output are standardised release tests including standardised methods for detection and characterisation of the released fragments. In addition, a testing strategy to cover all relevant exposure scenarios along the life cycle is required.

In order to achieve the envisioned output, the following actions are required:

» Identifying specific intentional and unintentional transformation processes during the life cycle of (products containing) AdMas for which no release test is existing.
» Further validation and standardisation of existing release tests for AdMas.

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11. Life Cycle Information as Part of Advanced Material Characterisation

Recommendation

Develop a guidance including information requirements and reporting standards for the description of life cycle history and fate of the material including its environment. This should allow characterisation of test objects at crucial life cycle stages for risk assessment, taking into account the information on previous life cycle stages and transformation steps between stages.

Issue

As AdMas may transform during their life cycle and use in products, a physico-chemical characterisation of an AdMa only at the beginning of its life cycle will often not be sufficient as identification for later life cycle stages. On the other hand, a thorough characterisation of the material throughout its life cycle will often also not be feasible. Thus, chemical identification and physico-chemical characterisation to unambiguously define a material should be enriched by a complementary characterisation approach, which is available in the form of material, sample and data provenance trails.

State-of-the-art and resources to be considered

Minimal (regulatory) information requirements and reporting standards provide a set of physico-chemical characteristics to uniquely identify an advanced (nano)material (see recommendation 1). However, for later life cycle stages additional specific characterisation may be needed for certain material classes or products. Life cycle stages, which considerably change the identity of the AdMas, may include product lifetime and use stage, end-of-life stages, and in some cases even the production stage. To optimise hazard and risk assessment, an evaluation on sameness and similarity appears essential to allow grouping approaches and effective use of test results not only when looking at different AdMas but also when deciding, which life cycle stages need to be considered separately. Sameness refers to a material being indistinguishable from another material in terms of physico-chemical characterisation, producer and specific batch. Similarity refers to materials that are sufficiently alike for read-across approaches, i.e. using (hazard) data from one material to assess a similar material. A thorough characterisation for each life cycle stage would extremely increase the costs for identification and characterisation of (the safety profile of) an AdMa, or might even not be possible at all because of the many properties that may influence the safety profile.

Specifically for nanomaterials, OECD Test Guidelines and Guidance Documents have been or are currently being adapted or newly developed to be able to uniquely identify and characterise them. Additionally, minimal sets of characterisation parameters have been proposed. These include a classification framework for graphene-based materials (see recommendation 5) or requirements to characterise nanoforms for the REACH Regulation [1].

Apart from testing just one species at a time in environmental safety testing, more complex multi-species mesocosm experiments can be performed that allow species interactions. To deal with this complexity, first the NIKC database [2] and later the EU projects NanoFASE and NanoCommons introduced a new way to characterise nanomaterials in their life cycle stages and in complex environments. Instead of (or not only based on) a direct physico-chemical characterisation, materials

are identified and characterised by reporting their history and fate, i.e. all stages they travelled through during their production and later modification (integration into product and biological matrices). This was visualised in so-called Instance Maps [3].

The MACRAMÉ project contributed to an updated version of this concept by describing Study Design Maps [4]. These Study Design Maps are supplemented by explicit descriptions of the transformation from one stage to the next (see recommendation 12). To present the links of a later life cycle stage to previous ones not only visually, the transformations can be covered in the (meta)data describing the material/test object.

Output / product(s) envisioned and action(s) required

A guidance is needed that defines a dual approach for AdMa characterisation based on direct measurements of (physico-chemical) properties combined with a life cycle description of the AdMa. Such a life cycle description should include the history and fate of the material and its environment (i.e. complex product and biological matrices) up to the life cycle stage under investigation. The guidance will then provide the minimal reporting checklist for physico-chemical characterisation of the current and previous life cycle stages, potentially combined with safety and sustainability aspects from earlier stages. Where possible, this should also address options on metadata schemes and standards for covering material, sample and data provenance trails describing life cycle stages and transformations between them. Potentially the guidance could integrate visualisation approaches like Instance / Study Design Maps, as well as guidance on using the dual system for categorisation and grouping of materials for group-based risk assessments. Finally, the guidance should also explore relationships with digital material/product passports as a way to connect and access information from both earlier and later life cycle stages.

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12. Sharing of Safety-relevant Information on Advanced Materials along the Life Cycle

Recommendation

Define and establish reporting and (meta)data standards for sharing safety-relevant information on earlier life cycle stages. This information needs to be integrated as it provides a valuable background for later analyses of life cycle stages (product use and end-of-life).

Issue

Effective identification and characterisation of individual components and material in a multi-component material or a product is challenging, in particular now with the increasing demand to integrate sustainability considerations in safety (and sustainability) assessment. For effective sharing of the information throughout the different life stages and avoiding unnecessary repetition of collecting information, the use of digital standards to organise data and information sharing should be explored.

State-of-the-art and resources to be considered

Information on early life cycle stages (i.e. synthesis and production stages treating including raw or pristine materials) becomes increasingly available. Sources of such information include chemical safety data sheets, chemical databases like PubChem and regulatory databases from e.g. ECHA and US EPA. However, required data for risk assessment are not easy to extract, since they are generally available only in hardly-machine-actionable formats. This complicates their extraction out of a huge amount of information and data, that may not be fully publicly available, and do not always provide the metadata to evaluate their regulatory relevance and reliability.

Digital material and product passports are currently under development as they become regulatory requirements, e.g. in the EU Batteries Act. These are meant to provide information to stakeholders downstream in the value chain and concerned with later life cycle stages. Information that includes the chemical composition and the necessary treatment e.g. during production and recycling. However, it is currently not clear whether the information will be provided in a harmonised way across all material sectors, nor whether it will provide all details needed for regulatory use. There are also uncertainties on how passports for materials that represent some intermediate stages in the value chain should provide information. These uncertainties include questions on how to access the information of upstream life cycle stages and integrate all this information in the downstream stages.

Instance Maps and their derivative Study Design Maps have been developed to document the history and fate of materials (see recommendation 11). In this way, they represent material, sample and data provenance trails. By linking information and data about specific instances to the Study Design Maps the full assessment of a specific life cycle stage can be constructed as the sum of the data generated for this stage combined with the information on all previous stages and transformations [1].

Effective reporting has the advantage that information can already be prepared for early life cycle stages. This can then be used to define the characterisation needs and realistic exposure scenarios for later stages. Additionally, the burden of data collection can be distributed between all players in a material value chain that are essential for a manageable risk assessment that integrates the product

and end-of-life stages. To support the aggregation of all this information, the MACRAMÉ knowledge management and sharing solutions are a mixed system of centralised and distributed components linked together with Instance / Study Design Maps [2].

Output / product(s) envisioned and action(s) required

A guidance should be created that describes the structuring of data collection and sharing along the value chain and life cycle stages. The guidance should address and assure interoperability between all this information. Minimal reporting and (meta)data standards as well as digital and secure data sharing options need to be defined to streamline the flow of characterisation and safety-related data along the value chain. All information needs to be available in digital, easily accessible and machine-actionable format and include details for unambiguous identification and characterisation including any material transformations.

To ensure the necessary data will be generated and reported, these data should be mandatory in digital chemical / material / product passports. The passports themselves should be equipped with documentation and visualisation of the relationships between data points and life cycle stages, e.g. in the form of study design easy-to-understand representation of the material flow and fate as available in maps.

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