Discussion on Existing Standards and Quality Criteria in Nanosafety Research

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Supplement: Mind Map – Catalogue of Criteria of the NanoS-QM Project

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Abstract

The partners of the research project NanoS-QM (Quality- and Description Standards for Nanosafety Research Data) identified and invited relevant experts from research institutions, federal agencies, and industry to evaluate the traceability of the results generated with the existing standards and quality criteria. During the discussion it emerged that numerous studies seem to be of insufficient quality for regulatory purposes or exhibit weaknesses with regard to data completeness. Deficiencies in study design could be avoided by more comprehensive use of appropriate standards, many of which already exist. The use of Electronic Laboratory Notebooks (ELNs) that allow for early collection of metadata and enrichment of datasets could be one solution to enable data re-use and simplify quality control. Generally, earlier provision and curation of data and metadata indicating their quality and completeness (e.g. guidelines, standards, standard operating procedures (SOPs) that were used) would improve their findability, accessibility, interoperability, and reusability (FAIR) in the nanosafety research field.

Document Guide

Introduction

Research data for nanosafety comprise data from various academic fields, spanning a range from the material sciences to toxicology and from basic science to regulatory processes. It is therefore essential that those data can be understood and their quality evaluated, re-used and enriched along their life-cycle by users with different scientific backgrounds. This remains a challenge for the interdisciplinary workflow and ultimately for the safety assessment of novel nanomaterials (NMs).

In the NanoS-QM project, the project partners of the Leibniz Research Alliance Nanosafety are developing quality standards for data in this field. In doing so, they are building the foundations for improved and comprehensible hazard identification and risk assessment of NMs.

The NanoS-QM project partners identified and invited relevant experts from research institutions, federal agencies, and industry to evaluate the traceability of the results generated with the existing standards and quality criteria. The critical feedback initiated discussions of crucial aspects and strategies for improvements. The results of the discussions will, together with internal round-robin tests, contribute to the further development of quality criteria in the project NanoS-QM.

The workflow of nanosafety research served as the red thread along which the discussion was guided. The characterisation of NMs properties is followed by testing their toxicology *in vitro* and *in vivo.* Data generated are used for risk assessment in the next step. The final question was which data and metadata are needed for smoother risk assessment processes.

During the workshop on the use of nanosafety research data across different fields, the discussions resulted in essential insights on: the description of data, the importance of target-oriented analysis of NMs' properties, rating the usability of data from certain studies, and the importance of data curation. The results of the discussions are summarised in this document.

Method

The workshop was held online in two sessions of 2.5 h on 17 June 2020. To initiate the discussions, the partners of the NanoS-QM project presented a state-of-the art on four topics. A transcript of the state-of-the-art had been made available to the participating experts in advance for commenting. The four topics are:

1. Characterisation of Nanomaterials in Biological Systems (Norbert Riefler - Leibniz-Institut für Werkstofforientierte Technologien)
2. *In vitro* and *in vivo* Data Generation, Definition of Test Systems (Roel Schins - Leibniz Research Institute for Environmental Medicine)

3. Endpoints used in regulatory toxicology (Christoph van Thriel - Leibniz Research Centre for Working Environment and Human Factors)
4. Required Data and Metadata (Kunigunde Binder, Christian Bonatto Minella - Leibniz Institute for Information Infrastructure, Linda Elberskirch - Leibniz-Institute for New Materials)

Both, the transcripts and the resulting discussions are presented in the chapters below. The discussion has been curated for flow and clarity. The comments in the text are not assigned to the individual workshop participants and may not represent the opinion of all participating experts. Italic type indicates statements by the experts.

Conclusions

The definition of NMs, like the one adopted by the EU, may not be generally valid. In addition the characterisation of basic properties, like the size of NMs, is anything but trivial. Precision and application area of frequently used techniques were questioned by the experts. Furthermore, the properties of the many different specific NMs vary greatly. Physicochemical characteristics of the materials, e.g. solubility/dissolution and other properties related to particokinetics are relevant for toxicological endpoints and should be analysed early in the innovation process.

Characterising NM properties in relevant media that mimic biologic conditions are even more challenging as relationships between different parameters in biological environments are very complex. Characterisation of NM properties therefore requires experience in the field.

There is still an urgent need for standardisation to improve assay validity and data reliability. Accurate dose analysis at NM deposition and target sites is of main relevance for the evaluation of cellular and molecular mechanisms of action. Standards should provide comprehensive information about the method and its validity in the nanosafety field. Existing standards should be reviewed and applied if they are appropriate for the chosen method. Use of appropriate standards should be made mandatory if studies are to be used for risk assessment.

Advanced *in vitro* models require detailed standard descriptions regarding (1) test conditions and acceptance criteria and (2) dosimetry and analysis of internal exposure and effects. There was disagreement whether advanced *in vitro* models are more difficult to standardise or not.

In general, the experts consider existing regulatory test strategies sufficient for NMs. However, the use of non-guideline studies for regulatory purposes should be considered. While the work of the Organisation for Economic Co-operation and Development (OECD) is regarded as the most valid and reliable information source, its update of the existing OECD Test Guidelines (TGs) and especially the introduction of new TGs can take long.

For regulatory purposes, data with insufficient quality seem to be provided (e.g. through IUCLID). Measures for quality assessment are only included during the approval procedure. Unfortunately, the interpretation of study results and the evaluation/classification of chemicals/NMs often depends largely on the “human factor” and therefore differs among stakeholders.

The reuse of research data on nanosafety is restricted due to insufficient or missing description of metadata. Curation of already available studies would be an enormous and time-consuming task that is deemed impractical.

To enable quality assessment of studies and facilitate data re-use, integrating ELNs in the routine scientific work was recommended, as it supports the necessary process digitisation within a laboratory as well as the automated metadata documentation. This could also encourage disseminating negative results (data where an effect is absent, important for dose-effect relationships) which otherwise might never find publication and could be useful for the community. However, not all ELNs allow the collection of metadata.

The question arises whether and how the data quality in the field of Nanosafety research can be improved using metadata.

Topic 1 - Characterisation of Nanomaterials in Biological Systems

NMs (NM) are most commonly defined as material units (particles) with a size between 1 and 100 nm in at least one dimension (ECHA). The current recommendation of the European Commission on the definition of a NM not only focuses on particle size, but more specifically gives a number size distribution, includes materials with a specific surface per unit volume above 60 m^2/cm^3 and states exceptions (single-walled carbon nanotubes, graphene flakes and fullerenes even if < 1 nm). Whereas the latter definition comprises materials independent of their origin, others exist that address only engineered or intentionally produced NM. All over the world, definitions are inconsistent with regard to specification or inclusion of a quantitative measure, origin, type of nanostructure or the presence of specific or novel materials properties. This example illustrates that a comprehensive set of characterisation techniques is necessary to merely determine, whether the material under consideration is a NM or not.

Although NMs fall under the existing REACH and CLP definition of a substance, from a scientific point of view, it is clear that NMs are neither simple chemicals nor can be treated like bulk matter. This is demonstrated by their size-dependent properties, such as unique optical, electronic or magnetic properties, which determine their application potential. In the biological context, the size and shape of NMs might determine their entry pathways into living organisms and their distribution down to the subcellular scale. In addition, properties like reactivity, surface structure or large specific surface area are known to influence chemical or physical processes at the NMs bio-interface, e.g. dissolution, redox-reactions, generation of reactive oxygen species or adsorption of molecules. These processes are expected to induce or modulate biological effects differently from larger particles or bulk materials (Auffan, 2009). For example, one recent study shows that Fe-doping of engineered CuO nanoparticles (NPs) can be used to control Cu ion release in the interior of tumor cells. This could be achieved by differential accumulation and controlled release kinetics that are neither possible by chemical substances nor bulk materials. By i*n vivo* studies, this approach - in combination with an immunosuppressive agent - was shown to be effective for cancer treatment (Naatz et al. 2020). Consequently, the nano-bio interface has been regarded as a new direction in science (Nel et al. 2009).

The characterisation of NMs in biological systems is a challenge on its own. Even the base characterisation of NMs, e.g. particle size, size distribution and morphology, requires in practice more than one measurement method: electron microscopy can be used to reveal the 2D particle morphology, but the 3D structure requires enhanced image processing skills, not to mention the labour-intensive evaluation of a multitude of aggregates to get a statistically relevant mean value. The size of

agglomerates and aggregates might be determined using measurement methods like Dynamic Light Scattering (DLS) or Differential Centrifugation Sedimentation (DCS), but without information about primary particle size and only with previous knowledge of appropriate input parameters. Determination of materials properties under biologically relevant conditions (dispersed in body fluids or relevant media) is even more sophisticated.

These considerations lead to a multi-method approach for a comprehensive characterisation of NMs. The parameters to be determined were divided into two groups: “physical particle properties” (e.g. particle mean diameter, particle size distribution, shape, dimensionality, surface area, aggregation and agglomeration, density, porosity, crystalline structure/phase, crystallite size, flammability, explosiveness) and “surface properties and activities” (e.g. zeta potential, surface chemistry, hydrophobicity, stability, including degradation/dissolution, redox potential, radical formation, photocatalytic activity, adsorption and desorption). The former involves the application of characterisation methods typical in the field of materials science, like the ones mentioned above. The latter parameters are significantly influenced by the surrounding conditions and should also be determined under (biologically) relevant conditions. In line, the decision-making framework for grouping and testing of NMs has proposed to consider intrinsic and system-dependent materials properties as well as bio-physical interactions in order to describe relevant materials properties and to allow for their grouping (Arts, 2015). Only at the beginning of this year, new information requirements for the registration of NMs in their REACH dossiers have come into operation. These include intrinsic and system-dependent materials properties. However, test methods to identify materials properties relevant for toxicological effects still need to be further defined in order to reach general acceptance.

In the frame of NanoS-QM, the following scientific and regulatory standards or guidelines were used to derive parameters to specify relevant properties of NMs:

- Minimum information reporting in bio-nano experimental literature (MIRIBEL): ‘Minimum information standard’ for experimental studies investigating nano-bio interactions. This guideline lists three categories to be reported: materials characterisation, biological characterisation, and details of experimental protocols (Faria et al. 2018).
- DaNa: The checklist ‘Methodology of selection, collection, and evaluation of toxicological publications in the DaNa project’ provides a collection of criteria supporting the generation, selection, and evaluation of nanotoxicological studies.
- ISO/TC: The Technical Report ‘Nanotechnologies — Guidance on physico-chemical characterisation of engineered nanoscale materials for toxicological assessment’. Provides guidance for the physico-chemical characterisation of NMs, including parameters and measurement methods.

- OECD: Series on the Safety of Manufactured Nanomaterials: The series compiled a Guideline/Regularia collection on physico-chemical properties of NMs.
- REACH **(**Registration, Evaluation, Authorisation and Restriction of Chemicals): The updated REACH regulation on nanoforms lists a range of physicochemical parameters necessary for the registration of NMs.

None of these standard and guideline collections are complete in that they deliver a complete set of parameters as well as specification of appropriate measurement approaches or methods. Therefore, starting from the identification of relevant parameters, an analysis of common characterisation methods for these parameters has been performed. The result shows fairly satisfying descriptions in case of physical particle properties, while surface properties (i.e. in particular parameters in biological relevant environments) are not well described. As a consequence, further studies are necessary to get concise metadata based on the following key requirements:

- **Consistency of data**; a literature research should be accomplished to find papers comparing different methods. Their results (e.g. “an underestimation of method X compared to method Y of Z percent”, see for instance (Anderson et al. 2013)) can be included in the already existing tabular overview as a kind of conversion factor.
- **Data from standardised biological environments**; the transferability of material characterisation within or outside biological environments is mentioned above as a gap, which can be bridged by studies where both cases (within and outside) are investigated in a slender step-by-step procedure to deliver highly resolved SOPs
- **Kinetic data**; NM stability must be included, for instance, by information about the medium (solvent) and the half-life period of NM therein, which is currently subject only in research programs and not in regulations

Questions to be discussed:

- Are there other relevant parameters which need to be taken into account?
- Are there other or further relevant regulatory or scientific standards that should be considered?
- Do you think that the mentioned parameters are equivalent for scientific and industrial research?
- Which additional information is required to support the use of the parameters?
- What data and metadata are required to support relevant data gathering for regulatory but also for scientific purposes?

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Summary report of comments and discussion to Topic 1: Characterisation of Nanomaterials in Biological Systems

Key points of discussion

Definitions like the EU definition for NPs may not be generally valid.

The selection of appropriate methods for NM characterization is challenging due to the variety of NM specific properties.

The relationships between different parameters in biological environments are very complex and require experience in this field.

Discussion

NM characterisation comprises the measurement and description of NP specific properties. The presentation focused on the determination of NM specific parameters based on scientific and regulatory standards or guidelines as well as industrial

norms. The results were discussed in the context of consistency of data with a focus on transferability and alignment of results of measurements. Furthermore, the characterisation of NMs in biological systems is a challenge on its own and was considered in terms of standardisation and the generation of kinetic data.

At the beginning of the presentation the EU definition of a NM was mentioned. Although the EU definition of NMs was questioned for scientific investigations within the transcript, the problem of base characterisation like the size of NPs was at the center of the discussion. In particular the precision of the frequently used Dynamic Light Scattering was questioned or is not applicable for every kind of particle. One expert remarked his experience that DLS devices from different manufacturers deliver different results and, therefore, conversion factors between different measurement principles are not realistic. However, studies show that comparisons are reasonable and feasible (e.g. Braun et al. 2011) with the restriction that these tricky characterisation methods require much experience ("Only specialized laboratories might do that"). Another expert remarked that conversion of geometric (EM) to hydrodynamic (DLS) diameter is possible, but that the estimated parameters should rather be selected towards biological characterisation, in particular the surface characteristics. Kinetic characterisations and the effective concentrations of NMs (including sedimentation of submersed and airborne particles) were highlighted, which is - according to another expert - very complex by including translocations and biopersistence (see Utembe et al. 2015). Characterisation under biological relevant conditions is important and the "protein corona" is a good concept, but it neglects other biomolecules like lipids, glycoproteins etc. One of the experts also stated that the base characterisation - important for the regulation according to an earlier comment - have to be performed with a combination of different methods and should be detached from toxicological approaches. The consistency of data cannot be comprehensively checked, however, a need is seen and a partition of the literature in subject areas (eg. resp. tox. of TiO2, or oral tox. of AgNP) and assignment of specialized experts to each topic is suggested.

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Topic 2 – *In vitro* and *in vivo* data generation and definition of test systems

Studies that address safety of NMs employ a wide range of test systems and experimental procedures. The establishment of the dose range of a NM that produces a biological response provides valuable data on its biocompatibility and potential toxicity associated with deliberate or unintentional exposures. The resulting data are useful for conventional risk assessment methodologies and regulatory toxicology (see also workshop Topic 3) but can also elucidate new cellular and molecular mechanisms of actions of NMs. *In vitro* and *in vivo* studies at realistic exposure scenarios can also contribute to the development of novel non-animal alternative *in vitro* systems or feed into adverse outcome pathways (AOP) that are increasingly embraced in regulatory toxicology (Halappanavar et al. 2020).

An *in-depth* analysis of the current state of available methods for the qualitative and quantitative detection of NMs in entrance/barrier organs as well as in secondary target organs has been performed in relation to data requirement needs and quality criteria. Principal approaches for their detection involve a wide range of light and electron microscopy methods and assays based on elementary analysis or cytometry. Each of these methods has pros and cons, for instance, regarding its detection limit or its ability to determine the NM in a qualitative versus quantitative manner and in a static versus dynamic fashion. Moreover, the exposure can be determined on the level of whole organs and tissues down to the level of single cells and their subcellular compartments. The importance of quantitative exposure analyses for risk assessment is obvious. For instance, whole organ burden analysis is used to determine clearance and dissolution kinetics in long-term *in vivo* studies in rodents (Bermudez et al. 2002; Bermudez et al. 2004; Creutzenberg et al. 1990; Wu et al. 2009; Eydner et al. 2012). Accurate dose analysis at NM deposition and target sites also supports *in vitro* to *in vivo* extrapolation exercises and dose range justification for *in vitro* studies. Methods that enable detection of NMs at the cellular and subcellular level are of main relevance for the identification of cellular and molecular mechanisms of action, related to kinetics and processes of cellular uptake, intracellular (re)distribution and dissolution. There is an urgent need for the further standardisation of these various assays to accurately determine (internal) dose, for *in vitro* as well as *in vivo* test systems. *In vitro* studies (e.g. Peuschel et al. 2015) have revealed that a lack in standardisation can lead to inconsistent and incomparable study outcomes. Standards are needed that provide information on the model system, the test method, the biological characterisation, the acceptance criteria of the test model, the administered dose, the study design and the analysis methods. Also TGs that are typically applied for regulatory purposes (e.g. the nanomaterial-adopted OECD TG 412 and ISO 10993) do not include any thorough description for the qualitative or quantitative analysis of NM at the target site. This is also the case

for recent “standardisation improvement” initiatives, including the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guideline (Kilkenny et al. 2010) and the NMs scientific guideline MIRIBEL (Minimum Information Reporting in Bio–Nano Experimental Literature) (Faria et al. 2018).

Also, with regard to the *in vivo* and *in vitro* evaluation of the biological or toxic effects of NMs, there is a need to further develop and improve test standards. A large set of OECD tests is available and major advances have been made regarding the evaluation of their applicability to the testing of NMs. The OECD Working Party on Manufactured Nanomaterials (WPMN) has been leading a global programme promoting the understanding of environment, health and safety aspects of NMs (http://www.oecd.org/chemicalsafety/) and several new OECD TGs for nano-relevant properties are being developed (Rasmussen et al. 2019). This will be discussed further in topic 3. While these TGs mostly provide in-depth information and recommendations regarding study design and test model acceptance criteria that are applicable or adaptable to NMs they often lack details regarding detailed data requirement needs and specific quality criteria.

In addition to the conventional tests used for regulatory purposes, there has also been a growing interest in the use of advanced non-animal alternative tests that more realistically reflect *in vivo* (physiological) conditions. Important models have been developed for the lung and gastrointestinal tract to allow for improved hazard assessment related to inhalation and ingestion as the two most relevant routes of exposure. Experimental systems in which human airway epithelial cells in mono or co-culture with further lung-relevant cell types, or lung microtissues, are cultured at the air liquid interface (ALI) have been developed and brought to further advancement in recent years (Lacroix et al. 2018; Herzog et al. 2014). As these systems concentrate on simulating the real-life exposure of the airways using highly sophisticated technology and cell models, there are specific needs for the reporting of metadata of the specific experimental setting, which should include an appropriate description of the cell model used. With regard to exposure characterisation and dosimetry such ALI systems can build on methods (exposure analyses, modelling) available from “*in vivo*" inhalation toxicology research. Besides the ALI-based approaches, *in vitro* models that incorporate mechanical strain are being developed in more recent time. In comparison, these models are less well developed due to the variety of innovative cell stretching systems currently used or under development (Schmitz et al. 2019; Zamprogno et al. 2019). The determination of defined quality criteria (i.e. biological model, instrument / test system/ membrane characterisation, culture conditions, exposition, and endpoints) should be an important step towards standardisation and improvement of data relevance. Similar to these and other innovative lung models, also for the GI-tract a variety of *in vitro* models have become available and are being used or considered in nanosafety research. These include co-culture models to reflect the intestinal mucus barrier, approaches to mimic effects of and interactions with the food matrix, digestion-relevant constituents, peristalsis

and the microbiota as well as more advanced organoid/organ-on-a chip approaches (Kaempfer et al., 2020). For the testing of NMs, each of these advanced *in vitro* models require, beyond their validation, detailed standard descriptions regarding their test conditions and acceptance criteria as well as dosimetry and analysis of internal exposure and effects.

A final major aspect regarding the testing of NMs, *in vitro* as well as *in vivo*, is the evaluation of the potential to cause artefacts in assay readouts in an assay and test specific manner. While this has been acknowledged for various assays, there is further need for standardisation to improve assay validity and data reliability.

Questions to be discussed:

- Determination of internal exposure to NMs: which methods do you regard as applicable at the organ level, cellular and subcellular level?
- Are you aware of standardised protocols describing such measurements?
- Are these methods widely accepted and available?
- Do you consider such measurements as valuable? In which context?
- Do you expect such measurements to be of relevance for the regulatory context? For science-based questions only?
- What parameters are necessary to apply these methods appropriately?
- Development of novel test systems: Which parameters would be needed to validate and use novel test systems, such as ALI or systems implementing mechanical strain for scientific as well as regulatory questions? Do you see limitations?
- Assay interference: what parameters would you regard as of main importance to exclude assay interference and to produce valid data using in vitro assays?

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Summary report of comments and discussion to Topic 2 - In vitro and in vivo data generation and definition of test systems

Key points of discussion:

There is still an urgent need for standardization to improve assay validity and data reliability. Existing standards should be reviewed and applied if appropriate.

Accurate dose analysis at NM deposition and target sites is of main relevance for the identification of cellular and molecular mechanisms of action. This is especially important if the data shall be used in a regulatory context.

Advanced *in vitro* models require further detailed descriptions of the specific experimental conditions, the acceptance criteria and the cell model used. There is disagreement whether advanced in vitro models are more difficult to standardize or not.

Use of appropriate standards should be made mandatory if studies are to be used for risk assessment.

Discussion

Establishing the dose range of NMs that produces a biological response requires detailed understanding of their interaction mechanisms at the organ or cellular and subcellular level. Therefore, quantification of NM internalization involving a wide range of light and electron microscopy methods and assays based on elementary analysis or cytometry is crucial to predict the potential impact of intracellular NM doses. Each method has its specific advantages as well as limitations, for instance, regarding its detection limit or its ability to determine the NM in a qualitative versus quantitative manner. It was commented, that the new methods of internal NM detection, for example stimulated emission depletion (STED) and stochastic optical reconstruction microscopy (STORM), makes nanoscale materials and components of the cell accessible inside cells for fluorescence-based investigations (van der Zwaag et al. 2016; Müller, Schumann, and Kraegeloh 2012). Both methods have a resolution below the diffraction limit but are not applicable for all particle and dye applications. Thus, the microscopical methods are good in theory but difficult to put into practice because specific dyes are required.

One expert pointed out that accurate dose analysis at NM deposition and target sites is of main relevance for the identification of cellular and molecular mechanisms of action. However, following the Adverse Outcome Pathways (AOPs) concept, there is no requirement to evaluate intracellular NM concentration. The AOP concept describes a mode of interaction of a substance / NM that induces the first key event of causally linked events at different levels of biological organisation (https://www.oecd-ilibrary.org/environment/oecd-series-on-adverse-outcome-pathways).

Besides the AOP concept, it was agreed on the urgent need for the further standardisation of the qualitative and quantitative detection of NMs to accurately determine (internal) dose, for in vitro as well as in vivo test systems. This was also commented by an expert with reference to his experiences (Krug 2014).

Standards are needed that provide information on the model system, the test method, the biological characterisation, the acceptance criteria of the test model, the administered dose, the study design and the analysis methods. In connection to this, an expert suggested distinguishing between administered and delivered dose, while another expert mentioned the international guidance on Good In vitro Method Practices (GIVIMP) for the development and implementation of in vitro methods for regulatory use aims to help describing such measurements to reduce uncertainties (OECD 2018).

With respect to data reliability, one expert estimates that more than 80% of published data are not appropriate for risk assessment. To enhance data reliability for the regulatory context, thorough description of the dose at the target is of greater importance than the characterization. There is a need to further develop and improve test standards. But, the more complex the (in vitro alternative) test system, the less easy it is to standardize.

To enhance reliability and fulfil lack of details, one expert regretted that SOPs from former nanosafety projects were rarely reused. It was further recommended that only data that are processed under guidelines should be used for risk assessment.

Besides conventional tests methods used for regulatory purposes, there has also been a growing interest in the use of advanced non-animal alternative tests related to inhalation and ingestion as the two most relevant routes of exposure. An expert commented on this, that intended exposure and I.V. administration for nanomedicines are also of high regulatory relevance and advanced in vitro models are currently under development to investigate these effects. Important models for the lung, in which human airway epithelial cells in mono or co-culture with further lung-relevant cell types, or lung micro-tissues, are cultured at the air liquid interface (ALI) have been developed and brought to further advancement in recent years (Herzog et al. 2014; Lacroix et al. 2018). Another expert mentioned that simulating real-life exposure should be validated while just looking similar would not be sufficient for reproducing in vivo responses.

Several experts commented on these developments of alternative test systems. It was stated that the more complex these advanced non-alternative test systems for simulating real-life exposure are, the more difficult it is to standardize them. However, it was doubted whether the standardization of ALI based testing is more difficult than standardization of the conventional "submersed" in vitro systems. It was pointed out that since the number of labs that use complex ALI systems is low, also the number of results will be low.

The experts agreed that for the testing of NMs, advanced in vitro models require detailed standard descriptions regarding (1) test conditions and acceptance criteria and (2) dosimetry and analysis of internal exposure and effects.

The importance of approaches to evaluate and exclude assay interference and to provide valid data using in vitro assays was emphasized: (1) to use two complementary methods for the same endpoint and (2) to include "spiking" controls. An expert referred to publications on the topic of assay interference (Bohmer et al. 2018; Elliott et al. 2017; Hirsch et al. 2011; Petersen et al. 2020; Roesslein et al. 2015; Roesslein et al. 2013).

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Topic 3 - Data and Metadata Related to Endpoints Used in Regulatory Toxicology

Key points of discussion:

In general, existing regulatory test strategies are sufficient for testing NMs.

There are two main challenges for the regulation of NMs:

1. A general lack of data mainly due to the insufficient quality of studies
2. The "human factor": The interpretation of study results and the evaluation/classification of chemicals/NM often differs between different stakeholders.

State-of-the art and discussion

Even though differences between NMs and chemicals have been identified and described in the scientific literature (Gebel et al. 2014) and by the OECD, the European Union observatory for nanomaterials (EUON) clearly stated on their website that "Nanomaterials are chemical substances". This European evaluation of the regulation of NMs is shared by the German agencies UBA, BfR, and BAuA. Together, they published a joined document saying that, while methodological adaptation seems to be necessary, the general testing methods and strategies for chemicals (provided on the ECHA website) also provide an appropriate framework for testing NMs (2013). Moreover, the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (DFG MAK-Commission) published a book about the toxicity of NMs. Based on their review of existing scientific literature, they concluded that for "risk assessment of NMs the same information is needed as for microscale materials".

> *During the workshop, none of the invited experts expressed any strong objections against these statements. This clearly indicates that existing regulatory strategies are sufficient for NMs. However, it was mentioned that updates of the existing OECD TGs take too long. That is partly caused by the nature of OECD (voluntary cooperation) and the way how these TGs are adjusted. Nevertheless, during the workshop the OECD efforts were acknowledged. It was further stated that non-guideline studies could be used in regulatory toxicology, too.*

In general, the methodological adaptations are thought to be related to the physico-chemical characterisation and data on absorption, distribution, metabolism and excretion (ADME) of NMs are expected to clearly differ from those of traditional chemicals and larger particles. These aspects will be presented and discussed during the two other sessions.

In general, endpoints and their respective data and metadata comparable to the regulation of chemicals are required for NMs. Here, the REACH legislation is mandatory and since 2010 it is possible to add nanospecific information into the IUCLID system.

During the discussion it was clarified that the IUCLID system is a closed data infrastructure used by producers that want to register their product under REACH. Several participants pointed out that the data entry is not subjected to any quality assessment. (...“more than 80% invalid publications”). One expert said that quality assessment procedures are only included during the approval procedure. It was restated that quality of the provided information is sometimes really insufficient (“Junk”). With respect to NMs it was brought up that data gaps will be normal. During this discussion “high dose”-studies and species differences were mentioned that always imply extrapolation steps with additional uncertainties or might render studies non-useable for human risk assessment due to unrealistic dosages. (see also discussion of topic 2)

This data management system plays a central role in the IT environments of all organisations that manage scientific data on chemicals in regulatory context. With respect to NMs under REACH, the concept “nanoform” was introduced into the regulation in Annex VI (published on 12/3/2018). Recently, ECHA published a guidance document that has been developed to provide advice to registrants preparing registration dossiers that cover “nanoforms”.

Thus, in the EU the safe use of NMs is subjected to REACH and here three guiding principles are important:

A. the required toxicity information is “tonnage”-dependent (more tonnage, more information)
B. OECD guideline tests are considered the most valid and reliable source of information
C. the Annex VI of REACH states that for nanoforms toxicity data should provide data from studies via inhalation route

While some of these “tonnage”-dependent toxicity information are only used/ needed for CLP (Classification, Labeling and Packaging of substances and mixtures) purposes, more details/data are needed to establish DNELs (Derived No-Effect Levels) as a safe level for humans. Such toxicological information usually provide a NOAEL (no-observed-adverse-effect level) from an *in vivo* study also needed in the context of occupational exposure limits (OELs) or tolerable weekly/daily intake (TWI or TDI) for food additives/contaminants.

It was noted that such toxicological studies might not assess susceptibility or second hit scenarios adequately. Thus, a NOAEL might underestimate the toxicity of a NM/ compound (a more general problem in toxicological risk assessment).

As a result the most prominent routes of exposure, namely ingestion and inhalation through the respiratory and the gastrointestinal tract, including the liver, are the most relevant targets of NM toxicity. Based on the existing knowledge about the general mechanisms underlying the toxicity of NMs (see Buchman et al. 2019) and conceptual frameworks such as the Adverse Outcome Pathway (AOP, Ankley et al. 2010) extrapolation from such molecular initiating events (MIE) to adverse health effects in humans chronic health effects such as pulmonary fibrosis (Labib et al. 2016), inflammatory diseases of the GI tract (Bettini et al. 2017), and hepatotoxicity (Tang et al. 2019) have to be expected. Therefore, data about comparable adverse outcomes from repeated-dose toxicity studies in rodents (OECD TG 412 [28-day study]/ 413 [90-day study] or TG 407 [28-day study]/ 408 [90-day study]) are needed to provide the necessary information/data to estimate a NOAEL that can be used as a point of departure (POD) in quantitative risk assessment. Here, it has to be mentioned that TG 412 and 413 were updated in 2018 to enable the testing and characterisation of effects of NMs. After screening the toxicity data in the dossiers of the 11 NMs summarised in the Testing Programme of Manufactured Nanomaterials of the OECD it became obvious that apical endpoints are related to:

- pathology and histopathology in various organs/tissue (respiratory and gastrointestinal tract most relevant)
- various markers of systemic and tissue inflammation (immune cells, cytokines, chemokines, etc.)
- functional tests (neurobehavioral, lung function, respiration rate)
- clinical observations, body weight, food and water consumption, or mortality

With respect to relevant endpoints (tests and data) it was emphasized during the workshop [BR] that material characteristics, e.g. solubility and other properties related to translocation (see topic 1), are relevant for these toxicological endpoints. Inflammation was considered as an important effect, especially if particles might be able to cause fibrosis [BR]. [CvT] pointed out that the requests for toxicity testing (acute, repeated-dose etc.) under REACH are related to lot size. However, there was agreement that in the context of NMs material characteristics, kinetics and biopersistence are relevant predictors for health effects. In response to [BR] it was said that surface modifications need to be considered when using such properties in the context of health effects extrapolation [HB].

As another relevant issue the use/ interpretation of study results/ data by different stakeholders was discussed. One expert mentioned the different conclusions of EFSA and ANSES about food-grade Ti_2O. It was remarked that the study quality as well as national evaluation and classification (e.g. carcinogenicity) standards in human health protection contribute to such differences.

Data and metadata describing such apical endpoints are extremely complex as various techniques, devices, scoring sheets, etc. are used. As histopathology and

inflammation of the respiratory and gastrointestinal tract are the most relevant and sensitive endpoints the development of Research Data Management in the context of nanosafety should focus on methods related to these apical endpoints. As these endpoints are also relevant for cell- or tissue-based *in vitro* systems a data and metadata structure should be developed for assays measuring these physiological processes in 2D- and 3D-cell cultures (e.g. spheroids, organoids), too.

However, the “result”-data of any particular *in vivo* or *in vitro* experiment must be linked to data/metadata about the experimental design, analytical methods, etc. usually provided by TGs or SOPs. In the context of nanosafety such SOPs are for instance available in the DaNa 4.0 database. Here, the SOP of the short-term rat inhalation study (STIS) can be found that has been developed as a shorter alternative to existing guideline studies TG 412 and 413 in the context of NM toxicity testing (Klein et al. 2012; Ma-Hock et al. 2009). This example shows that at least for some NMs the required data and metadata seem to be available. However, the information is not well linked to each other and a comprehensive nanosafety data management system should be developed and implemented into the ongoing research activities.

Questions to be discussed:

- What are the relevant agencies, commissions and stakeholders?

  Stakeholders/projects on the EU-level were named. Recently, the three NMBP-related projects (RiskGONE, NANORIGO, Gov4Nano) as well as the Malta2 initiative that are somehow related to Risk Governance Councils" and "Risk Governance Frameworks".

- Please describe a “core” set of endpoints and associated data/metadata that would be needed to evaluate the toxicity of NMs
- Which methods are needed/available to generate these datasets?
- Do you think the guidance of existing guidelines is suitable to generate standardised datasets to evaluate the toxicity of NMs?
- With respect do NMs and risk assessment: How could the standardisation of data management be realised? - e.g. specifications in guidelines, recommendation/ establishment of database(s)

  If projects are funded from governmental agencies with the objective to standardize the methods, why do they not use existing protocols and develop them further? So many European projects did not use the established SOPs which we have published in 2012!!! So much money has been wasted in establishing with each new project their own SOPs…

https://www.nanopartikel.info/files/methodik/NANOMMUNE_QHB_FINAL_2011.pdf

- Who should host such databases?

Should be open access, in any case.

- Would these databases/repositories assist read across and grouping of NMs?
- Who should/ will re-use research data related to the toxicity of NMs?

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Topic 4 - Required Data and Metadata

Key points of discussion:

The reuse of research data on nanosafety is restricted due to missing or insufficient metadata description.

Further information associated with the data, would increase transparency and comprehension for data-users.

The use of ELNs can help overcoming this problem: ELN could assist scientists in the transition from the current analogical (pen and paper) research processes documentation to a digital one. It could enable the storing, the managing and the publishing of research data.

4.1 Analysis of existing standards and guidelines

A literature investigation was conducted to analyse existing standards and guidelines describing the status of NM research and regulation with regards to nanosafety assessments. ***Regulatory standards or guidelines*** comprise documents from official authorities that are designed to identify and characterise potential risks posed by NMs with the aim of obtaining regulatory approval or their notification. ***Scientific standards or guidelines*** are not legal regulations in principal. These standards are more closely oriented to scientific practice with the aim of enhancing comparability, reproducibility and therefore the communication and exchange of results of NM studies or describing specific requirements for certain methods (method standards). The descriptive information (in the following named as parameters) were adapted from the standards or guidelines and grouped as follows:

- **Basic information** (Subgroups: Basic information, Test Material information)
- **Material characterisation** (Subgroups: Material description, Sample preparation, Physical particle properties, Surface properties and activity)
- **Study information** (Subgroups: Test method, Biological characterisation, Quality/Acceptance criteria, Dose metrics/Dosimetry, Experimental procedure/Study design)
- **Instrument information** (Subgroups: Instrument, Sample information, Method specific examples: FlowCyt specific, EM-Microscope specific, Imaging experiment)
- **Analysis and results** (Subgroups: Analysis and results, Discussion/ Limitation/Interpretation, Read out/Experimental outcomes)

Additionally, a proposal to distinguish between common and unique parameters was made (***Importance*** 1: Required, 2: Required if available, 3: Optional; schema according to LINCS (Library of Integrated Network-Based Cellular Signatures) data standards). With regards to the implementation of the parameters in a database, a first classification of the information into data and metadata was conducted. A generally valid differentiation between data and metadata could not be found by a literature query. Descriptions exist only for special cases, since the designation is a question of point of view. Metadata are often used to describe the data with the aim to increase their findability and reusability. This requires a minimum of standardisation, which consists of a metadata scheme with defined parameters to provide information and context of the data. For example:

Data: Measurement e.g. Microscopy image

Metadata: Information and context e.g. Size of the picture, magnification, image processing, and other

4.2 Collection of quality criteria from the perspective of users

In order to identify existing hurdles to data reuse, a structured interview was formulated and conducted with researchers from the institutes involved in the NanoS-QM project. The results of the interview served to establish a set of suitable quality criteria which will try to eliminate or at least reduce barriers for research data reuse within the nanosafety research community.

Sixteen questions were formulated in the interview: they span from general information on the research focus of the interviewees to metadata, data, repositories and current understanding of data-sharing and -reusing. The survey was addressed to scientists working in the field of omics, material science and toxicology.

The most important message which was extrapolated from the answers to the interview is that scientists are keen on reusing data however, only provided that those data are complemented by comprehensive metadata which, unfortunately, it is very often not the case. Aiming to improve the lack of comprehensive metadata and information, ELNs were proposed as a potential solution in the interview because they would assist scientists in the digital documentation of all the experimental steps and acquisition of information which are hardly retrievable at a later point. The scientists interviewed also considered as a key, the involvement of publishing houses in the discourse of data reuse and suggested that any scientific work must be published together with its original raw data which should be then made available to the scientific community. In addition, the interview highlighted that raw data generated by other scientists should always be guaranteed after a project ends, in particular, in the case of projects financed by public funds. Data curation was also suggested in the interview as a potential tool to bypass obstacles to data reuse.

Curation would also provide a further source of trust to the shared data and it would consequently promote data reuse.

> *The number of studies published on NMs and nanotoxicology is already very high. Therefore, it would be very demanding to summarise and review the published material which may not all be quality-controlled. The question would be who is willing to review these published material to create a common database? Where could a funding be granted in order to provide a sustainable database service? It all may fall into a work capacity issue.*

Hence, the fact that data reuse is not yet routine in the scientific community could be also associated with a reproducibility issue. In fact, the replication of experiment protocols does not often lead to the same results because the original data are rarely shared and/or data are rarely associated with comprehensive metadata standards.

> *Data reliability is not only a challenge in the fields of nanotoxicology or chemistry but rather a structural problem for all (biological) studies; most researchers consider mostly the publication number but not the quality and the reproducibility. It was also mentioned that data collected in research labs should be richer in either parameters or metadata in order to be re-used by industries since they are rarely collected using GLP-conditions. However, data produced in academy should not be ignored because they could represent a useful term of comparison or a reference for industry.*

Finally, accessibility to negative data was mentioned during the interview.

> *The workshop participants noted that negative data are nevertheless important for the derivation of dose values for evidence studies. However, scientists do not provide any support on this because the absence of effects is often unaccountable.*

In addition, on one side, one may question which could be a scientist´s reward in sharing results which does not end up in a publication. Nevertheless this was always a topic of discussion within several research communities and it might require attention.

The results of the initial research work of NanoS-QM led to a catalogue of quality criteria which is presented in the form of a Mind Map in section 4.3 (Supplement – Catalogue of criteria of the NanoS-QM Project). The Mind Map format was chosen because it provides a clear vision of the process and its sub-processes.

Most used data formats
Table: xls, csv, ascii, matlab
Code: py, ipyn (Jupyter & python)

Text: txt, pdf
Image: gif, png, jpeg, tiff, svg, manufacturer formats
Application: xml (Extensible Markup Language), hdf5 (Hierarchical Data Format)
Proprietary data format: CEL & FASTQ (gene array, sequence), FCS (flow cytometry standard), mzML/mzXML(mass spectrometry)
Molecular structure format: mol, pdb
Ontology data format: owl , *rdf

4.3 Introduction to the representation of the quality criteria

The Mind Map (Supplement – Catalogue of criteria of the NanoS-QM Project) represents a summary of relevant quality criteria for research data and corresponding metadata in the research field of nanosafety which were collected within the Task "Assessment of the status quo of quality criteria and disciplinary requirements" of the NanoS-QM project. The collected criteria need to be validated by round robin tests and the question remains which parameters may need to be added to the current criteria. For a better overview, the criteria were assigned to different categories, which deal with experimental (highlighted in green) and content-related aspects (highlighted in yellow) as well as legal (highlighted in red) and technical framework conditions (highlighted in blue).

> *One consideration was to determine which data and related metadata are essential in the area of nanosafety along the research data lifecycle.*

Every single step of the cycle, i.e. from planning a research project to collecting and analysing research data and associated metadata for publication, archiving and reuse, requires different data and metadata. Metadata can be understood as structured information that helps to describe and grant access to resources of all kinds by means of standardised schemes.

> *To develop a comprehensive metadata standard, different types of metadata must be observed. This way, the state of the data in the respective cycle step can be reproduced as precisely as possible. Within this project, metadata were categorised as subject-specific (e.g. basic information and materials, material characterisation, study information, instrument information, analysis and results, discussion/limitation, read out, experimental outcomes), bibliographic (e.g. title, creator, keywords, abstract/description, coverage/geolocation, format, identifier (i.e. doi), issue, journal name, language, pages, publication year, publisher, resource type, source, volume, contributor), administrative (e.g. manufacturer/record maintainer, terms of use, rights), technical (e.g. article history,*

data format, data type, download link to the file, file size, resolution, status) or structural (e.g. relation/publications references, version).

With regards to the provision of data for risk assessment and regulation, subject-specific parameters are of paramount importance for the validation of in vitro *tests by* in vivo *experiments and the development of regulatory relevant innovative test systems and predictive assays. Harmonization principles such as the Mutual Acceptance of Data (MAD) for non-clinical health and safety test study by the OECD ensure that test study data, that are generated in accordance with the OECD TGs and the OECD Principles of Good Laboratory Practice (GLP), should be accepted in all OECD member countries (MC) for risk assessment and other purposes with regard to the protection of human health and the environment. Interesting in the context of subject-specific metadata acquisition is the specification ISA-TAB-Nano developed by Thomas et al. (2013), an extension of the ISA-TAB standard, which comprises four spreadsheet-based file formats (Investigation, Study, Assay and Material) for representing and integrating various types of NM data. ISA-TAB-Nano considers the use of ontology terms to support standardised descriptions and to facilitate search and integration of data. This specification could possibly provide a good starting point for the development of the metadata standard in the framework of this project. In addition to subject-specific metadata, bibliographic information is generally used to describe data, e.g. publications. This type of metadata becomes important when a scientist is looking for reference information and data relevant to his planned study design or when he desires to publish his results. Among others, DataCite Metadata Schema and Dublin Core DCMI Metadata Element Set Version 1.1 are widely used for this purpose. If studies and corresponding data are to be stored in a database or in a repository, administrative and technical information are required in addition to the metadata already mentioned. In summary, the workshop participants added that it would be important to include metadata about dose-effect relationships as well as the human factor with regard to carrying out experiments (e.g. storage and handling of materials, suspension preparation, assay implementation and device performance).*

The collection of minimum information was carried out by analysing the current existing standards and ISO norms (for examples see “Analysis of existing standards or guidelines”) as well as the respective experiments on material characterisation and studies on the effects in biological systems. Finally the results of the interview performed on data reuse contributed to complete the list of minimum requirements.

4.3.1 Standardisation of data and metadata

With regard to scientifically comprehensive standardization, rules of conduct such as the “Guidelines for Safeguarding Good Scientific Practice” and “Empfehlungen zur gesicherten Aufbewahrung und Bereitstellung digitaler Forschungsprimärdaten” of

the German Research Foundation (DFG) provide appropriate standards for scientific work and are intended to promote the accessibility and reusability of research data and their related metadata. Another important aspect in addition to standardizing data is maintaining and increasing its quality. The position paper "The challenge of data quality" by the German Council for Scientific Information Infrastructures (RfII) examines, for example, current challenges in this context and derives recommendations. To facilitate curation of research data, guides such as DDCs "How to Appraise and Select Research Data for Curation" offer guidance on developing approaches for assessing and selecting data sets for curation.

In order to add more value to the data and to guarantee a cross-system common understanding of the terms used, metadata should be enriched as much as possible.

This can be achieved, for example, by using norm data.

> *Norm data are standardised data to avoid ambivalences (i.e. different names of a person or a place).*

They describe entities such as people, corporations, locations, events or subjects and are used by cultural and scientific institutions such as archives, libraries or museums but also by scientists in research projects. Each entity contains a unique stable identifier that can be used to link objects to each other and to external sources. Examples are represented by the GND-ID (Gemeinsame Normdatei-ID), which is assigned and administered by the German National Library or the ORCID-ID (Open Researcher and Contributor-ID) of the ORCID organisation *for the unique identification of scientific and other academic authors.* Another strategy to enrich metadata is to use controlled vocabularies, for example to assign research data to an area of expertise or to define their language and country of origin based on approved standards (ISO norms). Controlled vocabularies are a collection of defined terms for the uniform description of objects. These terms are identified to facilitate the comprehensive search and retrieval of data. In addition to controlled vocabularies, ontologies can also be used, which represent a knowledge area in the form of triples (subject - predicate - object), which in turn can be represented as a knowledge graph. Components for this are hierarchically ordered concepts, implemented as classes, instances or elements, properties as well as relations between concepts and restrictions. Each element consists of a name and a Uniform Resource Identifier (URI). Well known is, among others, the Open Biological and Biomedical Ontology (OBO) Foundry, which is dedicated to the creation and maintenance of ontologies in the field of biomedicine and has developed a set of principles for ontology development. Currently, there are more than one hundred ontologies which follow these principles.

4.3.2 Technical Requirements

In addition to the relevant criteria, the necessary technical requirements should also be considered in order to enable effective management of the research data. These

include the establishment of an authentication and authorisation infrastructure (AAI) in order to guarantee controlled access and thus prevent the misuse of research data. For assistance, generally known guidelines such as the FAIR principles ensure that data are findable, accessible, interoperable and reusable. However, an attempt should be made to avoid access restrictions (e.g. password requests or encryption) as far as possible in order not to make access unnecessarily difficult or impossible for third parties. Another important aspect is that technical dependencies (e.g. hard- and software) should be evaluated in advance, for example when planning to implement an application programming interface (API) that is needed to access data in databases or repositories. In order to ease the work in science, access to content-relevant databases or repositories should generally be made possible. It would also be helpful here to agree on a common export format based on defined criteria (e.g. hardware and software independent, human and machine readable, flexible storage capacity, etc.) in order to ensure the best possible interoperability.

Moreover, a concept for the integration of an ELN was recommended, as it supports the necessary digitisation of work processes within a laboratory and enables automated documentation of metadata which helps to save time and increase the data quality. During the workshop two examples were suggested: 1. Chemotion ELN, an open source modular system for researchers working in the field of chemical sciences. The web based application provides the basic functionalities necessary for the acquisition and processing of chemical data and can be extended with specification from other systems. A pilot project of Chemotion will also be carried out within the NanoS-QM project. 2. ElabFTW, an open source tool currently used by IWT-Bremen. Data can be stored in a structured way and protocols can be pre-fabricated. However, it does not offer the collection of metadata in the system and is therefore not an option for the NanoS-QM project. It can be concluded that the motivational aspect plays an important role in the readiness to use ELN: it should be a simple tool and the advantage of collecting metadata should be clear but also the reward in the form of an increased impact factor or the opportunity for collaborations.

4.3.3 Legal aspects

Finally, those aspects that regulate the handling of research data at the legal level should not be overlooked. Here, legal regulations on copyright, data protection and rights of use should be observed. To comply with copyright law, research data should be subject to licensing. In this context, the DDC's "How to License Research Data" guide provides, with a focus on the UK, guidance on how to apply license to research data and explains which one of them are most appropriate. Widely used for the publication of content are, for example, the licenses of the Creative Commons Organisation, which are internationally recognised and can be converted into rights of use or distribution.

The organization offers six standard license contracts that can be customized to provide a wide range of options for determining the further use of research data.

In the scientific research environment, for example, the Creative Commons License 4.0 (CC-BY 4.0) is widely used, which permits the sharing and processing of data under attribution. Compliance with data protection should, above all, play a role when personal data such as name, date of birth or patient data needs to be made available for reuse.

Here it is important to ensure that prior consent for the transmission and reuse of this data is obtained and that confidentiality is contractually guaranteed by the user.

Furthermore, terms of use should be defined to ensure transparency in the use of research data.

Questions to be discussed:

- Could parameters with general validity be identified?
- Are there other or further relevant regulatory or scientific standards that should be considered?
- Which do you think the barriers to data re-use are? (E.g. accessibility, ontology, data security and reliability, intellectual property among others)
- Which factors you think are relevant with respect to data reuse?
- Which databases/repositories are relevant in this research area?
- Do you think that the criteria collected within the NanoS-QM project should be completed with additional information in order to guarantee an optimal data reuse?
- Do you think that the mentioned parameters are equivalent for scientific and industrial research?

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