The Director General

Maisons-Alfort, 30 September 2021

OPINION
of the French Agency for Food, Environmental and Occupational Health & Safety

on a specific health risk assessment guide for nanomaterials in food products

ANSES undertakes independent and pluralistic scientific expert assessments.
ANSES primarily ensures environmental, occupational and food safety as well as assessing the potential health risks they may entail.
It also contributes to the protection of the health and welfare of animals, the protection of plant health and the evaluation of the nutritional characteristics of food.
It provides the competent authorities with all necessary information concerning these risks as well as the requisite expertise and scientific and technical support for drafting legislative and statutory provisions and implementing risk management strategies (Article L.1313-1 of the French Public Health Code).
Its opinions are published on its website. This opinion is a translation of the original French version. In the event of any discrepancy or ambiguity the French language text dated 30 September 2021 shall prevail.

On 17 October 2016, ANSES received a formal request from the Directorate General for Food (DGAL), the Directorate General for Health (DGS), the Directorate General for Labour (DGT), the Directorate General for Risk Prevention (DGPR) and the Directorate General for Competition, Consumer Affairs and Fraud Control (DGCCRF) to conduct the following expert appraisal: request for an opinion on nanomaterials in food products.

1. BACKGROUND AND PURPOSE OF THE REQUEST

Background

In many areas such as the agri-food sector, engineered nanomaterials are used for their unique nanoscale properties (optical, mechanical, etc.) and large specific surface area. Engineered nanomaterials may be added intentionally as food additives, or as technological additives in the formulation of food contact materials.

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Nanomaterials are not covered by any specific regulations, but are governed by various existing sectoral regulations (EC No 258/97[1], EU No 1169/2011[2], EU No 10/2011[3], etc.). The question of regulatory harmonisation is one of the issues and concerns raised by civil society organisations – mainly non-governmental organisations (NGOs) – regarding nanomaterials in general, and in food in particular.

The coexistence of different definitions of nanomaterial within these sectoral regulations contributes to confusion, mainly through the interpretation of the different terms used. In this context, as part of its expert appraisal, the Working Group (WG) on "Nano and Food" established a classification of the term "engineered nanomaterial" (Section 3.1), in order to clarify the scope of the analysis. As a preamble and in order to facilitate reading of the opinion, the WG provides some clarifications in the box below.

In the context of this formal request, the concept of "intentional production" refers to the deliberate production of nano-sized particles of the engineered nanomaterial (see classification Section 3.1). The concept of "unintentional production" refers to the unintended production of nano-sized particles of the engineered nanomaterial.

The WG uses the term "intentionally added" when a substance containing or likely to contain engineered nanomaterials (see classification Section 3.1) is added deliberately and has a technological purpose in food. It uses the term "unintentionally added" when the presence of a substance containing or likely to contain engineered nanomaterials does not have a technological purpose in food.

By way of illustration, E171[4] is a food additive that is intentionally added to food and for which the production of nano-sized particles is unintentional. E551[5] is a food additive that is intentionally added to food and for which the production of nano-sized particles is intentional. E551 is also used as a processing aid in the formulation of food, in which case it is not intended to be found in food.

<u>Purpose of the request and scope of the study</u>

In this context, the WG on "Nano and Food" was set up to respond to the following requests:

1) Carry out a detailed study of the agri-food sector;

2) Prioritise the substances and/or finished products according to relevant criteria;

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[1] Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients.
[2] Regulation (EU) No 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers.
[3] Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food.
[4] E171: food additive containing titanium dioxide.
[5] E551: food additive containing synthetic amorphous silicas.

3) Update the available data (data on toxicological effects and exposure data);

4) Study the feasibility of a health risk assessment of nanomaterials;

5) Depending on the results of 4), carry out a risk assessment of the prioritised nanomaterials.

In order to respond to these requests, ANSES suggested breaking the work down as follows:

- Phase 1: carry out a detailed study of the agri-food sector;
- Phase 2: conduct an analysis of the criticisms and contentions relating to the presence of nanomaterials in food and the different dimensions they raise (scientific, regulatory, economic, societal);
- Phase 3: examine the issue of assessing the risks of exposure to intentionally added engineered nanomaterials contained in human food products identified in Phase 1, considering the following points:
  - prioritisation of substances and/or end products of interest according to relevant criteria;
  - review of the available data (data on toxicological effects and exposure data) on the prioritised substances.

The scope of this formal request was limited to intentionally added nanomaterials in human food, which concerns:

- food additives;
- substances used in active and intelligent food contact materials (FCMs) deliberately brought into contact with food.

Engineered nanomaterials added intentionally during the processing of raw materials, food or water, but which do not have a technological purpose in food, were not considered in this work. This concerns:

- processing aid residues;
- residues from the migration of food contact materials (FCMs) other than active and intelligent packaging, and of water contact materials;
- residues from the treatment or supply of drinking water;
- veterinary medicinal product residues;
- plant protection product residues;
- residues of biocidal products used as disinfectants or for other purposes;
- other nanomaterials resulting from (or related to) accidental contamination of the food chain.

Some nanomaterials are used in food supplements. However, due to the lack of information on the type of nanomaterials used, the food supplements concerned and consumption habits, these products were not considered in this formal request.

Excluding these nanomaterials does not mean that there is no health concern. The nanomaterials in the above-mentioned residues, as well as those used in food supplements, may undergo a risk assessment as part of a later formal request.

In 2020, in the framework of this formal request, ANSES published an initial opinion[6] incorporating the conclusions and recommendations of the WG on "Nano and Food" and the CES ERCA relating to the first two phases of the expert appraisal, namely: (i) a detailed study of the agri-food sector and (ii) an analysis of the criticisms/contentions relating to the presence of nanomaterials in food and the different types of issue they raise (scientific, regulatory, economic, societal). Initial information for Phase 3, relating to the issue of assessing the risks of exposure to intentionally added engineered nanomaterials, was also discussed.

The objective of this opinion is to propose a scientific and technical guide describing the methodology to be used for assessing the risk of the nanoscale fraction of engineered nanomaterials used as food additives. Assessing the risk associated with engineered nanomaterials used in FCMs was not considered in this guide due to the use of different methodological concepts (especially for exposure calculations) and a lack of data.

Application of this guide to specific cases of engineered nanomaterials is the subject of ongoing expert appraisal work, which will be published in a separate opinion at a later stage.

2. ORGANISATION OF THE EXPERT APPRAISAL

The expert appraisal was carried out in accordance with French Standard NF X 50-110 "Quality in Expert Appraisals – General requirements of Competence for Expert Appraisals (May 2003)".

ANSES analyses interests declared by experts before they are appointed and throughout their work in order to prevent risks of conflicts of interest in relation to the points addressed in expert appraisals. The experts' declarations of interests are made public via the ANSES website (www.anses.fr).

ANSES entrusted examination of this request to the Working Group (WG) on "Nano and Food", reporting to the Expert Committees on "Assessment of the risks related to physical agents and new technologies" (CES AP) and "Assessment of physico-chemical risks in food" (CES ERCA).

The methodological and scientific aspects of the WG's work were regularly submitted to the CES AP and CES ERCA between September 2017 and May 2021.

The WG's work was adopted by the CES ERCA (lead CES) at its meetings on 15 April and 19 May 2021.

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[6] Nanomaterials in food products https://www.anses.fr/fr/system/files/ERCA2016SA0226Ra.pdf

## 3. ANALYSIS AND CONCLUSIONS OF THE WG ON "NANO AND FOOD" AND THE CES ERCA

3.1. EFSA's proposed methodology for risk assessment of nanomaterials: analysis and comments of the WG on "Nano and Food"

In 2018, EFSA published a scientific and technical guide (EFSA 2018) proposing a comprehensive, tiered methodology for the risk assessment of nanomaterials used in food, feed, novel foods, food contact materials and pesticides. This document provides guidance to applicants when applying for authorisation to use nanomaterials. It also provides guidance to assessors in their risk assessment process.

This guide details all the information on the definition of nanomaterials, physico-chemical characterisation, dissolution, exposure, hazard identification and characterisation that needs to be considered for assessing the risk of nanomaterials in the food and feed chain. The specificities of nanomaterials have been integrated by the EFSA Scientific Committee throughout the risk assessment methodology; it is therefore referred to as a "nanospecific" risk assessment methodology.

The tiered approach proposed by EFSA is illustrated in Figure 1 and addresses the following questions about the studied material:

- Is the material a nanomaterial?

- Does the material have properties that are characteristic of the nanoscale?

- Is the nanomaterial found in food?

- Does the material quickly degrade in digestive tract conditions?

- Is the material biopersistent?

- Does the material have any adverse effects?
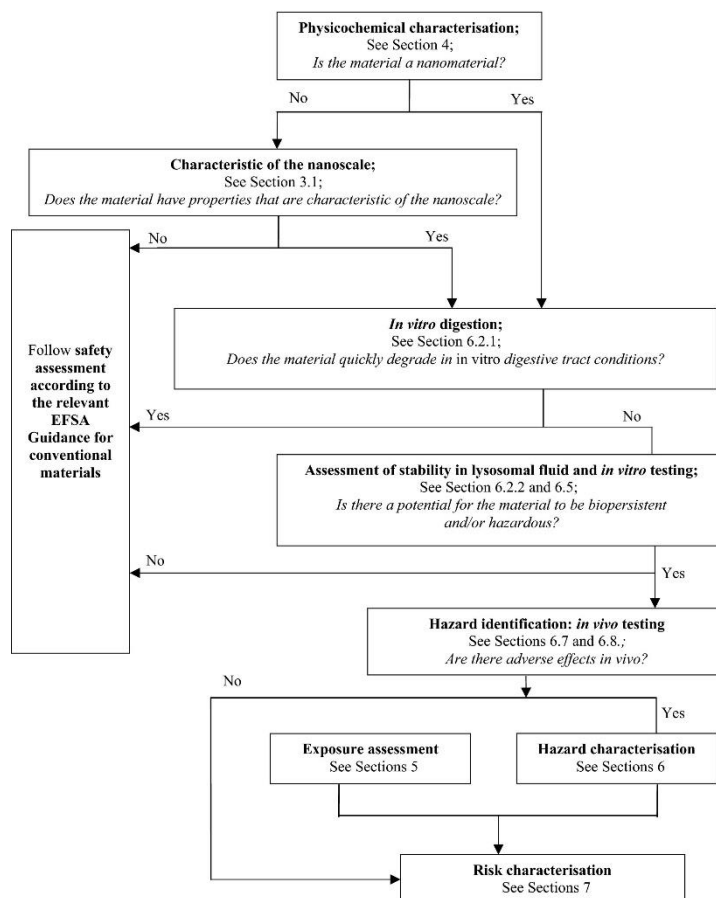
Figure 1. EFSA flowchart presenting the tiered approach to risk assessment of nanomaterials[7]

Comments of the WG on "Nano and Food"

The WG on "Nano and Food" reviewed the guidance document proposed by EFSA and identified some limitations, which are listed below and detailed in the following sections.

- Nanomaterials and definitions considered in the context of a nanospecific risk assessment;
- Analytical techniques used to measure the size of the constituent particles of engineered nanomaterials;
- Detection of nanomaterials in food;
- Nanomaterial dissolution in gastrointestinal tract conditions;
- Nanomaterial dissolution in lysosomal conditions (assessment of biopersistence and intracellular accumulation of nanomaterials);
- Toxicological studies considered in the context of a nanospecific risk assessment.

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[7] EFSA 2018. Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health.

- • Discussions on nanomaterials and definitions considered in the context of nanospecific risk assessments

The EFSA guide considers all the nanomaterials described below:

- engineered nanomaterials meeting the definition of the Novel Food Regulation (EU) No 2015/2283 and the INCO Regulation (EU) No 1169/2011 on information to consumers, i.e. nanomaterials that have particle sizes in the defined nanoscale (1-100 nm) (EFSA 2018);

- other materials that contain particles having a size above 100 nm and exhibiting characteristic nanoscale properties (such as a large specific surface area or different toxicokinetic behaviour compared to the non-nanoform) should also be considered (EFSA 2018);

- materials that are not engineered as nanomaterials but contain a fraction of particles, less than 50% in the number-size distribution, with one or more dimensions in the size range 1 to 100 nm (EFSA 2018);

- nanoscale entities made of natural materials that have been deliberately produced to have nano-enabled properties, or that have been modified for use in the development of other nanoscale materials (e.g. for encapsulating components) (EFSA 2018);

- nanomaterials having the same elemental composition but occurring in different morphological shapes, sizes, crystalline forms or surface properties, for example, as a consequence of different production processes (EFSA 2018);

- nanomaterials that meet the European Commission's recommendation on a definition (and future reviews) (although this recommendation is currently under review) (EFSA 2018).

As mentioned in the ANSES opinion of 2020 and reiterated in this document, the risk assessment methodology (HRA) proposed by the WG on "Nano and Food" is limited to engineered nanomaterials used as food additives. In this context, the WG established its own classification of the term engineered nanomaterial, because the concepts of intentionality and 50% particle number threshold, as mentioned in the regulatory definitions, were not considered relevant to or compatible with the WG's work (Section 3.2.2). It is important to remember that this classification has no regulatory value.

- • Discussions on analytical techniques used to measure the size of the constituent particles of engineered nanomaterials

Determining the size of the constituent particles is an essential step throughout the process of risk assessment of nanomaterials. In particular, it is used to verify whether the studied materials meet the physico-chemical criteria used for the regulatory definitions or, in the context of this formal request, for the classification of an engineered nanomaterial proposed by the WG. Measuring the size of the constituent particles is also essential for calculating the

mass fraction of particles in the nanoscale (Section 3.2.4.5) or for the grouping and read-across steps that may be used in the hazard characterisation (Section 3.2.5.1).

EFSA proposes the use of several independent techniques for measuring size, one of which is electron microscopy. If electron microscopy observation is not possible, alternative imaging techniques can be used. In addition, other non-imaging-based analytical techniques such as dynamic light scattering (DLS) and centrifugal liquid sedimentation (CLS) are also suggested.

The WG believes that only the results of measurements of the size of the constituent particles of engineered nanomaterials obtained by electron microscopy should be used. Indeed, this technique has the fewest preparation artefacts and assumptions in the processing of results. It is therefore the most suitable and most robust method for determining the absolute size and morphology of these particles.

Electron microscopy observations can also be used to distinguish between isolated particles and those embedded in aggregates and agglomerates[8]. The WG recommends systematically coupling electron microscopy observations with energy dispersive x-ray (EDX[9]) analysis, in order to limit the observation of artefacts (e.g. nanomaterials other than the one of interest).

- Discussion on the presence of nanomaterials in food

The EFSA Scientific Committee addresses the issue of nanomaterials in food at an early stage of its methodology. If nanomaterials are observed in food matrices, then the so-called "nanospecific" risk assessment should continue, and should study the dissolution phenomena in the conditions of the gastrointestinal tract. On the contrary, if no nanomaterials are observed in food matrices, then the risk assessment should follow a standard approach. If no quantitative data are available on the concentration of a nanomaterial in food or on dissolution phenomena, EFSA proposes drawing up a "worst case" scenario, i.e. assuming that all the nanomaterials initially added to food are ingested and then absorbed in the form of nanoscale particles.

According to the WG, quantification of nanomaterials in food is an essential step for calculating exposure (Section 3.2.4). However, the WG questions the relevance of incorporating restrictive, time-consuming and costly nanomaterial dissolution and quantification studies for all the foods concerned at an early stage of the risk assessment methodology. Indeed, according to the EFSA document, guiding the HRA towards either a standard or nanospecific approach implies measuring the dissolution phenomena of the nanomaterial considered. Because of the diversity of food matrices and therefore of physico-chemical environments, exhaustive screening for nanomaterials in all these matrices would need to be performed and the different analytical steps optimised according to the type of foods considered (Section 3.2.4.3). Moreover, nanomaterials may exhibit dynamic dissolution phenomena (e.g. the nanomaterial may be in dissolved form in the food and then in particulate form again *in vivo*, due to different physico-chemical conditions). The WG did not therefore include measurements of dissolution in food in its methodology.

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[8] ANSES Opinion 2018-SA-0168. Review of analytical methods available for characterising nano-objects and their aggregates and agglomerates, in order to meet regulatory requirements.
[9] EDX can be used to identify the chemical components of particles observed in electron microscopy.

- Discussion on dissolution phenomena in gastrointestinal tract conditions

In its 2020 opinion (ANSES 2020), ANSES had analysed the methodology concerning dissolution phenomena proposed by EFSA. Briefly, in the EFSA guidance document, the Scientific Committee stated that if less than 12%[10] by mass of the starting material was found in nanoparticle form after 30 minutes of incubation, then the risk assessment did not require the implementation of a "nanospecific" approach. The WG on "Nano and Food" had highlighted several limitations related to the parameters used in developing this threshold (half-life of nanomaterials, time to pass through the intestinal barrier, intrinsic properties of nanomaterials). Because of these limitations, the WG did not adopt the dissolution threshold proposed in the EFSA guide. In this context, an alternative analytical approach to characterising nanomaterial dissolution was proposed by the WG on "Nano and Food" in the ANSES opinion of 2020[11] and is set out in Section 3.2.3 of this document.

- Discussion on phenomena of biopersistence and intracellular accumulation

The study of biopersistence and accumulation as proposed by EFSA consists in measuring dissolution phenomena of nanomaterials in lysosomal conditions (i.e. in pH conditions simulating the internal environment within lysosomes). However, the absorption of nanomaterials by cells can occur through different mechanisms. These include "active" mechanisms that can lead to the formation of lysosomes in which acidic conditions and the presence of enzymes favour the degradation/dissolution of nanomaterials. Measuring the stability of nanomaterials in these conditions would therefore enable their persistence and intracellular accumulation to be assessed. As with the measurement of dissolution in the gastrointestinal tract, a threshold of 12% by mass of the starting material after 72 h of incubation was proposed by EFSA. Below this threshold and in the absence of any observed toxicity in the battery of *in vitro* tests proposed in the EFSA guide, continuation of the "nanospecific" HRA is no longer necessary and a standard approach can then be implemented. The WG on "Nano and Food" reiterates the previously mentioned limitations of the methodology used for determining the 12% mass threshold for measuring dissolution in the gastrointestinal tract. Moreover, intracellular accumulation phenomena are complex mechanisms and may follow different internalisation pathways, some of which are shown in Figure 2.

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[10] Calculation of the 12% mass threshold is explained in the EFSA 2018 guide (Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health) as well as in the ANSES 2020 opinion on nanomaterials in food.
[11] https://www.anses.fr/fr/system/files/ERCA2016SA0226Ra.pdf
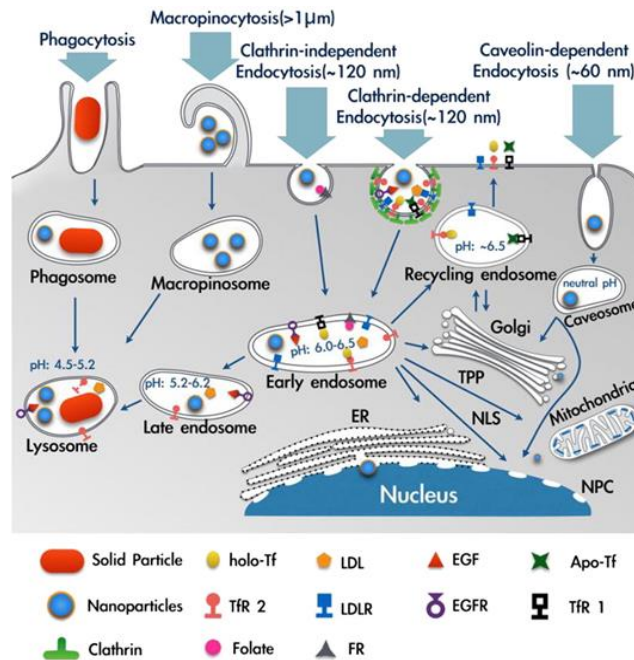
Figure 2. Illustration of the different cellular internalisation pathways of nanomaterials (Yameen *et al.* 2014)

These mechanisms are not yet fully understood. The illustration in Figure 2 indicates that there may be various intermediate steps before formation of the lysosome. These intermediate steps may direct the nanomaterials to other cell organelles such as the Golgi apparatus, mitochondria, endoplasmic reticulum or nucleus. For example, studies (Slowing *et al.* 2006) have shown that silica nanoparticles internalised within human cancer cells (HeLa) were able to escape from endosomes depending on the surface charge of the particles (the more negative the surface charge, the more the silica nanoparticles are able to escape). Lastly, other absorption pathways not involving lysosome formation, such as the paracellular pathway, have also been observed in the crossing of the intestinal barrier (Coméra *et al.* 2020). In this context, therefore, the WG did not adopt this step of dissolution within lysosomes and believes that only the concept of dissolution within the gastrointestinal tract should be considered.

- Discussions on the toxicological studies considered in the context of a nanospecific risk assessment

Overall, the hazard identification and characterisation methodologies proposed by EFSA and ANSES are similar and are both based on a tiered approach. The results of the required *in vitro*, toxicokinetic and subchronic studies determine whether further in-depth studies are needed. However, some changes have been made by the WG. The main differences in hazard identification and characterisation developed by the WG will be explained throughout the document and are summarised below:

- Biopersistence and intracellular accumulation (dissolution in lysosomes) are not considered;

- Testing for precancerous biomarkers (preneoplastic cells) in the gastrointestinal tract is systematically performed in the repeated dose 90-day oral toxicity study;
- Studies on the gut microbiota are not considered.

In summary, certain steps proposed in the EFSA guide were not adopted by the WG on "Nano and Food" as mentioned above and in the ANSES opinion of 2020. The main points not adopted by the WG are summarised below and in the table in the Conclusions:

- The WG did not use the same definitions as those used by EFSA. Indeed, the classification of "engineered nanomaterial" as described in this document is only used by the WG on "Nano and Food" in the context of this formal request;
- Analytical techniques other than electron microscopy were not used to measure the size of the constituent particles;
- Measurements of nanomaterial dissolution in food were not selected as a parameter to be considered when deciding whether or not to pursue the nanospecific HRA;
- The dissolution thresholds proposed by EFSA were not adopted and alternative analytical strategies for measuring dissolution in gastrointestinal tract conditions are suggested below by the WG;
- The concepts of biopersistence and intracellular accumulation as assessed through lysosomal dissolution were not considered in the WG's methodology;
- Changes were made regarding the studies to be considered for hazard identification and characterisation (see Conclusions).

The nanospecific risk assessment methodology proposed by the WG on "Nano and Food" is presented sequentially in the following sections and summarised in Figure 3.

3.2. Risk assessment methodology for the nanoscale fraction of engineered nanomaterials proposed by the WG on "Nano and Food"

3.2.1. Overall description of the nanospecific risk assessment methodology

The HRA of the nanoscale fraction of engineered nanomaterials is based on the same concepts as an HRA of so-called conventional substances. The three pillars of risk assessment are:

- Characterising the substances of interest from a physico-chemical perspective;
- Determining the levels of consumer exposure to these substances;
- Identifying and characterising the hazard associated with these substances.

Nanomaterials have specific physico-chemical characteristics that give them different properties (optical, mechanical, etc.) and behaviours (stability, adsorption, etc.) from those

observed in conventional substances. Moreover, engineered nanomaterials are made up of particles that vary greatly in terms of size, morphology, crystallinity, etc.

Ideally, the HRA should be conducted specifically on each of the constituent particles of the studied nanomaterial. However, this approach is not scientifically and technologically feasible. Nanomaterials should therefore be regarded as complex mixtures in which attempts at particle grouping and read-across can be implemented to carry out an assessment of the risks to consumers exposed to these engineered nanomaterials (Section 3.2.5).

The specificities of nanomaterials have been integrated at different steps of the risk assessment methodology proposed in this document, whether for developing the classification of an engineered nanomaterial, or during the steps to characterise dissolution, determine exposure or decide the toxicological studies to be considered. Figure 3 illustrates the main steps of this "nanospecific" methodology. These steps should be approached in a sequential manner:

- Step 1: Does the studied material qualify as an engineered nanomaterial?

- Step 2: Is the nanomaterial completely dissolved in gastrointestinal tract conditions?

- Step 3:

    a: What are the levels of consumer exposure to the nanoscale fraction of the engineered nanomaterial?

    b: What are the toxicological effects of the nanoscale fraction of the engineered nanomaterial?

- Step 4: Are there sufficient data on hazard characterisation and exposure levels to conduct the risk characterisation? If so, does the nanoscale fraction of the engineered nanomaterial pose a risk?

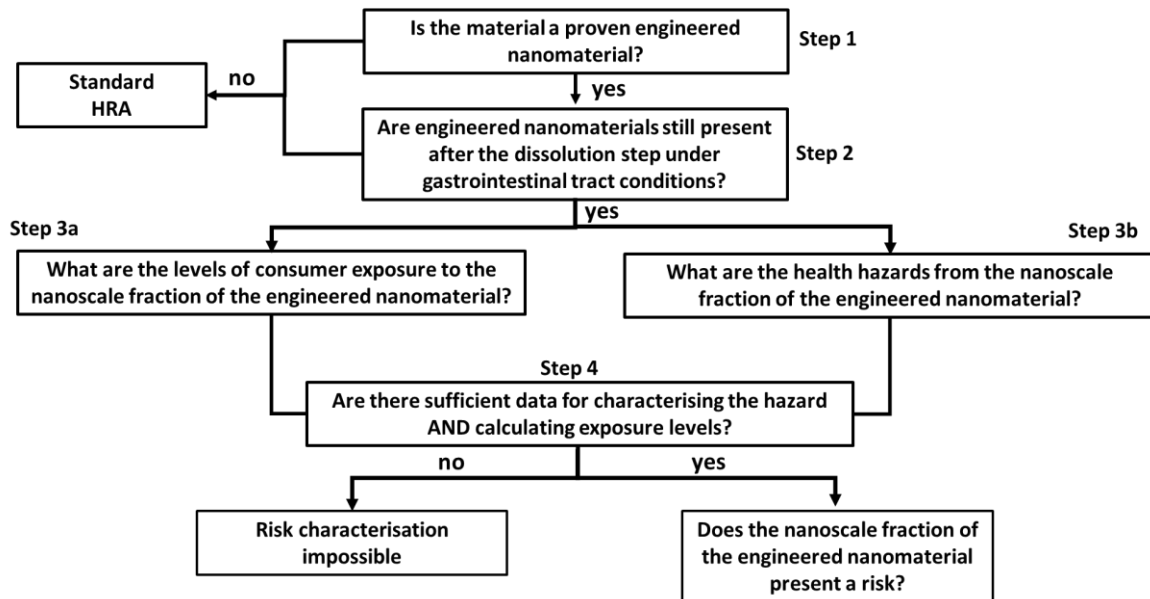All these steps are described in the sections below.

Figure 3. Flow chart presenting the overall methodology for nanospecific risk assessment developed by the WG on "Nano and Food". Details of the exposure calculation and hazard characterisation steps are given in Figure 6 and Figure 9.

3.2.2. Step 1: Does the material qualify as an engineered nanomaterial?

Methodology

The first step is to determine whether the studied material corresponds to a proven nanomaterial, i.e. whether it fits the description of the term "engineered nanomaterial" proposed by the WG and restated below.

Engineered nanomaterial: material of an organic, inorganic or composite nature, produced by humans for application purposes and comprised wholly or partly of constituent particles with at least one dimension between 1 and 100 nm (nanoscale).
The dimensions of the constituent particles may be greater than 100 nm if these particles have a large specific surface area or nanoscale properties.
Constituent particles can be found in the form of aggregates or agglomerates whose dimensions can be much larger than the nanoscale.
Materials for which the nanoscale fraction was not intentionally produced during the manufacturing process are included in the scope of this classification.

The details on the development of this classification were specified in the ANSES opinion of 2020 on the first phase of the expert appraisal in response to this formal request. The main points are briefly restated below.

The WG decided not to take into account the 50% particle number threshold as mentioned in the European Commission's recommendation on a definition[12]. This threshold is not based on any health, analytical or technological considerations.

Several techniques are currently used, particularly by official testing laboratories, to detect the presence of nanomaterials, such as electron microscopy (transmission or scanning), dynamic light scattering (DLS), single particle counting by inductively coupled plasma mass spectrometry (sp-ICP-MS) or by asymmetric flow field-flow fractionation (AF4). The WG believes that electron microscopy is currently the most suitable and most robust technique for measuring the size of the constituent particles.

For the same chemical composition, certain properties and behaviours of matter at the nanoscale can be fundamentally different from those of the same compound at a larger size or in molecular form. The properties specific to the nanoscale are associated with a large specific surface area compared to non-nanoscale forms (and therefore potentially high chemical or biological reactivity) as well as with particular physical or chemical properties (mechanical, optical, etc.).

Electron microscopy, considered by the WG to be the most suitable and most robust technique for measuring particle size, can be seen as a semi-quantitative technique (estimation of the number of nano-sized particles ≤ 100 nm). In addition, various electron microscopic observations have shown that nanomaterials used as food additives are often observed in the form of aggregates/agglomerates. This can be due to food product manufacturing processes, the physico-chemical nature of the nanomaterials and the sample preparation protocols prior to electron microscopy observation. An analytical limit of detection could not therefore be established by the WG.

However, in order to limit the difficulties in interpreting the results obtained by electron microscopy, the WG proposes implementing the following procedure. Electron microscopy observations should be based on three independent preparations from the same batch of the studied material. For each preparation, the operators should measure particle size from a population ranging from at least 100 particles to 300[13] particles observed on around 10 microscopic fields (chosen randomly from the entire surface of the preparation), to ensure better representativeness. All observable particles (with well-defined contours) in each of the fields of observation should be measured. On completion of this analysis, if at least one particle in the nanoscale, in the free state or embedded in an aggregate/agglomerate, is observed on each of the three preparations, then the material qualifies as an engineered nanomaterial. Otherwise, the material will not be regarded as a nanomaterial according to the classification proposed by the WG.

In order to limit ambiguities in interpretation, particularly for samples containing a small nanoscale fraction, a negative control consisting in observing in EM only the dispersion medium (i.e. without the studied material) will help detect possible sample contamination (nanomaterials found in the air, laboratory instruments, etc.). Combining EDX with EM observations ensures that the observed objects are made of the same chemical element as

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[12] Commission Recommendation 2011/696/EU of 18 October 2011 on the definition of nanomaterial.
[13] Above a population of 300 particles, the particle size distributions are only slightly affected by any increase in the number of particles.

the studied material. In the case of scanning EM observation, the WG recommends using the spin coating procedure as described in Section 3.2.4.5 for sample preparation.

Decisions

If the studied material does not meet the classification established by the WG, then the risk assessment should follow a standard approach. If the material is identified as an engineered nanomaterial according to the classification established by the WG, then Step 2 should be followed.

3.2.3. Step 2: Are engineered nanomaterials left over after the dissolution step in gastrointestinal tract conditions?

Step 2 serves to determine whether engineered nanomaterials are still present after a dissolution step in digestive tract simulants. The study by Avramescu *et al.* (2017) investigated the influence of parameters such as pH, temperature and crystal form on the dissolution behaviour of inorganic nanomaterials. In this study, two materials were tested: ZnO and TiO_2. For these two studied compounds and in both pH conditions (1.5 and 7), the tests showed that regardless of the pH condition, the ability of the non-nanoscale analogues of ZnO and TiO_2 to resist chemical/biochemical alterations was equal to or greater than that of the corresponding nanomaterials. The results also showed that the particle size and crystalline form of inorganic nanomaterials are important properties that influence dissolution behaviour and biodurability. Lastly, all the nanomaterials and non-nanoscale analogues displayed significantly higher solubility at acidic pH than at neutral pH.

In vitro digestion models have already been addressed in publications[14]. The operating conditions used in these models reflect the physiological conditions of the gastrointestinal tract, with varying degrees of complexity. Thus, digestion studies have been carried out on certain nanomaterials (Ag, SiO_2, ZnO) using these models and have been published in the NANoREG project (deliverable D5.02).

In view of the absence of standardised tests and the diversity of engineered nanomaterials, the WG is not currently able to recommend detailed protocols setting out precisely all the physico-chemical parameters to be considered that could be transposed to all the nanomaterials identified for this formal request. In this opinion, the WG proposes analytical strategies and lists the factors requiring vigilance, such as the intrinsic parameters of nanomaterials (size, chemical nature, etc.) and the experimental conditions (type of medium, ionic strength, initial concentration of (nano)materials, etc.).
The analytical strategies proposed by the WG should therefore be tailored to the specificities of each engineered nanomaterial, and the choice of experimental conditions implemented by the operators should be justified.

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[14] Report on the development of a solubility testing procedure, NANoREG, 2015.

The WG proposes two approaches for studying the dissolution phenomena of nanomaterials. Initially, the WG recommends following Strategy 1 (detailed in the next paragraph), which is simple to implement and provides a rapid response regarding the presence of engineered nanomaterials after the dissolution step. In the event of a negative result and in order to limit the number of false negative conclusions, Strategy 2 (detailed in the following paragraph) should be applied.

Methodology of Strategy 1
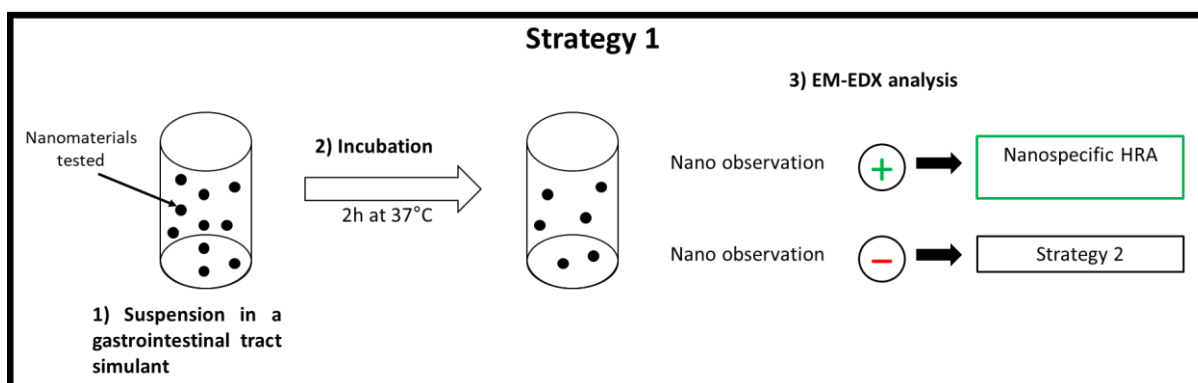
Figure 4. Analytical diagram of Strategy 1 for answering the question about dissolution of engineered nanomaterials. EM: electron microscopy, EDX: energy dispersive x-ray analysis, HRA: health risk assessment.

1)   The nanomaterials are suspended in a biological medium simulant. As part of a risk assessment for the oral route, the WG recommends studying nanomaterial dissolution in gastrointestinal tract simulants. Because there are no standard operating conditions, the WG leaves it to operators to determine the composition of the dispersion medium. However, the composition of the media should be clearly described and the choices justified. In order to expose the nanomaterials to the pH variations observed along the digestive tract, the nanomaterials should be suspended in two simulants: one at pH 1 and the other at pH 7, as these two pH values represent the extreme values observed from stomach to intestine. The concentration levels of the suspended nanomaterials should be high enough to allow observation under an electron microscope (scanning or transmission). However, the maximum concentrations should not reach those for which particle sedimentation phenomena are observed. This is because the WG believes that sedimentation phenomena, following the loss of the dispersed nature of the constituent particles, can influence dissolution phenomena. The choice of concentrations should therefore be justified.

2)   Incubation takes place while stirring at 37°C for 2 hours.

3)   Immediately after the incubation step (2 h), observation under the electron microscope (scanning or transmission) shows the presence of any residual particles. Electron microscope observation should be systematically coupled with energy dispersive x-ray (EDX) spectrometry to exclude artefacts that do not correspond to the studied nanomaterial.

The WG recommends avoiding lag times between each step in order to limit particle sedimentation.

Decisions

If nanomaterials are observed by electron microscopy coupled with EDX (EM-EDX) after the dissolution step, then the risk assessment should follow a "nanospecific" approach. If no nanomaterials are observed during EM-EDX, then Strategy 2, including a pre-concentration step, should be implemented. This step could facilitate the detection of nanomaterials initially found at low concentration levels. The analytical decision criteria in EM are the same as those described in Section 3.2.2.

Methodology of Strategy 2
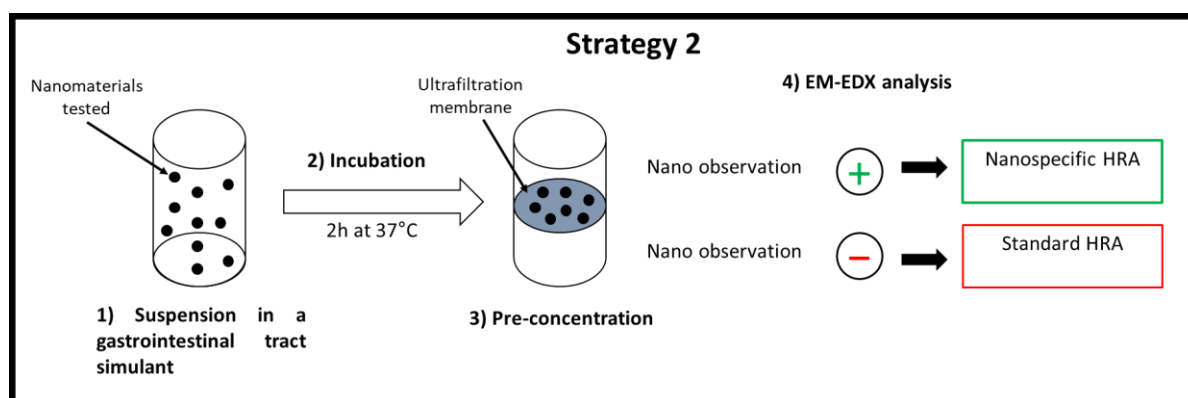
Figure 5. Analytical diagram of Strategy 2 answering the question about dissolution of engineered nanomaterials. EM: electron microscopy, EDX: energy dispersive x-ray analysis, HRA: health risk assessment.

The suspension and incubation steps are identical to Strategy 1. Following the incubation step, a membrane pre-concentration step (via an ultrafiltration technique) should be implemented if no nanomaterials are observed after Strategy 1. In order to assess the performance of this step, pre-concentration factors should be determined and clearly indicated.
The operator should ensure that the pore size of the ultrafiltration membrane (usually expressed in Dalton equivalent) is suited to the physico-chemical properties of the nanomaterial, particularly with a view to retaining the smallest particles constituting the nanoscale fraction of the initial material. Other pre-concentration techniques such as ultracentrifugation and dialysis are also available. In the case of ultracentrifugation, experimental parameters can be difficult to set, centrifugation times can be long and the pellet consisting of the particles can diffuse into the supernatant. Dialysis, although simple to implement, relies on long diffusion times. In this context, ultrafiltration techniques appear to be the fastest, most effective and simplest to implement.

If no nanomaterials are observed in the filtration membrane retentate, operators should ensure that the substances found in particulate form prior to the incubation step are found in dissolved form in the filtrate. This step ensures that particles have not been adsorbed on the wall of the tubes or trapped in the filtration membrane (these particles are no longer accessible for analysis). ICP-MS techniques can be used for this purpose.

Decisions

If nanomaterials are observed in EM-EDX after the dissolution and pre-concentration steps, then the risk assessment should follow a "nanospecific" approach. If no nanomaterials are found during the EM-EDX observation then the risk assessment should follow a standard approach. The analytical decision criteria in EM are the same as those described in Section 3.2.2.

3.2.4. Step 3a: What are the levels of consumer exposure to the nanoscale fraction of the engineered nanomaterial?

3.2.4.1. Preamble

As mentioned when setting out the scope of this formal request, the HRA methodology developed by the WG on "Nano and Food" focuses exclusively on engineered nanomaterials used as food additives. The methodologies presented in this document therefore focus on calculations of oral exposure of consumers to engineered nanomaterials used as food additives. As a reminder, engineered nanomaterials used as food additives are complex mixtures of polydisperse particles consisting partially (in the case of E171) or entirely (in the case of E551) of constituent particles in the nanoscale.

The WG therefore proposes initially determining the exposure levels of consumers to the engineered nanomaterial as a whole (i.e. to the corresponding food additive) and then subsequently determining, from the previously calculated levels of exposure to the engineered nanomaterial, the exposure level of consumers to the nanoscale fraction (i.e. to the constituent particles in the nanoscale (1-100 nm)) of the engineered nanomaterial. These steps are shown in Figure 6. Consumption data were combined with data on concentrations of food additives in food matrices using the Food Additives Intake Model (FAIM, details are provided in the following sections). This made it possible to calculate levels of exposure to food additives and then, in the context of this formal request, to engineered nanomaterials. Calculating levels of exposure to the nanoscale fraction consisted in multiplying the level of consumer exposure to the engineered nanomaterial by the mass percentage of constituent particles in the nanoscale. The details of these calculations are set out below.
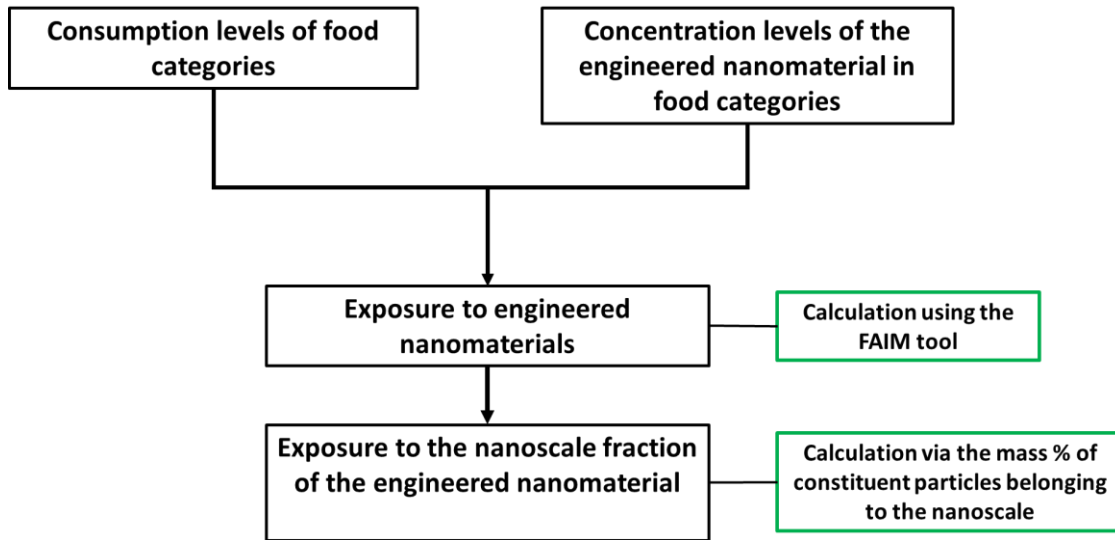
Figure 6. Methodology for calculating consumer exposure to engineered nanomaterials and to their nanoscale fraction. The green boxes indicate the models and calculation methods used.

3.2.4.2. Consumption levels

The WG's work focused solely on consumer exposure at the national level. The consumption levels of the French population considered in the FAIM software came from the INCA2 study (consumption data collected in 2006 and 2007)[15]. The consumption levels for each of the food categories considered in the FAIM software are indicated, in the case of France, for four age groups:

- Children (3-10 years)
- Adolescents (11-17 years)
- Adults (18-64 years)
- Elderly people (65 years and over)

During the first phase of its expert appraisal, the WG identified all food categories liable to contain engineered nanomaterials. Some of these categories concern a particular age group, namely the child population. Thus, in order to determine exposure levels for children under 3 years of age, it was necessary to base exposure calculations on the consumption levels from the 2005 "BEBE-SFAE" study, a joint TNS-Sofres-Dijon University Hospital study carried out for the French Association for Children's Food (Fantino and Gourmet 2008), as specified in the infant total diet study published by ANSES in 2016[16].

3.2.4.3. Concentration levels

- Food categories concerned

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[15] Individual and national study on food consumption: https://www.anses.fr/en/content/detailed-results-inca-2-study
[16] https://www.anses.fr/en/content/infant-total-diet-study-itds

As a first step, the WG recommends collecting concentration data on engineered nanomaterials for all food categories listed in the FAIM tool. This is because during its expert appraisal, the WG discovered that some nanomaterials, mainly E171, were found not only in food categories for which their use was authorised, but also in those for which their use was not authorised. The presence of engineered nanomaterials in food categories that are not supposed to contain them may be explained by the "carry over"[17] phenomenon.

- Data sources

In the course of this work, the WG identified two main sources of data:

- use levels reported by industry. These levels represent quantities of incorporation of food additives used in the formulation of foods. These data have been published in various studies, including by EFSA[18] and RIVM[19].

- analytical data from the testing of food products. Numerous scientific publications have explored the extraction and quantification of nanomaterials in different food matrices. These data can also come from inspection bodies (DGCCRF) or the grey literature (e.g. analyses carried out by NGOs).

The WG believes that concentration data on engineered nanomaterials, whether in the form of use levels or from analytical data, should be given equal importance. The WG also points out that where concentration levels are determined from different data sources and the amount of data from these sources differs, then the concentration data should be weighted (an example is provided in the footnote[20]).

- Extraction and preparation of the extract before characterisation

The physico-chemical characterisation (morphology, particle size distribution, etc.) of engineered nanomaterials used in food requires them to be extracted from the food matrices. These matrices have different physico-chemical properties that can influence extraction protocols. For example, E171 can be extracted from a cake in an aqueous medium, whereas extracting the same additive from chewing gum requires a more complex extraction protocol involving the use of liquid nitrogen. The WG emphasises that extraction protocols should not

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[17] Carry-over: the food additive is added to the final food via other ingredients and does not systematically appear in the list of ingredients of the finished product.

[18] Re-evaluation of titanium dioxide (E171) as a food additive: https://doi.org/10.2903/j.EFSA.2016.4545

[19] Sprong, C., *et al.*, Exposure assessment of the food additive titanium dioxide (E 171) based on use levels provided by the industry. RIVM letter report 2015-0195, 2016.

[20] Due to the heterogeneity of the amount of data from sources x and y, a weighted average of nanomaterial (NM) concentrations per food category should be calculated.

$[NM]_{food\ category} = (a[NM]_x + b[NM]_y) / (a+b)$

where $[NM]_{food\ category}$ is the average concentration of engineered nanomaterial per food category, a is the amount of data from source x, b the amount of data from source y, and $[NM]_x$ and $[NM]_y$ are the average concentrations of engineered nanomaterial from the data derived from sources x and y respectively.

introduce changes to the sample's initial particle size distribution. This could result from selective extraction according to particle size.

In general, the protocol for extracting nanomaterials from a food matrix combines the following steps:

- with complex food matrices, a first step of chemical digestion is performed to increase accessibility of the nanomaterials and improve the recovery rate;
- then there are repeated washings with different solvents chosen according to the food matrix and the type of particles to be extracted, in order to eliminate the food matrix residues still present. A washing step consists in centrifuging the suspension containing the particles, then removing the supernatant liquid and replacing it with a clean solvent;
- lastly, ultrasound is applied to the washed suspension to separate any particle agglomerates/aggregates present as much as possible, in order to reliably determine the size of the constituent particles.

- Quantification of nanomaterials in food

Different quantitative techniques can be used to determine concentration levels of engineered nanomaterials (in the context of this formal request, the engineered nanomaterials considered are food additives) in food matrices. This quantification of the food additive does not need to distinguish the form (particulate, molecular or ionic) in which the nanomaterial is present. Techniques such as ICP-MS, ICP-HRMS, ICP-OES, sp-ICP-MS or UV spectrophotometry can be used for this purpose. With the exception of sp-ICP-MS, these quantitative techniques are unable to distinguish particulate forms from molecular or ionic forms. Quantification of the additive (example: E551) using these techniques is based on quantification of the chemical element (example: silicon). This approach can lead to the concentration of the food additive being overestimated because the presence of the quantified element in food may be of natural origin.

On the other hand, calculations of exposure to the nanoscale fraction of the nanomaterial require the use of analytical techniques that can characterise particulate forms, measure their size and determine particle size. According to the WG, electron microscopy is the only technique currently suitable for this type of measurement (more details will be given in the following sections).

3.2.4.4. Calculation of exposure to engineered nanomaterials (food additives)

As mentioned above, calculations of consumer exposure to engineered nanomaterials are based on the use of FAIM software. This tool enables applicants, risk assessors and risk managers to estimate average and high (95th percentile) exposure to food additives for different age groups in several European countries.

Food additive occurrence data are entered for each of the food categories, and individual consumption levels, collected from the EU Member States (EFSA's comprehensive European food consumption database), are used for calculating exposure.

Consumer exposure is calculated by multiplying, for each food category, the concentrations of the nanomaterial by the consumption levels of each individual referenced in the database. The exposure levels calculated for each of the food categories are added together to estimate total daily exposure for each individual. These total daily exposure levels for each individual are averaged over the number of survey days. Single-day dietary surveys are excluded as they do not reflect repeated exposure. This approach makes it possible to obtain individual total daily exposure distributions and to calculate an average value and a 95th percentile of total daily exposure for each age group considered.

In the case of France, the FAIM tool calculates levels of exposure to food additives for four age groups: children (3 to 10 years), adolescents (11 to 17 years), adults (18 to 64 years) and the elderly (65 years and over).

3.2.4.5. Calculation of exposure to the nanoscale fraction of engineered nanomaterials

As mentioned in the preamble, calculating consumer exposure to the nanoscale fraction of the engineered nanomaterial considered is based on determining the mass fraction of constituent particles in the nanoscale (1-100 nm).

- Data sources and sampling

The mass fraction of constituent particles in the nanoscale can be determined directly on the nanomaterial before it is incorporated into food formulations. This approach enables a maximum mass fraction of the raw material to be determined because this raw material is not subjected to the physico-chemical conditions of the food, which could potentially, in certain cases, lead to particle dissolution phenomena.

A second approach is to determine the mass fraction from nanomaterials extracted from food matrices. This approach requires nanomaterials to be extracted from different food categories in order to consider the variability of the physico-chemical conditions observed in the different food matrices (see extraction protocol in previous sections). The WG recommends this second approach, which is more representative of the mass fractions to which consumers may be exposed.

- Sample preparation and observation

Sample preparation is an important step to facilitate observation of the particles by electron microscopy. With this in mind, the National Metrology and Testing Laboratory (LNE) has developed a method for depositing particles on a substrate after spreading a drop of suspension containing the particles in a thin layer. This approach also limits particle aggregation/agglomeration during deposition.

Briefly, the particles are suspended at a pH defined by their physico-chemical properties and then deposited on a silicon substrate. This substrate is then subjected to a velocity gradient by rotation allowing radial and isotropic diffusion of the liquid on the surface of the silicon substrate

via the spin coating technique. This method results in a homogeneous single-layer distribution of the particles before they are observed.

The centrifugal forces spread the liquid over the surface of the silicon substrate, with any excess being expelled from the substrate. The applied rotation times (30 s) and speeds (maximum 8000 rpm) are not sufficient to separate the particles according to their size. The proportions of particles before and after rotation are therefore unchanged due to the loss of liquid related to the centrifugal forces.

Studies have shown that the pH of the deposited suspension can influence the quality of particle dispersion. This is because the silicon substrate generally has a negative surface charge and the particles have a surface charge dependent on the pH value of the suspension, with their surface charge being neutral at the pH corresponding to the isoelectric point of the particles[21]. Thus, if the particles and the silicon substrate have surface charges of the same sign, adhesion of the particles to the substrate surface will be low and few particles will be observable in EM. Furthermore, if the pH is close to the isoelectric point, then the surface charge of the particles will be insufficient in absolute value to ensure electrostatic repulsion between the particles, which will then tend to agglomerate during deposition, making it difficult to observe and measure the size of the constituent particles in EM. Figure 7 illustrates the influence of pH in the case of E171. The more efficient dispersion of the particles at pH 2 can be explained by the fact that the isoelectric point of E171 is around 4. At pH 2, the surface charge of the particles is positive and the electrostatic repulsion between the particles limits the formation of aggregates/agglomerates. At pH 6 the surface charge of the particles is negative overall; however, the electrostatic repulsion is not sufficient to repel the particles from each other, thus favouring their aggregation/agglomeration.

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[21] If the pH > isoelectric point (pI) then the particles will be negatively charged. If pH < pI then the particles will be positively charged.
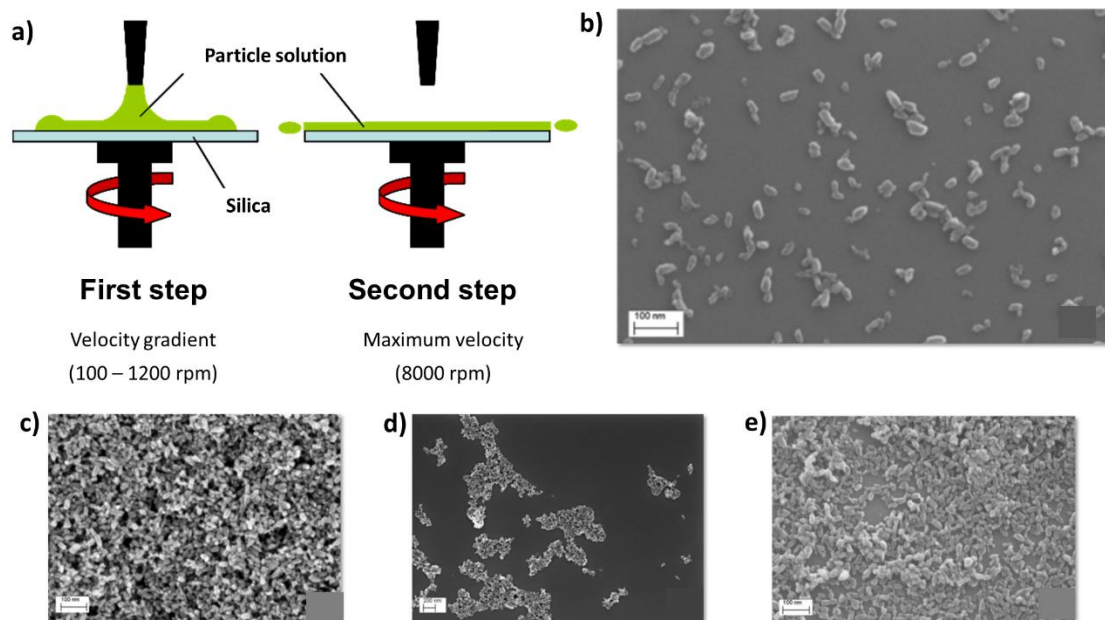
Figure 7. (a) Diagram illustrating the spin coating technique. A drop of solution containing the suspended particles is deposited and then spread evenly on the surface of a silicon substrate by applying a rotational velocity gradient. (b) Observation of E171 particles on the surface of the silicon substrate after application of the spin coating technique at pH 2. Results obtained for E171 particles at pH 6 without spin coating (c), at pH 6 with spin coating (d), and at pH 2 without spin coating (e).

The approach developed by the LNE is not standardised but offers an interesting analytical strategy for preparing samples prior to observation, as it is easy to implement and inexpensive. Some of the data used by the WG to determine the nanoscale fraction of E171 were based on this preparation technique (results not currently published). The operating conditions should be optimised by taking into account the physico-chemical properties of the nanomaterials (especially their surface charge and chemistry). This technique was validated with measurements made on reference particles (European Joint Research Centre – JRC) and via inter-comparisons with operators not using the spin coating technique. The protocols for this technique are specified in the study by Ghomrasni *et al.* (2020).

- Model and calculations

The first step is to determine the mass-based particle size distribution of the engineered nanomaterial considered. Two parameters can be measured for this purpose: the particle's surface area or the Feret diameter (Figure 8). In both cases, determination of these parameters requires observation of the samples by electron microscopy (scanning or transmission).
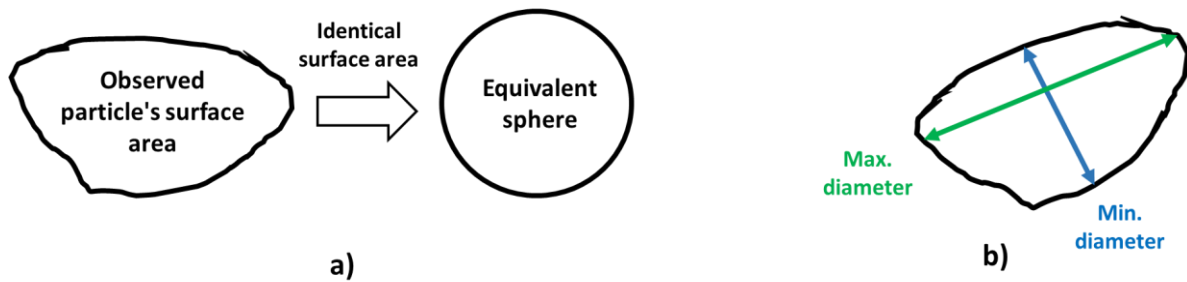
Figure 8. Determination of the mass fraction of particles in the nanoscale by measuring a) the equivalent sphere or b) the Feret diameter

- o Measuring the surface area

The constituent particles of nanomaterials do not generally have perfectly spherical shapes, but are often equated to spheres, and then referred to as spheroids. The first step is therefore to measure the surface area of the particles observed by electron microscopy. A disc with the same surface area as the measured one is then considered to determine the diameter of the equivalent disc and then the volume of the equivalent sphere. By considering the average density of the engineered nanomaterial in question, the mass of each particle within the observed sample can then be determined. The mass contribution of the constituent particles in the nanoscale can therefore be calculated.

- o Feret diameter measurement

As mentioned earlier, most of the particles that make up nanomaterials have morphological anisotropies. As illustrated in Figure 8, minimum and maximum Feret diameters can be measured for each particle. The WG believes that the smallest dimension of each particle should be considered for determining the particle size distribution and therefore recommends measuring the minimum Feret diameter. From this diameter, and assuming that the particle is spherical, the volume of the sphere and then its mass could be calculated as explained in the previous paragraph.

The WG believes that measuring the minimum Feret diameter is the best approach for determining the number of particles in the nanoscale, i.e. most suitable for determining the number of particles with at least one dimension smaller than 100 nm. Considering the smallest dimension then maximises the number of particles in the nanoscale.

However, using the minimum Feret diameter leads to an underestimation of the volume and therefore of the mass of the particles. This bias is exacerbated in the case of particles with high anisotropy (e.g. iron oxides).

Ideally, the WG recommends using initially the measurement of the Feret diameter to determine the population of particles in the nanoscale, and then the measurement of the particle surface area to estimate their mass.

- • Determination of the level of exposure to the nanoscale fraction

Levels of consumer exposure to the nanoscale fraction of the engineered nanomaterial (E_{NF}) considered are derived by multiplying the levels of exposure to the engineered nanomaterial (E_{EN}) by the average or maximum (depending on the exposure scenario considered) mass contribution (percentage) of the nanoscale fraction of the engineered nanomaterial (%$_{NF}$).

$$E_{NF} = E_{EN} \times \%_{NF}$$

3.2.5. Step 3b: What are the health effects of the nanoscale fraction of engineered nanomaterials?

3.2.5.1. Preamble

Hazard identification and characterisation of the nanoscale fraction of engineered nanomaterials is based on a tiered approach, outlined in Figure 9.
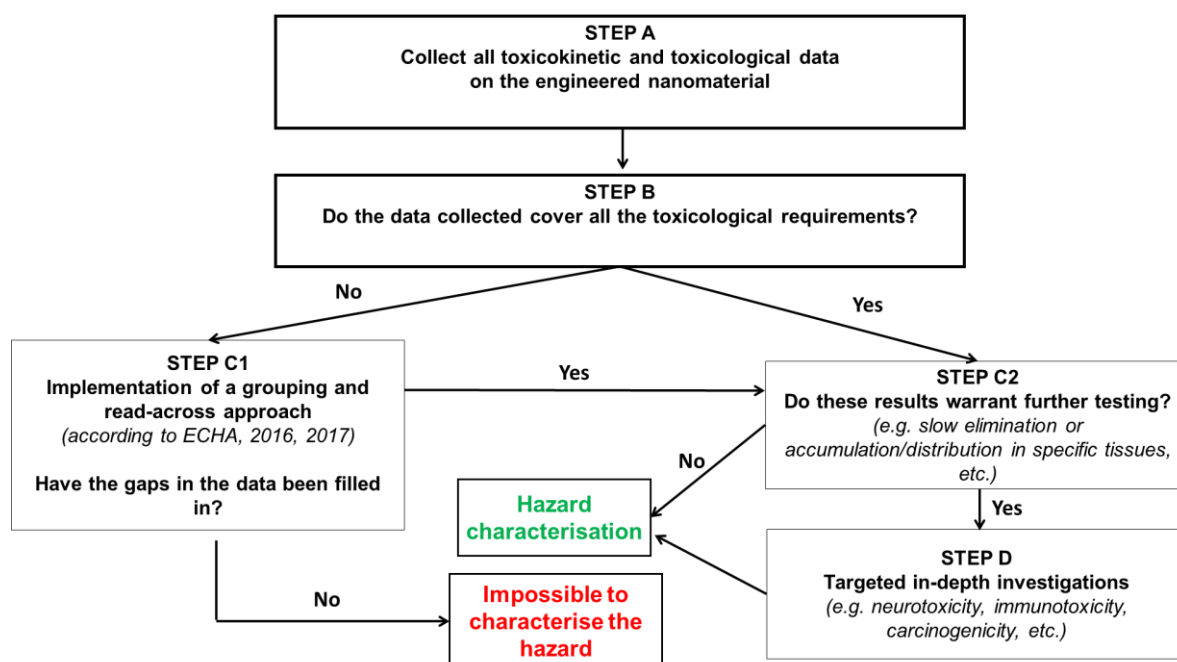
Figure 9. Tiered methodology for hazard assessment and characterisation of the nanoscale fraction of engineered nanomaterials

- **Step A: Collect all toxicokinetic and toxicological data on the engineered nanomaterial**

  This step involves collecting all *in vitro, in vivo* and *in silico* toxicokinetic and toxicological data (i.e. predictive studies using approaches such as QSAR, PBTK, etc.) relating to the nanoscale fraction of the studied engineered nanomaterial. If available,

epidemiological studies should also be considered. The collected data could include scientific publications, as well as study reports generated by industry. The selected studies should meet quality criteria, particularly concerning physico-chemical characterisation of the studied nanomaterials (crystallinity, size, particle size). The selected toxicological studies should at least include the average size of the particles used. As mentioned earlier in this document and in the ANSES opinion published in 2020, the WG believes that electron microscopy is currently the most suitable and most robust technique for measuring the size of constituent particles. Information on physico-chemical characterisation is important, especially for grouping/read-across purposes (Step C).

Toxicological data obtained from the engineered nanomaterial as a whole can also be considered. These studies should include at least the following selection criteria:
- measurement of the average size of the constituent particles
- nanoscale fraction by number and/or mass

The size and particle size distribution of nanomaterials are essential parameters in a nanospecific HRA. Other physico-chemical parameters such as the presence of impurities, morphology, surface charge and chemistry, crystallinity, specific surface area and porosity should also be considered. Analytical methods suitable for the study of these parameters are described in the EFSA guide[22].

Lastly, as well as including studies based on the nanoparticle forms of the engineered nanomaterial, the data collected should include the ionic and/or molecular forms of the corresponding element (e.g. the Ag+ ion in the case of nanomaterials containing silver).

The WG emphasises that if the ionic and/or molecular form of the nanomaterial considered has toxicological effects, then there should be a systematic study of these effects based on nanoparticle forms.

- **Step B: Do the data collected cover all the required toxicological data?**

  This step involves analysing the collected data to determine whether they cover all the required toxicological studies (Section 3.2.5.3).

  <u>Decisions</u>: If the required toxicological data are not covered, then the grouping and read-across approach should be implemented in Step C1. If not, Step C2 should be implemented.

- **Step C1: Implementation of a grouping and read-across approach**

  The variability of the physico-chemical properties of nanomaterials results in a wide variety of materials with potentially different biokinetic behaviours and hazards, and

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[22] EFSA 2018: Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health.

therefore risks. To limit the amount of testing needed for risk assessment or to attempt to compensate for unavailable biokinetic and toxicological information, grouping and read-across approaches can be used (Oomen *et al.* 2015).

In general, substances can be grouped according to their properties: physico-chemical, toxicological, ecotoxicological and/or their fate in the environment and in the human body. These properties are potentially comparable. Within a group of substances, a lack of data can be overcome by using the "read-across" approach. This method makes it possible to predict information about a given biological and/or biokinetic parameter for a substance to be assessed (target substance), using data on the same parameter described for one or more other substances in the same group (i.e. source substance(s)), without having to conduct new experiments. When used, read-across should be performed independently for each biological and/or biokinetic parameter. These grouping and read-across approaches make better use of the available information and help determine whether or not additional data need to be generated for a specific biological parameter.

Different grouping and read-across concepts have been suggested by researchers and were recently reviewed by Lamon *et al.* (2018). So far, these concepts have focused mainly on occupational health and safety aspects, with a focus on hazard assessments, while the environmental counterpart is still in its infancy (Wigger and Nowack 2019). In this context, an ECHA guide on the grouping of nanomaterials was recently developed as an appendix to Chapter R.6 of REACh (ECHA Appendix R.6-1, 2019)[23]. This document aims to offer a systematic and pragmatic approach that can ultimately reveal potential differences in the toxicological properties and fate of nanomaterials[24]. This is a stepwise approach, with nanomaterials grouped together on the basis of relevant physico-chemical parameters (which may vary depending on the assessment criterion considered). Application of this strategy will determine whether there are data on hazards and/or biokinetic behaviour available for the nanomaterials and whether these data are applicable to the group(s) formed. It is important to ensure the applicability and relevance of all available data on the hazards of existing nanoforms (or sets of nanoforms).

For the results to be usable, it is essential to demonstrate that the grouping of the nanomaterials and the read-across of studies between source and target nanomaterials is robust and justified. In order to facilitate data collection and the systematic and transparent documentation of the grouping/read-across approach, a stepwise approach is recommended (see Figure 10) for each biological and biokinetic parameter intended to be covered by the approach.

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[23] ECHA. Reference: ECHA-17-G-17-EN. Guidance on information requirements and chemical safety assessment. Appendix R.6-1 for nanomaterials applicable to the Guidance on QSARs and Grouping of Chemicals, Version 2.0, December 2019.
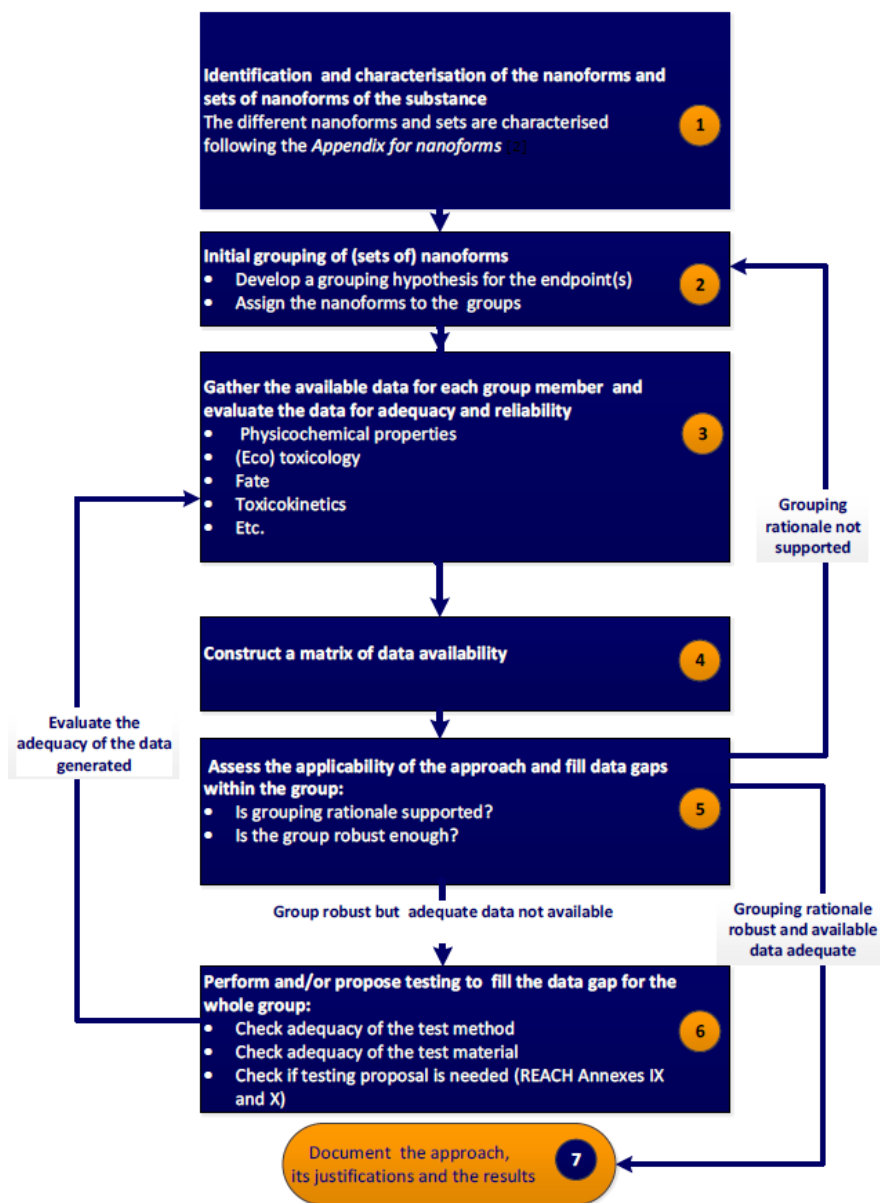[24] ECHA uses the term nanoform.

Figure 10. Stepwise approach for grouping/read-across (*from ECHA-17-G-17-EN*)

ECHA's Appendix R.6-1 was applied for a case study on grouping and read-across specifically targeting the genotoxicity of nano-TiO$_2$ (OECD 2018)[25]. The aim of this read-across exercise was to determine the genotoxic potential of two crystalline forms of nano-TiO$_2$ (rutile and anatase nano-TiO$_2$) on the basis of the results of the *in vitro* comet assay obtained from different TiO$_2$ nanoparticles (nano-TiO$_2$ NM100 to NM105).

The JRC also followed the guidance in ECHA's Appendix R.6-1 for the genotoxicity assessment of carbon nanotubes (Aschberger *et al.* 2019). In particular, grouping and read-across methods were applied to 19 types of multi-walled carbon nanotubes in order to make up for the lack of genotoxicity data.

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[25] ENV/JM/MONO(2018)28. 21 September 2018.

Thus, in order to make up the shortfall in data on a nanomaterial to be assessed, the WG recommends initially using the grouping/read-across approaches as described in ECHA's Appendix R.6-1 (2019).

Decisions: If the gaps in data have not been filled by grouping/read-across then the HRA cannot be finalised. If the lack of data has been resolved, Step C2 should be implemented.

- **Step C2: Do the results warrant further testing?**

  Analysis of the results of the required toxicological studies determines whether or not further studies of this kind are needed (Section 3.2.5.4).

  Decisions: If the results of the required toxicological studies show effects that call for further investigation, then additional targeted studies should be carried out in Step D. For example: positive *in vitro* genotoxicity tests determine whether or not further *in vitro* and/or *in vivo* genotoxicity studies are needed.
  If further studies are not necessary then the risk characterisation can be carried out.

- **Stage D: Targeted in-depth investigations**

  After analysis of the data generated during the additional studies, the risk characterisation can be carried out.

3.2.5.2. Changes and/or adaptations to the methodology in the toxicological assessment of engineered nanomaterials

The key steps in the toxicological assessment of engineered nanomaterials relate to physico-chemical characterisation, preparation methods (dispersion protocol), choice and justification of the test system(s) used and associated experimental conditions, and consideration of possible biases and interferences. It is therefore necessary to modify and/or adapt the methodologies used in the toxicological assessment of engineered nanomaterials.

- **Method of preparing engineered nanomaterials: dispersion protocol**

To study the toxicological effects of engineered nanomaterials *in vitro* or *in vivo*, a dispersion protocol should be implemented to identify certain hazards associated with the nanoparticle form. Ideal dispersion can be described as the state in which particles are completely separated from each other and there are no agglomerates/aggregates (Sager *et al.* 2007). Thus, implementing a dispersion step could help identify certain effects associated with the uses of a nanomaterial as a food additive. However, it can also seem unrepresentative of a food additive as found in food. Under these conditions, it may then be useful to compare any

observed effects of a dispersed nanomaterial with those of a nanomaterial added directly to a food matrix.

Any dispersion protocol can directly influence the quality and stability of the prepared nanoparticle suspension. For example, Donaldson (2010) found that a nanomaterial is very poorly dispersed in pure water and forms agglomerates, which is not the case when proteins, which form a corona[26] on the surface of the particles, are added.

The protocol chosen for dispersion, including the addition of compounds such as dispersants or proteins, is known to influence the physico-chemical characteristics of nanoparticle suspensions and therefore possibly the outcome of tests. For example, according to Magdolenova *et al.* (2012), a suspension of nano-TiO_2 dispersed by 3 min of sonication in an initial medium with no serum (obtaining large agglomerates) induced DNA damage in three cell lines, while the same nano-TiO_2 dispersed in the presence of serum in stock solution after 15 min of sonication (obtaining agglomerates of less than 200 nm) had no effect in terms of genotoxicity. This shows that using different procedures to prepare dispersions of the same nanomaterial can be a direct source of variation in the measured toxicity or ecotoxicity (Hartmann *et al.* 2015). It is therefore important, when assessing the hazard associated with engineered nanomaterials, to establish standardised test procedures.

These technical considerations were recently addressed in the NANoREG project (Oomen *et al.* 2015). Guides have been produced by the European Chemicals Agency (Lamon 2019), and guidelines on nanomaterial preparation and dosimetry have been published by the Organisation for Economic Cooperation and Development (OECD).

Within the framework of European projects on the toxicology of nanomaterials, standard protocols have been proposed[27,28]. However, these protocols are often limited to the characterisation of nanomaterials in dispersion solutions and do not address the questions asked after the nanomaterials have been added to cell culture or treatment media (distribution, corona, stability, sedimentation). Recently, Kaur *et al.* (2017, Figure 11) proposed a stepwise protocol for the dispersion of nanomaterials in aqueous media. This protocol, which is based on real-time physico-chemical characterisation, was able to identify optimal sonication conditions (intensity and duration), with a view to improving the stability and homogeneity of the nanoparticle suspensions without affecting the integrity of the sample (no modification, no degradation of the nanomaterial).

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[26] A crown of proteins and other elements that bind to the surface of the nanomaterial. Its composition depends on the medium in which the nanomaterial is dispersed. The corona obtained when preparing a nanoparticle suspension used in an *in vitro* test will therefore be very different from that produced during an *in vivo* test. In addition, it will differ *in vivo* depending on the route of entry, e.g. skin or lungs.
[27] Final protocol for producing suitable manufactured nanomaterial exposure media. The generic NANOGENOTOX dispersion protocol. 2011. https://www.anses.fr/en/system/files/nanogenotox_deliverable_5.pdf
[28] F Caputo, J Clogston, L Calzolai, M Rösslein, A Prina-Mello. Measuring particle size distribution of nanoparticle enabled medicinal products, the joint view of EUNCL and NCI-NCL. A step by step approach combining orthogonal measurements with increasing complexity. J. Control Release. 2019 Apr 10;299:31-43.
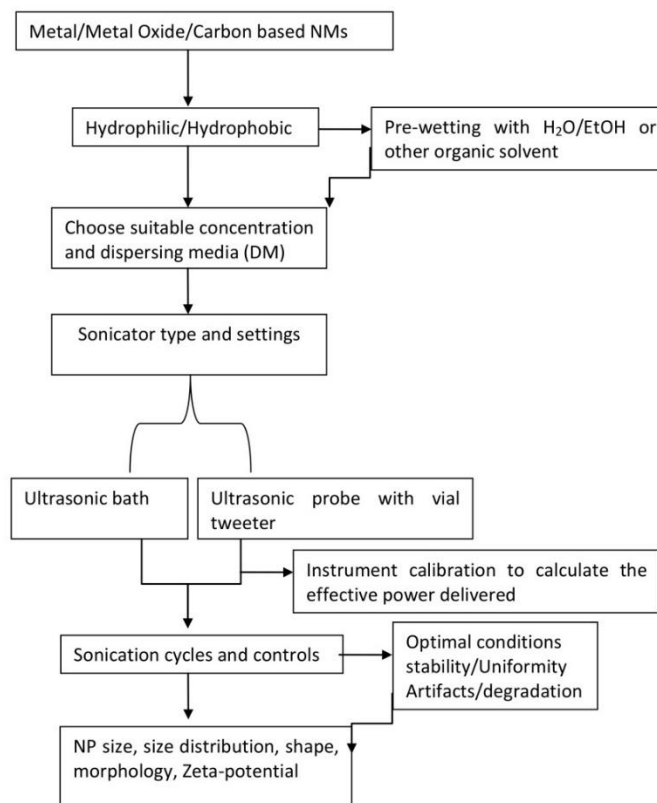
Figure 11. Flow chart describing the scheme and stepwise sequence of the nanomaterial dispersion protocol (*from Kaur et al. 2017*)

- **Choice of experimental conditions for toxicological tests**

Overall, the choice of experimental conditions determines the relevance of the results and the weight that can be attributed to them. The three major parameters are: *(i)* the treatment conditions (in terms of maximum dose studied and range of doses, duration, frequency, means of exposure, etc.); *(ii)* the model used (cell type or animal species); and *(iii)* possible interference with the biological parameter measured.

o Treatment conditions

For the same nanomaterial, the aggregation/agglomeration characteristics of the dispersions obtained can modify the level of exposure of cell models, whether they are in suspension or adherent, by adjusting the sedimentation rates of the different sizes of aggregates/agglomerates formed. This can then influence the intracellular concentration, and therefore possibly the test result. The same is true for the treatment conditions (number of cells/ml or cm^2, treatment volume, well surface area, etc.). It is therefore important, when assessing the hazard associated with engineered nanomaterials, to also establish standardised test procedures on these points.

The question of the most relevant metric is currently still a subject of debate. In *in vitro* tests, the nanomaterial's mass and surface area per unit area of the culture medium are most commonly used (while it may seem optimal, expression of the surface concentration, i.e.

particle surface area/unit area of the culture medium, is rarely used). Particle number concentration is more difficult to use because the analytical techniques applied to monitor dosimetry give very variable results (Peterson *et al.* 2019). In *in vivo* tests, because of the need to use a mass reference, the dose is always expressed in units of mass of the nanomaterial (e.g. mg) per kg of body weight of the studied species.

Dispersion preparations and exposure dosimetry can refer to the recommendations of DeLoid *et al.* (2017).

o Relevance of dose levels

The objective of hazard characterisation generally requires the use of high doses, which can make it difficult to compare responses obtained from *in vivo* and *in vitro* experiments. For example, with some nanomaterials, the doses used in *in vitro* experiments may be sufficient to induce pro-inflammatory, toxic and/or genotoxic effects in cell models, whereas the same dose level *in vivo* may only mobilise antioxidant defences without any subsequent adverse effect.

Overall, it can be difficult to extrapolate between *in vitro* and *in vivo* tests, since *in vitro* studies are often carried out on exposures with treatment times less than or equal to 24 hours at relatively high concentrations, whereas any *in vivo* effects are expected over the medium/long term, because of the time needed for absorption and/or potential accumulation of nanomaterials in certain organs. Thus, for the same biological parameter studied, the effects observed during *in vitro* and *in vivo* tests may be different or even contradictory, which raises the question of the predictivity level of *in vitro* experiments. This concept is particularly important since there is a growing trend towards alternatives to animal testing, and in particular towards cell culture models, which need to be as predictive as possible. It is therefore necessary to work on the experimental design of the *in vitro* experiment, taking care to implement chronic/repeated exposures at lower concentrations, with nanomaterials prepared in a dispersant that is representative of that encountered in *in vivo* conditions. For example, to improve the predictive power of *in vitro* tests with regard to the intestinal toxicity of nanomaterials, Marucco *et al.* (2020) emphasised the importance of using a simulant of the human digestive system as the dispersion medium.

If *in vitro* results have to be used to predict the *in vivo* effects of nanomaterials, their relevance will be all the more certain if the *in vitro* dose is extrapolated from a relevant *in vivo* concentration (availability of toxicokinetic data). A "starting point" dose for *in vitro* studies can be set by extrapolating *in vivo* organic burden data to determine the dose to be used *in vitro* (Lan Ma-Hock *et al.* 2021). This approach reduces the difficulties in setting exposure doses for *in vitro* models.

For *in vivo* tests in animals, it is also important to avoid the use of excessive doses, according to the type of administration, which could lead to irrelevant results. Firstly, the choice of maximum dose tested should be dictated by the quality (homogeneity and stability) of the dispersion. Indeed, beyond a certain initial concentration, the nanoparticle dispersion can become heterogeneous, and this saturation level should not be exceeded as it risks causing non-intrinsic and therefore biologically irrelevant effects. In all cases, a range of concentrations

should be used to assess the concentration level that can achieve a homogeneous and stable dispersion.

The choice of maximum concentration assessed can also draw on test results showing a saturation level of the intracellular dose; this threshold should not be exceeded as it risks generating non-specific results (Summers 2013; Lammel 2019).

Lastly, when available, data from toxicokinetic studies can also be used to set experimental doses, including *in vitro*, taking into account the nanoparticle load observed in certain organs and applying a multiplying factor.

In all cases, it is therefore necessary to justify the dose levels used in the assessment tests.

Thus, the dispersion methods used to assess nanomaterial toxicity need to be harmonised and standardised to ensure comparable data quality and minimise artefacts produced by changes in a nanomaterial during dispersion preparation. Such harmonisation and standardisation will also improve comparability between tests, laboratories and studies on different types of nanomaterials.

It therefore seems essential to justify, in all toxicological assessment tests:

- the method of preparing the nanoparticle suspension;

- the type of dispersant used;

- the addition of co-formulants (serum, mucus, etc.) where appropriate;

- the methodology used to disperse the nanoparticles;

- the metrology used to monitor dispersion.

- **Choice of test system**

  o Cell model

The choice of cell model is also a crucial parameter. Indeed, the cell line used (human, animal, etc.) and the tissue of origin will determine the level of sensitivity of the cells to exposure to engineered nanomaterials. Depending on their origin, they may use different metabolic pathways, may not have the same receptors on their membrane surface or the same DNA repair capabilities, and may have different antioxidant statuses.

For example, since the genotoxicity of nanomaterials depends largely on oxidative mechanisms, if cells with poor or no antioxidant systems are used, the effects may be exacerbated.

It is therefore important to choose a cell model:

- preferably of human origin and, as far as information is available (data from toxicokinetic studies), one that is representative of a potentially exposed organ;
- whose biological representativeness and performance have been characterised (origin, detoxification capacities, antioxidant systems, endo- and exocytosis, etc.).

Lastly, regarding the culture method and the treatment method, several choices are possible:

- in conventional 2D, single-layer fashion;
- in co-culture;
- on inserts to grow the cells in three dimensions, so as to work on several compartments.

The design of the culture method and the treatment method used should be justified.

o   Choice of animal model

In general, compared to cell-based models, there are fewer restrictions on the choice of animal model, and the species recommended in the guidelines can be used.

For toxicokinetic studies, the animal species chosen should be predictive for humans, which can be complex due to opsonisation phenomena[29], which vary from one species to another.

When conducting *in vivo* tests, therefore, the choice of animal model should also be justified, although for oral exposure, the recommendations in the guidelines can be applied.

- **Interference with measured parameters**

Nanomaterials can interfere with the constituents of the media used in (cyto)toxicity tests, particularly with the reagents used (dyes, fluorescent agents, etc.), which can lead to bias in the response.

Incompatibilities with certain experimental conditions may also be observed, particularly for the two commonly used genotoxicity tests: the comet assay (DNA fragmentation test), and the micronucleus test (chromosomal aberration test)[30].

Indeed, for the *in vitro* micronucleus test, cytochalasin B is often used in the standard protocol to block cells at a certain phase of mitosis[31]. However, this substance inhibits the endocytosis of nanomaterials, leading to a risk of obtaining a negative result that is not relevant. If the use of cytochalasin B is required, it should therefore be added after a few hours or at the end of treatment. Furthermore, depending on the type of nanomaterial, accumulation in the cytoplasm may interfere with the reading of the test, with the risk that micronuclei may be obscured by the nanomaterials (Charles *et al.* 2018; Jalili *et al.* 2018). However, in both cases, adaptations of the assay are possible.

For the comet assay, the presence of residual nanomaterials after treatment can lead to interactions with DNA during the electrophoresis phase, which may lead to an irrelevant positive or negative result.

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[29] Opsonisation: a biochemical process whereby a molecule coats the membrane of a target cell to promote phagocytosis.
[30] Chromosomal aberration: change in the chromosomes (abnormality may be structural or numerical).
[31] Mitosis: chromosomal events of cell division.

It therefore appears necessary to make changes and adaptations to improve the sensitivity and specificity of the tests, particularly *in vitro*, thus avoiding the need for *in vivo* tests, which are usually carried out in the event of a significant response in *in vitro* tests.

It is important to note that interference may also occur between certain nanomaterials and certain *in vitro* cytotoxicity tests (Guadagnini *et al.* 2015). To establish the cytotoxicity of nanomaterials without any ambiguity, therefore, the recommendations below should be followed:

- test for possible interference with the reagents and test conditions, starting with cell-free controls to ensure that the nanoparticles do not interfere with the reagents or with the type of measurement used in the test;
- use at least two types of tests in parallel, based on different principles.

It is also important to test for artefacts due to culture conditions, such as use of a biological fluid simulant, protein content of the medium, use of bovine versus human serum (Pisani *et al.* 2017) and possible interactions with nanomaterials.

In *in vitro* co-culture models, the use of inserts such as Transwell may hinder translocation by adsorption of nanomaterials on or within the filter. It is therefore necessary to carry out appropriate checks for possible retention of nanomaterials on the insert filter in cell-free conditions.

3.2.5.3. Toxicological data required

As mentioned in the preamble and described in Figure 9, the methodology for hazard identification and characterisation of the nanoscale fraction of nanomaterials is based on a tiered approach. As a first step, the WG drew up the list of toxicological data required. The results of these studies will determine whether further toxicological studies are needed. The interactions between these different studies are shown in Figure 12 and explained below.
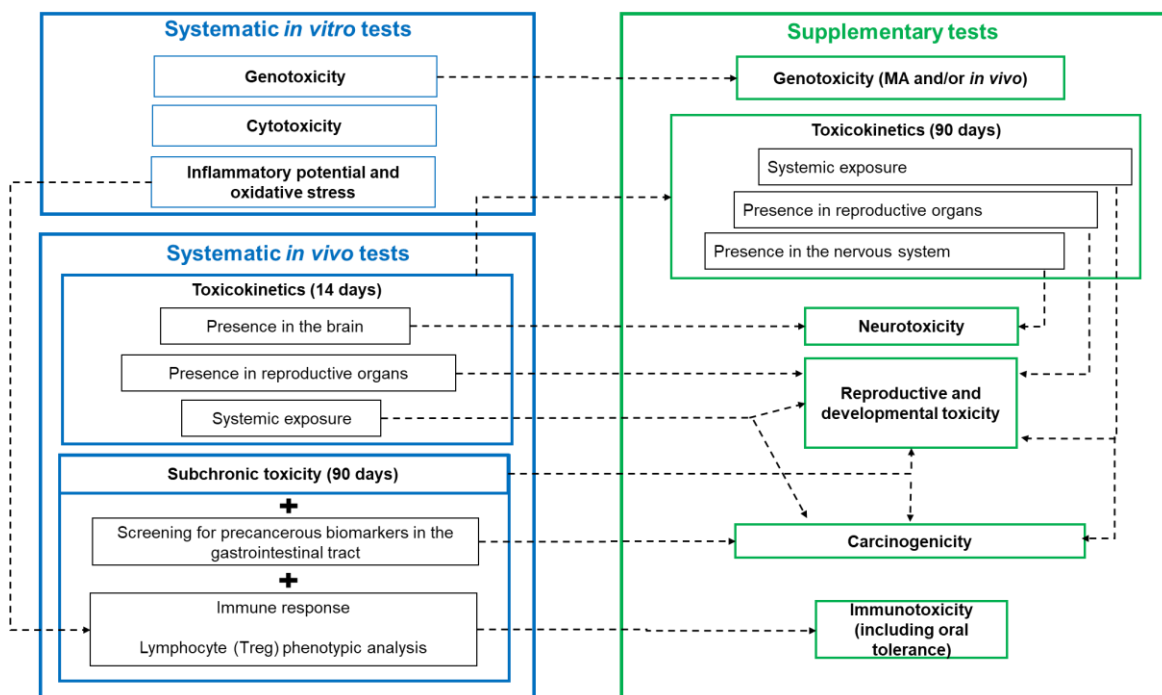
Figure 12. List of systematic toxicological data required (in blue) and supplementary toxicological studies (in green)

3.2.5.3.1. Cytotoxicity, inflammatory potential and oxidative stress (*in vitro*)

The results of *in vitro* tests for cytotoxicity, inflammatory potential and oxidative stress cannot be used directly for a nanospecific HRA. Nevertheless, exploitation of these results can help guide and develop the *in vivo* tests.

When ingested, nanomaterials from food can interact with the various organs of the digestive tract such as the mouth, oesophagus, stomach, small intestine and large intestine. Each of these organs has distinct physiological conditions and cell types. The potential impact (fate and effect) of the nanomaterials may therefore be exerted differently on each of these organs. Ideally, the *in vitro* toxicological assessment should be performed in cell models representative of each of these organs.

- **Cell systems**

A wide range of cell systems is available for the *in vitro* studies. Primary cells, directly isolated from organs, have several limitations: they are often technically difficult to grow/maintain over long periods of time, and may show variable responses from one individual to another.

Cell lines established by immortalisation or of cancer origin are easier to use, but these modified cells do not have all the geno/phenotypic characteristics of primary cells. The cells can be grown alone or in co-culture. Monocultures are easier to use and easier to standardise for cytotoxicity testing. However, care should be taken to limit the number of passages to avoid drift phenomena, i.e. the loss of geno/phenotypic traits.

The cells of the digestive tract used most often are those of the intestine, the major organ of contact. The best documented are epithelial cell monocultures. Extensive work has been done on human Caco-2 cells because they can differentiate into cells with many characteristics of enterocytes and can be maintained in differentiated culture for several days. They also enable permeability studies to be conducted (integrity measurements and transport kinetics through the intestinal barrier), thanks to the formation of a tightly organised intestinal epithelium. These cells can be grown either on plates to study accumulation, toxic effects, etc., or on semi-permeable insert membranes for transport studies.

More recently, *in vitro* models have become more complex in co-culture systems in order to better represent the intestinal epithelium. They typically combine Caco-2 cells with mucus-secreting epithelial cells (HT29-MTX) and/or macrophage-like immune cells, such as the human monocytic cell line THP-1 or RajiB cells, promoting differentiation into the "M" cells typical of the epithelium bordering Peyer's patches. The use of these co-cultures thus creates conditions closer to those existing *in vivo*, in particular a mucus-secreting epithelium such as that lining the ileum (Lehner *et al.* 2020) and the presence of M cells often associated with nanomaterial internalisation and passage from the intestinal lumen to the lymphoid follicle (des Rieux *et al.* 2005).

In nanoparticle transport studies, *ex vivo* protocols in which intestinal explants are placed in Ussing chambers can be used. These explants are viable for 2 to 4 h, and therefore allow the analysis of nanoparticle uptake over short periods (Brun *et al.* 2014).

There are also cell models of the oral cavity (TR146, SVpgC2a) and the stomach epithelium, although there are still only a few cytotoxicity studies on these models.

After passing through the intestinal barrier, nanomaterials are liable to enter the circulatory system and reach other organs such as the liver, spleen, kidneys, lungs and even the brain. If crossing of the intestinal barrier is suspected, the cytotoxicity tests should be completed on cell models representative of these systemic organs, such as hepatocytes. Similarly, the immune system is a potential target of nanomaterials. Immune cell models can be used, for example macrophages, which are in the front line during exposure to nanomaterials and orchestrate the local inflammatory response in the gut and within the body.

In each case, the cell lines should be characterised by specifying the origin of the cells, the culture media, the number of passages, the cell division time, their morphology and their state of differentiation before and during execution of the test[32].

- **Dosimetry**

It is important to assess the dose delivered to the cells and the dose internalised, in order to validate and correctly interpret the *in vitro* cytotoxicity data. Besides information on particle mass per incubation volume or particle mass per cell culture area, data should also be provided on the incubation volume, well surface area, number of cells seeded, specific surface area of nano-objects, sedimentation rate, etc. Internalisation of nanomaterials can be verified by EM-EDX observations of exposed cells. ICP techniques can be used to quantify this internalisation.

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[32] OECD: Guidance Document on Good In Vitro Method Practices (GIVIMP)

Approaches based on the modelling of nanoparticle sedimentation have been developed and allow the effective dose to be calculated. These include the ISDD and ISD3 models (Thomas *et al.* 2018).

- *In vitro* **toxicity tests to be implemented**

The effects of nanomaterials can be studied through cell viability/toxicity tests, but also through the cellular response to oxidative stress or pro-inflammatory responses by cytokine release.

The experimental design, including the treatment conditions (exposure times, serum concentration, changes to the medium, etc.) and measurement conditions, should be clearly defined and justified in relation to the different parameters tested. The exposure conditions should not cause indirect effects (hypoxia, pH change, etc.) that interfere with cellular responses. For culture systems using inserts, the integrity of the cell barrier can be verified by measuring the transepithelial electrical resistance (TEER) or by using a marker (e.g. Lucifer yellow, or labelled dextran).

Most tests can be performed using a wide variety of colorimetric or fluorimetric methods,

o Cell viability/death and cell metabolism measurement tests

A battery of tests are available for estimating cell viability by studying:
- membrane rupture, using the lactate dehydrogenase (LDH) leakage assay or the vital dye exclusion assay (e.g. Trypan blue);
- altered lysosomal activity (e.g. neutral red);
- altered mitochondrial activity (e.g. MTT, MTS, WST-1, WST-8, resazurin assays);
- intracellular energy, by measuring ATP;
- apoptosis (e.g. caspases, annexin V).

o Oxidative stress

There are many tests for measuring oxidative stress based on the characterisation of cellular accumulation of oxidative species or the impairment of cellular antioxidant systems.

Cell-free tests can be performed to assess the intrinsic oxidative potential of the nanomaterial under test. Reactive oxygen species (ROS) such as non-radical (hydrogen peroxide) and radical products (superoxide anion, hydroxyl radical, etc.) can induce changes in cellular macromolecules (oxidation of DNA, amino acids, proteins and polyunsaturated fatty acids). The detection of ROS and the resulting changes can be used as monitoring parameters. In addition, the measurement of antioxidant capacity (amount or activity of antioxidant enzymes, ratio of oxidised to reduced glutathione) can also be used as markers of oxidative stress.

o   <u>Immunotoxicity tests</u>

The assessment of immunotoxic effects can be addressed *in vitro* by studying inflammatory potential on epithelial or immune cells (Aiba *et al.* 2017). The aim is to assess the ability of nanomaterials to provoke an inflammatory response by measuring mediators of inflammation, classically the pro-inflammatory (e.g. TNF alpha, IL-1 beta, IL-6, IL-8, MIF) and anti-inflammatory (e.g. TGF beta, IL-10) cytokines that constitute innate immunity (rapid and non-specific defences). Some methods allow the simultaneous detection of several pro- and anti-inflammatory mediators (multiplex analysis based on cytokine profiling (Bhattacharya *et al.* 2017)). Activation of the transcription factor NF-kappaB (a cytoplasmic factor that activates the transcription of many inflammatory response genes) can also be considered as a marker of inflammatory activity.

In addition, observations in stimulated conditions of these same cell types (immune and epithelial), for example by naturally pro-inflammatory endotoxins (e.g. lipopolysaccharide or LPS), will help assess the potential adjuvant or repressive effects of nanomaterials on the responses previously induced by the toxins.

Regardless of the type of results obtained *in vitro*, they should be confirmed in a 90-day subchronic oral toxicity study. In addition, these *in vivo* studies can be used to assess the effect on cell-mediated and humoral adaptive immunity (e.g. oral tolerance to food antigens) linking different compartments of the immune system.

- **Outlook: integrated testing strategies**

In order to refine, reduce and/or replace the use of animals in traditional toxicological approaches, the promotion of alternative methods to animal testing is strongly encouraged by many international bodies (OECD, EFSA, European Commission, NIH, etc.).

To this end, integrated testing strategies (ITSs) are being developed. By combining *in vitro* tests, modelling methods (*in silico*), "*omics*" methods (genomics, proteomics, metabolomics), exploitation of existing data and use of thresholds of toxicological concern, as well as read-across approaches, toxicological properties can be documented to identify hazards.

Extensive work is currently under way to move towards a change to the standard system of human health risk assessment, in particular by proposing mode-of-action tests that can predict toxic effects. In addition, these mechanistic studies can be used to analyse the relevance of subsequent, more targeted *in vivo* testing. In this context, the possibility of using adverse outcome pathways (AOPs) as a mechanistic framework to assess the specific adverse effects of nanomaterials has been explored (Gerloff *et al.* 2017; Halappanavar *et al.* 2019; OECD). This process of identifying key events (KEs) associated with *in vitro* or *in vivo* exposure to nanomaterials is promising and should be further explored in the short, medium and long term before its application.

Lastly, regarding the choice of cell systems to be considered for *in vitro* testing, a higher level of complexity is achieved by using either reconstituted intestinal epithelia or 3-D intestinal organoids. Thus, "mini-guts", differentiated from intestinal crypts, were initially developed as self-organising units that contain all the cell types found in the intestinal epithelium (Sato *et al.*

2009). More recently, these organoids have been developed on supports fed by a microfluidic system to obtain a spread-out organisation reproducing the morphology of the intestinal epithelium (Nikolaev *et al.* 2020).

3.2.5.3.2. *In vitro* genotoxicity

Nanomaterials can potentially induce direct and indirect primary genotoxic effects, as well as secondary genotoxic effects, as shown in the figures below.
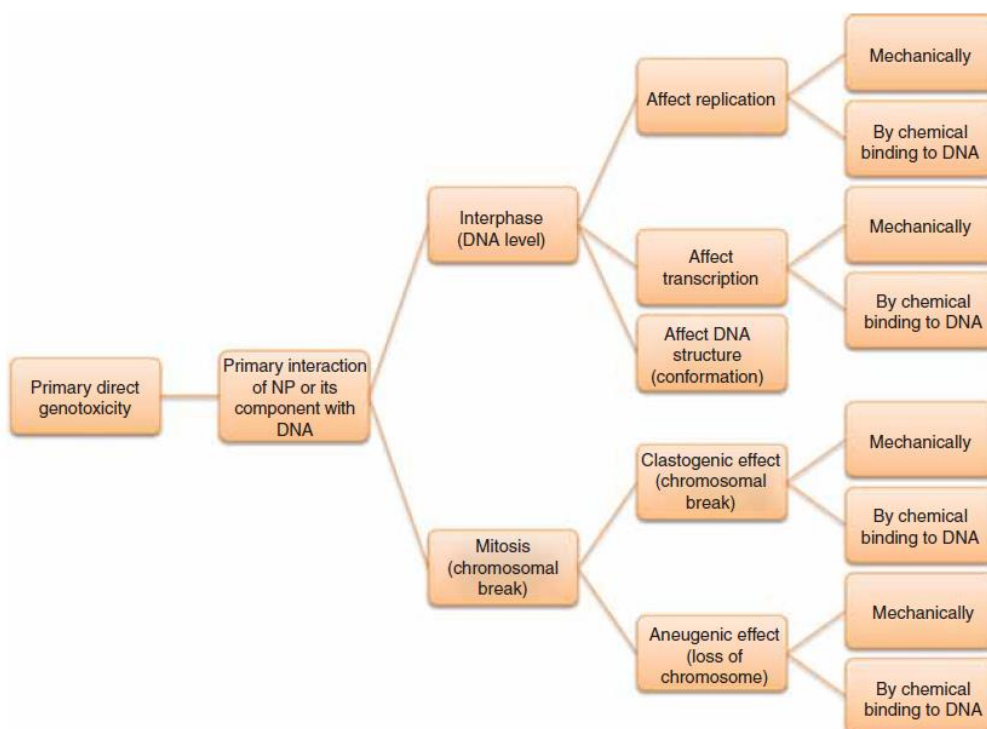

Figure 13. Diagram of potential induction of direct primary genotoxicity by nanomaterials (*from Magdolenova et al. 2014*)
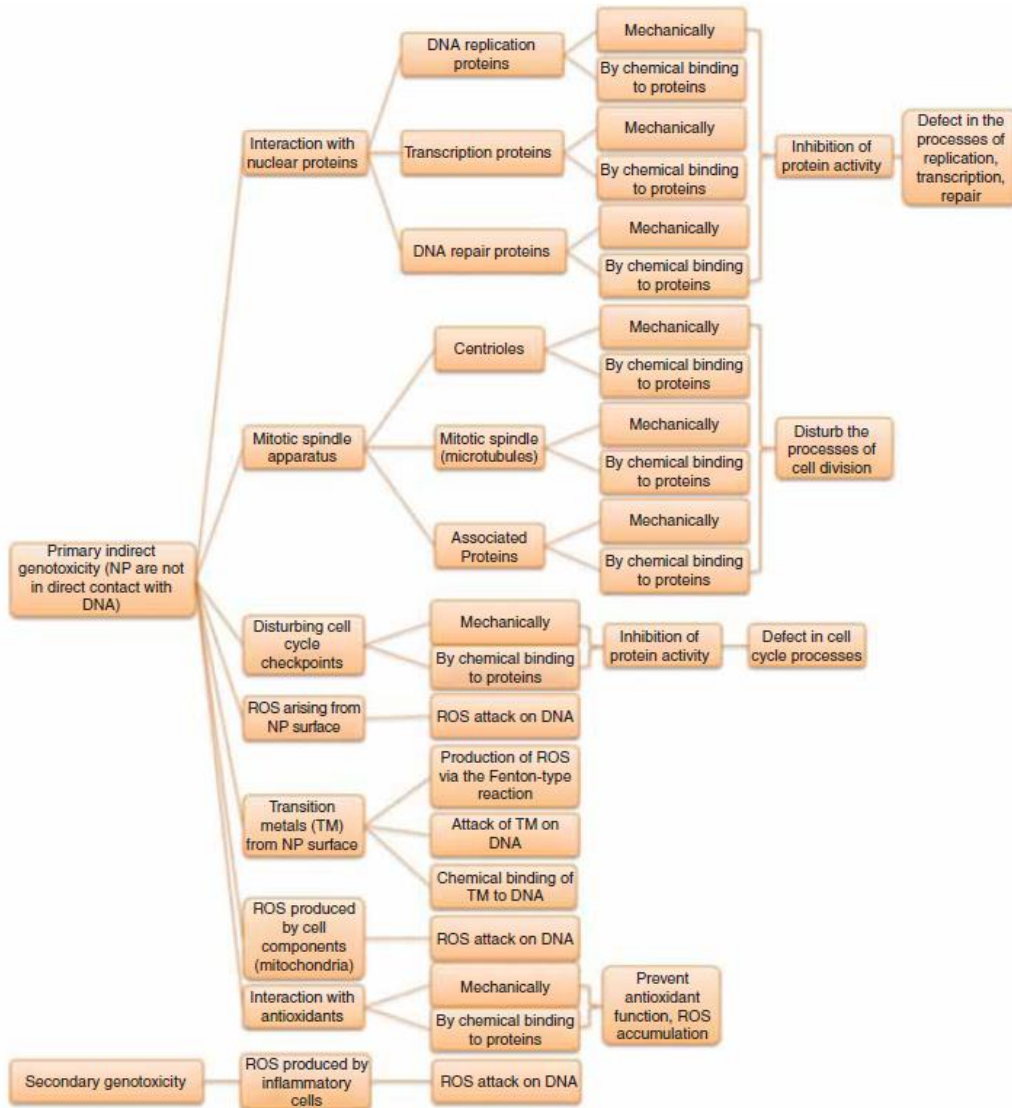
Figure 14. Diagram of potential induction of indirect primary and secondary genotoxicity by nanomaterials (*from Magdolenova et al. 2014*)

The genotoxic and mutagenic properties of nanomaterials are often closely tied to the increased production of reactive oxygen species (ROS) and nitrogen species (RNS). "Oxidative stress" can be defined as an imbalance between the production of ROS and the body's antioxidant capacity. This is probably the most widely accepted mechanism responsible for the potential genotoxic activity of nanomaterials, although it may not be the only one. The different pathways for the generation of oxidative phenomena mediated by (nano)materials and their involvement in primary and secondary genotoxicity processes have been known for a number of years, and were described by Schins and Knaapen (2007).

In order to cover the different genetic events possibly leading to genotoxicity, a minimum battery of tests is needed. EFSA (2011) therefore recommended combining two *in vitro* tests, the bacterial mutation test (Ames test, OECD TG 471) and the *in vitro* micronucleus test (OECD TG 487).

However, the Ames test performed on bacteria is considered inappropriate for assessing the mutagenicity of nanomaterials due to a high risk of false negative results (as it is likely that some nanomaterials are unable to pass through the bacterial wall). Doak *et al.* (2012) concluded that "although the Ames test is a reliable genotoxicity screen for the assessment of chemicals, it does not appear to be suitable for the assessment of nanomaterials". In order to assess the gene mutation induction parameter, a mammalian cell test should be performed (OECD TG 476 or 490).

Moreover, since some nanomaterials are capable of inducing structural (clastogenesis) and/or numerical (aneuploidy) chromosomal aberrations, the *in vitro* micronucleus test (OECD TG 487) appears to be well suited because it is capable of revealing both these types of effects (Pfuhler *et al.* 2013) and can be carried out on potential target cells, provided that internalisation of the nanomaterials by the cell line used has been demonstrated. This method was considered the most predictive in the Nanogenotox project[33]. Lastly, incompatibility with certain experimental conditions should be verified, for example the simultaneous co-exposure of nanomaterials and cytochalasin B, which inhibits endocytosis (Doak *et al.* 2009).

- **Standardised *in vitro* tests**

A critical assessment of knowledge on mutagenesis and genotoxicity of nanomaterials has revealed that some standard models are not well suited or even applicable to the study of nanomaterials. For example, as the bacterial cell wall can act as a barrier to many nanomaterials, bacterial test systems are probably insufficient to ensure DNA exposure and cannot therefore be regarded as sufficiently robust. While the Ames test, which is performed on a bacterial test system, is a reliable test for assessing chemicals, it does not seem to be suitable for assessing the mutagenesis of nanomaterials, due to the high risk of false negative results (Balasubramanyam *et al.* 2010) and its use is therefore not recommended (Doak *et al.* 2012 and OECD 2014).

As an alternative to the bacterial gene mutation test, *in vitro* mammalian cell gene mutation tests (OECD TG 476, 490)[34], for which no publications have yet identified any specific limitations for the assessment of nanomaterials, are recommended. However, although tests using thymidine kinase (TK) or hypoxanthine phosphoribosyltransferase (HPRT) systems have been adapted to type TK6 or WIL2-NS human lymphoblastoid cells (Canova *et al.* 2005; Wang *et al.* 2007; Kim *et al.* 2017), it should be recalled that by default, these tests mainly use rodent cells (L5178Y, CHO, V79), with deficiencies (small quantity of detoxification enzymes, deficient p53 expression, karyotypic instability, etc.) that could lead to an overestimation of the observed

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[33] Nanogenotox European Project (March 2010 – February 2013). http://www.nanogenotox.eu/

[34] OECD Guideline for the Testing of Chemicals No. 476: In Vitro Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes, 29 July 2016. OECD Guideline for the Testing of Chemicals No. 476: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene, 29 July 2016.

effects and an incorrect assessment. In addition, their non-human origin may also limit their relevance, as rodent cell lines are thought to be more likely to give false positive results that would not be observed with a more suitable test system (Fowler *et al.* 2012).

As a complement, and in order to investigate the possible induction of chromosomal aberrations (structural and numerical) by nanomaterials, the micronucleus test, for which an OECD guideline (OECD TG 487) is available, should be used. It should be noted that the protocol will need to be adapted, since in its most commonly used version, this test includes a cytokinesis blocking step using cytochalasin B, which could hinder the cellular internalisation of nanomaterials. The cells should therefore be exposed to the nanomaterials first and then to cytochalasin B afterwards, as described by Gonzalez *et al.* (2011). Furthermore, as mentioned above, the possible accumulation of nanomaterials in the cytoplasm could generate interference when analysing the slides under the microscope, with the risk that micronuclei could be obscured by the particles. The use of flow cytometry could overcome this limitation.

Exploitation of the results of the systematically required *in vitro* genotoxicity tests will be explained in Section 3.2.5.4.1 concerning further genotoxicity studies.

3.2.5.3.3. Toxicokinetics

Toxicokinetic studies aim to assess the behaviour of a compound after it enters an organism. They provide information on absorption, distribution, metabolism and excretion (ADME). By identifying the organs in which the compound (or its metabolites) is found, they help focus the toxicity studies on organs that may be potential targets. With regard to nanomaterials, toxicokinetic studies mainly focus on absorption and distribution phenomena, by assessing the ability of nanomaterials to pass through certain biological barriers (intestinal, pulmonary, blood-brain, for example) and become distributed in the various organs. Information can also be collected on the excretion of nanomaterials via their quantification in faeces, urine and bile. Toxicokinetic studies should take into account the principles of OECD Guideline 417. However, as this guideline is not tailored to nanomaterials, the specific points listed below should be considered.

Regarding the route of administration, it is generally accepted that gavage ensures that the correct dose is given. However, in this case, administration is intragastric, so the first organs of the digestive tract (mouth and oesophagus) are not directly exposed. To take account of these initial contact areas, the dose can be administered either in drinking water or in feed. In this case, it is more difficult to estimate the dose ingested by each animal. Furthermore, in these cases, the stability of the nanomaterials in water or feed needs to be verified.

In oral studies, nanomaterials are generally poorly absorbed through the intestinal barrier (only a few % of the administered dose by mass) (Shi *et al.* 2013; Jones *et al.* 2015; Geraets *et al.* 2014); however, accumulation in certain systemic organs – in particular the liver, spleen and kidneys – is often reported (Loeschner *et al.* 2011; Kermanizadeh *et al.* 2015; Brand *et al.* 2020; Krause *et al.* 2020; Heringa *et al.* 2018).

It therefore appears necessary to assess distribution after repeated administrations and over a long enough time (14 days). This exposure time should take into account the low intestinal absorption and the problems with the limit of detection of the analytical methods used.

Similarly, the doses tested should be estimated taking these limitations into account but without causing toxicity that could affect the kinetics.

Considering that some publications report a gender effect on the distribution, accumulation or toxicity of nanomaterials (Han *et al.* 2020; Tassinari *et al.* 2020; Mohammadpour *et al.* 2019; Chen *et al.* 2013; Kim *et al.* 2011; Kim *et al.* 2007), toxicokinetic studies should be performed in both sexes. It is important to note, however, that greater variability in quantification data can be expected for females, since the period of the oestrous cycle can influence gut permeability (Braniste *et al.* 2009). In order to reduce this variability, it may be necessary to synchronise the cycles of the females beforehand.

For the distribution of nanomaterials, special attention should be paid to the gastrointestinal tract, brain and reproductive organs (as this information will be essential for initiating further toxicity tests), as well as to organs where accumulation of nanomaterials – such as the spleen, liver and kidneys – has already been regularly observed. For the gastrointestinal tract, in addition to samples from the small and large intestine, it is important to have samples with lymphoid tissues (Peyer's patches and mesenteric lymph nodes), which are potential passage zones for nanomaterials (Bouwmeester *et al.* 2017; Janer *et al.* 2014; Geraets *et al.* 2014). Negative controls receiving only the vehicle must be included in the studies. It is also important to check that the doses used did not significantly alter the intestinal epithelium, which could call into question the results obtained for systemic distribution. Histological data on the gastrointestinal tract are therefore recommended. Additional data on intestinal permeability using markers (creatinine, different sizes of dextran, ^{51}Cr-EDTA) can also be generated.

If the results obtained in the 14-day kinetic study do not show distribution of the nanomaterials (gastrointestinal tract, brain, sexual organs, liver, spleen and kidneys), a longer-term analysis in the 90-day subchronic toxicity study will be required, to assess distribution and possible quantification in the organs listed above as a priority. If distribution results are obtained in the 14-day toxicokinetic study, a comparison of the distribution results from the 90-day study could show whether a redistribution effect in certain organs occurs over time.

The analytical parameters (extraction yield, LOD and LOQ) of the methods used for the assays should be indicated for each of the studied organs. In some cases, external contamination (e.g. via feed) may generate high baseline levels in negative controls, preventing detection of any increase in the systemic organs of treated animals (assuming low passage through the intestinal barrier). Although this is often difficult to achieve, some solutions can be proposed to lower this baseline as much as possible: for example, as silica is an element found in the diet of rodents but also in glass labware, special experimental conditions as well as suitable feed with a low silica content are needed (Aureli *et al.* 2020).

Conventional quantification methods are mainly limited to analyses using inductively coupled plasma (ICP) coupled with different detection systems such as mass spectrometry (ICP-MS) or optical emission spectrometry (ICP-OES). However, with the exception of single particle technology (sp-ICP-MS), these methods are unable to distinguish particulate forms from ionic forms. Therefore, in order to distinguish the constituent particulate forms of nanomaterials, additional analytical methods are required (e.g. electron microscopy, SAXS[35], Tof-SIMS[36]), but

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[35] Small angle X-ray scattering
[36] Time-of-flight secondary ion mass spectrometry

these can only analyse a limited number of samples. For some nanomaterials that cannot be analysed by ICP and/or in order to facilitate their detection, nanomaterials labelled by fluorescence or radioactivity techniques or consisting of metallic cores can be used. In this case, it is important to check beforehand that 1) the labelling does not affect the physico-chemical properties of the nanomaterials (it could modify their behaviour) and 2) the labelling is stable and is not lost during the study, which would lead to false conclusions.

Further studies to assess the persistence and excretion of nanomaterials can be performed by adapting the experimental design with recovery periods after repeated treatment.

- **Outlook**

Given the concept of the 3Rs (reduce, replace, refine) and the actions undertaken to limit animal testing, the outlook from the point of view of kinetics is organised around *in vitro* and *in silico* approaches.

For *in vitro* approaches, kinetic data on oral absorption can be based on passage studies using human intestinal barrier models. For this purpose, cultures of intestinal epithelial cells of varying complexity (possibility of co-cultures with mucus cells or with induction of M cells) are grown on porous membranes (inserts). While these intestinal cell studies have a guidance document produced by the EURL ECVAM[37], other *in vitro* barrier models are also available (e.g. placental, blood-brain, testicular). However, their use for nanomaterials remains controversial due to the limitations encountered (physical barrier of the insert, low passage through the barrier, and detection limit of the methods). For some barriers (intestine, placenta), *ex vivo* systems can be used, but these are more difficult to manipulate, their response varies depending on the donor and they can only be used to study a very limited number of nanomaterials at a time.

For *in silico* approaches, two complementary methods are recommended.

In vitro/in vivo extrapolation (IVIVE) enables the behaviour of nanomaterials *in vivo* in animals to be linked to data obtained *in vitro*, in order to simulate what happens in humans. *In vivo* kinetic data are then coupled with *in vitro* kinetic data collected from human cell models (e.g. passage through the intestinal barrier, hepatic and renal clearance).

In addition, physiologically-based toxicokinetics (PBTK) mathematical models can be generated by combining data from toxicokinetic studies, physiological data on the organism considered (such as tissue volumes, blood flow) and physico-chemical and biochemical parameters of the nanomaterials (Utembe *et al.* 2020). These models aim to predict the behaviour of nanomaterials in humans for a given exposure by simulating concentrations in various organs, tissues or biological fluids as a function of time. In addition, they may be able to take account of physiological changes in different populations (e.g. age, disease).

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[37] Tracking System for Alternative methods towards Regulatory acceptance https://tsar.jrc.ec.europa.eu/test-method/tm2013-01.

3.2.5.3.4. Repeated dose toxicity (subchronic)

Subchronic toxicity studies are *in vivo* studies that identify and characterise the hazard of a compound. Several groups of animals are exposed daily to the studied compound *via* gavage, feed or drinking water over a prolonged period of 90 days. This exposure covers the post-weaning, growth and adult periods of the test animals. During the exposure phase and/or at the end of the 90 days, the following are performed on the animal: measurement of weight, food and water consumption, behaviour and symptom monitoring, ophthalmological, haematological, clinical biochemistry and urine examinations, a general autopsy and histopathology. These studies should be conducted according to the latest version of the OECD guideline (TG 408). Compared to the previous version, this new one includes the monitoring of parameters related to endocrine effects (oestrous cycles, measurement of thyroid hormones, etc.). Analysis of the results of the subchronic study will determine whether further toxicological tests are needed. In order to obtain all the data necessary for performing these additional tests, the WG proposes integrating the monitoring of additional parameters as described below during this subchronic study. If these further tests are not necessary, the subchronic study should allow toxicological reference points such as the NOAEL[38] or the BMDL[39] to be established.

- o <u>90-day toxicokinetics</u>

> If the 14-day toxicokinetic studies do not result in any detection in the systemic organs, a longer exposure time (the 90-day subchronic toxicity study) is needed to verify passage through biological barriers and distribution of the nanomaterials.

For the 90-day toxicokinetic studies, the same attention should be paid to the organs to be considered as a priority (gastrointestinal tract, brain, reproductive organs, liver, spleen and kidneys), the analytical methods, and the baseline level in the negative controls.

- o Measurement of nanomaterials in the nervous system after 90 days

> If the results of the 14-day toxicokinetic studies do not show any detection of nanomaterials in the brain, then nanomaterials in the central and peripheral nervous system should be measured after 90 days of exposure.

The WG would also like to point out that it will be important to take samples of nerve tissue in order to quantify the nanomaterials found in these areas (Bolon *et al.* 2013). These tissues should only be collected after exsanguination or perfusion of the animals (using physiological serum) to ensure that the measurement carried out reflects the quantity of nanomaterials accumulated in the nervous system and not that found in the blood compartment. The brain, spinal cord and also the intestines, with a high density of nerve cells (enteric nervous system, Grundmann *et al.* 2015), should be sampled and weighed, to determine by ICP-MS or atomic

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[38] No observed adverse effect level
[39] Benchmark dose limit

absorption the quantity of nanomaterials present in relation to the controls. Thus, even if the study is limited to three months of exposure, which may be too short for translocation to the brain, these samples may be sufficient to reveal an accumulation of nanomaterials in the peripheral (Grundmann *et al.* 2015) (in the intestinal wall) and central (spinal cord) nervous compartments, which may be predictive of direct neurotoxicity through damage to nerve cells.

    o   <u>Immunotoxic potential</u>

While immunotoxicity can be assessed *in vitro* on cultured cells (often immortalised cell lines) exposed to nanomaterials, the immune response raised under these conditions is limited to markers of inflammation, essentially represented by cytokines of the rapid and non-specific response (innate immune response). However, the immunotoxicity of nanomaterials can be manifested as adverse reactions both on the innate response and on the ability of the immune system (IS) to organise a cell-mediated or humoral response (adaptive immune response) on the scale of the whole individual, such as allergy, tolerance to food antigens or autoimmune reactions. These alterations take time to develop and require at least a subchronic oral study.

It should be noted that the OECD TG 408 guideline (90-day study) already provides for the study of parameters regarded as indicative of an impact on the immune system, such as relative weight (organ weight to body weight ratio) for the spleen and thymus, as well as a histopathological study in these same organs extended to bone marrow, the intestine (including Peyer's patches) and the lymph nodes draining the regions concerned by the oral route (mesenteric lymph nodes for the intestine) as well as remote from them (other nodes testifying to systemic effects). Histopathological study of the intestinal mucosa should reveal neutrophilic infiltration, oedema formation and/or structural changes. In addition to these approaches, the type and levels of cytokine secretion (pro- and anti-inflammatory) in the gut mucosa should be determined. Gene expression could supplement these data to help demonstrate an immunosuppressive effect of nanomaterials. Cytokine profiles will serve as more precise indicators of the immunotoxic or immunomodulatory potential of ingested nanomaterials under conditions of long-term exposure *in vivo*, in the demonstration of a local inflammatory response[40] or of immunosuppression able to diminish the effectiveness of the gut's immune defences. This approach should be followed in the 90-day study even in the absence of nanoparticle absorption. This is because with the oral route there is potential for contact with lymphoid tissue (i.e. Peyer's patches, including uptake of particles by antigen-presenting cells) without the need for local accumulation of particles or systemic passage.

In addition to characterising the cytokine profile in the intestine, the multilevel approach should integrate a phenotypic analysis of T cells in the intestine, mesenteric lymph nodes and spleen after 90 days of oral exposure to the studied nanomaterial. Particular attention should be paid to the frequency of regulatory T cells (Tregs) involved in maintaining peripheral tolerance and suppressing immune responses. This is because Tregs can inhibit T cell proliferation and inflammatory cytokine production, playing an essential role in the prevention of autoimmunity (auto-tolerance) and homeostasis. In association with gut immunity, Treg cells also play a

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[40] For *in vivo* measurements, particular attention should be paid to the presence of bovine serum albumin (BSA) in the dispersion medium of the nanomaterials, given this protein's immunogenic potential.

central role in inducing immunisation or tolerance to food, with suppressive activity being exerted by the cytokines TGF-β and IL-10. The frequency of Treg cells should be assessed on the basis of expression of the transcription factor forkhead box P3 (FoxP3)[41] specific to this T cell sub-population. This measurement in a 90-day study will at least provide information on the immunotoxic potential of nanomaterials on adaptive immunity. If these results indicate that the nanomaterial is able to repress the expression level of FoxP3 used as a control for impaired T cell differentiation into Treg cells (e.g. Bettini *et al.* 2017 for E171), further studies will be required to characterise the potential breakdown in antigen tolerance (see additional oral tolerance tests).

    o   <u>Precancerous biomarkers of the gastrointestinal tract</u>

Carcinogenesis is a multi-step process involving the transformation of a normal cell into a tumour cell, with the following main steps: initiation, promotion, development of malignant cells and tumour progression. The biochemical and histological changes in the colonic mucosa observed during these different stages are now well documented. Thus, it is now possible to screen for early biomarkers of intestinal carcinogenesis (preneoplastic lesions) including the counting of aberrant crypt foci (ACF), β-catenin accumulated crypts (BCACs) and mucin-depleted foci (MDF).

The WG would like to point out that these preneoplastic lesions can occur spontaneously and do not systematically develop into malignant tumours. These lesions should be used as early biomarkers that determine the need for carcinogenesis studies based on the results obtained.

<u>ACF</u>

Aberrant crypt foci (ACF) were described by Bird *et al.* (1987) as lesions consisting of large, thick crypts in the colon of mice previously exposed to a carcinogenic substance: azoxymethane (AOM). In rats, several studies have shown that ACF formation increases after the animals have been exposed to substances known to promote carcinogenesis. These ACF have also been observed in patients (Roncucci *et al.* 1991) with familial adenomatous polyposis. Significant correlations have been found (Takayama *et al.* 1998) between the number of adenomas observed and the number and size of ACF (characterised by the number of crypts per focus). The results described in the literature suggest that ACF can be considered as precursors of adenomas and cancers. However, other studies have highlighted that certain compounds such as 2-carboxyphenyl retinamide are known to promote the development of colon cancer while preventing the occurrence of ACF (Zheng *et al.* 1999). It has also been shown that cholic acid, known to be a promoter of colon carcinogenesis, suppresses the formation of ACF while promoting the formation of another biomarker: BCACs (Hirose *et al.* 2003). Thus, it appears that in some cases, other biomarkers may be more suitable and more robust than ACF as a biomarker of early colon carcinogenesis.

There are currently no guidelines for the detection of these biomarkers. However, the protocols for observing ACF are based on simple methylene blue staining. In 2017, ANSES had stated,

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[41] FoxP3 is involved in the regulation, activation and differentiation of T cells as the main control gene for Treg cell development and function.

in its opinion on dietary exposure to titanium dioxide nanoparticles, that the protocol used by Bettini *et al.* (2017) had followed a well-established scientific model. The WG would like to clarify that the use of these biomarkers and their correlation with tumour incidence involves the identification of "large ACF". There does not seem to be a universally accepted definition of a "large ACF". Bettini *et al.* chose to set a threshold for defining a "large ACF" at more than three aberrant crypts per focus. However, other authors have set this threshold at 10 aberrant crypts (Maurin *et al.* 2006). The methodology used to identify these large ACF should therefore be clearly described and justified.

BCACs

In the event of colon cancer premalignant lesions, mutation of the β-catenin gene and accumulation of this protein in cells have been shown to be involved in the formation of small dysplastic crypts in the colons of rats previously exposed to AOM. These crypts containing excessive amounts of β-catenin are identified as β-catenin-accumulated crypts (BCACs). These display histological changes that differ from the typical appearance of ACF. The number of crypts per lesion appears to increase over time after exposure to a carcinogen (Mori *et al.* 2004) and the cell proliferation activity of BCACs appears to be more pronounced than that observed for ACF (Yamada *et al.* 2001). As with ACF, there are currently no guidelines for the study of this biomarker, however various studies have been published that can serve as references for the observation and characterisation of BCACs that are not identified directly on the surface of colonic mucosa but are observed on histological sections by immunohistochemistry (Hata *et al.* 2004; Yamada *et al.* 2000).

MDF

Mucin-depleted foci (MDF) were identified by Caderni *et al.* (2003) and are also considered an early biomarker of carcinogenesis. Indeed, MDF show similar dysplastic features to those seen in colon tumours. These lesions have also been observed in patients with colorectal carcinoma and familial adenomatous polyposis (Fernia *et al.* 2008). A few weeks after exposure of rodents to AOM, foci of crypts with very small amounts of mucin (glycosylated proteins involved in epithelial lubrication) were observed. Studies have shown that the number and size of MDF increase over time after exposure to a carcinogen (Hata *et al.* 2004). Some studies have highlighted the fact that MDF are preferentially located in the distal portion of the colon while ACF are mainly observed in the middle part (Fernia *et al.* 2005; Suzui *et al.* 2013). MDF are easily identified directly on colonic mucosa using Alcian blue staining techniques (Yoshimi *et al.* 2004).

The 90-day exposure times proposed for the subchronic studies are sufficient and appropriate for studying these three biomarkers. The choice of animal species and strain should be considered when preparing the experimental protocol, as not all species have the same chemoinduction properties (chemoinduction in the F344 rat is easier to implement than in the C57 mouse).

The analysis of biomarkers identified by the WG can provide further information. Thus, systematic screening for two biomarkers is required to confirm the appearance of preneoplastic lesions. The WG believes that there are methodological constraints to the search for BCACs (including the need to perform frontal sections of the colon prior to the observation step). In this context, the WG recommends ACF and MDF as the two biomarkers to be considered when screening for preneoplastic lesions.

3.2.5.4. Further toxicological data

3.2.5.4.1. *In vitro* and *in vivo* genotoxicity

If positive results are obtained in the basic battery of *in vitro* tests (mammalian cell gene mutation test and micronucleus test), all relevant data should be examined with a weight of evidence approach (dose-response relationship, response amplitude and minimum positive concentration, reproducibility of *in vitro* results, specificity of the material's response at the nanoscale, etc.). Depending on this assessment, the following steps may lead to a conclusion without further testing, or to the need for further *in vitro* testing (in which case methodological changes may be made), and/or the implementation of *in vivo* tests.

- **Further *in vitro* tests for mechanistic purposes**

While the comet assay is not part of the basic battery of tests, it may be useful to implement *in vitro*, for mechanistic purposes in the follow-up of positive *in vitro* results, particularly if previous *in vitro* toxicity results have shown an "oxidative stress" effect of nanomaterials. In these conditions, use of the modified protocol (use of repair enzymes, i.e. Fpg or hOGG1) can reveal oxidative DNA damage and assess whether or not it is exclusive.

To demonstrate a possible aneugenic effect, it may be necessary to perform an *in vitro* micronucleus test coupled with the fluorescence *in situ* hybridisation (FISH) methodology using, for example, pan-centromeric probes.

- **Further *in vivo* tests**

Further *in vivo* testing may be required. In this case, the *in vivo* tests carried out should allow the assessment of several genotoxicity parameters (endpoints) on different target organs. In these conditions, a combination of *in vivo* micronucleus tests and comet assays is a suitable choice. This study should be conducted on primary exposed target organs (e.g. organs of the gastrointestinal tract such as the stomach, colon, etc.), circulating blood or systemic organs exposed after translocation (e.g. liver).

The gene mutation assay on transgenic animals (OECD TG 488) carried out on the target organ(s) may also be of interest for complementary purposes, but as mentioned above, this test is very time-consuming to perform and is not widely available.

In any case, negative results obtained on systemic organs are only relevant when there is evidence of exposure.

In vivo tests are generally carried out with relatively short treatment times (usually no more than a few days). The experimental design is therefore questionable, especially with regard to systemic organs, which are only exposed to very small quantities of nanomaterials at each administration, given the probable low percentage passing through the intestinal barrier. In order to overcome this limitation, coupling genotoxicity tests with repeated dose toxicology studies (or the 14-day toxicokinetic study) could be considered.

- **Experimental conditions**

Many technical and methodological points can cause inconveniences that prevent *in vitro* tests on nanomaterials from being very predictive of *in vivo* effects. These include the use of very high doses (up to 2 mg/ml in cell systems); possible interactions of nanomaterial suspensions with test systems; possible interference with methods for determining cytotoxicity, etc. (Section 3.2.5.2).

In addition, to ensure that the responses are qualitatively and/or quantitatively "nanospecific", it would be useful to compare the genotoxicity response of materials at the nanoscale and at the microscale. The inclusion of positive and negative reference nanomaterials could also be of particular interest to demonstrate the sensitivity and specificity of the test systems used.

- **Outlook**

In addition to a proposed regulatory strategy, new *in vitro* tests should be developed and adaptations of existing tests should be proposed for assessing the genotoxicity of nanomaterials.

It may be necessary to begin the process to validate the micronucleus test carried out on target organs (colon and liver) following oral exposure to nanomaterials.

Similarly, versions of the *in vitro* comet assay with high-throughput screening offering the possibility of testing many nanomaterials rapidly and concomitantly have recently been developed (Nelson *et al.* 2017). Validation of these tests would enable them to be used for regulatory purposes.

The implementation of high-throughput methods allowing the simultaneous study of several parameters (e.g. cytotoxicity, biomarkers of genotoxicity, oxidation, apoptosis, interactions with DNA repair systems, etc.) could be particularly interesting for the rapid screening of nanomaterial toxicity. Due to the multiplicity of nanomaterials used, high-throughput methods could facilitate screening and then provide useful data for a read-across assessment. Similarly, modelling tools (quantitative nanostructure-toxicity relationship) should be developed.

Lastly, among these new *in vitro* tests, methods based on genomic and proteomic research could be useful for detecting effects related to oxidative stress, inflammation, etc. These technologies can also be used to define modes of action and signalling pathways, in order to assess interactions between these different pathways, etc. (Pfuhle *et al.* 2013).

3.2.5.4.2. Toxicity to the immune system

- **Test of oral tolerance to food antigens**

Oral tolerance depends on establishment of an appropriate immune response of the gut-associated lymphoid tissue and on a physiological process characterised by the absence of cellular and humoral response to oral administration of antigens (tolerisation) compared to the same antigens administered systemically (immunisation). Treg lymphocytes are a key element in the induction and maintenance of immune tolerance to food.

> If the required 90-day subchronic study tests show that the nanomaterial has an impact on Treg cell frequency (significant decrease), conducting a further *in vivo* test of oral tolerance to food antigens should be considered.

A study of oral tolerance to ovalbumin (OVA, a model food antigen) is classically used in rodents to assess the harmful potential of xenobiotics on the adaptive immune response. Various studies have been conducted using nanomaterials such as amorphous silica (Toda *et al.* 2016), silver (Xu *et al.* 2015) or carbon black (Fine *et al.* 2016). The duration of exposure to the nanomaterial in rodents will be the same as in the subchronic study, i.e. 90 days. In a physiological situation, oral tolerance is manifested by the absence of a response (anti-OVA serum immunoglobulin (IgG)) to oral administration of OVA antigen. Conversely, in the event of a breakdown in induction of the tolerogenic effect, production of anti-OVA serum IgG will be observed, indicating intolerance to the model food antigen[42]. There is no dedicated test in the OECD guidelines to specifically address this question.

- **Other further tests**

In addition to oral tolerance, the *in vivo* assessment of the immunotoxic or immunomodulatory potential of a nanomaterial on the adaptive immune response can be based on other simple functional tests involving the cells of the gut-associated lymphoid tissue (dendritic cells or DCs, macrophages, T cells) and their interrelationships (e.g. migration of antigen-presenting cells such as DCs) with those of the mesenteric lymph nodes and spleen.

Initial information on an individual's ability to develop a humoral immune response (i.e. antibody response) to a particular antigen may be obtained by following the additional developmental immunotoxicity tests described in the OECD extended one-generation reproductive toxicity study (EOGRTS TG No. 443, Section 3.2.5.4.4).

> If there is a significant effect on one or more of the cellular compartments of the immune system during the 90-day subchronic study (proliferation of spleen cells, with phenotypic analysis of T, B, NK cells), functional and mechanistic studies to identify the consequences on the individual's health (e.g. allergic risk, autoimmune reactions, developmental toxicity on the immune system), based mainly on disease models, could be considered.

However, there are no dedicated tests in the OECD guidelines to address each of these questions, so studies should be carried out on a case-by-case basis.

3.2.5.4.3. Neurotoxicity

From a regulatory perspective, the OECD has included guidelines for conducting tests specifically to assess the neurotoxic effects of chemicals[43]. The purpose of these guidelines is to identify chemicals that permanently or reversibly affect the nervous system. The alterations

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[42] To verify intolerance, the test may be supplemented by a study of the inflammatory response in the gut after oral re-stimulation with OVA.
[43] http://www.oecd.org/document/7/0,3343,en_2649_34377_37051368_1_1_1_1,00.html

to the nervous system taken into consideration are well-defined and therefore limited to those established so far.

These guidelines are based on animal studies, mainly using rodents (OECD 424 and 426). Several parameters are taken into account, including:

- clinical observations in the housing cage or in an open-field area;

- neurofunctional tests such as those assessing motor activity, learning and memory;

- neuropathology observations using perfusion-fixed tissue to estimate the integrity of nerve tissue on the basis of gross morphology, morphometry and histopathology.

Among these parameters, the behavioural assessment recommended is one of the leading tests. This is because the results of behavioural tests are considered to be among the most significant and sensitive criteria in chemical risk assessment. Individual neurobehavioural performance depends on characteristics relating to exposure (dose, chronicity) and specific to the individual (age and general state of health defining the individual's vulnerability), and therefore has an added predictive value compared to biochemical or neuropathological alterations alone, for example.

Given the importance of behavioural tests and the obvious concern that chemicals may be unsuitably classified as neurotoxic, implementation of these tests requires several critical issues to be addressed in order to achieve even greater reliability in their use and application (Slikker *et al.* 2005). All the critical points that need to be carefully considered when planning these studies have been identified and detailed. It should be noted that this rigour is lacking in many of the studies conducted outside these guidelines, which therefore calls for the greatest caution when interpreting the data reported on neurotoxic potential.

Neurotoxicity studies are not mandatory in Europe. However, such studies need to be performed when there is evidence of neurotoxicity from standard regulatory single-dose (OECD 402, 403, 420, 423 and 425) or repeated-dose (including OECD 407 and 408) toxicity tests[44] or from human data[45]. These guidelines for neurotoxicity studies have thus rarely been used, especially since, as with all toxicology studies, they are also very resource-intensive in terms of animals, time and overall cost (Rovida *et al.* 2009).

As with any chemical, it is only reasonable to initiate neurotoxicity studies for nanomaterials in specific cases.

It is currently not possible to link neurological signs in humans with exposure to nanomaterials after ingestion. In experimental *in vivo* studies, indicators of neurological impairment in rodents have been found after exposure to certain nanomaterials. However, interpretations of the causal links are most often limited, in particular and among other things, by a lack of information on the purity or the precise characterisation of the nanomaterials used, the route of exposure and the doses used, which do not mimic realistic exposure conditions that can be transposed to humans. Moreover, these studies rarely follow the recommendations for implementing *in vivo* tests and for interpreting the data collected, as described in the regulatory tests.

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[44] These tests can indicate functional and/or histopathological information gathered from the major organ systems, including the nervous system.
[45] Mostly on occupational exposure.

Under these conditions, it seems essential to propose an alternative framework based on other criteria for presuming neurotoxic properties, in order to determine the need for specific neurotoxicity studies. A strong criterion could be the presence of a nanomaterial in the nervous compartment. In general, translocation leading to accumulation within the nervous compartment is a slow process (Kreyling *et al.* 2017), partly because of the effectiveness of the brain's own physiological barriers to xenobiotic agents, such as the blood-brain barrier, although some pathways such as the olfactory nerve or the trigeminal nerve have been identified (Bencsik *et al.* 2018). Moreover, quantitatively speaking, the mass of nanomaterials accumulated in the brain is generally small (Kreyling *et al.* 2017).

However, studies indicate that the clearance of nanomaterials in the brain may also be a very slow process, thus favouring their long-term accumulation (Kreyling *et al.* 2017). This observation underlines the importance of considering the neurotoxicity of nanomaterials essentially over the long term, this question being all the more relevant when the type of nanomaterial considered is stable and poorly soluble, as is the case for TiO_2, for example.

> In the absence of toxicological data on behavioural impairment, the first simple criterion to be demonstrated is the presence of an accumulation of the studied nanomaterial in the tissues of the nervous system. If the nanomaterial is detected in the brain during the 14-day toxicokinetic study, then further neurotoxicity testing should be performed. In the absence of any nanomaterial in the brain, the 90-day toxicokinetic studies should be modified to include an assay for the nanomaterial in the nervous system (see section on 90-day toxicokinetic studies). This screening should be compatible with high-quality histological sampling and investigation. If the nanomaterial is found in the nervous system, then further neurotoxicity testing should be performed.

Thus, the factor relating to "accumulation of nanoparticles" in the central and/or peripheral nervous system is a prerequisite for triggering neurotoxicity tests, especially if it is combined with a nanomaterial type that favours biopersistence. This factor is of decisive value in health risk analysis calculations, even though this translocation to and accumulation in the nervous system is very low.

If these criteria are observed, this triggers the further neurotoxicity studies stipulated in the OECD guidelines. However, the specificity of the typical properties at this nanoscale (Bencsik *et al.* 2021) calls for more relevant methodologies and experimental designs to be taken into consideration.

Whenever possible, it would be worth implementing simultaneous oral exposure to a proven neurotoxic substance[46], in order to be able to draw on an effect benchmark, which would clarify interpretation and enable a distinction between a direct neurotoxic effect from a secondary

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[46] These substances with a proven neurotoxic effect could be selected based on the list of criteria proposed for alternative tests performed for developmental neurotoxicity. Ascher *et al.* Reference compounds for alternative test methods to indicate developmental neurotoxicity (DNT) potential of chemicals: example lists and criteria for their selection and use. ALTEX (2017) 34(1).doi.org/10.14573/altex.1604201

toxic effect[47]. The numerous transgenic models defining more sensitive biological environments could also make a useful contribution, especially to address specific vulnerabilities of the nervous system such as during ageing. For example, some models can be used to study neurodegenerative processes in a manner consistent with OECD guidelines 407 and 408.

To address the specific issue of neurotoxicity of a nanomaterial, the approach that involves studying the whole-organism response is the most relevant. The identification of behavioural, memory, motor or other neurological manifestations is the best marker of a neurotoxic effect. However, the collection of histopathological observations, advised by the various regulatory bodies (Bradley *et al*. 2011), could be better exploited in particular by using specific markers of glial cell activation (Escartin *et al*. 2021), including more recent ones such as those of immune cells of the nervous system (Kraft *et al.* 2011). These neuropathology assessments allow evidence of a neurotoxic effect to be identified and even confirmed. Data from complementary biological parameters should be accumulated, in particular by fully exploiting the current state of the art in neuropathology/neuroscience (van Thriel *et al.* 2019). In addition, it would be interesting to introduce a histopathological analysis of the enteric nervous system. In the context of nanomaterial ingestion, particularly in the case of nerve translocation, this is the first nervous compartment concerned in which neurotoxic impairment could be initiated, well before the brain, as has been shown for other ingested neurotoxic substances[48].

- **Outlook and developments in neurotoxicity studies**

The introduction of alternative models to the rodent, more ethically acceptable and representing an inevitable evolution in *in vivo* toxicological tests, offers new possibilities that are perfectly suited to neurotoxicology studies (Peterson *et al.* 2008). For example, the batteries of behavioural tests developed for zebrafish provide very similar assessments of sensorimotor, emotional and cognitive functions to those in rodents. These models have the advantage of producing data useful for establishing predictive toxicology, based on mechanistic toxicology (the principle of adverse outcome pathways, or AOPs, to link molecular and cellular neurotoxic mechanisms to behavioural dysfunction) and offer very promising opportunities for translating animal data to humans, and therefore have a place in risk assessment studies (Vorhees *et al.* 2021).

3.2.5.4.4. Reproductive and developmental toxicity

The potential toxicity of nanomaterials to exposed individuals and their offspring is of concern because of their ability to pass through biological barriers (placental, testicular, etc.).

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[47] The presence of circulating pro-inflammatory factors, resulting from damage to another peripheral organ, could secondarily initiate brain inflammation, with activation of microglial cells which, if it persists, has neurotoxic implications.
[48] As for pesticides, for example: Naudet N, Antier E, Gaillard D, Morignat E, Lakhdar L, Baron T, Bencsik A. Oral Exposure to Paraquat Triggers Earlier Expression of Phosphorylated α-Synuclein in the Enteric Nervous System of A53T Mutant Human α-Synuclein Transgenic Mice. J Neuropathol Exp Neurol. 2017 Dec 1;76(12):1046-1057. doi: 10.1093/jnen/nlx092.

Recently, ECHA published a review on the reproductive and developmental toxicity of engineered nanomaterials[49]. The hundred or so publications selected concerned *in vivo* studies on fertility and development conducted in rats and mice after oral and respiratory administration of nanomaterials. Nano-TiO_2 and silver were the two most frequently studied nanomaterials and together accounted for almost 50% of the works listed. Other nanomaterials also studied were zinc oxide, silicon oxide and nanomaterials containing carbon.

With regard to fertility, the studies focused almost exclusively on males and highlighted contradictory conclusions on the impact of nanomaterials on the reproductive organs and certain fertility parameters such as sperm count, morphology and motility. The ECHA document stated that there were few studies on fertility in females.

Studies following the OECD guidelines indicate little or no concern regarding the developmental toxicity of nano-TiO_2, zinc oxide, silicon dioxide and silver. However, these limited studies mainly focused on toxicity to the foetus and its growth. Other studies not following OECD guidelines have reported effects on the placenta or on the viability of offspring. As with fertility, the disparity of the results prevents definitive conclusions from being drawn about the studied nanomaterials.

For some nanomaterials, brain damage with developmental neurotoxicity is suggested (Amiri *et al.* 2018; Valdiglesias *et al.* 2015; Singh *et al.* 2019). Given the vulnerability of the developing brain and the fact that early brain damage can have irreversible effects in the adult (Cui *et al.* 2014), studies reporting developmental impairment, especially in the brain, should be systematically considered. These early indications of potential neurodevelopmental toxicity clearly show the need to focus on providing neurobehavioural and neuropathology assessment data, mainly through a study of sufficient length that covers the sensitive stages.

In general, the studies considered in the ECHA document show that nanomaterials can be distributed in different organs involved in reproduction and/or development, and that the distribution phenomena will depend on the chemical nature and size of these nanomaterials.

> For reproductive and developmental toxicity, the 90-day subchronic studies are limited to effects on the reproductive organs and the oestrous cycle. Effects on fertility, conception, gestation and development of organs and functions (including neurological) should be investigated in further tests if systemic exposure is demonstrated in toxicokinetic studies and/or if histomorphological damage to the reproductive organs is observed.

Different guidelines have been developed for the reproduction and development study.

These different studies are listed and described in the EFSA guidance document (2018). Briefly, these studies incorporate prenatal developmental toxicity tests (OECD TG 414) as well as an extended one-generation reproductive toxicity study (EOGRTS OECD TG 443) with specific exploration of cohorts of animals to perform reproductive and developmental toxicity tests (cohort 1) and developmental neurotoxicity tests (cohort 2). Prior to the EOGRTS, a combined repeated-dose toxicity study with the reproduction/developmental toxicity screening test (OECD TG 422) can be conducted. For specific neurodevelopmental studies, OECD Guideline 426 may also be considered.

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[49] ECHA 2020: A critical review of studies on the reproductive and developmental toxicity of nanomaterials

- **Outlook**

In the ECHA document on reproductive and developmental toxicity of engineered nanomaterials, various proposals and recommendations were made. In addition to the need to generate missing data on reproduction and development, methodological recommendations were made. The WG agrees with these recommendations, which are briefly restated below, and clarifies certain points:

- In females, the uptake and distribution of nanomaterials to different organs is likely to vary considerably during the oestrus cycle and during gestation. The exposure periods for treated animals should take these considerations into account.

- For the postnatal period, developmental toxicity studies should consider the fertility of the offspring as well as the function of other organs (neurological, cardiovascular, immune).

- Reproductive and developmental toxicity studies should follow the OECD guidelines as far as possible.

- Studies on the passage of nanomaterials through biological barriers (placenta, testicular barrier) should be carried out beforehand, during ADME studies, using sensitive analytical methods able to quantify nanoscale materials and differentiate them from non-nanoscale materials.

3.2.5.4.5.   Carcinogenicity

Nanomaterials are liable to induce carcinogenic effects due to their structure and their potential to interact with DNA and/or the mitotic apparatus, generate inflammatory reactions and accumulate in certain organs. Some nanomaterials have been classified as potential carcinogens by inhalation. The International Agency for Research on Cancer (IARC) has classified Mitsui-7 multi-walled carbon nanotubes (MWCNT) (Mitsui, Tokyo, Japan) as possibly carcinogenic to humans (Group 2B). Some studies show that while the probability of exhibiting carcinogenic activity depends on the composition of the material, other physico-chemical characteristics (e.g. size, morphology) may themselves have a significant influence on the acceleration of the neoplastic process, thereby playing an additional role in the mechanisms of pathogenesis.

There are very few carcinogenesis studies available in the literature, especially by the oral route. However, assessing the carcinogenic potential of a nanomaterial should not be systematic (cumbersome, time-consuming, ethical issues), but should be justified.

Assessing the carcinogenic potential of the nanomaterial is only required if tissue damage associated with possible carcinogenic effects (inflammation, proliferation, etc.) is observed and/or if there is an accumulation of the nanomaterial in systemic organs in 90-day repeated dose toxicity studies or in toxicokinetic tests. Similarly, if there is a significant occurrence of

precancerous biomarkers in organs of the gastrointestinal tract, a carcinogenesis study is also required.

As a reminder, if the nanomaterial belongs to a category (defined after grouping/read-across) for which one or more analogues are known to exhibit carcinogenic activity, then a carcinogenesis study is required. On the other hand, if a prior assessment of genotoxic potential reveals significant intrinsic genotoxic activity in animal studies, because of the very high probability of the nanomaterial's carcinogenic potential, it does not seem worthwhile conducting a complete carcinogenesis study, as the nanomaterial is then considered *de facto* to be possibly carcinogenic.

The investigation of carcinogenic activity can be carried out according to the recommendations of OECD Guideline 451 (2009)[50] or by conducting a combined study according to the recommendations of OECD Guideline 453 (2009)[51] without necessarily adapting it. The study is carried out on one species, usually the rat. Carcinogenicity studies in a second species may be required if the results obtained in the first species are ambiguous (or possibly species-specific), or if observations from other studies suggest possible carcinogenic activity in another species.

- **Outlook**

  o *In vivo* methods

Shorter studies using transgenic animals (including models over-expressing or having an activated oncogene, deficient in the tumour suppressor gene or DNA repair gene, etc.) may be considered. However, these models have not been validated in inter-laboratory trials and there are no guidelines for them, so they can only be carried out for second-line testing.

  o *In vitro* methods

While it may be premature at this stage to consider the total abolition of animal testing, for ethical reasons and in an evolving regulatory context incorporating animal welfare concepts, it is likely that animal testing for risk assessment, priority setting and classification will be replaced by one or more of the methods not involving *in vivo* testing. As most of these alternative methods cannot be used on their own, it will be necessary to incorporate them into a so-called integrated testing strategy (ITS), based on weight-of-evidence methods integrating several independent sources of information (grouping and read-across, thresholds of

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[50] OECD (Organisation for Economic Cooperation and Development), 2009. OECD Guidelines for the Testing of Chemicals. Test No. 451: Carcinogenicity studies. OECD Publishing, Paris, France, 15 pp.
[51] OECD (Organisation for Economic Cooperation and Development), 2009. OECD Guidelines for the Testing of Chemicals. Test No. 453: Combined chronic toxicity/carcinogenicity studies. OECD Publishing, Paris, France, 20 pp.

toxicological concern, exposure-based evidence and computational methods (SAR, QSAR, PBPK) and *in vitro* tests), as well as information on the mode and/or mechanisms of action.

In this context, the cell transformation assay (CTA) has been put forward as a possible alternative to animal models. This test is intended to investigate carcinogenic potential based on experimental evidence that the cellular and molecular processes involved in cell transformation *in vitro* appear to be similar to those supporting carcinogenesis *in vivo*, and occur as a result of a comprehensive cellular response to direct and indirect DNA damage (Combes *et al.* 2007; Corvi and Vanparys 2012; Mascolo *et al.* 2010; Rohrbeck *et al.* 2010; Vanparys *et al.* 2012; Vasseur and Lasne 2012). Technical guidelines for this assay on SHE, BALB/c 3T3 and Bhas 42 cells have been published by the OECD.

The CTA measures the morphological transformation of cells, either as transformed colonies or as foci derived from a single cell. Several models have been developed and implemented since the early 1960s; the three main ones are summarised below:

- Syrian hamster embryo (SHE) cells were the first to be used to set up a model for studying cell transformation *in vitro*. They are normal diploid, metabolically and p53-competent primary cells, with the ability to biotransform xenobiotics, as evidenced by studies with substances requiring metabolic activation (OECD ENV/JM/MONO(2015)18). Recently, the CTA using SHE was presented as a promising tool for the identification of non-genotoxic carcinogenic compounds (Colacci *et al.* 2014).
- The model using BALB/c 3T3 mouse embryonic fibroblasts was the first to be developed using established cell lines. The BALB/c 3T3 CTA can be performed using the standard protocol, which has been validated by the EURL ECVAM and is included in the list of methods for REACh (method B-21), or a protocol modified to reduce the cytotoxicity and improve the specificity of the test (Vaccari *et al.* 1999).
- Bhas 42 cells are derived from BALB/c 3T3 A31-1-1 cells transfected with a plasmid containing the v-Ha-ras gene (Sasaki *et al.* 1988). As these cells express an activated v-Ha-ras oncogene, they are regarded as initiated cells, according to the two-step paradigm of genotoxic carcinogenesis (Sasaki *et al.* 2015).

All CTA models provide an easily detectable criterion for malignant transformation. However, the subjectivity in identifying foci or morphologically transformed colonies has often been indicated as one of the main limitations of the CTA (Corvi *et al.* 2017). Furthermore, one of the main criticisms preventing the use of the CTA as a stand-alone test in a regulatory context is the lack of mechanistic information enabling a better understanding of the key events leading to oncotransformation (Mascolo *et al.* 2018). To overcome this limitation and improve the use of this assay in the integrated carcinogenesis testing strategy, it would be useful to combine it with a transcriptomic approach, to show the molecular steps leading to malignant transformation *in vitro* (Mascolo *et al.* 2018). Similarly, coupling the CTA with DNA methylation analysis for the detection of non-genotoxic carcinogens was recently advocated (Hwang *et al.* 2020).

Furthermore, the decision not to perform carcinogenicity tests on the basis of negative genotoxicity test results creates a regulatory vacuum for the identification of carcinogens acting through a non-genotoxic mode of action (epigenetic carcinogens). Thus, the development of

integrated approaches for testing and assessment (IATA) including a combination of *in vitro* (such as "*omics*") and *in silico* (such as QSARs) methods was recently proposed for assessing non-genotoxic carcinogenic activity (Jacobs *et al.* 2020). Key events involved in adverse outcome pathways (AOPs) have been identified for some cancer types (colon, bladder and breast (Jacobs *et al.* 2020)). Exploring the molecular characterisation of colorectal carcinogenesis using comprehensive systems biology approaches has been seen as promising (Maglietta *et al.* 2012).

3.2.6. Step 4: Risk characterisation of engineered nanomaterials

This scientific and technical guide proposes a tiered methodology to provide the data that are essential for characterising the risk of engineered nanomaterials. The risk characterisation of engineered nanomaterials is based on the same approach as that used for the risk characterisation of conventional substances. This approach is based on the use and comparison of data from exposure and hazard characterisation calculations. As pointed out in the EFSA (2018) guidance document, effectively conducting risk characterisation ideally requires a weight-of-evidence assessment to be performed in order to evaluate the relevance and quality of the data collected at each step of the risk assessment. A critical analysis of approaches to assessing levels of evidence at the hazard identification step was published by ANSES in 2016[52].

3.3. Conclusions and recommendations of the WG on "Nano and Food" and the CES ERCA

3.3.1. Conclusions

The WG on "Nano and Food" proposes a scientific and technical guide describing a risk assessment methodology tailored to engineered nanomaterials used in food. This document is not intended to be a guideline or regulation. In 2018, EFSA proposed a guide on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. The main risk assessment concepts proposed by the EFSA Scientific Committee and ANSES are similar; however, some methodological differences have been highlighted within this document and are summarised in the table below.

Topic	EFSA 2018	ANSES, 2021	Rationale
Definition of nanomaterials	The EFSA guide considered different existing definitions such as: the definition of engineered nanomaterial (Regulations (EU) 2015/2283 and (EU) No 1169/2011) and the Commission Recommendation on the	In its previous opinion published in 2020, the WG established its own classification of the term engineered nanomaterial (see Section 3.2.2). This classification has no regulatory value.	In order to consider all food additives with a nanoscale fraction and/or typical nanoscale properties, the WG proposed its own classification. In particular, the concepts of

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[52] ANSES Request no. 2015-SA-0089: Opinion on the progress report on the assessment of the weight of evidence at ANSES: critical literature review and recommendations at the hazard identification stage

	definition of nanomaterial (2011/696/EU).		intentionality and 50% particle number threshold were not considered relevant to the work of the WG.
Measurement of particle size	EFSA proposed the use of different techniques for measuring size, one of which was systematically electron microscopy (EM). If EM observation is not possible, alternative imaging techniques can be used. Other techniques such as dynamic light scattering (DLS) and centrifugal liquid sedimentation (CLS) were also suggested.	The WG believes that only electron microscopy (EM) should be used for measuring the size of the constituent particles of engineered nanomaterials. The WG recommends that EM observations be systematically coupled with energy dispersive x-ray (EDX) analysis, in order to limit the observation of artefacts.	The WG recommends the use of EM as being the most suitable and most robust technique for determining the size and morphology of the constituent particles. EM observations can also be used to distinguish between isolated particles and those embedded in aggregates and agglomerates.
Nanomaterial dissolution in gastrointestinal tract conditions	EFSA considered that if less than 12% by mass of the starting material was found in nanoparticle form after 30 minutes of incubation in conditions mimicking those of the gastrointestinal tract, then the HRA did not require the implementation of a "nanospecific" approach.	The WG did not adopt the 12% threshold proposed by EFSA. The WG proposed two alternative analytical strategies (see Section 3.2.3) for assessing the presence of particles via an EM-EDX approach after 2 h of incubation in pH conditions covering the extreme values observed in the gastrointestinal tract (pH1 and 7).	Determination of the 12% mass threshold and the incubation time was based on the concepts of half-life and time to pass through the intestinal barrier. The WG believes that the half-lives of nanomaterials and the time to pass through the intestinal barrier are highly variable due to their different physico-chemical characteristics and the surrounding environment.
Nanomaterial dissolution in lysosomal conditions, persistence and intracellular accumulation	In the same way as for the gastrointestinal tract, a dissolution threshold (12% by mass after 72 h of incubation) was also proposed in the lysosomal compartment. Below this threshold and in the absence of any observed toxicity in the battery of *in vitro* tests proposed, continuation of the nanospecific HRA is no longer necessary.	The WG did not consider dissolution phenomena in lysosomal conditions in its nanospecific HRA methodology.	Dissolution phenomena in lysosomal conditions were not adopted because: the analysis methodology is not well documented, organelles other than the lysosome may be exposed to the nanomaterials, and other cellular internalisation pathways may be involved, as well as other translocation pathways that do not justify internalisation in the cells (paracellular pathway)
Identification and quantification of nanomaterials in food	Screening for nanomaterials in food was applied at an early stage of the methodology to confirm or rule out the implementation of a nanospecific	Screening for nanomaterials and their dissolution in food were not considered as decision-making criteria	The presence of nanoscale particles in food depends mainly on the physico-chemical properties and complexity

	HRA. This screening was also performed for calculating exposure levels.	for the implementation of a nanospecific HRA. The WG believes that the presence of nanomaterials in food can be deduced early and quickly in Steps 1 and 2 proposed in this document.	of the food matrices. In view of this complexity and the multiplicity of engineered nanomaterials used in food, screening for nanoscale particles in food cannot be exhaustive.
Hazard identification and characterisation	The hazard identification and characterisation methodologies proposed by EFSA and ANSES are similar and are based on a tiered approach. The results of the *in vitro*, toxicokinetic and subchronic studies will determine whether further in-depth studies are needed. The main differences identified by the WG are summarised below: • The step of persistence and intracellular accumulation (dissolution in lysosomes) was not adopted by the WG (see explanations above). • The implementation of carcinogenicity studies in the EFSA guide was not clearly explained. The WG proposes systematically screening for precancerous biomarkers in the gastrointestinal tract during the 90-day subchronic study. Independently of systemic exposure, local adverse effects in the gastrointestinal tract may occur via non-genotoxic mechanisms. The early and systematic observation of precancerous biomarkers therefore determines whether further carcinogenesis studies are needed. • In its guide, EFSA mentions the possibility of conducting studies on identifying the effects of nanomaterials on microbiota. These studies are not, at this stage, adopted by the WG. Indeed, there are currently no representative guidelines or protocols for assessing the complexity of the microbiota as mentioned in the EFSA guide. Moreover, the difficulties in interpreting the results mean it is impossible to use this type of study effectively within an HRA. Studies have already been carried out on the interaction of certain nanomaterials (titanium dioxide[53], zinc oxide[54], silver[55]) with the bacteria of the intestinal flora (internalisation, bacterial growth, phylogenetic composition, etc.) and intestinal mucus. Although some of these studies were considered in the ANSES opinion of 2019 on the updating of E171 toxicological data, they were not conducted *in vivo* but on *in vitro* test systems including a few bacterial strains that do not represent the completeness or complexity of the human gut microbiota.		

3.3.2. Recommendations and outlook

Through this scientific and technical guide, the WG sought to propose pragmatic concepts and methodologies applicable to the context of risk assessment. However, some methodological and technical challenges still need to be addressed regarding the physico-chemical characterisation of engineered nanomaterials, the determination of consumer exposure levels to these nanomaterials, and the identification and characterisation of their hazards.

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[53] Z. Chen, S. Han, D. Zhou, S. Zhoub, G. Jia. Effects of oral exposure to titanium dioxide nanoparticles on gut microbiota and gut-associated metabolism in vivo. Nanoscale, 2019,11, 22398-22412.

[54] T. Xia, W. Lai, M. Han, M. Han, X. Ma, L. Zhang. Dietary ZnO nanoparticles alters intestinal microbiota and inflammation response in weaned piglets. Oncotarget, 2017;8(39):64878-64891.

[55] L.A. Wilding, C. M. Bassis, K. Walacavage, S. Hashway, P. R. Leroueil, M. Morishita, A. D. Maynard, M. A. Philbert, I. L. Bergin. Repeated dose (28 day) administration of silver nanoparticles of varied size and coating does not significantly alter the indigenous murine gut microbiome. Nanotoxicology. 2016; 10(5): 513–520.

Recommendations on physico-chemical characterisation

Recommendations on the physico-chemical characterisation of nanomaterials were formulated in the ANSES opinion published in 2020 and are reiterated below.

Briefly, the WG points out that electron microscopy is currently the most suitable technique for determining the size and morphology of nanomaterials. However, this observation technique does not have the same advantages as dynamic light scattering (DLS) or single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) in terms of throughput. The technique therefore needs to be optimised, particularly concerning image acquisition and processing, in order to adapt electron microscopy to routine measurements.

While electron microscopy is the only method currently recommended by the WG, the WG emphasises the existence of initiatives involving artificial intelligence to automate the analysis and cross-checking of data from different techniques to ensure metrological robustness. These initiatives could eventually make it easier to characterise nanomaterials. The WG encourages work to achieve this objective.

The WG incorporated the dissolution properties of nanomaterials in gastrointestinal tract conditions into its risk assessment methodology. Although exploratory studies mimicking physiological conditions of varying degrees of complexity have already been published, there are currently no standardised test conditions for assessing the dissolution of these nanomaterials in gastrointestinal tract conditions. In this context, the WG recommends that work be undertaken to develop a reference method or methods suitable for studying nanomaterials in gastrointestinal fluids.

Biomolecules found in food or biological fluids are able to adsorb on the surface of nanomaterials to form a coating (or corona). This corona can modify the surface charge and chemistry of the particles and can therefore have an impact on the *in vivo* fate of the nanomaterials. The WG therefore recommends that studies be performed to characterise corona formation on the surface of nanomaterials.

Recommendations on calculating exposure levels

Calculations of consumer exposure to the nanoscale fraction of engineered nanomaterials consider different parameters, such as the consumption levels of the different food categories, the concentration levels of the nanomaterials within these categories, and lastly the mass fraction of particles in the nanoscale.

Concerning consumption data, the FAIM software used to determine the levels of exposure to food additives takes into account French data from the INCA2 studies. The WG recommends that in future, the data updated during the INCA3 study be used.

Regarding the measurement of concentration levels of nanomaterials in food, the WG points out that nanomaterials have complex physico-chemical properties that require the use of suitable protocols and analytical techniques. Prior to food additive quantification and/or particle observation, the step of extracting the nanomaterials from their food matrices presents certain difficulties. Extraction protocols should therefore be developed, optimised and adapted to the type and complexity of the food matrices. For example, studies have already been carried out on the extraction of nanomaterials in alkaline media (Ojeda *et al.* 2020), by enzymatic hydrolysis (Taboada-López *et al.* 2019) or via the use of magnetic particles (Luo *et al.* 2020).

The mass fraction of particles in the nanoscale should be determined on the basis of representative sampling, which should take into account, for the same nanomaterial, the variability of the different batches available on the market. As there are different calculation methodologies for the mass fraction, the WG recommends a harmonised approach for calculating the mass fraction of particles in the nanoscale.

The WG also recommends implementing measurement campaigns for engineered nanomaterials used as food additives in the food categories identified in the first phase of the expert appraisal in response to this formal request[56].

Recommendations on hazard identification and characterisation

Various developments and outlooks have been suggested by the WG throughout this guide, in particular concerning hazard identification and characterisation. This is because advances in knowledge and analytical methods, the need to reduce animal testing, the spread of predictive and mechanistic toxicology, and the development of tools and equipment offering high-throughput analysis present new opportunities that will lead to changes in risk assessment methodologies in the coming years.

Some toxicological assessments were not included in this guide, in particular studies on gut microbiota (see details in the table in the conclusion section). In order to include the potential effects of nanomaterials on the gut microbiota in a nanospecific risk assessment methodology, a significant amount of research is required. This work should make it possible to optimise and harmonise tests in order to adapt them to assessing the effects of nanomaterials on the gut microbiota. In parallel, it will also be necessary to better understand the influence of microbiota changes (i.e. dysbiosis) on human health and to improve the processing of the masses of data from such studies[57]. Nevertheless, the 90-day studies of subchronic exposure to nanomaterials proposed by the WG can provide knowledge on these substances' impact on the composition and metabolic activity of the gut microbiota (analysis of the composition by 16S sequencing, production of metabolites such as short-chain fatty acids), particularly for nanomaterials with biocidal activities (Lamas *et al.* 2020).

Furthermore, due to the propensity of some nanomaterials to pass through the placenta and accumulate in the meconium (i.e. the first stool of the newborn), reflecting *in utero*

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[56] https://www.anses.fr/fr/system/files/ERCA2016SA0226Ra.pdf
[57] The need for microbiota studies is not specific to the case of nanomaterials

contamination in humans (Aengenheister *et al.* 2021; Guillard *et al.* 2020), developmental toxicology studies in animals could also include analysis of the gut microbiota of offspring exposed *in utero*. Such information could support new nanospecific risk assessment processes to address concerns about potential adverse effects on offspring, especially given the importance of bacterial-immune cell interactions for the development and maturation of immune functions, among other physiological functions (Lamas *et al.* 2020).

Lastly, there are currently no epidemiological data for the specific case of the oral route. The EPInano system supported by *Santé Publique France* was set up in 2014 for the epidemiological surveillance of workers potentially exposed to engineered nanomaterials. The WG believes that it would be worth drawing on the establishment of such a scheme as a basis for future epidemiological studies of consumer exposure to engineered nanomaterials via the oral route.

4. AGENCY CONCLUSIONS AND RECOMMENDATIONS

The French Agency for Food, Environmental and Occupational Health & Safety endorses the conclusions and recommendations of the WG on "Nano and Food" and the CES ERCA.

This opinion is the result of the second phase of ANSES's expert appraisal on the study of nanomaterials in food. The first phase of the expert appraisal (ANSES's 2020 opinion) investigated the debates and controversies related to the use of nanomaterials, the identification of engineered nanomaterials used as food additives and technological additives in food contact materials, and the identification of food categories that may contain these nanomaterials. Some initial methodological information for guiding the risk assessment towards a standard or nanospecific approach was also proposed.

This second opinion includes a scientific and technical guide whose objective is to propose a risk assessment methodology tailored to the nanoscale fraction of engineered nanomaterials used as food additives. This nanospecific risk assessment is therefore applicable only to oral exposure and consists of several steps in the following sequence: identification of engineered nanomaterials, characterisation of their dissolution properties, calculation of exposure levels, identification and characterisation of the hazard and lastly characterisation of the risk. With regard to characterisation of the hazard, this guide highlights some major methodological challenges (control of dispersion, organ dosimetry, relevance of certain established protocols, etc.) and discusses the results that can be obtained with different experimental protocols currently in use.

In 2018, EFSA proposed a technical guide on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. The methodology developed by the WG on "Nano and Food" is established on the same foundations as those developed by the EFSA Scientific Committee: the results of systematic *in vitro*, toxicokinetic and subchronic toxicity studies determine whether further in-depth and targeted toxicological tests are needed. However, through this guide ANSES proposes different parameters for certain aspects: the applicability of the proposed European regulatory definition, particle size measurements, dissolution properties, calculations of exposure to the nanoscale fraction and certain hazard identification parameters. With regard to the proposed definition, the Agency

also emphasises that it responded to the public consultation launched by the European Commission in anticipation of a possible revision, insisting on the need for it to change. An opinion is being prepared for 2022 to clarify the position that ANSES defended in this consultation.

The next phase of the expert appraisal will consist in applying the methodology established by this opinion in order to carry out, on the basis of the available data, a nanospecific risk assessment of the engineered nanomaterials identified during the first phase of the expert appraisal (ANSES 2020 opinion). About this subject, it should be noted that in 2021, in the context of an updated risk assessment based on new data, EFSA formulated an opinion[58] stating that titanium dioxide can no longer be considered safe when used as a food additive, mainly because genotoxic effects cannot be ruled out.

Pending finalisation of the expert appraisal and given the uncertainties about the risks of nanomaterials in food, ANSES reiterates and updates the recommendations made during the various expert appraisals on this subject since 2006, namely the need to:

• develop analytical and toxicological methodologies tailored to assessing the health risk of nanomaterials;

• limit the exposure of workers, consumers and the environment as part of a gradual approach, in particular by promoting the use of safe products that do not contain nanomaterials and are equivalent in terms of function, effectiveness and cost;

• strengthen the traceability of consumer products containing nanomaterials, essential for risk assessment work. ANSES calls for improved reporting via the national R-nano portal, in order to ensure a better description of the nanomaterials placed on the market, their uses and the associated exposure.

A risk assessment tailored to the nanoscale fraction of engineered nanomaterials, especially those used as food additives, raises methodological and technical challenges that need to be addressed. The Agency stresses the need to conduct physico-chemical characterisation studies as soon as possible, using electron microscopy approaches, for the suspected engineered nanomaterials identified in the first phase of the expert appraisal. This physico-chemical characterisation (in particular the measurement of size and particle size distribution) is an essential step for assessing the risk of these nanomaterials.

Similarly, ANSES supports the WG's recommendations on the standardisation of test conditions for physico-chemical characterisation, exposure calculations and toxicological studies with a view to generating suitable, usable data for assessing the risk of engineered nanomaterials.

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[58] Safety assessment of titanium dioxide (E171) as a food additive; EFSA Journal 2021;19(5):6585.

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ANNEX 1

Presentation of the participants

FOREWORD: The expert members of the Expert Committees and Working Groups or designated rapporteurs are all appointed in a personal capacity, *intuitu personae*, and do not represent their parent organisation.

WORKING GROUP ON "NANO AND FOOD"

Chair

Mr Fabrice NESSLANY – Manager of the Toxicology Department – *Institut Pasteur* in Lille – toxicology, genotoxicity and risk assessment.

Vice-Chair

Mr Emmanuel FLAHAUT – Research Director at the National Centre for Scientific Research (CNRS) – physical chemistry, ecotoxicology.

Members

Ms Anna BENCSIK – Research Director at ANSES – neurotoxicology, *in vivo* nanotoxicology, histopathology.

Ms Marie CARRIERE – Researcher at the French Alternative Energies and Atomic Energy Commission – nanotoxicology, cell biology, physical chemistry.

Mr Patrick CHASKIEL – Teacher and Researcher at Paul-Sabatier University, Toulouse III – economics, communication sciences. Mr CHASKIEL participated in the WG from June 2017 to June 2019.

Mr Laurent DEVOILLE – Research Engineer at the National Metrology and Testing Laboratory – physics, metrology.

Mr Fernand DORIDOT – Teacher and Researcher at the *Institut Catholique des Arts et Métiers* in Lille – history and philosophy of science and technology, governance of technology.

Ms Valérie FESSARD – Head of the Toxicology of Food Contaminants Unit at ANSES – *in vivo* toxicology.

Mr Eric HOUDEAU – Research Director at the National Institute for Agricultural Research (INRA) – physiology, food toxicology.

Mr Olivier JOUBERT – Teacher and Researcher at the University of Lorraine – molecular biology, biotechnologies, toxicology.

Ms Stéphanie LACOUR – Research Director at CNRS – law, regulations and control of new technologies. Ms LACOUR participated in the WG from June 2017 to June 2019.

Mr Stéphane PEYRON – Lecturer at the University of Montpellier – physical chemistry, food contact materials.

Ms Odette PRAT – Retired – Former Research Officer at the French Alternative Energies and Atomic Energy Commission – toxicogenomics.

Ms Marie-Hélène ROPERS – Research Officer at INRA – physical chemistry, nanomaterials in food.

EXPERT COMMITTEE

CES on "Assessment of chemical risks in food"

Chair

Mr Bruno LE BIZEC – University Professor, ONIRIS. Analytical chemistry and risk assessment.

Vice-Chairs

Mr Fabrice NESSLANY – Manager of the Toxicology Department – *Institut Pasteur* in Lille. Toxicology, genotoxicity and risk assessment.

Ms Karine TACK – Laboratory Manager, IRSN. Analytical chemistry.

Members

Mr Claude ATGIE – University Professor, Bordeaux-INP. Toxicology.

Mr Pierre-Marie BADOT – University Professor, Bourgogne Franche-Comté University. Contaminant transfer.

Ms Martine CLAUW – University Professor, National Veterinary School of Toulouse. Toxicology.

Ms Marie-Yasmine DECHRAOUI – Researcher in environmental toxicology. Biotoxins and environmental toxicology.

Mr Nicolas DELCOURT – Lecturer and Hospital Pharmacist, University of Toulouse. Clinical toxicology, biochemistry and neuroscience.

Ms Christine DEMEILLIERS – Lecturer, Grenoble-Alpes University. Toxicology, nutrition.

Mr Erwan ENGEL – Research Director, INRA. Analytical chemistry.

Mr Jérôme GAY-QUEHEILLARD – Lecturer, University of Picardie Jules Verne. Digestive impacts and metabolism.

Mr Petru JITARU – Head of Unit, ANSES, Laboratory for Food Safety. Analytical chemistry.

Ms Sonia KHIER – Lecturer, University of Montpellier. Pharmacokinetics.

Ms Emilie LANCE – Lecturer, University of Reims – expertise in biotoxins and ecotoxicology.

Ms Caroline LANIER – Lecturer, University of Lille – expertise in health risk assessment and food risk management.

Ms Raphaële LE GARREC – Lecturer, University of Western Brittany – expertise in toxicology, biotoxins and regulations.

Mr Ludovic LE HEGARAT – Deputy Head of Unit – ANSES, Fougères Laboratory. Predictive toxicology and genotoxicology.

Mr Nicolas LOISEAU – Research Officer, INRA. Metabolic biochemistry, chemistry and molecular biophysics.

Mr David MAKOWSKI – Research Director, INRA. Agronomy, statistics and modelling.

Mr Eric MARCHIONI – University Professor, Strasbourg University. Analytical chemistry.

Mr Jean-François MASFARAUD – Lecturer, University of Lorraine. Ecotoxicology and health risk assessment.

Mr César MATTEI – Lecturer, University of Angers. Toxicology.

Mr Alain-Claude ROUDOT – University Professor, University of Western Brittany. Mathematical modelling and exposure assessment.

Mr Yann SIVRY – Lecturer, University of Paris Diderot/IPGP. Environmental pollution and nanoparticles.

Ms Paule VASSEUR – Emeritus University Professor. Expertise in toxicology.

ANSES PARTICIPATION

Scientific coordination

Mr Bruno TESTE – Scientific Project Leader, Food Risk Assessment Unit (UERALIM) – ANSES.

Scientific contribution

Ms Eleni ANASTASI – EU-FORA Fellow (State General Laboratory of Cyprus/ANSES).

Mr Moez SANAA – Head of Unit, Food Risk Assessment Unit (UERALIM), ANSES.

Administrative secretariat
Ms Angélique LAURENT – ANSES.