MUT/2021/05

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Guidance on Genotoxicity Testing Strategies for Manufactured Nanomaterials

Background

1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) has a remit to provide UK Government Departments and Agencies with advice on the most suitable approaches to testing chemical substances for genotoxicity. The COM views regarding the most appropriate strategy for genotoxicity testing are outlined in full in the COM (2021) "*Guidance On A Strategy For Genotoxicity Testing Of Chemical Substances*".

2. In brief, the COM recommend a staged approach to genotoxicity testing. **Stage 0**, in the absence of test data from adequately designed and conducted genotoxicity tests, consists of preliminary considerations of the test chemical substance, including, physico-chemical properties, Structure Activity Relationships (SAR), and information from screening tests. **Stage 1** consists of *in vitro* genotoxicity tests that provide information on three types of genetic damage (namely, gene mutation, chromosomal damage and aneuploidy) and gives appropriate sensitivity to detect chemical genotoxins. **Stage 2** consists of *in vivo* genotoxicity tests which are chosen on a case-by-case basis to address any genotoxic endpoints identified in Stage1; investigate genotoxicity in tumour target tissue(s) and/or site of contact tissues; investigate potential for germ cell genotoxicity; and investigate potential genotoxicity for chemicals where high/moderate and prolonged exposure is anticipated, even if negative in Stage 1.

3. As part of an update of the full COM guidance (COM, 2021), a number of additional topics have been included for consideration. One such area addresses genotoxic testing strategies for manufactured nanomaterials (NMs). A summary of the COM opinion based on currently available information is provided in the paragraphs below. It is recognised by the Committee that this is a rapidly developing area and updates will be carried out as new information becomes available.

4. Nanomaterials generally refer to manufactured materials with one or more dimensions in the nanometer size range, most commonly between 1 and 100 nm (a diversity of definitions exists). They present challenges in safety evaluation owing to their small size, relatively large surface area, tendency for aggregation and unknown disposition in biological systems.

Evaluation of genotoxicity testing methodologies

1. A number of projects and initiatives have been, or are currently being, conducted in an attempt to evaluate and harmonise methodologies to assess the genotoxicity of nanomaterials. These are briefly described below:

- OECD nanomaterial research has been conducted through the Working Party on Manufactured Nanomaterials (WPMN) and the Working Group of National Coordinators of the Test Guidelines Programme (WNT). OECD research has focused on the development of regulatory test guidelines and guidance on nanosafety to support the regulatory safety needs of member countries (Steinhäuser *et al.*, 2017; OECD, 2018).
- In its 7th Framework Programme (EU FP7) the EU initiated the NanoSafetyCluster (NSC), including the Nanogenotox and NANoREG projects.
    - The NANOGENOTOX Joint Action (JA) collaborative project was completed in 2013. It was coordinated by ANSES and involved 16 associated partners and 15 collaborating partners from across Europe (ANSES, 2013c; OECD, 2014).
    - In the NANoREG project, over 85 institutional partners from EU member states, associated states, the Republic of Korea and Brazil collaborated in developing reliable, reproducible and relevant methods for testing and assessing the effects of nanomaterials on human health and the environment in a regulatory context. The project ended in 2017 and all results are available via the NANoREG Results Repository[1].
- Under EU Horizon 2020, the successor to EU FP7, the Dutch National Institute for Public Health and the Environment (RIVM) carried out the ProSafe project. Finishing in 2017, this collaborative project supported the review of regulatory relevant results from NANoREG, the OECD WPMN and other projects of the NanoSafety Cluster funded within EU FP7. A white paper was produced with recommendations for policy makers and regulators (ProSafe, 2017; Prosafe/OECD, 2017).

5. A full list of completed (and on-going projects) is available at the EU Nano Safety Cluster (https://www.nanosafetycluster.eu/).

6. The following sections outline COM's current understanding of the applicability of using standard *in vitro* and *in vivo* genotoxicity testing assays to assess the genotoxicity potential of NMs. Information provided draws on the critical review of published primary data evaluating the potential genotoxic effects of NMs using standard genotoxicity testing assays from the working group of the International Life Sciences Institute (ILSI) Genetic Toxicology Technical Committee (GTTC) (Elespuru *et al.,* 2018).

[1] (https://www.rivm.nl/en/about-rivm/mission-and-strategy/international-affairs/international-projects/nanoreg)

Applicability of *in vitro* assays for genotoxicity testing to nanomaterials

Bacterial (Ames) genotoxicity assay

7. The bacterial reverse mutation test (Ames test) (OCED TG 471) uses amino acid-requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, involving substitution, addition or deletion of DNA base pairs. The test is relatively easy to perform, is rapid and inexpensive, and is often used as an initial screen of genotoxicity - in particular, to detect point mutation-inducing activity. Extensive data have shown that many chemicals that are Ames-positive are also positive in other genotoxicity tests such as micronuclei tests. There are, however, some mutagenic chemicals that are not detected by the Ames test, mainly due to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability between bacterial and mammalian cells (OECD, 2020).

8. It is widely recognised that the standard bacterial Ames assay is not an adequate component of a genotoxicity testing strategy for NMs (Doak et al., 2012; OECD, 2014; ECHA, 2017; Elespuru et al., 2018). Modifications of the standard assay have been evaluated, including preincubation, extended exposure durations and concentrated exposures. However, these did not change the negative outcome for the NMs tested (for example, Butler et al., 2015).

9. It is considered that the gram-negative strains of bacteria used in the Ames assay may not be able to take up NMs as they lack the mechanisms of endocytosis, pinocytosis and phagocytosis present in mammalian cells. In addition, it is also possible that bacterial cells do not have the same type of response that causes positive effects of NMs in mammalian cells.

10. COM is in agreement with the conclusion reached by Elesperu *et al.* (2018) and other authoritative bodies that the bacterial assays using *S. typhimurium* and *E. coli* tester strains appear not to take up or respond to NMs and that previously reported negative results using these tests may not be definitive. Although bacterial assays may be useful in assessing soluble genotoxic agents released from NMs, overall, the use of an Ames assay is not recommended to assess the mutagenic potential of NMs due to their insoluble nature and the inability of bacteria to take up NMs, thus potentially leading to false negative results.

In vitro mammalian micronucleus assay

11. The *in vitro* micronucleus (MN) assay aims to detect micronuclei in the cytoplasm of interphase cells following exposure to the test substance, which represents DNA damage that has been transmitted to daughter cells. Both aneugens and clastogens can be detected in cells that have undergone cell division during or after exposure to the test chemical. Micronuclei may originate from acentric chromosome fragments, or from whole chromosomes that are unable to migrate to the spindle poles during cell division. Hence, the *in vitro* MN test is a comprehensive test for investigating the chromosome damaging potential of a test substance. To analyse

the induction of micronuclei, it is essential that mitosis has occurred in both treated and untreated cultures. The OECD TG (487) allows for the test to be carried out with or without cytokinesis block, providing there is evidence that mitosis has occurred in the cells being analysed (OECD, 2016a).

12. Micronuclei may also arise from lagging chromosomes[2] and the OECD TG 487 assay also allows the detection of substances that induce aneuploidy that are otherwise difficult to study using conventional chromosomal aberration tests (e.g. OECD TG 473). TG 487 specifies that techniques such as Fluorescence *In Situ* Hybridization (FISH) should be used to differentiate between substances inducing changes in chromosome number and/or ploidy from those inducing clastogenicity (OECD, 2018a). FISH can also provide additional information on the mechanisms of chromosome damage and MN formation (OECD, 2016a).

13. The *in vitro* MN assay is a component of the standard genotoxicity testing strategy, and it has been extensively documented how the test could be adapted for the evaluation of NMs, including the use of different cell lines, different treatment times and co-exposure with cytochalasin B. There is considerable variation in approaches used to date, particularly in terms of the chosen cell line, which does not allow for comparison and identification of trends.

14. The 3D human reconstructed skin micronucleus assay (RSMN) has been developed to assess the genotoxicity of dermally applied compounds incorporated into cosmetics, utilising two highly differentiated *in vitro* models of the human epidermis, EpiDerm™ and Episkin LM™. A global validation of the EpiDerm™ model has been carried out and a submission has been made to the OECD to include this assay into the Test Guideline programme. More information on these assays is included in the COM *Guidance on the use of 3D Tissue Models for genotoxicity testing* (COM, 2021).

15. COM is in agreement with the conclusion reached by Elesperu *et al.* (2018) and other authoritative reviews (for example, Doak et al., 2012) that a modified *in vitro* MN assay should be a recommended component of a toxicity testing battery for NMs. As the inclusion of a cytokinesis block can inhibit the uptake of NMs, it is advised that cytokinesis block should be applied following NM exposure, and cytotoxicity should be determined in parallel (SCCS, 2012; OECD, 2014). In the absence of a standardised assay, it is recommended that standard cell lines with suitably low background MN frequencies and stable genetic backgrounds are used, with an exposure time that allows at least one complete cell cycle to ensure direct contact of the NM with cellular DNA during mitosis.

In vitro chromosomal aberration assay

16. The *in vitro* chromosomal aberration test (OECD TG 473) aims to identify substances that cause structural chromosomal aberrations in cultured mammalian

[2] Lagging whole chromosomes are those that are not included in the daughter nuclei produced by mitosis due to incorrectly attaching to the spindle during the segregation of chromosomes in anaphase.

cells. Following exposure to the test chemical, the cells are treated with Colcemid® or colchicine to induce metaphase arrest after which cells are analysed for chromosomal aberrations. Two types of structural aberrations are seen, namely chromosome or chromatid aberrations. Although polyploidy[3] can also arise in chromosome aberration assays *in vitro,* alone this does not indicate aneugenic potential and could simply indicate cell cycle perturbation or cytotoxicity (OECD, 2016b).

17. Elespuru *et al.* (2018) concluded that cytogenetic damage is an important genotoxicity endpoint ,and the chromosome aberration assay was recommended for incorporation into a test battery. The authors highlighted that scoring of chromosomal aberrations should be undertaken by an experienced person and aberrations characterised according to typical categories (e.g., chromatid breaks), with chromatid and chromosome gaps being noted separately from aberrations. Although it was considered that modification of the assay was not needed for the assessment of NMs, the importance of confirming particle uptake was stressed.

18. COM is in agreement with the conclusion reached by Elesperu *et al.* (2018) that the chromosome aberration assay should be incorporated into a test battery with scoring undertaken by an expert, and although the current test method does not need modification it is important that confirmation of NM uptake is undertaken in parallel.

In vitro Comet assay

19. The Comet assay aims to detect single and double-stranded DNA damage and repair in eukaryotic cells following treatment with the test substance, by measuring the migration of DNA from individual nuclei. The comet pattern observed is considered to be due to the loops containing a break losing their supercoiling properties and becoming free to extend toward the anode. The alkaline version offers greater sensitivity than the standard test and is often the method of choice when low levels of DNA damage are being detected (OECD, 2018a).

20. Modification of the assay using lesion-specific endonucleases (e.g. formamido-pyrimidine-DNA glycosylase (Fpg) and endonuclease III (EndoIII, also known as Nth), can enable the detection of different oxidised and alkylated bases as well as cyclobutane pyrimidine dimers, mis-incorporated uracil and apurinic/apyrimidinic sites. Adding further enzymes to the Comet assay toolbox could potentially increase the variety of DNA lesions that can be detected. The enzyme-modified Comet assay can play a crucial role in the elucidation of the mechanism of action of both direct and indirect genotoxins, thus increasing the value of the assay in the regulatory context (Muruzabal *et al.,* 2021).

21. In their review, Elespuru *et al.* (2018) reported that the *in vitro* NM studies conducted to date have used either the standard alkaline Comet assay or the Comet assay with addition of Fpg) to provide evidence of the presence of oxidative lesions.

[3] Numerical chromosomal aberrations in cells or organisms involving entire set(s) of chromosomes, as opposed to an individual chromosome or chromosomes (aneuploidy).

Some of the NM studies compared both methods, which allows the proportion of strand breaks caused by oxidative damage to be estimated. However, due to the large variability in cell lines, concentrations, exposure time and dispersion protocols used, Elespuru *et al.* reported that comparison between studies was not possible.

22. COM is in agreement with the conclusion reached by Elesperu *et al*. (2018) regarding the uncertainty of using the current non-standardised *in vitro* Comet assay as a screening assay for NM genotoxicity and hence do not currently recommended the assay for screening of NMs.

In vitro mammalian gene mutation assay

23. The *in vitro* mammalian cell gene mutation test aims to detect gene mutations induced by the test substance in cultured mammalian cells through the measurement of forward mutations in reporter genes. The standard assay (OECD TG 490) measures mutations in the endogenous thymidine kinase gene (referred to as TK or Tk for human and rat respectively) in two cell lines: L5178Y TK+/- -3.7.2C mouse lymphoma cell line (generally called L5178Y) and the TK6 human lymphoblastoid cell line (generally called TK6). The assay can detect both gene mutations (point mutations, frame-shift mutations, small deletions, etc.) and chromosomal events (large deletions, chromosome rearrangements and mitotic recombination) (OECD (2016c).

24. The *in vitro* mammalian cell gene mutation test (OECD TG 476) using the hypoxanthine-guanine phosphoribosyl transferase (HPRT; Hprt in rodent cells, HPRT in human cells), and the xanthine-guanine phosphoribosyl transferase (XPRT) reporter genes also detects a range of different mutations. The HPRT assay is the most widely used for regulatory purposes and detects base pair substitutions, frameshifts, small deletions and insertions. Mutations resulting from large deletions and possibly mitotic recombination can also be detected (OECD, 2016d).

25. Both the standard assay and the Hprt assay have been used to evaluate NMs. Elespuru *et al.* (2018) concluded that, overall, the information available was not sufficient to fully evaluate the mutagenicity of NMs in mammalian cells nor draw any conclusions on the relative sensitivity of the various reporter genes to the potential mutagenic action of NMs. The updated REACH annex, as well as papers by Doak et al (2012) and Elespuru et al. (2018) all clearly state that the forward mutation assay should replace the Ames test for the mutagenicity testing of nanomaterials.

26. COM is in agreement with the conclusion reached by Elesperu *et al.* (2018) to recommend the inclusion of an *in vitro* mammalian gene mutation assay in the test battery for NMs. Assay modifications, beyond the general recommendations for the testing of all NMs (see paragraphs 26 – 43), are not currently considered to be needed and the standard protocols for Tk locus (OECD TG 490) and Hprt locus (OECD TG 476) should be followed.

Applicability of *in vivo* assays for genotoxicity testing to nanomaterials

27. There is generally much less data available on the assessment of the genotoxicity of NMs *in vivo*. However, *in vivo* assessments are considered important for assessing genotoxicity of NMs as these reflect the impact of *in vivo* metabolism, toxicokinetics and DNA repair processes, which contribute to the overall genotoxic response. *In vivo* assays are usually undertaken when a positive finding has been detected in in vitro assays (COM, 2021). Elespuru et al. (2018) carried out a highly detailed review of *in vivo* studies used for the assessment of NMs.

In vivo bone marrow MN assay and chromosomal aberration assay

28. The mammalian *in vivo* MN test (e TG, 474) evaluates MN formation in erythrocytes sampled either in the bone marrow or peripheral blood cells of animals (usually rodents). The purpose of the assay is to identify substances that cause cytogenetic damage which results in the formation of micronuclei containing either lagging chromosome fragments or whole chromosomes (OECD, 2016e).

29. The mammalian *in vivo* chromosomal aberration test (OECD TG, 475) detects structural chromosome aberrations in bone marrow cells of animals (usually rodents). Both chromatid- and chromosome-type structural aberrations are detected (OECD, 2016f).

30. Both assays have been used to evaluate the genotoxic potential of a range of NMs. Elespuru *et al.* (2018) concluded that, overall, the data reviewed show that both the *in vivo* MN assay and the *in vivo* chromosome aberration assay can be used in the standard form for evaluating the genotoxicity of NMs. The authors caution that attention needs to be paid to certain mechanistic aspects, including relevant tissue exposure and potential particle overload effects.

In vivo Comet assay

31. The *in vivo* alkaline Comet (single cell gel electrophoresis) assay (or *in vivo* Comet assay) (OECD TG 489) is used for the detection of single and double stranded DNA breaks in cells or nuclei isolated from multiple tissues of animals (usually rodents) that have been exposed to potentially genotoxic material(s) (also see paragraph 19). Breaks occur due to direct interactions with DNA, alkali labile sites or as a consequence of transient DNA strand breaks resulting from DNA excision repair. These strand breaks may be repaired, resulting in no persistent effect, they may be lethal to the cell, or may be fixed into a mutation resulting in a permanent viable change. They may also lead to chromosomal damage (OECD, 2016g).

32. A large number of different types of NMs have been evaluated using the *in vivo* Comet assay. Elespuru *et al.* (2018) concluded that the standard form of OECD TG 489 can be used to assess NMs. However, the authors highlighted the potential artifacts that can occur where residual particles are present during the DNA electrophoresis stage of the assay, the need to consider a relevant exposure route for humans, and the selection of a maximum dose that does not trigger particle overload conditions. In addition, they reported that increased sensitivity can be achieved

through the addition of 8-oxoguanine DNA glycosylase (OGG1) or Fpg glycosylases, which recognise particular oxidative DNA adducts.

In vivo gene mutation assays in transgenic rodents

33. A small number of mutagenicity studies of NMs have also been reported in transgenic rodent (TGR) models with mutation reporter systems including gpt-delta, lacZ and myh$^{-/-}$ mice; F344 and C57BL/6 rats with K-RAS codons 8 and 12. Following review of the data, Elespuru *et al.* (2018) reported that exposure to NMs was associated with a 2 to 3 fold higher positive response over that of background. Differences in response were noted for some NMs between species, suggesting the importance of including different test species in genotoxicity testing of NMs.

34. In addition, where measured, significant shifts were seen in spectra compared with untreated control or background spectra. However, these were substantially lower than the shifts induced with standard mutagenic agents (for example, N-ethyl-N-nitroso urea (ENU) and 7,12-Dimethylbenz[a]anthracene (DMBA)). Elespuru et al. (2018) concluded that additional studies were needed to characterise the shifts detected to see if there was a correlation with the MoA of the NM.

Special considerations for the genotoxicity testing of nanomaterials

35. A number of factors are considered to influence the genotoxicity of NMs including size, surface properties, chemical composition and shape, in addition to dose and exposure duration (Kohl *et al*., 2020). These aspects are considered below.

Physical characteristics

36. NMs are generally grouped into broad categories of materials, including: metals (silver, gold, copper); metal oxides (titanium dioxide, iron oxide, zinc oxide); carbon based NMs (single- or multi-walled carbon nano tubes); and different types of polymers and advanced NMs (complex, hybrid, multi-component or multi-structure NMs) (Kohl et al., 2020).

37. The physical characterisation of NMs in terms of size, shape and inherent properties is recognised as a key step prior to their assessment. Evaluation of the fate of NMs when added to a biological test system is also being recognised as essential, as the conditions used in the test system may not reflect those in which the NMs were characterised. Many studies have been carried out to assess the effects of different media, pH, surface charge, coatings and proteins on the fate, action and toxic properties of NMs. In addition, effects resulting from experimental handling of NMs, and interference with endpoints of widely used assays such as alamarBlue™, Neutral Red or WST-1, have also been reported as issues. It has therefore been recommended that the characterisation of NMs should be carried out under the test conditions in the genotoxicity testing assay (as discussed in paragraph 37) (Elespuru *et al*., 2018; Kohl *et al*., 2020).

Exposure route and dose

38. The route of exposure of humans to NMs is a key consideration when assessing genotoxic potential, and it is essential to provide assurance that the NMs reach the cells in the test system. This can be achieved by characterising the physicochemical properties of the NM under the test system conditions and the behaviour of the NM within the system, particularly whether the NM gets into the cell/nucleus. Should the NM not gain access to the cell, genotoxicity can only occur through the release of genotoxic moieties which have the ability to penetrate the cell.

39. There remains some uncertainty about an "appropriate" dose for the genotoxic testing of NMs. Current OECD TGs for genotoxicity testing generally recommend higher levels than would be typically experienced during human exposure, as this compensates for "sensitive populations, statistically small samples, and extended time of exposure". However, this could lead to "particle overload" and the generation of artefactual positives. As it is not currently possible to address artefact generation within the available assays, Elespuru *et al.* (2018) considered that dose response assessment over a range of doses would be beneficial in risk assessment.

Mode of action (MoA) and secondary toxicity

40. NM genotoxicity has been reported as a result of primary (direct or indirect) or secondary genotoxicity. Primary direct genotoxicity requires physical contact of NMs with DNA in the nucleus and can lead to DNA breaks and other DNA lesions, large DNA malformation, or chromosomal damage. The primary indirect mechanism can arise as a result of reactive oxygen species (ROS) generation following reactions at the surface of the NM or via the release of redox-active transition ions such as Fe2+, Ag+, Cu+, Mn2+, and Ni2+ leading to the production of ROS via the Fenton-type reaction. It has been shown that for some NMs, genotoxicity results from only one of these mechanisms whilst for others both mechanisms can occur simultaneously after NM exposure (Kohl *et al.,* 2020).

41. Secondary genotoxicity is considered to be the major mechanism associated with NMs. This is defined as genetic damage mediated by ROS or reactive nitrogen species (RNS) (and possibly other mediators) produced as a consequence of an oxidative burst from phagocytes, activated as a result of the presence of the NM (Kohl *et al.,* 2020). It has been suggested that secondary genotoxic mechanisms may be best derived using multi-cell type models as they better mimic the in vivo environment (Evans et al., 2019).

42. In their review of published data, Elespuru *et al.* (2018) did not identify common modes of action for the toxicity and genotoxicity of NMs. The conclusions drawn by the authors were as follows:

- Nanomaterials, when positive in a genotoxicity assay, do not generally induce the large increases in genotoxic responses that are characteristic of many classical DNA damaging agents. This might not be surprising, since NMs typically do not interact directly with DNA (i.e., do not involve covalent interactions such as alkylation, or intercalation).

- The observations in this analysis are consistent with the concept that the genotoxicity of most NMs is likely to be indirect, e.g., via generation of oxidative species or indirect consequences of inflammation (Landsiedel et al., 2009; Xia et al., 2013; and many others).
- Another possible mechanism involves direct physical interaction with the spindle apparatus during cell division (Sargent et al., 2010; Siegrist et al., 2014).
- Based on this, it appears appropriate to apply the principles of indirect versus direct genotoxic effects of NMs in risk assessments.

43. At this time, it is not possible to confirm that the oxidative stress MoA is the key mechanism associated with the genotoxicity of NMs, as the evaluation of other mechanisms (for example, perturbation of DNA repair or DNA synthesis) has not been widely investigated. In addition, the lack of Comet assay standardisation has resulted in variable responses in studies of oxidative stress. However, Elespuru *et al*. (2018) note that if the major mechanism of genotoxicity of NMs is indirect, then assessment of the toxicity may be better integrated into a broader evaluation of systems toxicology.

44. Several approaches have been proposed for categorising NMs which may help with understanding their MoA. These include:

- Tox21 high content approach targeting defined pathways of toxicity involving pulmonary inflammation, ROS and membrane effects, as related to the physical and chemical properties of NMs (Goodwin *et al*., 2015).
- Development of a decision-making framework (DF4) that groups and tests NMs through consideration of the intrinsic material and system-dependent properties, biopersistence and uptake and biodistribution, as well as cellular and apical toxic effects derived from *in vitro* studies. Categories of (1) soluble NMs, (2) biopersistent high aspect ratio NMs, (3) passive NMs, and (4) active NMs have been defined (Arts *et al*., 2015; 2016).
- Use of the decision-making framework in combination with “read across” (Oomen *et al*., 2015) which could minimise testing where there is no human relevance.

Current recommend strategy of the COM

45. The COM is in agreement with the strategy proposed by Elespuru *et al*. (2018) for the assessment of the genotoxicity of NMs as shown in Figure 1.

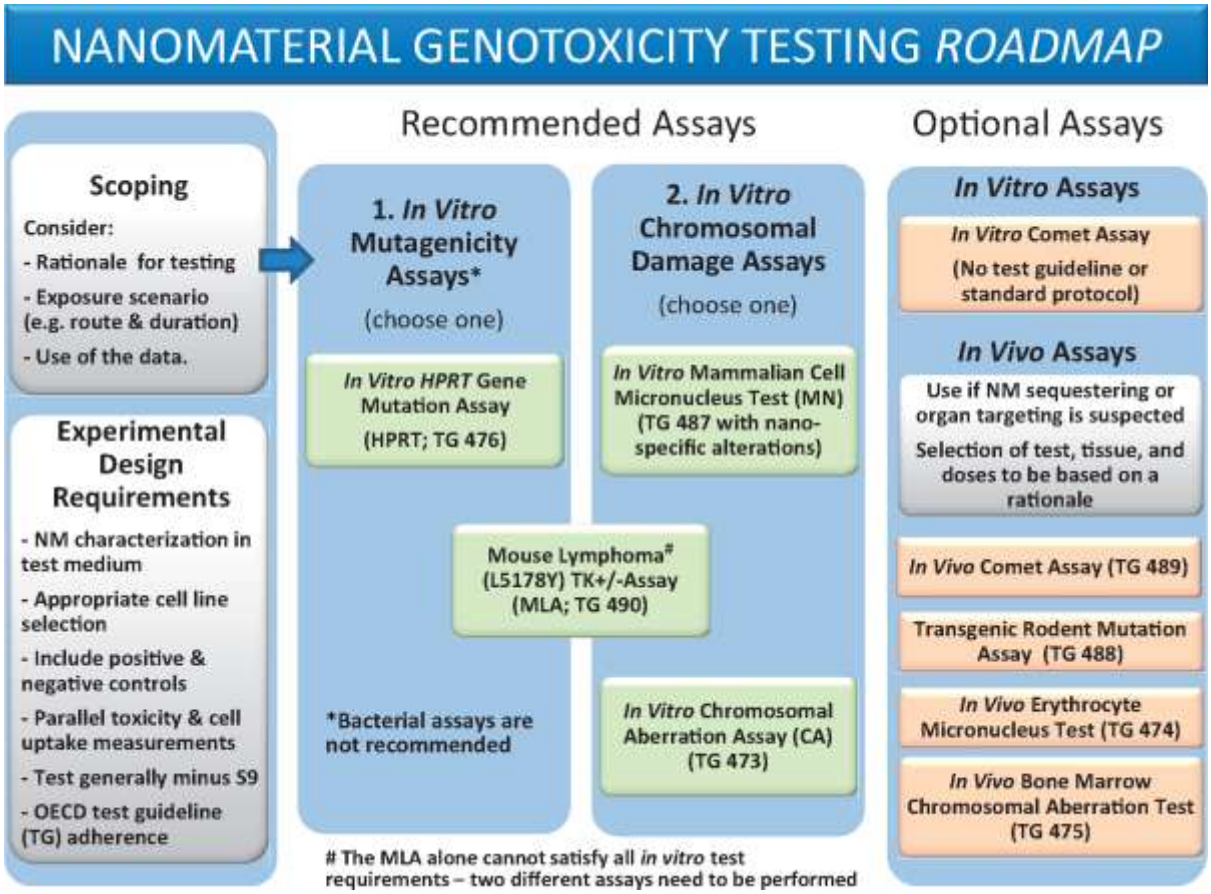

Figure 1: Nanomaterial genotoxicity testing roadmap (Elespuru *et al*., 2018).

46. The recommended battery of tests for the genotoxicity testing of NMs is comprised of two types of assessment, one to detect mammalian gene mutation and the other to detect chromosomal damage.

47. To detect gene mutation, the *in vitro* mammalian mutagenicity assay is used in place of the bacterial mutation assay (AMES; OECD 471), and either the mouse lymphoma (L5178Y) TK6 Assay (MLA) (OECD TG 490) or HPRT gene mutation assay (HPRT) (OECD TG 476) are considered appropriate. These assays are included as they detect similar small scale changes (for example, single base pair changes and frameshifts) as the bacterial assay, but the MLA also provides information on a number of additional broader changes including chromosome rearrangements, deletions (both small and large) and mitotic recombination. The choice of assay will depend on the scale of changes being evaluated; for example, the MLA may be more appropriate for hazard identification of NMs than the HPRT assay.

48. Three *in vitro* assays are recommended to detect chromosome damage, namely the chromosomal aberration assay (OECD TG 473), the MN assay with modification (OECD TG 487) and the MLA (OECD TG 490). These are included as they detect large scale genetic damage affecting chromosomes, particularly breaks, rearrangements, or whole chromosome loss, with the MLA also detecting small scale damage (paragraph 3). The addition of S9 is recommended only where the NM is

comprised of materials likely to be affected by mammalian metabolism (Elespuru *et al*., 2018).

49. Due to the lack of an OECD guideline, the *in vitro* comet assay, which detects DNA strand breaks, is only recommended for inclusion if an assessment of oxidative damaging effects is being undertaken. Consideration needs to be given to analytical handling issues that affect the quantitative endpoint.

50. *In vivo* assays are only recommended where targeting/sequestering of NMs to a specific target organ has been previously demonstrated, or where further information regarding *in vivo* risk is needed. Recommended assays include the *in vivo* Comet assay (OECD TG 489), the *in vivo* transgenic rodent mutation assay (OECD TG 488) and the bone marrow chromosomal aberration test (OECD TG 475). The *in vivo* MN assay is only recommended when evidence has shown that the NM is distributed systemically or a target organ has been demonstrated (Elespuru *et al*., 2018).

Future directions

51. Horizon 2020 projects currently being undertaken include: RiskGONE[4], led by ANSES, on the governance of nanomaterials, especially on *in vitro* genotoxicity and high throughput methods; the NanoSolveIT[5], project, which is developing a validated, multi-scale nanoinformatics integrated approach to testing and assessment (IATA) for assessment of potential adverse effects of NM on human health and the environment; and PATROLS[6], which aims to establish a set of laboratory techniques and computational tools to predict potential human and environmental hazards resulting from engineered nanomaterial exposures. A full list of on-going projects is available at the EU Nano Safety Cluster (https://www.nanosafetycluster.eu/).

52. Gene expression and epigenetics are two new endpoints that have been identified for the genotoxicity testing of NMs. Exposure to NMs has been associated with the de-regulation of genes involved in the DNA damage response and DNA repair, cell cycle progression, oxidative stress and inflammatory responses; some of these also leading to secondary genotoxicity. Kohl *et al*. (2020) proposed that following interaction of cells with NMs (or chemicals), gene expression is regulated through the activation of molecular signaling.

53. Some epigenetic responses have also been proposed as potential biomarkers of exposure to NM, or disease risk following exposure. However, relevant mechanisms for this have yet to be established. Several metallic, non-metallic and carbon-based NMs have been reported to affect epigenetic mechanisms, resulting in epigenetic responses including DNA methylation, non-coding microRNAs (miRNAs), and histone modifications (Kohl *et al*., 2020).

[4] https://riskgone.wp.nilu.no/
[5] https://www.nanosolveit.eu/
[6] https://www.patrols-h2020.eu/about-us/index.php

54. High through-put screening (HTS) methods are defined as “the use of automated tools to facilitate rapid execution of a large number and variety of biological assays that may include several test substances in each assay” (Nel *et al*., 2013). These *in vitro* methods were developed as a rapid way of identifying unwanted effects of novel compounds by the pharmaceutical and chemical industries. They are also viewed as a potential screening method for novel NMs to inform hazard identification and risk assessment.

55. Kohl *et al.* (2020) reported that two of the existing genotoxicity testing methods are amenable to HTS approaches, namely the *in vitro* Comet assay and *in vitro* MN assay. The former has minaturised versions available which, when combined with an automated slide evaluation system, allow testing within minutes as opposed to hours for the standard assay. In a similar way, a HTS version of the micronucleus assay is also available using 96-well plates, robotic auto-sampling and automatic scoring.

56. Advanced 3D *in vitro* models are considered to have the potential to serve as alternatives to *in vivo* testing and are considered to better represent the *in vivo* situation than 2D *in vitro* models for key parameters such as cell viability, proliferation, differentiation, morphology, gene and protein expression and function (Kohl *et al*., 2020).

57. Genotoxicity testing of NMs has been reported using spheroids constructed from primary hepatocytes, HepG2 hepatocellular carcinoma cells and the HepaRG cell line, linked to the Comet assay or MN assay, and airway models (e.g. MucilAir™, Epithelix and EpiAirway, MatTek) also linked with the Comet assay. However, these are at an early stage of development and require further modifications before they can be used to test the genotoxicity of NMs. Commercial reconstructed skin tissues (EpiDerm™, Phenion® FT, EpiSkin™) have been utilised to develop the RSMN (see paragraph 14) and the reconstructed skin comet assay (RS comet assay) for genotoxicity testing. These have only been used in a very limited way for evaluating NMs and require further development (Pfuhler *et al.,* 2020).

58. Organ-on-a-chip (OOC) technologies have been developed for screening the cytotoxicity and genotoxicity of NMs. The cytokinesis block micronucleus assay has been linked to micro-array (micro-fluidic) based cell sorting to evaluate the genotoxicity of NMs on human primary lymphocyte subtypes (Vecchio *et al.,* 2014), and in a similar way a microarray based approach has been linked with the comet assay to produce CometChip® Technology (Watson *et al*., 2014). Chip technologies can therefore facilitate high throughput level screening of NMs in combination with 3D models that have more physiologically relevant features.

Questions for the Committee

Members are asked to review this draft document as attached and consider the following questions:

- Do members agree with the strategy presented?
- Are there any other aspects which should be included in the updated guideline document?
- Do members have any additional data for inclusion?
- Do members consider that the paper can be published as COM guidance?

IEH Consulting under contract supporting the PHE COM Secretariat.
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