Genetic toxicity testing of a metal oxide nanoform to meet regulatory requirements: shared experience to aid progress in an evolving scientific and regulatory landscape

Bryony L Ross* and Kai B Paul
Blue Frog Scientific Ltd. Edinburgh

*** Corresponding author:**
bryony.ross@bluefrogscientific.com | www.linkedin.com/in/bryonyross
Abstract 3700

Via application of up-to-date additions and amendments to traditional test guidelines, we realized a robust, guideline and guidance compliant *in vitro* mammalian cell gene mutation test to support registration of a nanoform under EU REACH.

INTRODUCTION:

- Additional considerations are required to execute guideline *in vitro* genotoxicity studies for hazard identification of nanoforms under regulatory frameworks.
- Testing was undertaken to satisfy EU REACH requirements for mutagenicity hazard identification of a metal oxide nanoform manufactured at <10 tonnes per annum (Annex VII, Fig. 1, Table 1).
- The relevant legal text, associated guidelines, scientific understanding and best practice are evolving rapidly (Table 2). However, there is little shared experience in the public/industry domain on the practicalities of trying to implement this.
- Here we share:
  - our approach to undertaking a robust, guideline- and guidance- compliant *in vitro* mammalian gene mutation test.
  - some of the key outstanding challenges and limitations.
  - How practicability and the context of "what the test was designed for" (*i.e.* as a screening study) was considered.

METHODS:

- We utilised OECD test guideline 490 (*in vitro* Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene), in a format amended for nanoforms.
- We included specific considerations for:
  - Sample preparation
  - Avoiding interference
  - Validity criteria
  - Cell line
- Additional characterisation included:
  - Dissolution
  - Particle size
  - Measurements in relevant medium
- Deviations from the test guideline were reported

KEY ISSUES (Table 3):

- Lack of clear technical/practical guidance: real potential for data to be generated which is neither reliable, robust nor meaningful without considerable expert input to study design.
- Variance between requirements of legal text, associated guidance, latest scientific understanding and best practise.

LIMITATIONS (Table 4):

- Ability of single testing laboratory to undertake additional aspects of testing required for nanoforms seriously limits ability to place such studies and/or ensure their comprehensiveness in line with current guidance.
- Prohibitive time/cost implications: requirements for testing nanoform far exceed typical timeline or budget expected for this type of regulatory submission costing one third to double more, not including external expert time.
- Decisions made on the design of this study sought to strike a balance between the ideal and the practical (*i.e.* what was possible within the testing facility).

CONCLUSIONS:

- We were able to undertake a robust, guideline- and guidance- compliant study to support registration of the nanoform under EU REACH.
- The cost (time and monetary) of studies on nanoforms is far in excess of that on bulk materials. This is further emphasised when considering that, in effect, mutagenicity testing at Annex VII/VIII under EU REACH is very much a screening level study and there is a tendency to try and overinterpret such data for nanoforms.
- A rapidly changing scientific and regulatory landscape means guidance relating to study design is somewhat fluid.
- To avoid significant barriers to realising the benefits of nano and other advanced materials, a pragmatic approach balancing cost and practicability of the additional regulatory testing requirements for nanoforms is needed.

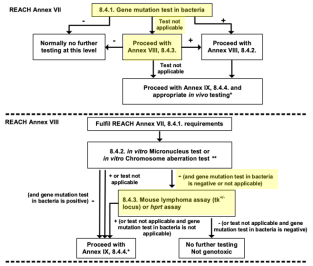

Fig. 1: Testing requirements for Annex VII registration of a nanoform, according to the EU REACH mutagenicity testing strategy. Adapted from Chapter R.7a: Endpoint specific guidance (Draft v7, 2023).

Nanoform: Inorganic metal oxide	
Purity	97%
Particle size distribution	~10 nm
Solubility	0.1 to 10 mg/L (pH dependent)

Table 1: Basic characterisation of the nanoform undergoing registration at EU REACH Annex VII.

Table 2: Key literature to support design of the study. in total, over 20 separate resources were required to inform design, far more than for standard chemicals when undertaking an in *vitro* mammalian gene mutation (OECD 490)

Reference	Year (publication or latest update)
ECHA EU REACH Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7a - Endpoint specific guidance	Sections related to mutagenicity: draft Version 7.0, June 2023
ECHA EU REACH Guidance on information requirements and chemical safety assessment, Appendix R7-1 for nanomaterials applicable to Chapter R7a - Endpoint specific guidance	Version 4.0, December 2022
OECD Series on the Safety of Manufactured Nanomaterials, No. 90: Physical-Chemical Decision Framework to Inform Decisions for Risk Assessment of Manufactured Nanomaterials	2019
OECD Series on the Safety of Manufactured Nanomaterials, No. 36: Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials	2012
OECD Guideline for the Testing of Chemicals, Test No. 318, adopted 09. Oct. 2017 "Dispersion stability of Nanomaterials in simulated environmental media"	2017
Nanogenotox Standard operating procedures for characterisation of the selected manufactured nanomaterials and dispersions thereof	2011
NanoReg D2.08 Standard operating protocol (SOP 05) for test item preparation and comparability of results during in vitro testing	2018
OECD Guideline for the Testing of Chemicals, Part 490, adopted 29. Jul. 2016 "In vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene"	2016
Council Regulation (EC) No. 440/2008, last amended by Commission Regulation (EU) 2023/464, EU Method B.17: "Mutagenicity - In Vitro Mammalian Cell Gene Mutation Test", adopted 06. Mar. 2023	2023
Doak, S.H. *et. al.* (2023), Current status and future challenges of genotoxicity OECD Test Guidelines for nanomaterials: a workshop report, Mutagenesis, Volume 38, Issue 4	2023
Chen T., Dusinska A. and Elespuru R. (2022): Thymidine Kinase+/- Mammalian Cell Mutagenicity Assays for Assessment of Nanomaterials. Front. Toxicol. 4:864753	2022

Table 3: Considerations for testing nanoforms, and how we approached their inclusion into the study design.

Nanoform testing related consideration	Commentary on approach
Suitability of selected test guideline	Originally, the assay was planned as an *in vitro* mammalian cell gene mutation test using the Hprt genes following OECD 476. However, a decision was made to move to the OECD 490 *in vitro* mammalian cell gene mutation test using the thymidine kinase gene. This was due to i) a high background mutant frequency in the cell line (V79) and ii) the fact that the morphologically different colonies counted within the OECD 490 test system allow differentiation between gross chromosomal aberrations and point mutations, thus providing greater sensitivity. Uptake into cell lines must also be proven.
Characterisation of nanomaterial in the test medium	The following characterisation was included: To determine dissolution: ICP-OES (Inductively Coupled Plasma Optical Emission spectroscopy) to measure concentration (soluble and total fraction) at three timepoints: i) start of the treatment, ii) after 3h and iii) after 4h (end of treatment). To ensure an understanding of dose and dissolved vs. ionic fraction. To determine agglomeration: DLS (Dynamic Light Scattering) to measure hydrodynamic diameter of particulates at start and end of incubation. Comparing changes in particle size distribution allows a qualitative assessment or proxy measure of the state of dispersion.
Metabolic activation (S9)	*In vitro* mutagenicity studies such as the OECD 490 include an experiment in the presence and absence of metabolic activation system (S9 mix). In the case of organic nanomaterials, it is recommended to perform the study only in the presence of S9, due to the changes S9 makes to culture medium protein content. In this case, the nanomaterial being tested was no inorganic, therefore it was possible to include experimental conditions with and without S9 (+/- S9).
Cytotoxicity	Cytotoxicity evaluation was designed in such a way that interference from the nanomaterial would not become a confounding factor *i.e.*, avoiding colourimetric or fluorometric assays.
Uptake into target cells	There exists some evidence in the scientific literature to support cellular uptake of nanoparticles by the L517BY cell line. However, due to the nature of the nanomaterial being tested, it was not possible to undertake additional testing to ascertain whether the particles were present intracellularly. However, we emphasise that this is a screening test, and no such process would be conducted for standard organic substances.
Sample preparation	EU REACH Appendix R7-1 for nanomaterials applicable to Chapter R7a stipulates that no generally applicable standard operating procedure is available for dispersion of dry powder in liquid. As such, the NANOGENOTOX and NanoReg protocols were used as guidance for a deviation from the test guideline for sample preparation.
Dosimetry	Consideration was made for use of favourable alternative dose metrics (*i.e.*, surface area) as an addition to mass based alone.
Acceptability criteria	As the nanoform is of low solubility and a slow dissolution rate the preparation was a suspension, a deviation from the test guideline was included to remove the criterion stipulating that in the case of precipitation, the highest analysed concentration should be the lowest concentration where precipitation is visible to the unaided eye.

Table 4: Balancing the ideal with the practical – recognized limitations of the study

When considering the low tonnage band of this registration alongside the time and funding required to complete a screening level study for this endpoint, decisions had to be made to balance achieving the most robust study possible with the practicability of additions to accommodate for the test item being nanoform. As a result, the following limitations were recognized.

Limitation	Commentary
Cell & nuclear uptake	Under conditions of the study, it was not possible to make a conclusive statement on whether the nanoparticles were able to pass cellular/nuclear membranes to interact either directly with DNA, or via indirect mechanisms (*e.g.*, DNA associated proteins, mitotic spindle apparatus, oxidative stress etc.). **Context:** this is a screening assay; no such process is required for other substances.
Interaction with components of biological media	It was not possible from this study to establish whether the nanomaterial interacted with the components of the biological media, yielding partially or totally soluble or dispersed transformation products (that may influence the overall toxicity and fate processes). **Context:** this is a screening assay, moreover, if hazard is identified it is irrelevant what the cause is for this regulatory step, as the introduction of the nanoform led to the hazard regardless of transformation.
Characterisation	Characterisation was not undertaken in the presence of cells, or S9. In the presence of cells gradual dissolution/transformation/degradation or temporal changes in particle size distribution and surface charge may be possible. **Context:** characterization in complex medium is not possible and there is a risk of introducing artifacts when using e.g., TEM imagery.
Cost (time and financial)	The study took far longer than a standard OECD 490 test and required considerably higher funding to meet the additional requirements for testing on nanoforms. **Context:** 1/3 to double more than usual not including external expert time.