

Appendix E – Advice of the ccWG on Nanotechnology: Nanoscale considerations for the assessment of the study design and study results of TiO₂ toxicity studies

1. Background

This advice was requested by the EFSA FIP unit to support the FAF Panel WG Titanium dioxide (E 171) in the context of their mandate to re-evaluate E 171 (EFSA-Q-2020-00262). In particular, scientific criteria for implementing the provisions of the EFSA Scientific Committee Guidance on Nanoscience and Nanotechnologies in the food and feed chain (EFSA Scientific Committee, 2018a¹) on this material were requested. The purpose of this advice and the support, is to indicate when the design of a study with this material (TiO₂) is adequate for a hazard characterisation of small particles, including nanoparticles. As different types/levels of information may be available depending on each study, the ccWG Nanotechnology distinguished possible situations and developed a corresponding scoring system for consideration by the FAF Panel WG Titanium dioxide (E 171).

2. Advice

2.1.1. Solubility and dissolution rate

The EFSA ANS Panel (2016) opinion indicates that titanium dioxide is insoluble in water, without a quantitative estimation, and that solubility at gastric pH is expected to be very low (EFSA ANS Panel, 2016²).

No solubility or dissolution rate studies have been provided.

The information from the ECHA database³ confirms that, at pH higher than 2, TiO₂ does not dissolve in water but, similarly to other metal oxides, may suffer a chemical transformation of the dioxide to a soluble ionic or other metal-bearing species. Transformation/dissolution (T/D) studies according to the OECD GD 29 ENV/JM/MONO(2001) are available for micro-sized (Klawonn et al. 2017a-c⁴) and nano-sized (Klawonn et al. 2017d-f⁴) TiO₂ in the ECHA database.

In the above-mentioned studies (Klawonn et al., 2017 a-f⁴), the mean background-corrected dissolved Ti concentration (after filtration through a 0.2 µm membrane and centrifugal filtration) on day 1 was 0.024 µg Ti/L, which corresponds to 0.04 µg TiO₂/L. No identification of the dissolved chemical species is provided.

These data indicate that following the exposure to TiO₂, measurement of the levels of Ti (i.e. 'total titanium') in tissues or cells can be used as a marker for internal exposure to TiO₂ (nano)particles.

Dispersion assessment and scoring

Table 1 presents the criteria proposed by the ccWG Nanotechnology for scoring the capacity of the study design and study results for addressing the hazard of the fraction of small particles including

¹EFSA Scientific Committee, 2018a. Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: part 1, human and animal health. EFSA Journal 2018;16(7):5327, 95 pp. <https://doi.org/10.2903/j.efsa.2018.5327>

²EFSA ANS Panel (EFSA Panel on Food Additives and Nutrients Sources added to Food), 2016. Re-evaluation of titanium dioxide (E 171) as a food additive. EFSA Journal 2016;14(9):4545, 83 pp. <https://doi.org/10.2903/j.efsa.2016.4545>

³<https://echa.europa.eu/registration-dossier/-/registered-dossier/15560/4/9>

⁴Klawonn et al., 2017 a-f. Study summaries reported in the registration dossier under the REACH Regulation available at: <https://echa.europa.eu/es/registration-dossier/-/registered-dossier/15560/4/9>

nanoparticles. The Guidance on nanotechnologies in the food and feed chain (EFSA Scientific Committee, 2018a) stipulates in Section 6.9 that *In both in vitro and in vivo testing, it is recommended to check the structure/properties of the nanomaterial in the test medium (e.g. particle agglomeration/aggregation)*. This is to aid the interpretation of the results in line with the indication from the nano guidance (EFSA Scientific Committee, 2018) *Critical to the interpretation of studies (especially those with negative results) is the demonstration that cells (in vitro) and tissues (in vivo) were exposed to the nanomaterial, i.e. that the nanomaterials actually came into contact with the cells/tissues*.

TiO₂ nanoparticles have a high tendency to form agglomerates. The level of agglomeration depends on the media and the protocol used for preparing the samples as well as the concentration. Even the best currently available dispersion protocols cannot ensure that 100% of the material would be as non-agglomerated constituent particles, especially at high TiO₂ concentrations in liquid suspensions. In addition, the study design should address the potential for dispersed particles (as constituent particles or small agglomerates) to start re-agglomerating. Lag-time after dispersion is also important and the samples prepared and used fresh will more likely be better dispersed. The scoring method in this advice assesses the level of information provided in the study publication or report. A score of 1 indicates that the evaluators assessing the reliability of the results have sufficient information for checking to what extent the results cover the fraction of small particles, including nanoparticles. The highest score can be achieved by: a) application of a dispersion protocol suitable to bring particles in suspension in a well dispersed state, covering also the stability of the dispersion and reporting the final level of agglomeration in the administered doses (*in vivo* studies) or final concentrations in the cell media (*in vitro* studies); b) the confirmation of exposure (internalisation of TiO₂ particles); c) a combination of both. Lack of information or caveats in the methodology trigger lower scores. It should be noted that a high degree of agglomeration may decrease the level of absorption, but there still can be some absorption; in case of confirmed internal exposure, this information should be used to assess the toxicological information.

A high level of agglomeration/aggregation is always present in pristine TiO₂ powders. In liquid media, the level of agglomeration of TiO₂ particles is pH dependent and increases with the concentration. After assessing the available information, the ccWG Nanotechnology has proposed a set of thresholds for TiO₂ high doses/concentrations at which, without a specific protocol on dispersion and stability, a very high level agglomeration is expected in liquid dispersions.

Table 1. Scheme for scoring for nanoscale considerations in the study design (dispersion and/or confirmation of internal exposure)

Criteria for scoring	Considerations for interpreting the results for the hazard characterisation of small particles including nanoparticles: Positive results: indications of toxicity observed Negative results: no indications of toxicity or differences with untreated control observed
<p>Score 1: At least one of the following criteria is met:</p> <ul style="list-style-type: none"> • Dispersion covered by a verified SOP or a systematic approach (e.g. reference to NANOGENOTOX or ENPRA protocols or to verified protocols specific for titanium dioxide (e.g. Guiot and Spalla, 2013⁵), ISO or OECD guidelines for dispersion of nanomaterials, or equivalent reference*); or • Sonication applying energy densities from 600 J/ml to 2500 J/ml sample volume plus confirmation of stability for at least 30 min and/or stability ensured until at least administration or application is completed; or • Specific confirmation of sufficient level of dispersion and of the stability of the dispersion (options include EM, DLS**, zeta potential higher than 25 mV or lower than -25 mV in the dispersion media); or • Use of demonstrably effective dispersing agents or surfactants with a proper justification (and inclusion of solvent control); or • In case of administration in treated feed, information on the level of agglomeration in the stock suspension/powder mixed with the feed and in the treated feed, or • Confirmation of cell/tissue exposure during execution of the test (e.g. ICP-MS or ultrastructural localisation by EM, enhanced dark-field microscopy, etc.); including evidence that the particles correspond to Ti. 	<p>Score 1: both positive and negative results are relevant and reliable from the perspective of assessing small particles, including nanoparticles. The evaluators have sufficient information on the level of agglomeration (i.e. according to the SOP or through the reporting of the different levels of agglomeration observed at different doses/concentrations) for assessing the role of the fraction of small particles including nanoparticles when considering the reliability of the results and in the assessment of the dose–response relationships. If the score is based on confirmation of internal exposure, the evaluators when assessing the results should consider in the Weight of Evidence (WoE) and assessment of uncertainties whether the confirmation is only qualitative (e.g. microscopy supported by verification of Ti in the particles) or, as preferable, there is a quantitative assessment (e.g. Ti measurement) of the level of internal exposure at each dose/concentration.</p>

⁵ Guiot C, Spalla O. Stabilization of TiO₂ nanoparticles in complex medium through a pH adjustment protocol. Environ Sci Technol. 2013 Jan 15;47(2):1057-64. doi: 10.1021/es3040736. Epub 2012 Dec 26. PMID: 23240597

<p>Score 2: None of the criteria above fulfilled, but there are some indications in the study design and although incomplete, some information on dispersion is presented. This score also applies when there are some limitations in the confirmation of exposure of cells in <i>in vitro</i> studies (i.e. microscopic confirmation of cell internalisation without confirmation that the particles correspond to Ti).</p>	<p>Score 2: The relevance of the results for assessing small particles, incl. nanoparticles may be affected by agglomeration. Therefore, the reliability of the dose–response relationship must be checked case-by-case. In the WoE, negative results should be considered carefully before use to balance or rebut positive results from other studies.</p>
<p>Score 3: No information or mention of dispersion of nanoparticles, but the test design included normal approaches for testing poorly soluble chemicals (e.g. OECD recommendations for each TG***)</p>	<p>Score 3: The relevance of the results for assessing small particles incl. nanoparticles cannot be verified (i.e. cannot be confirmed but cannot be excluded); this situation creates uncertainty regarding the capacity of the study results to cover the fraction of small particles including nanoparticles. Specifically, negative results may be due to the lack of exposure to the fraction of small particles. The evaluators should consider the lack of information on the level of agglomeration when assessing the reliability of positive results.</p>
<p>Score 4: criterion 1 or 2 are not met and only high doses used:</p> <ul style="list-style-type: none"> • for <i>in vitro</i> all doses at 100 µg/ml or above; • for <i>in vivo</i> all doses at 50 (liquid forms)-100 (food matrix) mg/kg bw ****or above <p>without further information on dispersion and stability) or verifiable information that the study design simulate actual consumer exposure to E 171*****.</p> <p>This score also applies when there are observations confirming massive aggregation/agglomeration of the material in non-nano clusters or the study design is inadequate for poorly soluble chemicals.</p>	<p>Score 4: When a study is designed for testing only high doses/concentrations, and lacks specific considerations on dispersion, presence of large agglomerates of small particles must be considered. The results may still be informative for larger particles and large agglomerates, but not for the fraction of small particles including nanoparticles. In the case of <i>in vivo</i> studies, such a study design should trigger a high likelihood for reduced bioavailability of the fraction of small particles including nanoparticles due to extensive agglomeration. Negative results are therefore not informative for assessing consumers exposure to the fraction of small particles including nanoparticles. In case of positive results, the evaluators should consider that the dose may not represent the actual fraction of nanoparticles available for absorption. For <i>in vitro</i> studies, a similar concern should be considered for negative results. For positive results, artefacts due to local effects of precipitation should be considered.</p>

Notes:

* These protocols are often designed for ensuring a good level of dispersion in the liquid stock suspension, and are directly applicable in the assessment of dispersion for *in vivo* studies when substances are prepared in liquid vehicles for administration or as a liquid stock added to the diet; additional considerations are needed for *in vitro* studies as the dispersion/agglomeration may be affected by the characteristics of the test media.

**DLS is relevant for confirming stability of dispersion through the measurement of the hydrodynamic diameter (not for assessing the particle size distribution of E 171) and for assessing dispersion of monodisperse materials.

*** A document compiling the indications in the relevant TGs for *in vivo* and *in vitro* tests was compiled for the draft Guidance on technical requirements for regulated food and feed product applications⁶.

**** These threshold doses are calculated on the basis of the maximum concentration of TiO₂ that can be dispersed according to the protocols mentioned above (See list under Score 1) for preparation of suspensions for *in vivo* toxicological studies for the hazard assessment of small particles.

***** According to the EFSA Scientific Committee Guidance (2018a¹) (Section 6.9), bolus gavage administration may be the method of choice for the identification of the hazards associated with the nanomaterial; mixing with the feed is possible, but requires specific considerations. From the perspective of nanospecific risk assessment worst-case use patterns are those maximising the exposure of consumers to constituent particles and small agglomerates. According to the available information (Meacock et al., 1997⁷; Phillips and Barbano, 1997⁸; Pichot et al., 2015⁹; JECFA, 2006¹⁰; US3839074¹¹; US5571334¹²; US5800601¹³; US6893671¹⁴; US9907325¹⁵), mixing of TiO₂/E171 as powder with the animal diet in *in vivo* studies (i.e. without prior dispersion in a liquid medium) does not cover all technological procedures in use in the food industry, and creates uncertainty regarding its capacity for simulating consumer exposure to E171 nanoparticles via food.

Glossary

Agglomerate	Agglomerate refers to a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components.
Constituent particle	Constituent particles are the (morphologically) identifiable particles, including those inside an aggregate or agglomerate. In agglomerates the constituent particles are only weakly bound. In aggregates the constituent particles are strongly bound. Mobility-based techniques cannot be used to measure the size of constituent particles in aggregates and agglomerates (from Rauscher et al., 2019 ¹⁶)
Pristine material	Original, pure material (before it is processed).

⁶ <http://www.efsa.europa.eu/sites/default/files/consultation/consultation/Draft-Nano-Technical-Guidance-For-Public-Consultation.pdf>

⁷ Meacock G, Taylor KDA, Knowles MJ and Himonides A, 1997. The improved whitening of minced cod flesh using dispersed titanium dioxide. *Journal of the Science of Food and Agriculture*, 73, 221 E225.

⁸ Phillips LG and Barbano DM, 1997. The influence of fat substitutes based on protein and titanium dioxide on the sensory properties of lowfat milks. *Journal of Dairy Sciences*, 80, 2726.

⁹ Pichot R, Duffus L, Zafeiri I, Spyropoulos F and Norton IT, 2015. Particle-Stabilized Food Emulsions. *Particle Stabilized Emulsions and Colloids. Formation and Applications*, RS.

¹⁰ JECFA (Joint FAO, WHO Expert Committee on Food Additives), 2006. Titanium dioxide: Chemical and Technical Assessment. First draft prepared by P.M. Kuznesof, reviewed by M.V. Rao.

¹¹ United States Patent 3,839,074, Taylor J.S. 1974. Opaque Composite Film.

¹² United States Patent 5,571,334, Dunn J.M., Gross A.T., Finocchiaro E.T. 1996. Starch-Based Opacifying Agent for Foods and Beverages.

¹³ United States Patent 5,800,601, Zou W.K., Siddiqui M.W., Xiao F., Morelos A.C., Vega J.G. Dong Q.Q., Aguilar J. 1998. Food Grade Jet Inks

¹⁴ United States Patent 6,893,671 B2, Ben-Yoseph E.M., Collins T.M., Shastry A.V., Willcocks N.A., Narine S.S., Suttle J.M. 2005. Chocolate Confectionery Having High Resolution Printed Images on an Edible Image-Substrate Coating.

¹⁵ United States Patent 9,907,325 B2, Piorkowski D.T. 2018. Encapsulated Weighting Agents For Beverage Emulsions.

¹⁶ Rauscher H, Roebben G, Mech A, Gibson N, Kestens V, Linsinger TPJ and Riego Sintes J, 2019. An overview of concepts and terms used in the European Commission definition of nanomaterials. EUR 29647 EN, European Commission, JRC, Ispra, , doi:10.2760/459136