Scientific Committee on Consumer Safety

SCCS

Scientific Advice on Titanium dioxide (TiO_2)

(CAS/EC numbers 13463-67-7/236-675-5, 1317-70-0/215-280- 1, 1317-80-2/215-282-2)

The SCCS adopted this document
by written procedure 13 May 2024

ACKNOWLEDGMENTS

SCCS members listed below are acknowledged for their valuable contribution to the finalisation of this Scientific Advice.

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This document has been subject to a commenting period of eight weeks after its initial publication (from 5 December to 6 February 2024). Comments received during this period were considered by the SCCS. Main changes occurred in the following sections: Abstract & conclusions (last paragraph from conclusion #5 has been moved to the end of conclusion #1 and the product type "mouthwashes" has been removed from conclusion #5), Table 3.1.4.A1, sections 3.1.10-11, section 3.2 Toxicokinetics, section 3.4.1.1 Mutagenicity / genotoxicity *in vitro* (Table with study details on *in vitro* study, the information on RM11, and the SCCS comments on the *in vitro* study #2), section 3.4.1.2 Mutagenicity / genotoxicity *in vivo* (the SCCS comments on the *in vivo* study #1 & the inclusion criteria for TiO2 particulate forms), Tables 3.4.1.3.B-D and Annex X. For Titanium dioxide nanogrades, the information on the particle fraction with an aspect ratio larger than 3 for all the nano grades has been introduced (Table 3.1.9.1., Table 3.1.9.1.B1 (Annex L).

All Declarations of Working Group members are available on the following webpage:
Register of Commission expert groups and other similar entities (europa.eu)

1. ABSTRACT

The SCCS concludes the following:

1. In light of the EFSA Opinion on genotoxicity concerns for E171, does the SCCS consider Titanium dioxide safe in oral cosmetic products?

From the provided information, the SCCS has noted that the titanium dioxide (TiO_2) materials evaluated in this Scientific Advice belong to a wide range of grades[1] (44 pigmentary and 40 nano grades) used in cosmetic products. The pigmentary grades differ from the food additive E171 in terms of crystalline forms, particle sizes, coatings, etc., with the exception of 13 uncoated pigmentary grades that can be considered as equivalent to E171.

Having considered all the information (including that evaluated by EFSA, 2021), the SCCS considers that the available evidence is not sufficient to exclude the genotoxicity potential of almost all of the types of TiO_2 grades used in oral cosmetic products. The only exception are two nano grades (RM09 and RM11) for which the provided genotoxicity data indicate no genotoxicity concern. More information is, however, needed on the potential uptake and cellular effects of the nano grades in the oral mucosa to consider them safe for use in oral-care products.

More experimental data are needed from studies carried out under valid protocols and appropriate testing guidelines to exclude the genotoxicity potential of the selected representatives of the other grades of TiO_2 (both pigmentary and nano) used in oral cosmetic products.

It is worth highlighting that the SCCS approach to risk assessment of TiO_2 ingredients in orally-used cosmetic products is slightly different from that of EFSA. This is because cosmetic products are not meant to be ingested orally, and any ingestion via the oral route can only be unintended and incidental. Keeping this in mind, the amounts of orally-ingested cosmetic ingredients can only be expected to be far lower than the amounts ingested when a TiO_2 material is used as a food additive, which is consumed via intake of the food products. For the SCCS, the potential absorption/retention, translocation and adverse effects of nanoparticles in the buccal mucosa are therefore important considerations for safety evaluation.

The SCCS recommends that safety assessment of the pigmentary TiO_2 grades used in cosmetics should also take account of the fact that some of them contain a sizeable proportion of the particles in the nano size scale – some over 50% (in terms of particle number, median constituent particle size).

2. In light of the EFSA Opinion, does the SCCS consider that previous Opinions issued by the SCCS on inhalation and dermal exposure to Titanium dioxide need to be revised?

The conclusions drawn in previous SCCS Opinions on dermally applied cosmetic products (SCCS/1516/13, SCCS/1580/16) remain unchanged for the TiO_2 grades and the coatings evaluated in those Opinions. New data on dermal absorption will be required for other

[1] The term "grade" is used to describe the type of materials by the Applicants. The SCCS decided to keep the term "grade" for the whole SA for the sake of consistency of the text.

types of TiO_2 grades and coatings that are not covered in the Cosmetics Regulation 1223/2009, nor by entry 27a in Annex VI.

According to the Cosmetics Regulation 1223/2009, the nanoform of TiO_2 is already restricted under entry 27a of Annex VI as not to be used in applications that may lead to exposure of the end-user's lungs by inhalation. The conclusions drawn in the previous Opinions (and SCCS/1583/17, SCCS/1617/20) on the safety of TiO_2 used in specific cosmetic products that may lead to exposure by inhalation also remain unchanged.

3. In the event that the estimated exposure to Titanium dioxide from cosmetic products is found to be of concern, SCCS is asked to recommend safe concentration limits for each category of products and types of use.

Since the genotoxicity hazard of almost all of the grades of titanium dioxide could not be excluded (with the exception of RM09 and RM11), the SCCS cannot recommend any safe limits for the materials when used in cosmetic products that could lead to oral or inhalation exposure, other than those already indicated in the previous SCCS Opinions (SCCS/1516/13, SCCS/1580/16 and SCCS/1617/20).

4. In light of the potential removal of the E 171 purity specification from the food additives Regulation, the SCCS is requested to review and indicate the respective specifications for Titanium dioxide when used in cosmetics.

In view of the concerns on the potential genotoxicity of the TiO_2 grades considered in this Scientific Advice, the SCCS is of the opinion that the Applicants should draw up a proposal for specifications of the different TiO_2 grades used in those cosmetic products that could lead to oral and inhalation exposure. The SCCS will be able to assist the Commission in reviewing the proposal.

5. Does the SCCS have any further scientific concerns regarding the use of Titanium dioxide in cosmetic products?

Studies have indicated that oral mucosal cells are prone to the uptake of nanoparticles (including TiO_2 nanoparticles), as they may penetrate the mucous layer and may be internalised by the epithelial cells. Considering that some oral products containing TiO_2 nanoparticles, such as toothpastes, will be used every day and potentially more than once a day, further investigations are needed to exclude the risk to the consumer from long-term repeated exposures of the oral mucosa to TiO_2 nanoparticles.

Keywords: SCCS, scientific advice, Titanium dioxide (TiO_2), Regulation 1223/2009, CAS/EC numbers 13463-67-7/236-675-5, 1317-70-0/215-280- 1, 1317-80-2/215-282-2.

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Scientific Advice on Titanium dioxide (TiO_2), CAS/EC numbers 13463-67-7/236-675-5, 1317-70-0/215-280- 1, 1317-80-2/215-282-2, preliminary version of 4 December 2023, final version of 13 May 2024, SCCS/1661/23

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In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide Opinions on questions concerning health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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SCCS - Opinions (europa.eu)

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2. MANDATE FROM THE EUROPEAN COMMISSION

Background

Titanium dioxide (TiO_2) (CAS/EC No. 13463-67-7/236-675-5, 1317-70-0/215-280-1, 1317-80-2/215-282-2) is a white, insoluble, inert substance with a high refractive index. In its microcrystalline form, it is used as a white pigment or opacifying agent in make-up, skin care, hair and oral products. In addition, since TiO_2 absorbs and scatters both UVA and UVB rays, it is also used as inorganic UV-filter primarily in sunscreens, but also in day creams, foundations and lip balms, to provide protection against UV radiation. The introduction of colourless, ultrafine nanoparticles of TiO_2 improved its application on the skin while maintaining and enhancing its UV-filter properties.

TiO_2 is authorised both as colourant under entry 143 of Annex IV and as UV-filter under entries 27 and 27a (nano form) of Annex VI to Regulation (EC) No. 1223/2009 (Cosmetics Regulation). In light of its classification as a Carcinogen Category 2 (*i.e.* suspected human carcinogen) by inhalation route only and its inclusion in Annex VI to Regulation (EC) No. 1272/2008 (CLP Regulation) TiO_2 was re-assessed by the SCCS[2]. Subsequently, entry 321 in Annex III was introduced and additional provisions in the existing entries of 143 of Annexes IV and 27 and 27a of Annex VI were added that further restricted the use of TiO_2 in cosmetic products.

In March 2021, the Panel on Food Additives and Flavourings (FAF Panel) of the European Food Safety Authority (EFSA) issued an Opinion on the safety of TiO_2 (E171) as a food additive[3]. In particular, based on new relevant scientific evidence considered by the panel to be reliable, including data obtained with TiO_2 nanoparticles and data from an extended one-generation reproductive toxicity (EOGRT) study, the panel indicated that a concern for genotoxicity could not be ruled out. In light of this and given the many uncertainties, the panel concluded that E171 should no longer be considered as safe when used as a food additive.

In May 2022, the Commission services received a dossier submission by industry accompanied by a comprehensive and up to date review of the genetic toxicity database for TiO_2 providing scientific evidence to demonstrate the safety of non-nano (pigmentary) and nano form of TiO_2 in cosmetic products.

The Commission requests the SCCS to re-assess the safety of TiO_2, focusing on genotoxicity and exposure via the inhalation and oral route (lip care, lipstick, toothpaste, loose powder, hair spray), since the currently available scientific evidence supports an overall lack of dermal absorption of TiO_2 particles[4].

[2] https://ec.europa.eu/health/sites/default/files/scientific_committees/consumer_safety/docs/sccs_o_238.pdf
[3] https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2021.6585
[4] https://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_136.pdf

Terms of reference

1. In light of the EFSA Opinion on genotoxicity concerns for E171, does the SCCS consider Titanium dioxide safe in oral cosmetic products?

2. In light of the EFSA Opinion, does the SCCS consider that previous Opinions issued by the SCCS on inhalation and dermal exposure to Titanium dioxide need to be revised?

3. In the event that the estimated exposure to Titanium dioxide from cosmetic products is found to be of concern, SCCS is asked to recommend safe concentration limits for each category of products and type of use.

4. In light of the potential removal of the E 171 purity specification from the food additives Regulation. The SCCS is requested to review and indicate the respective specifications for Titanium dioxide when used in cosmetics.

5. Does the SCCS have any further scientific concerns regarding the use of Titanium dioxide in cosmetic products?

3. OPINION

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Titanium Dioxide

3.1.1.2 Chemical names

From Applicant
Dioxotitanium, TiO_2
Titanium dioxide, COLIPA No. S75

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final

For some specific RM TiO_2 grades which have been tested, *i.e.* RM09 and RM11
RM09 (Molecular formula: TiO_2 (SiO_2)
Chemical name: Titanium dioxide (and silicium dioxide)

Ref.: 4023311_final Report.pdf, 4023313_final_report.pdf

RM11 (Molecular formula: TiO_2 (Al_2O_3 and $[C_2H_6OSi]n$)
Chemical name: Titanium dioxide (and aluminium oxide and silicone)
Synonym: Titanium dioxide (and alumina and dimethicone)

Ref.: 4023312_final Report.pdf, 4023314_final_report.pdf

3.1.1.3 Trade names and abbreviations

No information provided by the Applicant. Any available information in this regard has already been indicated in the previous SCCS Opinions relating to TiO_2 material.

3.1.1.4 CAS / EC number

From Applicant
CAS Number: 13463-67-7*
* Also, Anatase CAS 1317-70-0; Rutile CAS 1317-80-2
EC n°: 236-675-5**
** Also, Anatase EC 215-280-1; Rutile EC 215-282-2

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final

For some specific RM TiO_2 grades which have been tested, *i.e.* RM09 and RM11
RM09 (Molecular formula: TiO_2 (SiO_2)
CAS No.: 13463-67-7 (and 7631-86-9)
EC No: 236-675-5

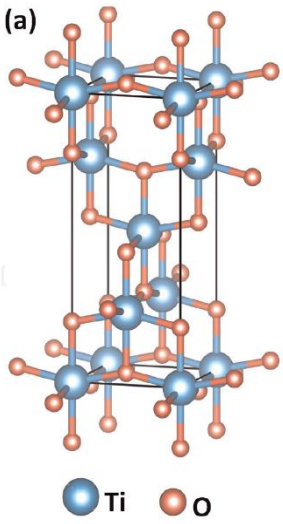
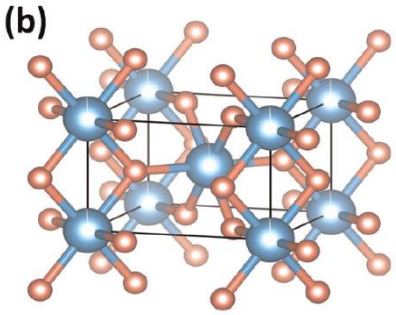
Ref.: 4023311_final Report.pdf, 4023313_final_report.pdf

RM11 (Molecular formula: TiO_2 (Al_2O_3 and $[C_2H_6OSi]n$)
CAS No.:13463-67-7 (and 1344-28-1 and 63148-62-9)
EC No: 236-675-5

Ref.: 4023312_final Report.pdf, 4023314_final_report.pdf

3.1.1.5 Structural formula

From SCCS

Anatase form	Rutile form
	

From Ref.: Modification of Physical and Chemical Properties of Titanium Dioxide (TiO_2) by Ion Implantation for Dye Sensitized Solar Cells
Hafsa Siddiqui – DOI :10.5772/intechopen.83566

i) Pigmentary Grades

Anatase Form: RM01, RM03, RM04, RM05, RM06, RM07, RM19, RM26, RM27, RM67, RM67b, RM68, RM70a, RM70b, RM70c, RM70d, RM70e, RM70f

Rutile Form: RM02, RM08, RM28, RM29, RM30, RM31, RM32, RM33, RM34, RM35, RM36, RM37, RM38, RM39, RM69, RM69b, RM72a, RM72b, RM72c, RM72d, RM72e, RM72f, RM72g, RM72k, RM72i, RM72j-bis

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final Table page 8/28 – Column: 6.6) / N7) Crystalline Form

ii) Nano Grades

Rutile form:

RM09, RM40, RM41, RM42, RM43, RM44, RM45, RM46, RM47, RM48, RM49, RM51, RM52, RM53, RM55, RM56, RM59, RM74d, RM80

Rutile with up to 1% anatase:
RM57, RM58, RM60, RM61
Rutile with 1% anatase:
RM82

Rutile with up to 5% anatase:
RM10, RM11, RM62, RM63, RM64, RM65, RM74a, RM74b, RM74c, RM74e, RM75, RM76, RM77, RM78, RM79, RM81

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final
Table from Page 15/28, Column N7a) % anatase

From Applicant
The anatase % is derived from the relative intensity of well separated X-ray diffraction lines of anatase and rutile using a calibration curve. Suitable reflections may be 36.5°for rutile and 48° for anatase.

From **Ref.**: CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

SCCS comment
This Opinion is limited to the TiO_2 crystalline forms comprised of rutile, anatase or a mixture of the two forms. Other crystalline forms of TiO_2 have not been assessed.

3.1.1.6 Empirical formula

TiO_2

3.1.2 Physical form

From Applicant
Titanium dioxide grades used in cosmetics may be divided into two groups
- pigmentary grades with median primary particle size >100nm whose primary function is to provide whiteness and opacity as well as some UV protection and
- nano grades with median primary particle size <100nm whose primary function is to provide UV attenuation without excessive whiteness.

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final

SCCS comment
In line with the JRC report (2023), the SCCS recommends the use of the term "constituent particle" instead of "primary particle".

3.1.3 Molecular weight

From Applicant
79.866 g/mol

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final

3.1.4 Purity, composition and substance codes

From Applicant

Purity, composition (Pigmentary and Nano Grades):
All the raw materials that are described in the submission are in compliance with the relevant specifications. However, as is common with quality control testing against pharmacopeia and similar regulatory specifications, in many cases the analytical measurements are only recorded as a pass or fail against the specification. Therefore, it has not proved possible for all suppliers to ascertain actual values from the analytical laboratories for all of the raw materials. They are only able to obtain confirmation that they meet the relevant specifications as some of the equipment available within the suppliers may not have a precision for the exact measurement however can detect whether it fits the specification or not. This is one of the reasons why it is a challenge to submit exact measured values to the SCCS.

Given that TiO_2 is manufactured from naturally occurring ores, there can be variability within these different ores accounting for a different impurity analytical profile (specifically heavy metals) within the specification limits. In the case of heavy metals, the specification is a maximum value. The principal raw material ores for manufacturing TiO_2 include ilmenite (iron titanium oxide, $FeTiO_3$), naturally occurring rutile (TiO_2) or titanium slag which all contain naturally occurring heavy metals in variable amounts depending on the nature and geographic source of these raw materials. This results in heavy metals being present as unavoidable trace elements in the manufactured titanium dioxide product even though GMP are applied for cosmetics ingredients. Depending on the raw material sourcing and the manufacturing process, the heavy trace metals for cosmetics ingredients products are reduced by a significant factor for some elements like lead, arsenic and antimony compared to products marketed for "technical" applications. These trace elements are embedded in the lattice of the TiO_2 and are not bioavailable. Therefore, rather than give a potentially unrepresentative single data point, the ranges of values presented give an accurate account of this natural variability.

Whilst we have validated methods to confirm the specification of our products, we must stress that the values we have (particularly for total composition) are based on calculations, so there is automatically some level of uncertainty. Considering this then it is difficult to obtain a 100% absolute value. As per the analytical methods description, metal/metalloid components are analysed for their metal/metalloid content and further expressed as oxides. Under this practice it is almost impossible to achieve a 100% composition. For example, it is not possible to know whether aluminium is present as Al_2O_3 or $Al(OH)_3$ or similarly if the analysed elemental silicon is related to silica, silicones or silanes. Only an approximation can be made based on the manufacturing process.

From Ref.: CE-TiO2-23-003.0 - CE Response to clarifications requested by SCCS 10 03 23 – final.pdf

Surface Coatings (Pigmentary and Nano Grades)
Where coatings are present, they are all homogeneous and, where there is more than one, they are multi-layered.

The aluminium species in the coatings of titanium dioxide materials are not crystalline alumina but poorly characterised oxyhydroxide species which can variously be described as AlOOH and $Al(OH)_3$ but are generally described as $Al(OH)_3$. The description of the coating as alumina is purely an analytical convention. Where alumina is referred to in addition to aluminium hydroxide e.g., "Alumina 0.3%, Aluminium Hydroxide 2.0%", then this is aluminium that is contained in the lattice of the titanium dioxide having been added as a processing aid at calcination (Calcination Salts) to control the crystal phase and primary particle size. This alumina is part of the core titanium dioxide and is not a coating.

From **Ref.:** CE-TiO2-23-003.0 – CE
Response to clarifications requested by SCCS 10 03 23 – final.pdf

Pigmentary grades

Pigment grades of titanium dioxide (CPR, Annex IV entry 143), must comply with the "purity criteria as set out in Commission Directive 95/ 45/EC (E 171)", which was replaced by Commission Regulation (EU) No 231/2012 of 9 March 2012 [4] laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. In addition, it is specified that "Titanium dioxide in powder form containing 1% or more of particles with aerodynamic diameter <10 µm, to be used in compliance with Annex III, No 321".
For cosmetics applications, organic and inorganic surface treatments that have been approved for cosmetics use may also be applied to the titanium dioxide.

The raw materials may be grouped into categories by composition as follows (specifications are given in Table 3.1.4.A1):

Table 3.1.4.A1: Pigmentary grades - Categories by composition (from **Ref.:** January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf) and Structure (from **Ref.:** January 2023_PhysChem data on Cosmetics TiO2 grades_final, Table page 8/28 – Column:6.6) / N7) Crystalline Form)

	Composition	**Crystalline structure**	**Pigmentary grades**
a	Titanium Dioxide	Anatase	RM01, RM03, RM04, RM26, RM67, RM67b, RM68, RM70c
		Rutile	RM02, RM28, RM69, RM69b, RM72c
b1	Titanium Dioxide with up to 2% alumina and/or silica	Rutile	RM30
b2	Titanium Dioxide with more than 2% alumina and/or silica	Rutile	RM31, RM37
c1	Titanium Dioxide with organics added	Anatase	RM27, RM70a, RM70b, RM70d, RM70e, RM70f
		Rutile	RM29, RM72a, RM72b, RM72d, RM72e, RM72f, RM72g, RM72k
c2	Titanium Dioxide with up to 2% alumina and/or silica with organics added	Anatase	RM05, RM06, RM07, RM19,
		Rutile	RM08, RM32, RM33, RM34, RM35, RM36, RM72i, RM72j-bis
c3	Titanium Dioxide with inorganics (including >2% alumina and/or silica) with organics added	Rutile	RM38**,** RM39

Table 3.1.4.A2: Pigmentary grades / Proposed specifications for titanium dioxide cosmetics grades Titanium Dioxide Pigments used in cosmetics (from **Ref.:** January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf - Table 1.1 Proposed specifications for titanium dioxide cosmetics grades)

Titanium Dioxide Pigments used in cosmetics						
Category name	**(a)**	**(b1)**	**(b2)**	**(c1)**	**(c2)**	**(c3)**
Composition (Titanium Dioxide +…)	None	Alumina / silica (<2%)	Inorganics (incl. Alumina/ Silica >2%)	Organics only	Alumina / silica (<2%) + organics	Inorganics (incl. Alumina/ Silica >2%) + organics
Constituent particle size			Median >100 nm (<50% of <100nm particles by number)			
Loss on drying (105°C, 3 hours)	≤0.5%	≤0.5%	≤0.5%	≤0.5%	≤2.0%	≤0.5%
Loss on ignition on a volatile matter free basis (800°C)	≤1.0%	≤1.0%	≤1.5%	≤21%	≤11%	≤2.5%
Total alumina and silica	Total ≤0.5%	≤2.0%	≤8%	Total ≤0.5%	≤2.0%	≤8%
Matter soluble in 0.5 N HCl	≤0.5%	≤ 1.5%	≤2%	≤0.5%	≤1.5%	≤2%
Matter soluble in 0.5 N HCl on the basis of the product as sold	N/A	N/A	≤2%	≤1.5%	≤5.0%	≤4.0%
Water soluble matter	≤0.5%	≤0.5%	≤1%	≤0.5%	≤4.0%	≤1%
Cadmium*	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg
Antimony*	≤2 mg/kg	≤2 mg/kg	≤2 mg/kg	≤2 mg/kg	≤2 mg/kg	≤2 mg/kg
Arsenic*	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg
Lead*	≤10 mg/kg	≤10 mg/kg	≤10 mg/kg	≤10 mg/kg	≤10 mg/kg	≤10 mg/kg
Mercury*	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg	≤1 mg/kg

*After an extraction with 0.5 N HCl

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
Table 1.1 Proposed specifications for titanium dioxide cosmetics grades

The full detailed formula compositions of the 44 pigmentary titanium dioxide grades are reported in Annex A *"Formula Composition and coatings of the Pigmentary and Nano titanium dioxide grades"* - Table 3.1.4.A3: and Table 3.1.4.A4.

Coating of Pigmentary titanium dioxide grades

The full information on the coatings of the pigmentary grades is given in Annex A "Formula compositions and coatings of the pigmentary and nano titanium dioxide grades":

- for the composition, in Table 3.1.4.A5,
- for the multilayer sequence, in Table 3.1.4.A6

Among the 44 pigmentary titanium dioxide grades, the following 13 pigmentary titanium dioxide grades are reported to be uncoated:
RM01, RM02, RM03, RM04, RM26, RM28, RM67, RM67b, RM68, RM69, RM69b, RM70c, RM72c.

Surface Contamination (Pigmentary grades)

A surface contamination by TMP (trimethylolpropane or 2-Ethyl-2-(hydroxymethyl) propane-1,3-diol) is noted for the two following pigmentary grades: RM72i, RM72j-bis. No surface contamination has been reported for the other pigmentary grades.

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
Table from Page 9/28 - Column N9.4) Surface Contamination

From Applicant
Nano Grades

Nano titanium dioxide grades for use as UV filters must meet the following criteria (SCCS Opinion 1516/13 (2014), CPR Annex VI entry 27a):

- purity >99%,
- rutile form, or rutile with up to 5% anatase, with crystalline structure and physical appearance as clusters of spherical, needle, or lanceolate shapes,
- median particle size based on number size distribution >30nm[5]
- aspect ratio from 1 to 4.5, and volume specific surface area <460m^2/cm^3,
- coated with Silica, Hydrated Silica, Alumina, Aluminium Hydroxide, Aluminium Stearate, Stearic Acid, Trimethoxycaprylylsilane, Glycerin, Dimethicone, Hydrogen Dimethicone, Simethicone; or coated with one of the following combinations:
- Silica at a maximum concentration of 16% and Cetyl Phosphate at a maximum concentration of 6%,
- Alumina at a maximum concentration of 7% and Manganese Dioxide at a maximum concentration of 0.7%,
- Alumina at a maximum concentration of 3% and Triethoxycaprylylsilane at a maximum concentration of 9%,
- photocatalytic activity <10 % compared to corresponding non-coated or non-doped reference, nanoparticles are photostable in the final formulation.

Nano TiO_2 typically complies with USP and FDA criteria (21 CFR 73.1575) required for attenuation grades which are (all tests conducted on uncoated, untreated material):

- Titanium dioxide contains not less than 99% and not more than 100.5% TiO_2
- Loss on ignition (at 800°C) <13%
- Water soluble substances <0.25%
- Acid soluble substances <0.5%
- Arsenic (HCl soluble) <1ppm
- Lead (HCl soluble) <10ppm
- Antimony (HCl soluble) <2ppm
- Mercury <1ppm

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf

The full formula compositions of the 40 nano titanium dioxide grades are noted in Annex A *"Formula compositions and coatings of the pigmentary and nano titanium dioxide grades"* – in Table 3.1.4.B1 and Table 3.1.4.B2.

[5] From Applicant: Note According to a previous SCCS Opinion (SCCS/1516/13) "…whilst primary particle size may be smaller (around 10 nm), the median particle size of TiO_2 nanomaterials in a cosmetic formulation must not be smaller than 30 nm in terms of number-based size distribution". This median measurement is based on I) CPS, II) Lumisizer and III) DLS particle size distribution measurements only. SEM or TEM measurements with median particle size based on number size distribution <30nm, are not in contradiction to the Cosmetic Products Regulation (EC) No 1223/2009 and fully in line with the SCCS opinion and science-based expectations.

As reported in the Table 3.1.4.B2, the TiO_2 content ranges from 99.0% up to more than 99.9%. The loss on ignition is noted to be less or equal to 13% (RM09, RM10, RM11, RM64, RM65, RM75, RM76, RM78, RM79, RM80). The lowest loss on ignition is equal to 0.1% (RM81)

Coatings of the nano titanium dioxide grades

The 40 nano titanium dioxide grades are coated with Silica, Hydrated Silica, Alumina, Aluminium Hydroxide, Aluminium Stearate, Stearic Acid, Trimethoxycaprylylsilane, Glycerin, Dimethicone, Hydrogen Dimethicone, Simethicone; or coated with one of the following combinations:
- Silica at a maximum concentration of 16% and Cetyl Phosphate at a maximum concentration of 6%,
- Alumina at a maximum concentration of 7% and Manganese Dioxide at a maximum concentration of 0.7%,
- Alumina at a maximum concentration of 3% and Triethoxycaprylylsilane at a maximum concentration of 9%,

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf -

The full detailed information on the coatings of the nano titanium dioxide grades are reported in Annex A *"Formula compositions and coatings of the pigmentary and nano titanium dioxide grades"*:
- for the composition, in Table 3.1.4.B3,
- for the multilayer sequence, in Table 3.1.4.B4

For the 40 nano Titanium dioxide grades for which the coating Section has been reported as applicable by Applicant, all the 40 nano Titanium dioxide grades are reported to be coated.

Surface Contamination (Nano grades)
No surface contamination has been reported for any nano grades.

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
Table from Page 17/28 - Column 9.4) Surface contamination

Dispersing agents / Additives (Nano grades)
For the nano grade RM77, Sodium Hexametaphosphate as dispersing agent and Phenoxyethanol, Sodium Methylparaben as additive have been reported.

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
Table from Page 14/28 - Column N2.7) Dispersing agents and Column N2.8) Additives

Doping (Nano grades)
The following nano grades are doped with 1000 ppm Fe: RM 75, RM 76, RM77, RM80. The RM66 nano grade is doped with Manganese (< 1%).

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
Table from Page 14/28 – Column "N2.5) Doping material"

Table 3.1.4.: Summary of the information on the outermost layer for the pigmentary and the nano titanium dioxide grades (noted by SCCS)

Outermost Layer	**Pigmentary grades* (Product Code)**	**Nano grades** (Product Code)**
No surface treatment	RM01, RM02, RM03, RM04, RM26, RM28, RM67, RM67b, RM68, RM69, RM69b	
No surface treatment (silica is separate processing aid)	RM70c, RM72c	/
Silica	/	RM09 (10%), RM74d (20%), RM78 (17%)
Hydrated silica	RM31 (5.0%)	RM47 (30%)
Al_2O_3	RM06 (1.3%)	RM77 i), RM81 (6%)
Aluminium hydroxide	RM30 (2.3%), RM37 (3.7%) RM72i (0 – 5%)	RM41 (13.5%), RM45 (17%), RM46 (10.5%), RM55 (3.0%), RM59 (11%)
Manganese dioxide	/	RM80 (1%)
Glycerin	RM05 (0.6%), RM08 (0.6%), RM19 (0.3%)	/
Triethoxycaprylylsilane	RM07 (0.8%), RM70a (5%) RM70b (5%), RM72a (< 5%) RM72b (< 5%), RM72j-bis (< 6%)	RM74c (6%)
Methicone	RM27 (2%)	/
Dimethicone	RM36 (3.8%), RM39 (1.0%)	RM11 (3%), RM44 (15.4%), RM58 (2.9%), RM74e (6%), RM82 (2.0 – 4.5%)
Hydrogen Dimethicone	RM29 (1.5%), RM35 (2.0%)	RM10 (11%), RM43 (5.7%), RM51 (3.4%), RM52 (4.7%), RM57 (1.9%), RM61 (2.0%) RM74a (< 10%)
Simethicone	/	RM75 (2%)
Algin	RM32 (9.1%)	/
Stearic Acid	/	RM40 (20%), RM42 (11%), RM48 (8.0%), RM49 (13%), RM53 (15%), RM60 (4.7%) RM56 (4.0), RM62 (4.7%), RM63 (13.5%), RM64 (6.5%), RM65 (4.6%), RM74b (15% max), RM76 (10%)
Isostearic Acid	RM33 (3.8%), RM38 (1.0%)	/
Isopropyl Titanium Triisostearate	RM72e (0 – 5%)	/
Phytic Acid	RM72f (0 – 5%)	/
Hexadecyl dihydrogen phosphate	/	RM79 (6%)
Lauroyl Lysine 4.8%	RM34	/

Sodium Glycerophosphate	RM70e (< 5%)	/
Hydrogenated Lecithin	RM70f	/
Tocopherol	RM72d (0 – 5%)	/
Arginine	RM72g (0 – 5%)	/
Rosa Damascena Flower Cera	RM70d (0 – 5%)	/
Aloe Barbadensis Leaf Extract	RM72k (1% max)	/

* From **Ref.:** Multi-layer coating sequence – Pigment.xls – 30 June 2023
** From **Ref.:** Multi-layer coating sequence – Nano.xls – 30 June 2023

3.1.5 Impurities / accompanying contaminants

From Applicant

The Applicant have provided the impurity profiles of the Raw materials on the Water-soluble substances (%), Acid-soluble substances (%), Arsenic (HCl-soluble) (mg/kg), Lead, (HCl-soluble) (mg/kg), Antimony (HCl-soluble) (mg/kg), Mercury (HCl-soluble) (mg/kg), Cadmium (HCl-soluble) (mg/kg).
This information is discussed and reported in Annex B *"Impurity profile of the Raw Materials – Pigmentary and Nano Titanium Dioxide Grades"*:

- for pigmentary titanium dioxide grades in Table 3.1.5 - A: Pigmentary grades – Impurity Profile of Raw Materials.
- for nano titanium dioxide grades in Table 3.1.5 - B: Nano grades – Impurity profile of Raw materials.

Based on the information provided by Applicant, the SCCS has summarised maximum impurities levels in the following Table 3.1.5.

Table 3.1.5.: Impurities for Pigmentary and Nano Titanium dioxide grades.

Impurities	Pigmentary grades	Nano grades
Water soluble substance	≤ 0.5%	<0.25%
Acid soluble substance	≤ 1.5%	<0.5%
Arsenic (HCl-soluble)	≤ 1 mg/kg	<1ppm
Lead (HCl-soluble)	≤ 10 mg/kg	<10ppm
Antimony (HCl-soluble)	≤ 2 mg/kg	<2ppm
Mercury (HCl-soluble)	≤ 1 mg/kg	<1ppm
Cadmium (HCl-soluble)	≤ 1 mg/kg	not provided

3.1.6 Solubility

From Applicant
Insoluble in water and organic solvents

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf

3.1.7 Partition coefficient (Log Pow)

From Applicant

The information provided by Applicant on the Partition coefficient is reported in Annex C *"Partition Coefficient – Pigmentary and Nano titanium dioxide grades"*:

- For the pigmentary titanium dioxide grades: Table 3.1.7.A
- For the nano titanium dioxide grades: Table 3.1.7.B

Table 3.1.7. Summary of the information provided by Applicant related to partition coefficient (prepared by the SCCS)

	Pigmentary grades	**Nano grades**
n/a (no organic components):	16 grades: RM01, RM02, RM03, RM04, RM26, RM28, RM30, RM31, RM37, RM67, RM67b, RM68, RM69, RM69b, RM70c, RM72c.	/
Hydrophilic	4 grades: RM04, RM05, RM19, RM72f,	11 grades: RM09, RM41, RM45, RM46, RM47, RM55, RM59, RM74d, RM77, RM78, RM80
Hydrophobic	13 grades: RM27, RM29, RM33, RM34, RM35, RM38, RM70d, RM70e, RM70f, RM72d, RM72e, RM72g, RM72k.	26 grades: RM10, RM11, RM40, RM42, RM43, RM44, RM48, RM49, RM51, RM52, RM53, RM56, RM57, RM60, RM61, RM62, RM63, RM64, RM65, RM74a, RM74b, RM74c, RM74e, RM76, RM79, RM82
Amphiphylic	/	2 grades: RM75, RM81
K_{OW} Measured – Calculated*	9 Grades: RM07: 9* at 20°C, RM08: - 1.75* at 25°C, RM32: - 2.6 – 1.9, RM36, RM39: 2.6 – 4.3, RM72a, RM72b: 1.1 at 20°C, RM72i: - 0.47 at 26°C, RM72j-bis: 3.9 at 20°c	RM58: 2.6 – 4.3.

3.1.8 Additional physical and chemical specifications

3.1.8.1. Organoleptic properties (colour, odour, taste if relevant)

i) Pigmentary Grades: White Odourless Tasteless

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final Table from Page 7/28 – Column 6.2) Organoleptic properties

ii) Nano grades

/

3.1.8.2. Melting point

Rutile: > 1800°C
Anatase: Does not melt but transforms to rutile (MP >1800°C)

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final

3.1.8.3. Boiling point

/

3.1.8.4. Flash point

Not applicable

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final

3.1.8.5 Vapour pressure

/

3.1.8.6. Density

From Applicant

The information on the density, porosity pour density and tap density has been reported by Applicant. The information has been noted in Annex D *"Density of the pigmentary and nano titanium dioxides grades"*:

- for the pigmentary titanium dioxide grades: Table 3.1.8.6.A
- for the nano titanium dioxide grades: Table 3.1.8.6.B

Table 3.1.8.6.: Summary table of the density, porosity, pour density and tap density for the pigmentary and nano titanium dioxide grades (formulated by the SCCS based on the information from Tables 3.1.8.6.A and 3.1.8.6.B in Annex D)

	Pigmentary grades	**Nano grades**
Density (g/cm^3)	3.62 (RM27) to 4.34 (RM28)	2.51 (RM44) to 4.26 (RM82)
Porosity	1.01 (RM33) to 2.27 (RM31) Not reported: RM19, RM67, RM67b, RM68, RM69, RM69b, RM70a, RM70b, RM70c, RM70d, RM70e, RM70f, RM72a, RM72b, RM72c, RM72d, RM72e, RM72f, RM72g, RM72i, RM72j-bis, RM72k	1.20 (RM78) to 3.22 (RM57) Not reported: RM74a, RM74b, RM74c, RM74d, RM74e
Pour Density (g/cm^3)	0.31 (RM 31) to 1.11 (RM72j-bis) Not reported: RM70a, RM70b, RM72g.	0.10 (RM78) to 0.63 (RM64)
Tab density (g/cm^3)	0.595 (RM01) to 1.80 (RM39) Not reported: RM19, RM67, RM67b, RM68, RM69, RM69b, RM70a, RM70b, RM70c, RM70d, RM70e, RM70f, RM72a, RM72b, RM72c, RM72d, RM72e, RM72f, RM72g, RM72i, RM72j-bis, RM72k	0.12 (RM78) to 0.99 (RM57) Not reported: RM74a, RM74b, RM74c, RM74d, RM74e.

3.1.8.7. Viscosity

/

3.1.8.8. pKa

From Applicant
The pKa data is not available. The Applicant proposed to replace this data item with the pH value at isoelectric point.

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf

The value of pH at isoelectric point are reported in Annex E *"pH value at isoelectric point – Pigmentary and Nano titanium dioxide grades".*

3.1.8.9. pH

From Applicant
Typical method: TiO_2 dispersions were prepared by adding the 1 wt. % of TiO_2 powder to deionised water. The dispersions were placed on magnetic stirrer (1500 rpm) for 15 minutes at ambient temperature to ensure that the powder is fully dispersed. The pH is measured using a pH meter calibrated with standard buffers prior to use.

Ref.: CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

The pH values are reported in Annex F *"pH values – Pigmentary and Nano Titanium dioxide grades"*:
- For the pigmentary grades: Table 3.1.8.9.A
- For the nano grades: Table 3.1.8.9.B.

Table 3.1.8.9. Summary of the pH values (noted by the SCCS)

	Pigmentary grades	**Nano grades**
pH	3.9 (RM04, RM72j-bis) to 8.5 (RM03, RM08). Not reported: RM07, RM27, RM29, RM33, RM34, RM35, RM36, RM38, RM39, RM70d, RM72.	4.1 (RM74b) to 9.0 (RM45) Not reported: RM10, RM11, RM40, RM42, RM43, RM44, RM48, RM49, RM51, RM52, RM53, RM56, RM57, RM58, RM60, RM61, RM62, RM76, RM82

3.1.8.10. Refractive index

/

3.1.8.11. UV/visible light absorption spectrum

From Applicant
The information provided by Applicant on the UV/visible light absorption spectrum are reported in Annex G *"UV/Visible light absorption spectrum"*:
- For the pigmentary grades: Table 3.1.8.11.A
- for the nano grades: Table 3.1.8.11.B

Table 3.1.8.11. Summary of the UV absorption values for the pigmentary and the nano titanium dioxide grades as a function of the wavelengths (formulated by the SCCS based on Tables 3.1.8.11.A and 3.1.8.11.B)

UV Absorption	Pigmentary grades	Nano grades
At 308 nm	5.7 (RM01) to 90 (RM07)	9.07 (RM82, RM01) to 92 (RM09, RM10)
At 360 nm	7.2 (RM02) to 88 (RM08)	10 (RM45) to 85 (RM09)
At 400 nm	4 (RM38) to 89.9 (RM01)	2 (RM44) to 62.99 (RM82)

3.1.8.12. Photocatalytic Activity

The information provided by Applicant on the photocatalytic activity is reported in Annex H *"Photocatalytic activity – pigmentary and nano titanium dioxide grades"*.

- For pigmentary grades: Table 3.1.8.12.A
- For Nano grades: Table 3.1.8.12.B

Nano grades:
The photocatalytic activity compared to the uncoated / undoped material is ranging from 0.019 % (RM63) to less or equal to 10% (RM09, RM10, RM11, RM74a, RM74b, RM74c, RM74d, RM74e, RM75, RM76, RM77, RM78, RM80, RM81, RM79, RM82).

3.1.8.13. RedOx Potential

From Applicant:
Redox potential is not a differentiating characteristic between different titanium dioxide materials. The range of redox potentials measured across the most diverse grades which were selected was only 323-406mV and the measurement variability was up to +/-10mV on each sample tested.

Ref.: Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024)

The RedOx potential values are reported in Annex I *"RedOx potential – pigmentary and nano titanium grades"*:

- For the pigmentary grades: see Table 3.1.8.13.A
- For the nano grades: see Table 3.1.8.13.B

Pigmentary grades
Among the 44 pigmentary grades, the redox potential has been measured for 5 grades RM01; 377 mV, RM28; 325 mV, RM30; 406 mV, RM31; 323 mV, RM70a; 349 mV. For RM70e, it is noted as not measurable, too hydrophobic.

Nano grades
Among the 40 nano grades, the redox potential has been measured for 2 grades: RM09; 350 mV, RM41; 300 mV.

SCCS comments
No information on the RedOx potential has been provided for 39 pigmentary grades or for 38 nano grades.

3.1.9. Particle Shape, particle size and distribution

From Applicant

Data on primary particle size of Pigmentary Titanium Dioxide Raw Materials for Cosmetics measured by Scanning Electron Microscopy (SEM) was submitted by Cosmetics Europe to the SCCS in March 2023. Additional data on primary particle size has now been generated using Transmission Electron Microscopy (TEM) at the request of the SCCS.

From **Ref.:** PS TEM Pigment - Annexes 9 and 10 (April 2023)

The methods used by Applicant for the determination of the Primary Particle Size Distribution and Shape by SEM – Applicant #1 method (used for Pigmentary Titanium Dioxide), by SEM – Applicant #2 method (used for Nano Titanium Dioxide) and by TEM have been reported (see related Annex K *"Measurement methods – Appendix 1, 2 and 3*)".

The method used by Applicant for the determination of Secondary Particle Size Distribution (Aggregates/Agglomerates) by Disc Centrifuge has been reported (see related Annex K *"Measurement methods – Appendix 4")*

From **Ref.:** Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third Package - Report 2 (31 March 2023)

3.1.9.1 Particle shape, Aspect ratio

The full sets of data provided by Applicant, related to the particle shapes and the aspect ratio values, are reported in Annex L *"Particle shape, Aspect Ratio – Pigmentary and nano titanium dioxide grades"*

- For the pigmentary grades: see Table 3.1.9.1.A1 (SEM observations) and Table 3.1.9.1.A2 (TEM observations).
- For the nano grades: see Table 3.1.9.1.B1

Table 3.1.9.1. Summary of the shape and aspect ratio for the pigmentary and nano titanium dioxides grades (SEM and TEM observations) (formulated by SCCS based on Tables 3.1.9.1.A1 and 3.1.9.1.B1)

	Pigmentary grades	**Nano grades**
Shape	**Spheroidal (SEM, TEM):** all grades	**Spheroidal** (RM09, RM11, RM55, RM56, RM57, RM58, RM59, RM60, RM61, RM62, RM64, RM65, RM74a, RM74b, RM74c, RM74d, RM74e, RM78, RM81, RM82) **Lanceolate** (RM10, RM40, RM41, RM42, RM43, RM44, RM45, RM46, RM47, RM48, RM49, RM51, RM52, RM53, RM63, RM75, RM76, RM77, RM79, RM80)
Aspect ratio (SEM)	**1.25** (RM05, RM06, RM07, RM19, RM26, RM32, RM67, RM70b, RM70c, RM70e, RM70f) **to 1.33** (RM37, RM38)	**/**
Aspect ratio (TEM)	**1.20** (RM01) **to 1.55** (RM37)	**1.4** (RM60, RM62) **to 4.4** (RM75)

% particles (number based) with aspect ratio larger than 3		0.4 % (RM09) to 78.8% (RM75)

3.1.9.2. Particle size and distribution

Pigmentary titanium dioxide grades

High Resolution Transmission Electron Microscopy Investigation (HR-TEM)

For the pigmentary titanium grades, some typical high resolution TEM images (HR-TEM) of pigmentary grades that illustrate particular features for the different categories are shown (see Annex J *"HR TEM and TEM images"*):

- Category a / pigmentary *(Surface of Untreated Titanium Dioxide)*: Anatase RM01, Rutile RM02
- Category b1 / pigmentary *(Surface of Titanium Dioxide Treated with Low Levels of Inorganics (<2% Alumina and/or Silica) only)*: RM 30 - Rutile treated with 0.3% Alumina and 2.3% Aluminium Hydroxide
- Category b2 / pigmentary *(Rutile treated with 0.3% Alumina, 2.3% Aluminium Hydroxide and 5% Hydrated Silica)*: RM31 - Rutile treated with 0.3% Alumina, 2.3% Aluminium Hydroxide and 5% Hydrated Silica.
- Category c1 / pigmentary (Surface of Titanium Dioxide Treated Only with Organics): RM70f - Anatase with <5% Hydrogenated Lecithin
- Category c2 / pigmentary *(Surface of Titanium Dioxide Treated with Low Levels of Inorganics (<2% Alumina and/or Silica) and also with Organics):* RM 35 -Rutile treated with 0.3% Alumina 0.3%, 2.2%vAluminium Hydroxide, 2% Hydrogen Dimethicone 2.0% (RM35)
- Category c3 / pigmentary *(Surface of Titanium Dioxide Treated with Inorganics (Including >2% Alumina and/or Silica) and with Organics Added)*: RM38 - Rutile treated with 0.2% Alumina, 3.7%, Aluminium Hydroxide, 0.4%, Zinc Oxide and 1% Isostearic Acid.

Ref.: CE Cons TD_Phys-chem second data package_23 03 2023.pdf

Pigmentary titanium dioxide grades

Transmission electron Microscopy Investigations (TEM)

TEM images have been provided for every pigmentary grade analysed ((see Annex J *"HR-TEM and TEM images"*)

Ref.: CE Cons TD_Phys-chem second data package_Annex 1 and 2_Pigment_23 02 2023.pdf

Pigmentary titanium dioxide grades

Constituent particles sizes, agglomerates / aggregates sizes, % nano, aspect ratio

The full-size distribution of all the various pigmentary titanium grades have been provided by Applicant.

The two sets of data provided, related to the particle sizes, are reported in Annex L *"Particle shape, Aspect Ratio – Pigmentary and nano titanium dioxide grades":*

- Table 3.1.9.1.A1: Constituent particle sizes determined by SEM expressed by number and by mass, % nano and aspect ratio determined by SEM, particle size of agglomerates / aggregates measured by CPS DC expressed by mass and by number.
- Table 3.1.9.1.A2: The data related to the Primary particle sizes and aspect ratio values determined by TEM are reported.

Table 3.1.9.2.A3. Summary of the constituent particle sizes (mean and median, $Feret_{min}$), % nano (size below 100 nm, number based) determined by SEM and TEM observations (formulated by the SCCS, based on Tables 3.1.9.1.A1 and 3.1.9.1.A2 from Annex L)

Pigmentary grades constituent Particles	Mean size Particle size	Median Size Particle size	% nano
SEM	108 - 388 nm	103 - 360 nm	0.0 - 45.9%
TEM	88 - 427 nm	85 - 406 nm	0.0 - 66.7%

Table 3.1.9.2.A4. Summary of the agglomerate / aggregate sizes of the Titanium pigmentary grades (mass and number based) (formulated by the SCCS, based on Tables 3.1.9.1.A1 and 3.1.9.1.A2 from Annex L)

Pigmentary grades Agglomerates / Aggregates	Mean size (Mass based)	Median Size (Mass based)	Mean size (Number based)	Median Size (Number based)
CPS DC	408 – 1295 nm	309 – 979 nm	101 – 874 nm	166 – 550 nm

Comparison of the size distribution (% nano) obtained by SEM and TEM observations and measurements (RM26 and RM67)

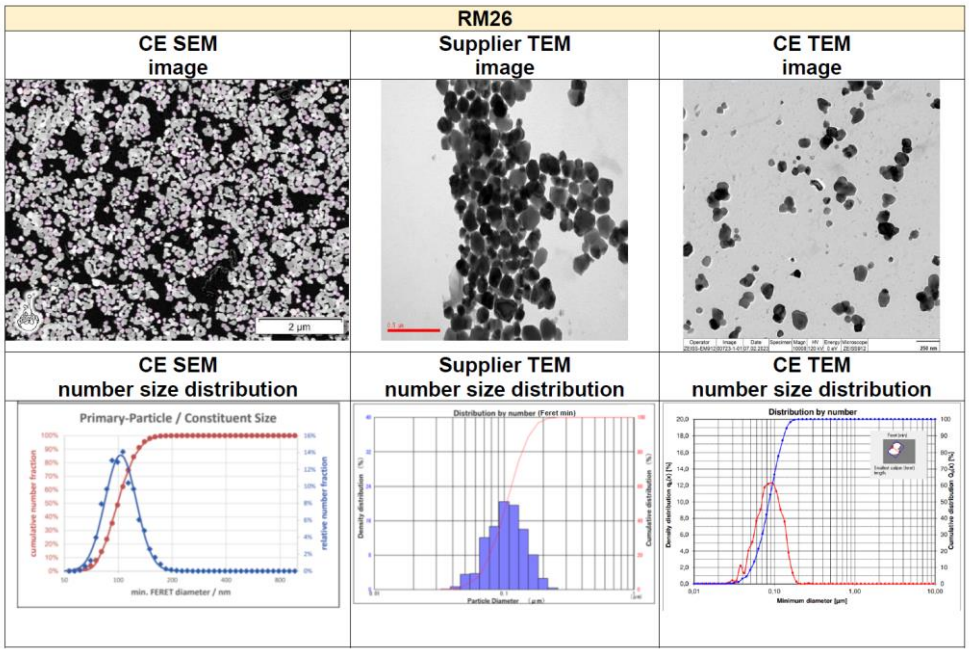
From Applicants

It can be noted that some differences are found between the data generated using different methods due to the dispersion protocols used to prepare the samples for imaging, the nature of the imaging methodology and the software used for image analysis. This can give rise to different categorisations where materials are close to a categorisation threshold e.g., the definition of a nanomaterial as >50% of primary particles <100nm. All the samples for which significant differences are found are anatase which is less robust than rutile and it is even possible that the more aggressive rubout technique has caused some crystal damage resulting in generation of fine fragments.

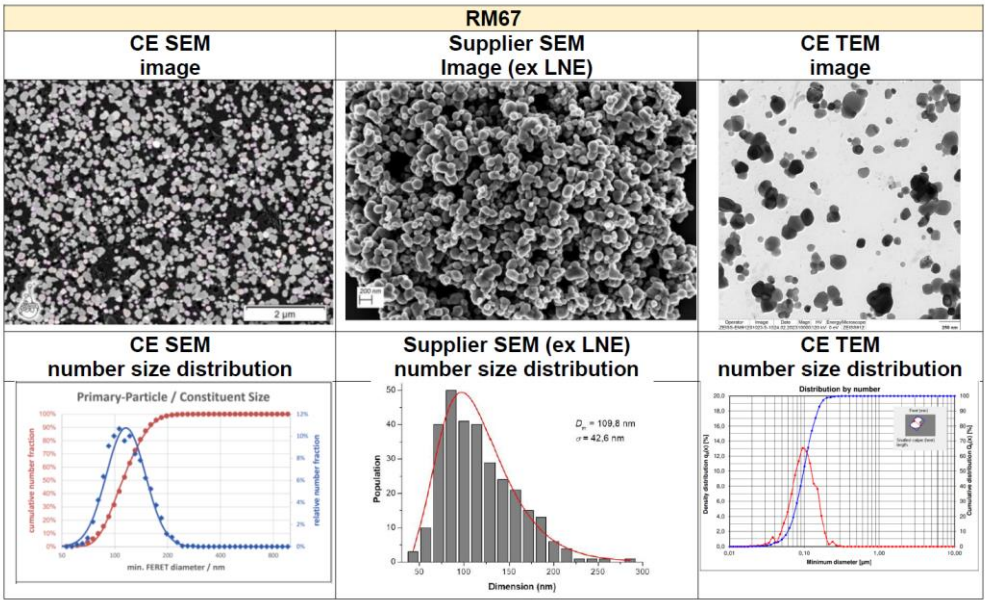
There are six samples that give differing results, but they derive from only two base titanium dioxide materials. RM26 is treated with dimethicone to make RM27; RM67 is treated with triethoxycaprylylsilane to derive RM70a and RM70b, and RM70c is RM67 additioned with nano silica as a processing aid.

Comparison of the images and the data derived from analysis of the images is shown below for the base pigmentary materials, RM26 and RM67, and demonstrates the differences that can arise. Hence it is important to consider more than one protocol and method before reaching a definitive conclusion.

In each case, as two methods show that the % of primary particles <100nm is <50%, these materials would not be categorised as nanomaterials under the EU recommendation 2011/696.



		Primary Particle Size by number (Feret min)		
Product Code	**Measurement Method**	**Mean size [nm]**	**Median size [nm]**	**%Nano % by number < 100 nm**
RM26	CE SEM data	106	103	45.9%
	Supplier TEM data	109	104	45.2%
	CE TEM data	88	85	66.7%



		Primary Particle Size by number (Feret min)		
Product Code	**Measurement Method**	**Mean size [nm]**	**Median size [nm]**	**%Nano % by number < 100 nm**
RM67	CE SEM data	120	115	30.5%
	Supplier SEM data ex LNE	110	102	48%
	CE TEM data	101	96	53.2%

From **Ref.:** PS TEM Pigment – Annexes 9 and 10.pdf (April 2023)

SCCS comments

For the RM26 pigmentary grade:

- the SCCS noted a significant difference for the % nano between the two TEM data provided: 45.2% and 66.7% from Supplier TEM data and Ce TEM data, respectively
- The SCCS noted the difference between % nano (number-based) measured by SEM (45.9 %) and TEM (45.2 up to 66.7%)

For the RM67 pigmentary grade:

- the SCCS noted a significant difference for the % nano between the two SEM data provided: 30.5% and 48% from CE SEM data and Supplier SEM Data, respectively.
- the % nano provided by CE TEM data is higher than 50.0% (53.2%). Therefore, the SCCS does not agree with the sentence from Applicants: "In each case, as two methods show that the % of constituent particles <100nm is <50%, these materials would not be categorised as nanomaterials under the EU recommendation 2011/696."

Nano titanium dioxide grades

High Resolution Transmission Electron Microscopy Investigation (HR-TEM)

Some typical high-resolution TEM (HR-TEM) images for nano grades have been provided by Applicants (for detailed images, see Annex J *"HR-TEM and TEM images"*):

- Surface of Nano Titanium Dioxide Treated with Inorganics: RM60 – Nano Titanium dioxide 91.2%, Aluminium Hydroxide 4.1%, Stearic Acid 4.7%, RM74d – Nano Titanium Dioxide with Silica coating.
- Different morphologies are studied for the following grades: RM46, RM53, RM59, RM62, RM78, RM74d.
- Further HR-TEM images show that a variety of morphologies and sizes can be produced by a single process (Sulfate Process) and the same is true of the Chloride Precipitation Process (RM63, RM64).

Ref.: CE Cons TD_Phys-chem second data package_23 03 2023.pdf

Nano titanium dioxide grades

Transmission electron Microscopy Investigations (TEM)

TEM images have been provided for every nano titanium grade analysed (see Annex J *"HR-TEM and TEM images"*)

Ref: CE Cons TD_Phys-chem second data package_Annex 3 and 4_Nano_23 02 2023.pdf

Nano titanium dioxide grades:

Constituent particle sizes, agglomerates / aggregates sizes

The full-size distribution curve of the various nano titanium dioxide grades has been provided by Applicants.

The full set of data related to the particle sizes of the nano titanium dioxide grades (constituent particles, agglomerates/aggregated particles) is reported in Annex L – Table 3.1.9.1.B1.

Table 3.1.9.2.B2. Summary of the constituent particle sizes (mean and median) for nano titanium dioxide grades (TEM observations and measurements), (formulated by SCCS, based on Tables 3.1.9.1.B1 from Annex L)

Nano grades Constituent Particles	**Mean size Particle size (by number)**	**Median Size Particle size (by number)**
TEM	10 – 86 nm	9 – 81 nm

Table 3.1.9.2.B3. Summary of the mean and the median ranges of agglomerates / aggregates of the nano titanium dioxide grades determined by CPS DC

Mean size (number)	**Median size** (number)	**Mean size** (mass)	**Median size** (mass)
46 – 168 nm	43 - 162 nm	118 - 1156 nm	59 – 832 nm

3.1.9.3. Aerodynamic diameter

The information on Aerodynamic diameter provided by Applicants have been reported in Annex M *"Aerodynamic diameter – Pigmentary and Nano titanium dioxide grades"*:

- For the pigmentary grades: see Table 3.1.9.3.A
- For the nano grades: see Table 3.1.9.3.B

Table 3.1.9.3. Summary of the Aerodynamic diameter (%<10 µm) as a function of the nano titanium dioxide grades (formulated by SCCS based on Tables 3.1.9.3.A and 3.1.9.3.B. from Annex M)

Aerodynamic diameter	**Pigmentary grades**	**Nano Grades**
0% below 10 µm	RM03, RM04, RM05, RM07, RM08, RM30, RM32	RM40, RM78, RM79, RM81
Less than 1% below 10 µm	The other 37 grades	The other 36 grades

3.1.9.4. Surface (SSA, VSSA)

The information provided by Applicants on the Specific Surface Area (SSA) and Volume Specific Surface Area (VSSA) have been reported in Annex N *"Specific Surface Area (SSA) and Volume Specific Surface Area (VSSA) – Pigmentary and Nano titanium dioxide grades"*:

- For the pigmentary grades: see Table 3.1.9.4.A
- For the nano grades: see Table 3.1.9.4.B

Table 3.1.9. Summary of the information related to constituent particles sizes (SEM/TEM), Aspect ratio, % Nano, Agglomerates/Aggregates sizes (CPS DC) (formulated by the SCCS)

		Pigmentary grades	Nano grades
Constituent particle (SEM / TEM)	**Mean size** (by number)	88 - 427 nm (TEM) 108 - 388 nm (SEM)	10 – 86 nm (TEM)
	Median size (by number)	85 - 406 nm (TEM) 103 - 360 nm (SEM)	9 – 81 nm (TEM)
	Aspect ratio	1.20 – 1.55 (TEM) 1.25 – 1.33 (SEM)	1.4 – 4.4 (TEM)
	% AR > 3	/	0.4 - 78.8% (TEM)
	% Nano	0.0 - 66.7% (TEM) 0.0 - 45.9% (SEM)	100% (TEM)
Agglomerates / Aggregates (CPS DC)	**Mean size** (by number)	101 – 874 nm	46 – 168 nm
	Median size (by number)	166 – 550 nm	43 - 162 nm
	Mean size (by mass)	408 – 1295 nm	118 - 1156 nm
	Median size (by mass)	309 – 979 nm	59 – 832 nm
	Specific Surface Area	2 - 15.8 m^2/g	8 - 117 m^2/g
	Volumic Specific Surface Area	8 - 68.4 $m^2.cm^3$	34 - 402 $m^2.cm^3$

3.1.9.5. Surface Components / Surface reactivity

From Applicants:
The identity of the surface components and functional groups are not measured but inferred from a knowledge of the chemical moieties that have been used to treat the surface. All surface treatments are cosmetic ingredients that are widely used in cosmetic formulations. Some of the surface species could be determined by methods such as infra-red spectroscopy.

From Ref.: CE response to SCCS Request of 13 June 2023_29062023.pdf

The information related to Surface Components / Surface reactivity provided by Applicants are reported in Annex O *"Surface Components / Surface reactivity – Pigmentary and Nano Titanium dioxide grades"*:

- For the Pigmentary grades: see Table 3.1.9.5.A
- For the Nano grades: see Table 3.1.9.5.B

Table 3.1.9.5. Summary of the information provided for the surface components and/or functional groups for the pigmentary and nano titanium dioxide grades (noted by SCCS based on Table 3.1.9.5.A and Table 3.1.9.5.B from Annex O)

Surface components, functional groups	Pigmentary titanium dioxide grades	Nano titanium dioxide grades
Uncoated	RM01, RM02, RM03, RM04, RM26, RM28, RM67, RM67b, RM68, RM69, RM69b, RM70c, RM72c.	All the 40 nano titanium dioxide grades are coated.
Alkyl chain, Carboxyl group	RM33, RM38	RM40, RM42, RM48, RM49, RM53, RM56, RM60, RM62, RM63, RM64, RM65, RM74b, RM76
-OH	RM26, RM28	RM09, RM77, RM78, RM80
-OH; -PO42-	RM01, RM02, RM03, RM04, RM06, RM67, RM67b, RM68, RM69, RM69b, RM70c, RM72c	
-OH; -(C3H5(OH)3)	RM19	
-OH; -(C3H5(OH)3); -PO42-	RM05, RM08	
Methyl group	RM29, RM35, RM36, RM 39	RM10, RM11, RM43, RM44, RM51, RM52, RM57, RM58, RM61, RM74a, RM74e, RM82
Methyl group, - OH		RM75
Hydroxyl group	RM30, RM31, RM37, RM72i	RM41, RM45, RM46, RM47, RM55, RM59, RM66, RM73, RM74d, RM81
Caprylylsilane group	RM70a, RM70b, RM72a, RM72b	RM74c
Carboxyl group, Hydroxyl group	RM32	
Carboxyl group, Amino group	RM34	
Hydrogenated Lecithin	RM70f	
Hydroxyl, Caprylylsilane:	RM72j-bis	
Cetyl group		RM79
Sodium Glycerophosphate	RM70e	
Phytic Acid, Hydroxyl group	RM72f	
Cocos Nucifera (Coconut) Oil, Aloe Barbadensis Leaf Extract	RM72k	
Sodium Cocoyl Glutamate, Cystine, Lauric Acid, Arginine	RM72g	
Bis-PEG-15 Dimethicone/ IPDI Copolymer, PEG-2-Soyamine, Isopropyl Titanium Triisostearate	RM72e	
Persea Gratissima (Avocado) Oil, Hydrogenated Vegetable Oil, Tocopherol	RM72d	
Rosa Centifolia Flower Wax, Rosa Damascena Flower Cera, Cera Alba	RM70d	

SCCS comments
For the pigmentary and the nano titanium dioxide grades, the Applicant did not provide explanation on the tested media, or on the stability of the surface components.

3.1.10 Homogeneity and Stability

From Applicants
The coating materials are applied to the surface to improve particle dispersion, inhibit or abolish photoactivity and improve compatibility with other ingredients present in cosmetics formulations. The coating materials are not UV absorbers and all these materials are common cosmetic ingredients which are widely used for different purposes in cosmetic products.

Stability of the coating on the particle is important for the technical properties of TiO_2-containing formulas (stability of emulsion, colour, segregation of particles).

Complete stability of coating materials on the TiO_2 particle has been demonstrated with variation in pH, temperature, shear force and time (up to 180 days) in studies previously submitted to the SCCS in 1998 (references 62, 63), in 1999 (references 68 and 72), 2000 (reference 96), 2009 (references 113 and 116) and 2014.
Hence, it can be concluded that the coatings are stable under the conditions and timespan of the *in vitro* tests performed.

Ref.: CE-TiO2-23-003.0 - CE Response to clarifications requested by SCCS 10 03 23 - final

The information on the homogeneity and the stability provided by Applicants is reported in Annex P *"Homogeneity and Stability – Pigmentary and Nano Titanium Dioxide grades"*.

SCCS comments
The provided references (62, 63, 68, 72, 96, 113) are related to the stability studies of just a few specific coatings on TiO_2 particles.
Furthermore, for references (68, 72, 96), no indication has been provided on the size, structure, or shape of the tested coated-TiO_2 particles.
The set of reported data on the stability of the coatings of TiO_2 particles does not cover the full diversity of the coatings listed by Applicants for this Opinion.

3.1.11 Dispersibility

From Applicants
Nanogenotox guidance as well as EFSA guidance are available for methods of dispersion and were used as reference for dispersion of materials in (key) studies. Therefore, the consortium has looked at dispersibility of representative materials in conditions mimicking the ones applied during toxicological testing utilizing both:

- the Nanogenotox protocol (with Bovine Serum Albumin dispersant) (see Annex K "*Measurement methods - Appendix 8"*)
- and the method used for the SCCS evaluation of Titanium Dioxide (nano) with small changes regarding the dispersant and the fact that all material was prewetted to obtain optimal results for both hydrophobic and hydrophilic material (so called by Applicants *"modified SCCS method"*) (see Annex K *"Measurement methods - Appendix 9").*

The results for a representative selection of grades (one from each of the six categories a-c3 for pigments and three nano grades of different polarity) which are highlighted in Annex Q *"Dispersibility"*, Tables 3.1.11.A1 and A2, and Table 3.1.11.B1 and B2 for pigmentary and nano grades respectively.

SCCS comments
The method noted by the Applicants as *"modified SCCS method"* is in fact a specific method developed by the Applicants for providing information relating to the evaluation by the SCCS of a former Titanium dioxide (nano) Dossier (SCCS/1516/13). As such, it is not a method proposed or modified by the SCCS.

Dispersibility of Pigmentary grades
From Applicant
The histograms for particle size (agglomerate / aggregates particles) (both by number and mass) determined using the so-called by Applicants *"modified SCCS method"* have been provided. The particle size data reported by Applicant have been reported in the Table 3.1.11.A1 and in Table 3.1.11.A2 (Annex Q) for the so-called by Applicant *"modified SCCS dispersibility method"* and the "Nanogenotox dispersibility" protocol, respectively.

Table 3.1.11.A3 from Annex Q *"Dispersibility"* compares the particle sizes of TiO_2 cosmetics grades dispersed using the Nanogenotox protocol and the so called by Applicants *"Modified SCCS protocol"* (described in the March 2023 submission) to establish the effect of dispersion energy and measured using CPS DC The median sizes derived using the Nanogenotox protocol are around 10% larger than those obtained using the so-called by Applicant *"modified SCCS protocol"* (difference is even larger for the hydrophobic grade RM70a).

SCCS comments

Among the 44 pigmentary titanium dioxide grades, one pigmentary grade for each of the 6 categories has been tested. RM01 (a), RM30 (b1), RM31 (b2), RM70a (c1), RM05 (c2), RM39 (c3).

The SCCS notes the influence of the so-called by Applicant *"modified SCCS Dispersibility"* and of the "Nanogenotox dispersibility" methods on the particle sizes (mean and median particles sizes by mass and by number), as compared with initial ones reported in Annex Q *"Dispersibility"* - Table 3.1.9.A1. The comparisons are reported in Tables Annex Q *Dispersibility"* - Table 3.1.11.A1: Table 3.1.11.A2.

Dispersibility of Nano grades

From Applicant

The histograms for particle size (agglomerate / aggregates particles) (both by number and mass) determined using the so called by Applicant *"modified SCCS method"* have been provided.

The particle size data provided by Applicant have been reported in Annex Q *"Dispersibility"*, Table 3.1.11.B1 and Table 3.1.11.B2 for the so-called by Applicants *"modified SCCS dispersibility method"* and the Nanogenotox dispersibility protocol, respectively.

In Annex Q *"Dispersibility"*, Table 3.1.11.B3 compares the particle sizes of TiO_2 cosmetics grades dispersed using the Nanogenotox protocol and the so called by Applicants *"Modified SCCS protocol"* to establish the effect of dispersion energy and measured using CPS DC.

The median sizes by number are close for the different protocols (the Nanogenotox protocol sizes always being larger), with the greatest difference being for the hydrophobic sample, RM11. The median sizes by mass are much larger using the Nanogenotox protocol.

All of the nano samples measured are well above the 30nm threshold for secondary particle size set by the SCCS Opinion of 2014, irrespective of the dispersion protocol applied.

Ref.: Dispersibility Nanogenotox – Report, 4th Data Package, 21 April 2023

SCCS comments

It is noted that among the 40 nano-titanium dioxide grades, the dispersibility of 3 nano-grades have been tested: RM09, RM11 and RM75.

The SCCS notes (see in Annex Q *"Dispersibility"* - Table 3.1.11.B1) that the particle sizes reported by the Applicants as being obtained using what they call the *"modified SCCS dispersibility method"*) are the same as the ones corresponding to the initial state provided in Annex Q - Table 3.1.9.1.B1

Stability of the dispersed RM09 and RM11 during the following genotoxicity tests:

- Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)
- Micronucleus Test in Chinese Hamster V79 Cells *in vitro*

From Applicants

RM09:

The stability of the dispersion and the agglomeration/aggregation behavior as well as cellular uptake of the test item were investigated in the parallel study ICCR Study Number 4023311 "RM09: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)" performed under comparable conditions: In the accelerated stability study, it was demonstrated via

dynamic light scattering (DLS) measurements that the test item RM09 showed stable particle sizes without increased aggregation/agglomeration for at least 24 hours.

From **Ref.:** 4023313_final_report.pdf
(RM09: Micronucleus Test in Chinese Hamster V79 Cells in vitro)

RM11:
To reflect the stability of the dispersion and the agglomeration/aggregation behavior of the test material during cell culture exposure in the genotoxicity experiment, particle size determination of the test dispersion using dynamic light scattering (DLS) was performed in the parallel study (ICCR Study Number 4023312 "RM11: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)") as well (external assignment under non-GLP). In the V79/HPRT study, the test item preparation and exposure were performed under comparable conditions, and thus, the results from the TEM and DLS analyses are considered transferable between the two studies.

From **Ref.:** 4023314_final_report
(RM11: Micronucleus Test in Chinese Hamster V79 Cells in vitro)

The DLS measurements of RM09 and RM11 performed in gene mutation assay in Chinese hamster V79 cells *in vitro* (V79 / HPRT) and micronucleus test in Chinese hamster V79 Cells *in vitro* are reported in Annex S.

SCCS comments

Dispersion protocols

For the dispersion protocols used by Applicants for the V79/HPRT tests on RM09 and RM11 and for the parallel DLS study (see Annex S), the SCCS has noted the following parameters in Table 3.1.11. Applicants have provided revised information for the dispersion protocol during the consultation period (Att.1 -CE TiO_2 - Response and Comments - 06.02.2024.pdf (February 2024).

Table 3.1.11. Dispersion protocols parameters for the V79/HPRT tests on RM09 and RM11 and for the parallel DLS study (from **Ref.**: Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024))

	RM09[i)]	**RM11[ii)]**	**Both RM09 and RM11[iii)]**
From Report	**V79/HPRT test**	**V79/HPRT test**	**DLS measurement**
Quantity	0.0126 g (12.6 mg)	18 mg	6 mg
Ethanol	60 uL	90 uL	60 / 90 uL
Volume	11.94 mL	17.9 mL	11.94 / 17.9 mL
Total Volume	**12 mL**	**18 mL**	**12/18 mL**
Probe sonicator	Bandelin SonoPlus Ultraschall Homogenisator HD 2200	Bandelin SonoPlus Ultraschall Homogenisator HD 2200	Sonics Vibra Cell VC505
Power	200 W	200 W	200 W
Duration	32 min	32 min	32 min
Amplitude	10%	10%	10%
Energy	**3200 J/mL ***	**2133 J/mL ***	**3200/2133 J/mL ***

i) from **Ref.:** 4023311_final Report.pdf - Report: RM09: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)
i)* 200 W x (32 x 60 seconds) x 0.1 (amplitude) / 12 mL = 3200 J/mL sample volume (from SCCS)

ii) from **Ref.:** 4023312_final Report Report -RM11: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)
ii)* (200 W x 0.1 (amplitude) x (32 x 60)) / 18 mL = 2133 J/mL sample volume (from SCCS)

iii) from **Ref.:** from **Ref.**: Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024)

SCCS comments:
Dispersion energy value
Considering the above Table, the SCCS has noted that two different values for dispersion energy per volume, 3200 J/mL and 2133 J/mL, have been used for RM09 and RM11, respectively.

Only the dispersion energy used for the gene mutation assays applied to RM11 (*i.e.* 2133 J/mL) is in the range of the typical probe sonication dispersion conditions noted by SCCS between 600 J/mL and 2,500 J/mL sample volume (SCCS/1655/23 - Guidance on the Safety Assessment of Nanomaterials in Cosmetics).
The highest dispersion energy (*i.e.* 3200 J/mL) is 28% higher than the highest range limit of the typical dispersion conditions noted by SCCS in SCCS/1655/23.

Centrifugation step before DLS measurements (not used for preparation of RM09 and RM11 suspensions for the V79/HPRT tests)
The SCCS noted that the centrifugation step was used for DLS measurements but was not applied to the RM09 and RM11 dispersions used for the V79/HPRT tests. This type of centrifugation step introduced a change in the agglomerates/aggregates size distribution by decreasing the number of the larger aggregates and the concentration was modified compared to the original one by extracting the largest agglomerates/aggregates (from Ref. CE response to SCCS Request of 13 June 2023_29062023.pdf).
Therefore, the agglomerates/aggregates size distribution obtained in the parallel accelerated dispersion study is not expected to be representative of the agglomerates/aggregates size distributions of the performed gene mutation assays on RM09 and RM11.

3.2 TOXICOKINETICS

3.2.1 Dermal / percutaneous absorption

SCCS comment from the Opinions SCCS/1583/17 and SCCS/1516/13, 22 July 2013, Revision of 22 April 2014:
The three coated nano-TiO_2 materials under evaluation were tested *in vitro* for dermal/percutaneous absorption using dermatomed pig skin. The SCCS has accepted the results of the studies that have shown that none of the test materials penetrated in any significant amount through the dermatomed pig skin. Imaging of the skin sections using transmission electron microscopy also did not show any nanoparticles of TiO_2 beyond the uppermost layers of the stratum corneum.
The studies and literature information evaluated in the previous SCCS Opinion on coated and uncoated nano forms of TiO2 (SCCS/1516/13, 22 July 2013, Revision of 22 April 2014) indicated that TiO2 nanoparticles do not penetrate (simulated) sunburnt skin. However, it was pointed out that such information on flexed or damaged skin is not available; the evaluated studies were not directed towards hazard identification using either a dose-response approach or a worst-case scenario (overdosing situation), and there were certain knowledge gaps in relation to the possible dermal penetration of nano-TiO2 on repeated or long-term use of cosmetic products, which may not only be used on flexed healthy skin but also on skin that may have lesions or cuts.

3.2.2 Other studies on toxicokinetics

SCCS comment from the Opinions SCCS/1580/16 and SCCS/1516/13, 22 July 2013, Revision of 22 April 2014:
The limited available evidence suggests that if TiO2 nanoparticles become systemically available, they may accumulate mainly in liver with a very slow clearance.

SCCS comments from the Opinions SCCS/1617/20 and SCCS/1583/17:
The information on kinetics and deposition of inhaled TiO_2 in the lungs and other organs is insufficient and therefore a more extensive evaluation of kinetics/deposition of the particles is needed.

3.3 EXPOSURE ASSESSMENT

3.3.1 Function and uses

From Applicant:

Titanium dioxide grades used in cosmetics may be divided into two groups:

- pigmentary grades with median primary particle size >100nm whose primary function is to provide whiteness and opacity as well as some UV protection and
- nano grades with median primary particle size <100nm whose primary function is to provide UV attenuation without excessive whiteness.

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final

The different types of titanium dioxide, product types, target consumers and intended use concentrations in the Cosmetics Europe Titanium Dioxide oral products are presented in the following Table:

Table 3.3.1. Functions and cosmetic uses of titanium dioxide (Pigmentary and Nano grades)

Type of titanium dioxide	Product type	Target consumer	Intended use concentrations
Pigmentary	Toothpaste	Adult, Children	3%
Pigmentary	Lip care or lipstick	Adult, Children	15%
Nano	Lip care or lipstick (with SPF)	Adult, Children	8%

Ref.: SCCS request July 2023_ConsTD resp_16082023.pdf (Augsut 2023)

3.4 TOXICOLOGICAL EVALUATION

In view of the mandated questions, the toxicological evaluation in this Scientific Advice mainly focused on assessment of the available evidence on mutagenicity/genotoxicity. As such, other toxicological aspects that had already been evaluated in the relevant previous SCCS Opinions were not considered in this Scientific Advice.

3.4.1 Mutagenicity / genotoxicity

OVERVIEW OF THE ASSESSMENT BY THE SCCS

In order to conclude on potential genotoxicity of TiO_2 when used in cosmetics products, the SCCS collected and analysed all the available data, either provided by the Applicants or from published scientific literature on the assessment of genotoxic effects of TiO_2 nano- and pigmentary materials both in *in vitro* and *in vivo* models. To this end, the SCCS (re)reviewed the information from the following sources:

1. Several genotoxicity studies on TiO_2 grades used in cosmetics submitted by the Applicant. The analysis is presented in paragraphs "3.4.1.1 Mutagenicity / genotoxicity in vitro" and "3.4.1.2 Mutagenicity / genotoxicity *in vivo*".

The Applicants submitted several file packages between April 2022 – August 2023, containing numerous documents, including the following study reports:

i) IN VITRO:

1. ToxTracker study
2. Gene mutation assay in Chinese Hamster V79 cells in vitro (V79/HPRT) on RM09
3. Gene mutation assay in Chinese Hamster V79 cells in vitro (V79/HPRT) on RM11
4. Micronucleus test in Chinese Hamster V79 cells in vitro on RM09
5. Micronucleus test in Chinese Hamster V79 cells in vitro on RM11
6. Micronucleus test in human peripheral blood mononuclear cells in vitro on E171-E
7. The alveolar macrophage assay
8. MucilAir-Rat-RF

ii) IN VIVO:

9. An *in vivo* study in rats instilled intratracheally with 11 commercial TiO_2 samples (Creutzenberg, 2022)
10. The study in rats exposed by inhalation to nanograde TiO_2 (6 nm), published by Akagi *et al.* (2023).

As the *in vitro* study, reports #7 and #8 did not contain genotoxicity endpoints; the results were not considered by the SCCS in the WoE, and only shortened descriptions of the results were included in the Annex V. The analysis of the second *in vivo* study (Akagi *et al.*, 2023) is included in the analysis of the published literature data in "Annex X. SCCS and EFSA analysis of studies on TiO_2 genotoxicity".

2. The SCCS considered all the evidence that had already been assessed by the SCCS in previous Opinions, and by EFSA in the Opinion on E171 (EFSA, 2021).

3. Other papers published on genotoxicity of TiO_2 particles complementing the analysis performed by the SCCS and EFSA. The SCCS analysis of publications up to April 2023 is presented in paragraph "3.4.1.3. The overall SCCS assessment of the genotoxicity of TiO_2 grades used in cosmetic products".

3.4.1.1 Mutagenicity / genotoxicity *in vitro*

The general conclusions on mutagenicity/genotoxicity study results (both *in vitro* and *in vivo*) of TiO_2 grades used in cosmetics as provided by the Applicant are presented below, as well as in Annex T (Tables 8-12). References to the general conclusions provided by the Applicant are included in the References section of this Scientific Advice.

The general conclusions by the Applicant on mutagenicity/genotoxicity study results of TiO_2 grades used in cosmetic products:

i) The Cosmetic Europe Titanium Dioxide Consortium (Applicants) submitted in May 2022 a report on the human safety evaluation of titanium dioxide (TiO_2) in cosmetics with a focus on genotoxicity for consideration by the SCCS (CE, 2022). That report summarised the findings of a scientific evaluation on the genotoxicity of titanium dioxide used in cosmetic products, thereby addressing in detail the genotoxicity concerns raised by EFSA in its most recent review (2021) without relying on any (pre- or post-2009) *in vivo* genotoxicity studies.
The Applicant's assessment report compliments the scientific work done in parallel by an independent expert panel on the genotoxicity of titanium dioxide which has also been submitted for consideration by the SCCS by the Titanium Dioxide Manufacturer Association (TDMA, 2022). The expert panel conducted a weight of evidence (WoE) assessment of the genotoxicity of titanium dioxide based on all available *in vitro* and *in vivo* data (up to December 2021) irrespective of the titanium dioxide grades. Also, the expert panel review included the available data identified in the EFSA evaluation as well as additional studies available since the initial EFSA review including data generated in industrial and contract research laboratories on behalf of titanium dioxide producers.
This assessment by the expert panel constitutes one of the most comprehensive and up to date reviews of the genotoxicity database for titanium dioxide. The expert panel has recently published their WoE assessment on the genotoxicity of titanium dioxide (Kirkland *et al.*, 2022).
In the expert panel review, datasets from publications and study reports were reviewed for reliability using the ToxR Tool (Schneider *et al.*, 2009) which applies modified Klimisch scores (Klimisch, 1997). The publications and the study reports used in the expert panel review included the most relevant test systems and endpoints, as described in the Guidance Document on Revisions to OECD Genetic Toxicology Test Guidelines (OECD, 2015).
Each study dataset was assigned a modified Klimisch reliability score of 1 (reliable without restrictions), 2 (reliable with restrictions) or 3 (unreliable) using the principles of the ToxR Tool, together with expert judgement. The standard ToxR Tool template was modified to include nanoparticle (NP) characterisation as detailed in Card and Magnuson (2010). They were then reviewed for quality, study design and acceptability of the data using expert judgement (WoE evaluations).
The publication therefore reviewed in detail a total of 192 datasets (*in vitro* and *in vivo* studies) from relevant test systems and endpoints, out of which only those considered of sufficient quality, reliability, and relevance (i.e., "moderate" or "higher" weight based on WoE evaluations) for the assessment of genotoxic hazard (a final total of 34 datasets) were taken into account. The numbers of datasets in the different categories are given in Table 8.
Since the Ames test is not recommended for testing insoluble particles, Ames tests were not included under *in vitro* studies for the final evaluation. *In vitro* studies measuring formation of reactive oxygen species, epigenetic DNA methylation and cell transformation were discussed in the EFSA opinion, but not included in the expert panel review (see table above) since they are considered to provide only supporting information rather than direct evidence of genotoxic effects (OECD, 2015; Expert panel report on genotoxicity, 2022; Kirkland *et al.*, 2022).
As further explored below (*in vitro*, Table 9; *in vivo*, Table 11), many of the studies were performed with NPs of titanium dioxide. Some comments on the characterisation of the NPs are provided in both tables. It is clear that whilst some studies included quite extensive characterisation (nano scores of 8-10), others did not (nano scores of 1-3), and this variability in characterisation was seen for datasets giving both negative and positive results.
Additionally, the Applicant provided data from mutagenicity (HPRT assay) and cytogenicity *in vitro* micronucleus test) studies performed (See section *In vitro* studies and Table 10) according to OECD guideline and GLP-compliant. These studies incorporated the most recent genotoxicity testing requirements for nanomaterials as outlined in SCCS (2019), ENV/JM/MONO(2014), and the OECD (Draft 2021). They were performed with two representative titanium dioxide nano grades as typically used in cosmetic products (i.e., RM09, RM11). As required by entry 27a of Annex VI to R 1223/2009, the crystal phase of

both test materials was rutile based, with hydrophilic and hydrophobic coatings. Both selected materials had a primary particle size of 20-25 nm which is typical for nano titanium dioxide materials used in cosmetics (i.e., the median primary particle size of the 42 samples assessed is 25.5 nm).
To comply with the Cosmetic Regulation provisions on animal testing (Article 18), in this dossier the applicant considered only *in vivo* studies which were conducted before March 2013. However, studies conducted post-2013 for other purposes than cosmetics are also presented in Annex I.

ii) In vitro studies - Expert panel WoE of data until 2021

Of the 93 *in vitro* datasets reviewed in the quality assessment of all *in vitro* data, only 14 (comprising 9 MN, 3 CA, a single HPRT and a single TK gene mutation data set) with a weighting of "moderate", "moderate to high" or "high" from publications and study reports were considered relevant for the expert panel assessment. Ten out of the 14 *in vitro* data sets were conducted with nano-grade titanium dioxide.
Kirkland *et al.* (2022) reported that there was no evidence of induction of gene mutations *in vitro*, although only 2 mammalian cell gene mutation studies achieved a final weight of "moderate". Most *in vitro* tests for MN and CA were negative. Only 2 *in vitro* MN studies in Table 9 were positive or weakly positive, and the concentrations at which these effects were seen induced oxidative damage, apoptosis, and necrosis, although these changes were also seen in negative studies. Therefore, it is highly likely that the increases in MN were secondary to oxidative stress and cytotoxicity.
It should be noted that there was much variability across the different datasets in terms of the particle concentrations tested in mammalian cells *in vitro*. This may be due to different forms of titanium dioxide being tested, cell type, method of formulation, etc., but it makes comparison of any effects between studies very challenging.
In line with OECD Guidance, failure to expose mammalian cells for at least 1 cell cycle, or, for shorter exposures, failure to clearly demonstrate that the particles entered the cells, was not considered acceptable by Kirkland *et al.* (2022), particularly when negative results were obtained. Therefore, some *in vitro* MN, CA and gene mutation studies that gave positive or equivocal results with short treatments suggested that intracellular exposure had occurred, so were considered reliable and retained a "moderate" weight (so were considered relevant to the assessment of genotoxic potential and were included in Table 9). The studies that gave negative results with short treatments and with no clear demonstration of cellular uptake were considered unreliable and were given "low to moderate" or "low" weights and not considered relevant (and were excluded from Table 9). 10 (ten) *in vitro* MN/CA and 2 (two) *in vitro* mammalian cell gene mutation studies that were negative did include sufficiently long exposures (prior to cytochalasin B treatment in the MN studies) to provide robust negative results. Table 9 below summarises those *in vitro* studies achieving moderate weight after WoE assessment (CE, 2022; TDMA, 2022; Kirkland *et al.*, 2022).

iii) *In vitro* studies- Newly conducted *in vitro* studies

In order to generate high-weight *in vitro* data on representative titanium dioxide nano grades, HPRT and micronucleus tests were performed according to current OECD guidelines, which were specifically tailored for the testing of nanomaterials. Both RM09 and RM11 were tested in both assays up to a concentration of 100 µg/mL based on the recommendations set out for the *in vitro* genotoxicity testing of manufactured nanomaterials. This maximum concentration was selected, because higher concentrations of poorly soluble nanomaterials are considered not physiologically relevant and because artefactual effects may result from the precipitate (OECD TGs 476 and 487). The V79 cells were exposed to the RM09 without exogenous metabolic activation. The cells were not exposed to the test substance in presence of a metabolic activation system since both the test substance core and the coating are inorganic and not metabolised by enzymes. In contrast, RM11 was tested both in absence and presence of a metabolic activation (Elespuru *et al.*, 2018 and Doak *et al.*, 2012). In order to demonstrate cellular nanoparticle uptake, transmission electron microscopic analysis was

included in the HPRT assay, which was performed under comparable conditions as the micronucleus test. Due to the organic coating of RM11 and the inclusion of a metabolic activation system in the assay, the test substance was additionally tested using a 4-hour exposure. In the micronucleus assay, the treatment with the cytokinesis blocker cytochalasin B was not carried out in parallel to the test item as described in the current OECD TG 487 (2016), but in succession as described in OECD TG 487 (2010) (Chapter 40, Table 1: -S9 Extended exposure, Option B), since cytochalasin B has been shown to inhibit uptake of nanoparticles by endocytosis (Elespuru *et al.*, 2018). Dynamic light scattering (DLS) analyses were performed to demonstrate the stability of the dispersion. Summary of the recently conducted *in vitro* studies with representative titanium dioxide nano grades are presented below.

iv) In vivo studies

Kirkland *et al.* (2022) reviewed in detail a total of 20 studies comprising 11 MN (bone marrow and peripheral blood), 2 CA, 2 transgenic rodent (TGR) mutation studies (gpt and Spi mutants), 3 comet assays (2 in liver and lung and a single study in liver) and two 8-OHdG adduct studies in the lung, in their review. However, only 7 studies were pre-2013 and are therefore summarised in Table 11. Post-2013 studies are presented in Annex I.

The SCCS note: the Annex I of the Applicant's document (ADDITIONAL STUDIES CONDUCTED POST 2013, page 73/84) contains a summary of additional collateral and confirmatory evidence not used by the Applicant in the final safety assessment.

The seven pre-2013 studies comprised 5 *in vivo* MN/CA studies, 1 comet and 1 8-OHdG adduct study. Of the 5 MN/CA studies, 3 studies showed positive or weakly positive (approximately 2-fold) increases in MN. These positive responses were associated with inflammation, oxidative stress and/or apoptosis. In addition, one study scored a Klimisch 3 in the ToxR tool and was thus considered unreliable. Therefore, there are reasons to question whether any of these positive *in vivo* MN/CA responses are biologically relevant and indicative of a direct DNA-damaging effect of titanium dioxide.
As per Kirkland *et al.* (2022), two positive MN studies used oral gavage dosing and one used drinking water administration, however absorption via the oral route has been shown to be very low. With such low oral bioavailability, bone marrow exposure would be negligible, therefore, the plausibility of the positive MN results is questionable.
One *in vivo* 8-OHdG study used a single intratracheal instillation of doses up to 1.2 mg and the study outcome was negative. Table 11 summarises the "moderate", "moderate-high" or "high" weight *in vivo* studies conducted pre-2013.
Overall, the genetic toxicity of pigmentary and nanograde titanium dioxide was assessed in various *in vitro* and *in vivo* studies using both rutile and anatase forms. Of the 21 relevant datasets reviewed (i.e., 14 *in vitro* and 7 pre-2013 *in vivo)*, only 5 (24%) were positive. All were from chromosomal damage studies (MN or CA assays), and it is accepted by many regulatory guidelines that chromosome breakage can be secondary to physiological stress (ICH, 2013; Kirkland *et al.*, 2007). Since, as discussed above, all the positive findings were associated with high cytotoxicity, oxidative stress, inflammation, apoptosis or combinations of these, it is highly likely that the observed genotoxic effects of titanium dioxide, including those with nano particles, are secondary to physiological stress, as has been described recently in a comparable review (Krug, 2022). There were no positive results from gene mutation studies which is consistent with DNA/chromosomal damage being secondary to physiological stress, although data from robust *in vivo* gene mutation studies would be useful in reaching firm conclusions. Further, four recently conducted OECD guideline compliant *in vitro* genotoxicity tests (HPRT and micronucleus tests) with two representative nano titanium dioxide grades have demonstrated negative results. As shown in Table 12, the profile of genotoxicity results from the most robust studies with titanium dioxide does not fit the response pattern which would be expected for a genotoxic carcinogen (CE, 2022; 2023; TDMA, 2022; Kirkland *et al.*, 2022).

Applicant's conclusion on genotoxicity

Overall, the conclusion from the robust datasets reviewed, that achieved "moderate", "moderate to high" or "high" weight, did not support a direct DNA-damaging mechanism for titanium dioxide in either the nano or pigmentary form. This conclusion is in line with the outcomes of the recent reviews by Food Safety Authorities of England, Canada, Australia, and New Zealand (COT, 20229; Health Canada, 2022; FSANZ, 2022). Additionally, four recent high-weight studies (CE, 2023) with two representative titanium dioxide nano grades have demonstrated negative results in OECD guideline compliant *in vitro* genotoxicity tests, which were specifically tailored for the testing of nanomaterials. These studies confirm the conclusion drawn on the lack of a direct genotoxic potential.

Ref.: Dossier on the Human Safety Evaluation of Titanium Dioxide in Cosmetic Products (CAS No. 13463-67-7, 12026-28-7, 1317-70-0, 1317-80-2, 20338-08-3/ EC No. 236-675-5, 243-744-3, 1317-70-0, 215-282-2, 234-711-4). (Submission I with focus on potential oral exposure). COSMETICS EUROPE INGREDIENT N° S75. 28 April 2023", pages 37-53/84.

Description of the study reports submitted by the Applicant and comments by the SCCS

***IN VITRO* STUDY #1. ToxTracker**

Guideline:	none
Test system:	mouse embryonic stem (mES) reporter cell lines
Test substance:	11 TiO_2 test substances: E, G1-1, G2-5, G3-1, G4-19, G5-4, G6-3, G7-5, G8-2, G9-5, G10-4
Batch (Purity):	not provided
Vehicle:	cell culture medium (undisclosed)
Assay medium:	cell culture medium (undisclosed)
Concentrations:	0, 0.125, 0.25, 0.5, 1, 2 mg/mL
Treatment:	4 h exposure, without and with metabolic activation; 24 h exposure, only without metabolic activation
S9:	Aroclor 1254-induced rat liver S9 (Moltox)
Positive controls:	cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded protein response) and aflatoxin B1 (metabolic activation of progenotoxins by S9)
Negative control:	Vehicle
GLP:	No
Study period:	13/03/2019 and 22/03/2019

Cytotoxicity testing/dose range finding

To prepare the test substances for exposing mES cells, provided powders were mixed in cell culture medium at a concentration of 2 mg/ml for 24 hours at 37ºC. For substance testing, first a dose range finding was performed using wild-type mES cells (strain B4418). Wild type mES cells were exposed to 20 different concentrations of the 11 TiO_2 test substances (E, G1-1, G2-5, G3-1, G4-19, G5-4, G6-3, G7-5, G8-2, G9-5, G10-4) or positive reference coumpunds, with a maximum concentration of 2 mg/ml. Cytotoxicity was estimated by cell count after 24 h exposure using a flow cytometer and is expressed as the percentage of viable cells after 24 h exposure compared to vehicle control exposed cells. From this dose range finding, 5 concentrations were selected.

Toxtracker assay

The six independent mES reporter cell lines were seeded in gelatin-coated 96-well cell culture plates in 200 µl mES cell medium (50.000 cells per well). 24 h after seeding the cells in the 96-well plates, medium was aspirated and fresh mES cell medium containing 10% fetal calf serum and the diluted chemicals was added to the cells. For each tested compound, five concentrations were tested in 2-fold dilutions (0.125, 0.25, 0.5, 1, 2 mg/mL).

Induction of the GFP reporters was determined after 24 h exposure using a flow cytometer. Only GFP expression in intact single cells was determined. Mean GFP fluorescence was measured and used to calculate GFP reporter induction compared to a vehicle control treatment. Cytotoxicity was estimated by cell count after 24 h exposure using a flow cytometer and was expressed as percentage of intact cells after 24 h exposure compared to vehicle exposed controls. For cytotoxicity assessment in the ToxTracker assay, the relative cell survival for the six different reporter cell lines was averaged, because the cytotoxicity levels are very similar. Metabolic activation was included in the ToxTracker assay by addition of S9 liver extract from aroclor 1254-induced rats (Moltox). Cells were exposed to five concentrations of the test samples in the presence of 0.25% S9 and required co-factors (RegenSysA+B, Moltox) for 24 h.
Positive reference treatments with cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded protein response) and aflatoxin B1 (metabolic activation of progenotoxins by S9) were included in all experiments. Solvent concentration was the same in all wells and never exceeded 1% for DMSO. In case auto-fluorescence of the test substances was observed in the dose range finding, wild type mES cells were exposed to the test samples at the same concentrations as used in the ToxTracker. The mean fluorescence caused by the compound was then subtracted from the ToxTracker results of the respective compound.
This experiment was conducted as a non-GLP study, however general principles to conduct proper scientifically correct *in vitro* experiments were adhered to, and in particular care was taken for proper handling of test article (stock) solutions to prevent/minimise degradation of the test articles based on instructions/compound information from the sponsor. For all ToxTracker analyses, Toxys strictly follows the Good Cell Culture Practice guidelines from the OECD.

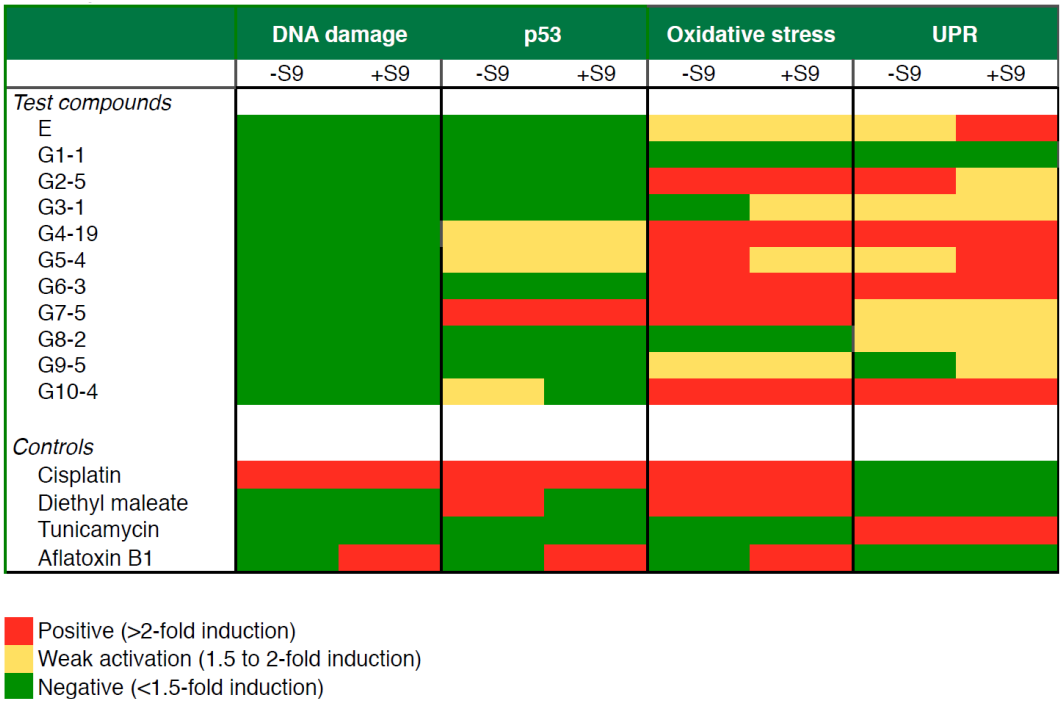
TOXTRACKER results and discussion (from the study report)
The validity of the ToxTracker assay was confirmed using exposure to the reference compounds specific for the pathways evaluated. The genotoxic compound cisplatin showed induction of the DNA damage response (Bscl2, Rtkn) and p53-mediated cellular stress (Btg2). Diethyl maleate (DEM) induced primarily the oxidative stress related reporters Srxn1 and Blvrb, tunicamycin induced the unfolded/misfolded protein stress response (Ddit3). The positive control compound aflatoxin B1, which requires metabolic activation to become genotoxic, selectively induced the Bscl2 and Rtkn reporters when tested in the presence of S9 liver extract. Generally, the controls showed GFP induction levels compliant with historical data and demonstrated the functionality of the mES reporter cell lines.
Cytotoxicity
The test substances did not dissolve in the cell culture medium and at the highest tested concentrations, precipitation was observed at the end of the treatment. At the maximum tested concentrations in the absence of a metabolising system cytotoxicity was observed for all test samples. In the presence of a metabolizing system, there was no increase in cytotoxicity observed for any of the samples. The six ToxTracker reporter cell lines showed a comparable cytotoxic response to the test samples. For this reason, the cell survival graphs in the GFP induction figures show the average cytotoxicity of the six different cell lines.
Genotoxicity
None of the tested substances activated the Bscl2-GFP or Rtkn-GFP markers for DNA damage more than 2-fold and therefore none of the test materials were classified as genotoxic. Btg2-GFP, the reporter for p53 activation, was activated in response to exposure to test substance G7-5, both in the absence and presence of S9. For test substances G4-19 and G5-4, a weak activation (>1.5 fold) of the Btg2-GFP reporter was observed in the absence and presence of S9, but induction levels did not reach the 2-fold threshold for a positive ToxTracker result. Test substance G10-4 weakly activated the Btg2-GFP reporter only in the absence of S9.
Oxidative stress
Induction of the Srxn1-GFP reporter is associated with activation of the Nrf2 antioxidant response and activation of the Blvrb-GFP reporter is associated with the Hmox1 antioxidant response. Activation of the Srxn1-GFP reporter was observed for test substances G2-5, G4-19, G6-3, G7-5 and G10-4 in absence and presence of a S9 metabolising system. For test

substance G5-4, Srxn1-GFP was activated more than 2-fold in the absence of S9, but in the presence of S9 the induction was weak (>1.5 fold) and did not reach the 2-fold threshold for a positive ToxTracker result. Test substances E and G9-5 weakly activated the Srxn1-GFP reporter both in the absence and presence of S9, while test substance G3-1 only weakly activated Srxn1-GFP in the presence of S9. For the titanium dioxide samples, we only observed activation of Blvrb-GFP in one instance, after after exposure to test substance G2-5 in the presence of S9, but induction levels did not reach the 2-fold threshold for a positive ToxTracker result.

Protein damage

The Ddit3-GFP reporter, associated with protein damage and the unfolded protein response, was activated by test substances G4-19, G6-3 and G10-4 in the absence and presence of S9. A weak activation (>1.5 fold) of Ddit3-GFP in both the absence and presence of S9 was observed for test substances G3-1, G7-5 and G8-2, but induction levels did not reach the 2-fold threshold for a positive ToxTracker result. Test substances E and G5-4 weakly induced (>1.5 fold) the Ddit3-GFP reporter in the absence of S9, but in the presence of S9 the induction exceeded the 2-fold threshold for a positive ToxTracker result. Test substance G2-5 activated Ddit3-GFP in the absence of S9, but in the presence of S9 only a weak activation (>1.5 fold) of the reporter was observed. For test substance G9-5, a weak activation (>1.5 fold) of the protein stress reporter was observed only in the presence of S9, but induction levels did not reach the 2-fold threshold for a positive ToxTracker result.

The Applicant's summary of the ToxTracker assay results:



Ref.: ToxTracker Test report. Draft version 1.0. Toxys project code: 18032. 26 March 2019. Hendriks G., Derr R. & Brandsma I.

The SCCS note:

In response to the SCCS request, the Applicant provided the following information on the correspondence of TiO_2 samples used in the Toxtracker study to the TiO_2 raw materials used in cosmetic products:

G-sample	Description	Exact equivalent RM (same grade)	Analogous RMs (similar PhysChem characteristics)
G6-3	Nano rutile coated with alumina and stearic acid	**RM42**	*RM40, RM48, RM49, RM56, RM60, RM62, RM76, RM63, RM64, RM65, RM74b*
G8-2	Nano rutile coated with silica	**RM09**	*RM47, RM74d, RM78*
E171-E	Uncoated pigmentary anatase (E171 spec)	**RM67**	*RM01, RM03, RM04, RM26, RM67b, RM68*
G3-1	Uncoated pigmentary rutile		*RM02, RM28, RM69, RM69b*
G4-19	Pigmentary rutile coated with alumina		*RM06, RM30, RM72i*

G6-3, G8-2 and E171-E are cosmetics grade TiO_2.
G3-1 and G4-19 are not marketed for use in cosmetics but have similar physicochemical characteristics to some cosmetics grades.

The SCCS comments on the results from ToxTracker study report

Although the ToxTracker methodology looks promising for genotoxicity assessment, it still needs to be validated, especially when applied to nanomaterials.
In the opinion of the SCCS, the ToxTracker study results are of limited value because the methodology is barely described, and no reference is made to a protocol for dispersion used for sample preparation.
As for the DNA damage reporter assays, although the results for all materials tested were negative, no proof of cell internalisation was provided. The SCCS therefore considers the study as inconclusive.

The SCCS has also noted that:

- No reference is made to the use of serum in exposure medium, nor is the biological medium used for NPs dispersion indicated. It is stated that the test substances did not dissolve in the cell culture medium and at the highest tested concentrations, precipitation was observed at the end of the treatment. For preparation of test item, no reference to sonication or dilutions were provided.
- Cytotoxicity was observed for all test samples at the maximum tested concentrations in the absence of a metabolising system. In the presence of a metabolising system, no increase in cytotoxicity was observed for any of the samples.
- The results concerning genotoxicity were reported as negative, because none of the eleven tested titanium dioxide samples activated the Bscl2-GFP or Rtkn-GFP markers for genotoxicity more than 2-fold, while the result for positive controls did. Activation of the cellular stress reporter gene was observed for only one test substance, G7-5, and activation of both the oxidative stress reporter and the reporter for protein damage was observed after exposure to several test substances.

***IN VITRO* STUDY #2.** Gene mutation assay in Chinese Hamster V79 cells *in vitro* (V79/HPRT) on RM09, ICCR 4023311

Draft report	Sokolowski, A., 2023
Evaluation status:	**New study**
Title:	RM09: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)
Document No:	ICCR Study Number: 4023311
Guideline followed in study:	OECD 476 (2016)
Current guideline:	OECD 476 (2016)
Guideline and deviations from guideline in force at that time:	OECD 476 (2016) Deviations: - 24-hour treatment to ensure sufficient particle uptake
	- Without metabolic activation only, since test item core and coating are inorganic materials, which are not metabolised by S9 fraction
GLP:	Yes
Testing Facility:	ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany
Test material:	NANO: RM09 (purity ≥99%; surface modification: coated with amorphous silica; particle number size distribution: number weighted median x50: 20 nm measured by SEM, Feret min)
Test material preparation:	Following the Nanogenotox protocol (Jensen *et al*. 2011); suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication
Test system:	Chinese hamster lung fibroblast V79 cell line
Negative controls:	Solvent control and negative control (deionised water)
Positive controls:	Ethylmethane sulfonate
Test concentrations:	0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µg/mL
Number of experiments and replicates:	2 independent experiments (first trial and repeat experiment) using duplicate cultures
Exposure (duration):	24 hours
Particle uptake analysis:	Yes, uptake was analysed via TEM.
Dispersion analysis:	Yes, dispersion was analysed via DLS.

The gene mutation potential of RM09 was examined in a HPRT assay in V79 Chinese hamster lung fibroblasts in the absence of metabolic activation (Sokolowski, A., 2023). In order to get a well dispersed and stable suspension, RM09 was prepared following the recommendations of the Nanogenotox protocol (Jensen *et al.*, 2011). The cell cultures were treated with RM09 for 24 hours. A short-term treatment as outlined by the current OECD TG 476 (2016) is considered inadequate for nanomaterials as cellular uptake of the test item needs to be demonstrated. The exposure duration of 24 hours was selected in order to expose the cells for at least one cell cycle to ensure sufficient cellular uptake as recommended by OECD Nanomaterials Working Party recommendation (OECD, 2014) and as published previously (Elespuru *et al.*, 2018 and Doak *et al.*, 2012). The test material was tested up to a concentration of 100 µg/mL, based on the recommendations set out for the *in vitro* genotoxicity testing of manufactured nanomaterials (OECD, 2021). The maximum concentration (100 µg/mL) was selected since higher concentrations of poorly soluble nanomaterials are considered not physiologically relevant (OECD, 2021) and to avoid artefactual effects resulting from precipitate (OECD TG 476). The V79 cells were exposed to RM09 without exogenous metabolic activation. The cells were not exposed to the test item in presence of a metabolic activation system, since both the test item core and the coating are inorganic and not metabolized by enzymes. Solvent, negative, and positive control cultures were run concurrently.

The test material was tested up to precipitating concentrations as observed microscopically and by the unaided eye. Cytotoxicity as determined by the relative survival was not observed at any concentration tested. The HPRT test with RM09 showed statistically significantly increased mutation rates at some precipitating concentrations, i.e., at 6.3, 25.0, and 100 µg/mL. However, no such effect was observed at 0.8, 1.6, 3.1, 12.5, and 50 µg/mL. All values obtained were within the 95% confidence interval of the historical negative control data range. Trend analysis revealed that the combined duplicate cultures show a positive concentration-response relationship. However, this effect was mainly due to culture 2 and was not reproduced in culture 1. The outcome was considered to be negative as per expert judgement. However, in order to confirm this test outcome, a repeat experiment was performed under the same conditions. In the repeat experiment, the mutation frequencies observed in the treatment group did not show a statistically significant difference from the solvent control culture and showed no concentration-response relationship. Thus, the outcome of the repeat experiment confirmed the outcome of the first experiment. The positive control induced distinct and statistically significant increases in the mutant frequency confirming the sensitivity of the test system Solvent and negative control cultures showed mutant frequencies that fell within acceptable ranges of the historical control data base and demonstrated the validity of the assay.
In the accelerated stability study, it was demonstrated via DLS measurements that RM09 showed stable particle sizes without increased aggregation/agglomeration for at least 24 hours. The cellular uptake of RM09 nanoparticles by V79 cells was demonstrated at all concentrations evaluated (i.e., 25, 50, and 100 µg/mL) and the test item was observed exclusively in cytoplasmic vesicles but not in the cell nucleus.
In conclusion, under the experimental conditions reported, the test item did not induce gene mutations at the HPRT locus in V79 cells. Therefore, RM09 is considered to be non-mutagenic in this HPRT assay, when tested in the absence of S9 up to the top recommended concentration for nanomaterials.

TEM Observations of Internalization of Nanoparticles in V79 Cells
Cross-sections of V79 cells could be examined by chemical staining with osmium tetroxide (enhancement of contrast) and ultramicrotomy with a transmission electron microscope.
For all three concentrations examined (25, 50, 100 µg/mL), the TEM ultra-thin sections revealed V79 cell in which the RM09 nanoparticles could be detected.
The nanoparticles are almost entirely found with the cells. Most of the observed V79 cells showed agglomeratres of RM09 nanoparticles. Only occasionally separated particles or single small agglomerates can be observed.
In general, no RM09 nanoparticle agglomerates were observed in the nuclei of the cells.
In conclusion, cellular uptake of RM09 was demonstrated at all concentrations evaluated and observed exclusively in cytoplasmic vesicles but not in the cell nucleus.

Short Report Nano characterization of the test solution with dynamic light scattering (DLS) (non-GLP) (detailed report in Annex S)
Four samples were measured in three replicates via DLS at 37°C for 24 hours with one data point per hour.
For sample 24h RM09 0.8 µg/mL – S9 mix the z – average diameter at T0 (first measurement point after preparation of the sample mixture) was 50 nm and 57 nm at Tend (last measurement point of the accelerated stability measurement). Signal intensity was approximately 1-fold above the formulation signal level. The higher intensity of the sample signal in comparison with the background signal of the formulation buffer, the less likely an impact of background noise on the experiment data. 24 h RM09 100 µg/mL – S9 mix had a z-average of 135 nm at T0 and 137 nm at Tend. An interference of the FBS with DLS measurements could not be observed.
Samples were centrifugated before the experiment, as an initial intensity test showed high scattering due to large particles in the samples, which led to abortion of data collection. For neither of the samples, a clear trend toward larger particle sizes could be measured within the tested time frame.

Ref.: Sokolowski A., ICCR Study Number: 4023311, 2023. RM09: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)

The SCCS note:

The following complementary information was provided by the Applicant on the representativeness of RM09 and RM11 used in the genotoxicity studies (document: "CE response to SCCS Request of 13 June 2023_29062023.pdf" and From Att.1 -CE TiO_2 - Response and Comments - 06.02.2024.pdf (February 2024):

"*To be representative it was decided to have one hydrophobic grade and one hydrophilic grade and also to have one grade coated with silica and one grade coated with alumina. The primary particle size of each sample chosen was in the range 20-30 nm which is typical of the entire dataset (mean size of the 42 samples is 26.5 nm and median is 25.5 nm).*

- RM11 (20 nm mean primary particle size Feret min by number, 6% alumina and 3% dimethicone) is representative of hydrophobic cosmetic nano grades and is coated with alumina (it has also been extensively studied by OECD WPMN and Nanogenotox since it is the same grade as NM103).

- RM09 (26 nm mean primary particle size Feret min by number, 10% silica) is representative of hydrophilic cosmetic nano grades – coated with amorphous silica (this grade has been extensively characterised by TDMA and used in their studies as G8-2).

Although marketed typically as an intermediate any additional treatment is optional and it can also be used directly in sunscreens in appropriate (hydrophilic) formulations. If used in hydrophobic formulations, an appropriate formulation step to improve compatibility is necessary. During such formulating steps RM09 itself remains unchanged though dispersants may become adsorbed on the surface to improve the compatibility with a particular formulation phase. (Therefore, RM09 is not an intermediate in REACH terms)".

SCCS comments on the *in vitro* study #2: ICCR 4023311

Based on the analysis of the study results, the SCCS is of the opinion that the results on RM09 testing in the *in vitro* gene mutation test, despite some shortcomings (as noted below), are negative.

The SCCS has noted that:

- The study design is not fully compatible with OECD TG 476 because it does not use a short incubation time and does not include application of S9 mix. However, using such an approach in case of TiO_2 particles coated with inorganic substance(s) may be justified and in line with the SCCS/1655/23 Guidance on the Safety Assessment of Nanomaterials in Cosmetics. The SCCS is also aware that there is work ongoing on adapting new TG for genotoxicity testing with new exposure conditions, including applying only prolonged incubation period and recommendations specific for using S9mix for nanomaterials testing.
- As described in ICCR Study Number 4023311, the negative and solvent control as well as the stability of the highest and lowest test item concentrations were measured by DLS each hour for 24 hours to analyse the stability of the dispersion and the agglomeration/aggregation behaviour of the test item over the time. For TEM analysis, RM09 at 25, 50 and 100 µg/mL for 24 h cell exposure was used.
- Based on the analysis of Annex 2 to ICCR Study Number 4023311, the SCCS is of the opinion that cellular uptake of RM09 was convincingly demonstrated, however, at RM09 concentrations higher than those recommended by the OECD TG 490 (paragraph 29). According to the information on precipitation provided by the Applicant, the highest acceptable concentration tested should be 6.3 µg/mL (Exp I) or 12.5 µg/mL (Exp IA), and these concentrations were not tested for cellular uptake, *i.e.* the lowest concentration tested by the Applicant for uptake was 25 µg/mL.
- The results of the first experiment (24h treatment) showed significantly higher mutation frequency (MF) in the highest analysed concentration, with linear regression analysis showing a borderline trend ($p = 0.058$). However, in the repetition experiment, negative results were obtained.

***IN VITRO* STUDY #3.** Gene mutation assay in Chinese Hamster V79 cells *in vitro* (V79/HPRT) on RM11, ICCR 4023312

Draft Report:	Sokolowski, A., 2023
Evaluation status:	**New study**
Title:	RM11: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)
Document No:	ICCR Study Number: 4023312
Guideline followed in study:	OECD 476 (2016)
Current guideline:	OECD 476 (2016)
Guideline and deviations from guideline in force at that time:	OECD 476 (2016) Deviations: None
GLP:	Yes
Testing Facility:	ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany
Test material:	NANO: RM11 (purity ≥99%; surface modification: coated alumina and dimethicone; particle number size distribution: Number weighted median x50: 19 nm measured by SEM, Feret min)
Test material preparation:	Following the Nanogenotox protocol (Jensen et al. 2011); suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication
Test system:	Chinese hamster lung fibroblast V79 cell line
Negative controls:	Solvent control and negative control (deionised water)
Positive controls:	Ethylmethane sulfonate (without metabolic activation) and 7,12-dimethylbenz(a)anthracene (with metabolic activation)
Test concentrations:	0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µg/mL

Sokolowski, A. (2023) investigated the potential of RM11 to induce gene mutation at the Hprt locus (OECD TG 476, 2016) in V79 Chinese hamster lung fibroblasts in both the absence and presence of metabolic activation. In order to get a well dispersed and stable suspension, RM11 was prepared following the recommendations of the Nanogenotox protocol (Jensen *et al.*, 2011). The test material was tested up to a concentration of 100 µg/mL based on the recommendations set out for the *in vitro* genotoxicity testing of manufactured nanomaterials (OECD, 2021). The maximum concentration (100 µg/mL) was selected since higher concentrations of poorly soluble nanomaterials are considered not physiologically relevant (OECD, 2021) and to avoid artefactual effects resulting from precipitate (OECD TG 476). The cell cultures were treated with RM11 for 24 hours. A short-term treatment as outlined by the current OECD TG 476 (2016) is considered inadequate for nanomaterials as cellular uptake of the test item needs to be demonstrated. The exposure duration of 24 hours was selected in order to expose the cells for at least one cell cycle to ensure sufficient cellular uptake as recommended by OECD Nanomaterials Working Party recommendation (OECD, 2014) and as published previously (Elespuru *et al.*, 2018 and Doak *et al.*, 2012). Due to the organic coating of RM11 and the inclusion of a metabolic activation system in the assay, the test material was additionally tested using a 4-hour exposure. RM11 was tested both in absence and presence of a metabolic activation system, since the coating is of organic nature and could potentially be metabolised by enzymes of the S9 fraction. Solvent, negative, and positive control cultures were run concurrently.
The test material was tested up to precipitating concentrations as observed microscopically and by the unaided eye. Cytotoxicity as determined by the relative survival was not evident at any concentration tested. In the 4-hour experiments with RM11 in absence and presence of metabolic activation, statistically significantly increased mutation frequencies were not observed at any concentrations tested when compared to the concurrent solvent control. In the 24-hour experiment without metabolic activation, the mutation frequency was

sporadically statistically significantly increased. However, all values obtained with both treatment schedules (4- and 24-hour exposure) were clearly within the 95% confidence interval of the historical negative control data range. Moreover, the trend tests did not indicate a positive concentration-response relationship under the conditions tested. Thus, the sporadic statistically significant increases were considered to be of no biological relevance and to be chance findings. The positive controls induced distinct and statistically significant increases in the mutant frequency. Thus, the sensitivity of the test system was demonstrated. Solvent and negative control cultures showed mutant frequencies that fell within acceptable ranges of the historical control data base, and thus, demonstrated the validity of the assay.
In the accelerated stability study, it was demonstrated via DLS measurements that RM11 showed stable particle sizes without increased aggregation/agglomeration for at least 24 hours. The cellular uptake of RM11 nanoparticles by V79 cells was demonstrated at all concentrations evaluated (i.e., 25, 50, and 100 µg/mL) and the test item was observed exclusively in cytoplasmic vesicles but not in the cell nucleus.
In conclusion, under the experimental conditions reported, the test item did not induce gene mutations at the HPRT locus in V79 cells. Therefore, RM11 is considered to be non-mutagenic in this HPRT assay, when tested in the absence and presence of S9 up to the top recommended concentration for nanomaterials.

TEM Observations of Internalization of Nanoparticles in V79 Cells
Cross sections of V79 cells could be examined by chemical staining with osmium tetroxide (enhancement of contrast) and ultramicrotomy with a transmission electron microscope.
For all three concentrations examined (25, 50 and 100 µg/mL), the TEM ultra-thin cuts show V79 cell in which the RM11 nanoparticles could be detected. Nevertheless, many cells show no obvious internalization of RM11 nanoparticles and many of the RM11 nanoparticle agglomerates can be observed outside the cells. The majority of the RM11 nanoparticles (inside and outside the cells) are present in agglomerated form. Only occasionally separated particles or single smaller agglomerates can be seen.
In general, no RM11 nanoparticle agglomerates were observed in the nuclei of the cells.
In conclusion, cellular uptake of RM11 nanoparticles was demonstrated at all concentrations evaluated and observed exclusively in cytoplasmic vesicles but not in the cell nucleus.

Short Report Nano characterization of the test solution with dynamic light scattering (DLS) (non-GLP) (detailed report in Annex S)
For sample 4h RM11 0.8 µg/mL – S9 mix, the z-average diameter at T0 was ca. 183.3 nm and 290 nm at Tend, with a high standard deviation for both data points due to a signal intensity that was approximately 1-fold above the scattering level of the formulation buffer. 4h RM11 100 µg/mL – S9 mix had a z-average diameter of 168 nm at T0 and 176 nm at Tend.
All samples containing S9 mix showed comparable z-average diameters at T0 and at Tend, when compared to each other, as well as comparable scattering intensities, including the Water and LM samples. The normalized intensities of the solvent control sample with S9 mix (T0: 1.0 x 10^6 kCnt/s and Tend: 1.7 x 10^6 kCnt/s) were in a comparable range to the values measured for the samples containing the test material and S9 mix (0.8 µg/mL: T0: 1.0 x 10^6 kCnt/s and Tend: 1.7 x 10^6 kCnt/s – 100 µg/mL: T0 1.2 x 10^6 kCnt/s and Tend: 1.7 x 1010^6 kCnt/s). Therefore, the data possibly reflects the z-average diameter of the S9 components instead of the z-average diameter of the nanoparticles.

24 h RM 11 0.8 µg/mL – S9 had a z-average diameter of approx. 24 nm at T0 and 32 nm at Tend, wit a low signal amplitude. An interference of the FBS with the DLS measurements could not be observed. RM 11 24 h + 10 % FBS Konz 8 had a z-average diameter of 109 nm at T0 and of 118 nm at Tend.
Samples were centrifuged before the experiment, as an initial intensity test at 20°C showed high scattering due to large particles in the samples, which led to abortion of data collection. For neither of the samples, but the samples containing the S9 mix, a clear trend toward larger particles sizes could be measured with the tested time frame.

Ref.: Sokolowski, A., ICCR Study Number: 4023312, 2023. RM11: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)

SCCS comments on *in vitro* study #3: ICCR 4023312

Based on the analysis of the study results, the SCCS is of the opinion that the results on RM11 testing in the *in vitro* gene mutation test, despite some shortcomings (as noted below), are negative.

The SCCS has noted that:

- As described in ICCR Study Number 4023312, the negative and solvent control and the stability of the highest and lowest test item concentrations were measured by DLS each hour for 24 hours in order to analyse the stability of the dispersion and the agglomeration/aggregation behaviour of the test item over the time. For TEM analysis, RM11 at 25, 50 and 100 µg/mL for 24 h cell exposure was used.
- Based on the analysis of Annex 3 to ICCR Study Number 4023312, the SCCS is of the opinion that cellular uptake of RM11 was convincingly demonstrated, however, only at RM11 concentrations higher than those recommended by the OECD TG 490 (paragraph 29). According to the information on precipitation provided by the Applicant, the highest acceptable concentration tested should be 6.3 µg/mL (4 or 24 h of exposure), and these concentrations were not tested for cellular uptake, *i.e.* the lowest concentration tested by the Applicant for uptake was 25 µg/mL.
- Significantly higher MF frequency was observed in two analysed concentrations compared to the solvent control after 24h treatment, but these were within the 95% confidence interval of the historical negative control data range.

***IN VITRO* STUDY #4.** Micronucleus test in Chinese Hamster V79 cells *in vitro* on RM09, ICCR 4023313

Draft report:	Naumann, S., 2023
Evaluation status:	**New study**
Title:	RM09: Micronucleus Test in Chinese Hamster V79 Cells *in vitro*
Document No:	ICCR Study Number: 4023313
Guideline followed in study:	OECD 487 (2016)
Current guideline:	OECD 487 (2016)
Guideline and deviations from guideline in force at that time:	OECD 487 (2016) Deviations: - 24-hour treatment only to ensure sufficient particle uptake - Without metabolic activation only, since test item core and coating are inorganic materials, which are not metabolised by S9 fraction
GLP:	Yes
Testing Facility:	ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany
Test material:	NANO: RM09 (purity ≥99%; surface modification: coated with amorphous silica; particle number size distribution: number weighted median x50: 20 nm measured by SEM, Feret min)
Test material preparation:	Following the Nanogenotox protocol (Jensen et al. 2011); suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication
Test system:	Chinese hamster lung fibroblast V79 cell line
Negative controls:	Solvent control and negative control (deionised water)
Positive controls:	Mitomycin C (clastogenic control) and Griseofulvin (aneugenic control)
Test concentrations:	1.1, 3.5, 10.7, 18.7, 57.1, and 100 µg/mL
Number of experiments and replicates:	1 experiment using duplicate cultures
Exposure (duration):	24 hours
Particle uptake analysis:	Yes, uptake was analysed via TEM. The TEM study was performed within the context of ICCR Study Number: 4023311. However, the test item preparation and exposure were performed under comparable conditions, i.e., same test material preparation protocol as well as the same test system (V79 Chinese hamster lung fibroblasts) cultured in MEM (minimal essential medium) with 10% foetal bovine serum and the same exposure time (24 hours and the same top concentration tested.)
Dispersion analysis:	Yes, dispersion was analysed via DLS. The DLS study was performed within the context of ICCR Study Number: 4023311. However, the test item preparation and exposure were performed under comparable conditions, i.e., same test material preparation protocol as well as the same test system (V79 Chinese hamster lung fibroblasts) cultured in MEM (minimal essential medium) with 10% foetal bovine serum and the same exposure time (24 hours and the same top concentration tested.)

Naumann, S. (2023) examined the clastogenic and aneugenic potential of RM09 in an *in vitro* micronucleus test (OECD 487, 2016) with V79 Chinese hamster lung fibroblast. In order to get a well dispersed and stable suspension, the test material was prepared following the recommendations of the Nanogenotox protocol (Jensen *et al.*, 2011). The test material was tested up to a concentration of 100 µg/mL, based on the recommendations set out for the *in vitro* genotoxicity testing of manufactured nanomaterials (OECD, 2021). The maximum concentration (100 µg/mL) was selected since higher concentrations of poorly soluble nanomaterials are considered not physiologically relevant (OECD, 2021) and to avoid artefactual effects resulting from precipitate (OECD TG 487, 2016). The cells were exposed

to RM09 only without exogenous metabolic activation, since both the test item core and the coating are inorganic and not metabolized by enzymes. The cell cultures were treated with RM09 for 24 hours only. A short-term treatment as outlined by the current OECD TG 487 (2016) is considered inadequate for nanomaterials as cellular uptake of the test item needs to be demonstrated. The exposure duration of 24 hours was selected in order to expose the cells for at least one cell cycle to ensure sufficient cellular uptake as recommended by OECD Nanomaterials Working Party recommendation (OECD, 2014) and as published previously (Elespuru *et al.*, 2018 and Doak *et al.*, 2012). The treatment with the cytokinesis blocker cytochalasin B was not carried out in parallel to the test item as described in the current OECD TG 487 (2016), but in succession as described in OECD TG 487 (2010) (Chapter 40, Table 1: -S9 Extended exposure, Option B), since cytochalasin B has been shown to inhibit uptake of nanoparticles by endocytosis (Elespuru *et al.*, 2018). Solvent, negative, and positive control cultures were run concurrently.
In the main experiment, RM09 was tested up to precipitating concentrations as observed microscopically and by the unaided eye. Cytotoxicity, as determined by the cytokinesis-block proliferation index, was not evident at any concentration tested. Under all conditions tested, RM09 did not show statistically significant increases in the micronucleus rate, when compared to the concurrent solvent control. Moreover, the micronucleus frequencies observed were all well within the 95% confidence interval of the historical control data range and the values did not show a positive concentration-response relationship. The solvent control values were comparable to the concurrent negative control values and well within the 95% confidence interval of the historical control data. The positive controls induced distinct and statistically significant increases in the micronucleus frequency, when compared to the solvent controls. Thus, the sensitivity of the test system and the validity of the assay was demonstrated.
In an accelerated stability study, it was demonstrated via DLS measurements that RM09 showed stable particle sizes without increased aggregation/agglomeration for at least 24 hours (please refer to Sokolowski, A., 2023 [ICCR Study Number: 4023311]). The cellular uptake of RM09 nanoparticles by V79 cells was demonstrated in a TEM study (please refer to Sokolowski, A., 2023 [ICCR Study Number: 4023311]) at all concentrations evaluated (i.e., 25, 50, and 100 µg/mL) and the test item was observed exclusively in cytoplasmic vesicles but not in the cell nucleus.
In conclusion, under the experimental conditions reported, the test item did not induce micronuclei as determined by the *in vitro* micronucleus test in Chinese hamster V79 cells. Therefore, RM09 is considered to be non-clastogenic and non-aneugenic in this *in vitro* micronucleus test, when tested in the absence of S9 up to the top recommended concentration for nanomaterials.

Ref.: Naumann, S., ICCR Study Number: 4023313, 2023. Report RM09: Micronucleus Test in Chinese Hamster V79 Cells in vitro

SCCS comment on the *in vitro* study #4: ICCR 4023313

Based on the analysis of the study results, the SCCS is of the opinion that the results on RM09 testing in the *in vitro* micronucleus test are negative.

The SCCS has noted that:

- The study design is not fully compatible with OECD TG 487 because it does not use a short incubation time and does not include application of S9 mix. However, using such an approach in case of TiO_2 particles coated with inorganic substance(s) may be justified, and in line with the SCCS/1655/23 Guidance on the Safety Assessment of Nanomaterials in Cosmetics. The SCCS is also aware that there is work ongoing on adapting new TG for genotoxicity testing with new exposure conditions, including applying only a prolonged incubation period and recommendations specific for using S9 mix for nanomaterials testing.
- Information on the stability of the dispersions and the cellular uptake of the test item is provided in ICCR Study Number 4023311, where identical RM and the same conditions of suspension preparation and V79 cells exposure for TEM analysis were used.

- As described in ICCR Study Number 4023311 the negative and solvent control and the stability of the highest and lowest test item concentrations were measured by DLS each hour for 24 hours in order to analyse the stability of the dispersion and the agglomeration/aggregation behaviour of the test item over the time. For TEM analysis RM09 at 25, 50 and 100 µg/mL for 24 h cell exposure was used. Based on the analysis of Annex 2 to ICCR Study Number 4023311, the SCCS is of the opinion that cellular uptake of RM09 was convincingly demonstrated however, at RM09 concentrations were higher than recommended by OECD TG 487. According to the information on precipitation provided by the Applicant, the highest acceptable concentration tested (based on OECD TG 487 recommendation) should be 10.7 µg/mL, and this concentration was not tested for cellular uptake, *i.e.* the lowest concentration tested by the Applicant for uptake was 25 µg/mL.
- The SCCS noted that positive control cell cultures treated with Griseofulvin showed a mean micronucleus frequency of 3.75%, which was below the minimum value of the historical positive control range for Griseofulvin (4.10 - 19.60%).

***IN VITRO* STUDY #5.** Micronucleus test in Chinese Hamster V79 cells *in vitro* on RM11, ICCR 4023314

Draft report:	Naumann, S., 2023
Evaluation status:	**New study**
Title:	RM11: Micronucleus Test in Chinese Hamster V79 Cells *in vitro*
Document No:	ICCR Study Number: 4023314
Guideline followed in study:	OECD 487 (2016)
Current guideline:	OECD 487 (2016)
Guideline and deviations from guideline in force at that time:	OECD 487 (2016) Deviations: None
GLP:	Yes
Testing Facility:	ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany
Test material:	NANO: RM11 (purity ≥99%; surface modification: coated alumina and dimethicone; particle number size distribution: Number weighted median x50: 19 nm measured by SEM, Feret min)
Test material preparation:	Following the Nanogenotox protocol (Jensen et al. 2011); suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication
Test system:	Chinese hamster lung fibroblast V79 cell line
Negative controls:	Solvent control and negative control (deionised water)
Positive controls:	Mitomycin C (clastogenic control; without metabolic activation), Griseofulvin (aneugenic control; without metabolic activation), and cyclophosphamide (clastogenic control; with metabolic activation)
Test concentrations:	1.1, 3.5, 10.7, 18.7, 57.1, and 100 µg/mL
Number of experiments and replicates:	2 independent experiments using duplicate cultures
Exposure (duration):	4 and 24 hours
Particle uptake analysis:	Yes, uptake was analysed via TEM. (The TEM study was performed within the context of ICCR Study Number: 4023312. However, the test item preparation and exposure were performed under comparable conditions, i.e., same test material preparation protocol as well as the same test system (V79 Chinese hamster lung fibroblasts) cultured in MEM (minimal essential medium) with 10% foetal bovine serum and the same exposure time (24 hours and the same top concentration tested.)
Dispersion analysis:	Yes, dispersion was analysed via DLS. The DLS study was performed within the context of ICCR Study Number: 4023312. However, the test item preparation and exposure were performed under comparable conditions, i.e., same test material preparation protocol as well as the same test system (V79 Chinese hamster lung fibroblasts) cultured in MEM (minimal essential medium) with 10% foetal bovine serum and the same exposure time (24 hours and the same top concentration tested.)

The test substance, RM11, was evaluated for its ability to induce clastogenic or aneugenic effects in V79 Chinese hamster lung fibroblasts, in the absence and presence of a metabolic activation system using the *in vitro* micronucleus assay (OECD 487, 2016). In order to get a well dispersed and stable suspension, the test material was prepared following the recommendations of the Nanogenotox protocol (Jensen *et al.* 2011). The test material was tested up to a concentration of 100 µg/mL based on the recommendations set out for the *in vitro* genotoxicity testing of manufactured nanomaterials (OECD, 2021). The maximum concentration (100 µg/mL) was selected since higher concentrations of poorly soluble nanomaterials are considered not physiologically relevant (OECD, 2021) and to avoid

artefactual effects resulting from precipitate (OECD TG 487). RM11 was tested both in absence and presence of a metabolic activation system, since the coating is of organic nature and could potentially be metabolised by enzymes of the S9 fraction. The cell cultures were treated with RM11 for 24 hours. A short-term treatment as outlined by the current OECD TG 487 (2016) is considered inadequate for nanomaterials as cellular uptake of the test item needs to be demonstrated. The exposure duration of 24 hours was selected in order to expose the cells for at least one cell cycle to ensure sufficient cellular uptake as recommended by OECD Nanomaterials Working Party recommendation (OECD, 2014) and as published previously (Elespuru *et al.*, 2018 and Doak *et al.*, 2012). Due to the organic coating of RM11 and the inclusion of a metabolic activation system in the assay, the test material was additionally tested using a 4-hour exposure. The treatment with the cytokinesis blocker cytochalasin B was not carried out in parallel to the test item as described in the current OECD TG 487 (2016), but in succession as described in OECD TG 487 (2010) (Chapter 40, Table 1: -S9 Extended exposure, Option B), since cytochalasin B has been shown to inhibit uptake of nanoparticles by endocytosis (Elespuru *et al.*, 2018). Solvent, negative, and positive control cultures were run concurrently.
The test material was tested up to precipitating concentrations as observed microscopically and by the unaided eye. Cytotoxicity, as determined by the cytokinesis-block proliferation index, was not evident at any concentration tested. Under all conditions tested, RM11 did not show statistically significant increases in the micronucleus rate, when compared to the solvent control. Moreover, the micronucleus frequencies observed were all well within the 95% confidence interval of the historical control data range and the values did not show a positive concentration-response relationship. The solvent control values were comparable to the concurrent negative control values and well within the 95% confidence interval of the historical control data. The positive controls induced distinct and statistically significant increases in the micronucleus frequency, when compared to the solvent controls. Thus, the sensitivity of the test system and the validity of the assay was demonstrated.
In an accelerated stability study, it was demonstrated via DLS measurements that RM11 showed stable particle sizes without increased aggregation/agglomeration for at least 24 hours (please refer to Sokolowski, A., 2023 [ICCR Study Number: 4023312]). The cellular uptake of RM11 nanoparticles by V79 vells was demonstrated in a TEM study (please refer to Sokolowski, A., 2023 [ICCR Study Number: 4023312]) at all concentrations evaluated (i.e., 25, 50, and 100 µg/mL) and the test item was observed exclusively in cytoplasmic vesicles but not in the cell nucleus.
In conclusion, under the experimental conditions reported, the test item did not induce micronuclei as determined by the *in vitro* micronucleus test in Chinese hamster V79 cells. Therefore, RM11 is considered to be non-clastogenic and non-aneugenic in this *in vitro* micronucleus test, when tested in the absence and presence of S9 up to the top recommended concentration for nanomaterials.

Ref.: Naumann, S., ICCR Study Number: 4023314, 2023. Report RM11: Micronucleus Test in Chinese Hamster V79 Cells in vitro

SCCS comment on the *in vitro* study #5: ICCR 4023314

Based on the analysis of the study results, the SCCS is of the opinion that the results on RM11 testing in the *in vitro* micronucleus test are negative.

The SCCS has noted that:

- Information on the stability of the dispersions and the cellular uptake of the test item is provided in ICCR Study Number 4023312, where identical RM and the same conditions of suspension preparation and V79 cells exposure for TEM analysis were used.
- As described in ICCR Study Number 4023312, the negative and solvent control as well as stability of the highest and lowest test item concentrations were measured by DLS every hour for 24 hours in order to analyse the stability of the dispersion and the

agglomeration/aggregation behaviour of the test item over the time. For TEM analysis, RM11 at 25, 50 and 100 µg/mL for 24 h cell exposure was used.

- Based on the analysis of Annex 2 to ICCR Study Number 4023312, the SCCS is of the opinion that cellular uptake of RM11 was convincingly demonstrated however, at RM11 concentrations higher than recommended by OECD TG 487. According to the information on precipitation provided by the Applicant, the highest acceptable concentration tested (based on OECD TG 487 recommendation) should be 6.1 µg/mL, but this concentration was not tested for cellular uptake, *i.e.* the lowest concentration tested by the Applicant for uptake was 25 µg/mL.

***IN VITRO* STUDY #6.** Micronucleus test in human peripheral blood mononuclear cells *in vitro* on E171-E

The SCCS note:
The Applicant provided two GLP reports on testing of E171-E material in the Ames test and the micronucleus test. As the SCCS considers the Ames test as not relevant for genotoxicity testing of particulate materials containing a nanofraction, the study report was not analysed nor taken in the WoE approach.
The SCCS analysis of study results on the micronucleus test *in vitro* on E171-E is presented below.

Guideline:	OECD Guideline 487 (July 2016)
Test system:	Human peripheral blood mononuclear cells
Test substance:	Food-grade TiO_2 (E171-E; anatase); Particle size (ECD) (number measurement, primary particle size): x10 = 0.070 µm x50 = 0.110 µm x90 = 0.180 µm
Batch (Purity):	not provided
Vehicle:	water
Assay medium:	RPMI-1640 containing 15% heat inactivated fetal bovine serum
Concentrations:	0.3, 1, 10, and 30 µg/mL for all three exposure groups (dark conditions)
Treatment:	4 h exposure, without and with metabolic activation 24 h exposure, only without metabolic activation
S9:	Aroclor 1254-induced rat liver S9
Positive controls:	Mitomycin C, Cyclophosphamide, Vinblastine
Negative control:	Vehicle
GLP:	Yes
Study period:	23 September - 30 November 2020

Methods
Cells were cultured in complete medium (RPMI-1640 containing 15% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units penicillin, 100 µg/mL streptomycin) by adding 0.5 mL heparinized blood to a centrifuge tube containing 5 mL of complete medium with 2% phytohemagglutinin.
After the 4-hour treatment (-/+ S9), the cells were centrifuged, the treatment medium was removed, the cells were washed, re-fed with complete medium containing Cytochalasin B (cytoB) at 6.0 µg/mL and returned to the incubator under standard conditions. For the 24-hour treatment in the non-activated study, cyto B (6.0 µg/mL) was added at the beginning of the treatment.
Cells were collected after being exposed to cytoB for 24 hours. The cells were stained with acridine orange.
A minimum of 2000 binucleated cells from each concentration (if possible, 1000 binucleated cells from each culture) were examined and scored for the presence of micronuclei. At least

1,000 cells (500 cells per culture) were evaluated to determine the CBPI at each dose level and the control.
Samples were collected and sent for electron microscopy analysis for cellular uptake analysis. The results of cellular uptake analysis were not provided for inclusion in the report.

Results
In the preliminary toxicity assay, the doses tested ranged from 0.01 to 100 µg/mL; the maximum concentration was tested due to the low solubility of the test substance and expected turbidity. Cytotoxicity [55 ± 5% reduction in cytokinesis-blocked proliferation index (CBPI) relative to the vehicle control] was not observed at any dose in any of the three treatment groups. At the conclusion of the treatment period, visible precipitate could be observed with the unaided eye at doses ≥ 3 µg/mL in all three exposure groups. During evaluation of cytotoxicity, visible precipitate was observed on the slides at doses ≥ 30 µg/mL in all three exposure groups. Based upon these results, the doses chosen for the micronucleus assay ranged from 0.3 to 30 µg/mL for all three exposure groups.
In the micronucleus assay, cytotoxicity (55 ± 5% CBPI relative to the vehicle control) was not observed at any dose in any of the three treatment groups. At the conclusion of the treatment period, visible precipitate could be observed with the unaided eye at doses ≥ 2 µg/mL in all three exposure groups. During evaluation of cytotoxicity, visible precipitate was observed on the slides at doses ≥ 3 µg/mL in the non-activated 4-hour exposure group; at doses ≥ 2 µg/mL in the S9-activated 4-hour exposure group; and at doses ≥ 10 µg/mL in the non-activated 24-hour exposure group. The doses selected for evaluation of micronuclei were 0.3, 1, 10, and 30 µg/mL for all three exposure groups.
Neither statistically significant nor dose-dependent increases in micronuclei induction were observed at any dose in treatment groups with or without S9 ($p > 0.05$; Fisher's Exact and Cochran-Armitage tests). The results were within the 95% control limit of the historical negative control data.

Ref.: Roy S. *In Vitro* Mammalian Cell Micronucleus Assay in Human Peripheral Blood Lymphocytes (HPBL). BioReliance Corporation, USA. Study Number AG28TA.348.BTL. 19 October 2021

SCCS comment on the *in vitro* study #6: Micronucleus test
Based on the analysis of the study results, the SCCS is of the opinion that the results on E171-E material testing in the *in vitro* micronucleus test are inconclusive.

The SCCS has noted that:
- The test material E171-E should be classified as E171-similar, not E171-equivalent, as the details on physicochemical properties and nature of impurities are unknown to the SCCS and these may differ from E171 specification,
- The study results indicate no significant increase in MN frequency after exposure to E171-E. However, internalisation of TiO_2 particles was not confirmed,
- For the 24-hour treatment in the non-activated study, cyto B was added at the start of the treatment (which might decrease internalisation of the particles),
- Details were not provided on suspension preparation or on its stability.

3.4.1.2 Mutagenicity / genotoxicity *in vivo*

Additional studies submitted by the Applicant

***IN VIVO* STUDY #1. Creutzenberg, O. (2022) Toxicological characterisation of eleven Titanium Dioxide powders.**

The SCCS note:
In response to the SCCS request for information about which legislation authorised the use of the *in vivo* study by Creutzenberg, 2022, (this information was required considering the ban on animal testing of cosmetic products and ingredients), the Applicant informed the SCCS that:
The 2022 Creutzenberg study was a range-finding study that was performed as preliminary work to meet the requirements of the REACH Substance Evaluation of titanium dioxide – details of the Substance Evaluation decision can be found at: https://echa.europa.eu/documents/10162/b1dd5108-5268-c7fd-2d70-c2d29ef9251e.
Based on the justification of the testing request, ECHA refers to a large number of uses that are within scope of REACH. These include cosmetic and non-cosmetic uses. This study was therefore not conducted for the purpose of the cosmetic product regulation requirements only but for any use of titanium dioxide in consumer products (*i.e.* multipurpose). Thus, from a legal perspective, it was considered appropriate for CE to rely on this study for the purpose of the dossier submission to the SCCS.

This screening study was conducted in rats using intratracheal instillation, with the objectives:
- to investigate lung toxicity of 11 commercial titanium dioxide samples with a rapidly executed bronchoalveolar lavage fluid (BALF) analysis; this first ranking step was necessary prior to starting further testing with a more profound experimental design.
- to perform BALF analysis 3 and 28 days after intratracheal instillation of the 11 titanium dioxide samples at a single dose.
- to assess the genotoxic potential in BALF cells 3 days after intratracheal instillation using the *in vivo* alkaline comet assay.

Titanium dioxide samples used in the *in vivo* instillation experiments in rats:
1. Uncoated mixed phase nano titanium dioxide [G1-1]: non-cosmetic grade
2. Uncoated nano anatase titanium dioxide (5 nm) [G2-5]: non-cosmetic grade
3. Uncoated pigmentary rutile titanium dioxide [G3-1]: (potential) cosmetics grade
4. Pigmentary rutile titanium dioxide coated with alumina and TMP [G4-19]: (potential) cosmetics grade
5. Pigmentary rutile titanium dioxide coated with alumina, zirconia and TMP [G5-4]: non-cosmetic grade
6. Nano rutile titanium dioxide coated with alumina and hydrophobic organic [G6-3]: (potential) cosmetics grade
7. Pigmentary rutile titanium dioxide coated with high SSA silica and alumina (40 m^2/g) [G7-5]: non-cosmetics grade
8. Nano rutile titanium dioxide coated with silica (40 m^2/g) [G8-2]: (potential) cosmetics grade
9. Pigmentary rutile titanium dioxide coated with aluminium phosphate [G9-5]: non-cosmetics grade
10. Nano anatase titanium dioxide (5nm) with tungsten trioxide as co-catalyst [G10-4]: non-cosmetic grade
11. Pigmentary uncoated anatase titanium dioxide [E171-E]: (potential) cosmetics grade

The well-established inert dust titanium dioxide ("Bayertitan T") and the strongly inflammogenic quartz DQ12 ("Dörentrup DQ12") were used as particle-like negative and

positive reference items, respectively, and the known clastogen ethyl methanesulfonate (EMS) served as methodological positive control for the alkaline comet assay with BAL cells. Animals treated with 0.9% saline were used as vehicle control group.

The study design and procedures are described in brief as follows (scheme in the Table below):
The test and particle-like reference materials were suspended in saline by gentle stirring (exposure to light was minimised as far as feasible). The total dose (1 mg/rat) was instilled in two aliquots, each suspended in a volume of 0.3 mL and administered on two consecutive days to achieve a homogeneous distribution of the test/reference materials in the lungs.
All samples, except G6-3, were prepared with saline as vehicle (1.67 mg test item/mL). In contrast, G6-3 was prepared with 0.05 % Tween 80® in saline, due to its hydrophobic nature (see also Driscoll *et al*., 2000). After gentle shaking all samples were sonicated for 5 minutes to guarantee homogeneous suspensions. Additionally, G6-3 was stirred with a magnetic stirrer for 30 minutes. For the other samples vortexing was used to perpetuate the homogeneity until administration to the animals. Sonication device consisted of a Bandelin Sonorex RK 510H with HF performance of 160/320 W (160 W average), and HF frequency of 35 kHz. Samples were sonicated for 5 min. Under these conditions, any detachment of coating material was considered as negligible.
Concurrent controls were treated with the vehicle saline only or 0.05 % Tween 80® in vehicle. The rats were anaesthetised by CO_2/O_2 67/33 (v/v) for some seconds to perform the intratracheal instillation. This is the shortest and most gentle anaesthesia for this kind of dosing, as compared to intraperitoneally or inhalatory administered narcotic agents. The intratracheal instillation of the particle suspensions was followed by a post-treatment observation period for up to 28 days.
Bronchoalveolar lavage was performed in all rats at days 3 or 28 after the last instillation. The lung lavage fluid was collected and characterised using total and differential cell counts and biochemical endpoints (lactate dehydrogenase (LDH) activity, β-glucuronidase (ß-Glu) activity, and total protein (TP) level) in the BALF as well as determination of DNA strand break induction in BAL cells on day 3.

Intratracheal instillation study – Overview of treatment groups

Group	Treatment	Initial dose (mg)	Number of animals day 3	Number of animals day 28	Number of animals in total
1	Vehicle control A (0.9 % Saline)	-	8 (+3)	8	16 (+3)
2	G1-1	1	6	6	12
3	G2-5	1	6	6	12
4	G3-1	1	6	6	12
5	G4-19	1	6	6	12
6	G5-4	1	6	6	12
7	G6-3	1	6	6	12
8	G7-5	1	6	6	12
9	G8-2	1	6	6	12
10	G9-5	1	6	6	12
11	G10-4	1	6	6	12
12	E171-E	1	6	6	12
13	Bayertitan T Negative control	1	6	6	12
14	Quartz DQ12 Positive control	1	6	6	12
15	Vehicle control B (0.9 % Saline + 0.05% Tween)	-	1	-	1
				Total number of animals	176

Administration of total dose in two halves, each test/reference item suspended in a vol. of 0.3 ml saline and administered, on two consecutive days (day -1 and day 0)

***In vivo* mammalian alkaline comet assay with BAL cells**

For three out of the 6-8 animals per treatment group (treatment groups 1-14), as well as for 3 additional animals treated with vehicle control A and one with vehicle control B (treatment group 15), three coded comet assay slides were prepared under red light to avoid unspecific DNA damage. The *in vivo* alkaline comet assay was subsequently performed under red light according to the respective SOPs of Fraunhofer ITEM and by considering the OECD Guideline for the Testing of Chemicals No. 489 (*In Vivo* Mammalian Alkaline Comet Assay).

As a methodological positive control, an aliquot of 150,000 cells of a concurrent negative control lavagate was transferred to 1.5 ml reaction cups, spun down and the supernatant was discarded. The cell pellet was subsequently resuspended in 1 ml of DMEM cell culture medium, containing 1 µl/ml of the known clastogen EMS, and cells were incubated for 1 h in a heat block at 37 °C. At least two positive control samples were generated per sacrifice day.

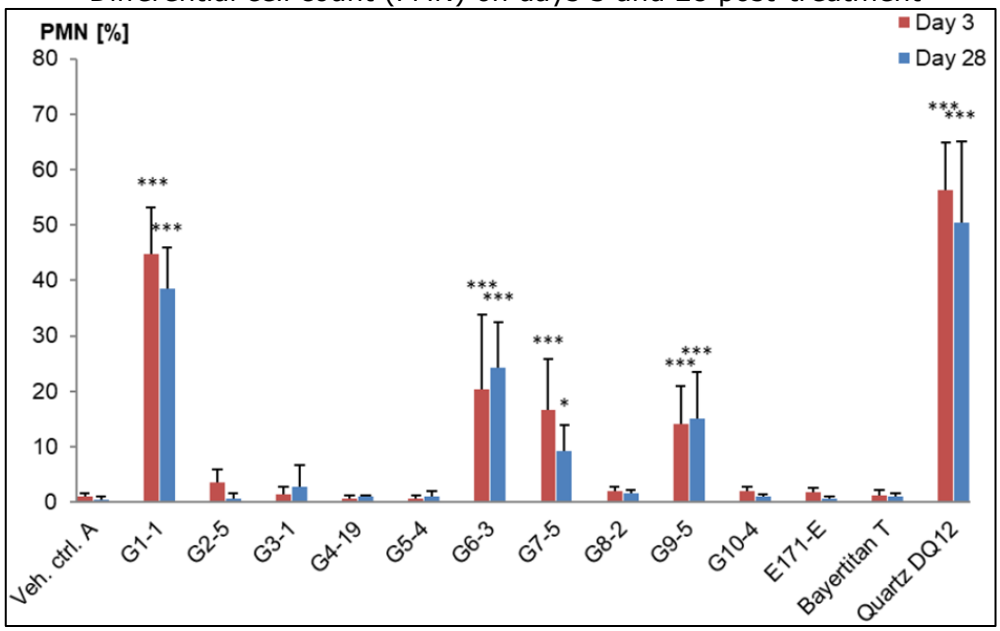
Three aliquots of BAL cells per animal were centrifuged for 5 min at 900 rpm (Heraeus Biofuge® 15, Thermo Scientific, Germany), re-suspended in pre-heated 0.75% low melting agarose (peqlab, Erlangen, Germany), applied to agarose pre-coated slides, using an agarose sandwich technique, and lysed for 2 h at 4°C to liberate the DNA (lysis buffer: 2.5 M NaCl, 100 mM Na2EDTA, 200 mM NaOH, 1 % Triton X-100, 10 % DMSO, pH 10). Subsequent DNA-unwinding (20 min) and electrophoresis (20 min, 32 V, 320 mA) were both done in an electrophoresis chamber (PERFECT BLUE™ 41-2340, peqlab, Darmstadt, Germany; capacity: 40 slides) on ice, in 4 °C cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). In every electrophoresis run both methodological positive control slides and slides from

vehicle control animals were included. After electrophoresis, slides were neutralized using a 0.4 M Tris HCl (pH 7.4) buffer and then stained with ethidium bromide (0.002 %).
DNA strand break induction was finally analysed for two slides per animal or methodological positive control, using a Zeiss Axioskop (fluorescence microscope) and the Comet assay III Software from Perceptive Instruments (Steeple Bumpstead, Haverhill, UK). As the main and recommended (OECD 489) endpoint, the tail intensity (TI) of 100 nuclei per slide and two slides per animal/sample (200 nuclei in total) were analysed. So-called "hedgehogs" and overlapping nuclei/comets were excluded from analysis, but "hedgehogs" were documented. The tail intensity (TI) is a direct measure of the amount of broken DNA. This measure can be standardised among various studies and laboratories and is linear over a wide range. The comet assay analyses were all performed in a blinded manner, without knowledge of the concrete identity of the test items.

For the comet assay, as recommended, the arithmetic means of the two medians of the 100 nuclei analysed per slide were calculated per animal, followed by calculation of the group means (generally 3 animals per particle treated group) ± SD from the arithmetic means of the single animals.

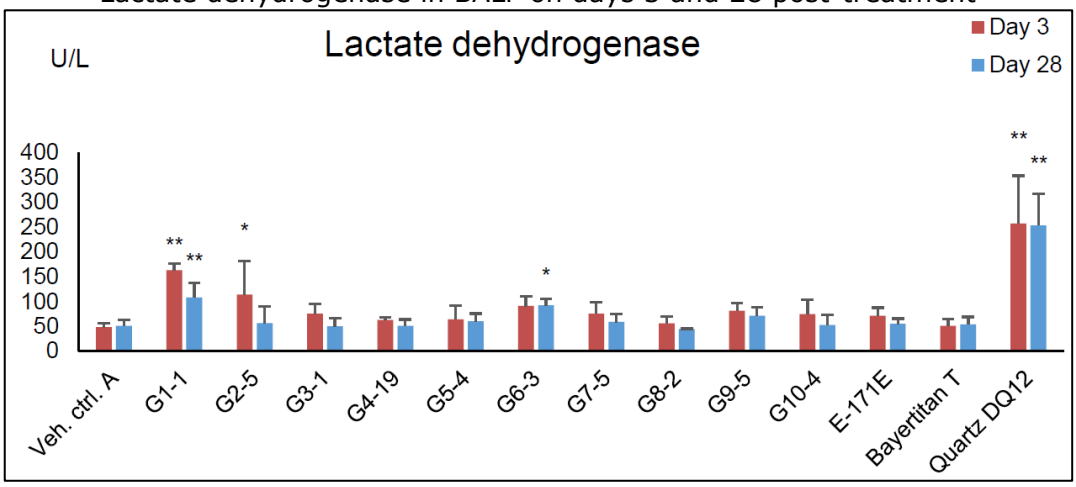
Results:
In the titanium dioxide-treated groups, no statistically significant increases in lung weight were detected. Only the positive control group, treated with quartz DQ12, showed statistically significant increases in lung weights in comparison with the vehicle treated control group.
Macroscopic examination showed treatment-related findings in the quartz DQ12 (positive control) treated animals, where moderately enlarged lung associated lymph nodes were observed.
Bronchoalveolar lavage fluid analysis showed mid to high levels of polymorphonuclear neutrophil (PMN) influx with statistical significance (in comparison with the vehicle control group) up to 56% PMN with quartz DQ12 (positive control) and to a lesser extent in G1-1 (45%). Other samples showed much lower PMN levels:

Differential cell count (PMN) on days 3 and 28 post-treatment

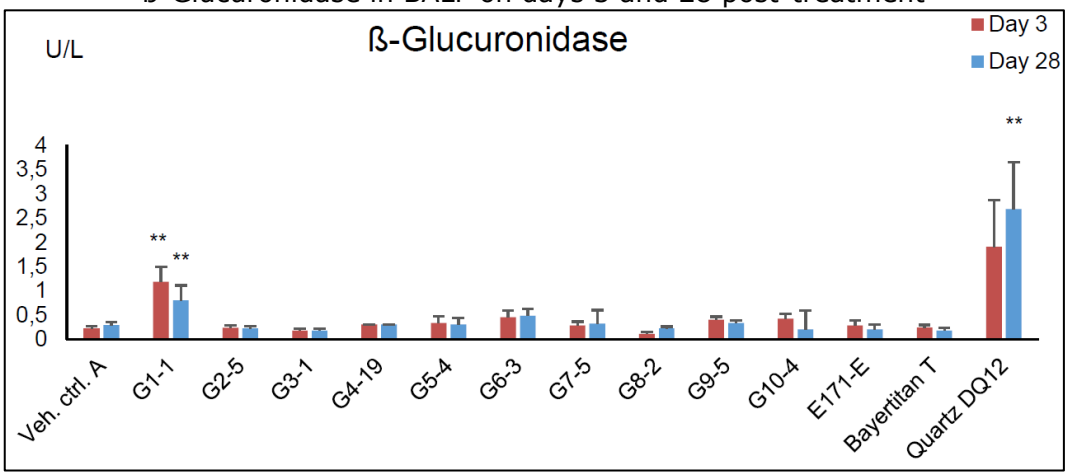


The ranking of the PMN parameters is mirrored in the biochemical parameters of the BALF, in which only samples DQ12 and G1-1 showed consistent elevated levels in lactate dehydrogenase (LDH) activity, ß-glucuronidase activity and total protein (TP) level, G6-3 occasionally elevated LDH activity and TP level, and G9-5 only increased TP level on Day 28.

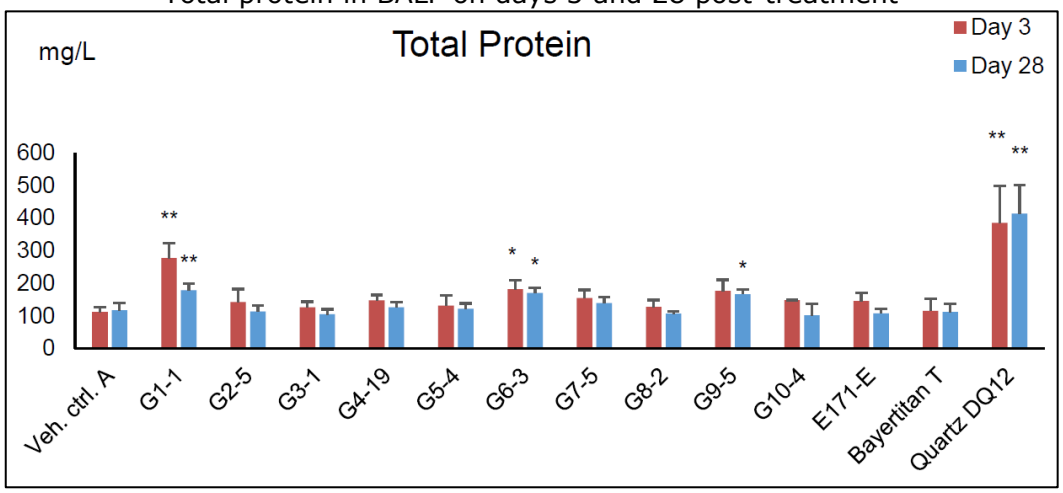
Lactate dehydrogenase in BALF on days 3 and 28 post-treatment



ß-Glucuronidase in BALF on days 3 and 28 post-treatment



Total protein in BALF on days 3 and 28 post-treatment

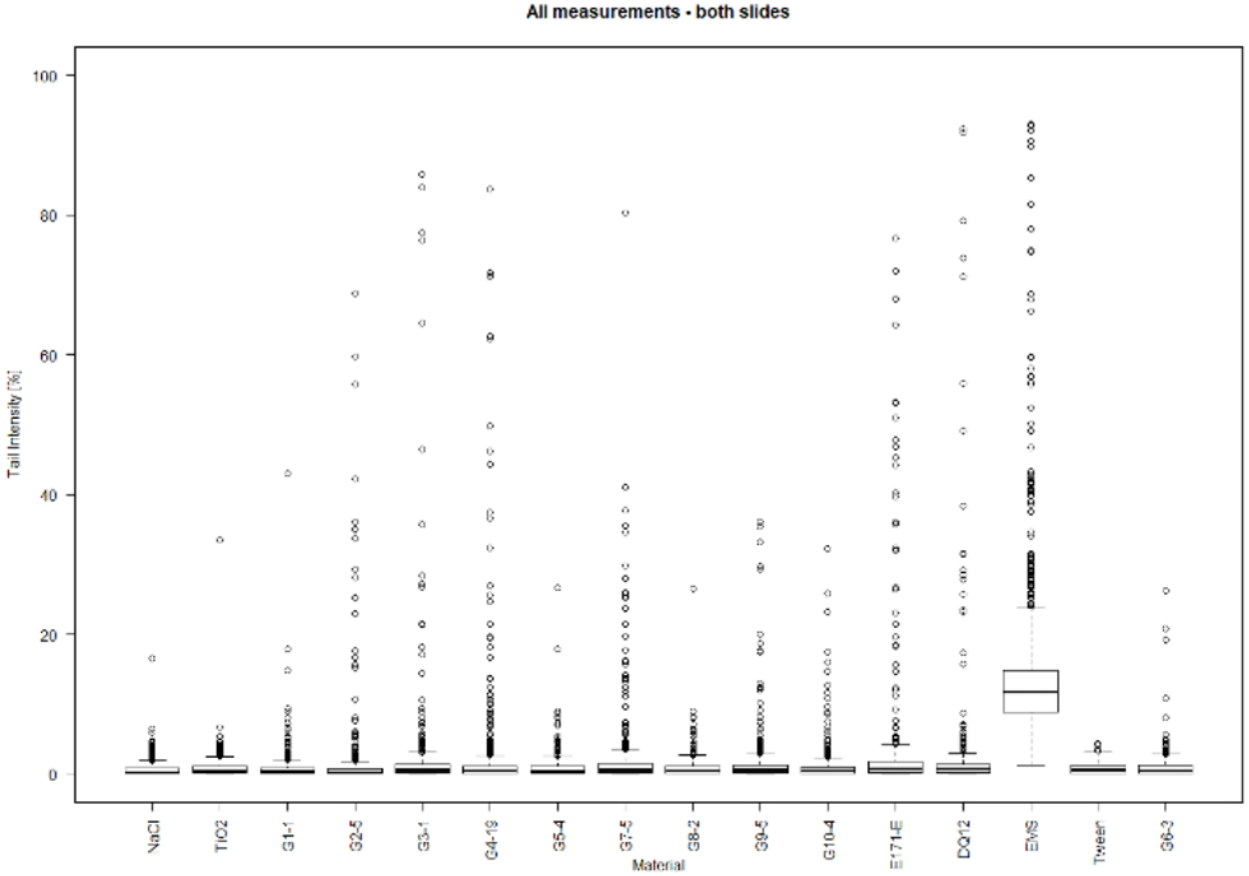


The cytological and biochemical parameters show differences between the different titanium dioxide samples with regard to their biological reactivity. The sample G1-1 shows consistently the highest reactivity.

***In vivo* alkaline comet assay:** The DNA damaging potential of the eleven titanium dioxide samples was investigated in BAL cells, using the *in vivo* alkaline comet assay. As the main

and recommended (OECD 489, 2016) endpoint, the tail intensity (TI) of 100 nuclei per slide and two slides per animal/sample (200 nuclei in total) were analysed. So-called ''hedgehogs'' and overlapping nuclei/comets were excluded from analysis, but "hedgehogs" were documented. Hedgehogs were only observed for E171-E-treated animals and in the methodological positive controls (4 – 5 per sample; negative control cells treated *in vitro* with EMS). For all other treatments no hedgehogs were present.
When evaluating the TI values [%] on a single cell level (200 events per sample/animal and 1 to 7 animals per treatment, mostly 3 animals) for the different test materials (see Figure below), it can clearly be seen that the methodological positive control EMS is associated with markedly higher TI values, compared with the other test materials, whereas the vehicle controls demonstrated almost no heavily damaged cells, thus, indicating appropriate performance of the test. For nearly all particulate test items, small populations of cells with slightly higher DNA damage were noted. These highly damaged cells are most likely a result of the mechanical interference of the test items, as cells were in most cases highly loaded with titanium dioxide particles.

In vivo alkaline comet assay with BAL cells (day 3 post-exposure). Data distribution on the single cell level for the different materials.



Conclusions on the DNA damage (by the Report's Authors):
Bronchoalveolar lavage fluid (BALF) analysis indicated no biologically relevant increases in arithmetic group mean tail intensity (TI), compared to the respective vehicle controls, when using the median tail intensity as summarizing slide measure and the arithmetic means of the medians of two slides per animal, according to OECD 489. Thus, neither one of the TiO_2 samples nor Quartz DQ12 seemed to exhibit a relevant DNA damaging potential.

Ref.: Creutzenberg, O. (2022) Toxicological characterisation of eleven Titanium Dioxide powders. Fraunhofer ITEM Study no. 02 N 19 538 (non-GLP) - Module I-1. Unpublished study report

SCCS comments on the *in vivo* study #1

The SCCS, after analysis of the results for all TiO_2 test materials, considers them as inconclusive for the following reasons:

- The study design has major deviations from the recommendations by the OECD TG 489:
1. There were 3 animals used per treatment group, while according to OECD 489: "group sizes at study initiation (and during establishment of proficiency) should be established with the aim of providing a minimum of 5 analysable animals of one sex, or of each sex if both are used, per group". Due to the limited number of animals used, the statistical significance of the results, especially with E171 sample, is difficult to assess.

2. The animals were sampled 3 days after the last (second) exposure, while according to OECD 489: "Animals should be given daily treatments over a duration of 2 or more days (*i.e.* two or more treatments at approximately 24 hour intervals), and samples should be collected once at 2-6 h (or at the Tmax) after the last treatment". It cannot be excluded that sampling after 3 days might be too long to detect DNA damaging effect in some cases of test materials (due to, *e.g.* clearance or induction of DNA repair mechanisms).

3. Comet assay was performed on BALF cells and not on lung tissues, which must be analysed according to the OECD TG 489 with and without specific modification to detect oxidative damage.
- The Applicant has provided morphological analysis of BALF cells by light microscopy stating that for almost all test items, the cellular condition was described as "cells partially filled with particles" or "cells filled with particles". Although, this analysis may be regarded as an indication of uptake, information provided on cells other than alveolar macrophages or granulocytes would be more informative. In any case, confirmation by TEM would be necessary.
- Only 5 out of 11 TiO_2 materials tested are potential cosmetic grades. For the information on correspondence of the tested grades of TiO_2 G-samples to the TiO_2 raw materials used in cosmetic products, please see the chapter on "The SCCS analysis of the study reports submitted by the Applicant. *IN VITRO* STUDY #1. ToxTracker".
- The following statement by the Applicant: "Over the variety of all grades tested, the sample G1-1 (also known as Aeroxide P25 or NM-105) showed the highest inflammogenic potential, with all other grades showing lower biological reactivity. Based on the grade with the highest inflammogenic potential still showing a negative *in vitro* genetic toxicity, it is justified to assume that all other titanium dioxide grades are intrinsically covered as they will exhibit a lesser biological reactivity and a negative *in vitro* genetic toxicity" is in contradiction to Table 2.1. and A1.1., where P25 is stated as being not used in cosmetics formulations, and several publications have indicated its positive genotoxic effects.

***IN VIVO* STUDY #2**

The Applicant drew the attention of the SCCS to a very recent publication by Akagi *et al*. (Particle and Fibre Toxicology. 2023 Jun 20;20(1):23. doi: 10.1186/s12989-023-00533-x). The SCCS analysis of the study is included in the "Annex X. SCCS and EFSA analysis of studies on TiO_2 genotoxicity". In brief, in the study oral administration of TiO_2 anatase nanoparticles with a crystallite size of 6 nm to rats up to 1000 mg/kg bw/day for 90 days showed no toxic effects such as general toxicity, titanium accumulation in the liver, kidneys, and spleen, or colonic crypts abnormalities. Micronucleus test in isolated hepatocytes, as well as γ-H2AX staining in bone marrow cells, nasal cavity, BALT, trachea, Peyer's patches, cervical and mediastinal lymph nodes tissues, were negative.
The Applicant has not clarified if the TiO_2 nanoparticles used in the study by Akagi *et al.* (2023) are relevant to the cosmetic grades.
Considering that distribution of the TiO_2 NPs to internal organs after p.o. administration was not convincingly demonstrated in this study, the SCCS considered the results as inconclusive. Moreover, as only coated rutile phase TiO_2 material with up to 5% anatase is indicated for

potential use in cosmetic products, the nanoparticles used in the study by Akagi *et al*. (2023) are not relevant for the range of TiO_2 materials used in cosmetic products.

3.4.1.3 The overall SCCS assessment of the genotoxicity of TiO_2 grades used in cosmetic products

The SCCS evaluated the genotoxicity of TiO_2 grades used in cosmetic products based on data available from 2 sources:

1. Study reports on TiO_2 grades genotoxicity submitted by the Applicant
2. Published literature search

3.4.1.3.1. The SCCS evaluation of genotoxicity of selected TiO_2 raw materials based on the original study reports provided by the Applicant

The SCCS evaluated the original study reports on genotoxicity of selected TiO_2 raw materials provided by the Applicant, *i.e.* 8 *in vitro* and 2 *in vivo* studies (the second study by Akagi *et al*., 2023, indicated by the Applicant, included in the analysis of the published literature data in "Annex X. SCCS and EFSA analysis of studies on TiO_2 genotoxicity" in the MS Excel file).

Based on the analysis, the results of genotoxicity testing of RM09 and RM11 were considered negative. However, for the other TiO_2 grades, the results were considered inconclusive based on different limitations identified. Detailed SCCS comments to each of the studies are presented in paragraphs 3.4.1.1 and 3.4.1.2. and a summary of the SCCS evaluation of the studies in presented in Table 3.4.1.3.A.

Table 3.4.1.3.A. The SCCS evaluation of genotoxicity of selected TiO_2 raw materials based on the original study reports provided by the Applicant. Titanium dioxide raw materials used in cosmetics are highlighted in bold (information according to the Applicant).

	TiO_2 samples tested	**(Potential) cosmetic grade**	**Creutzenberg (2022)** *in vivo* comet in BAL cells	**ToxTracker** (Biomarker gene: Bscl2, Rtkn)	**Mammalian cell gene mutation assay *in vitro***	**Micronucleus test *in vitro***
1	**Pigmentary uncoated anatase TiO_2 [E171-E] Equivalent to RM67**	**Yes**	Inconclusive	Inconclusive	NA	Inconclusive (BioReliance)
2	**Nano rutile TiO_2 coated with alumina and hydrophobic organic [G6-3] Equivalent to RM42**	**Yes**	Inconclusive	Inconclusive	NA	NA
3	**Nano rutile TiO_2 coated with silica (40 m^2/g) [G8-2] Equivalent to RM09**	**Yes**	Inconclusive	Inconclusive	Negative	Negative
4	**RM11**	**Yes**	NA	NA	Negative	Negative
5	**Uncoated pigmentary rutile TiO_2 [G3-1]**	**Not marketed for use in cosmetics** but have similar PhysChem characteristics to some	Inconclusive	Inconclusive	NA	NA

		cosmetics grades				
6	**Pigmentary rutile TiO_2 coated with alumina and TMP [G4-19]**	**As above**	Inconclusive	Inconclusive	NA	NA
7	Uncoated mixed phase nano TiO_2 [G1-1]	no	Inconclusive	Inconclusive	NA	NA
8	Pigmentary rutile TiO_2 coated with high SSA silica and alumina (40 m^2/g) [G7-5]	no	Inconclusive	Inconclusive	NA	NA
9	Pigmentary rutile TiO_2 coated with aluminium phosphate [G9-5]	no	Inconclusive	Inconclusive	NA	NA
10	Uncoated nano anatase TiO_2 (5 nm) [G2-5]	no	Inconclusive	Inconclusive	NA	NA
11	Pigmentary rutile TiO_2 coated with alumina, zirconia and TMP [G5-4]	no	Inconclusive	Inconclusive	NA	NA
12	Nano anatase (5 nm) with tungsten trioxide as co-catalyst [G10-4]	no	Inconclusive	Inconclusive	NA	NA

NA - not available

3.4.1.3.2. Published literature search carried out by the SCCS

The SCCS carried out a search of the published literature to obtain any further information that might be relevant for consideration in the current safety assessment. The parameters used to search the published literature were:

- the period to be covered as the update of the EFSA Opinion on TiO_2 (2021): 1 January 2021 to 16 April 2023 (the last EFSA search was done on December 2020); 2005-2023: additional search on other TiO_2 grades not analysed by the EFSA,
- English language. If a relevant text was provided in another language, it was translated into English.
- No specific restrictions to geographical area.
- The types of documents analysed were peer-reviewed articles, journal entries, book chapters, government funded publications, etc.
- Terms were searched in: title, abstract, key word and text field.
- Databases searched: Web of Science, Pub-Med.

Criteria of evaluation of the genotoxicity results in the open literature:
The literature analysis and the conclusions by the EFSA have been updated by the SCCS by extending for the analysis of the TiO_2 materials that had been excluded from the EFSA analysis because they were not relevant for the assessment of E171.
The following inclusion criteria for TiO_2 particulate forms (irrespective of size) have been used by the SCCS during preparation of the current Scientific Advice:

Pigmentary grades	Nanoparticle grades
E171, food-grade (anatase/rutile) non-coated	Rutile coated (or rutile with up to 5% anatase) - the Applicant provided information that all nanoforms used in cosmetics were rutile, coated)
E171-similar pigmentary grades non-coated	
Pigmentary grades other than E171 non-coated	

coated	

For assessment of the available information, the SCCS adopted the same approach as EFSA on the genotoxicity analysis. A comparative overview of the approaches used by EFSA, Kirkland *et al.*, 2022 and the SCCS is provided in Annex W.
The Tables presented in the EFSA Appendices to the Opinion on TiO_2 (2021), were used as a starting point, and basic information from these tables was merged into the one SCCS/EFSA database on TiO_2 materials relevant for cosmetic products (Annex X. SCCS and EFSA analysis of studies on TiO_2 genotoxicity).
Four of the current SCCS experts participating in this task had also participated in the preparation of the EFSA Opinion on TiO_2 (2021).

Number of records retrieved from the published literature search

Web of Science 2023-01-09
Keywords: titanium dioxide AND nanoparticle* AND mutagenic* genotoxic* >> number of records 285; sorted by the most relevant
Keywords: titanium dioxide AND nanoparticle* AND mutagenic* genotoxic* >> number of records 129

Web of Science 2023-04-16
Keywords: titanium dioxide AND nanoparticle* AND mutagenic* genotoxic* >> number of records 288
Keywords: titanium dioxide AND nanoparticle* AND mutagenic* genotoxic* >> number of records 130.

For a detailed list of publications selected for analysis, please see "Annex V. List of publications on TiO_2 particles genotoxicity analysed by the SCCS".

Detailed analysis of genotoxicity of TiO_2 materials based on the review of the published literature

The detailed information with analysis and evaluation scores with sorting and filtering options is presented in "Annex X. SCCS and EFSA analysis of studies on TiO_2 genotoxicity", the MS Excel file.

Total number of records (combinations "TiO_2 material-test system" for the SCCS evaluation and "TiO_2 form" for the EFSA data) is 353. After excluding records not taken into consideration during the WoE for different reasons presented in the sheet "NOT taken into consideration" (35 records), the number of records taken into consideration during the WoE (sheet "TAKEN into consideration") was 318.
The main reasons for excluding some records were:
- no information provided on crystalline form tested,
- insufficient methodology description,
- TiO_2 form tested was not relevant.

After further excluding the records with low relevance (34 records in the sheet "RELEVANCE - LOW) the number of records curated for the final analysis was 284.
The main reasons for scoring some records as of low relevance were:
- unacceptable level of cytotoxicity,
- no positive control used in the experiment,
- excessively high concentrations used,
- no or insufficient data on dispersion,
- no proof on internalisation,
- short time of exposure used, etc.

In view of the large number of TiO_2 grades used in cosmetic products, the SCCS segregated them into 4 categories for the purpose of the current assessment. These were:

1. E171-equivalent materials [6]
The E171-equivalent material was defined by the SCCS based on the specifications given in the scientific opinion by EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings): Scientific opinion on the proposed amendment of the EU specifications for titanium dioxide (E 171) with respect to the inclusion of additional parameters related to its particle size distribution. EFSA Journal 2019;17(7):5760, 23 pp. https://doi.org/10.2903/j.efsa.2019.5760):
- It consists of anatase or rutile generally containing small amounts of the other phase (rutile or anatase, 2% m/m) and it may also contain small quantities (< 0.5%) of constituent particle growth and crystal phase control agents (alumina, sodium or potassium in combination with phosphate).
- The average median Feret min diameter of the constituent particles obtained by three laboratories using SEM was reported, for the five brands of anatase, to range between 104 and 166 nm and the percentage of particles by number 100 nm ranges from 11.4 to 45.6%. For the rutile sample the <median Feret min diameter was 151 nm and the percentage of particles by number 100 nm was <5.4%.

2. E171-similar materials
The E171-similar material was defined as particles comparable to the E171-equivalent material in terms of crystalline phase, size, lack of coating. However, firm conclusions on the similarity with the E171-identical material could not be drawn, due to incomplete or missing data on the physicochemical properties.

3. PIGMENTARY MATERIALS other than E171-equivalent or E171-similar materials

4. NANOMATERIALS
subcategory: Anatase
subcategory: Rutile
subcategory: Anatase/Rutile

The summary of the SCCS final evaluation is presented below (Tables 3.4.1.3.B-D) for the following groups of TiO_2 materials:

1. TiO_2 E171-equivalent (anatase/rutile, <2%) AND E171-similar materials – analysis of the published literature data – TABLE 3.4.1.3.B
2. TiO_2 PIGMENTARY Materials other than E171-equivalent or E171-similar materials – analysis of the published literature data – TABLE 3.4.1.3.C
3. TiO_2 NANOMATERIALS – analysis of the published literature data – TABLE 3.4.1.3.D

[6] To name the categories, the SCCS prefers to use the term „material" instead of "grade" used by the Applicant.

The SCCS conclusions on safety of TiO_2 E171-equivalent and E171-similar materials used in cosmetic products

E171-equivalent materials
Considering all the available relevant information summarised in Table 3.4.1.3.B below, the SCCS is of the opinion that genotoxic hazard of the pigmentary TiO_2 materials equivalent to E171 cannot be excluded. This is based on analysis of the compiled SCCS/EFSA published literature data review, indicating overall genotoxic hazard *in vitro* (1 positive micronucleus test, 5 positive Comet assays, 1 positive γH2AX assay), compared to 2 negative Comet assays after oral exposure and 1 inconclusive Comet assay in BAL cells after *in vivo* exposure (Creutzenberg *et al.*, 2022). In the opinion of the SCCS, the Comet assay *in vitro* is an indicator test for genotoxicity and can be used as supporting evidence in WoE. Therefore, a safe use of these pigmentary TiO_2 materials in cosmetic products cannot be confirmed with the currently available weight of evidence. Considering some limitations of the positive *in vitro* micronucleus study (Proquin *et al*., 2017), a valid *in vitro* micronucleus or chromosomal aberration test (assuring all nanotoxicology state-of-the-art principles are applied) with adequately selected E171-equivalent material(s) would be needed to overrule the current conclusion.

E171-similar materials
The SCCS is of the opinion that a genotoxic hazard of pigmentary TiO_2 E171-similar materials cannot be excluded. This is based on the analysis of one *in vitro* GLP study (micronucleus test on the material notified as E171-E with inconclusive result) provided by the Applicant and the compiled SCCS/EFSA published literature data review, indicating 1 positive Comet assay after oral exposure, as well as 1 equivocal micronucleus test after intraperitoneal exposure, and 1 negative chromosomal aberration assay after intraperitoneal exposure. Hence, safe use of these pigmentary TiO_2 materials in cosmetic products cannot be confirmed. Additional valid *in vitro* micronucleus or chromosomal aberration test (assuring all nanotoxicology state-of-the-art principles are applied) with adequately selected E171-similar material(s) would be needed to overrule the current conclusion.

TABLE 3.4.1.3.B. TiO_2 E171-equivalent AND E171-similar materials – analysis of the published literature data merged in the SCCS/EFSA database

TiO_2 material tested	**Description**	**Number of records identified** among the total n = 284	**Number of records and outome** (positive; negative; inconclusive or equivocal)	
			Analysis by EFSA (until December 2020)*	**Analysis by SCCS** (2020-2023)**
E171-equivalent	Total	11		
	In vitro	9		
	Micronucleus *in vitro*	1	1 Positive: Proquin *et al*., 2017	
	Comet *in vitro*	6	2 Positive: Brown *et al*., 2019; Proquin *et al*., 2017 1 Negative: Dorier *et al*., 2019	3 Positive: Ferrante *et al*., 2023; Vignard *et al*., 2023
	Other genotoxicity endpoints *in vitro* – H2AX	1		1 Positive: Vignard *et al*., 2023
	Other genotoxicity endpoints *in vitro* – ToxTracker	1	1 Negative: Brown *et al*., 2019	
	In vivo:	2		
	Comet *in vivo*	2	2 Negative: Bettini *et al*., 2017 Jensen *et al*., 2019	
E171-similar	Total	3		
	In vitro	0		One GLP study report on micronucleus test submitted by the Applicant, on E171-E with inconclusive result
	In vivo	3		
	Micronucleus *in vivo*	1	1 Equivocal: Shelby and Witt, 1995	
	Chromosomal aberrations *in vivo*	1	1 Negative: Shelby and Witt, 1995	
	Comet *in vivo*	1	1 Positive: Sycheva *et al*., 2011	

* Only final result (*i.e.* negative, positive, equivocal) from an EFSA Appendix was included;
** TiO_2 material-test system combination included

The SCCS conclusions on safety of TiO_2 PIGMENTARY MATERIALS other than "E171-equivalent or E171-similar materials" used in cosmetic products

Considering all the available relevant information, summarised in Table 3.4.1.3.C below, the SCCS is of the opinion that genotoxic hazard of TiO_2 PIGMENTARY MATERIALS, used in cosmetic products both uncoated and coated, that fall into the category of "other than E171-equivalent or E171-similar material" cannot be excluded. This is based on analysis of the compiled SCCS/EFSA published literature data review up to April 2023, indicating that:

- pigmentary materials anatase, uncoated, can induce genotoxic effects *in vitro* (mainly represented by the positive Comet assay results), and *in vivo* (1 positive Comet assay after oral exposure). Although 5 studies in the published literature reported negative results in *in vitro* micronucleus test using similar pigmentary anatase materials, indicating safety of these materials, positive results from *in vitro* and *in vivo* Comet assays make it difficult to conclusively exclude genotoxicity hazard of these materials.
- pigmentary materials rutile, uncoated, can induce DNA damaging effects (5 positive Comet assay results) and cell transformation. Although 4 studies in the published literature reported negative results in *in vitro* micronucleus test and 1 in *in vitro* chromosomal aberration test using similar pigmentary rutile materials, indicating

safety of these grades, the relevance of the test materials to the cosmetic grades cannot be conclusively determined. The positive results from *in vitro* Comet assays make it difficult to conclusively exclude genotoxicity hazard of the pigmentary rutile materials.

- pigmentary materials anatase/rutile, uncoated, show DNA damaging effect (1 positive Comet assay result), but not induction of micronuclei. Based on the results it is not possible to conclusively exclude genotoxicity hazard of the pigmentary anatase/rutile materials.
- Based on the collective information safe use of pigmentary materials in oral cosmetic products cannot be confirmed with the currently available weight of the evidence. Therefore, the Applicant should provide further evidence from studies according to the Scheme of testing strategy for genotoxicity/mutagenicity of cosmetic ingredients presented in the SCCS Notes of Guidance (SCCS/1647/22) and Guidance on the Safety Assessment of Nanomaterials in Cosmetics (SCCS/1655/23).

Another Scientific Opinion on the safety of TiO_2 in toys has been published recently (June 2023) by the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) (https://health.ec.europa.eu/publications/scheer-scientific-opinion-safety-titanium-dioxide-toys-0_en). Although the SCHEER Opinion relates to the safety of TiO_2 in toys, it concluded that such a use can only be considered safe "when the absence of an ultrafine fraction (*nanoscale/nanosized particles (1-100 nm) indicated as ultrafine particles in line with conventions in inhalation toxicology*) in the TiO_2 pigments can be demonstrated by an appropriate methodology." The SCHEER Opinion is therefore not in contradiction to the conclusions drawn in this Opinion, because the physicochemical data evaluated by the SCCS have shown that most of the pigmentary TiO_2 grades used in cosmetic products contain a varying proportion of the constituent particles in the nano range.particles in the nano range.

Table 3.4.1.3.C. TiO_2 PIGMENTARY MATERIALS other than "E171-equivalent or E171-similar material" – analysis of the published literature data merged in the SCCS/EFSA database

TiO_2 material tested	**Description**	**Number of records identified** among the total n = 284	**Number of records and outome** (positive; negative; inconclusive or equivocal)	
			Analysis by EFSA (until December 2020)*	**Analysis by SCCS** (2020-2023)**
Pigmentary material other than "E171-equivalent or E171-similar"	Total	42		
Anatase	Surface chemistry/Coating	0		
	Surface chemistry/No coating	27		
	In vitro assays	26		
	Mammalian cell gene mutations *in vitro*	1	1 Negative: Xu *et al*., 2009	
	Micronucleus *in vitro*	5	4 Negative: Di Bucchianico *et al*., 2017; Andreoli *et al*., 2018; Uboldi *et al*., 2016; Guichard *et al*. 2012	1 Negative: Zijno *et al*., 2020
	Comet *in vitro*	17	10 Positive: El Yamani *et al*., 2017; Di Bucchianico *et al*., 2017; Zijno *et al*., 2020; NANOGENOTOX Project, 2013; Andreoli *et al*., 2018; Jugan *et al*., 2012; Guichard *et al*. 2012; Nakagawa *et al*., 1997; Murugadoss *et al*., 2020; Kumar *et al*., 2020 2 Negative: Vila *et al*., 2018; Brzicova *et al*., 2019	4 Positive: Gea *et al*., 2019; Murugadoss *et al*., 2021; Petkovic *et al*., 2011b; Zijno *et al*., 2020 1 Negative: Hamzeh *et al*., 2013
	Other genotoxicity endpoints *in vitro* – H2AX	2	1 Positive: Toyooka *et al*., 2012 1 Negative: Barillet *et al*. 2010	
	Cell transformation assay	1	1 Negative: Uboldi *et al*., 2016	
	In vivo assays	1		
	Comet *in vivo*	1	1 Positive: Murugadoss *et al*., 2020	
Rutile	Surface chemistry/Coating	0		
	Surface chemistry/No coating	13		
	In vitro assays	12		
	Micronucleus *in vitro*	4	4 Negative: Andreoli *et al*., 2018 Uboldi *et al*., 2016 Falck *et al*. 2009 Guichard *et al*. 2012	1 Negative: Kang *et al*., 2011
	Chromosomal aberrations *in vitro*	1		

	Comet *in vitro*	6	5 Positive: Nakagawa *et al*., 1997 Guichard *et al*. 2012 Karlsson *et al*., 2009 Andreoli *et al*., 2018 Falck *et al*. 2009 1 Negative: Nakagawa *et al*., 1997	
	Cell transformation assay	1	1 Positive: Uboldi *et al*., 2016	
	In vivo assays	1		
	Other genotoxicity endpoints *in vivo* - DNA binding	1	1 Negative: Jin *et al*., 2013	
Anatase/Rutile	Surface chemistry/Coating	0		
	Surface chemistry/No coating	2		
	In vitro assays	2		
	Micronucleus *in vitro*	1	1 Negative: Andreoli *et al*., 2018	
	Comet *in vitro*	1	1 Positive: Andreoli *et al*., 2018	
	In vivo assays	0		

* Only final result (*i.e.* negative, positive, inconclusive or equivocal) from an EFSA Appendix was included;
** TiO_2 material-test system combination included

The SCCS conclusions on safety of TiO_2 NANOMATERIALS used in cosmetic products

The Applicant provided required genotoxicity testing results using mammalian cell gene mutation and micronucleus tests on RM09 (rutile, coated with amorphous silica, hydrophilic) and RM11 (rutile, coated with alumina and dimethicone, hydrophobic), with negative results. The SCCS conducted analysis of the available published literature data on TiO_2 nanomaterials composed of rutile coated (only such nanomaterials are used in cosmetic products). Based on the analysis of only 4 *in vitro* studies found on alumina coated TiO_2 grades tested in micronucleus assay and Comet assay with negative results, there is reasonable evidence that alumina coated TiO_2 nanomaterial grades (rutile) are not genotoxic (all combinations have been investigated in one study by Jalili *et al.,* 2018). 3-aminopropyltriethoxysilane coated rutile (the same material NRCWE-002) was tested negative and positive in two Comet assay studies after intratracheal administration conducted by the same group of researchers. Most probably the discrepancy in the results could depend on different suspension medium used for dispersion of NRCWE-002.
In conclusion, only limited information is available for the rutile nanomaterials with 2 types of coating, whereas TiO_2 nanomaterials intended for use in cosmetic products are coated with a number of chemicals, and in some cases as multiple coatings (please see Table 3.4.1.3.D). For the rest of the rutile coated nanomaterials used in cosmetic products (except RM09 and RM11), the genotoxicity hazard is not known. Hence safe use of such rutile coated nanomaterials in cosmetic products cannot be confirmed with the currently available weight of the evidence.

Table 3.4.1.3.D. TiO_2 NANOMATERIALS – analysis of the published literature data merged in the SCCS/EFSA database

TiO_2 material tested	**Description**		**Number of records and outome** (positive; negative; inconclusive or equivocal)	
		Number of records identified among the total n = 284	**Analysis by EFSA** (until December 2020)**	**Analysis by SCCS** (2020-2023)***
TiO_2 Nanomaterials	Total	228		
RUTILE	Surface chemistry/No coating:	29*		
	Surface chemistry/Coating:	5		
	Alumina coating	4		
	In vitro assays	4		
	Micronucleus *in vitro*	2		2 Negative: Jalili *et al.*, 2018
	Comet *in vitro*	2		2 Negative: Jalili *et al.*, 2018
	In vitro assays	1		
	Positively charged coating (3-aminopropyltriethoxysilane)	1		
	In vivo assays	1		
	Comet *in vivo*	1	1 Positive: Wallin *et al.*, 2017	1 Negative: Hadrup *et al.*, 2017

* Rutile non-coated nanomaterials (N=29) are not used in cosmetic products, hence were not considered in this assessment. The remaining ones relate to anatase or anatase/rutile materials.
** Only final result (*i.e.* negative, positive, inconclusive or equivocal) from an EFSA Appendix was included.
*** TiO_2 material-test system combination included

3.4.2. Potential uptake of TiO_2 nanoparticles by oral mucosa cells

Although cosmetic products are not intended to be orally ingested, some incidental exposure takes place when oral product categories like toothpaste are used. Therefore, for cosmetic products containing nanomaterials intended to be used orally, it is important to consider that it will be the oral mucosa that will be exposed to nanoparticles in the first place before any ingestion can take place.
In this regard, it is important to keep in mind that, unlike dermal cells that are protected from entry of particulate materials by *stratum corneum*, the mucosal epithelium is only covered under a layer of mucous and therefore more prone to exposure of nanoparticles.
In this regard, a number of studies have indicated that oral mucosal cells are particularly prone to uptake of nanoparticles as they are able to penetrate the mucous layer and may be internalised by the epithelial cells. These studies range from *in vitro* studies in cell lines (Best *et al*., 2015) and 3D buccal mucosa models (Konstantinova *et al.* 2017) to ex-vivo in porcine buccal tissue sections (Teubl *et al.*, 2014, 2015; Vignard *et al*., 2023). The particles tested in these studies range from fluorescently-labelled carboxyl polystyrene nanoparticles to titanium dioxide nanoparticles, as well as food grade TiO_2 particles (E171) (Vignard *et al*., 2023). The available evidence from these studies has suggested that the penetration of nanoparticles to the oral mucosal cells can be a relatively rapid process (within a few minutes – according to Teubl *et al.*, 2015). The internalised particles have been found to reach up to 1/3 of the epithelium (up to *stratum superficiale*) – with some evidence that they can also reach the connective tissue (Teubl *et al*., 2014, 2015) and submandibular lymph nodes from pigs exposed to food-grade TiO_2 particles (E171) (Vignard *et al*., 2023).

The available evidence so far has however not clearly indicated a dependency of particle penetration on either size or hydrophobicity/hydrophilicity of the nanoparticles, although smaller nanoparticles seem to be more internalised compared to larger particles/ agglomerates. There are also indications from the studies that the intracellular distribution of hydrophilic and hydrophobic nanoparticles within the mucosal cells is different, as the hydrophilic ones are more freely distributed in the cytoplasm, whilst the hydrophobic ones tend to end up in vesicles. There is also some indication that nanoparticles (TiO_2) internalised by TR146 human buccal mucosal cells induce the generation of reactive oxygen species *in vitro* (Teubl *et al.*, 2014, 2015). Apart from oxidative stress induction, TiO_2 NM-102 and E171 were shown to induce genotoxic effect (γH2AX staining) in TR146 cells (Vignard *et al*., 2023). However, it should be emphasised that γH2AX staining test is considered a genotoxicity indicative test.

It is known that the oral mucosal epithelium depending on the region of oral cavity has a continuous turn-over around 14 days for buccal mucosa to 24 days for hard palate (Squier and Kremer, 2001). However, considering that some oral products, such as toothpastes will be used every day, and potentially more than once a day, it needs further investigations to exclude the concern over the uptake of TiO_2 nanoparticles in the buccal mucosa from long-term repeated exposures to orally used cosmetic products.

4. CONCLUSION

The SCCS concludes the following:

1. In light of the EFSA Opinion on genotoxicity concerns for E171, does the SCCS consider Titanium dioxide safe in oral cosmetic products?

From the provided information, the SCCS has noted that the titanium dioxide (TiO_2) materials evaluated in this Scientific Advice belong to a wide range of grades[7] (44 pigmentary and 40 nano grades) used in cosmetic products. The pigmentary grades differ from the food additive E171 in terms of crystalline forms, particle sizes, coatings, etc., with the exception of 13 uncoated pigmentary grades that can be considered as equivalent to E171.

Having considered all the information (including that evaluated by EFSA, 2021), the SCCS considers that the available evidence is not sufficient to exclude the genotoxicity potential of almost all of the types of TiO_2 grades used in oral cosmetic products. The only exception are two nano grades (RM09 and RM11) for which the provided genotoxicity data indicate no genotoxicity concern. More information is, however, needed on the potential uptake and cellular effects of the nano grades in the oral mucosa to consider them safe for use in oral-care products.

More experimental data are needed from studies carried out under valid protocols and appropriate testing guidelines to exclude the genotoxicity potential of the selected representatives of the other grades of TiO_2 (both pigmentary and nano) used in oral cosmetic products.

It is worth highlighting that the SCCS approach to risk assessment of TiO_2 ingredients in orally-used cosmetic products is slightly different from that of EFSA. This is because cosmetic products are not meant to be ingested orally, and any ingestion via the oral route can only be unintended and incidental. Keeping this in mind, the amounts of orally-ingested cosmetic ingredients can only be expected to be far lower than the amounts ingested when a TiO_2 material is used as a food additive, which is consumed via intake of the food products. For the SCCS, the potential absorption/retention, translocation and adverse effects of nanoparticles in the buccal mucosa are therefore important considerations for safety evaluation.

The SCCS recommends that safety assessment of the pigmentary TiO_2 grades used in cosmetics should also take account of the fact that some of them contain a sizeable proportion of the particles in the nano size scale – some over 50% (in terms of particle number, median constituent particle size).

2. In light of the EFSA Opinion, does the SCCS consider that previous Opinions issued by the SCCS on inhalation and dermal exposure to Titanium dioxide need to be revised?

The conclusions drawn in previous SCCS Opinions on dermally applied cosmetic products (SCCS/1516/13, SCCS/1580/16) remain unchanged for the TiO_2 grades and the coatings evaluated in those Opinions. New data on dermal absorption will be required for other

[7] The term "grade" is used to describe the type of materials by the Applicants. The SCCS decided to keep the term "grade" for the whole SA for the sake of consistency of the text.

types of TiO_2 grades and coatings that are not covered in the Cosmetics Regulation 1223/2009, nor by entry 27a in Annex VI.

According to the Cosmetics Regulation 1223/2009, the nanoform of TiO_2 is already restricted under entry 27a of Annex VI as not to be used in applications that may lead to exposure of the end-user's lungs by inhalation. The conclusions drawn in the previous Opinions (and SCCS/1583/17, SCCS/1617/20) on the safety of TiO_2 used in specific cosmetic products that may lead to exposure by inhalation also remain unchanged.

3. In the event that the estimated exposure to Titanium dioxide from cosmetic products is found to be of concern, SCCS is asked to recommend safe concentration limits for each category of products and types of use.

Since the genotoxicity hazard of almost all of the grades of titanium dioxide could not be excluded (with the exception of RM09 and RM11), the SCCS cannot recommend any safe limits for the materials when used in cosmetic products that could lead to oral or inhalation exposure, other than those already indicated in the previous SCCS Opinions (SCCS/1516/13, SCCS/1580/16 and SCCS/1617/20).

4. In light of the potential removal of the E 171 purity specification from the food additives Regulation, the SCCS is requested to review and indicate the respective specifications for Titanium dioxide when used in cosmetics.

In view of the concerns on the potential genotoxicity of the TiO_2 grades considered in this Scientific Advice, the SCCS is of the opinion that the Applicants should draw up a proposal for specifications of the different TiO_2 grades used in those cosmetic products that could lead to oral and inhalation exposure. The SCCS will be able to assist the Commission in reviewing the proposal.

5. Does the SCCS have any further scientific concerns regarding the use of Titanium dioxide in cosmetic products?

Studies have indicated that oral mucosal cells are prone to the uptake of nanoparticles (including TiO_2 nanoparticles), as they may penetrate the mucous layer and may be internalised by the epithelial cells. Considering that some oral products containing TiO_2 nanoparticles, such as toothpastes, will be used every day and potentially more than once a day, further investigations are needed to exclude the risk to the consumer from long-term repeated exposures of the oral mucosa to TiO_2 nanoparticles.

5. MINORITY OPINION

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ANNEX: Safety concerns for titanium dioxide grades used in cosmetic products

From the provided information, the SCCS has noted that the titanium dioxide (TiO_2) materials evaluated in this Scientific Advice belong to a wide range of grades (44 pigmentary and 40 nano grades) used in cosmetic products. The pigmentary grades differ from the food additive E171 in terms of crystalline forms, particle sizes, coatings, etc., with the exception of 13 uncoated pigmentary grades that can be considered as equivalent to E171.

In view of the currently available evidence being insufficient to exclude genotoxicity of the different grades of TiO_2 materials used in cosmetics, the SCCS has identified the following scientific aspects that constitute the basis for a concern over the safety of the use of these materials in cosmetic products that could lead to consumer exposure via the oral or inhalation route:

PHYSICOCHEMICAL ASPECTS
The physicochemical characteristics of the TiO_2 grades used in cosmetic products are very wide ranging. A number of discrepancies and data gaps (including stability) have been identified that need addressing, without which it is not possible to relate many of the grades to the materials tested in toxicological studies.

To avoid a case-by-case assessment of all the materials considered in this Scientific Advice, narrow groups of the materials with similar characteristics need to be formed and justified on the basis of physicochemical characterisation data on each of the materials. In this regard, it is important that rigorous specifications are drawn by the Applicant for each group/grade used in cosmetic products that may lead to oral and/or inhalation exposure. Toxicological test data for one or more representative material(s) from each group/grade can then be justified for use in read-across to other member of the group.

GENOTOXICITY/MUTAGENICITY
The SCCS considers that the currently available weight of the evidence is not sufficient to exclude mutagenicity/ genotoxicity potential of almost all of the TiO_2 grades to be used in cosmetic products that have been assessed in this Scientific Advice. Without excluding the mutagenicity/genotoxicity potential, the SCCS cannot recommend a safe level of use for the TiO_2 materials in oral cosmetic products.
Further evidence from valid *in vitro* testing protocols and guidelines on genotoxicity/mutagenicity would therefore be needed for at least one representative of each type of the TiO_2 grades used in cosmetic products.

EXPOSURE ASPECTS
Potential dermal absorption
Some of the materials assessed in this SA have different characteristics including coating compared to those materials that have been assessed in the previous SCCS Opinions (SCCS/1516/13, SCCS/1580/16). The potential dermal absorption of those types of coatings which have not been evaluated before is not known, and therefore excluding the consumer risk on the basis of the lack of exposure is not possible without further experimental data on representative coated materials.

Uptake in the oral mucosa
Studies have indicated that oral mucosal cells are prone to uptake of nanoparticles (including TiO_2 nanoparticles) as they are able to penetrate the mucous layer and may be internalised by the epithelial cells. Considering that some oral products containing TiO_2 nanoparticle grades, such as toothpastes, will be used every day, and potentially more than once a day, further evidence is needed to exclude the concern over the uptake/retention, potential translocation and adverse effects of TiO_2 nanoparticles in the oral mucosa from long-term repeated exposures to orally used cosmetic products.

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For complete references list used by CEFIC Expert Panel please see the publication: Kirkland D, Aardema MJ, Battersby RV, Beevers C, Burnett K, Burzlaff A, Czich A, Donner M, Fowler P, Johnston HJ. 2022. A weight of evidence review of the genotoxicity of titanium dioxide (TiO_2). Regulatory Toxicology and Pharmacology 136: 105263.

iii) REFERENCES used for analysis of genotoxicity by the SCCS:

Please see ANNEX V. List of publications on TiO_2 particles genotoxicity analysed by the SCCS

iv) REFERENCES used by the SCCS in the section on oral mucosa uptake:

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7. GLOSSARY OF TERMS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158

8. LIST OF ABBREVIATIONS

See SCCS/1647/22, 12th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – Appendix 15 - from page 158

Annex A: Formula composition of the pigmentary and nano titanium dioxide grades

Table 3.1.4.A3: Pigmentary Grades – Formula Composition as a function of the categories noted a, b, c, d *(from **Ref.:** January 2023_PhysChem data on Cosmetics* TiO_2 *grades_final.pdf - Table 1.2 Physico-chemical data for Pigmentary Titanium Dioxide used in Cosmetics and (*) completed from **Ref.:** CE-* TiO_2*-23-003.0 - Att 2_March 2023 update to Physchem data tables CE Jan 2023 submission to SCCS – Pigment – final.xls)*

Product Code		Formula/ Composition	Product Code		Formula/ Composition	Product Code		Formula/ Composition
RM01	a	Titanium Dioxide	RM37	b2	Titanium dioxide 95.7%, Alumina 0.2%, Aluminium Hydroxide 3.7%, Zinc Oxide 0.4%	RM05	c2	Titanium Dioxide 97.5%, Al2O3 1.3 %, Glycerin 0.6 %
RM02	a	Titanium Dioxide	RM27	c1	Titanium dioxide 98.0%, Methicone: 2.0%	RM06	c2	Titanium Dioxide 98.2%, Al2O3 1.3%
RM03	a	Titanium Dioxide	RM29	c1	Titanium dioxide 98.5%, Hydrogen Dimethicone 1.5%	RM07	c2	Titanium Dioxide 97.5%, Al2O3 1.1% Triethoxycaprylylsilane 0.8%
RM04	a	Titanium Dioxide	RM70a	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	RM08	c2	Titanium Dioxide 97.9%, Al2O3 1.3%, Glycerin 0.6%
RM26	a	Titanium Dioxide 100%	RM70b	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	RM19	c2	Titanium Dioxide*, Alumina 1.2%*, Glycerin 0.3%*
RM28	a	Titanium Dioxide 100%	RM70d	c1	Titanium Dioxide >95%, Cera Alba 0-5% Rosa Centifolia Flower Wax 0-5% Rosa Damascena Flower Cera 0-5%	RM32	c2	Titanium dioxide 88.6%, Alumina 0.3%, Aluminium Hydroxide 2.0%, Algin 9.1%
RM67	a	Titanium Dioxide	RM70e	c1	Titanium Dioxide >95%, Sodium Glycerophosphate <5%	RM33	c2	Titanium dioxide 93.7%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Isostearic Acid 3.8%
RM67b	a	Titanium Dioxide	RM70f	c1	Titanium Dioxide >95%, Hydrogenated Lecithin <5%	RM34	c2	Titanium dioxide 92.7%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Lauroyl Lysine 4.8%
RM68	a	Titanium Dioxide	RM72a	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	RM35	c2	Titanium dioxide 95.5%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Hydrogen Dimethicone 2.0%
RM69	a	Titanium Dioxide	RM72b	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	RM36	c2	Titanium dioxide 93.7%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Dimethicone 3.8%
RM69b	a	Titanium Dioxide	RM72d	c1	Titanium Dioxide >85% Persea Gratissima (Avocado) Oil 0-5% Hydrogenated Vegetable Oil 0-5%, Tocopherol 0-5%	RM72i	c2	Titanium Dioxide >94%, Aluminium Hydroxide 0-5%
RM70c	a	Titanium Dioxide >95%, Silica 0-5%**(6)**	RM72e	c1	Titanium Dioxide >95%, PEG-2-Soyamine 0-5%, Bis-PEG-15 Dimethicone / IPDI Copolymer 0-5% Isopropyl Titanium Triisostearate 0-5%	RM72j-bis	c2	Titanium Dioxide >87%, Aluminium Hydroxide <5% Trimethoxycaprylylsilane <6%
RM72c	a	Titanium Dioxide >95%, Silica 0-5%**(6)**	RM72f	c1	Titanium Dioxide >95%, Phytic Acid 0-5% Sodium Hydroxide 0-5%	RM38	c3	Titanium dioxide 94.7%, Alumina 0.2%, Aluminium Hydroxide 3.7%, Zinc Oxide 0.4%, Isostearic Acid 1.0%
RM30	b1	Titanium dioxide 97.4%, Alumina 0.3%, Aluminium Hydroxide 2.3%	RM72g	c1	Titanium Dioxide >85%, Sodium Cocoyl Glutamate 0-5% Cystine 0-5%, Lauric Acid 0-5%, Arginine 0-5%	RM39	c3	Titanium dioxide 94.7%, Alumina 0.2%, Aluminium Hydroxide 3.7%, Zinc Oxide 0.4%, Dimethicone 1.0%
RM31	b2	Titanium dioxide 92.5%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Hydrated Silica 5.0%	RM72k	c1	Titanium Dioxide >85% Cocos Nucifera (Coconut) Oil: Max 11% Aloe Barbadensis Leaf Extract: Max 1%			

(6): *Silica is present as a processing aid not as a coating*

Ref.: *January 2023_PhysChem data on Cosmetics* TiO_2 *grades_final.pdf*
Table 1.2 Physico-chemical data for Pigmentary Titanium Dioxide used in Cosmetics

CE-TiO2-23-003.0 - Att 2_March 2023 update to Physchem data tables CE Jan 2023 submission to SCCS – Pigment – final.xls
Footnote (6) only applies to the grades that are denoted with a superscript 6 – specifically RM70c and RM72c.

From **Ref.:** CE-TiO2-23-003.0 - CE Response to clarifications requested by SCCS 10 03 23 – final.pdf

Table 3.1.4.A4: Pigmentary Grades – Formula Composition *(from **Ref.:** January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf - Table 1.2 Physico-chemical data for Pigmentary Titanium Dioxide used in Cosmetics)*

Product Code	Category	Formula/ Composition	TiO_2 (%)	Loss on drying (%) (1)	Loss on ignition (%) (2)	Al_2O_3 and/or SiO_2 (%) (3)	Al_2O_3 (%)	SiO_2 (%)
RM01	a	Titanium Dioxide	99.4	0.09	0.06	no	<0.01	<0.01
RM02	a	Titanium Dioxide	99.2	0.12	0.05	no	<0.01	<0.01
RM03	a	Titanium Dioxide	≥99	≤0.5	≤ 1.0	≤0.5	0.05	≤0.05
RM04	a	Titanium Dioxide	≥ 99	≤0.5	≤ 1.0	≤0.5	0.12	0.12
RM26	a	Titanium Dioxide 100%	99.2	0.26	0.11	0	0	0
RM28	a	Titanium Dioxide 100%	99.3	0.11	0.07	0	0	0
RM67	a	Titanium Dioxide	>99	≤0.5	≤0.5	0	0	0
RM67b	a	Titanium Dioxide	>99	≤0.5	≤0.5	0	0	0
RM68	a	Titanium Dioxide	>99	≤0.5	≤0.5	0	0	0
RM69	a	Titanium Dioxide	>99	≤0.5	≤0.5	0	0	0
RM69b	a	Titanium Dioxide	>99	≤0.5	≤0.5	0	0	0
RM70c	a	Titanium Dioxide >95%, Silica 0-5%6	>95	≤0.5	≤0.5	<5	0	<0.3
RM72c	a	Titanium Dioxide >95%, Silica 0-5%6	>95	≤0.5	≤0.5	<2.3	<2	<0.3
RM30	b1	Titanium dioxide 97.4%, Alumina 0.3%, Aluminium Hydroxide 2.3%	99.2	0.1	0.2	1.8	1.8	0
RM31	b2	Titanium dioxide 92.5%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Hydrated Silica 5.0%	99.2	0.1	0.2	6.8	1.8	5
RM37	b2	Titanium dioxide 95.7%, Alumina 0.2%, Aluminium Hydroxide 3.7%, Zinc Oxide 0.4%	99.2	0.01	0.2	2.7	2.7	0
RM27	c1	Titanium dioxide 98.0%, Methicone: 2.0%	99.2	0.26	0.11	0	0	0
RM29	c1	Titanium dioxide 98.5%, Hydrogen Dimethicone 1.5%	99.3	0.11	0.07	0	0	0
RM70a	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	>95	≤0.5	≤0.5	<2	<2	<2
RM70b	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	>95	≤0.5	≤0.5	<2	<2	<2
RM70d	c1	Titanium Dioxide >95%, Cera Alba 0-5% Rosa Centifolia Flower Wax 0-5% Rosa Damascena Flower Cera 0-5%	>95	≤0.5	≤0.5	<2	<2	<2
RM70e	c1	Titanium Dioxide >95%, Sodium Glycerophosphate <5%	>95	≤0.5	≤0.5	<2	<2	<2
RM70f	c1	Titanium Dioxide >95%, Hydrogenated Lecithin <5%	>95	≤0.5	≤0.5	<2	<2	<2
RM72a	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	>94	≤0.5	≤0.5	<2.3	<2	<0.3

RM72b	c1	Titanium Dioxide >95%, Triethoxycaprylylsilane <5%	>95	≤0.5	≤0.5	<2.3	<2	<0.3
RM72d	c1	Titanium Dioxide >85% Persea Gratissima (Avocado) Oil 0-5% Hydrogenated Vegetable Oil 0-5%, Tocopherol 0-5%	>85	≤0.5	≤0.5	<2.3	<2	<0.3
RM72e	c1	Titanium Dioxide >95%, PEG-2-Soyamine 0-5%, Bis-PEG-15 Dimethicone / IPDI Copolymer 0-5% Isopropyl Titanium Triisostearate 0-5%	>95	≤0.5	≤0.5	<2.3	<2	<0.3
RM72f	c1	Titanium Dioxide >95%, Phytic Acid 0-5% Sodium Hydroxide 0-5%	>95	≤0.5	≤0.5	<2.3	<2	<0.3
RM72g	c1	Titanium Dioxide >85%, Sodium Cocoyl Glutamate 0-5% Cystine 0-5%, Lauric Acid 0-5%, Arginine 0-5%	>85	≤0.5	≤0.5	<2.3	<2	<0.3
RM72k	c1	Titanium Dioxide >85% Cocos Nucifera (Coconut) Oil: Max 11% Aloe Barbadensis Leaf Extract: Max 1%	>85	≤0.5	≤0.5	<2	<2	<2
RM05	c2	Titanium Dioxide 97.5%, Al2O3 1.3 %, Glycerin 0.6 %	≥ 99	≤0.5	≤1,0	≤2.0	1.3	0.3
RM06	c2	Titanium Dioxide 98.2%, Al2O3 1.3%	≥ 99	≤0.5	≤1,0	≤2,0	1.2	0.4
RM07	c2	Titanium Dioxide 97.5%, Al2O3 1.1% Triethoxycaprylylsilane 0.8%	≥ 99	≤0.5	≤1,0	≤2.0	1.2	0.3
RM08	c2	Titanium Dioxide 97.9%, Al2O3 1.3%, Glycerin 0.6%	≥ 99	≤0.5	≤1,0	≤2.0	1.4	0.38
RM19	c2	Titanium Dioxide, Alumina, Glycerin	>96	<0.5	<1.0	~1.3	~1.3	0
RM32	c2	Titanium dioxide 88.6%, Alumina 0.3%, Aluminium Hydroxide 2.0%, Algin 9.1%	99.2	0.1	0.2	1.6	1.6	0
RM33	c2	Titanium dioxide 93.7%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Isostearic Acid 3.8%	99.2	0.1	0.2	1.8	1.8	0
RM34	c2	Titanium dioxide 92.7%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Lauroyl Lysine 4.8%	99.2	0.1	0.2	1.8	1.8	0
RM35	c2	Titanium dioxide 95.5%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Hydrogen Dimethicone 2.0%	99.2	0.1	0.2	1.8	1.8	0
RM36	c2	Titanium dioxide 93.7%, Alumina 0.3%, Aluminium Hydroxide 2.2%, Dimethicone 3.8%	99.2	0.1	0.2	1.8	1.8	0
RM72i	c2	Titanium Dioxide >94%, Aluminium Hydroxide 0-5%	>94	≤0.5	≤0.5	<6	< 5	<0.3
RM72j-bis	c2	Titanium Dioxide >87%, Aluminium Hydroxide <5% Trimethoxycaprylylsilane <6%	>87	≤0.5	≤0.5	<6	< 5	<0.3
RM38	c3	Titanium dioxide 94.7%, Alumina 0.2%, Aluminium Hydroxide 3.7%, Zinc Oxide 0.4%,	99.2	0.01	0.2	2.7	2.7	0

		Isostearic Acid 1.0%						
RM39	c3	Titanium dioxide 94.7%, Alumina 0.2%, Aluminium Hydroxide 3.7%, Zinc Oxide 0.4%, Dimethicone 1.0%	99.2	0.01	0.2	2.7	2.7	0

Ref.: *January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf*
Table 1.2 Physico-chemical data for Pigmentary Titanium Dioxide used in Cosmetics

(1) Loss on drying (%): Take 1 to 2 g of the sample, previously well mixed and accurately weighed. Take a glass-stoppered, shallow weighing bottle that has been dried at 105°C for 30 min. Transfer the sample into the bottle, replace the cover, and weigh the bottle and the sample. Distribute the sample as evenly as practicable to a depth of about 5 mm, and not over 10 mm in the case of bulky materials. Place the bottle with its contents in the drying chamber, removing the stopper and leaving it also in the chamber, and dry the sample at 105°C for 3 hours. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator before weighing.

(2) Loss on ignition (%): Proceed as directed for Loss on Drying above. However, unless otherwise directed, ignite the sample at a temperature of 800°C and use a platinum, quartz or porcelain dish instead of the weighing bottle.

(3) Aluminium oxide and/or Silicon Dioxide (%) (JECFA method): Weigh about 0.5 g of the sample to the nearest 0.1 mg, in a platinum or nickel crucible, add 5 g potassium hydroxide and 2 g boric acid, mix and melt completely using a torch burner and allow to stand at room temperature. Place the reaction product along with crucible into 150 ml hot deionized water in a 250-ml PTFE beaker and dissolve residue by agitation. Wash the crucible with hot deionized water and remove it. Add 50 ml hydrochloric acid and transfer the contents into a 250-ml polypropylene volumetric flask. Wash the beaker three times with hot deionized water, transfer the washings to the volumetric flask and make up to volume (Solution A). Prepare the test solution by 5 times dilution of Solution A with 2% hydrochloric acid. Analyse aluminium and silica in the test solution by ICP-AES technique. Set instrument parameters as specified by the instrument manufacturer. Use analytical lines for Al (396.152 nm) and Si (251.611 nm) and construct standard curve using standard solutions 0.2 – 5.0 µg/ml each. Read the concentration of Al and Si in sample solution (as µg/ml) and calculate the aluminium oxide and silicon dioxide content of the sample using the formula:

$\%Al_2O_3 = (1.889 \times C \times 250 \times 5 \times 100) / (W \times 10^6)$

$\%SiO_2 = (2.139 \times C \times 250 \times 5 \times 100) / (W \times 10^6)$

Where: C is concentration of Al or Si in the test solution (µg/ml),
W is weight of sample, g

TiO_2 **(%) (JECFA assay method):** Prepare the test solution by 1000 times dilution of Solution A (prepared in the test for Aluminium oxide and Silicon dioxide – see above) with 2% hydrochloric acid, taking care that dilution factor in each dilution step shall not be more than 20. Analyse Titanium in the test solution by ICP-AES technique. Set instrument parameters as specified by the instrument manufacturer. Use the analytical line for Ti (334.941 nm) and construct standard curve using Ti standard solutions: 0.5 - 1.5 µg/ml. Read the concentration in the sample solution (as µg/ml) and calculate the titanium dioxide content of the sample using the formula:

$\%\ TiO_2$ (on the dried basis) = $(1.668 \times C \times 250 \times 1000 \times 100) / (W \times 10^6 \times (100 - \%LOD - \%Al2O3 - \%SiO2)/100)$

Where: C is concentration of Ti in the test solution, µg/ml
W is weight of sample, g
%LOD is % loss on drying
%Al2O3 and %SiO2 are content (%) of Aluminium oxide and silicon dioxide

Alternatively for UV filters a method according to DIN EN ISO 591-1 may be used, and for pigments the XRFA referred to DIN EN ISO 591-1 may be used

From **Ref**.: CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Table 3.1.4.A5: Pigmentary grades / Coatings / Surface Moieties (***Ref.:*** *January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf – Table from Page 6/28 – Columns "N2.4) Coatings / Surface moieties" and "N2.5) Doping material*)")

Product Code	Coatings / Surface moieties[1]	Product Code	Coatings / Surface moieties[1]	Product Code	Coatings / Surface moieties[1]
RM01	None	RM32	Alumina:0.3%, Aluminium Hydroxide: 2%, Algin*: 9.1%	RM70c	None
RM02	None	RM33	Alumina:0.3%, Aluminium Hydroxide: 2.2%, Isostearic Acid:3.8%	RM70d	Rosa Centifolia Flower Wax, Rosa Damascena Flower Cera, Cera Alba
RM03	None	RM34	Alumina:0.3%, Aluminium Hydroxide: 2.2%, Lauroyl Lysine: 4.8%	RM70e	Sodium Glycerophosphate
RM04	None	RM35	Alumina:0.3%, Aluminium Hydroxide: 2.2% Hydrogen Dimethicone: 2%	RM70f	Hydrogenated Lecithin
RM05	Alumina	RM36	Alumina:0.3%, Aluminium Hydroxide: 2.2% Dimethicone: 3.8%	RM72a	Triethoxycaprylylsilane
RM06	Alumina	RM37	Alumina:0.2%, Aluminium Hydroxide: 3.7%, Zinc Oxide: 0.4%	RM72b	Triethoxycaprylylsilane
RM07	Alumina	RM38	Alumina:0.2%, Aluminium Hydroxide: 3.7%, Zinc Oxide: 0.4% Isostearic Acid: 1%	RM72c	None
RM08	Alumina	RM39	Alumina:0.2%, Aluminium Hydroxide: 3.7%, Zinc Oxide: 0.4% Dimethicone: 1%	RM72d	Persea Gratissima (Avocado) Oil, Hydrogenated Vegetable Oil, Tocopherol
RM19	Glycerin (~0.3%)	RM67	None	RM72e	Bis-PEG-15 Dimethicone/ IPDI Copolymer, PEG-2-Soyamine, Isopropyl Titanium Triisostearate
RM26	None	RM67b	None	RM72f	Phytic Acid & Sodium Hydroxide
RM27	Methicone: 2%	RM68	None	RM72g	Sodium Cocoyl Glutamate, Cystine, Lauric Acid, Arginine
RM28	None	RM69	None	RM72i	Aluminium Hydroxide
RM29	Hydrogen Dimethicone: 1.5%	RM69b	None	RM72j-bis	Aluminium Hydroxide, Trimethoxycaprylylsilane
RM30	Alumina:0.3%, Aluminium Hydroxide: 2.3%	RM70a	Triethoxycaprylylsilane	RM72k	Cocos Nucifera (Coconut) Oil, Aloe Barbadensis Leaf Extract
RM31	Alumina:0.3%, Aluminium Hydroxide: 2.2%, Hydrated Silica: 5%	RM70b	Triethoxycaprylylsilane	/	/

Algin = sodium alginate*

Ref.: *January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf Table from Page 6/28 – Columns "N2.4) Coatings / Surface moieties" and "N2.5) Doping material*

And (*) From **Ref.:** CE-TiO2-23-003.0 - CE Response to clarifications requested by SCCS 10 03 23 – final.pdf

Table 3.1.4.A6: Sequence of the multi-layers for the pigmentary titanium dioxide grades (From Multi-layer coating sequence – Pigment.xls – 30 June 2023)

	Innermost Layer	---------->		**Outermost Layer**
Product Code	**A**	**B**	**C**	**D**
RM01	No surface treatment			
RM02	No surface treatment			
RM03	No surface treatment			
RM04	No surface treatment			
RM05	Al_2O_3 1.3%	Glycerin 0.6%		
RM06	Al_2O_3 1.3%			
RM07	Al_2O_3 1.1%	Triethoxycaprylylsilane 0.8%		
RM08	Al_2O_3 1.3%	Glycerin 0.6%		
RM19	Al_2O_3 1.2%	Glycerin 0.3%		
RM26	No surface treatment			
RM27	Methicone 2%			
RM28	No surface treatment			
RM29	Hydrogen Dimethicone 1.5%			
RM30	Alumina 0.3%	Aluminium hydroxide 2.3%		
RM31	Alumina 0.3%	Aluminium hydroxide 2.2%	Hydrated silica 5.0%	
RM32	Alumina 0.3%	Aluminium hydroxide 2.0%	Algin 9.1%	
RM33	Alumina 0.3%	Aluminium hydroxide 2.2%	Isostearic Acid 3.8%	
RM34	Alumina 0.3%	Aluminium hydroxide 2.2%	Lauroyl Lysine 4.8%	
RM35	Alumina 0.3%	Aluminium hydroxide 2.2%	Hydrogen Dimethicone 2.0%	
RM36	Alumina 0.3%	Aluminium hydroxide 2.2%	Dimethicone 3.8%	
RM37	Zinc oxide 0.4%	Alumina 0.2%	Aluminium hydroxide 3.7%	

RM38	Zinc oxide 0.4%	Alumina 0.2% 3.7%	Aluminium hydroxide	Isostearic Acid 1.0%
RM39	Zinc oxide 0.4%	Alumina 0.2%	Aluminium hydroxide 3.7%	Dimethicone 1.0%
RM67	No surface treatment			
RM67b	No surface treatment			
RM68	No surface treatment			
RM69	No surface treatment			
RM69b	No surface treatment			
RM70a	Triethoxycaprylylsilane 5%			
RM70b	Triethoxycaprylylsilane 5%			
RM70c	No surface treatment (silica is separate processing aid)			
RM70d	Cera Alba 0 – 5%	Rosa Centifolia Flower Wax 0 – 5%	Rosa Damascena Flower Cera 0 – 5%	
RM70e	Sodium Glycerophosphate < 5%			
RM70f	Hydrogenated Lecithin			
RM72a	Triethoxycaprylylsilane < 5%			
RM72b	Triethoxycaprylylsilane < 5%			
RM72c	No surface treatment (silica is separate processing aid)			
RM72d	Persea Gratissima (Avocado) Oil 0 – 5%	Hydrogenated Vegetable Oil 0 – 5%	Tocopherol 0 – 5%	
RM72e	PEG-2-Soyamine 0 – 5%	Bis-PEG-15 Dimethicone/IPDI Copolymer 0 – 5%	Isopropyl Titanium Triisostearate 0 – 5%	
RM72f	Phytic Acid 0 – 5%			
RM72g	Sodium Cocoyl Glutamate 0 – 5%	Cystine 0 – 5%	Lauric Acid 0 – 5%	Arginine 0 – 5%
RM72i	Aluminium Hydroxide 0 – 5%			
RM72j-bis	Aluminium Hydroxide < 5%	Trimethoxycaprylylsilane < 6%		
RM72k	Cocos Nucifera (Coconut) Oil 11% max	Aloe Barbadensis Leaf Extract 1% max		

Ref.: Multi-layer coating sequence – Pigment.xls – 30 June 2023

Table 3.1.4.B1: Nano grades - Formula/ Composition *(from **Ref.:** January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf - Table 1.3 Physico-chemical data for Nano Titanium Dioxide used in Cosmetics, completed from **Ref.:** CE-TiO2-23-003.0 - Att 3_ March 2023 update to Physchem data tables CE Jan 2023 submission to SCCS – Nano – final.xls)*

Product Code	Formula / Composition	Product Code	Formula / Composition	Product Code	Formula / Composition
RM09 Hydrophilic	Titanium Dioxide*, Silica 10%*	RM52 Hydrophobic	Titanium dioxide 82.4%, Aluminium Hydroxide 10.0%, Hydrated Silica 2.9%, Hydrogen Dimethicone 4.7%	RM74a Hydrophobic	Titanium Dioxide ≥75%, Hydrogen Dimethicone <10%, Alumina <20%
RM10 Hydrophobic	Titanium Dioxide*, Silica 10%*, Hydrogen Dimethicone 11%*	RM53 Hydrophobic	Titanium dioxide 85.0%%, Stearic Acid 15.0%	RM74b Hydrophobic	Titanium Dioxide ≥ 70%, Alumina Max 15, Stearic Acid Max 15%
RM11 Hydrophobic	Titanium Dioxide*, Alumina 6%*, Dimethicone 3%*	RM55 Hydrophilic	Titanium dioxide 91.5%, Aluminium Hydroxide 3.0%, Hydrated Silica 5.5%	RM74c Hydrophobic	Titanium Dioxide Min 94%, Triethoxycaprylylsilane Max 6%
RM40 Hydrophobic	Titanium dioxide 66.7%, Aluminium Hydroxide 13.3%, Stearic Acid 20%	RM56 Hydrophobic	Titanium dioxide 89.0%, Aluminium Hydroxide 7.0%, Stearic Acid 4.0%	RM74d Hydrophilic	Titanium Dioxide, Silica <20%
RM41 Hydrophilic	Titanium dioxide 82%, Aluminium Hydroxide 13.5%, Hydrated Silica 4.5%	RM57 Hydrophobic	Titanium dioxide 89.8%, Aluminium Hydroxide 2.9%, Hydrated Silica 5.4%, Hydrogen Dimethicone 1.9%	RM74e Hydrophobic	Titanium Dioxide Min 80%, Silica Max 15%, Dimethicone Max 6%
RM42 Hydrophobic	Titanium dioxide 73.0%, Aluminium Hydroxide: 16.0%, Stearic Acid: 11%	RM58 Hydrophobic	Titanium dioxide 88.8%, Aluminium Hydroxide 3.0%, Hydrated Silica 5.3%, Dimethicone 2.9%	RM75 Amphiphilic	Titanium dioxide*, Alumina 11%*, Simethicone 2%*
RM43 Hydrophobic	Titanium dioxide 77.4%, Aluminium Hydroxide 12.7%, Hydrated Silica 4.2%, Hydrogen Dimethicone 5.7%	RM59 Hydrophilic	Titanium dioxide 87.0%, Aluminium Hydroxide 11.0%, Hydrated Silica 2%	RM76 Hydrophobic	Titanium dioxide*, Alumina 9%*, Stearic acid 11%*
RM44 Hydrophobic	Titanium dioxide 65.6%, Aluminium Hydroxide 10.8%, Hydrated Silica 3.6%, Dimethicone 15.4%, Hydrogen Dimethicone 4.6%	RM60 Hydrophobic	Titanium dioxide 91.2%, Aluminium Hydroxide 4.1%, Stearic Acid 4.7%	RM77 Aqueous Dispersion	Titanium dioxide*, Alumina 3%*, Sodium hexametaphosphate 2%*, 2-Phenoxyethanol 0.7%*, Sodium methylparaben 0.18%*
RM45 Hydrophilic	Titanium dioxide 76.0%, Aluminium Hydroxide 17.0%, Hydrated Silica 7%	RM61 Hydrophobic	Titanium dioxide 98.0%, Hydrogen Dimethicone 2.0%	RM78	Titanium dioxide*, Silica 18%*
RM46 Hydrophilic	Titanium dioxide 86.5%, Aluminium Hydroxide 10.5%, Hydrated Silica 3%	RM62 Hydrophobic	Titanium dioxide 91.2%, Aluminium Hydroxide 4.1%, Stearic Acid 4.7%	RM79	Titanium dioxide*, Silica 17%*, Hexadecyl dihydrogen phosphate 6%*
RM47 Hydrophilic	Titanium dioxide 70.0%, Hydrated Silica 30.0%	RM63 Hydrophobic	Titanium dioxide (76.5%), Alumina (10%), Stearic acid (13.5%)	RM80	Titanium dioxide*, Alumina 11%*, Manganese dioxide 1%*
RM48 Hydrophobic	Titanium dioxide 83.0%, Aluminium Hydroxide 9.0%, Stearic Acid 8.0%	RM64 Hydrophobic	Titanium dioxide (88.5%), Alumina (5%), Stearic acid (6.5%)	RM81	Titanium dioxide*, Silica 6%*, Alumina 6%*
RM49 Hydrophobic	Titanium dioxide 74.0%, Aluminium Hydroxide: 13.0%, Stearic Acid: 13%	RM65 Hydrophobic	Titanium dioxide (91.9%), Alumina (3.5%), Stearic acid (4.6%)	RM82	Titanium Dioxide 82-87%, Silica 10.5-14.5%, Dimethicone 2.0-4.5%
RM51 Hydrophobic	Titanium dioxide 83.6%, Aluminium Hydroxide 10.1%, Hydrated Silica 2.9%, Hydrogen Dimethicone 3.4%				

Ref.: i) January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
Table 1.3 Physico-chemical data for Nano Titanium Dioxide used in Cosmetics
ii) CE-TiO2-23-003.0 - Att 3_ March 2023 update to Physchem data tables CE Jan 2023 submission to SCCS – Nano – final.xls)

Table 3.1.4.B2: Nano grades - Formula/ Composition *(from **Ref.:** January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf - Table 1.3 Physico-chemical data for Nano Titanium Dioxide used in Cosmetics)*

Product Code	Grouping	Formula / Composition	TiO2 (%)	Loss on ignition (%)	Product Code	Grouping	Formula / Composition	TiO2 (%)	Loss on ignition (%)
RM09	Hydrophilic	Titanium dioxide, Silica	≥ 99	≤13	RM60	Hydrophobic	Titanium dioxide 91.2%, Aluminium Hydroxide 4.1%, Stearic Acid 4.7%	99.7	0.25
RM10	Hydrophobic	Titanium Dioxide, Silica, Hydrogen Dimethicone	≥99	≤13	RM61	Hydrophobic	Titanium dioxide 98.0%, Hydrogen Dimethicone 2.0%	99.7	0.25
RM11	Hydrophobic	Titanium Dioxide, Alumina, Dimethicone	≥99	≤13	RM62	Hydrophobic	Titanium dioxide 91.2%, Aluminium Hydroxide 4.1%, Stearic Acid 4.7%	99.7	0.25
RM40	Hydrophobic	Titanium dioxide 66.7%, Aluminium Hydroxide 13.3%, Stearic Acid 20%	99.1	4.25	RM63	Hydrophobic	Titanium dioxide (76.5%), Alumina (10%), Stearic acid (13.5%)	≥99	<13
RM41	Hydrophilic	Titanium dioxide 82%, Aluminium Hydroxide 13.5%, Hydrated Silica 4.5%	99.1	4.25	RM64	Hydrophobic	Titanium dioxide (88.5%), Alumina (5%), Stearic acid (6.5%)	≥99	<13
RM42	Hydrophobic	Titanium dioxide 73.0%, Aluminium Hydroxide: 16.0%, Stearic Acid: 11%	99.1	4.25	RM65	Hydrophobic	Titanium dioxide (91.9%), Alumina (3.5%), Stearic acid (4.6%)	≥99	<13
RM43	Hydrophobic	Titanium dioxide 77.4%, Aluminium Hydroxide 12.7%, Hydrated Silica 4.2%, Hydrogen Dimethicone 5.7%	99.1	4.25	RM74 a	Hydrophobic	Titanium Dioxide ≥75%, Hydrogen Dimethicone <10%, Alumina <20%	≥99	≤2.5
RM44	Hydrophobic	Titanium dioxide 65.6%, Aluminium Hydroxide 10.8%, Hydrated Silica 3.6%, Dimethicone 15.4%, Hydrogen Dimethicone 4.6%	99.1	4.25	RM74 b	Hydrophobic	Titanium Dioxide ≥ 70%, Alumina Max 15, Stearic Acid Max 15%	≥99	≤2.5
RM45	Hydrophilic	Titanium dioxide 76.0%, Aluminium Hydroxide 17.0%, Hydrated Silica 7%	99.5	4.14	RM74 c	Hydrophobic	Titanium Dioxide Min 94%, Triethoxycaprylylsilane Max 6%	≥99	≤2.5
RM46	Hydrophilic	Titanium dioxide 86.5%, Aluminium Hydroxide 10.5%, Hydrated Silica 3%	99.5	4.14	RM74 d	Hydrophilic	Titanium Dioxide, Silica	≥99	<2.5
RM47	Hydrophilic	Titanium dioxide 70.0%, Hydrated Silica 30.0%	99.5	4.14	RM74 e	Hydrophobic	Titanium Dioxide Min 80%, Silica Max 15%, Dimethicone Max 6%	≥99	≤2.5
RM48	Hydrophobic	Titanium dioxide 83.0%, Aluminium Hydroxide 9.0%, Stearic Acid 8.0%	99.5	4.14	RM75	Amphiphilic	Titanium dioxide, Alumina, Simethicone	99.7	≤13
RM49	Hydrophobic	Titanium dioxide 74.0%, Aluminium Hydroxide: 13.0%, Stearic Acid: 13%	99.5	4.14	RM76	Hydrophobic	Titanium dioxide, Alumina, Stearic acid	99.7	≤13
RM51	Hydrophobic	Titanium dioxide 83.6%, Aluminium Hydroxide 10.1%, Hydrated Silica 2.9%, Hydrogen Dimethicone 3.4%	99.5	4.14	RM77	Aqueous Dispersion	Titanium dioxide, Alumina, Sodium hexametaphosphate, 2-Phenoxyethanol, Sodium methylparaben	99.7	≤13
RM52	Hydrophobic	Titanium dioxide 82.4%, Aluminium Hydroxide 10.0%, Hydrated Silica 2.9%, Hydrogen Dimethicone 4.7%	99.5	4.14	RM78	Hydrophilic	Titanium dioxide, Silica	99.8	≤13

RM53	Hydrophobic	Titanium dioxide 85.0%%, Stearic Acid 15.0%	99.4	2.68	RM79	Hydrophobic	Titanium dioxide, Silica, Hexadecyl dihydrogen phosphate	99.8	≤13
RM55	Hydrophilic	Titanium dioxide 91.5%, Aluminium Hydroxide 3.0%, Hydrated Silica 5.5%	99.9	4.71	RM80	Hydrophilic	Titanium dioxide, Alumina, Manganese dioxide	99.8	≤13
RM56	Hydrophobic	Titanium dioxide 89.0%, Aluminium Hydroxide 7.0%, Stearic Acid 4.0%	99.9	4.71	RM81	Amphiphilic	Titanium dioxide, Silica, Alumina	99.5	0.1
RM57	Hydrophobic	Titanium dioxide 89.8%, Aluminium Hydroxide 2.9%, Hydrated Silica 5.4%, Hydrogen Dimethicone 1.9%	99.9	4.71	RM82	Hydrophobic	Titanium Dioxide 82-87%, Silica 10.5-14.5%, Dimethicone 2.0-4.5%	≥99	≤13
RM58	Hydrophobic	Titanium dioxide 88.8%, Aluminium Hydroxide 3.0%, Hydrated Silica 5.3%, Dimethicone 2.9%	99.9	4.71					
RM59	Hydrophilic	Titanium dioxide 87.0%, Aluminium Hydroxide 11.0%, Hydrated Silica 2%	99.7	0.25					

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf

Table 3.1.4.B3: Nano grades / Coatings / Surface Moieties (***Ref.:*** *January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf – Table from Page 14/28 - N2.4) Coatings / Surface moieties and Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024))*

Product Code	Coatings / Surface moieties[1]	Product Code	Coatings / Surface moieties[1]
RM09	Silica: 10%	RM60	Aluminium Hydroxide: 4.1%/ Stearic Acid: 4.7%
RM10	Silica, Hydrogen Dimethicone	RM61	Hydrogen Dimethicone: 2%
RM11	Alumina, Dimethicone	RM62	Aluminium Hydroxide 4.1%, Stearic Acid: 4.7%
RM41	Aluminium Hydroxide: 13.5%, Hydrated Silica: 4.5%	RM63	Alumina, Stearic acid
RM40	Aluminium Hydroxide: 13.3%/ Stearic Acid: 20%	RM64	Alumina, Stearic acid
RM42	Aluminium Hydroxide: 16%/ Stearic Acid: 11%	RM65	Alumina, Stearic acid
RM43	Aluminium Hydroxide: 12.7%, Hydrated Silica: 4.2%/ Hydrogen Dimethicone: 5.7%	RM74a	Hydrogen Dimethicone & Alumina
RM44	Aluminium Hydroxide: 10.8%, Hydrated Silica: 3.6%/ Dimethicone: 15.4%, Hydrogen Dimethicone: 4.6%	RM74b	Alumina, Stearic Acid
RM45	Aluminium Hydroxide: 17%, Hydrated Silica: 7%	RM74c	Triethoxycaprylylsilane
RM46	Aluminium Hydroxide: 10.5%, Hydrated Silica: 3%	RM74d	Silica
RM47	Hydrated Silica: 30%	RM74e	Silica & Dimethicone
RM48	Aluminium Hydroxide: 9%, Stearic Acid: 8%	RM75	Alumina, Simethicone
RM49	Aluminium Hydroxide: 13%/ Stearic Acid: 13%	RM76	Alumina, Stearic Acid
RM51	Aluminium Hydroxide: 10.1%, Hydrated Silica: 2.9%, Hydrogen Dimethicone: 3.4%	RM77	Alumina
RM52	Aluminium Hydroxide: 10%, Hydrated Silica: 2.9%, Hydrogen Dimethicone: 4.7%	RM78	Silica
RM53	Stearic Acid: 15%	RM79	Silica, Cetyl Phosphate
RM55	Aluminium Hydroxide: 3%, Hydrated Silica: 5.5%	RM80	Alumina, Manganese Dioxide
RM56	Aluminium Hydroxide: 7%/ Stearic Acid: 4%	RM81	Silica, Alumina
RM57	Aluminium Hydroxide 2.9%, Hydrated Silica 5.4%, Hydrogen Dimethicone 1.9%	RM82	Silica, Dimethicone
RM58	Aluminium Hydroxide: 3%, Hydrated Silica 5.3%/ Dimethicone: 2.9%		
RM59	Aluminium Hydroxide: 11%, Hydrated Silica: 2%		

1. Alumina (Al_2O_3) in surface coatings is actually a mixture of Alumina and Aluminium Hydroxide

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf Table from Page 14/28 - Column N2.4) Coatings / Surface moieties and Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (Februray 2024)

Table 3.1.4.B4: Sequence of the multi-layers for the nano titanium dioxide grades. (From Ref.: Multi-layer coating sequence – Nano.xls – 30 June 2023)

	Innermost Layer	---------->		**Outermost Layer**
Product Code	**A**	**B**	**C**	**D**
RM09	Silica 10%			
RM10	Silica 10%	Hydrogen Dimethicone 11%		
RM11	Alumina 6%	Dimethicone 3%		
RM40	Aluminium Hydroxide 13.3%	Stearic Acid 20%		
RM41	Hydrated Silica 4.5%	Aluminium Hydroxide 13.5%		
RM42	Aluminium Hydroxide 16.0%	Stearic Acid 11%		
RM43	Hydrated Silica 4.2%	Aluminium Hydroxide 12.7	Hydrogen Dimethicone 5.7%	
RM44	Hydrated Silica 3.6%	Aluminium Hydroxide 10.8%	Hydrogen Dimethicone 4.6%	Dimethicone 15.4%
RM45	Hydrated Silica 7%	Aluminium Hydroxide 17%		
RM46	Hydrated Silica 3%	Aluminium Hydroxide 10.5%		
RM47	Hydrated Silica 30%			
RM48	Aluminium Hydroxide 9.0%	Stearic Acid 8.0%		
RM49	Aluminium Hydroxide 13.0%	Stearic Acid 13%		
RM51	Hydrated Silica 2.9%	Aluminium Hydroxide 10.1%	Hydrogen Dimethicone 3.4%	
RM52	Hydrated Silica 2.9%	Aluminium Hydroxide10.0%	Hydrogen Dimethicone 4.7%	
RM53	Stearic Acid 15.0%			
RM55	Hydrated Silica 5.5%	Aluminium Hydroxide 3.0%		
RM56	Aluminium Hydroxide 7.0%	Stearic Acid 4.0%		
RM57	Hydrated Silica 5.4%	Aluminium Hydroxide 2.9%	Hydrogen Dimethicone 1.9%	
RM58	Hydrated Silica 5.3%	Aluminium Hydroxide 3.0%	Dimethicone 2.9%	
RM59	Hydrated Silica 2%	Aluminium Hydroxide 11%		
RM60	Aluminium Hydroxide 4.1%	Stearic Acid 4.7%		
RM61	Hydrogen Dimethicone 2.0%			
RM62	Aluminium Hydroxide 4.1%	Stearic Acid 4.7%		
RM63	Alumina 10%	Stearic acid 13.5%		
RM64	Alumina 5%	Stearic acid 6.5%		
RM65	Alumina 3.5%	Stearic acid 4.6%		
RM74a	Alumina < 20%	Hydrogen Dimethicone <10%		
RM74b	Alumina 15%	Stearic Acid 15% max		
RM74c	Triethoxycaprylylsilane 6%			
RM74d	Silica (20%)			
RM74e	Silica 15% max	Dimethicone 6%		
RM75	Alumina 10%	Simethicone 2%		
RM76	Alumina 9%	Stearic Acid 10%		
RM77 i)	Alumina			
RM78	Silica 17%			
RM79	Silica 16%	Hexadecyl dihydrogen phosphate 6%		

RM80	Alumina 10%	Manganese dioxide 1%		
RM81	Silica 6%	Alumina 6%		
RM82	Silica 10.5-14.5%	Dimethicone 2.0 – 4.5%		

i) RM77: Titanium dioxide, Alumina 3%, Sodium hexametaphosphate* 2%, 2-Phenoxyethanol* 0.7%, Sodium methylparaben* 0.18% - **Please note that the components marked are dispersing agents and should not be considered as a layer on the TiO_2 surface, although to a certain extent they do interact with the surface.*

Annex B: Impurity profile of the Raw Materials – Pigmentary and nano titanium dioxide grades

From Applicants

Given that TiO_2 is manufactured from naturally occurring ores, there can be variability within these different ores accounting for a different impurity analytical profile (specifically heavy metals) within the specification limits. In the case of heavy metals, the specification is a maximum value. The principal raw material ores for manufacturing TiO_2 include ilmenite (iron titanium oxide, $FeTiO_3$), naturally occurring rutile (TiO_2) or titanium slag which all contain naturally occurring heavy metals in variable amounts depending on the nature and geographic source of these raw materials. This results in heavy metals being present as unavoidable trace elements in the manufactured titanium dioxide product even though GMP are applied for cosmetics ingredients. Depending on the raw material sourcing and the manufacturing process, the heavy trace metals for cosmetics ingredients products are reduced by a significant factor for some elements like lead, arsenic and antimony compared to products marketed for "technical" applications. These trace elements are embedded in the lattice of the TiO_2 and are not bioavailable. Therefore, rather than give a potentially unrepresentative single data point, the ranges of values presented give an accurate account of this

From Ref.: CE-TiO2-23-003.0 - CE Response to clarifications requested by SCCS 10 03 23 – final.pdf

Acid-soluble substances (%)
Suspend 5 g of the sample in 100 ml 0.5 N hydrochloric acid and place on a steam bath for 30 min with occasional stirring. Filter through a Gooch crucible fitted with a glass fibre filter paper. Wash with three 10-ml portions of 0.5 N hydrochloric acid, evaporate the combined filtrate and washings to dryness, and ignite at a dull red heat to constant weight. The similar USP method may be used.

Water soluble substances (%)
Method is same as for acid-soluble substances (above) but using water in place of 0.5 N hydrochloric acid. The USP method is similar, but the suspension is not heated, and stands overnight at ambient conditions

HCl-soluble antimony, arsenic, cadmium and lead
Transfer 10.0 g of sample into a 250-ml beaker, add 50 ml of 0.5 N hydrochloric acid, cover with a watch glass, and heat to boiling on a hot plate. Boil gently for 15 min, pour the slurry into a 100- to 150-ml centrifuge bottle, and centrifuge for 10 to 15 min, or until undissolved material settles. Decant the supernatant through Whatman No. 4 filter paper, or equivalent, (or direct from supernatant if clear) collecting the filtrate in a 100-ml volumetric flask and retaining as much as possible of the undissolved material in the centrifuge bottle. Add 10 ml of hot water to the original beaker, washing off the watch glass with the water, and pour the contents into the centrifuge bottle. Form a slurry, using a glass stirring rod, and centrifuge. Decant through the same filter paper and collect the washings in the volumetric flask containing the initial extract. Repeat the entire washing process two more times. Finally, wash the filter paper with 10 to 15 ml of hot water. Cool the contents of the flask to room temperature, dilute to volume with water, and mix. Determine cadmium, and lead using an AAElectrothermal atomization technique, antimony by ICP-AES technique and arsenic using atomic absorption hydride technique.

Mercury
Determine using AAS (Cold vapour generation technique) after digestion with sulfuric and nitric acids in a closed vessel microwave digestion system.

From **Ref.**: CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Pigmentary grades

The Applicants have provided the impurity profiles of the Raw materials for Pigmentary titanium dioxide grades on the Water soluble substances (%), Acid-soluble substances (%), Arsenic (HCl-soluble) (mg/kg), Lead, (HCl-soluble) (mg/kg), Antimony (HCl-soluble) (mg/kg), Mercury (HCl-soluble) (mg/kg), Cadmium (HCl-soluble) (mg/kg). These informations are reported in the following Table (Table 3.1.5 - A: Pigmentary grades – Impurity Profile of Raw Materials).

Table 3.1.5 - A: Pigmentary grades – Impurity Profile of Raw Materials (from **Ref.:** January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf - Table 5)

Product Code	Category	Water soluble substances (%)	Acid-soluble substances (%)	Arsenic (HCl-soluble) (mg/kg)	Lead (HCl-soluble) (mg/kg)	Antimony (HCl-soluble) (mg/kg)	Mercury (HCl-soluble) (mg/kg)	Cadmium (HCl-soluble) (mg/kg)
RM01	a	0.31	0.32	<0.1	0.3	<0.1	<0.1	<0.1
RM02	a	0.26	0.27	<0.1	0.8	<0.1	<0.1	<0.1
RM03	a	≤0.5	≤0.5	≤1	≤10	≤2	≤1	≤1
RM04	a	≤0.5	≤0.5	≤1	≤10	≤2	≤1	≤1
RM05	c2	≤0.5	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1	≤ 1
RM06	c2	≤0.5	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1	≤ 1
RM07	c2	≤0.5	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1	≤ 1
RM08	c2	≤0.5	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1	≤ 1
RM19	c2	<0.3	<1.5	<1	<10	<2	<1	<1
RM26	a	0.21	0.32	≤1	≤5	≤0.1	<0.1	<0.1
RM27	c1	0.21	0.32	≤ 1	≤ 5	≤0.1	≤0.1	≤0.1
RM28	a	0.06	0.18	≤1	≤5	≤0.1	<0.1	<0.1
RM29	c1	0.06	0.18	≤ 1	≤ 5	≤0.1	≤0.1	≤0.1
RM30	b1	0.02	0.13	≤ 1	≤ 5	≤0.1	≤0.1	≤0.1
RM31	b2	0.02	0.13	≤ 1	≤ 5	≤0.1	≤0.1	≤0.1
RM32	c2	0.02	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1	≤ 0.1
RM33	c2	0.02	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1	≤ 0.1
RM34	c2	0.02	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1	≤ 0.1
RM35	c2	0.02	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1	≤ 0.1
RM36	c2	0.02	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1	≤ 0.1
RM37	b2	0.04	0.3	≤ 1	≤ 5	≤0.1	≤0.1	≤0.1
RM38	c3	0.04	0.3	≤ 1	≤ 5	≤ 0.1	≤ 0.1	≤ 0.1
RM39	c3	0.04	0.3	≤ 1	≤ 5	≤ 0.1	≤ 0.1	≤ 0.1
RM67	a	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM67b	a	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM68	a	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM69	a	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM69b	a	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM70a	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM70b	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM70c	a	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM70d	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM70e	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM70f	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72a	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72b	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72c	a	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72d	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72e	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72f	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72g	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72i	c2	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72j-bis	c2	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1
RM72k	c1	≤0.3	≤0.5	≤1	≤10	≤2	≤1	≤1

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf – Table 5

Based on the information provided by Applicants, the maximum impurities levels for the Pigmentary Titanium dioxide grades are reported below:

- Water soluble substances ≤ 0.5%
- Acid soluble substances < 1.5%
- Arsenic (HCl soluble) ≤ 1ppm
- Lead (HCl soluble) ≤ 10ppm
- Antimony (HCl soluble) ≤ 2ppm
- Mercury ≤ 1ppm
- Cadmium ≤ 1 ppm

From Applicants

Nano Titanium dioxide Grades

The Applicants have provided the impurity profiles of the Raw materials for Nano titanium dioxide grades on the Water soluble substances (%), Acid-soluble substances (%), Arsenic (HCl-soluble) (mg/kg), Lead, (HCl-soluble) (mg/kg), Antimony (HCl-soluble) (mg/kg), Mercury (HCl-soluble) (mg/kg), Cadmium (HCl-soluble) (mg/kg). These informations are reported in the following Table (Table 3.1.5 - B: Nano grades – Impurity profile of Raw materials)

The Applicants have reported the following impurities levels.

- Water soluble substances < 0.25%
- Acid soluble substances < 0.5%
- Arsenic (HCl soluble) < 1ppm
- Lead (HCl soluble) < 10ppm
- Antimony (HCl soluble) < 2ppm
- Mercury < 1ppm

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf

Table 3.1.5 - B: Nano grades – Impurity profile of Raw materials (from **Ref.:** January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf - Table N2.3 and Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024))

Product Code	Grouping	Water soluble substances (%)	Acid-soluble substances (%)	Arsenic (HCl-soluble) (mg/kg)	Lead (HCl-soluble) (mg/kg)	Antimony (HCl-soluble) (mg/kg)	Mercury (mg/kg)
RM75	Amphiphilic	≤0.1	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM81	Amphiphilic	≤0.25	0.2	<1	3	0.5	0.1
RM78	Hydrophilic	≤0.1	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM80	Hydrophilic	≤0.1	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM46	Hydrophilic	0.04	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM47	Hydrophilic	0.04	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM09	Hydrophilic	≤0.25	≤0.5	≤1	≤10	≤2	≤1
RM41	Hydrophilic	0.07	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM45	Hydrophilic	0.04	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM55	Hydrophilic	0.1	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM74d	Hydrophilic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM59	Hydrophilic	0.07	0.11	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM40	Hydrophobic	0.07	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM42	Hydrophobic	0.07	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM43	Hydrophobic	0.07	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM44	Hydrophobic	0.07	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM48	Hydrophobic	0.04	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM49	Hydrophobic	0.04	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM51	Hydrophobic	0.04	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM52	Hydrophobic	0.04	0.06	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM53	Hydrophobic	0.04	0.14	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM56	Hydrophobic	0.1	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM57	Hydrophobic	0.1	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM58	Hydrophobic	0.1	0.13	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM60	Hydrophobic	0.07	0.11	≤ 1	≤ 5	≤ 0.1	≤ 0.1

RM61	Hydrophobic	0.07	0.11	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM62	Hydrophobic	0.07	0.11	≤ 1	≤ 5	≤ 0.1	≤ 0.1
RM76	Hydrophobic	≤0.1	≤ 0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM79	Hydrophobic	≤0.1	≤ 0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM10	Hydrophobic	≤0.25	≤0.5	≤1	≤10	≤2	≤1
RM11	Hydrophobic	≤0.25	≤0.5	≤1	≤10	≤2	≤1
RM63	Hydrophobic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM64	Hydrophobic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM65	Hydrophobic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM74a	Hydrophobic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM74b	Hydrophobic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM74c	Hydrophobic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM74e	Hydrophobic	≤0.25	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1
RM82	Hydrophobic	≤0.25	≤0.5	≤1	≤10	≤2	≤1
RM77	Aqueous Dispersion	≤0.1	≤0.5	≤ 1	≤ 10	≤ 2	≤ 1

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
From Table N2.3,

Annex C: Partition Coefficient – Pigmentary and nano titanium dioxide grades

From Applicants

There is no standard method for measuring partition coefficient of particulate materials. Where an organic is present the literature value of the partition coefficient is given.

From **Ref.:** CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Table 3.1.7.A: Partition coefficient (log KoW) of Pigmentary Titanium dioxide grades

Product Code	Partition coefficient (log K_{ow})	Product Code	Partition coefficient (log K_{ow})	Product Code	Partition coefficient (log K_{ow})
RM01	n/a -no organic component	RM32	-2.6 - -1.9	RM70c	n/a -no organic component
RM02	n/a -no organic component	RM33	Hydrophobic	RM70d	Hydrophobic
RM03	n/a -no organic component	RM34	Hydrophobic	RM70e	Hydrophobic
RM04	n/a -no organic component	RM35	Hydrophobic	RM70f	Hydrophobic
RM05	Hydrophilic	RM36	2.6 - 4.3	RM72a	1.1 at 20°C
RM06	Hydrophilic	RM37	n/a -no organic component	RM72b	1.1 at 20°C
RM07	9 (calc) at 20°C	RM38	Hydrophobic	RM72c	n/a -no organic component
RM08	-1.75 (calc) at 25°C	RM39	2.6 - 4.3	RM72d	Hydrophobic
RM19	Hydrophilic	RM67	n/a -no organic component	RM72e	Hydrophobic
RM26	n/a -no organic component	RM67b	n/a -no organic component	RM72f	Hydrophilic
RM27	Hydrophobic	RM68	n/a -no organic component	RM72g	Hydrophobic
RM29	Hydrophobic	RM69	n/a -no organic component	RM72i	-0.47 at 26°C
RM28	n/a -no organic component	RM69b	n/a -no organic component	RM72j-bis	3.9 at 20°C
RM30	n/a -no organic component	RM70a	1.1 at 20°C	RM72k	Hydrophobic
RM31	n/a -no organic component	RM70b	1.1 at 20°C		

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
Table from Page 7/28 – Column 6.4) Partition coefficient (log K_{ow})

Table 3.1.7.B : Partition coefficient (log KoW) of Nano Titanium Dioxide grades

Product Code	K_{OW} for surface modified NMs (organic)	Product Code	K_{OW} for surface modified NMs (organic)	Product Code	K_{OW} for surface modified NMs (organic)
RM09	Hydrophilic	RM52	Hydrophobic	RM74a	Hydrophobic
RM10	Hydrophobic	RM53	Hydrophobic	RM74b	Hydrophobic
RM11	Hydrophobic	RM55	Hydrophilic	RM74c	Hydrophobic
RM40	Hydrophobic	RM56	Hydrophobic	RM74d	Hydrophilic
RM41	Hydrophilic	RM57	Hydrophobic	RM74e	Hydrophobic
RM42	Hydrophobic	RM58	2.6 - 4.3	RM75	Amphiphilic
RM43	Hydrophobic	RM59	Hydrophilic	RM76	Hydrophobic
RM44	Hydrophobic	RM60	Hydrophobic	RM77	Hydrophilic
RM45	Hydrophilic	RM61	Hydrophobic	RM78	Hydrophilic
RM46	Hydrophilic	RM62	Hydrophobic	RM79	Hydrophobic
RM47	Hydrophilic	RM63	Hydrophobic	RM80	Hydrophilic
RM48	Hydrophobic	RM64	Hydrophobic	RM81	Amphiphilic
RM49	Hydrophobic	RM65	Hydrophobic	RM82	Hydrophobic
RM51	Hydrophobic				

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final
From Table Page 17/28 – Column N10.2) K_{OW} for surface modified NMs (organic)

Annex D: Density of the Pigmentary and nano titanium dioxide grades

The density, pour density and tap density have been reported by Applicants.

Density (g/cm^3)
Helium pycnometry using a method similar to ASTM B923-22 (Standard Test Method for Metal Powder Skeletal Density by Helium or Nitrogen Pycnometry)

Pour Density (g/cm^3)
Pour a known mass of powder into a graduated measuring cylinder and measure the volume. Pour density is mass/volume. Nano UV-filters have low density and adhere to the vessel walls due to electrostatic forces. Therefore, to minimise variability, the following method may be used: Weigh measuring cylinder, pour material in, wait 5 minutes, read the poured volume and weigh back, calculate material mass and hence density.

Tap density (g/cm^3)
Proceed as for pour density but then tap the cylinder 100-1,000 times to settle powder, measure volume occupied and calculate tap density.
Method ISO 787/11; Proceed as for pour density. Then tap the cylinder 1,250 times with atamping volumeter to settle powder, measure the volume and repeat until volume does not change any more. Calculate the tap density.

From **Ref.:** CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Pigmentary Dioxide Grades

Table 3.1.8.6.A: Density, pour density and tap density for the Pigmentary grades

Product Code	Density (g/cm^3)	Porosity (Hausner ratio)	Pour Density (g/cm^3)	Tap density (g/cm^3)	Product Code	Density (g/cm^3)	Porosity (Hausner ratio)	Pour Density (g/cm^3)	Tap density (g/cm^3)
RM01	3.8	1.40	0.424	0.595	RM39	4.08	1.67	1.08	1.80
RM02	4.2	1.27	0.65	0.827	RM67	3.91	/	0.54	/
RM03	3.9	1.41	0.59	0.83	RM67b	4	/	0.44	/
RM04	3.85	1.30	0.53	0.69	RM68	4.02	/	0.53	/
RM05	3.81	1.11	0.75	0.83	RM69	4.47	/	0.67	/
RM06	3.84	1.38	0.6	0.83	RM69b	4.29	/	0.64	/
RM07	3.73	1.48	0.95	1.41	RM70a	3.83	/	0.60	/
RM08	4.1	1.38	0.61	0.84	RM70b	3.84	/	/	/
RM19	4.01	/	0.8	/	RM70c	3.99	/	/	/
RM26	3.79	1.63	0.56	0.91	RM70d	3.48	/	0.46	/
RM27	3.62	2.00	0.50	1.00	RM70e	3.96	/	0.43	/
RM28	4.34	1.61	0.69	1.11	RM70f	3.73	/	0.7	/
RM29	4.13	2.14	0.59	1.26	RM72a	4.14	/	0.93	/
RM30	4.28	1.32	0.76	1.00	RM72b	4.16	/	0.71	/
RM31	4.09	2.27	0.37	0.84	RM72c	4.33	/	0.32	/
RM32	3.67	2.26	0.50	1.13	RM72d	3.67	/	0.97	/
RM33	3.77	1.01	1.30	1.31	RM72e	3.85	/	0.95	/
RM34	3.73	1.24	1.08	1.34	RM72f	4.25	/	0.44	/
RM35	4.03	2.16	0.50	1.08	RM72g	3.93	/	/	/
RM36	3.80	1.88	0.58	1.09	RM72i	4.3	/	0.89	/
RM37	4.22	1.54	0.95	1.46	RM72j-bis	3.75	/	1.11	/
RM38	4.05	1.69	0.89	1.50	RM72k	3.27	/	1.02	/

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
Table from Page 10/28
Columns N15.1) Density (g/cm3),
N15.2 Porosity (Hausner ratio),
N15.3) Pour Density (g/cm3),

N15.3) Tap density (g/cm3)

Nano Titanium Dioxide Grades

Table 3.1.8.6.B: Density, pour density and tap density for the Nano grades

Product Code	Density (g/cm^3)	Porosity (Hausner ratio)	Pour Density (g/cm^3)	Tap density (g/cm^3)	Product Code	Density (g/cm^3)	Porosity (Hausner ratio)	Pour Density (g/cm^3)	Tap Density (g/cm^3)
RM09	3.66	1.35	0.43	0.58	RM60	3.70	2.52	0.29	0.73
RM10	3.13	1.38	0.16	0.22	RM61	4.21	1.81	0.41	0.74
RM11	3.66	1.46	0.26	0.38	RM62	3.70	2.17	0.40	0.88
RM40	2.52	1.46	0.20	0.29	RM63	2.79	1.35	0.43	0.58
RM41	3.69	1.93	0.22	0.43	RM64	3.37	1.41	0.63	0.89
RM42	2.86	1.33	0.37	0.49	RM65	3.54	1.64	0.59	0.97
RM43	3.30	2.19	0.27	0.60	RM74a	2.80	/	0.4	/
RM44	2.51	2.46	0.26	0.63	RM74b	3.10	/	0.7	/
RM45	3.41	2.10	0.34	0.72	RM74c	3.8	/	0.5	/
RM46	3.70	2.55	0.25	0.63	RM74d	4.20	/	0.1	/
RM47	3.51	1.52	0.15	0.24	RM74e	3.50	/	0.2	/
RM48	3.19	1.95	0.24	0.48	RM75	3.43	1.46	0.13	0.19
RM49	2.96	1.93	0.21	0.41	RM76	2.87	1.35	0.17	0.23
RM51	3.04	2.32	0.42	0.97	RM77	3.20	n/a	0.52	n/a
RM52	3.44	2.41	0.25	0.60	RM78	3.37	1.20	0.10	0.12
RM53	2.84	1.62	0.41	0.67	RM80	3.11	1.46	0.13	0.19
RM55	4.01	1.56	0.41	0.63	RM81	3.44	2.05	0.22	0.45
RM56	3.09	1.65	0.36	0.59	RM79	4.20	1.35	0.17	0.23
RM57	3.73	3.22	0.31	0.99	RM82	4.26	1.30	0.23	0.3
RM58	3.77	2.63	0.34	0.89					
RM59	4.09	1.36	0.44	0.60					

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
Table from Page 16/28, Column N8.3) Density (g/cm^3)
Table from page 18 / 208, Columns N15.2) Porosity (Hausner ratio)
N15.3) Pour Density (g/cm^3)
N15.3) Tap density (g/cm^3)

Annex E: pH value at isoelectric point – Pigmentary and nano titanium dioxide grades

From Applicants

The pKa data is not available. The Applicants has proposed to replace this data item with the pH value at isoelectric point.

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf

The pH at iep (isoelectric point) is the pH at which there is zero charge (zeta potential is zero). This pH has also been described as the "apparent pKa" as it is the pH at which the numbers of ionized (protonated) and deionized groups are equal in the system.

Ref.: Pratikkumar Patel, Nurudeen Mohammed Ibrahim, Kun Cheng, The Importance of Apparent pKa in the Development of Nanoparticles Encapsulating siRNA and mRNA, Trends in Pharmacological Sciences, Volume 42, Issue 6, 2021, Pages 448-460, https://www.sciencedirect.com/science/article/abs/pii/S0165614721000493 and also in Guidance Document on Testing Nanomaterials using OECD TG No. 312 "Leaching in Soil Columns" Series on Testing and Assessment, No. 342.

The detailed methods used by Applicants for the determination of iso-electric Point pH have been reported (see Annex K *"Measurement methods – Appendix 5"*).

The iso-electric point pH values are reported below in Table 3.1.8.8.A and Table 3.1.8.8.B for the pigmentary and the nano grades, respectively.

From **Ref.:** Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023)

Table 3.1.8.8.A: Iso-electric pH values as a function of the pigmentary titanium dioxide grades (from PS and Surface Property - Pigment Final.xlx - Third package (31 March 2023)).

Grade	pH at iep	Grade	pH at iep	Grade	pH at iep
RM01	3,3	RM32	~1	RM70c	2,8
RM02	3,7	RM33	(*)	RM70d	(*)
RM03	2,5	RM34	(*)	RM70e	3
RM04	4,9	RM35	(*)	RM70f	(*)
RM05	7,2	RM36	(*)	RM72a	(*)
RM06	7,7	RM37	7	RM72b	(*)
RM07	(*)	RM38	(*)	RM72c	2,3
RM08	8,4	RM39	(*)	RM72d	(*)
RM19	7,6	RM67	3,6	RM72e	(*)
RM26	3,4	RM67b	3,4	RM72f	<1
RM27	(*)	RM68	3,1	RM72g	(*)
RM28	4,5	RM69	2,6	RM72i	(**)
RM29	(*)	RM69b	3,4	RM72j-bis	(*)
RM30	8,3	RM70a	(*)	RM72k	(*)
RM31	<1	RM70b	(*)		

(*): N/A (hydrophobic)

(**): Not measured

Ref.: PS and Surface Property - Pigment Final.xlx - Third package (31 March 2023)

Table 3.1.8.8.B: Iso-electric pH values as a function of the nano titanium Dioxide grades (from PS and Surface Property - Nano Final.xlx - Third package (31 March 2023)).

Nano-grade	pH at iep	Nano-grade	pH at iep	Nano-grade	pH at iep
RM09	<1	RM52	(*)	RM74a	(*)
RM10	(*)	RM53	(*)	RM74b	(*)
RM11	(*)	RM55	4,8	RM74c	(*)
RM40	(*)	RM56	(*)	RM74d	4,1
RM41	8,6	RM57	(*)	RM74e	(*)
RM42	(*)	RM58	(*)	RM75	9,3
RM43	(*)	RM60	(*)	RM76	(**)
RM44	(*)	RM61	(*)	RM77	4,3
RM45	8	RM59	8,5	RM78	1,2
RM46	8,5	RM62	(*)	RM79	<1
RM47	2,3	RM63	(*)	RM80	9,2
RM48	(*)	RM64	(*)	RM81	4,8
RM49	(*)	RM65	(*)	RM82	(*)
RM51	(*)				

(*): N/A (hydrophobic)
(**): Not measurable

Ref.: PS and Surface Property - Pigment Final.xlx - Third package (31 March 2023)

Annex F: pH values – Pigmentary and nano titanium dioxide grades

From Applicants

Typical method: TiO_2 dispersions were prepared by adding the 1 wt. % of TiO_2 powder to deionised water. The dispersions were placed on magnetic stirrer (1500 rpm) for 15 minutes at ambient temperature to ensure that the powder is fully dispersed. The pH is measured using a pH meter calibrated with standard buffers prior to use.

From **Ref.:** CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

The pH values are reported in:

-For the pigmentary grades: Table 3.1.8.9.A

-For the nano grades: Table 3.1.8.9.B.

Table 3.1.8.9.A: pH values as a function of the pigmentary titanium dioxide grades (from **Ref.:** January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf)

Product Code	pH	Product Code	pH	Product Code	pH
RM01	7.3	RM32	8.3	RM70c	6
RM02	7.4	RM33	NA (Hydrophobic)	RM70d	NA (Hydrophobic)
RM03	6.5-8.5	RM34	NA (Hydrophobic)	RM70e	5.2
RM04	3.9-5.6	RM35	NA (Hydrophobic)	RM70f	5.5
RM05	6.7-8.3	RM36	NA (Hydrophobic)	RM72a	5.2
RM06	6.7-8.3	RM37	6.7	RM72b	6.3
RM07	NA (Hydrophobic)	RM38	NA (Hydrophobic)	RM72c	7.2
RM08	6.7-8.5	RM39	NA (Hydrophobic)	RM72d	5.1
RM19	6-9	RM67	7.5	RM72e	4.5
RM26	6.8	RM67b	6.9	RM72f	6.6
RM27	NA (Hydrophobic)	RM68	6.6	RM72g	NA (Hydrophobic)
RM28	7.9	RM69	6.2	RM72i	7.7
RM29	NA (Hydrophobic)	RM69b	5.9	RM72j-bis	3.9
RM30	6.7	RM70a	4.2	RM72k	4.2
RM31	7.3	RM70b	6		

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
Table from Page 10/28 – Column N17) pH

Table 3.1.8.9.B: pH values as a function of the nano titanium dioxide grades

Product Code	pH	Product Code	pH	Product Code	pH
RM09	6.9	RM52	NA (Hydrophobic)	RM74a	5.5
RM10	NA (Hydrophobic)	RM53	NA (Hydrophobic)	RM74b	4.1
RM11	NA (Hydrophobic)	RM55	7.5	RM74c	5.7
RM40	NA (Hydrophobic)	RM56	NA (Hydrophobic)	RM74d	4.9
RM41	7.0	RM57	NA (Hydrophobic)	RM74e	4.8
RM42	NA (Hydrophobic)	RM58	NA (Hydrophobic)	RM75	5.8 - 7.8
RM43	NA (Hydrophobic)	RM59	5.5	RM77	7.0 - 7.9
RM44	NA (Hydrophobic)	RM60	NA (Hydrophobic)	RM78	7.5 - 10.0
RM45	9.0	RM61	NA (Hydrophobic)	RM79	4.5 - 7.0
RM46	6.0	RM62	NA (Hydrophobic)	RM80	6.5 - 8.0
RM47	7.3	RM63	5-8	RM81	7.0 -10.0
RM48	NA (Hydrophobic)	RM64	5-8	RM76	NA (Hydrophobic)
RM49	NA (Hydrophobic)	RM65	5-8	RM82	NA (Hydrophobic)
RM51	NA (Hydrophobic)				

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
Table from Page 19/28 – Column N17) pH

Annex G: UV/visible light absorption spectrum

From Applicants

The TiO_2 is dispersed in a suitable medium (depending on whether it is hydrophobic or hydrophilic) and the UV absorbance measured at 308, 360 and 400nm in a UV-Visible spectrophotometer with correction for the absorbance of the suspending liquid.

Alternatively, reflectance from powder pellets can be measured and absorbance calculated. The results may be expressed as % absorbance, % transmittance or in in $L.mol^{-1}.cm^{-1}$.

From **Ref.**: CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Table 3.1.8.11.A: UV absorption as a function of the pigmentary titanium dioxide grades

	UV - Absorption				UV - Absorption		
Product Code	**308 nm**	**360 nm**	**400 nm**	**Product Code**	**308 nm**	**360 nm**	**400 nm**
RM01	5.7	16.5	89.9	RM39	8	8	4
RM02	8.4	7.2	41.5	RM67	58.1*	55*	54*
RM03	86	81	12	RM67b	59.7*	57.6*	57.9*
RM04	88	79	8	RM68	71*	68.1*	68.1*
RM05	89	79	8	RM69	62.7*	60.5*	60*
RM06	88	81	10	RM69b	65.3*	62.7*	63.2*
RM07	90	78	11	RM70a	48.7*	45.6*	44*
RM08	87	88	52	RM70b	43.2*	39.6*	38.2*
RM19	74	72	17	RM70c	48.3*	45.2*	44.1*
RM26	19	15	8	RM70d	72.9*	69.7*	69.2*
RM27	18	14	7	RM70e	50.1*	46.8*	45.6*
RM28	15	15	9	RM70f	67.2*	64*	63.4*
RM29	14	14	9	RM72a	62.8*	61.3*	61.8*
RM30	18	18	10	RM72b	50.5*	48.6*	47.3*
RM31	14	14	8	RM72c	50.7*	47.9*	45.8*
RM32	12	12	7	RM72d	68.4*	66.7*	66*
RM33	14	14	8	RM72e	71.6*	70*	69.8*
RM34	18	18	10	RM72f	62.8*	61*	60.2*
RM35	14	14	8	RM72g	73.6*	72.2*	71.9*
RM36	14	14	8	RM72i	28.3*	25.8*	23.2*
RM37	8	8	5	RM72j-bis	78.8*	75.7*	75.8*
RM38	8	8	4	RM72k	69.7*	66.8*	66.2*

(data marked * is %transmittance of 0.000495mol/L solution)

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf from Table on Page 8/28, Column 6.9) UV-Absorption)
(*) from CE-TiO2-23-003.0 - Att 2_March 2023 update to Physchem data tables CE Jan 2023 submission to SCCS – Pigment – final.xlx, March update to First data package January 2023.
and Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024)

Table 3.1.8.11.B: UV absorption as a function of the nano titanium dioxide grades

	UV Absorption				UV Absorption		
Product Code	**308nm**	**360nm**	**400nm**	**Product Code**	**308nm**	**360nm**	**400nm**
RM09	92	85	43	RM60	47	39	14
RM10	92	81	26	RM61	37	39	19
RM11	91	83	37	RM62	30	31	14
RM40	63	13	4	RM63	54	9.5	3.7
RM41	64	11	3	RM64	56.9	27.9	12.7
RM42	64	12	3	RM65	46	34.5	21.8
RM43	64	11	3	RM74a	19.5*	15.9*	20.4*
RM44	31	8	2	RM74b	46.2*	41.7*	40.8*
RM45	55	10	3	RM74c	2.5*	1.9*	5.3*
RM46	44	14	4	RM74d	16*	12*	16.4*
RM47	64	11	4	RM74e	44.8*	39.1*	38.2*
RM48	78	17	4	RM75	45	13	5
RM49	86	23	6	RM77	55	16	7
RM51	41	13	4	RM78	26	12	5
RM52	41	13	4	RM79	50	14	6
RM53	65	21	6	RM80	41	12	5
RM55	58	29	10	RM81	49.7	35.2	21.75
RM56	68	32	9	RM76	55	16	7
RM57	67	28	8	RM82	9.07	17.02	62.99
RM58	68	28	8				
RM59	47	32	12				

(data marked * is % transmittance of 0.000495mol/L solution)

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
From Table on Page 19/28 – Column 6.9 UV – absorption
(*) from CE-TiO2-23-003.0 - Att 3_ March 2023 update to Physchem data tables CE Jan 2023 submission to SCCS – Nano – final.xlx - March update to First data package January 2023
and Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024)

Based on the information provided by Applicants, the SCCS noted that:

Pigmentary titanium dioxide grades

For the 40 pigmentary titanium grades, the UV absorption is noted to range:

- at 308 nm, from 5.7 (RM01) to 90 (RM07),
- at 360 nm, from 7.2 (RM02) to 88 (RM08),
- at 400 nm, from 4 (RM38) to 89.9 (RM01)

Nano titanium dioxide grades

The UV absorption is noted to range:

- at 308 nm, from 9.07 (RM82) (RM01) to 92 (RM09, RM10),
- at 360 nm, from 10 (RM45) to 85 (RM09),
- at 400 nm, from 2 (RM44) to 62.99 (RM82)

Annex H: Photocatalytic Activity – Pigmentary and nano titanium dioxide grades

From Applicants

The detailed method used by Applicants for the determination of Photocatalytic Activity of Pigmentary Titanium Dioxide for the gas phase oxidation of nitric oxide has been reported (see Annex K *"Measurement methods - Appendix 6"*). The results are listed in the following Table 3.18.12.A.

From **Ref.:** Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023)

Pigmentary titanium dioxide grades

Table 3.1.8.12.A: Photocatalytic activity as a function of the pigmentary titanium dioxide grades

Product	NO removed (µmol)	Relative removal (%)	NO_2 generated (µmol)	NOx removed (µmol)	NOx adsorbed (µmol)	NOx desorbed (µmol)	Product	NO removed (µmol)	Relative removal (%)	NO_2 generated (µmol)	NOx removed (µmol)	NOx adsorbed (µmol)	NOx desorbed (µmol)
RM01	2,91	8	1,89	1,02	0,01	0	RM39	0,05	0,1	0,03	0,03	0,01	0
RM02	2,22	6,1	0,95	1,27	0,01	0	RM67	3,62	9,8	3,05	0,57	0,01	0
RM03	1,26	3,4	1,09	0,18	0,01	0	RM67b	2,57	6,9	2,17	0,41	0,01	0
RM04	2,75	7,4	2,2	0,55	0,01	0	RM68	1,79	4,8	1,55	0,25	0,01	0
RM05	2,77	7,4	1,35	1,42	0,01	0	RM69	4,22	11,3	3,58	0,65	0,01	0
RM06	1,27	3,4	1,01	0,26	0,01	0	RM69b	1,25	3,4	1,22	0,04	0,01	0
RM07	0,73	1,2	0,22	0,51	0,01	0	RM70a	12,73	34	6,15	6,59	0,01	0
RM08	1,15	3,1	0,46	0,7	0,01	0	RM70b	16,99	45,1	7,1	9,89	0,01	0
RM19	1,03	2,9	0,13	0,9	0,01	0	RM70c	3,27	8,9	2,72	0,56	0,01	0
RM26	1,95	5,4	1,14	0,82	0	0	RM70d	8,41	23	3,79	4,63	0,01	0
RM27	1,75	4,9	0,67	1,09	0,01	0	RM70e	3,63	9,9	2,74	0,89	0,01	0
RM28	0,85	2,3	0,39	0,46	0,01	0	RM70f	0,32	0,9	0,04	0,29	0,01	0
RM29	0	0	0	0,01	0,01	0	RM72a	0,51	1,4	0,12	0,39	0,01	0
RM30	0,59	1,6	0,32	0,27	0,01	0	RM72b	0,14	0,4	0,04	0,1	0,01	0
RM31	0,07	0,2	0,07	0,01	0,01	0	RM72c	0,94	2,5	0,71	0,24	0,01	0
RM32	0,02	0,1	0,02	0,01	0,01	0	RM72d	0,28	0,8	0,05	0,24	0,01	0
RM33	0,05	0,2	0,04	0,02	0,01	0	RM72e	0,66	1,8	0,15	0,51	0,01	0
RM34	0,01	0	0,01	0,01	0,01	0	RM72f	0,15	0,4	0,04	0,12	0,01	0
RM35	0,02	0,1	0,02	0,01	0,01	0	RM72g	0,05	0,1	0,02	0,04	0,01	0
RM36	0,04	0,1	0,03	0,02	0	0	RM72i	0,18	0,5	0,02	0,17	0,01	0
RM37	0,12	0,3	0,01	0,12	0,01	0	RM72j-bis	0,14	0,4	0,04	0,1	0,01	0,01
RM38	0,04	0,1	0,04	0,01	0,01	0	RM72k	0,18	0,5	0,02	0,17	0,01	0

From Ref.: PS and Surface Property - Pigment Final.xlx - Third Package (31 March 2023)

Nano Titanium dioxide grades

Photocatalytic Activity compared to the uncoated/undoped Material (%) (see Table 3.1.8.12.B): Typical method: A 5% TiO_2 formulation irradiated in a Suntest CPS+ solar simulator for 30 minutes at 300 W/m^2. Sample measured before and after using a

colourimeter and compared to the colour change of the uncoated/undoped material exposed under the same condition.

From **Ref.:** CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Table 3.1.8.12.B: Photocatalytic activity as a function of the nano titanium grades, compared to the uncoated / undoped materials (%)

Product Code	Photocatalytic Activity Compared to the uncoated/undoped Material (%)	Product Code	Photocatalytic Activity Compared to the uncoated/undoped Material (%)
RM09	≤10*	RM60	0.3
RM10	≤10	RM61	0.6
RM11	≤10	RM62	0.3
RM40	2.7	RM63	0.019
RM41	1.5	RM64	0.024
RM42	5.9	RM65	0.051
RM43	7	RM74a	≤ 10
RM44	3.3	RM74b	≤ 10
RM45	1.2	RM74c	≤ 10
RM46	1.8	RM74d	≤ 10
RM47	0.3	RM74e	≤ 10
RM48	8.1	RM75	≤ 10
RM49	1.2	RM76	≤ 10
RM51	1.8	RM77	≤ 10
RM52	2.4	RM78	≤ 10
RM53	0.9	RM80	≤ 10
RM55	1.2	RM81	≤10
RM56	0.6	RM79	≤ 10
RM57	0.6	RM82	≤10
RM58	1.2		
RM59	0.9		

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
From Table on Page 19/28 – Column 12) Photocatalytic Activity Compared to the uncoated/undoped Material (%)

(*) From Applicant: *"RM09 (26nm mean primary particle size Feret min by number, 10% silica) is representative of hydrophilic cosmetic nano grades – coated with silica but no organic (this grade has been extensively characterised by TDMA and used in their studies as G8-2). Although marketed typically as an intermediate any additional treatment is optional and it can also be used directly in sunscreens in appropriate (hydrophilic) formulations. If used in hydrophobic formulations, an appropriate formulation step to improve compatibility is necessary. During such formulating steps RM09 itself remains unchanged though dispersants may become adsorbed on the surface to improve the compatibility with a particular formulation phase. (Therefore, RM09 is not an intermediate in REACH terms)"*
from Ref.: Physchem data tables Jan 2023 submission - Nano (corrected) – 30 June 2023

Annex I: RedOx potential – pigmentary and nano titanium dioxide grades

The method used by Applicants to determine the RedOx potential has been reported (see Annex K "*Measurement methods – Appendix 7*).

From **Ref.:** Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023)

Pigmentary Titanium dioxide grades
The values of the RedOx potential for the pigmentary titanium grades are reported below in Table 3.1.8.13.A.

Table 3.1.8.13.A: RedOx Potential of the Pigmentary grades (from Ref.: PS and Surface Property - Pigment Final – Third Package (31 March 2023))

Product	Redox Potential (mV)	Product	Redox Potential (mV)	Product	Redox Potential (mV)
RM01	377	RM32	/	RM70c	/
RM02	/	RM33	(*)	RM70d	/
RM03	/	RM34	/	RM70e	(*)
RM04	/	RM35	/	RM70f	/
RM05	/	RM36	/	RM72a	/
RM06	/	RM38	/	RM72b	/
RM07	/	RM39	/	RM72c	/
RM08	/	RM67	/	RM72d	/
RM19	/	RM67b	/	RM72e	/
RM26	/	RM68	/	RM72f	/
RM27	/	RM69	/	RM72g	/
RM28	325	RM69b	/	RM72i	/
RM29	/	RM37	/	RM72j-bis	/
RM30	406	RM70a	349	RM72k	/
RM31	323	RM70b	/		

(*): Not measurable - too hydrophobic

Ref.: PS and Surface Property - Pigment Final – Third Package (31 March 2023)

Nano Titanium dioxide grades
The values of the RedOx potential for the nano titanium dioxide grades are reported below in Table 3.1.8.13.B.

Table 3.1.8.13.B: RedOx Potential of the Nano grades (from Ref.: PS and Surface Property - Nano Final – Third Package (31 March 2023)

Nano grade	Redox Potential (mV)	Nano-grade	Redox Potential (mV)	Nano-grade	Redox Potential (mV)
RM09	359	RM52	/	RM74a	/
RM10	/	RM53	/	RM74b	/
RM11	(*)	RM55	/	RM74c	/
RM40		RM56	/	RM74d	/
RM41	399	RM57	/	RM74e	/
RM42	/	RM58	/	RM75	/
RM43	/	RM59	/	RM76	/
RM44	/	RM60	/	RM77	/
RM45	/	RM61	/	RM78	/

RM46	/	RM62	/	RM79	(*)
RM47	/	RM63	/	RM80	/
RM48	/	RM64	/	RM81	/
RM49	/	RM65	/	RM82	/
RM51	/				

(*): Not measurable - too hydrophobic

Ref.: PS and Surface Property - Nano Final – Third Package (31 March 2023)

Annex J: HR-TEM and TEM images

High Resolution Electron Microscopy on Titanium Dioxide Grades used in Cosmetics
(from CE Cons TD_Phys-chem second data package_23 03 2023.pdf

From Applicants

This report shows the structure of the titanium dioxide raw materials visible with High Resolution Transmission Electron Microscopy (TEM) (up to 300,000x magnification). Primary particle morphology and inorganic surface coatings are visible as well as, in some cases, the crystalline lattice planes of the titanium dioxide. There are no lattice planes visible in the surface coating layers as the alumina/aluminium hydroxide and silica are amorphous.
Powder specimens were dispersed in ethanol using an ultrasonic bath. The High Resolution TEM images were taken on a JEOL JEM 2200fs operated at 200kV.
The images in this report are purely qualitative as only a small number of primary particles can be imaged at such high resolutions. Quantitative analysis of the primary particle and agglomerate size distributions and aspect ratio requires analysis of 300-600 particles per sample at lower magnification and this assessment will be reported separately.

Ref.: CE Cons TD_Phys-chem second data package_23 03 2023.pdf

Pigmentary titanium grades

For Pigmentary titanium grades, some typical high resolution TEM images of pigmentary grades that illustrate particular features for the different categories are shown (see Annex – TEM images), and those for every pigmentary grade analysed can be found below.

- Category a / pigmentary *(Surface of Untreated Titanium Dioxide)*: Anatase RM01, Rutile RM02
- Category b1 / pigmentary *(Surface of Titanium Dioxide Treated with Low Levels of Inorganics (<2% Alumina and/or Silica) only)* : RM 30 - Rutile treated with 0.3% Alumina and 2.3% Aluminium Hydroxide
- Category b2 / pigmentary *(Rutile treated with 0.3% Alumina, 2.3% Aluminium Hydroxide and 5% Hydrated Silica)* : RM31 - Rutile treated with 0.3% Alumina, 2.3% Aluminium Hydroxide and 5% Hydrated Silica.
- Category c1 / pigmentary (Surface of Titanium Dioxide Treated Only with Organics) : RM70f - Anatase with <5% Hydrogenated Lecithin
- Category c2 / pigmentary *(Surface of Titanium Dioxide Treated with Low Levels of Inorganics (<2% Alumina and/or Silica) and also with Organics)* : RM 35 -Rutile treated with 0.3% Alumina 0.3%, 2.2%vAluminium Hydroxide, 2% Hydrogen Dimethicone 2.0% (RM35)
- Category c3 / pigmentary *(Surface of Titanium Dioxide Treated with Inorganics (Including >2% Alumina and/or Silica) and with Organics Added)* : RM38 - Rutile treated with 0.2% Alumina, 3.7%, Aluminium Hydroxide, 0.4%, Zinc Oxide and 1% Isostearic Acid.

HR-TEM images:

Categorie a / pigmentary: Surface of Untreated Titanium Dioxide

It can be seen that the lattice planes extend right up to the surface of the primary particle with no surface species visible.

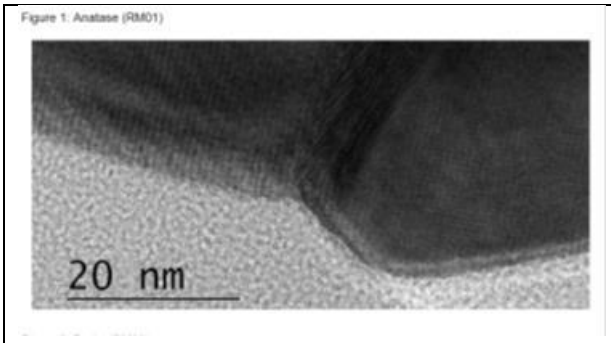


Figure 1: Anatase (RM01)

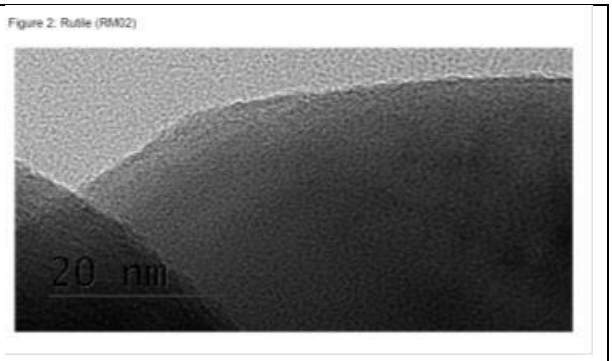


Figure 2: Rutile (RM02)

Categorie b1 / pigmentary: Surface of Titanium Dioxide Treated with Low Levels of Inorganics (<2% Alumina and/or Silica) only : At such low levels of inorganic coating, it is very difficult to see any evidence of the coating at the surface as shown in Figure 4.

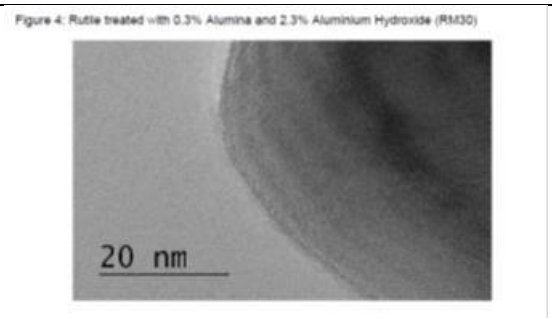


Figure 4: Rutile treated with 0.3% Alumina and 2.3% Aluminium Hydroxide (RM30)

Category b2 / pigmentary: Surface of Titanium Dioxide Treated Only with More than 2% Alumina and/or Silica:

In the most heavily surface treated raw materials, a layer of a few nanometres is visible at the surface especially with silica.

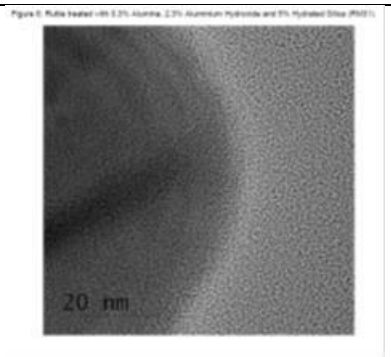
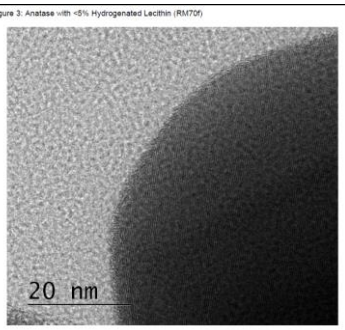


Figure 6: Rutile treated with 0.3% Alumina, 2.3% Aluminium Hydroxide and 5% Hydrated Silica (RM31)

Category c1 / pigmentary: Surface of Titanium Dioxide Treated Only with Organics:

The lattice planes extend right up to the surface of the primary particle with no surface species visible

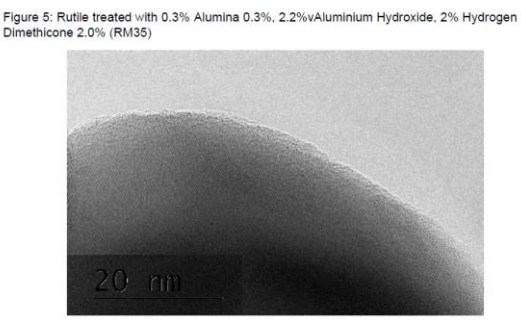
Figure 3: Anatase with <5% Hydrogenated Lecithin (RM70f)



Category c2 / pigmentary: Surface of Titanium Dioxide Treated with Low Levels of Inorganics (<2% Alumina and/or Silica) and also with Organics

At such low levels of inorganic coating, it is very difficult to see any evidence of the coating at the surface as shown in Figure 5.

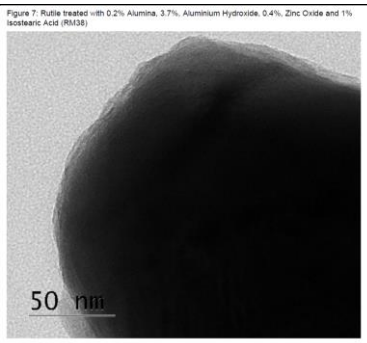
Figure 5: Rutile treated with 0.3% Alumina 0.3%, 2.2%vAluminium Hydroxide, 2% Hydrogen Dimethicone 2.0% (RM35)



Category c3 / pigmentary: Surface of Titanium Dioxide Treated with Inorganics (Including >2% Alumina and/or Silica) and with Organics Added (Category c3)

In the most heavily surface treated raw materials, a layer of a few nanometres is visible at the surface

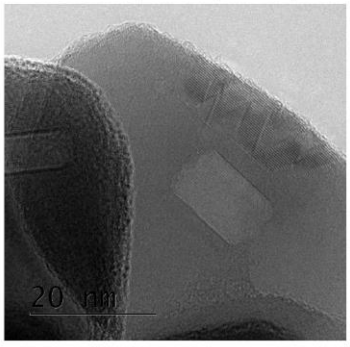
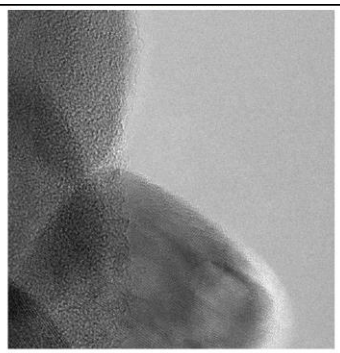
Figure 7: Rutile treated with 0.2% Alumina, 3.7%, Aluminium Hydroxide, 0.4%, Zinc Oxide and 1% Isostearic Acid (RM38)

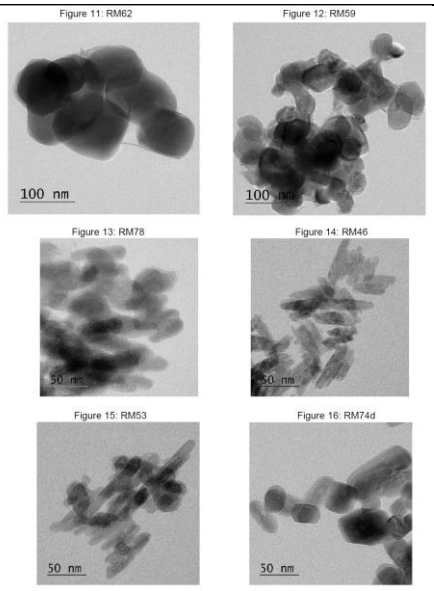


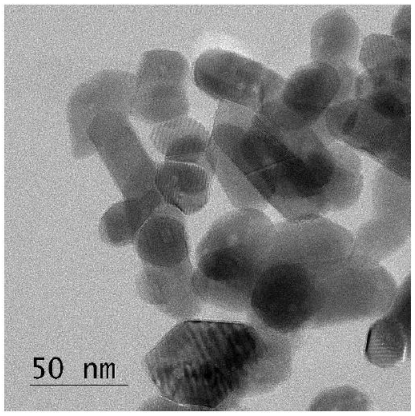
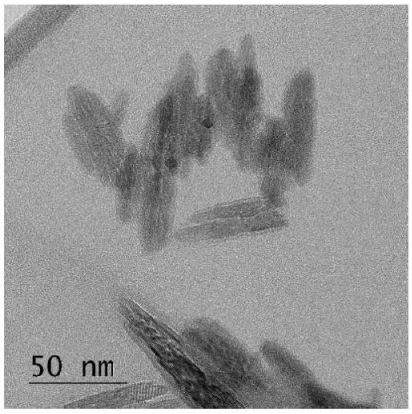
Nano titanium dioxide grades

From Applicants

Some typical high resolution TEM images for nano grades are shown below and those for every grade analysed can be found below:

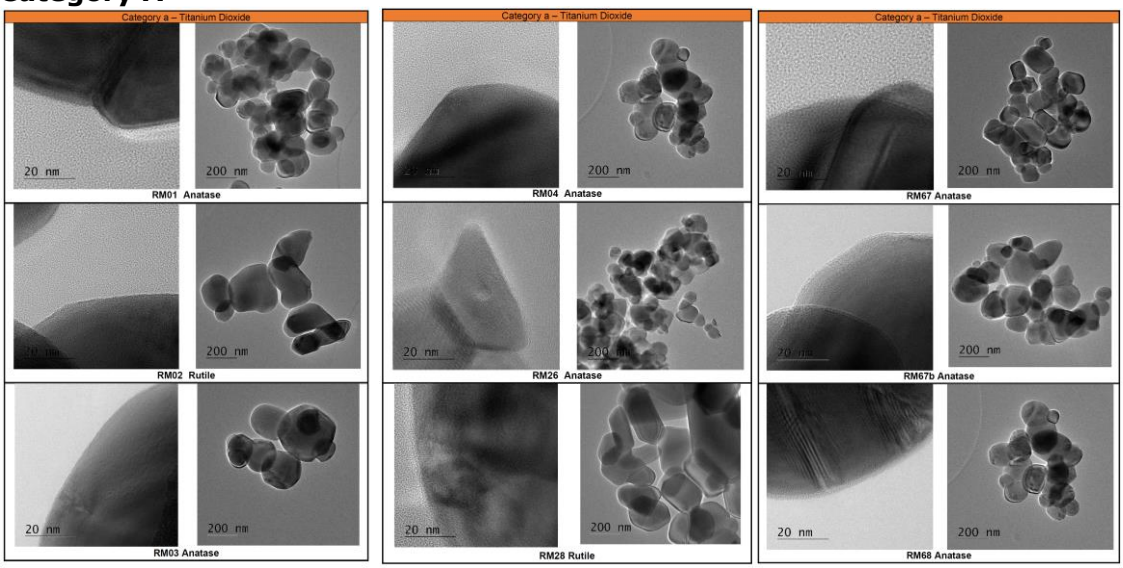
Surface of Nano Titanium Dioxide Treated with Inorganics In the most heavily surface treated raw materials, a layer of a few nanometres is visible at the surface especially with silica.		
	Figure 9: Nano Titanium dioxide 91.2%, Aluminium Hydroxide 4.1%, Stearic Acid 4.7% (RM60)	**Figure 10:** Nano Titanium Dioxide with Silica coating (RM74d)

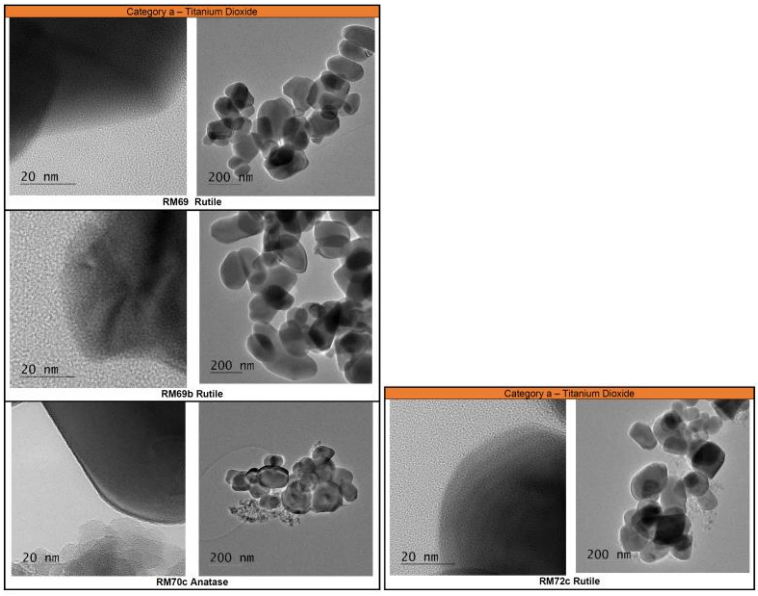
Different Morphologies of Nano Titanium Dioxide The primary particle sizes and morphologies of the nano titanium dioxide raw materials vary more than the pigmentary grades as shown below (all made by the Sulfate Process).	

Different Production Processes for Nano Titanium Dioxide Figures 11-16 show that a variety of morphologies and sizes can be produced by a single process (Sulfate Process) and the same is true of the Chloride Precipitation Process (see Figures 17 and 18).	Figure 17: RM64  Figure 18: RM63 

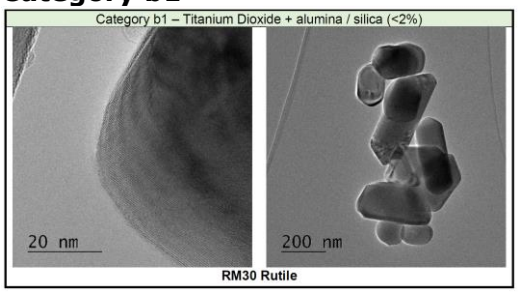
Pigmentary titanium dioxide grades – TEM Images (from Ref. : CE Cons TD_Phys-chem second data package_Annex 1 and 2_Pigment_23 02 2023.pdf)

Category A

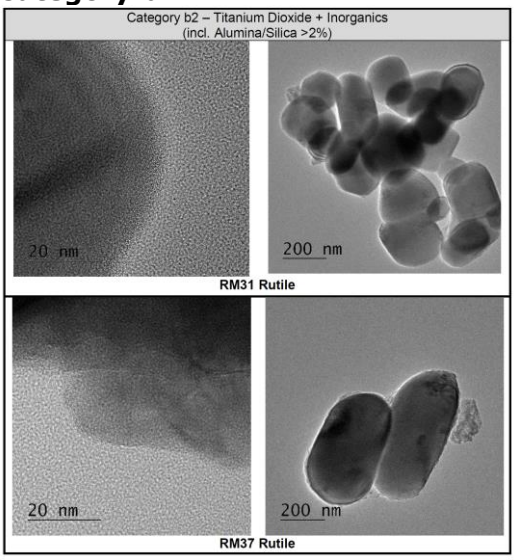




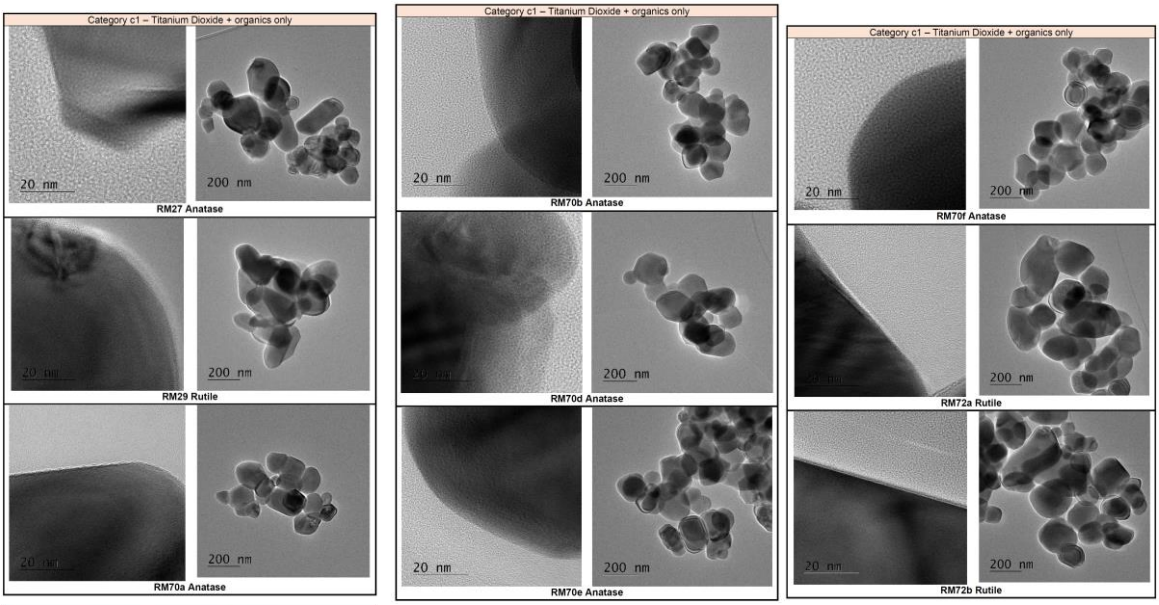
Category b1

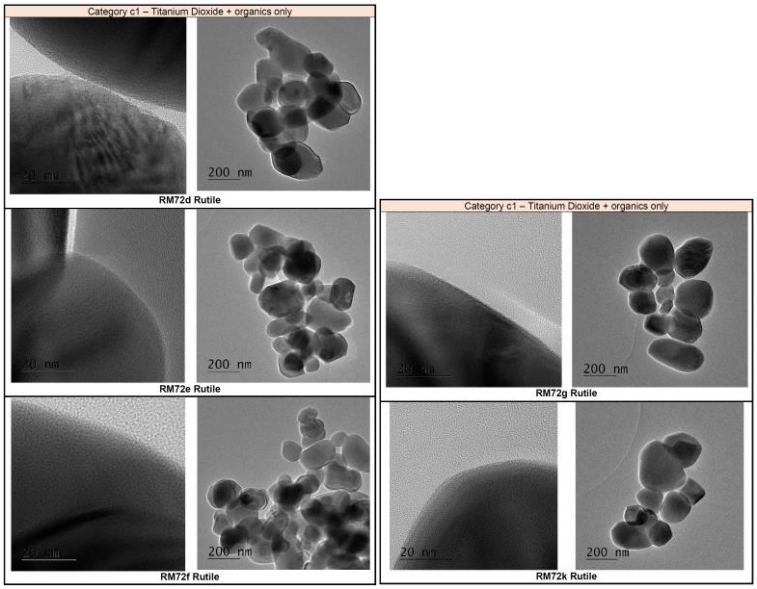


Category b2

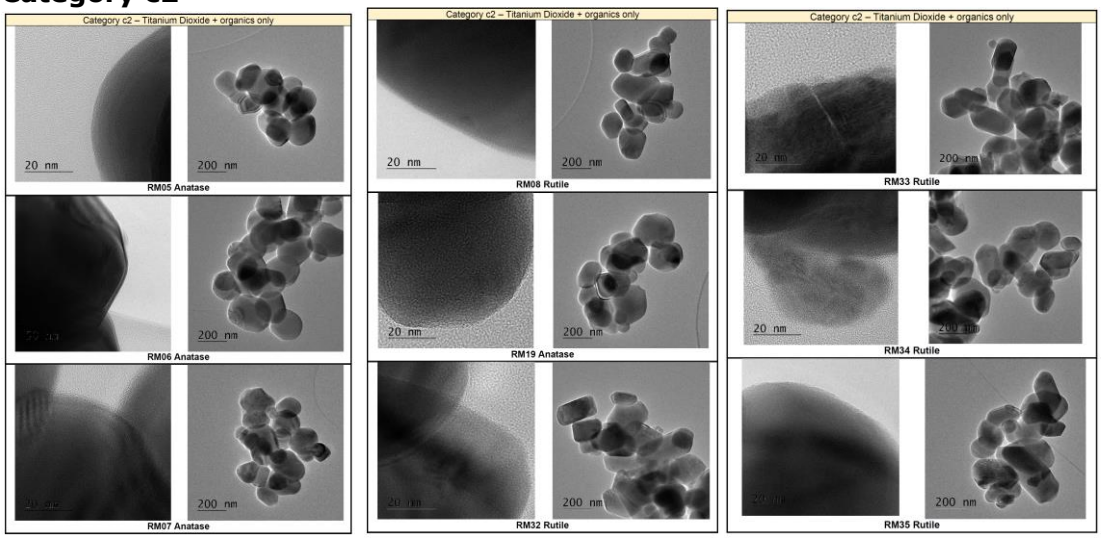


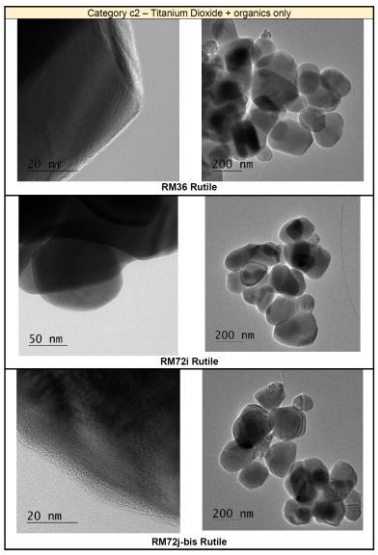
Category c1


Category c1 – Titanium Dioxide + organics only
RM27 Anatase
RM29 Rutile
RM70a Anatase
RM70b Anatase
RM70d Anatase
RM70e Anatase
RM70f Anatase
RM72a Rutile
RM72b Rutile

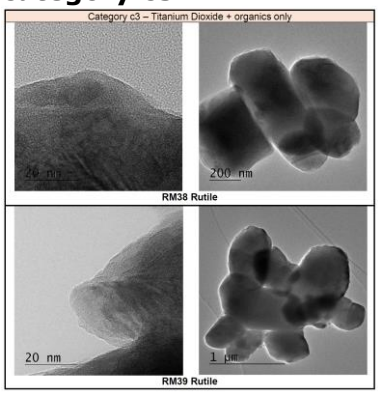

Category c1 – Titanium Dioxide + organics only
RM72d Rutile
RM72e Rutile
RM72f Rutile
RM72g Rutile
RM72k Rutile

Category c2

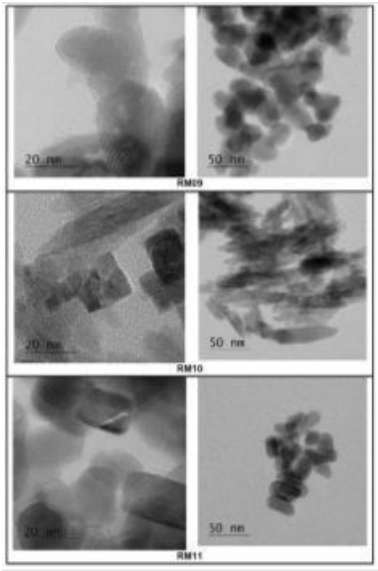

Category c2 – Titanium Dioxide + organics only
RM05 Anatase
RM06 Anatase
RM07 Anatase
RM08 Rutile
RM19 Anatase
RM32 Rutile
RM33 Rutile
RM34 Rutile
RM35 Rutile


Category c2 – Titanium Dioxide + organics only
20 nm
200 nm
RM36 Rutile
50 nm
200 nm
RM72i Rutile
20 nm
200 nm
RM72j-bis Rutile

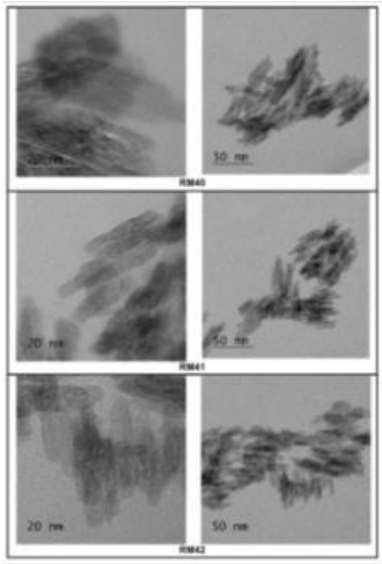
category c3


Category c3 – Titanium Dioxide + organics only
20 nm
200 nm
RM38 Rutile
20 nm
1 µm
RM39 Rutile

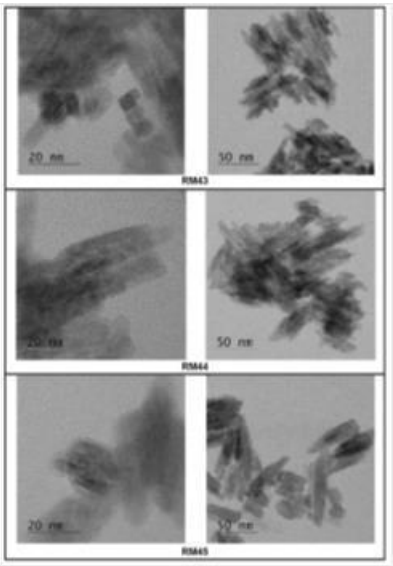
Nano titanium dioxide grades – HR-TEM images (from CE Cons TD_Phys-chem second data package_Annex 3 and 4_Nano_23 02 2023.pdf)



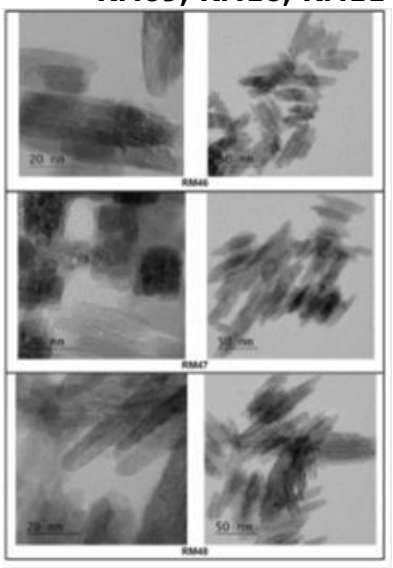
RM09, RM10, RM11



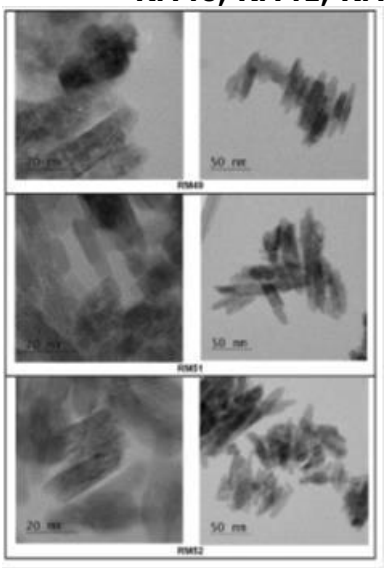
RM40, RM41, RM42



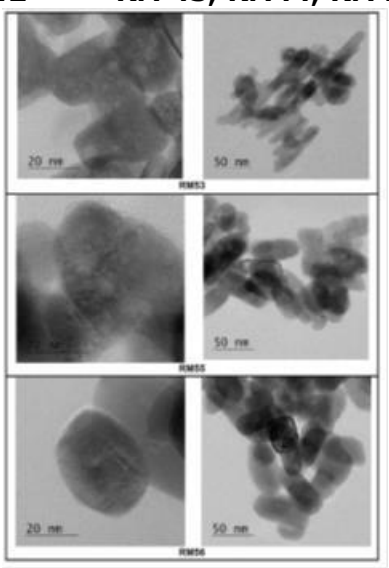
RM 43, RM44, RM45



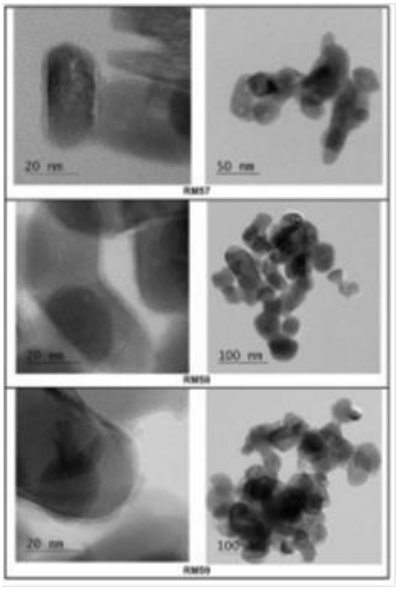
RM46, RM47, RM48



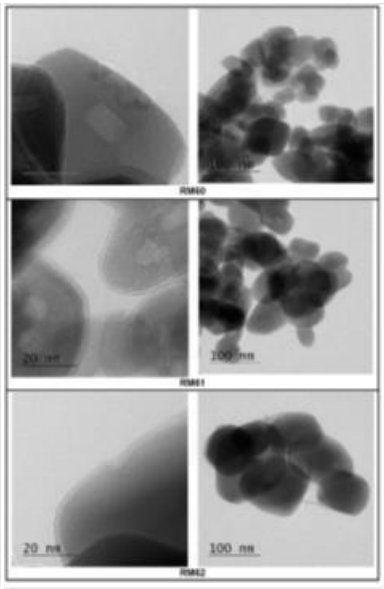
RM49, RM51, RM52



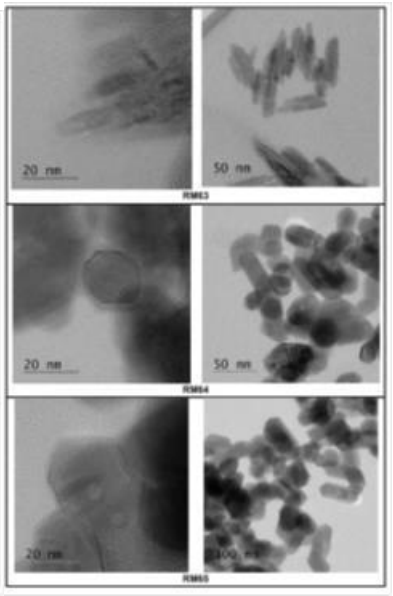
RM53, RM55, RM56



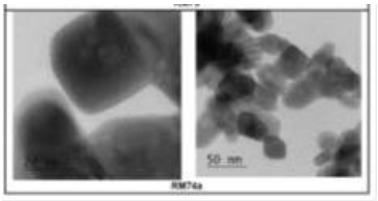
RM57, RM58, RM59



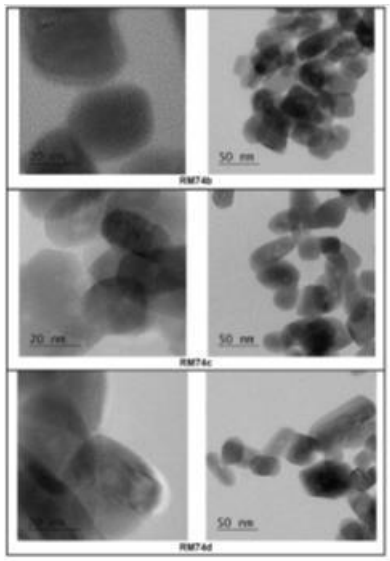
RM60, RM61, RM62



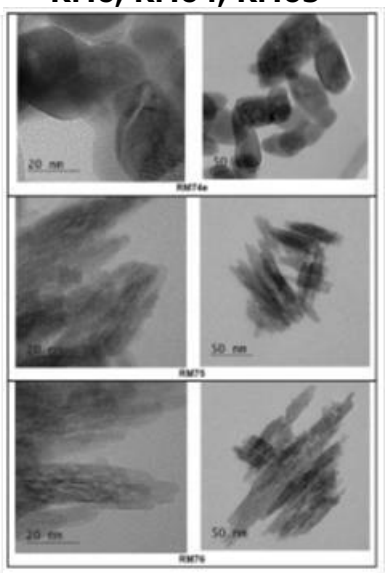
RM6, RM64, RM65



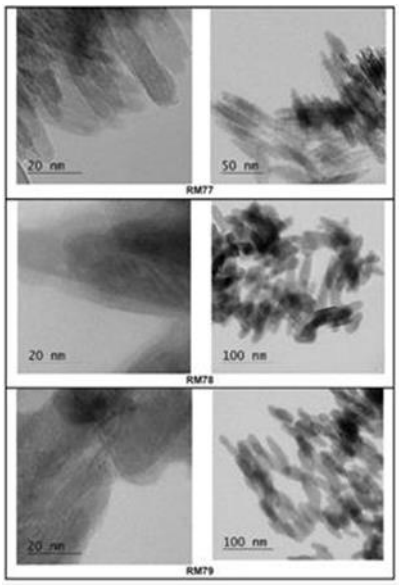
RM74a



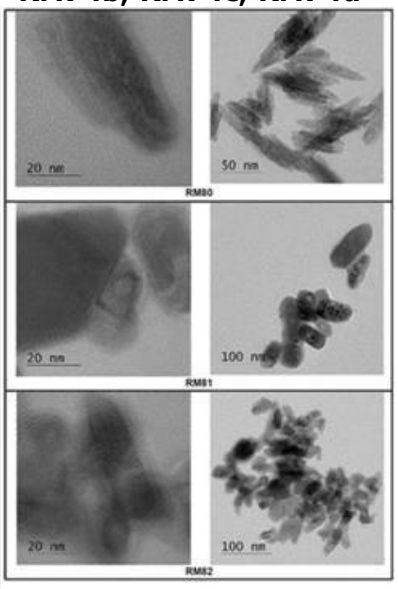
RM74b, RM74c, RM74d



RM74e, RM75, RM76



RM77, RM78, RM79



RM80, RM81, RM82

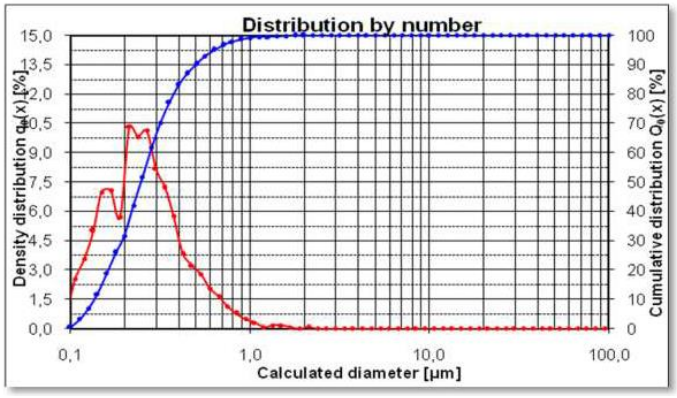
Annex K: Measurement methods - Appendix 1: Determination of constituent particle size distribution and shape by TEM

From Report 2 (Corrected) 30 June 2023: Titanium Dioxide Grades used in Cosmetics Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions Section Appendix 1 Determination of Primary Particle Size Distribution and Shape by TEM
(Informations similar as the ones provided in Ref.: Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023) - Ref. CE-TiO2-23-005.0)

The method determines size data of primary particles, such as number, volume and shape information. For this purpose, electron micrographs are evaluated with image analysis software and a touch-sensitive screen by drawing the crystal edges. All samples whose primary particle boundaries can be clearly identified in a microscopic image are suitable in principle for evaluation. All information on the volume distribution is derived from a sphere of the determined diameter. If the primary particle boundaries are difficult to recognize for the operator or if there is a margin of discretion, this has an unfavourable effect on the measurement uncertainty.

From the powder three spatula tips are taken from different locations of the sample vessel and a rubout is performed. The RM77 sample (aqueous dispersion) was previously dewatered in the vacuum drying cabinet. A drop of rubbing was transferred to a TEM grid. The TEM images were taken at different locations on the grid. Depending on the crystal size, magnifications of between 16,000x and 40,000x were selected. All TEM images were taken on the Zeiss AB 912 at a high voltage of 100kV. The recordings are evaluated with the software Image Pro Plus and a touch sensitive monitor. The individual primary particles are bypassed with the contact pin at the outer edges. If possible, only clearly recognizable primary particle lines were used for the evaluation. The data thus obtained are evaluated via an Excel template. A frame correction according to International Standard ISO 13322-1 Particle size analysis - Image analysis methods S.9 was performed.

Distribution:



x90 [µm]:	0,50
x50 [µm]:	0,25
x10 [µm]:	0,14
Mean [µm]:	0,29

Red line = left y-axis, Blue line = right y-axis
The X-axis is shown logarithmically and divided into classes.

The red line represents a normalized density distribution of the particles. The following applies to the y-axes:
With the number distribution q0: Percentage of the number of particles in the corresponding class, without units
For volume distribution q3: Percentage of the particle volume in the corresponding class, unit: [µm]
The blue line is the cumulative distribution. Here the particles are summed up class by class.
The x90; x50; x10 values are to be understood as follows:
e.g. x90 [µm]: 0.50 => 90% of all particles are smaller than 0.50µm
e.g. x50 [µm]: 0.25 => 50% of all particles are smaller than 0.25µm
The mean value is the mean value, with a perfect Gaussian distribution this is identical to the x50.

Annex K: Measurement methods - Appendix 2: Determination of constituent particle size distribution and shape by SEM – Applicant #2 method (used for nano titanium dioxide)

From Report 2 (Corrected) 30 June 2023: Titanium Dioxide Grades used in Cosmetics Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions Section Appendix 2 Determination of Primary Particle Size Distribution and Shape by SEM – Applicant #2 method (used for Nano Titanium Dioxide)
(Informations similar as the ones provided inRef.: Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023) - Ref. CE-TiO2-23-005.0)

From the powder three spatula tips are taken from different locations of the sample vessel and dispersed 5min in a test tube together with ethanol in the ultrasonic bath. The suspension is immediately dropped on a Si-wafer, dried and were examined without any metal coating. Depending on the crystal size, magnifications of between 25,000x and 10,000x were selected. All SEM images were taken on the FE-Hitachi SU 70 with the aid of an in-lens detector and at a high voltage of 5kV.
It is possible to determine primary particle size distributions by image analysis of SEM images of materials with dense and well-defined primary particle boundaries. For example, in Image 1 (TEM) and Image 2 (SEM) below the material has very clear boundaries which are easy to distinguish in the images prepared using both techniques.

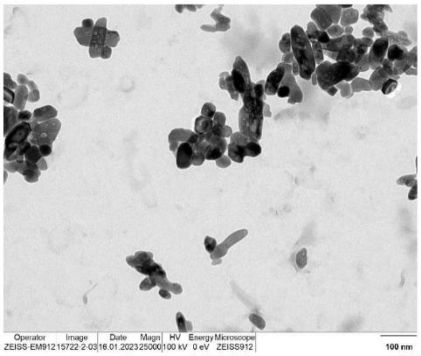
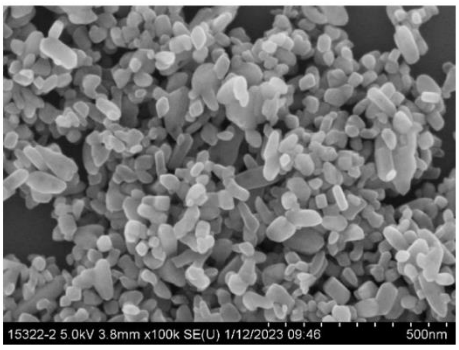


Image 1 TEM



RM74c Image 2 SEM

Nevertheless, due to the translucent effect of the TEM picture it is easier to define the primary particle boundaries than in the comparable SEM pictures with the same magnification. Additionally, the resolution of the SEM is not as high as for TEM, which makes the image evaluation even more difficult. Therefore, the primary particle size based on SEM pictures typically gives larger sizes than that based on TEM pictures whilst the aspect ratio determined by SEM is typically smaller than that based on TEM image analysis as shown in the table below.

Sample no.	Value / obtained by	TEM	SEM
RM57	x50 ECD [nm]	39	44
	Aspect Ratio	1.76	1.49
	x50 Feret min [nm]	30	38
RM58	x50 ECD [nm]	41	48
	Aspect Ratio	1.55	1.37
	x50 Feret min [nm]	33	42
RM61	x50 ECD [nm]	56	66
	Aspect Ratio	1.47	1.28
	x50 Feret min [nm]	48	59
RM74c	x50 ECD [nm]	40	47
	Aspect Ratio	1.53	1.47
	x50 Feret min [nm]	34	40

However, for some nanomaterials the resolution of the SEM is not adequate to enable the primary particle boundaries to be distinguished sufficiently to allow the image analysis software to function adequately whilst satisfactory images for analysis can be obtained with TEM (see Images 3 and 4).
The limitation lies with the resolution of SEM and the ability to distinguish the primary particle boundaries and therefore is not improved even with better dispersion techniques such as those described in N. B. Ghomrasni *et al.* ("Challenges in sample preparation for measuring nanoparticles size by scanning electron microscopy from suspensions, powder form and complex media", Powder Technology, Volume 359, 2020, Pages 226-237)

Image 3 TEM RM76

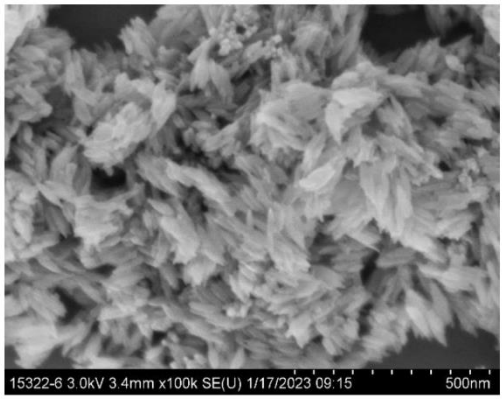


Image 4 SEM

As is clearly seen from Image 4, it is not possible to quantitatively analyse the SEM images of many of the nanomaterials and also, as shown in the comparative table, that for those nanomaterials where analysis is possible, the SEM primary particle size is always larger than the TEM size and the aspect ratio is always lower for SEM than TEM. Therefore, the primary particle size analysis of nanomaterials has only been done by TEM for all the nanomaterials.

Annex K: Measurement methods - Appendix 3: Determination of constituent particle size distribution and shape by SEM – Applicant #1 method (used for pigmentary titanium dioxide)

From Report 2 (Corrected) 30 June 2023: Titanium Dioxide Grades used in Cosmetics Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions Section Appendix 3 Determination of Primary Particle Size Distribution and Shape by SEM – Applicant #1 method (used for Pigmentary Titanium Dioxide)
(Information similar as the ones provided in Ref.: Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023) - Ref. CE-TiO2-23-005.0)

Microscope Hitachi Regulus 8230
Operating conditions Working Distance: 8 mm
High Voltage: 2.5 kV
Deceleration: 1.5 kV
Landing Voltage: 1.0 kV
Detector: PDBSE (Backscatter electron detector)
Sample Preparation: Cross-Section
Image Evaluation: Automated (user independent)

The method was first described in a peer reviewed publication in 2013[1], the relevant information is summarized in the following sections.

Sample Preparation:
The first step is to riffle[2] the as delivered sample to isolate a representative fraction of 2g, which is then dry-mixed[3] with a hot-mounting resin[4]. The mixture is hot-mounted at 180°C and 125bar for 12 minutes[5]. The cross-section is prepared by a five-step grinding and polishing process[6], which is completed with a polishing step using colloidal silica[7] and thorough cleaning of the sample surface.

Measurement:
Measurement is performed under standardized conditions: A series of 8 images is acquired, the image size is 2560 x 1920 pixels; the pixel size was chosen according to the size of the constituent particles with most samples measured with a pixel size of 3.3nm; but 10nm is used for RM39 with a d50 of 360nm, for example.

Image evaluation:
Image evaluation is done with the image analysis software "analySIS" from Olympus[8] using exclusively the implemented functions. The different steps of the procedure are fixed in an input sequence (macro) that is applied in the same way to each of the acquired images. The carefully tested assumptions underlying the evaluation procedure are as follows:
1. The constituent particles are convex particles with a non-complex shape.
2. The observed grey-values are a good approximation of a Gaussian distribution.

The steps of the automated image evaluation are as follows:
Automated Brightness and contrast adjustment
Preparation of a masking image:
- Noise filtering
- Automated thresholding
- Binarization
- Removal of isolated pixels
- Separation of touching/bound particles (separation of aggregates and agglomerates)

Applying the mask to the original image
Detecting the particles (including size, shape and gray scale features)
Filtering of detected particles (removal of false detections)

- Shape filtering (convexity > 0.90 and formfactor[9] > 0.86)
- Grey-value filtering (making mean and standard-deviation symmetric)

The described procedure leads to a reproducible, user-independent evaluation of several thousand particles and thus to a well-founded statistical description of the examined pigment.

1 https://doi.org/10.3762/bjnano.5.192
2 Micro Rotary Riffler, Quantachrome
3 MM400, Retsch
4 Polyfast, Struers
5 CitoPress5, Struers
6 Tegramin, Struers
7 Standard Colloidal Silica Suspension, Struers
8 Meanwhile Analysis is replaced by Stream and Olympus is now called Evident.
9 Sphericity according to the definition of Hakon Wadell

Annex K: Measurement methods - Appendix 4: Determination of secondary particle size distribution by Disc Centrifuge

From Report 2 (Corrected) 30 June 2023) – Titanium Dioxide Grades used in Cosmetics Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions - Section Appendix 4 Determination of Secondary Particle Size Distribution by Disc Centrifuge and from **Ref.** Primary and Secondary PS and Surface Properties - Report (corrected).docx – 30 June 2023
(Information similar as that provided in Ref.: Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023) - Ref. CE-TiO2-23-005.0)

Nano Titanium Dioxide
All samples are dispersed and measured in the same way whether hydrophilic or hydrophobic.

0.4 g of the powder sample is pre-wetted with ethanol and 2 drops of Disperbyk 190 and the paste is sonicated in an ultrasonic bath for a few seconds.

After the pre-wetting of the sample, 50 ml of 24% propanediol in 1 g/L Calgon N solution is added and the suspension is dispersed for 10 minutes with a Sonics ultrasonic horn at an amplitude of 57%.

For the measurement of the particle size, 0.1ml of the dispersion is injected into the disc centrifuge (CPS DC) operating at a speed of 20,000 rpm and a UV light source at 470nm.

The calculation of the results is done by the device software.

Pigmentary Titanium Dioxide
Dispersing agent:
- Hydrophilic materials - HMP Solution: 0.6g Sodium Hexametaphosphate made up to 1,000g with ultra-pure water.
- Hydrophobic materials - Imbentin Solution: 0.5g Imbentin-SG/45/AG + 0.05g Potassium Tripolyphosphate (KTTP) made up to 1,000g with ultra-pure water.

Preparation of dispersion:
- 2g of pigment + 80g dispersing agent.
- 1min dispersing by Ultra Turrax at 9,500 rpm
- 1:25 dilution in dispersing agent

Measurement:
For the measurement of the particle size, 0.1ml of the dispersion is injected into the disc centrifuge (CPS DC) operating at a speed of 3,000rpm and a UV light source at 405nm.
The calculation of the results is done by the device software.

Annex K: Measurement methods - Appendix 5: Determination of Zeta potential and iso-electric point pH

(From **Ref.:** Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023) - Ref. CE-TiO2-23-005.0 and from Ref.: and from **Ref.** Primary and Secondary PS and Surface Properties - Report (corrected).docx – 30 June 2023).

Measurement limitations

During the determination of the Zeta Potential, it is necessary to maintain a constant ionic strength for comparability of the measured values. For this reason, the measurement was performed in a 1 mM potassium chloride (KCl) solution. This concentration was chosen to stabilize the ionic strength satisfactorily but at the same time not to interfere with the actual measurement. However, such a concentration only effectively stabilizes the ionic strength in the pH range from 4 to 10, so it is essential to consider the measured Zeta Potentials at the extremes of pH as not entirely reliable. Other processes, such as increased solubility of TiO_2 or coating materials may play a role in the potential inaccuracy of measurements at pH values greater than 10 and less than 4.

Experimental

A solution of 1 mM KCl in deionized water was filtered through a 0.2 µm pore size filter membrane. Then, 20 mg of TiO_2 sample was dispersed in 200 ml of KCl solution using an ultrasonic bath (DT255H, Bandelin) for 5 min to form a 0.01% (w/v) dispersion. The dispersion was then stirred on a magnetic stirrer while adjusting the pH with NaOH or HCl, always at a concentration of 0.1 M or 0.01 M in deionized water. pH was measured using a pH meter (HI 5521, HANNA Instruments) calibrated with pH standards before use. The pH of the sample was adjusted to 6 and then gradually increased to pH 11 using NaOH. 0.8 ml sample was taken for Zeta Potential measurement at each desired pH value. In the next step, a fresh dispersion of the test sample was prepared, and the pH was adjusted to 5 and then gradually decreased to pH 1 using HCl. The measurement was carried out in the same way as the previous sample. Zeta potentials were recorded using a Zetasizer Nano ZS (Malvern). A new disposable folded capillary cell was used for each sample.

The Zeta Potential of the sample at each pH was recorded in three instrumental runs and plotted as a graph where the error bars represent the standard deviation between the three measurements. Experimental data points were fitted using the polynomial function Poly4 in Origin 2018 software. The isoelectric point (IEP) was calculated from the fitted curve as the pH value at which Zeta Potential = 0.

Annex K: Measurement methods - Appendix 6: Determination of photocatalytic activity of pigmentary titanium dioxide for the gas phase oxidation of nitric oxide

(From Ref.: Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023) - Ref. CE-TiO2-23-005.0 and from **Ref.** Primary and Secondary PS and Surface Properties - Report (corrected).docx – 30 June 2023)

The photocatalytic activity of Pigmentary Titanium Dioxide for the gas phase oxidation of nitric oxide (NO) under illumination with UV light has been determined according to ISO 22197-1.

Measurement procedure
Each sample powder was placed in a sample holder (5 x 10 cm^2 dimensions) and pressed slightly with a plunger. Afterwards the sample was mounted into the photocatalytic reactor. The height of each sample was adjusted to 5 mm distance from the glass reactor cover.

A gas mixture of Synthetic Air/NO (C(NO)=1ppm; ca. 50% relative humidity) was fed in the system, at first by-passing the reactor until a stable signal was achieved. At the beginning of each experiment the gas mixture was directed through the reactor over the sample without UV light illumination, resulting in a dark adsorption NO uptake. After NO signal reached constant level again, UV light (365 nm) was switched on and the sample was illuminated for 300 minutes. Upon switching off the light source the signal returned to its origin without illumination. After stopping NO and feeding only Synthetic Air over the sample, a desorption branch of the signal was observed for several minutes at the end of the experiment.NO oxidation rate was determined according to the ISO 22191-1 standard. Table 1 lists the relevant parameters during the tests.

Table 1: Parameters during the NO oxidation tests.

Parameter	Value
Temperature gas phase	21 °C
Gas flow rate	3 L/min
Gas composition	1 ppm NO in N_2
Gas humidity	50 % relative humidity
Reaction vessel	According to ISO 22191-1, material: PEEK, gas volume over the sample: 25 ml (0.5 ml)
Sample size	5 x 10 cm^2
Light intensity	10 W/m^2 (peak wavelength 365 nm)
Hydrodynamic residence time	0.5 s
Gas Analyzer	Horiba APNA-370

The results of NO oxidation are summarized as the total absolute amount of NO removed from the gas phase in the 5 h test interval given in µmol and also the relative removal, in relation to the maximum attainable in the test.

Additionally, according to ISO 22197-1, also the generated NO2 as well as absorbed, desorbed, and removed NOx are calculated and are reported.

Annex K: Measurement methods - Appendix 7: Determination of Redox Potential

(From Ref.: Titanium Dioxide Grades used in Cosmetics, Data on Primary and Secondary Particle Size and Surface Properties and Measurement Method Descriptions. Third data package - Report 2 (31 March 2023) and from **Ref.** Primary and Secondary PS and Surface Properties - Report (corrected).docx – 30 June 2023).

Sample Preparation
0.1g made up to 1L with demineralised water in a round bottomed flask of which 250 ml transferred to a 400 ml tall glass flask. Glass electrode inserted and sample mixed for 10 minutes reading taken when stable for 1 min.

Standardising Electrode
200 ml plastic container filled with fresh redox standard test solution and electrode immersed until stable reading observed.
The reading should be within 30 mV of the value expected for the standard test solution.
Measurement repeated with fresh solution.
The second reading should not differ from the first by more than 10 mV.

Procedure
- After the electrode/meter assembly has been standardized as described above, electrode was rinsed three times using a demineralised wash bottle.
- Sample was poured into in a clean glass beaker and electrode immersed into solution supported by a lab stand.
- Adequate agitation throughout the measurement period achieved using a magnetic stirrer.
- Millivolt potential of the solution recorded after allowing to mix for 10 minutes.
- Second portion of the sample measured as stated in above procedure and test deemed complete when two successive portions differ by no more than 10 mV.

Summary of test conditions

Sample concentration (gpl)	0.1
Temperature	20 °C
Volume ml	250 ml
ORP instrument	WTW pH1970i
Electrode type	Glass electrode SenTix ORP
Electrode SN	8230907045
Electrode fill solution	KCl

Annex K: Measurement methods – Appendix 8: Dispersibility with Bovine Serum Albumin (BSA) dispersant

From Ref. "Report 1 (corrected)" 30 June 2023 – Titanium Dioxide Grades used in Cosmetics Data on Dispersibility and Measurement Method Descriptions **-**Section Appendix 2 Dispersibility with Bovine Serum Albumin (BSA) dispersant as used for in vitro genotoxicological studies (following the Nanogenotox method) and Ref.: Dispersibility data on Cosmetics TiO2 grades - Report (corrected).docx – 30 June 2023

The titanium dioxide sample is formulated in 0.05% w/v BSA-water solution. The solvent was chosen according to the Nanogentox protocol. The dispersion protocol is based in the recommendations of the Nanogenotox protocol. A sterile 0.05% w/v BSA in Milli water solution is used to prepare the TiO_2 dispersion. For preparing a 1 mg/mL stock dispersion, 6 mg of the nanomaterial is prewetted with approx. 0.03 mL pure ethanol (purity ≥ 99%) and dispersed in 5.97 mL BSA- MilliQwater (0.05% w/v).
In order to obtain a homogeneous dispersion, this mixture is ultrasonicated with a probe sonicator (Sonics Vibra Cell VC505) for approx. 13 minutes at 500 W and approx. 10% amplitude. The plastic vial is cooled in an ice water bath during sonification.

Dispersion protocol

Final sample volume	6 mL
Final sample concentration	1 mgmL stock dispersion
Solvent	0.05 wt% BSA-water
Prewetting	In 0.5 vol% ethanol (purity ≥ 99%)
Dispersing agent	BSA
Sonication power	500 W at 10% amplitude 6500 J/mL sample volume (500 W x 780 s x 0.1 (amplitude) / 6 mL)
Sonication time	13 min
Sonication type	Probe sonication
Max stability time	30 min

Maximum stability time is defined to be 30 min.
Particle sizing by centrifugal sedimentation is conducted on a CPS-instruments DC 24000 UHR, with the following settings:

- Medium: Density gradient of 0 to 8% sucrose in water topped with 1 ml dodecane
- Rotation speed: 15,000 rpm
- Calibration with 196 or 184 nm PMMA standard, 225 µl in 50 ml water
- Measurement range: 0.03 µm to 2 µm
- Particle density: 4.1 g/ml
- Particle Refractive index @ 405 nm (detector wavelength) n = 2.6820 (https://refractiveindex.info/?shelf=main&book=TiO2&page=Bodurov)
- Particle absorption @ 405 nm k = 0
- Fluid density: 1.01 g/ml
- Fluid Refractive index: 1.34
- Fluid viscosity: 0.95 cP

References

Nanogenotox: Final protocol for producing suitable manufactured nanomaterial exposure media. The generic NANOGENOTOX dispersion protocol July 2011.

Two other former distinct reports have been provided by Applicants for describing the dispersibility method with Bovine Serum Albumin (BSA)

A/ Dispersibility with Bovine Serum Albumin (BSA) dispersant as used for in vitro genotoxicological studies (following the Nanogenotox method) (From Ref.: Dispersibility Nanogenotox – Report, Fourth data package – 21 April 2023)

General Description

The titanium dioxide sample is formulated as a 2.56 mg/ml prewetted (ethanol) dispersion in BSA-solution (0.05% wt), dispersed using 16 minutes sonication with 10% amplitude (ultrasonic sonotrode) in small glass vials and cooled in an ice water bath during sonification. Particle measurement is performed with a CPS Disc Centrifuge (DC24000).
BSA= Bovine Serum Albumin

Preparation details

Vials:	20 ml scintillation vials
Total volume of dispersion	6 ml
Total sample weight (6ml)	15.3 mg
Total prewetting ethanol	0.03 ml (3 portions of 0.01 ml)
Total BSA-solution volume	5.97 ml (2 portions : 0.97 ml after prewetting and additional 5 ml)
Ice bath:	Isolated Box filled with 80-90% ice and 10-20% water (inside the box as vial platform = a 250 ml glas filled with ice and upside down)
Ultrasonic horn/sonotrode	Sonifier S-450 (analog) with a standard 13 mm disruptor horn, (UF), manufacturer: Branson Ultrasonics (now Emerson)
Energy ultrasonic horn:	according to Nanogenotox protocol = 3,136 MJ/m^3 (16 minutes with 10% amplitude)
Dispersing amplitude:	10%
Dispersing time:	16 minutes
Optical Data used:	BI=2.75 AI=0.05
Gradient in DC:	Sugar based density gradient
DC (disc centrifuge):	CPS Instruments DC24000 (settings as shown below)

CPS DC24000 Settings

- Medium: Density gradient of 0 to 8% sucrose in water topped with 1 ml dodecane
- Rotation speed: 20,000 rpm
- Calibration with 710nm standard
- Measurement range: 0.03 µm to 3 µm
- Particle density: 4.1 g/ml
- Particle Refractive index @ 405 nm (detector wavelength) n = 2.75 (https://refractiveindex.info/?shelf=main&book=TiO2&page=Bodurov)
- Particle absorption @ 470 nm k = 0.05
- Fluid density: 1.075 g/ml
- Fluid Refractive index: 1.3706
- Fluid viscosity: 2.0cps
- Shape Factor: 1.5

DiscCentrifuge (DC) Technique

The disc centrifuge measures particle size distributions using the differential sedimentation method. Particles settle in a sugar-based density gradient under a gravitational field according to Stokes' Law. Depending on their size, particles take different times to pass through the gradient in the disc. In the outer range of the rotor a light source and a detector is positioned. The attenuation of light by particles is measured and according to Stokes' Law and Mie-Theory a particle size distribution (mass and number) may be calculated.

All measurement preparations are done accurately (but non-sterile) by the NANOGENOTOX dispersion protocol, Standard Operation Procedure (SOP) and background documentation, July, 2011, WP 4: Physicochemical Characterisation of Manufactured Nanomaterials (MNs) and Exposure Media (EMs), Deliverable 3: Final protocol for producing suitable MN exposure media, Keld Alstrup Jensen, *et al.* (The National Research Centre for Working Environment/CEA/INRS), V.2 (Final), Creation 31.08.2010, Completion 09.07.2011

B/ Dispersibility with Bovine Serum Albumin (BSA) dispersant as used for in vitro genotoxicological studies (Following the Nanogenotox method) (from Ref. From Report 1 – Titanium Dioxide Grades used in Cosmetics - Data on Dispersibility and Measurement Method Descriptions - Third package – 31 March 2023)

The titanium dioxide sample is formulated in 0.05% w/v BSA-water solution. The solvent was chosen according to the Nanogenotox protocol. The dispersion protocol is based in the recommendations of the Nanogenotox protocol. A sterile 0.05% w/v BSA in Milli water solution is used to prepare the TiO2 dispersion. For preparing a 1 mg/mL stock dispersion, 6 mg of the nanomaterial is prewetted with approx. 0.03 mL pure ethanol (purity ≥ 99%) and dispersed in 5.97 mL BSA- MilliQwater (0.05% w/v).

In order to obtain a homogeneous dispersion, this mixture is ultrasonicated with a probe sonicator (Sonics Vibra Cell VC505) for approx. 13 minutes at 500 W and approx. 10% amplitude. The plastic vial is cooled in an ice water bath during sonification.

Maximum stability time is defined to be 30 min.
Particle sizing by centrifugal sedimentation is conducted on a CPS-instruments DC 24000 UHR, with the following settings:
• Medium: Density gradient of 0 to 8% sucrose in water topped with 1 ml dodecane
• Rotation speed: 15,000 rpm
• Calibration with 196 or 184 nm PMMA standard, 225 µl in 50 ml water
• Measurement range: 0.03 µm to 2 µm
• Particle density: 4.1 g/ml
• Particle Refractive index @ 405 nm (detector wavelength) n = 2.6820 (https://refractiveindex.info/?shelf=main&book=TiO2&page=Bodurov)
• Particle absorption @ 405 nm k = 0
• Fluid density: 1.01 g/ml
• Fluid Refractive index: 1.34
• Fluid viscosity: 0.95 cP
References
Nanogenotox: Final protocol for producing suitable manufactured nanomaterial exposure media. The generic NANOGENOTOX dispersion protocol July 2011.

Annex K: Measurement methods - Appendix 9: Dispersibility in water (following the SCCS/1516/13 protocol) (so called by Applicant "modified SCCS method")

From Ref. "Report 1 (corrected)" 30 June 2023 – Titanium Dioxide Grades used in Cosmetics Data on Dispersibility and Measurement Method Descriptions **-** Section Appendix 1 Dispersibility in water (following the SCCS/1516/13 protocol) ("modified SCCS method")
(Informations similar as the ones provided in Ref. Report 1 – Titanium Dioxide Grades used in Cosmetics - Data on Dispersibility and Measurement Method Descriptions - Third package – 31 March 2023)

The dispersibility of a material is based on its inherent properties, so that it is not always possible to disperse all materials in the same solution or under the same conditions.

Applicant #2 uses a dispersibility protocol which is typically very useful to disperse a broad range of nano titanium dioxide in water. It follows the method used for data submitted to SCCS/1516/13 Revision of the Opinion on Titanium Dioxide, nano form and is also consistent with the EFSA guideline for the preparation of nanomaterials.

The SCCS method had to be changed to obtain validated results:

The concentration of 8mg/ml is relatively high in order to obtain sufficient intensity when measuring by the optical disc centrifuge (DC) method.

The prewetting and the dispersing aids have been changed to obtain optimal results. In this method the dispersants consist of Polyphosphate and PDO (1,3 Propanediol) and the material is prewetted with Ethanol and Disperbyk 190 to obtain optimal results with hydrophobic and hydrophilic grades.

The dispersion energy input is 600 J/ml and the quality of the dispersion is measured by DC.

The adjustment of the DC is optimised to show the quality of the dispersion for nano and pigmentary material.

The stability of the dispersion is not the main goal of the experiment, but the material is stable for two to three hours and can be redispersed by mixing with a magnetic stirrer. It is always advisable to check for settling, even after some minutes, depending on the material's particle size. Fine particles stay in the suspension while coarse particles settle more quickly.

The final pH value is dependent on the material dispersed and neither pH nor ionic strength have been measured.

Dynamic Light Scattering (DLS) is not feasible for the measurement of the dispersibility of pigmentary material and to obtain comparability, all dispersions (of nano- and of pigmentary material) have been measured by disc centrifuge.

Annex L: Particle shape, Aspect Ratio – Pigmentary and nano titanium dioxide grades

Table 3.1.9.1.A1: Pigmentary Titanium Dioxide physico-chemical data (**from ref.** PS and Surface Property - Pigment Final.xlsx) – Constituent particle sizes determined by SEM expressed by number and by mass, Particle size of agglomerates / aggregates measured by CPS DC expressed by mass and by number, % nano determined by SEM expressed by number and by mass, shape and aspect ratio determined by SEM

		Constituent Particle Size by number ($Feret_{min}$)			Constituent Particle Size by mass (Equivalent Circular Diameter)					Particle Size of Agglomerates /Aggregates by CPS DC by mass		Particle Size of Agglomerates /Aggregates by CPS DC by number	
Product Code	Category	Mean size (SEM) [nm]	Median size (SEM) [nm]	%Nano (SEM)% by number < 100 nm	Mean size (SEM) [nm]	Median size (SEM) [nm]	%Nano (SEM)% by mass < 100 nm	Shape Description	Shape Aspect Ratio[1]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]
RM01	a	126	120	27,2%	159	153	6,0%	Spheroida	1,29	424	364	271	255
RM02	a	147	142	8,7%	179	174	1,4%	Spheroida	1,31	424	380	250	300
RM03	a	212	200	3,1%	303	289	0,0%	Spheroida	1,26	542	517	607	403
RM04	a	138	130	19,2%	180	172	3,3%	Spheroida	1,30	577	528	318	374
RM05	c2	125	121	24,8%	155	149	6,2%	Spheroida	1,25	470	410	262	275
RM06	c2	182	176	8,5%	249	240	0,1%	Spheroida	1,25	690	625	404	412
RM07	c2	128	123	23,8%	159	154	5,1%	Spheroida	1,25	435	347	120	201
RM08	c2	131	126	21,9%	162	157	3,9%	Spheroida	1,28	408	352	235	252
RM19	c2	133	126	22,9%	173	166	4,3%	Spheroida	1,25	458	410	294	277
RM26	a	106	103	45,9%	121	118	22,9%	Spheroida	1,25	812	567	112	166
RM27	c1	108	104	42,2%	122	119	20,5%	Spheroida	1,27	1062	916	308	379
RM28	a	149	144	15,1%	195	187	1,4%	Spheroida	1,30	589	509	326	311
RM29	c1	147	141	17,5%	196	188	1,2%	Spheroida	1,31	777	699	335	439
RM30	b1	143	137	17,3%	185	178	1,8%	Spheroida	1,31	484	431	270	309
RM31	b2	148	143	14,7%	192	185	1,2%	Spheroida	1,30	769	671	299	375
RM32	c2	135	127	22,9%	180	172	4,0%	Spheroida	1,25	361	309	155	204
RM33	c2	146	140	16,5%	191	184	1,2%	Spheroida	1,31	1295	979	179	333
RM34	c2	144	139	19,7%	194	186	1,8%	Spheroida	1,31	443	408	293	320
RM35	c2	145	140	16,2%	188	181	1,6%	Spheroida	1,30	710	653	341	463
RM36	c2	147	142	15,6%	191	184	1,3%	Spheroida	1,31	1058	948	379	450
RM37	b2	375	345	0,0%	533	503	0,0%	Spheroida	1,33	891	838	341	464
RM38	c3	388	357	0,0%	551	521	0,0%	Spheroida	1,33	912	864	574	449
RM39	c3	379	360	0,0%	541	516	0,0%	Spheroida	1,32	919	887	874	550
RM67	a	120	115	30,5%	147	142	9,1%	Spheroida	1,25	511	356	169	208
RM67b	a	125	119	26,8%	155	150	6,4%	Spheroida	1,26	485	402	240	261
RM68	a	197	189	5,9%	275	264	0,0%	Spheroida	1,29	563	540	652	411
RM69	a	131	125	24,7%	170	163	4,0%	Spheroida	1,28	453	374	278	256
RM69b	a	135	131	18,3%	167	162	2,8%	Spheroida	1,30	492	407	229	285
RM70a	c1	120	114	32,0%	150	144	9,1%	Spheroida	1,26	476	330	120	186
RM70b	c1	125	118	27,6%	161	154	7,3%	Spheroida	1,25	457	324	113	176
RM70c	a	118	113	31,9%	142	138	10,4%	Spheroida	1,25	486	389	213	237
RM70d	c1	129	123	24,9%	164	158	5,1%	Spheroida	1,26	796	735	185	293
RM70e	c1	122	116	29,3%	153	147	8,7%	Spheroida	1,25	471	375	194	228
RM70f	c1	135	127	22,9%	180	172	4,0%	Spheroida	1,25	568	467	206	264
RM72a	c1	144	140	15,7%	183	177	1,2%	Spheroida	1,29	540	369	211	227
RM72b	c1	135	129	21,5%	175	168	2,9%	Spheroida	1,28	442	324	102	178
RM72c	a	135	129	21,8%	174	168	3,1%	Spheroida	1,28	442	376	296	269
RM72d	c1	135	131	19,2%	169	164	2,5%	Spheroida	1,30	473	364	209	245
RM72e	c1	135	129	22,6%	174	167	3,0%	Spheroida	1,28	472	348	152	205
RM72f	c1	134	127	22,9%	173	166	3,4%	Spheroida	1,28	453	334	101	186

RM72g	c1	147	144	15,5%	188	182	0,9%	Spheroida	1,29	456	334	179	219
RM72i	c2	135	129	21,9%	175	168	3,2%	Spheroida	1,30	623	437	226	254
RM72j-bis	c2	163	155	13,0%	224	214	0,4%	Spheroidal	1,26	637	535	297	323
RM72k	c1	135	129	21,9%	175	168	3,2%	Spheroida	1,30	623	437	226	254

Table 3.1.9.1.A2: Pigmentary Titanium Dioxide physico-chemical data (**from Ref.** PS TEM tables – Pigment.xls) - Constituent particle sizes determined by TEM expressed by number and by mass, % nano determined by TEM expressed by number and by mass, shape and aspect ratio determined by TEM

		Constituent Particle Size by number ($Feret_{min}$)			**Constituent Particle Size by mass (Equivalent Circular Diameter)**				
Product code	**Category**	**Mean size (TEM) [nm]**	**Median size (TEM) [nm]**	**%Nano (TEM) % by number < 100 nm**	**Mean size (TEM) [nm]**	**Median size (TEM) [nm]**	**%Nano (TEM) % by mass < 100 nm**	**Description**	**Aspect Ratio by TEM**
RM01	a	121	115	35,1%	180	180	3,8%	Spheroidal	1,20
RM02	a	167	163	12,0%	260	263	0,4%	Spheroidal	1,38
RM03	a	194	183	7,4%	311	310	0,2%	Spheroidal	1,22
RM04	a	130	126	27,5%	200	195	2,1%	Spheroidal	1,23
RM05	c2	130	126	27,2%	196	193	2,2%	Spheroidal	1,23
RM06	c2	188	178	9,9%	298	300	0,3%	Spheroidal	1,22
RM07	c2	122	115	33,1%	182	180	3,2%	Spheroidal	1,22
RM08	c2	137	132	24,9%	223	221	1,6%	Spheroidal	1,31
RM19	c2	139	135	21,9%	206	206	1,5%	Spheroidal	1,23
RM26	a	88	85	66,7%	130	131	19,8%	Spheroidal	1,24
RM27	c1	95	93	58,7%	136	136	15,1%	Spheroidal	1,24
RM28	a	187	183	5,5%	309	301	0,1%	Spheroidal	1,46
RM29	c1	181	176	9,6%	289	294	0,2%	Spheroidal	1,40
RM30	b1	169	165	8,0%	286	285	0,2%	Spheroidal	1,49
RM31	b2	162	161	13,1%	264	261	0,5%	Spheroidal	1,38
RM32	c2	170	169	9,5%	290	288	0,2%	Spheroidal	1,48
RM33	c2	175	172	9,2%	291	292	0,2%	Spheroidal	1,41
RM34	c2	173	170	11,1%	313	307	0,2%	Spheroidal	1,49
RM35	c2	164	161	11,5%	276	271	0,4%	Spheroidal	1,42
RM36	c2	160	155	12,2%	267	262	0,4%	Spheroidal	1,42
RM37	b2	332	276	4,1%	897	893	0,0%	Spheroidal	1,55
RM38	c3	376	351	1,0%	798	813	0,0%	Spheroidal	1,50
RM39	c3	427	406	0,0%	742	748	0,0%	Spheroidal	1,42
RM67	a	101	96	53,2%	156	154	10,6%	Spheroidal	1,22
RM67b	a	114	108	40,8%	170	171	5,5%	Spheroidal	1,24
RM68	a	211	210	5,5%	365	371	0,1%	Spheroidal	1,22
RM69	a	119	110	38,9%	211	207	3,5%	Spheroidal	1,34
RM69b	a	145	140	18,1%	228	226	0,8%	Spheroidal	1,41
RM70a	c1	98	94	55,3%	149	149	11,9%	Spheroidal	1,23
RM70b	c1	103	98	51,5%	163	159	9,4%	Spheroidal	1,24
RM70c	a	93	87	63,5%	141	139	17,2%	Spheroidal	1,21
RM70d	c1	116	110	38,7%	174	174	5,0%	Spheroidal	1,27
RM70e	c1	110	106	42,3%	167	162	6,5%	Spheroidal	1,24
RM70f	c1	123	114	33,8%	199	195	3,4%	Spheroidal	1,26
RM72a	c1	165	162	11,7%	254	257	0,3%	Spheroidal	1,41
RM72b	c1	133	128	28,0%	212	214	1,7%	Spheroidal	1,33
RM72c	a	123	114	35,3%	207	206	2,9%	Spheroidal	1,32
RM72d	c1	156	154	14,1%	244	245	0,5%	Spheroidal	1,40
RM72e	c1	132	125	28,8%	217	214	2,0%	Spheroidal	1,30
RM72f	c1	132	127	30,8%	221	216	2,1%	Spheroidal	1,31
RM72g	c1	164	159	12,9%	259	258	0,5%	Spheroidal	1,32

RM72i	c2	158	155	15,5%	250	251	0,6%	Spheroidal	1,39
RM72j-bis	c2	169	163	15,9%	274	279	0,5%	Spheroidal	1,32
RM72k	c1	158	155	15,5%	250	251	0,6%	Spheroidal	1,39

Based on the information provided by Applicants for the pigmentary titanium dioxide grades, the SCCS noted the following points:

i) Shape

The shape of all the constituent particles of the pigmentary titanium dioxide grades (SEM and TEM Observations) are noted to be spheroidal.

ii) Aspect ratio

The aspect ratio values of the pigmentary grades determined by SEM observations are found to range from:
- 1.25 (RM05, RM06, RM07, RM19, RM26, RM32, RM67, RM70b, RM70c, RM70e, RM70f)
- up to 1.33 (RM37, RM38)

The aspect ratio values determined by TEM observations are noted to range from:
- 1.20 (RM01)
- up to 1.55 (RM37)

iii) the Constituent Particle size and % nano (size below 100 nm) of Pigmentary titatium dioxide grades (SEM and TEM observations and measurements)

SEM observations and measurements
The mean constituent particle size of the pigmentary titanium dioxide grades (SEM observations) is noted to range from 108 nm (RM27) to 388 nm (RM38), with the Median constituent size (SEM observations) from 103 nm (RM26) to 360 nm (RM39).
The fraction of the particles (number based) with size below 100 nm (SEM observations) is noted to range from zero (RM37, RM38, RM39) to 45.9% (RM26).

TEM observations and measurements
The mean constituent particle size of the pigmentary titanium dioxide grades (TEM) is noted to range from 88 nm (RM26) to 427 nm (RM39), with the median constituent particle size of the pigmentary titanium dioxide grades (TEM) from 85 nm (RM26) to 406 nm (RM39).
The fraction of the particles (number based) with size below 100 nm (TEM) is noted to range from zero (RM39) to 66.7% (RM26).

Table 3.1.9.2.A3: Summary of the constituent particle sizes (mean and median), % nano (size below 100 nm) determined by SEM and TEM observations.

Pigmentary grades constituent Particles	Mean size Particle size	Median Size Particle size	% nano
SEM	108 - 388 nm	103 - 360 nm	0.0 - 45.9%
TEM	88 - 427 nm	85 - 406 nm	0.0 - 66.7%

Agglomerates / Aggregates sizes of Pigmentary grades measured by CPS DC
The mean size of Agglomerates / Aggregates by mass of the pigmentary titanium dioxide grades is found to range from 408 nm (RM08 – category c2) to 1295 nm (RM33 – category c2).
The median size of Agglomerates / Aggregates by mass is found to range from 309 nm (RM32 – category c2) to 979 nm (RM33 – category c2)

The mean size of Agglomerates / Aggregates by number of the pigmentary titanium dioxide grades is found to range from 101 (RM72f – category c1) to 874 (RM39 – category c3))
The median size of Agglomerates / Aggregates by number is found to range from 166 nm (RM26 – category a) up to 550 nm (RM39 – category c3)

Table 3.1.9.2.A4: Summary of the agglomerate / aggregate sizes of the Titanium dioxide pigmentary grades (mass and number based)

Pigmentary grades Agglomerates / Aggregates	Mean size (Mass based)	Median Size (Mass based)	Mean size (Number based)	Median Size (Number based)
CPS DC	408 – 1295 nm	309 – 979 nm	101 – 874 nm	166 – 550 nm

Table 3.1.9.1.B1: Nano Titanium dioxide grades: Size of Constituent Particle, Shape, Aspect ratio, Size of Agglomerates/Aggregates (from **Ref.:** PS and Surface Property - Nano Final.xlsx and from Ref. PS and Surface Property - Nano (corrected).xlsx -30 June 2023 and Att.2- Nano TEM size and AR data.xls (February 2024)) : Constituent particle sizes (mean and median ones) determined by TEM expressed by number and by mass, shape, aspect ratio and % AR > 3 (number based) determined by TEM, particle size of agglomerates / aggregates measured by CPS DC expressed by mass and by number.

	Constituent Particle Size by number ($Feret_{min}$)		Constituent Particle Size by mass (Equivalent Circular Diameter)		Shape			Particle Size of Agglomerates /Aggregates by number (CPS DC)		Particle Size of Agglomerates /Aggregates by mass (CPS DC)	
Product Code (nano)	Mean size (TEM) [nm]	Median size (TEM) [nm]	Mean size (TEM) [nm]	Median size (TEM) [nm]	Description	Aspect Ratio[1]	% AR > 3	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]
RM09	26	25	40	40	Spheroidal	1.6	0.4	58	53	238	96
RM10	18	17	36	36	Lanceolate	2.7	31.8	56	52	137	76
RM11	21	20	41	37	Spheroidal	1.6	1.8	60	55	296	116
RM40	10	10	24	24	Lanceolate	3.2	51.7	56	50	428	116
RM41	10	9	26	25	Lanceolate	3.6	62.4	47	44	604	414
RM42	10	9	23	23	Lanceolate	3.3	57.3	85	75	921	632
RM43	11	11	23	23	Lanceolate	3.0	42.3	48	44	685	532
RM44	12	11	25	25	Lanceolate	3.3	56.0	99	72	1000	717
RM45	13	13	33	32	Lanceolate	3.3	55.6	46	43	537	66
RM46	12	12	30	30	Lanceolate	3.6	69.6	54	48	1074	823
RM47	29	27	63	60	Lanceolate	2.8	34.2	66	56	227	180
RM48	20	18	55	49	Lanceolate	3.1	45.6	50	47	438	69
RM49	21	20	51	47	Lanceolate	3.1	48.2	56	51	207	80
RM51	17	16	53	43	Lanceolate	2.6	28.5	53	48	693	555
RM52	16	15	44	42	Lanceolate	2.8	36.0	49	45	372	146
RM53	25	23	56	49	Lanceolate	2.4	20.4	54	48	287	179
RM55	29	27	50	48	Spheroidal	1.8	6.2	74	64	1156	805
RM56	35	34	59	58	Spheroidal	1.6	1.5	77	70	348	130
RM57	31	30	51	50	Spheroidal	1.8	3.9	65	60	985	417
RM58	34	33	60	57	Spheroidal	1.5	2.4	76	71	423	114
RM59	46	44	81	78	Spheroidal	1.5	0.6	102	94	302	166
RM60	55	53	90	88	Spheroidal	1.4	1.0	112	109	187	162
RM61	51	48	90	87	Spheroidal	1.5	2.7	125	121	206	191
RM62	86	81	145	135	Spheroidal	1.4	1.8	168	162	337	262
RM63	14	13	34	33	Lanceolate	3.5	56.8	47	43	218	67

RM64	27	26	43	42	Spheroidal	1.6	1.1	68	63	612	113
RM65	28	28	49	48	Spheroidal	1.5	0.9	77	72	362	111
RM74a	34	33	56	54	Spheroidal	1.6	2.5	75	65	457	165
RM74b	33	32	61	57	Spheroidal	1.7	4.7	66	60	488	137
RM74c	35	34	60	56	Spheroidal	1.5	2.2	67	64	256	89
RM74d	26	25	51	48	Spheroidal	1.5	1.7	63	61	118	77
RM74e	28	27	57	53	Spheroidal	1.6	2.0	70	65	535	125
RM75	14	13	36	36	Lanceolate	4.4	78.8	47	45	314	59
RM76	20	20	45	45	Lanceolate	3.7	75.7	50	47	626	394
RM77	10	10	28	28	Lanceolate	4.2	69.5	50	47	198	62
RM78	27	26	45	45	Spheroidal	2.1	14.7	64	58	212	117
RM79	21	20	39	39	Lanceolate	2.8	36.8	63	58	265	112
RM80	17	17	39	39	Lanceolate	3.8	75.1	47	45	151	59
RM81	38	36	62	60	Spheroidal	1.7	2.8	71	65	678	152
RM82	22	21	39	39	Spheroidal	1.7	3.3	58	52	170	94

(1) Aspect ratio based on Equivalent Circular Diameter measurements by TEM

Based on the information provided by Applicants, the SCCS noted for the nano titanium dioxide grades that

i) **Shape**

The shapes of the constituent particles are
- spheroidal (RM09, RM11, RM55, RM56, RM57, RM58, RM59, RM60, RM61, RM62, RM64, RM65, RM74a, RM74b, RM74c, RM74d, RM74e, RM78, RM81, RM82)
- lanceolate (RM10, RM40, RM41, RM42, RM43, RM44, RM45, RM46, RM47, RM48, RM49, RM51, RM52, RM53, RM63, RM75, RM76, RM77, RM79, RM80)

ii) **Aspect ratio**

The aspect ratio values of the nano grades are noted to range:
- from 1.4 (RM60, RM62)
- up to 4.4. (RM75)

The % of particles (number based, determined by TEM) with an aspect ratio over than 3 is ranging from 0.4 (RM09) up to 78.8% (RM75)

iii) **Constituent particle sizes** (by number) of the nano titanium dioxide grades (TEM),

The mean constituent size (TEM) is ranging from 10 nm (RM40, RM41, RM42, RM 77) to 86 nm (RM62), with a median constituent size (TEM) from 9 nm (RM41, RM42) to 81 nm (RM62).

Table 3.1.9.2.B2: Summary of the constituent particle sizes (mean and median) for nano titanium dioxide grades (TEM observations and measurements)

Nano grades Constituent Particles	Mean size Particle size (by number)	Median Size Particle size (by number)
TEM	10 – 86 nm	9 – 81 nm

iv) agglomerates/aggregates by number

- The mean size of agglomerates/aggregates by number (CPS DC measurements) is found to range from 46 nm (RM45) to 168 nm (RM62), with Median size of agglomerates/ aggregates by number from 43 nm (RM45, RM63) up to 162 nm (RM62)
- The mean size of agglomerates/aggregates by mass (CPS DC measurements) is found to range from 118 nm (RM74d) to 1156 nm (RM55), with the median size of agglomerates/aggregates from 59 nm (RM80) to 823 nm (RM46).

Table 3.1.9.2.B3: Summary of the mean and the median ranges of agglomerates / aggregates of the nano titanium dioxide grades determined by CPS DC.

Mean size (number)	**Median size** (number)	**Mean size** (mass)	**Median size** (mass)
46 – 168 nm	43 - 162 nm	118 - 1156 nm	59 – 832 nm

Annex M: Aerodynamic diameter – Pigmentary and nano titanium dioxide grades

From Applicants

Rotating drum method as per DIN 55992-1:2006 ("Determination of a parameter for the dust formation of pigments and extenders - Part 1: Rotation method") or ISO EN 15051-2:2013.

From **Ref.**: CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Pigmentary grades

Table 3.1.9.3.A: Aerodynamic diameter **(%<10 µm)** as a function of the pigmentary titanium dioxide grades

Product Code	Aerodynamic diameter (%<10 µm)i	Product Code	Aerodynamic diameter (%<10 µm)i	Product Code	Aerodynamic diameter (%<10 µm)i
RM01	0.0037	RM32	0	RM70c	<1
RM02	0.0013	RM33	0.001	RM70d	<1
RM03	0	RM34	0.001	RM70e	<1
RM04	0	RM35	0.002	RM70f	<1
RM05	0	RM36	0.001	RM72a	<1
RM06	0.002	RM37	0.002	RM72b	<1
RM07	0	RM38	0.001	RM72c	<1
RM08	0	RM39	0.005	RM72d	<1
RM19	<0.001/<0.0002	RM67	<1	RM72e	<1
RM26	0	RM67b	<1	RM72f	<1
RM27	0.001	RM68	<1	RM72g	<1
RM28	0.001	RM69	<1	RM72i	<1
RM29	0.001	RM69b	<1	RM72j-bis	<1
RM30	0	RM70a	<1	RM72k	<1
RM31	0.002	RM70b	<1		

i. Aerodynamic diameter (%<10 µm) - Method: ISO EN 15051-2 / EN 15051-3

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
Table from Page 10/28, Column N14) Aerodynamic diameter (%<10 µm)

Nano grades

Table 3.1.9.3.B: Aerodynamic diameter **(%<10 µm)** as a function of the nano titanium dioxide grades

Product Code	Aerodynamic diameter (%< 10 µm)	Product Code	Aerodynamic diameter (%< 10 µm)	Product Code	Aerodynamic diameter (%< 10 µm)
RM09	0.00735	RM52	0.239	RM74a	<1
RM10	0.016	RM53	0.009	RM74b	<1
RM11	0.012	RM55	0.006	RM74c	<1
RM40	0	RM56	0.011	RM74d	<1
RM41	0.048	RM57	0.022	RM74e	<1
RM42	0.016	RM58	0.015	RM75	0.0470
RM43	0.122	RM59	0.006	RM76	0.0080
RM44	0.089	RM60	0.002	RM77	0.0470
RM45	0.016	RM61	0.005	RM78	0.0000
RM46	0.026	RM62	0.006	RM79	0.0000
RM47	0.038	RM63	<1	RM80	<1
RM48	0.012	RM64	<1	RM81	0.0000
RM49	0.051	RM65	<1	RM82	≤1
RM51	0.024				

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
From Table on Page 18/28 – Column 14) Aerodynamic diameter (%< 10 µm)

Based on the information provided by Applicants (Tables 3.1.9.3.A and 3.1.9.3.B), the SCCS noted that for:

Pigmentary grades
The 7 pigmentary titanium dioxide grades with 0% of particles with aerodynamic diameter below 10 µm are the following: RM03, RM04, RM05, RM07, RM08, RM30, RM32.
The other 37 pigmentary titanium dioxide grades are noted to exhibit a fraction of particles with aerodynamic diameter below 10 µm, less than 1%.

Nano grades:
The 4 (four) nano titanium dioxide grades with 0% of particles with aerodynamic diameter below 10 µm are the following: RM40, RM78, RM79, RM81.
The other 36 nano titanium dioxide grades are noted to exhibit a fraction of particles with aerodynamic diameter below 10 µm less than 1%.

Annex N: Specific Surface Area (SSA) and Volume Specific Surface Area (VSSA) – Pigmentary and nano titanium dioxide grades

From Applicants

Specific Surface Area measurement is performed according to protocols inspired by the standard NF ISO 9227 on the following points:
- Measurement method: volumetric method
- Exploitation of measurement data: Multipoint determination (5 points) in the relative pressure range where the BET equation is valid, either between 0.05 and 0.3.
Sample Degassing: under vacuum
- Time: about 16 hours
- Temperature: ambient (optionally then additional 1 hour at 180°C)
Analysis gas: Nitrogen
Tolerance on the relative pressure, P/Po: 0.245 %
5 mm Hg
Po (saturation pressure) measurement interval: 90-120 min.

From Ref.: CE-TiO2-23-003.0 - Att 1_Generic Description of Analytical Methods – final.pdf

Pigmentary titanium dioxide grades

Table 3.1.9.4. A: Specific surface Area and Volume-specific Surface Area (VSSA) as a function of the pigmentary titanium dioxide grades.

Product Code	Specific Surface Area (BET, m^2/g)	VSSA ($m^2.cm^3$)	Product Code	Specific Surface Area (BET, m^2/g)	VSSA ($m^2.cm^3$)	Product Code	Specific Surface Area (BET, m^2/g)	VSSA ($m^2.cm^3$)
RM01	9.6	36.5	RM32	5.9	22	RM70c	15.5	61.9
RM02	6.6	27.7	RM33	3	11	RM70d	9.7	33.7
RM03	6.7	26	RM34	6	22	RM70e	9.9	39.3
RM04	9.6	37	RM35	4.3	17	RM70f	8.5	31.8
RM05	10.2	39	RM36	3.4	13	RM72a	6.6	27.3
RM06	6.8	26	RM37	8	34	RM72b	6.6	27.5
RM07	10.1	38	RM38	2	8	RM72c	15.8	68.4
RM08	10.1	41	RM39	2	8	RM72d	4	14.7
RM19	8.6	34.5	RM67	9.5	37.1	RM72e	4.9	18.9
RM26	12	46	RM67b	9.1	36.4	RM72f	7.2	30.6
RM27	8.6	31	RM68	6.5	26	RM72g	6.2	24.4
RM28	7	30	RM69	6.3	27.9	RM72i	15.5	66.7
RM29	5	21	RM69b	8.5	36.6	RM72j-bis	5.3	19.9
RM30	9.4	40	RM70a	6.5	24.9	RM72k	5.8	18.9
RM31	12.8	52	RM70b	6.5	24.9			

Ref.: January 2023_PhysChem data on Cosmetics TiO2 grades_final.pdf
from Table on Page 9/28
Columns N8.1) Specific Surface Area (BET, m^2/g),
N8.2) VSSA ($m^2.cm^3$)

Nano Titanium dioxide Grades

Table 3.1.9.4. B: Specific surface Area and Volume-Specific Surface Area as a function of the nano titanium dioxide grades.

Product Code	Specific Surface Area (BET, m^2/g)	VSSA ($m^2.cm^3$)	Product Code	Specific Surface Area (BET, m^2/g)	VSSA ($m^2.cm^3$)	Product Code	Specific Surface Area (BET, m^2/g)	VSSA ($m^2.cm^3$)
RM09	60.7	222	RM52	68	234	RM75	99.2	340
RM10	63.4	198	RM53	35	100	RM78	63.8	215
RM11	48.6	178	RM55	48	193	RM80	117	402
RM40	75	189	RM56	32	99	RM81	8.0	34
RM41	110	406	RM57	32	119	RM76	63.6	183
RM42	72	206	RM58	27	102	RM79	45.1	140
RM43	87	287	RM59	50	205	RM74a	27.86*	78*
RM44	33	83	RM60	15	56	RM74b	17.11*	53*
RM45	110	375	RM61	15	63	RM74c	29.59*	112.4*
RM46	51	189	RM62	10	37	RM74d	60.84*	255.4*
RM47	57	200	RM63	107.5	300	RM74e	39.26*	137.4*
RM48	62	198	RM64	36.2	122	RM77	84	269
RM49	48	142	RM65	31.1	110	RM82	50	213
RM51	68	207						

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf - Table from Page 16/28,
Columns N8.1) Specific Surface Area (BET, m^2/g) and N8.2) VSSA ($m^2.cm^3$)
(*) BET and VSSA values corrected from initial file according to **Ref** Update of Data TiO2 SCCS Dossier - Sensient to EU Commission_20230202.pdf

Based on the data provided for Specific Surface Area (SSA) and Volumic Specific Surface Area (VSSA), the SCCS noted that for:

Pigmentary grades

- the SSA of the pigmentary titanium dioxide grades ranges from 2 m^2/g (RM38, RM39) up to 15.8 m^2/g (RM72c),
- the VSSA of the pigmentary titanium dioxide grades ranges from 8 $m^2.cm^3$ (RM38, RM39) up to 68.4 $m^2.cm^3$ (RM72c).

Nano grades

- the SSA of the nano titanium dioxide grades ranges from 8 m^2/g (RM81) up to 117 m^2/g (RM80),
- the VSSA of the nano titanium dioxide grades ranges from 34 $m^2.cm^3$ (RM81) up to 402 $m^2.cm^3$ (RM80).

	Specific Surface Area (BET, m^2/g)	Volume -Specific Surface Area ($m^2.cm^3$)
Pigmentary Titanium dioxide Grades	2 - 15.8	8 - 68.4
Nano Titanium dioxide Grades	8 - 117	34 - 402

Annex O: Surface Components / Surface reactivity – Pigmentary and Nano Titanium dioxide grades

From Applicants:
The identity of the surface components and functional groups are not measured but inferred from a knowledge of the chemical moieties that have been used to treat the surface. All surface treatments are cosmetic ingredients that are widely used in cosmetic formulations. Some of the surface species could be determined by methods such as infra-red spectroscopy
From **Ref.:** CE response to SCCS Request of 13 June 2023_29062023.pdf

Pigmentary titanium dioxide grades

Table 3.1.9.5.A: Surface components / Surface reactivity as a function of the pigmentary titanium dioxide grades

Product Code	Surface components, functional groups	Reactive sites / Surface Reactivity	Product Code	Surface components, functional groups	Reactive sites / Surface Reactivity	Product Code	Surface components, functional groups	Reactive sites / Surface Reactivity
RM01	-OH; -PO42-	-OH; -PO42- / low	RM32	Carboxyl group, Hydroxyl group	-OH; / low	RM70 c	-OH; -PO42-	-OH; -PO42- / low
RM02	-OH; -PO42-	-OH; -PO42- / low	RM33	Alkyl chain, Carboxyl group	None/low	RM70 d	Rosa Centifolia Flower Wax, Rosa Damascena Flower Cera, Cera Alba	None/low
RM03	-OH; -PO42-	-OH; -PO42- / low	RM34	Carboxyl group, Amino group	None/low	RM70 e	Sodium Glycerophosphate	-OH; -PO42- / low
RM04	-OH; -PO42-	-OH; -PO42- / low	RM35	Methyl group	None/low	RM70 f	Hydrogenated Lecithin	None
RM05	-OH; -(C3H5(OH)3); -PO42-	-OH; -PO42-/ low	RM36	Methyl group	None/low	RM72 a	Caprylylsilane	None/low
RM06	-OH; -PO42-	-OH; -PO42-/ low	RM37	Hydroxyl group	-OH / low	RM72 b	Caprylylsilane	None/low
RM07	-C8H17	None/low	RM38	Alkyl chain, Carboxyl group	None/low	RM72 c	-OH; -PO42-	-OH; -PO42- / low
RM08	-OH; -(C3H5(OH)3); -PO42-	-OH; -PO42-/ low	RM39	Methyl group	None/low	RM72 d	Persea Gratissima (Avocado) Oil, Hydrogenated Vegetable Oil, Tocopherol	None/low
RM19	-OH; -(C3H5(OH)3)	-OH / low	RM67	-OH; -PO42-	-OH; -PO42- / low	RM72 e	Bis-PEG-15 Dimethicone/ IPDI Copolymer, PEG-2-Soyamine, Isopropyl Titanium Triisostearate	None/low
RM26	-OH	-OH/ low	RM67 b	-OH; -PO42-	-OH; -PO42- / low	RM72 f	Phytic Acid, Hydroxyl group	-OH / low
RM27	Methyl group	None/low	RM68	-OH; -PO42-	-OH; -PO42- / low	RM72 g	Sodium Cocoyl Glutamate, Cystine, Lauric Acid, Arginine	None/low
RM28	-OH	-OH/ low	RM69	-OH; -PO42-	-OH; -PO42- / low	RM72 i	Hydroxyl group	-OH / low
RM29	Methyl group	None/low	RM69 b	-OH; -PO42-	-OH; -PO42- / low	RM72 j-bis	Hydroxyl, Caprylylsilane	-OH / low
RM30	Hydroxyl group	-OH / low	RM70 a	Caprylylsilane	None/low	RM72 k	Cocos Nucifera (Coconut) Oil, Aloe	None/low

							Barbadensis Leaf Extract	
RM31	Hydroxyl group	-OH / low	RM70 b	Caprylylsilane	None/low			

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
from Table on Page 9/28
Columns N9.1) Surface components, functional groups
N9.3) Reactive sites / Surface Reactivity

Nano titanium dioxide grades

Table 3.1.9.5.B: Surface components / Surface reactivity as a function of the nano titanium dioxide grades

Product Code	Surface components, functional groups	Reactive sites/ Surface reactivity	Product Code	Surface components, functional groups	Reactive sites/ Surface reactivity	Product Code	Surface components, functional groups	Reactive sites/ Surface reactivity
RM09	-OH	-OH / low	RM52	Methyl group	none / low	RM74 a	Methyl group	none / low
RM10	Methyl group	none / low	RM53	Alkyl chain, Carboxyl group	none / low	RM74 b	Alkyl chain, Carboxyl group	none / low
RM11	Methyl group	none / low	RM55	Hydroxyl group	-OH / low	RM74 c	Caprylylsilane group	none / low
RM40	Alkyl chain, Carboxyl group	none / low	RM56	Alkyl chain, Carboxyl group	none / low	RM74 d	Hydroxyl group	-OH / low
RM41	Hydroxyl group	-OH / low	RM57	Methyl group	none / low	RM74 e	Methyl group	none / low
RM42	Alkyl chain, Carboxyl group	none / low	RM58	Methyl group	none / low	RM75	Methyl group, -OH	-OH / low
RM43	Methyl group	none / low	RM59	Hydroxyl group	-OH / low	RM76	Alkyl chain, Carboxyl group	none / low
RM44	Methyl group	none / low	RM60	Alkyl chain, Carboxyl group	none / low	RM77	-OH	none / low
RM45	Hydroxyl group	-OH / low	RM61	Methyl group	none / low	RM78	-OH	-OH / low
RM46	Hydroxyl group	-OH / low	RM62	Alkyl chain, Carboxyl group	none / low	RM79	Cetyl-group	none / low
RM47	Hydroxyl group	-OH / low	RM63	Alkyl chain, Carboxyl group	none / low	RM80	-OH	-OH / low
RM48	Alkyl chain, Carboxyl group	none / low	RM64	Alkyl chain, Carboxyl group	none / low	RM81	Hydroxyl group	-OH / low
RM49	Alkyl chain, Carboxyl group	none / low	RM65	Alkyl chain, Carboxyl group	none / low	RM82	Methyl group	none / low
RM51	Methyl group	none / low						

Ref.: January 2023_PhysChem data on Cosmetics TiO_2 grades_final.pdf
From Table on Page 17/28
Columns N9.1) Surface components, functional groups
N9.3) Reactive sites/ Surface reactivity

Annex P: Homogeneity and Stability – Pigmentary and nano titanium dioxide grades

From Applicants
The coating materials are applied to the surface to improve particle dispersion, inhibit or abolish photoactivity and improve compatibility with other ingredients present in sunscreen formulations. The coating materials are not UV absorbers and all these materials are common cosmetic ingredients which are widely used for different purposes in cosmetic products.

Stability of the coating on the particle is important for the technical properties of TiO2-containing formulas (stability of emulsion, colour, segregation of particles).
Complete stability of coating materials on the TiO_2 particle has been demonstrated with variation in pH, temperature, shear force and time (up to 180 days) in studies previously submitted to the SCCS in 1998 (references 62, 63), in 1999 (references 68 and 72), 2000 (reference 96), 2009 (references 113 and 116) and 2014.

Hence it can be concluded that the coatings are stable under the conditions and timespan of the *in vitro* tests performed.

Ref.: CE-TiO2-23-003.0 - CE Response to clarifications requested by SCCS 10 03 23 - final

Reference 62
The object of the investigations was the emulsion 408.259 placed at disposal by L'Oreal containing 5% of coated titanium dioxide UV-Titan M 160, produced by Kemira. This product contains 7% alumina (Al2O3) and 10% stearic acid (CH3(CH2)16COOH) as coating materials. The constituent particle size is ranging from 7 to 10 nm, with an aspect ratio ~ 4

Ref.: Reference 62: Investigations of coated Titanium Dioxide – Final Report – Berlin, May 1997 and Att.1 -CE TiO2 – Response and Comments – 06.02.2024.pdf (February 2024)

Reference 63
Summary
The mechanical stability of aluminium oxide coating on the titanium dioxide particles was characterised by the ratio of the aluminium and titanium concentration in different samples. The method of laser induced plasma spectroscopy is suited for the determination of the Ti/Al ratio in liquid and solid samples. This method was used to determine the relative titanium / aluminium ratio in the investigated sunscreen systems.
As expected, the Al/Ti ratio is constant comparing the titanium dioxide dispersion Tioviel AQ-N, lot PRAQN 0051 with the sunscreen emulsion containing the Titanium dioxide AQ-N, lot 40.280. The Ti/Al ratio was found to be unchanged in different tapes strips taken after application of the sunscreen emulsion, 403.280. Instabilities of the alumina coating could not be detected, when the sunscreen components were handled under real conditions.
The product contains 6% silica, 16% alumina as coating materials. The constituent particle size is equal to 15nm, with an aspect ratio ~4.

Ref.: Reference 63 – Investigation of Alumina/silica coated titanium dioxide particles – TIOVEiL AQ-N (Tioxide Specialities LTD) – Final Report – Berlin, November 1997 and Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024)

Reference 68

Category A Samples.

Treatment	Company 1 % Al_2O_3/TiO_2	Company 2* % Al_2O_3/TiO_2	Company 3 % Al_2O_3/TiO_2	Company 3 % SiO_2/TiO_2	Company 4 % Al_2O_3/TiO_2	Company 4 % SiO_2/TiO_2
None	11.6	7.0	16.7	7.3	4.6	17.3
pH 5	11.1	7.0	16.8	7.4	4.6	17.1
pH 7	11.5	7.0	16.7	7.3	4.6	17.7
pH 9	11.3	7.0	16.5	7.4	4.6	17.3
Dispersion Shear	11.3	7.0	16.7	7.4**	4.6	17.4
80°C ,1 hour	11.4	7.0	16.4	7,4	4.6	17.4

* Measured on 5% (w/v) samples.
** 30 minutes only

Category B Samples

Treatment	Company 1 Type 1 %C	Company 1 Type 2 %C
None	2.80	3.70
pH 5	2.80	3.70
pH 7	2.81	3.68
pH 9	2.80	3.70
Dispersion Shear	2.80	3.70
80°C ,1 hour		

Ref. 68: Stability test for coatings applied to ultra-fine, cosmetic grade, titanium dioxide 1999 (Stability_Al2O3_TiO2, SiO2_TiO2, C_TiO2)

SCCS comments on Reference 68

No indication has been provided on the size of the Titanium core particles.

Reference 72

Ref. 72: Coating of Titanium Dioxide, H. Driller, 1999(Stability_Al2O3_TiO2, SiO2_TiO2, C_TiO2)

SCCS comments on Reference 72

Same results as Reference 68: No indication has been provided on the sizes of the Titanium core particles.

Reference 96

TYPICAL RESULTS

Test Material:	PSMA 2	PSMA 3	PSMA 4[1]	PSMA 5[2]	PSMA 6
Coating:	AL_2O_3	$AL_2O_3 : SiO_2$	AL_2O_3	AL_2O_3	$AL_2O_3 : SiO_2$
Treatment:					
None	11.6%	4.6% : 17.3%	7.0%	6.8%	16.7% : 7.3%
pH5	11.1%	4.6% : 17.1%	7.0%	6.8%	16.8% : 7.4%
pH7	11.5%	4.6% : 17.7%	7.0%	6.8%	16.7% : 7.3%
pH9	11.3%	4.6% : 17.3%	7.0%	6.9%	16.5% : 7.4%
Dispersion Shear	11.3%	4.6% : 17.4%	7.0%	6.9%	16.7% : 7.4%[3]
Temperature (80°C, 1 hour)	11.4%	4.6% : 17.4%	7.0%	--[2]	16.4% : 7.4%

Ref. 96: Stability test for surface treatments applied to fine particle, 2000(Stability-Al2O3_TiO2, Al2O3-SiO2_TiO2)

SCCS comments on Reference 96

No indication has been provided on the size of the Titanium core particles or on the thickness (or the composition) of the coatings. The stability of some specific coatings has been studied (Al_2O_3, Al_2O_3-SiO_2).

Reference 113

The stabilities of hydrophobic, Al_2O_3 coated grade and hydrophilic Al_2O_3-Glycerin coated grade have been studied.

HYDROPHOBIC, Al_2O_3 COATED GRADE

1. XRF-analysis

5 % suspension in water/methanol (20:80), parallel suspensions
After the treatment the suspension was centrifuged 10 min (G-value 6000).
The sediment was dried at 105 °C over night (constant weight) and ignited at 900 °C to constant weight.

Al_2O_3 (%) and TiO_2 (%) contents were measured with XRF.

	1st suspension			2nd suspension		
	Al_2O_3 (%)	TiO_2 (%)	Al_2O_3 / TiO_2	Al_2O_3 (%)	TiO_2 (%)	Al_2O_3 / TiO_2
Original suspension before centrifugation	6.2	81.6	0.08	6.2	81.8	0.08
after centrifugation	6.1	81.7	0.07	6.2	81.8	0.08
Mixed 60 min at 70 °C	6.1	81.6	0.07	6.2	81.8	0.08
Mixed 10 min/10 000 rpm with Ultra Turrax	6.1	81.8	0.07	6.2	81.8	0.08
Mixed 2 h at pH 5	6.1	81.6	0.07	6.1	81.8	0.07
Mixed 2 h at pH 7	6.1	81.7	0.07	6.2	81.8	0.08
Mixed 2 h at pH 9	6.2	82.9	0.07	6.2	82.3	0.08

Deviation of the test method: Al_2O_3 ± 0.1 % and TiO_2 ± 0.3 %

2. Carbon-analysis

5 % suspension in water/methanol (20:80), parallel suspensions
After the treatment the suspension was centrifuged 10 min (G-value 6000).
The sediment was dried at 105 °C over night (constant weight).

C (%) content was measured with Leco carbon analyser.

	1st suspension C (%)	2nd suspension C (%)
Original suspension before centrifugation	6.1	5.9
after centrifugation	6.1	6.0
Mixed 60 min at 70 °C	6.1	6.1
Mixed 10 min/10 000 rpm with Ultra Turrax	6.0	6.1
Mixed 2 h at pH 5	5.9	6.1
Mixed 2 h at pH 7	6.1	6.1
Mixed 2 h at pH 9	5.0	5.8

Deviation of the test method: C (%) ± 0.2

HYDROPHILIC, Al_2O_3-GLYCERIN COATED GRADE

1. XRF-analysis

5 % suspension in water, parallel suspensions
After the treatment the suspension was centrifuged 10 min (G-value 6000).
The sediment was dried at 105 °C over night (constant weight) and ignited at 900 °C to constant weight.

Al_2O_3 (%) and TiO_2 (%) contents were measured with XRF.

	1st suspension			2nd suspension		
	Al_2O_3 (%)	TiO_2 (%)	TiO_2/ Al_2O_3	Al_2O_3 (%)	TiO_2 (%)	TiO_2/ Al_2O_3
Original suspension before centrifugation	6.3	89.5	14.2	6.2	89.4	14.4
after centrifugation	6.3	89.6	14.2	6.2	89.4	14.4
Mixed 60 min at 70 °C	6.1	89.6	14.7	6.1	89.4	14.7
Mixed 10 min/10 000 rpm with Ultra Turrax	6.2	89.6	14.5	6.2	89.5	14.4
Mixed 2 h at pH 5	6.1	89.4	14.7	6.2	89.4	14.4
Mixed 2 h at pH 7	6.2	89.3	14.4	6.3	89.6	14.2
Mixed 2 h at pH 9	6.2	89.4	14.4	6.2	89.6	14.5

Deviation of the test method: Al_2O_3 ± 0.1 % and TiO_2 ± 0.3 %

2. Carbon-analysis

5 % suspension in water
After the treatment the suspension was centrifuged 10 min (G-value 6000).
The sediment was dried at 105 °C over night (constant weight).

C (%) content was measured with Leco carbon analyser.

	suspension C (%)
Original suspension before centrifugation	0.4
after centrifugation	0.2
Mixed 30 min at 80 °C	0.2
Mixed 10 min/10 000 rpm with Ultra Turrax	0.2
Mixed 2 h at pH 5	0.4
Mixed 2 h at pH 7	0.3
Mixed 2 h at pH 9	0.2

Deviation of the test method: C (%) ± 0.2

Both grades are containing 6% alumina. The grades are coated with 1% glycerin and 3% dimethicone respectively. The constituent particle size is equal to 20nm with an aspect ratio ~1.7.

Ref. 113: Stability studies for coatings of ultrafine titanium dioxide products, 2009 and Att.1 -CE TiO2 - Response and Comments - 06.02.2024.pdf (February 2024)

SCCS comments of Ref. 113

Two specific coatings on TiO_2 have been studied (Al_2O_3 and Al_2O_3-Glycerin).

Annex Q: Dispersibility – Pigmentary and nano titanium dioxide grades

Dispersibility of Pigmentary grades

Table 3.1.11.A1: Particle size by the so-called by Applicants "modified SCCS dispersibility method" (from Ref.: Dispersibility – Pigmentary.xlx, Third data package - 31 March 2023)

		Particle Size by so-called modified SCCS Dispersibility method				Initial particle size (from Table Table 3.1.9.1.A1)			
Product Code	Category	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]
		by number		by mass		by number		by mass	
RM01	a	204	195	343	298	271	255	424	364
RM30	b1	234	225	332	322	270	309	484	431
RM31	b2	309	297	472	446	299	375	769	671
RM70a	c1	210	202	388	321	120	186	476	330
RM05	c2	206	196	339	292	262	275	470	410
RM39	c3	549	545	789	775	874	550	919	887

Ref.: Dispersibility – Pigmentary.xlx, Third data package - 31 March 2023

Table 3.1.11.A2: Particle size by modified NanoGenotox method (from Ref.: Dispersibility Nanogenotox – Pigment.xlx: Fourth data package, 21 April 2023)

		Particle Size by Nanogenotox Dispersibiity method				Initial particle size (from Table Table 3.1.9.1.A1)			
Product Code	Category	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]
		by number		by mass		by number		by mass	
RM01	a	221	215	345	316	271	255	424	364
RM30	b1	243	244	368	358	270	309	484	431
RM31	b2	361	339	631	563	299	375	769	671
RM70a	c1	291	264	605	484	120	186	476	330
RM05	c2	218	214	365	319	262	275	470	410
RM39	c3	522	543	863	824	874	550	919	887

Ref.: Dispersibility Nanogenotox – Pigment.xlx: Fourth data package, 21 April 2023)

Comparaison of the particle size after dispersion using the Nanogenotox protocol and the Modified SCCS protocol (From Ref.: Dispersibility Nanogenotox – Report, 4th Data Package, 21 April 2023)

Table 3.1.11.A3 compares the particle sizes of TiO_2 cosmetics grades dispersed using the Nanogenotox protocol and the Modified SCCS protocol (described in the March submission) to establish the effect of dispersion energy and measured using CPS DC.

Table 3.1.11.A3: Comparison of Secondary Particle Size after Different Dispersion Protocols (measured by CPS DC) for Representative Titanium Dioxide pigments (From Ref. Dispersibility Nanogenotox – Report.pdf - 4th data package, 21 April 2023).

Product Code	Particle Size by Modified SCCS Dispersibility protocol		Particle Size by Nanogenotox Dispersibility protocol	
	Median size [nm]	Median size [nm]	Median size [nm]	Median size [nm]
	by number	by mass	by number	by mass
RM01	195	298	215	316
RM30	225	322	244	358
RM31	297	446	339	563
RM70a	202	321	264	484
RM05	196	292	214	319
RM39	545	775	543	824

The median sizes derived using the Nanogenotox protocol are around 10% larger than those obtained using the modified SCCS protocol (difference is even larger for the hydrophobic grade RM70a).

Ref.: Dispersibility Nanogenotox – Report.pdf - 4th data package, 21 April 2023

Dispersibility of Nano grades

The histograms for particle size (agglomerate / aggregates particles) (both by number and mass) determined using the modified SCCS method have been provided. The particle size data provided by Applicants have been reported in Table 3.1.11.B1 and Table 3.1.11.B2 for the modified SCCS dispersibility method and the Nanogenotox dispersibility protocol, respectively.

Table 3.1.11.B1: Particle size by modified SCCS dispersibility method (from Ref.: Dispersibility – Nano.xlx - Third data package 31 March 2023 and Dispersibility - Nano (corrected).xlx – 30 June 2023)

Product Code	Particle Size by so-called by Applicants "Modified SCCS Dispersibility method"				Initial Particle Size extracted from Table 3.1.9.1.B1			
	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]
	by number		by mass		by number		by mass	
RM09	58	53	238	96	58	53	238	96
RM11	60	55	296	116	60	55	296	116
RM75	47	45	314	59	47	45	314	59

Table 3.1.11.B2: Particle size by modified Nanogenotox dispersibility method (from Ref.: Dispersibility – Nanogenotox.xlx - Fourth data package 21 April 2023 and Ref.: Dispersibility Nanogenotox - Nano (corrected).xlx – 30 June 2023)

	Particle Size by Nanogenotox Dispersibiity method				Initial Particle Size extracted from Table 3.1.9.1.B1			
Product Code	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]	Mean size [nm]	Median size [nm]
	by number		by mass		by number		by mass	
RM09	65	59	854	187	58	53	238	96
RM11	99	81	361	242	60	55	296	116
RM75	56	53	643	98	47	45	314	59

Comparison of the particle size after dispersion using the Nanogenotox protocol and the Modified SCCS protocol (From Ref.: Dispersibility Nanogenotox – Report, 4th Data Package, 21 April 2023)
Table 3.1.11.B3 compares the particle sizes of TiO_2 cosmetics grades dispersed using the Nanogenotox protocol and the Modified SCCS protocol to establish the effect of dispersion energy and measured using CPS DC.

Table 3.1.11.B3: Comparison of Secondary Particle Size after Different Dispersion Protocols (measured by CPS DC) for Representative Titanium Dioxide (nano) UV filters (From Ref.: Dispersibility Nanogenotox – Report, 4th Data Package, 21 April 2023)

	Particle Size by Modified SCCS Dispersibility protocol		Particle Size by Nanogenotox Dispersibility protocol	
Product Code	Median size [nm]	Median size [nm]	Median size [nm]	Median size [nm]
	by number	by mass	by number	by mass
RM09	53	96	59	187
RM11	55	116	81	242
RM75	45	59	53	98

Table 3.1.11.B3 compares the particle sizes of TiO_2 cosmetics grades dispersed using the Nanogenotox protocol and the Modified SCCS protocol to establish the effect of dispersion energy and measured using CPS DC.
The median sizes by number are close for the different protocols (the Nanogenotox protocol sizes always being larger), with the greatest difference being for the hydrophobic sample, RM11. The median sizes by mass are much larger using the Nanogenotox protocol.
All of the nano samples measured are well above the 30nm threshold for secondary particle size set by the SCCS Opinion of 2014 irrespective of the dispersion protocol applied

Ref.: Dispersibility Nanogenotox – Report, 4th Data Package, 21 April 2023

Annex R: TEM Observations of internalization of nanoparticles in V79 Cells

Report: RM09: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT) - 4023311_final Report
From Applicants:
Cross-sections of V79 cells could be examined by chemical staining with osmium tetroxide (enhancement of contrast) and ultramicrotomy with a transmission electron microscope.
For all three concentrations examined (25, 50, 100 ug/mL), the TEM ultra-thin sections revealed V79 cell in which the RM09 nanoparticles could be detected.
The nanoparticles are almost entirely found with the cells. Most of the observed V79 cells showed agglomerates of RM09 nanoparticles. Only occasionally separated particles or single small agglomerates can be observed.
In general, no RM09 nanoparticle agglomerates were observed in the nuclei of the cells.
In conclusion, cellular uptake of RM09 was demonstrated at all concentrations evaluated and observed exclusively in cytoplasmic vesicles but not in the cell nucleus.

Concentration: RM09 - 100 ug/mL
Magnification: Fig. 1 – x 2000, Fig. 2: x 4000, Fig. 3: x 5000, Fig. 4: x 20000, Fig. 5: x 50000

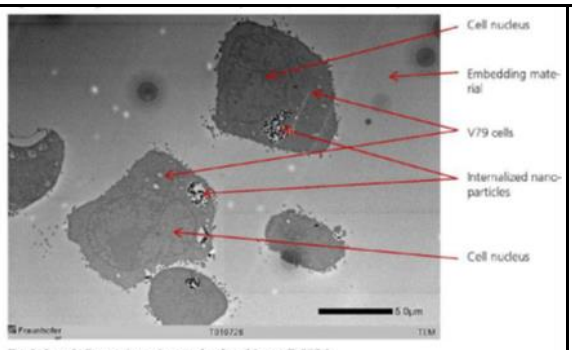


Fig. 1: Sample 3, overview micrograph, ultra-thin cut (2,000x)

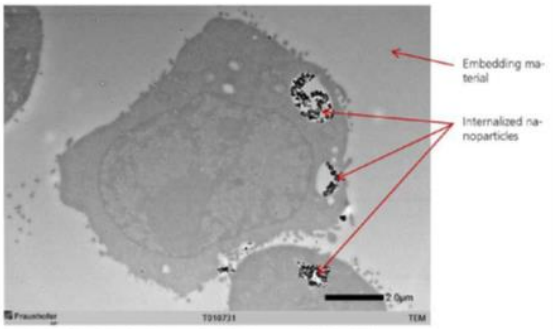


Fig. 2: Sample 3, V79 cell with internalized RM09 nanoparticles (4,000x)

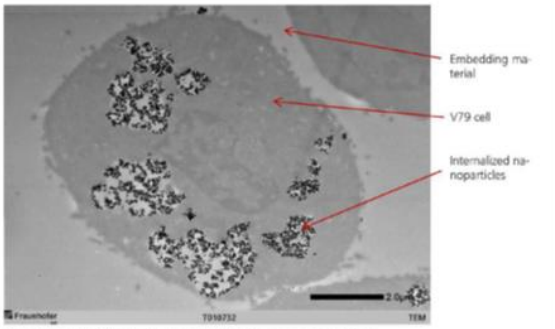


Fig. 3: Sample 3, V79 cell with internalized RM09 nanoparticles (5,000x)

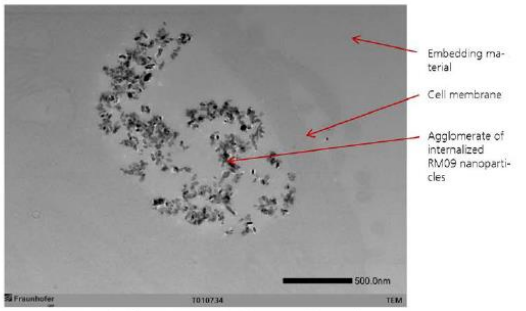


Fig. 4: Sample 3, Details of RM09 nanoparticles in the V79 cell (20,000x)

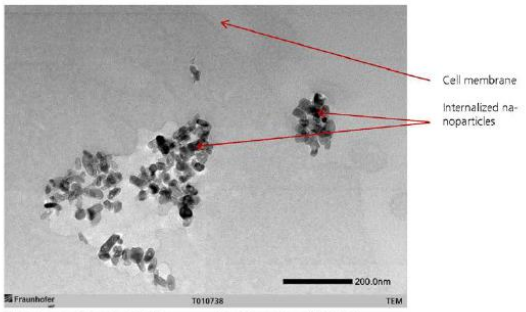


Fig. 5: Sample 3, Details of RM09 nanoparticles in the V79 cell (50,000x)

Concentration: RM09 - 50 ug/mL

Magnification: Fig. 6: x 2000, Fig. 7: x 4000, Fig. 8: x 5000, Fig. 9: x 20000, Fig. 10: x 50000

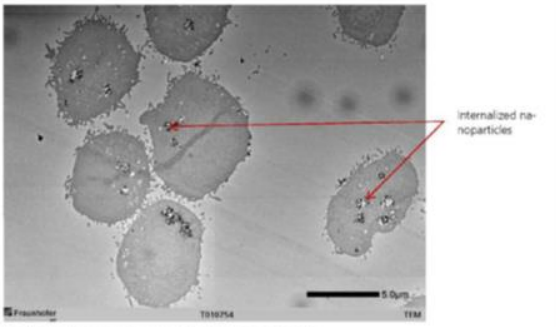


Fig. 6: Sample 2, overview micrograph, ultra-thin cut (2,000x)

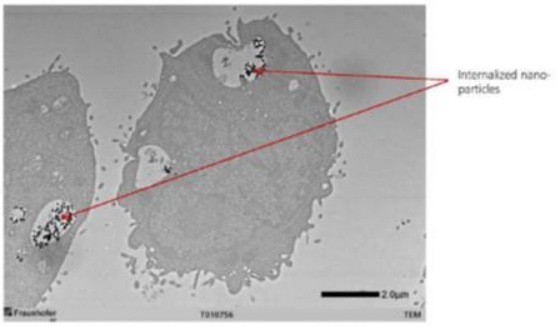


Fig. 7: Sample 2, V79 cells with internalized RM09 nanoparticles (4,000x)

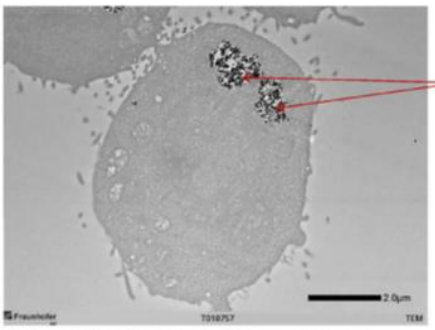


Fig. 8: Sample 2, V79 cell with internalized RM09 nanoparticles (5,000x)

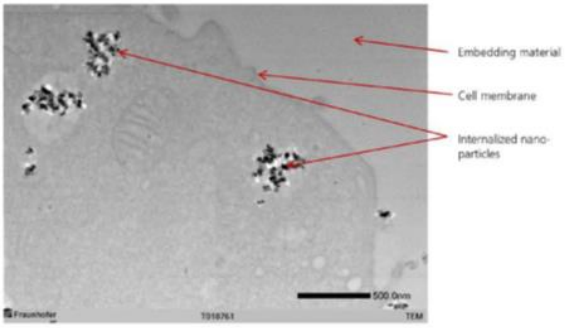


Fig. 9: Sample 2, Details of RM09 nanoparticles in the V79 cell (20,000x)

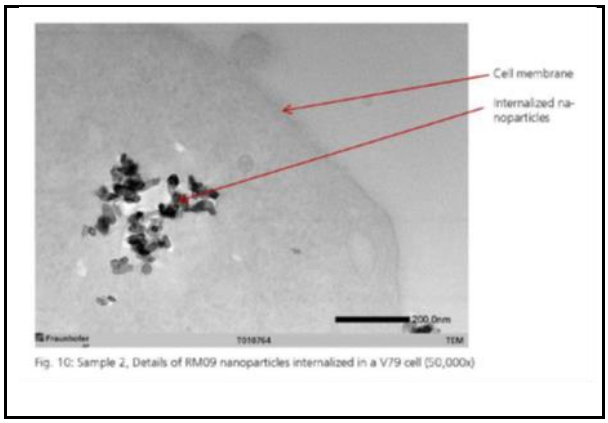


Fig. 10: Sample 2, Details of RM09 nanoparticles internalized in a V79 cell (50,000x)

Concentration: RL09 - 25 ug/mL
Magnification: Fig. 11 – x 2000, Fig. 12: x 4000

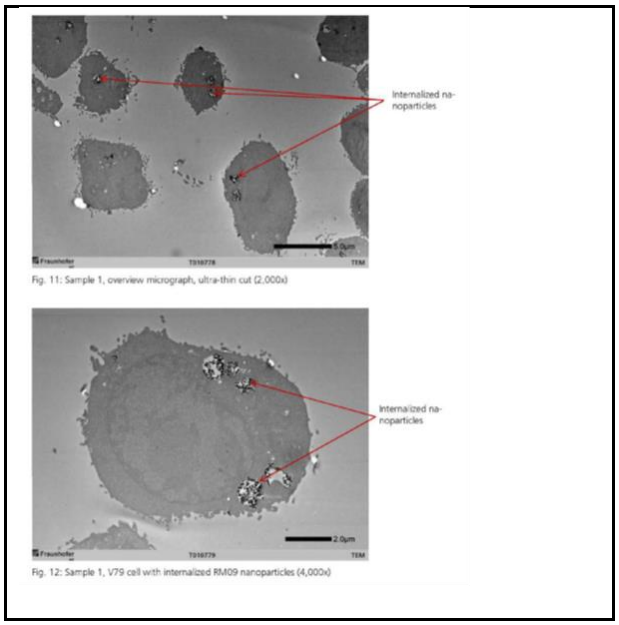


Fig. 11: Sample 1, overview micrograph, ultra-thin cut (2,000x)

Fig. 12: Sample 1, V79 cell with internalized RM09 nanoparticles (4,000x)

Report: RM11: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) - 4023312_final Report

From Applicants

Cross sections of V79 cells could be examined by chemical staining with osmium tetroxide (enhancement of contrast) and ultramicrotomy with a transmission electron microscope.
For all three concentrations examined (25, 50 and 100 ug/mL), the TEM ultra-thin cuts show V79 cell in which the RM11 nanoparticles could be detected. Nevertheless, many cells show no obvious internalization of RM11 nanoparticles and many of the RM11 nanoparticle agglomerates can be observed outside the cells. The majority of the RM11 nanoparticles (inside and outside the cells) are present in agglomerated form. Only occasionally separated particles or single smaller agglomerates can be seen.
In general, no RM11 nanoparticle agglomerates were observed in the nuclei of the cells.
In conclusion, cellular uptake of RM11 nanoparticles was demonstrated at all concentrations evaluated and observed exclusively in cytoplasmic vesicles but not in the cell nucleus.

Concentration: RM11 - 100 ug/mL
Magnification: Fig. 1 – x 2000, Fig. 2: x 2000, Fig. 3: x 4000, Fig. 4: x 20000, Fig. 5: x 50000

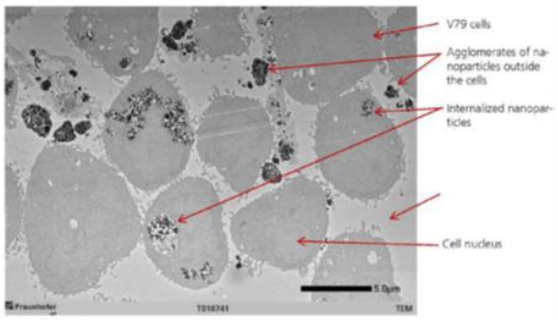


Fig. 1: Sample 6, overview micrograph, ultra-thin cut (2,000x)

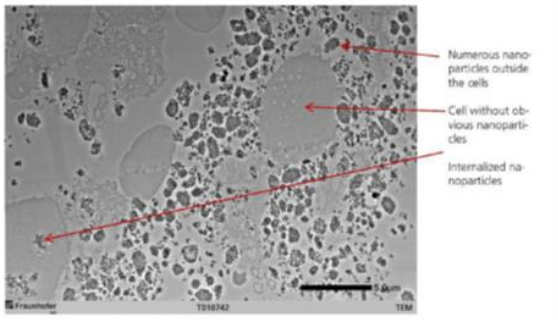


Fig. 2: Sample 6, V79 cell with internalized RM09 nanoparticles (2,000x)

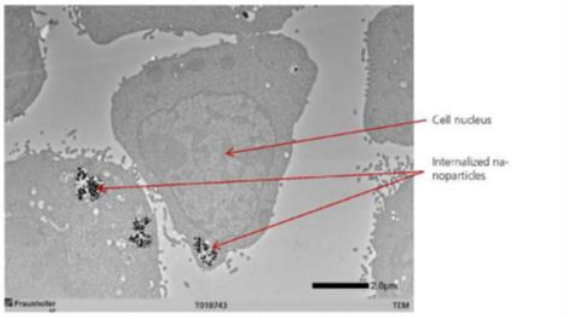


Fig. 3: Sample 6, V79 cell with internalized RM11 nanoparticles (4,000x)

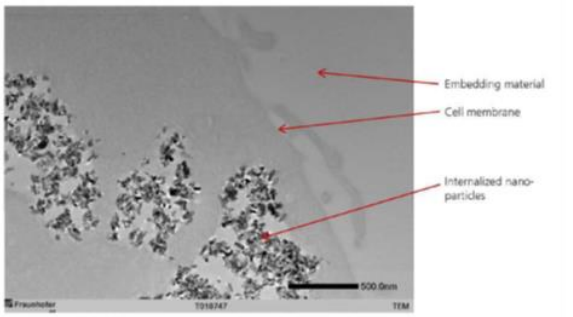


Fig. 4: Sample 6, Details of RM11 nanoparticles in a V79 cell (20,000x)

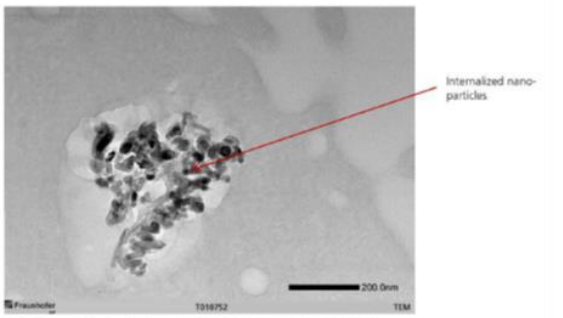


Fig. 5: Sample 6, V79 cells with internalized RM11 nanoparticles (50,000x)

Concentration RM11 - 50 ug/mL

Magnification: Fig. 6 – x 2000, Fig. 7: x 4000, Fig. 8: x 50000.

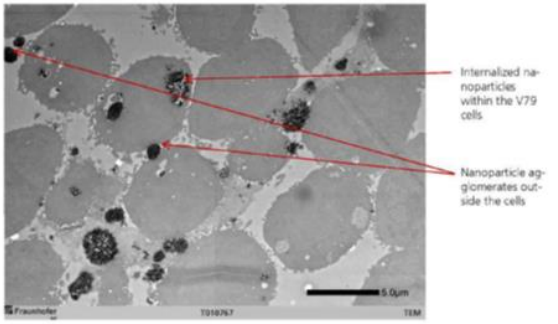


Fig. 6: Sample 5, overview micrograph, ultra-thin cut (2,000x)

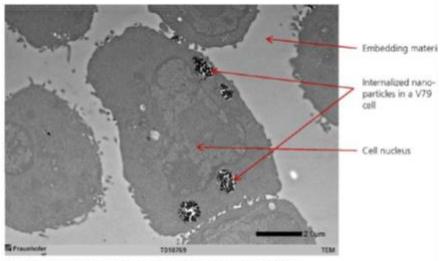


Fig. 7: Sample 5, V79 cell with internalized RM11 nanoparticles (4,000x)

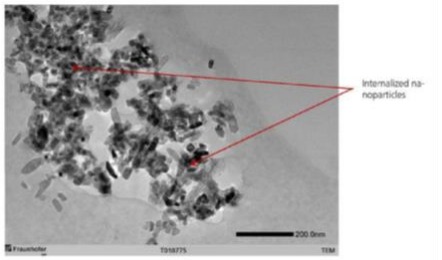


Fig. 8: Sample 5, V79 cells with internalized RM11 nanoparticles (50,000x)

Concentration RM11 - 25 ug/mL

Magnification: Fig. 9 – x 2000, Fig. 10: x 4000

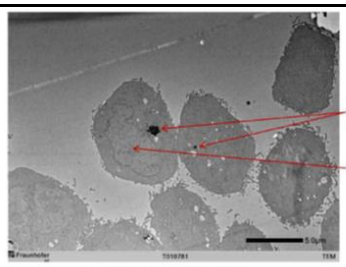


Fig. 9: Sample 4, overview micrograph, ultra-thin cut (2,000x)

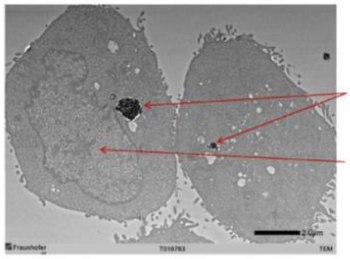


Fig. 10: Sample 4, V79 cell with internalized RM11 nanoparticles (4,000x)

Annex S: DLS measurements of RM09 and RM11 - Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT) and micronucleus test in Chinese hamster V79 cells in vitro

RAW MATERIAL 09 (RM09)

RM09 - Summary and conclusion of DLS measurements from Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

Four samples were measured in three replicates via DLS at 37°C for 24 hours with one data point per hour.
For sample 24h RM09 0.8 ug/mL – S9 mix the z – average diameter at T0 (first measurement point after preparation of the sample mixture) was 50 nm and 57 nm at Tend (last measurement point of the accelerated stability measurement). Signal intensity was approximately 1-fold above the formulation signal level. The higher intensity of the sample signal in comparison with the background signal of the formulation buffer, the less likely an impact of background noise on the experiment data. 24 h RM09 100 ug/mL – S9 mix had a z-average of 135 nm at T0 and 137 nm at Tend. An interference of the FBS with DLS measurements could not be observed.
Samples were centrifugated before the experiment, as an initial intensity test showed high scattering due to large particles in the samples, which led to abortion of data collection. For neither of the samples, a clear trend toward larger particle sizes could be measured within the tested time frame.

Ref.: 4023311_final Report – Report RM09: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

Detailed Results of the DLS experiments

From: 4023311_final Report – Report RM09: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

From Applicants:
To reflect the stability of the dispersion and the agglomeration/aggregation behavior of the test material during cell culture exposure in the genotoxicity experiment, particle size determination of the test dispersion using dynamic light scattering (DLS) was performed.

3.6.3. Nano characterization of the test solution with dynamic light scattering (DLS) (non-GLP):
The DLS measurement was performed at:
ZentriForce Pharma Research GmbH Dr. Marius Schmid Carl-Friedrich-Gauß-Ring 5 69124 Heidelberg

The stock solution of the test item and the application medium was prepared at ZentriForce Pharma Research GmbH.
The solutions were prepared on the day of measurement according to chapter 3.3 (Test item preparation).
The negative and solvent control as well as the highest and lowest test item concentrations was measured by DLS 24 hours with a measurement each hour in order to analyze the stability of the dispersion and the agglomeration/aggregation behaviour of the test item over the time. This data was used to reflect the stability of the dispersion and agglomeration/aggregation behaviour of the test material during the cell culture exposure in the genotoxicity experiment.

As stated in the Short Report (non-GLP) of ZentriForce Pharma Research GmbH: "For neither of the samples a clear trend toward larger sizes could be measured within the tested time frame." (cf. Annex 3).

3.6.4 Data Recording

The data generated were recorded in the raw data. The results are presented in tabular form, including experimental groups with the test item, solvent, and positive controls.

Materials and methods (extracted from Annex 3)

Samples

Sample was provided by the customer. Preparation of sixteen sample mixtures to be analyzed via DLS was conducted by the customer in ZentriForce Laboratory 2N21. A list of all samples prepared by the customer and analyzed with in project RICC001a is given in Table 1.

Table 1: Samples for RICC001a

Sample Name	Samle Type	Description	Storage conditions at ZentriForce Pharma
24 h RM09 Negative control – S9 mix	RM09 24 h MEM − 10% FBS Water	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
24 h Solvent control – S9 mix	RM09 24h + MEM 10% FBS LM	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
24 h RM09 0.8 µg/mL – S9 mix	RM09 24h + MEM 10% FBS Konz 1	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
24 h RM09 100 µg/mL – S9 mix	RM09 24h + MEM 10% FBS Konz 8	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation

Preparation of samples for DLS measurements

Sample mixtures (see Table 1) were prepared by the customer in the ZentriForce Laboratories. Subsequently, 1 mL of each sample were transferred into a microtube and centrifugated at 2767 RCF for 5 minutes. Supernatant was transferred to an Aurora 384 well plate for DLS Measurements.

As a preliminary test, samples 24 h Solvent control – S9 mix, 4 h Solvent control – S9 mix, 24 h RM09 0.8 ug/mL – S9 mix, 24 h RM09 100 ug/mL – S9 mix, *4 h RM11 0.8 ug/mL – S9 mix, *4 h RM11 100 ug/mL – S9 mix and *4 h Solvent control + S9 mix were measured with and without previous centrifugation at 2767 g for 5 minutes.
*Samples are covered in report RICC001b.

For sample centrifugation, 1 mL of each sample were transferred into a 1.5 mL microtube and centrifuged at 2767 RCF for 5 minutes. Supernatant was transferred to an Aurora 384 well plate for DLS accelerated stability measurement.
Due to high scatter intensities for uncentrifugated samples 24 h RM09 100 ug/mL – S9 mix (no data) and RM11 0.8 ug/mL – S9 mix, (incomplete data), the accelerated stability study was conducted on samples that were centrifugated before transfer to the well plate.

DLS measurements

All light scattering services were executed on a DynaPro@Plate Reader III (Wyatt Technology). Each sample was measured in triplicate (n= 3). The adequate performance of the instrument regarding its intended application was verified via a systema suitability test (SSF) prior to sample measurement. The software Dynamics (V.7.10.21, Wyatt Technology) was used for sample measurements and data evaluation. Measurement parameters for the SST are depicted in Table 3.

Laser power and attenuation for sample measurement were set to auto-attenuation to adjust to potential formation of larger particles during accelerated stability experiment. Sample-

specific measurement parameters are listed in Table 4. The well plate was centrifugated at 3.000 rpm for 2 min after sample loading, following a standard procedure to remove air bubbles from the wells.

One data point per hour was recorded for each sample replicate. Normalized intensities are calculated by the Dynamics software and reported for comparability between samples. In all cases, a standard deviation (sample) was used.

Reported parameters are listed in Table 5 – Table 10 in section 3.

SST parameters
An SST was performed before sample measurement. 1.4 mg/mL in BSA in 100 mM NaCl, stored at -80°C, were thawed before the SST. SST experiment parameters are listed in Table 3. SST results are shown in the Appendix. SST was passed for RICC001a sample measurement

Table 3: DLS SST measurement parameters.

DLS acquisition time (s)	DLS acquisitions per measurement	Laser power (%)	Attenuation level (%)	Measurements per well within a scan	Number of scans	Temperature (°C)	Plate sealant
5	5	100	0	1	1	20	none

DLS Sample measurements
Table 4: DLS sample measurement parameters.

DLS acquisition time (s)	DLS acquisitions per measurement	Laser power (%)	Attenuation level (%)	Measurements per well within a scan	Number of scans	Temperature (°C)	Plate sealant
5	3	auto	auto	1	24	37	sealing tape

Results
Four samples were measured in three replicates via DLS at 37°C for 24 hours with one data point per hour. Results for the first and last data point of the experiment for each sample are listed in Table 5 – Table 10.

For sample 24 h RM09 0.8 ug/mL – S9 mix, the z-average diameter at T0 (first measurement point after preparation of the sample mixture) was 50 nm and 57 nm at Tend (last measurement point of the accelerated stability measurement). Signal intensity was approximatively 1-fold above the formulation signal level. The higher intensity of the sample signal in comparison with the background signal of the formulation buffer, the less likely an impact of background noise on the experiment data.

24 h RM09 100 ug/mL – S9 mix had a z-average diameter of 135 nm at T0 and of 137 nm at Tend.

The z-average diameter in relation to incubation time is shown in Figure 1 to Figure 4 for each sample respectively.

Samples were centrifugated before the experiment, as an initial intensity test at 20°C showed high scattering dure to large particles in the samples, which led to abortion of data collection.

Detailed results and intensity distributions of all replicates of the measured samples are shown in the Appendix.

Table 5: Averages and standard deviations of z-average and intensity-based D10, D50 and D90 radii, T0. Samples were measured in triplicate.

Sample name	z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
24 h RM09 Negative control – S9 mix	16.6 ± 0.4	8.3 ± 0.2	3.3 ± 0.1	12.0 ± 0.6	62.2 ± 3.4
24 h Solvent control – S9 mix	16.3 ± 0	8.1 ± 0.1	3.2 ± 0.1	13.1 ± 2.7	41.4 ± 13.7
24 h RM09 0.8 µg/mL – S9 mix	50.0 ± 9.1	25.0 ± 4.5	5.0 ± 0.8	45.7 ± 5.7	56.3 ± 9.8
24 h RM09 100 µg/mL – S9 mix	134.5 ± 1.2	67.2 ± 0.6	43.8 ± 2.6	72.7 ± 2.6	115.3 ± 6.0

Table 6: Averages and standard deviation of mass-based D10, D50 and D90, T0. Samples were measured in triplicate.

Sample name	D10 (nm)	D50 (nm)	D90 (nm)
24 h RM09 Negative control – S9 mix	2.3 ± 0.4	3.0 ± 0.2	4.7 ± 0.5
24 h Solvent control – S9 mix	2.3 ± 0.4	3.0 ± 0.3	4.7 ± 0.2
24 h RM09 0.8 µg/mL – S9 mix	3.2 ± 1.0	3.7 ± 1.1	5.4 ± 0.6
24 h RM09 100 µg/mL – S9 mix	34.9 ± 43.4	108.1 ± 0.8	114.5 ± 0.0

Table 7: Averages and standard deviations of normalized intensities, T0. Samples were measured in triplicate.

Sample name	Normalized Intensity (kCnt/s)
24 h RM09 Negative control – S9 mix	26155 ± 723
24 h Solvent control – S9 mix	26063 ± 813
24 h RM09 0.8 µg/mL – S9 mix	46978 ± 8739
24 h RM09 100 µg/mL – S9 mix	1582471 ± 97289

Table 8: Averages and standard deviations of z-average and intensity-based D10, D50, D90 radii, Tend. Samples were measured in triplicate.

Sample name	z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
24 h RM09 Negative control – S9 mix	15.3 ± 0.4	7.7 ± 0.2	3.2 ± 0.1	11.2 ± 1.5	55.8 ± 17.6
24 h Solvent control – S9 mix	15.5 ± 0.5	7.8 ± 0.3	3.2 ± 0.0	10.7 ± 1.0	67.6 ± 29.1
24 h RM09 0.8 µg/mL – S9 mix	57.1 ± 10.8	28.6 ± 5.4	5.1 ± 0.6	49.9 ± 3.3	156.2 ± 155.1
24 h RM09 100 µg/mL – S9 mix	136.5 ± 2.3	68.2 ± 1.2	49.4 ± 8.1	71.8 ± 1.9	103.6 ± 18.1

Table 9: Averages and standard deviations of mass-based D10, D50 and D90 radii, Tend. Samples were measured in triplicate.

Sample name	D10 (nm)	D50 (nm)	D90 (nm)
24 h RM09 Negative control – S9 mix	2.1 ± 0.3	2.9 ± 0.3	4.8 ± 0.1
24 h Solvent control – S9 mix	2.1 ± 0.2	2.9 ± 0.2	4.8 ± 0.2
24 h RM09 0.8 µg/mL – S9 mix	2.4 ± 1.2	3.0 ± 1.2	5.5 ± 0.4
24 h RM09 100 µg/mL – S9 mix	23.6 ± 31.0	95.6 ± 19.0	105.6 ± 14.8

Table 10: Averages and standard deviation of normalized intensities, Tend. Samples were measured in triplicate.

Sample name	Normalized Intensity (kCnt/s)
24 h RM09 Negative control – S9 mix	30767 ± 483
24 h Solvent control – S9 mix	31431 ± 2171
24 h RM09 0.8 µg/mL – S9 mix	70631 ± 13550
24 h RM09 100 µg/mL – S9 mix	1593815 ± 50867

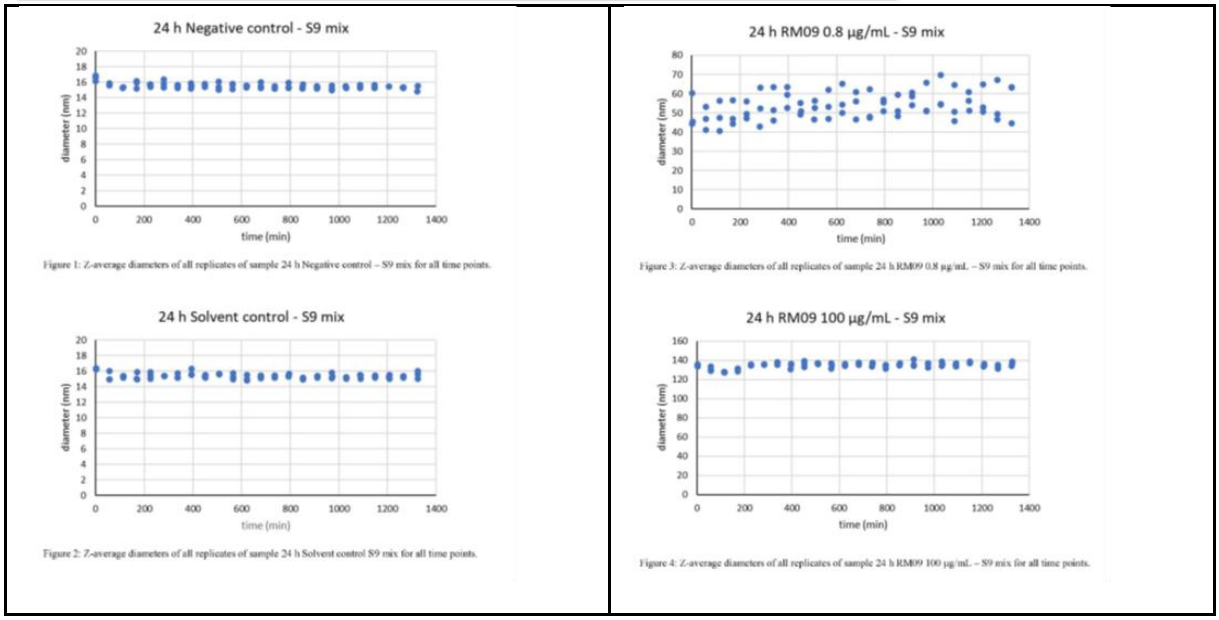


Figure 1: Z-average diameters of all replicates of sample 24 h Negative control – S9 mix for all time points.

Figure 2: Z-average diameters of all replicates of sample 24 h Solvent control S9 mix for all time points.

Figure 3: Z-average diameters of all replicates of sample 24 h RM09 0.8 µg/mL – S9 mix for all time points.

Figure 4: Z-average diameters of all replicates of sample 24 h RM09 100 µg/mL – S9 mix for all time points.

Summary and conclusion of DLS measurements from Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

Four samples were measured in three replicates via DLS at 37°C for 24 hours with one data point per hour.
For sample 24 RM09 0.8 ug/mL – S9 mix the z – average diameter at T0 (first measurement point after preparation of the sample mixture) was 50 nm and 57 nm at Tend (last measurement point of the accelerated stability measurement). Signal intensity was approximately 1-fold above the formulation signal level. The higher intensity of the sample signal in comparison with the background signal of the formulation buffer, the less likely an impact of background noise on the experiment data. 24 h RM09 100 ug/mL – S9 mix had a z-average of 135 nm at T0 and 137 nm at Tend. An interference of the FBS with DLS measurements could not be observed.
Samples were centrifugated before the experiment, as an initial intensity test showed high scattering due to large particles in the samples, which led to abortion of data collection. For neither of the samples, a clear trend toward larger particle sizes could be measured within the tested time frame.

Appendix

Table 12: z-average and intensity-based D10, D50 and D90 radii, T0

Sample name	Replicate	z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
24 h RM09 0.8 µg/mL – S9 mix	1	16.2	8.1	3.3	12.1	60.1
	2	16.9	8.5	3.3	11.4	60.4
	3	16.7	8.3	3.2	12.6	66.1
	AVG ± STD	16.6 ± 0.4	8.3 ± 0.2	3.3 ± 0.1	12.0 ± 0.6	62.2 ± 3.4
24 h RM09 0.8 µg/mL – S9 mix	1	16.3	8.2	3.3	16.1	29.5
	2	16.3	8.1	3.2	11.1	56.3
	3	16.3	8.1	3.1	12.0	38.3
	AVG ± STD	16.3 ± 0	8.1 ± 0.1	3.2 ± 0.1	13.1 ± 2.7	41.4 ± 13.7
24 h RM09 0.8 µg/mL – S9 mix	1	44.2	22.1	4.3	39.1	45.0
	2	60.4	30.2	4.8	49.1	61.2
	3	45.3	22.7	5.9	49.0	62.7
	AVG ± STD	50 ± 9.1	25.0 ± 4.5	5.0 ± 0.8	45.7 ± 5.7	56.3 ± 9.8
24 h RM09 0.8 µg/mL – S9 mix	1	134.5	67.2	45.3	75.4	114.1
	2	133.4	66.7	40.8	72.4	121.9
	3	135.7	67.8	45.3	70.3	109.9
	AVG ± STD	134.5 ± 1.2	67.2 ± 0.6	43.8 ± 2.6	72.7 ± 2.6	115.3 ± 6.0

Table 13: z-average and intensity-based D10, D50 and D90 radii, Tend

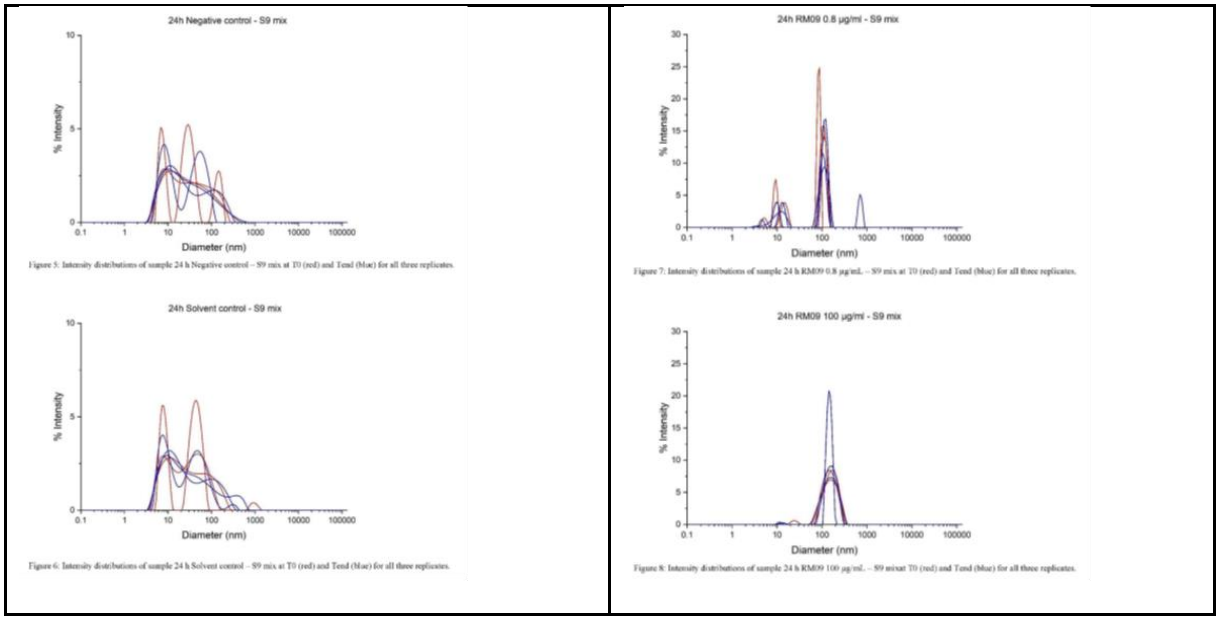
Sample name	Replicate	z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
Konz124 h RM09 0.8 µg/mL – S9 mix	1	14.8	7.4	3.1	10.3	59.7
	2	15.5	7.8	3.2	12.9	36.6
	3	15.5	7.8	3.2	10.3	71.3
	AVG ± STD	15.3 ± 0.4	7.7 ± 0.2	3.2 ± 0.1	11.2 ± 1.5	55.8 ± 17.6
Konz124 h RM09 0.8 µg/mL – S9 mix	1	15	7.5	3.2	9.7	67.8
	2	16	8.0	3.2	10.8	96.6
	3	15.6	7.8	3.1	11.7	38.4
	AVG ± STD	15.5 ± 0.5	7.8 ± 0.3	3.2 ± 0.0	10.7 ± 1.0	67.6 ± 29.1
Konz124 h RM09 0.8 µg/mL – S9 mix	1	63.2	31.6	5.7	49.4	335.3
	2	63.5	31.8	4.8	53.5	65.6
	3	44.7	22.3	4.7	46.9	67.7
	AVG ± STD	57.1 ± 10.8	28.6 ± 5.4	5.1 ± 0.6	49.9 ± 3.3	156.2 ± 155.1
Konz124 h RM09 0.8 µg/mL – S9 mix	1	133.9	66.9	42.0	72.2	118.0
	2	138.4	69.2	48.0	73.6	109.5
	3	137.1	68.6	58.1	69.8	83.3
	AVG ± STD	136.5 ± 2.3	68.2 ± 1.2	49.4 ± 8.1	71.8 ± 1.9	103.6 ± 18.1

Table 14: mass-based D10, D50 and D90 radii, T0

Sample name	Replicate	D10 (nm)	D50 (nm)	D90 (nm)
24 h RM09 0.8 µg/mL – S9 mix	1	2.2	3.0	5.0
	2	2.0	2.8	5.0
	3	2.7	3.2	4.2
	AVG ± STD	2.3 ± 0.4	3.0 ± 0.2	4.7 ± 0.5
24 h RM09 0.8 µg/mL – S9 mix	1	2.8	3.4	4.5
	2	2.0	2.8	4.9
	3	2.2	2.9	4.7
	AVG ± STD	2.3 ± 0.4	3.0 ± 0.3	4.7 ± 0.2
24 h RM09 0.8 µg/mL – S9 mix	1	3.8	4.3	4.9
	2	3.8	4.4	5.3
	3	2.1	2.5	6.1
	AVG ± STD	3.2 ± 1.0	3.7 ± 1.1	5.4 ± 0.6
24 h RM09 0.8 µg/mL – S9 mix	1	14.3	108.4	114.4
	2	5.8	107.2	114.5
	3	84.8	108.8	114.5
	AVG ± STD	34.9 ± 43.4	108.1 ± 0.8	114.5 ± 0.0

Table 15: Mass-based D10, D50 and D90 radii, Tend

Sample name	Replicate	D10 (nm)	D50 (nm)	D90 (nm)
24 h RM09 0.8 µg/mL – S9 mix	1	2.0	2.7	4.7
	2	2.4	3.1	4.7
	3	1.9	2.7	4.9
	AVG ± STD	2.1 ± 0.3	2.9 ± 0.3	4.8 ± 0.1
24 h RM09 0.8 µg/mL – S9 mix	1	2.0	2.8	5.0
	2	2.1	2.8	4.9
	3	2.4	3.1	4.6
	AVG ± STD	2.1 ± 0.2	2.9 ± 0.2	4.8 ± 0.2
24 h RM09 0.8 µg/mL – S9 mix	1	2.0	2.2	5.9
	2	3.8	4.4	5.3
	3	1.5	2.3	5.2
	AVG + STD	2.4 + 1.2	3.0 + 1.2	5.5 + 0.4
24 h RM09 0.8 µg/mL – S9 mix	1	6.2	107.5	114.4
	2	5.1	105.7	113.9
	3	59.4	73.7	88.4
	AVG ± STD	23.6 ± 31.0	95.6 ± 19.0	105.6 ± 14.8



From Report: 4023313_final_report - RM09: Micronucleus Test in Chinese Hamster V79 Cells in vitro

From Applicants

To reflect the stability of the dispersion and the agglomeration/aggregation behavior of the test material during cell culture exposure in the genotoxicity experiment, particle size determination of the test dispersion using dynamic light scattering (DLS) was performed in the parallel study (ICCR Study Number 4023311 "RM09: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)") as well (external assignment under non-GLP). In the V79/HPRT study, the test item preparation and exposure were performed under comparable conditions, and thus the results from the TEM and DLS analysis are considered transferable between the two studies.

In the accelerated stability study, it was demonstrated via dynamic light scattering (DLS) measurements that the test item RM09 showed stable particle sizes without increased aggregation/agglomeration for at least 24 hours. Moreover, samples from the test item exposure were sent for transmission electron microscopy analysis. The cellular uptake of

RM09 nanoparticles was demonstrated at all concentrations evaluated and the test item was observed exclusively in cytoplasmic vesicles but not in the cell nucleus

RAW MATERIAL 11

RM11 - Summary and conclusion of DLS measurements from Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

For sample 4h RM11 0.8 ug/mL – S9 mix, the z-average diameter at T0 was ca. 183.3 nm and 290 nm at Tend, with a high standard deviation for both data points due to a signal intensity that was approximately 1-forld above the scattering level of the formulation buffer. 4h RM11 100 ug/mL – S9 mix had a z-average diameter of 168 nm at T0 and 176 nm at Tend.
All samples containing S9 mix showed comparable z-average diameters at T0 and at Tend, when compared to each other, as well as comparable scattering intensities, including the Water and LM samples. The normalized intensities of the solvent control sample with S9 mix (T0: 1.0 x 10E6 kCnt/s and Tend: 1.7 x 10E6 kCnt/s) were in a comparable range to the values measured for the samples containing the test material and S9 mix (0.8 ug/mL: T0: 1.0 x 10E6 kCnt/s and Tend: 1.7 x 10E6 kCnt/s – 100 ug/mL: T0 1.2 x 10E6 kCnt/s and Tend: 1.7 x 10E6 kCnt/s). Therefore the data possibly reflects the z-average diameter of the S9 components instead of the z-average diameter of the nanoparticles.

24 h RM 11 0.8 ug/mL – S9 had a z-average diameter of approx. 24 nm at T0 and 32 nm at Tend, wit a low signal amplitude. An interference of the FBS with the DLS measurements could not be observed. RM 11 24 h + 10 % FBS Konz 8 had a z-average diameter of 109 nm at T0 and of 118 nm at Tend.
Samples were centrifuged before the experiment, as an initial intensity test at 20°C showed high scattering due to large particles in the samples, which led to abortion of data collection.

For neither of the samples, but the samples containing the S9 mix, a clear trend toward larger particles sizes could be measured with the tested time frame.

Ref. 4023312_final Report - RM11: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

Detailed Results of the DLS experiments

From 4023312_final Report - RM11: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

Introduction
The aim of this study was the analytical testing of nanoparticles by dynamic light scattering (DLS). For this purpose, an accelerated stability study at 37°C was conducted for a total of approximately 24 hours.

Samples
Sample was provided by the customer. Preparation of sixteen sample mixtures to be analyzed via DLS was conducted by the customer in ZentriForce Laboratory 2N21. A list of all smaple mixtures prepared by the customer and analyzed within project RICC001b in given in Table 1.

Table 1: Samples from RICC001b.

Sample Name	Sample Type	Description	Storage conditions at ZentriForce Pharma
24 h Negative control – S9 mix	RM11 24 h – 10% FBS + Water	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
24 h Solvent control – S9 mix	RM11 24 h + 10% FBS – LM	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
24 h RM11 0.8 µg/mL – S9 mix	RM11 24 h + 10% FBS + Konz 1	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
24 h RM11 100 µg/mL – S9 mix	RM11 24 h + 10% FBS + Konz 8	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h Negative control – S9 mix	RM11 4 h + Water	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h Solvent control – S9 mix	RM11 4 h + LM	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h RM11 0.8 µg/mL - S9 mix	RM11 4 h + Konz 1	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h RM11 100 µg/mL - S9 mix	RM11 4 h + Konz 8	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h Negative control + S9 mix	RM11 4 h + S9 + Water	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h Solvent control + S9 mix	RM11 4 h + S9 + LM	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h RM11 0.8 µg/mL + S9 mix	RM11 4 h + S9 + Konz 1	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation
4 h RM11 100 µg/mL + S9 mix	RM11 4 h + S9 + Konz 8	Mixture was prepared by customer in ZentriForce laboratory	Sample was measured immediately after preparation

Preparation of samples for DLS measurements
Sample mixtures (see Table 1) were prepared by the customer in the ZentriForce Laboratories.
As a preliminary test, samples 24h Solvent control – S9 mix, 4 h Solvent control – S9 mix,

As a preliminary test, samples 24 h Solvent control – S9 mix, 4 h Solvent control – S9 mix, *24 h RM09 0.8 ug/mL – S9 mix, *24 h RM09 100 ug/mL – S9 mix, 4 h RM11 0.8 ug/mL – S9 mix, 4 h RM11 100 ug/mL – S9 mix and 4 h Solvent control + S9 mix were measured with and without previous centrifugation at 2767 RCF for 5 minutes in a Thermo Fisher Heraeus Megafuge 8
*Samples are covered in report RICC001a

For sample centrifugation, 1 mL of each sample were transferred into a 1.5 mL microtube and centrifuged at 2767 RCF for 5 minutes. Supernatant was transferred to an Aurora 384 well plate for DLS accelerated stability measurement.
Due to high scatter intensities for uncentrifugated samples 24 h RM09 100 ug/mL – S9 mix (no data) and RM11 0.8 ug/mL – S9 mix, (incomplete data), the accelerated stability study was conducted on samples that were centrifugated before transfer to the well plate.

DLS measurements
All light scattering services were executed on a DynaPro@Plate Reader III (Wyatt Technology). Each sample was measured in triplicate (n= 3). The adequate performance of the instrument regarding its intended application was verified via a systema suitability test (SSF) prior to sample measurement. The software Dynamics (V.7.10.21, Wyatt Technology) was used for sample measurements and data evaluation. Measurement parameters for the SST are depicted in Table 3.

Laser power and attenuation for sample measurement were set to auto-attenuation to adjust to potential formation of larger particles during accelerated stability experiment. Sample-specific measurement parameters are listed in Table 4. The well plate was centrifugated at 3.000 rpm for 2 min after sample loading, following a standard procedure to remove air bubbles from the wells.

One data point per hour was recorded for each sample replicate. Normalized intensities are calculated by the Dynamics software and reported for comparability between samples. In all cases, a standard deviation (sample) was used.

Reported parameters are listed in Table 5 – Table 10 in section 3.

SST parameters
An SST was performed before sample measurement. 1.4 mg/mL in BSA in 100 mM NaCl, stored at -80°C, were thawed before the SST. SST experiment parameters are listed in Table 3. SST results are shown in the Appendix. SST was passed for RICC001a sample measurement

Table 3: DLS SST measurement parameters.

DLS acquisition time (s)	DLS acquisitions per measurement	Laser power (%)	Attenuation level (%)	Measurements per well within a scan	Number of scans	Temperature (°C)	Plate sealant
5	5	100	0	1	1	20	none

DLS Sample measurements
Table 4: DLS sample measurement parameters.

DLS acquisition time (s)	DLS acquisitions per measurement	Laser power (%)	Attenuation level (%)	Measurements per well within a scan	Number of scans	Temperature (°C)	Plate sealant
5	3	auto	auto	1	24	37	sealing tape

Results
Twelve samples were measured in three replicates via DLS at 37°C for 24 hours with one data point per hour. Results for the first and last data point of the experiment for each sample are listed in Table 5 – Table 10.

For sample 4h RM11 0.8 ug/mL – S9 mix, the z-average diameter at T0 (first measurement point after the preparation of the sample mixture) was 183 nm and 290 nm at Tend (last measurement point of the accelerated stability measurement), with a high standard deviation for both data points. Signal intensity was approximately 1-fold above the formulation signal level. The higher intensity of the sample signal in comparison with the background signal of the formulation buffer, the less likely an impact of background noise on the experiment data.

4 h RM11 100 ug/mL – S9 mix had a z-average diameter of 168 nm at T0 and 176 nm at Tend.

All samples with S9 mix showed comparable z-average diameters at T0 and Tend when compared to each other, as well as comparable scattering intensities, including the Water and LM samples.

24 h RM11 0.8 ug/mL – S9 mix had a z-average diameter of approx. 24 nm at T0 and 32 nm at Tend, with a low signal – to – noise ratio. 24 h RM11 100 ug/mL – S9 mix had a z-average diameter of ca. 109 nm at T0 and of 118 nm at Tend.

The z-average diameter in relation to incubation time in shown in Figure 1 to Figure 12 for each sample, respectively.

Samples were centrifugated before the experiment, as an initial intensity test at 20°C showed high scattering due to large particles in the samples, which led to abortion of data collection.

Detailed results and intensity distributions of all replicates of the measured samples are shown in the Appendix.

Table 5: Averages and standard deviation of z-average and intensity-based D10, D50 and D90 radii. Samples were measured in triplicate.

Sample name	z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
4 h Negative control – S9 mix	5.7 ± 7.6	2.8 ± 3.8	114.9 ± 53.6	161.8 ± 68.7	230.3 ± 89.4
4 h Solvent control – S9 mix	6.3 ± 0.7	3.2 ± 0.4	1.8 ± 0.2	4.0 ± 0.4	72.0 ± 60.6
4 h RM11 0.8 µg/mL – S9 mix	183.3 ± 153.2	91.7 ± 76.6	4.1 ± 3.3	53.5 ± 39.8	56.9 ± 42.5
4 h RM11 100 µg/mL – S9 mix	168.1 ± 4.6	84.1 ± 2.2	62.7 ± 2.1	73.0 ± 3.2	85.3 ± 7.5
4 h RM11 Negative control + S9 mix	167.7 ± 4.8	83.8 ± 2.4	48.2 ± 5.2	87.1 ± 6.6	158.2 ± 26.8
4 h RM11 Solvent control + S9 mix	168.0 ± 1.3	84.0 ± 0.7	44.5 ± 2.1	92.4 ± 3.0	182.0 ± 21.4
4 h RM11 0.8 µg/mL + S9 mix	166.0 ± 1.5	83.0 ± 0.8	42.7 ± 2.5	93.6 ± 7.5	197.4 ± 26.3
4 h RM11 100 µg/mL + S9 mix	169.9 ± 4.4	84.9 ± 2.2	55.7 ± 12.4	87.7 ± 1.7	140.6 ± 36.0
24 h Negative control – S9 mix	15.3 ± 0.2	7.7 ± 0.1	3.1 ± 0.1	11.9 ± 1.4	60.2 ± 12.1
24 h Solvent control – S9 mix	15.6 ± 0.7	7.8 ± 0.3	2.9 ± 0.2	10.0 ± 0.2	57.1 ± 11.9
24 h RM11 0.8 µg/mL – S9 mix	23.7 ± 10.4	11.8 ± 5.2	3.0 ± 0.1	13.2 ± 2.7	92.4 ± 53.2
24 h RM11 100 µg/mL – S9 mix	109.4 ± 9.0	54.7 ± 4.5	49.5 ± 4.2	67.3 ± 3.0	84.4 ± 8.6

Table 6: Averages and standard deviations of mass-based D10, D50 and D90 radii, T0. Samples were measured in triplicate.

Sample name	D10 (nm)	D50 (nm)	D90 (nm)
4 h Negative control – S9 mix	129.2 ± 45.2	162.4 ± 85.9	223.3 ± 108.6
4 h Solvent control – S9 mix	36.4 ± 61.1	40.0 ± 66.3	67.5 ± 111.4
4 h RM11 0.8 µg/mL – S9 mix	4.0 ± 3.4	4.3 ± 3.7	4.5 ± 3.8
4 h RM11 100 µg/mL – S9 mix	65.3 ± 2.9	79.6 ± 10.8	90.3 ± 12.4
4 h RM11 Negative control + S9 mix	97.0 ± 2.6	112.6 ± 1.5	2814.3 ± 4546.4
4 h RM11 Solvent control + S9 mix	98.4 ± 0.6	112.2 ± 0.9	199.2 ± 24.1
4 h RM11 0.8 µg/mL + S9 mix	97.8 ± 1.3	112.4 ± 0.9	220.9 ± 52.4
4 h RM11 100 µg/mL + S9 mix	94.9 ± 2.5	110.1 ± 1.5	142.4 ± 47.6
24 h Negative control – S9 mix	2.2 ± 0.5	2.9 ± 0.4	4.5 ± 0.2
24 h Solvent control – S9 mix	2.0 ± 0.1	2.5 ± 0.0	4.5 ± 0.2
24 h RM11 0.8 µg/mL – S9 mix	2.5 ± 0.3	3.0 ± 0.2	3.9 ± 0.3
24 h RM11 100 µg/mL – S9 mix	3.5 ± 1.1	3.9 ± 1.2	5.6 ± 0.4

Table 7: Averages and standard deviations of normalized intensities, T0. Samples were measured in triplicate.

Sample name	Normalized Intensity (kCnt/s)
4 h Negative control – S9 mix	491 ± 18
4 h Solvent control – S9 mix	873 ± 265
4 h RM11 0.8 µg/mL – S9 mix	1568 ± 649
4 h RM11 100 µg/mL – S9 mix	87409 ± 6594
4 h RM11 Negative control + S9 mix	1028178 ± 52698
4 h RM11 Solvent control + S9 mix	954261 ± 21475
4 h RM11 0.8 µg/mL + S9 mix	976951 ± 56708
4 h RM11 100 µg/mL + S9 mix	1179233 ± 63976
24 h Negative control – S9 mix	25920 ± 185
24 h Solvent control – S9 mix	27324 ± 710
24 h RM11 0.8 µg/mL – S9 mix	29530 ± 4786
24 h RM11 100 µg/mL – S9 mix	131965 ± 12189

Table 8: Averages and standard deviation of z-average and intensity-based D10, D50 and D90 radii, Tend. Samples were measured in triplicate.

Sample name	z-average z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
4 h Negative control – S9 mix	263.4 ± 263.6	131.7 ± 131.8	93.8 ± 34.9	181.0 ± 44.3	394.8 ± 151.5
4 h Solvent control – S9 mix	4.5 ± 0.5	2.3 ± 0.3	1.9 ± 0.0	3.4 ± 0.2	6.0 ± 0.4
4 h RM11 0.8 µg/mL – S9 mix	289.8 ± 146.4	144.9 ± 73.2	39.3 ± 18.2	41.0 ± 18.3	42.9 ± 18.8
4 h RM11 100 µg/mL – S9 mix	175.6 ± 9.4	87.8 ± 4.7	60.9 ± 5.4	69.0 ± 5.1	78.2 ± 7.8
4 h RM11 Negative control + S9 mix	235.5 ± 84	140.9 ± 3.4	83.4 ± 15.1	135.9 ± 6.7	258.2 ± 100.3
4 h RM11 Solvent control + S9 mix	265.6 ± 4.2	132.8 ± 2.1	64.8 ± 18.1	146.6 ± 14.2	235.9 ± 15.1
4 h RM11 0.8 µg/mL + S9 mix	272.5 ± 6.8	136.3 ± 3.4	74.6 ± 8.6	129.3 ± 36.6	223.0 ± 121.0
4 h RM11 100 µg/mL + S9 mix	266.6 ± 3.6	133.3 ± 1.8	70.0 ± 24.4	149.5 ± 16.3	237.7 ± 44.2
24 h Negative control – S9 mix	15.4 ± 0.2	7.7 ± 0.1	3.8 ± 1.2	8.6 ± 2.8	32.1 ± 23.1
24 h Solvent control – S9 mix	15.2 ± 0.2	7.6 ± 0.1	3.2 ± 0.1	11.4 ± 2.5	62.1 ± 4.3
24 h RM11 0.8 µg/mL – S9 mix	31.6 ± 24.6	15.8 ± 12.3	3.6 ± 1.1	18.5 ± 20.8	91.2 ± 99.5
24 h RM11 100 µg/mL – S9 mix	118.3 ± 6.3	59.2 ± 3.1	42.9 ± 28.5	74.5 ± 5.4	96.0 ± 17.6

Table 9: Averages and standard deviations of mass-based D10, D50 and D90 radii. Samples were measured in triplicate.

Sample name	D10 (nm)	D50 (nm)	D90 (nm)
4 h Negative control – S9 mix	107.6 ± 2.7	201.9 ± 35.4	362.7 ± 94.2
4 h Solvent control – S9 mix	1.4 ± 0.1	2.0 ± 0.1	3.5 ± 0.1
4 h RM11 0.8 µg/mL – S9 mix	39.3 ± 18.2	41.0 ± 18.4	42.8 ± 18.8
4 h RM11 100 µg/mL – S9 mix	61.6 ± 5.2	70.9 ± 6.2	80.2 ± 10.1
4 h RM11 Negative control + S9 mix	104.4 ± 2.0	142.1 ± 39.6	4369.2 ± 876.3
4 h RM11 Solvent control + S9 mix	105.2 ± 2.4	151.2 ± 31.9	250.0 ± 14.9
4 h RM11 0.8 µg/mL + S9 mix	72.4 ± 57.9	105.3 ± 90.0	233.0 ± 130.3
4 h RM11 100 µg/mL + S9 mix	106.6 ± 3.3	152.7 ± 38.6	246.7 ± 50.4
24 h Negative control – S9 mix	3.1 ± 1.8	3.6 ± 1.5	5.0 ± 0.5
24 h Solvent control – S9 mix	2.3 ± 0.6	3.0 ± 0.5	4.6 ± 0.3
24 h RM11 0.8 µg/mL – S9 mix	1.9 ± 0.5	2.4 ± 1.0	5.4 ± 0.5
24 h RM11 100 µg/mL – S9 mix	2.6 ± 1.2	3.0 ± 1.1	18.6 ± 22.6

Table 10: Averages and standard deviations of normalized intensities, Tend. Samples were measured in triplicate.

Sample name	Normalized Intensity (kCnt/s)
4 h Negative control – S9 mix	581 ± 218
4 h Solvent control – S9 mix	636 ± 10
4 h RM11 0.8 µg/mL – S9 mix	1455 ± 186
4 h RM11 100 µg/mL – S9 mix	81240 ± 11116
4 h RM11 Negative control + S9 mix	1650677 ± 46392
4 h RM11 Solvent control + S9 mix	1673810 ± 25857
4 h RM11 0.8 µg/mL + S9 mix	1702196 ± 43908
4 h RM11 100 µg/mL + S9 mix	1684673 ± 54586
24 h Negative control – S9 mix	26819 ± 1056
24 h Solvent control – S9 mix	28015 ± 1734
24 h RM11 0.8 µg/mL – S9 mix	40310 ± 10775
24 h RM11 100 µg/mL – S9 mix	168958 ± 21979

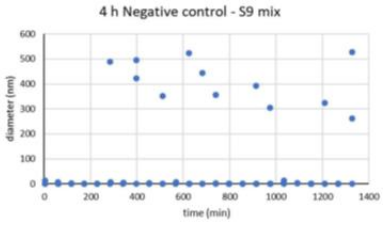


Figure 1: Z-average diameters of all replicates of sample 4 h Negative control – S9 mix for all time points.

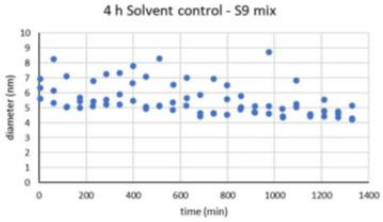


Figure 2: Z-average diameters of all replicates of sample 4 h Solvent control – S9 mix for all time points.

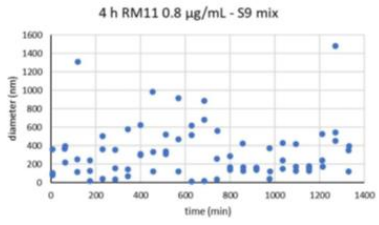


Figure 3: Z-average diameters of all replicates of sample 4 h RM11 0.8 µg/mL – S9 mix for all time points.

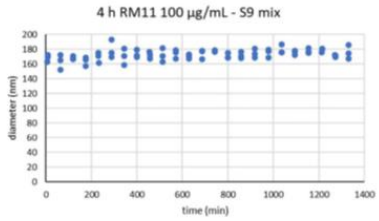


Figure 4: Z-average diameters of all replicates of sample 4 h RM11 100 µg/mL – S9 mix for all time points.

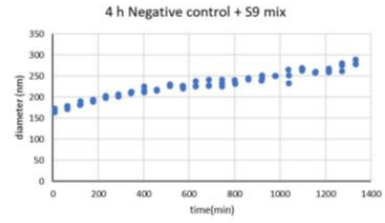


Figure 5: Z-average diameters of all replicates of sample 4 h Negative control + S9 mixfor all time points.

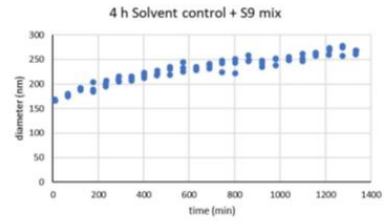


Figure 6: Z-average diameters of all replicates of sample 4 h Solvent control + S9 mix for all time points.

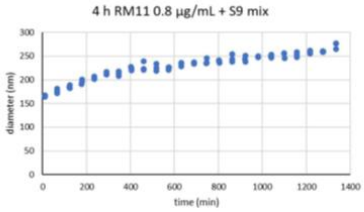


Figure 7: Z-average diameters of all replicates of sample 4 h RM11 0.8 µg/mL + S9 mix for all time points.

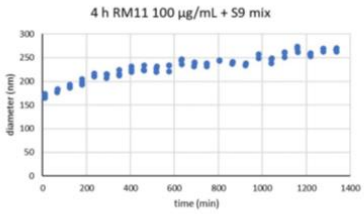


Figure 8: Z-average diameters of all replicates of sample 4 h RM11 0.8 µg/mL + S9 mix for all time points.

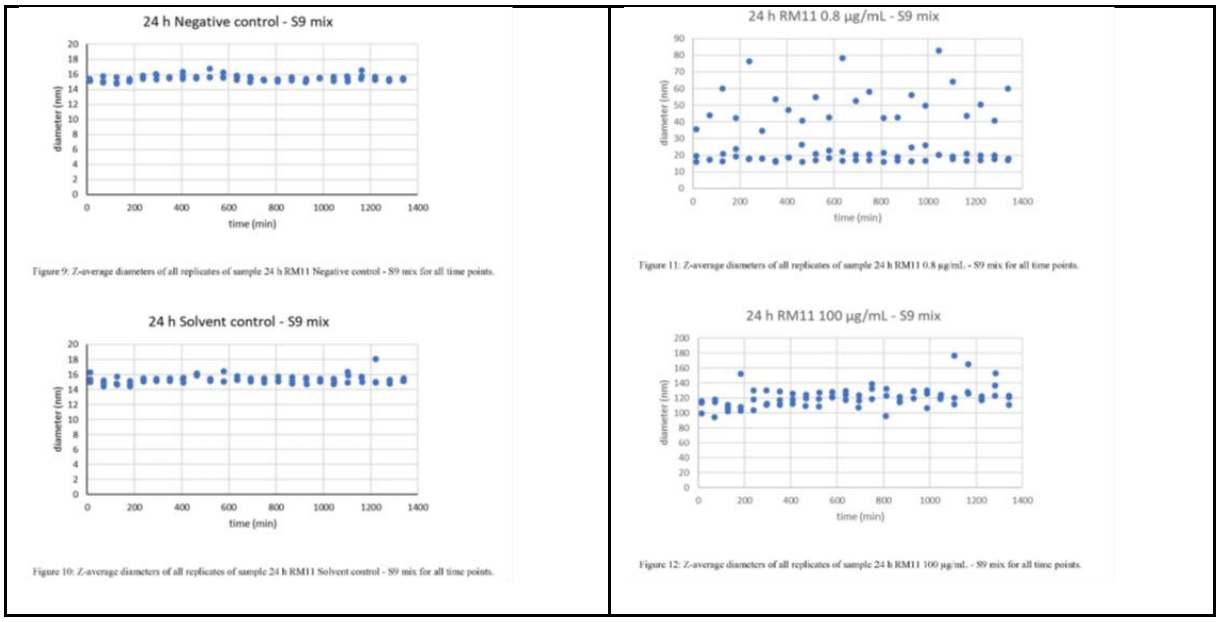


Figure 9: Z-average diameters of all replicates of sample 24 h RM11 Negative control - S9 mix for all time points.

Figure 10: Z-average diameters of all replicates of sample 24 h RM11 Solvent control - S9 mix for all time points.

Figure 11: Z-average diameters of all replicates of sample 24 h RM11 0.8 µg/mL - S9 mix for all time points.

Figure 12: Z-average diameters of all replicates of sample 24 h RM11 100 µg/mL - S9 mix for all time points.

Summary and conclusion - From Report: 4023312_final Report - RM11: Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

For sample 4h RM11 0.8 ug/mL – S9 mix, the z-average diameter at T0 was ca. 183.3 nm and 290 nm at Tend, with a high standard deviation for both data points due to a signal intensity that was approximately 1-forld above the scattering level of the formulation buffer. 4h RM11 100 ug/mL – S9 mix had a z-average diameter of 168 nm at T0 and 176 nm at Tend.
All samples containing S9 mix showed comparable z-average diameters at T0 and at Tend, when compared to each other, as well as comparable scattering intensities, including the Water and LM samples. The normalized intensities of the solvent control sample with S9 mix (T0: 1.0 x 10E6 kCnt/s and Tend: 1.7 x 10E6 kCnt/s) were in a comparable range to the values measured for the samples containing the test material and S9 mix (0.8 ug/mL: T0: 1.0 x 10E6 kCnt/s and Tend: 1.7 x 10E6 kCnt/s – 100 ug/mL: T0 1.2 x 10E6 kCnt/s and Tend: 1.7 x 10E6 kCnt/s). Therefore the data possibly reflects the z-average diameter of the S9 components instead of the z-average diameter of the nanoparticles.

24 h RM 11 0.8 ug/mL – S9 had a z-average diameter of approx. 24 nm at T0 and 32 nm at Tend, with a low signal amplitude. An interference of the FBS with the DLS measurements could not be observed. RM 11 24 h + 10 % FBS Konz 8 had a z-average diameter of 109 nm at T0 and of 118 nm at Tend.
Samples were centrifuged before the experiment, as an initial intensity test at 20°C showed high scattering due to large particles in the samples, which led to abortion of data collection.

For neither of the samples, but the samples containing the S9 mix, a clear trend toward larger particles sizes could be measured with the tested time frame.

DLS detailed results

Table 12: z-average and intensity-based D10, D50 and D90 radii, T0

Sample name	Replicate	z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
4 h Negative control – S9 mix	1	14.5	7.2	105.4	149.0	213.5
	2	1.7	0.8	172.5	235.9	326.8
	3	1	0.5	66.7	100.4	150.4
	AVG ± STD	5.7 ± 7.6	2.8 ± 3.8	114.9 ± 53.6	161.8 ± 68.7	230.3 ± 89.4
4 h Solvent control – S9 mix	1	6.3	3.2	1.5	3.7	128.0
	2	5.6	2.8	2.0	3.9	7.7
	3	6.9	3.5	1.7	4.5	80.4
	AVG ± STD	6.3 ± 0.7	3.2 ± 0.4	1.8 ± 0.2	4 ± 0.4	72.0 ± 60.6
4 h RM11 0.8 µg/mL – S9 mix	1	84	42.0	3.2	67.9	72.6
	2	359.8	179.9	1.4	84.1	89.3
	3	106.2	53.1	7.8	8.4	8.8
	AVG ± STD	183.3 ± 153.2	91.7 ± 76.6	4.1 ± 3.3	53.5 ± 39.8	56.9 ± 42.5
4 h RM11 100 µg/mL – S9 mix	1	163.1	81.6	61.6	69.3	78.2
	2	172.1	86.0	61.5	75.3	93.1
	3	169.2	84.6	65.1	74.3	84.7
	AVG ± STD	168.1 ± 4.6	84.1 ± 2.2	62.7 ± 2.1	73.0 ± 3.2	85.3 ± 7.5
4 h Negative control + S9 mix	1	164	82.0	44.0	94.6	171.8
	2	165.9	82.9	54.0	82.4	127.3
	3	173.1	86.6	46.7	84.2	175.5
	AVG ± STD	167.7 ± 4.8	83.8 ± 2.4	48.2 ± 5.2	87.1 ± 6.6	158.2 ± 26.8
4 h Solvent control + S9 mix	1	168.5	84.3	42.9	93.0	203.1
	2	168.9	84.5	46.9	89.1	160.4
	3	166.5	83.2	43.7	95.0	182.4
	AVG ± STD	168 ± 1.3	84.0 ± 0.7	44.5 ± 2.1	92.4 ± 3.0	182.0 ± 21.4
4 h RM11 0.8 µg/mL + S9 mix	1	164.3	82.1	39.8	99.8	184.5
	2	166.6	83.3	44.1	95.8	180.1
	3	167.1	83.6	44.2	85.2	227.7
	AVG ± STD	166 ± 1.5	83.0 ± 0.8	42.7 ± 2.5	93.6 ± 7.5	197.4 ± 26.3
4 h RM11 100µg/mL + S9 mix	1	165.5	82.7	41.7	86.5	182.1
	2	170	85.0	65.4	89.7	118.4
	3	174.2	87.1	60.0	87.0	121.4
	AVG ± STD	169.9 ± 4.4	84.9 ± 2.2	55.7 ± 12.4	87.7 ± 1.7	140.6 ± 36
24 h Negative control – S9 mix	1	15.3	7.7	3.1	10.8	69.4
	2	15.4	7.7	3.0	11.6	46.4
	3	15.1	7.6	3.3	13.5	64.7
	AVG ± STD	15.3 ± 0.2	7.7 ± 0.1	3.1 ± 0.1	11.9 ± 1.4	60.2 ± 12.1
24 h Solvent control – S9 mix	1	15	7.5	3.1	10.3	70.8
	2	15.4	7.7	2.8	9.9	50.1
	3	16.3	8.1	2.9	9.8	50.3
	AVG ± STD	15.6 ± 0.7	7.8 ± 0.3	2.9 ± 0.2	10.0 ± 0.2	57.1 ± 11.9
24 h RM11 0.8 µg/mL – S9 mix	1	19.6	9.8	3.0	12.7	139.4
	2	35.5	17.7	3.1	16.1	103.1
	3	16	8.0	2.9	10.7	34.7
	AVG ± STD	23.7 ± 10.4	11.8 ± 5.2	3.0 ± 0.1	13.2 ± 2.7	92.4 ± 53.2
24 h RM11 100 µg/mL – S9 mix	1	99.1	49.6	44.7	69.9	93.6
	2	113.5	56.7	51.8	68.0	83.2
	3	115.7	57.8	52.0	63.9	76.5
	AVG ± STD	109.4 ± 9	54.7 ± 4.5	49.5 ± 4.2	67.3 ± 3.0	84.4 ± 8.6

Table 13: z-average and intensity-based D10, D50 and D90 radii, Tend.

Sample name	Replicate	z-average diameter (nm)	z-average radius (nm)	D10 (nm)	D50 (nm)	D90 (nm)
4 h Negative control – S9 mix	1	0.9	0.4	101.2	225.6	563.1
	2	528	264.0	124.4	180.5	269.3
	3	261.2	130.6	55.8	137.1	352.0
	AVG ± STD	263.4 ± 263.6	131.7 ± 131.8	93.8 ± 34.9	181.0 ± 44.3	394.8 ± 151.5
4 h Solvent control – S9 mix	1	5.1	2.6	2.0	3.3	5.8
	2	4.3	2.1	2.0	3.6	6.5
	3	4.2	2.1	1.9	3.5	5.9
	AVG ± STD	4.5 ± 0.5	2.3 ± 0.3	1.9 ± 0.0	3.4 ± 0.2	6.0 ± 0.4
4 h RM11 0.8 µg/mL – S9 mix	1	352.6	176.3	41.1	42.7	45.3
	2	122.4	61.2	20.3	21.9	23.0
	3	394.3	197.2	56.6	58.4	60.3
	AVG + STD	289.8 ± 146.4	144.9 ± 73.2	39.3 ± 18.2	41.0 + 18.3	42.9 + 18.8
4 h RM11 100 µg/mL – S9 mix	1	167.1	83.6	58.1	63.5	69.3
	2	174.1	87.0	57.5	69.9	84.1
	3	185.7	92.9	67.2	73.6	81.0
	AVG + STD	175.6 ± 9.4	87.8 ± 4.7	60.9 ± 5.4	69.0 ± 5.1	78.2 ± 7.8
4 h Negative control + S9 mix	1	278.4	139.2	66.1	142.9	373.5
	2	289.5	144.8	90.1	135.1	210.1
	3	138.7	138.7	94.1	129.6	191.0
	AVG ± STD	235.5 ± 84	140.9 ± 3.4	83.4 ± 15.1	135.9 ± 6.7	258.2 ± 100.3
4 h Solvent control + S9 mix	1	267.5	133.8	74.6	130.2	228.0
	2	260.8	130.4	76.0	154.2	253.3
	3	268.5	134.3	43.9	155.3	226.4
	AVG + STD	265.6 ± 4.2	132.8 ± 2.1	64.8 ± 18.1	146.6 ± 14.2	235.9 ± 15.1
4 h RM11 0.8 µg/mL + S9 mix	1	264.6	132.3	84.4	87.4	90.3
	2	276.4	138.2	68.6	155.2	327.4
	3	276.5	138.3	70.7	145.2	251.3
	AVG ± STD	272.5 ± 6.8	136.3 ± 3.4	74.6 ± 8.6	129.3 ± 36.6	223.0 ± 121.0
4 h RM11 100µg/mL + S9 mix	1	266.6	133.3	47.5	168.0	251.6
	2	270.1	135.0	66.4	143.3	273.3
	3	263	131.5	96.0	137.2	188.3
	AVG ± STD	266.6 ± 3.6	133.3 ± 1.8	70.0 ± 24.4	149.5 ± 16.3	237.7 ± 44.2
24 h Negative control – S9 mix	1	15.5	7.7	3.0	10.3	43.4
	2	15.2	7.6	3.1	10.2	47.4
	3	15.4	7.7	5.1	5.3	5.6
	AVG + STD	15.4 + 0.2	7.7 + 0.1	3.8 + 1.2	8.6 – 2.8	32.1 + 23.1
24 h Solvent control – S9 mix	1	15.1	7.6	3.2	9.6	65.3
	2	15.1	7.6	3.1	10.3	57.2
	3	15.5	7.7	3.4	14.2	63.7
	AVG ± STD	15.2 ± 0.2	7.6 ± 0.1	3.2 ± 0.1	11.4 ± 2.5	62.1 ± 4.3
24 h RM11 0.8 µg/mL – S9 mix	1	17.1	8.5	3.9	6.6	31.7
	2	60	30.0	4.5	42.5	206.1
	3	17.8	8.9	2.4	6.4	35.8
	AVG ± STD	31.6 ± 24.6	15.8 ± 12.3	3.6 ± 1.1	18.5 ± 20.8	91.2 ± 99.5
24 h RM11 100 µg/mL – S9 mix	1	122.8	61.4	10.1	75.2	112.8
	2	121.1	60.5	60.7	79.5	97.5
	3	111.1	55.6	58.1	68.7	77.7
	AVG + STD	118.3 + 6.3	59.2 + 3.1	42.9 + 28.5	74.5 + 5.4	96.0 + 17.6

From Report: 4023314_final_report – RM11: Micronucleus Test in Chinese Hamster V79 Cells in vitro

From Applicants

To reflect the stability of the dispersion and the agglomeration/aggregation behavior of the test material during cell culture exposure in the genotoxicity experiment, particle size determination of the test dispersion using dynamic light scattering (DLS) was performed in

the parallel study (ICCR Study Number 4023312 "RM11: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)") as well (external assignment under non-GLP). In the V79/HPRT study, the test item preparation and exposure were performed under comparable conditions, and thus, the results from the TEM and DLS analyses are considered transferable between the two studies.

In the accelerated stability study, it was demonstrated via dynamic light scattering (DLS) measurements that the test item RM11 showed stable particle sizes without increased aggregation/agglomeration for at least 24 hours. Moreover, samples from the test item exposure were sent for transmission electron microscopy analysis. The cellular uptake of RM11 nanoparticles was demonstrated at all concentration evaluated and the test item was observed exclusively in cytoplasmic vesicles but not in the cell nucleus.

Annex T. Tables and references from the document "Dossier on the Human Safety Evaluation of Titanium Dioxide in Cosmetic Products (CAS No. 13463-67-7, 12026-28-7, 1317-70-0, 1317-80-2, 20338-08-3/ EC No. 236-675-5, 243-744-3, 1317-70-0, 215-282-2, 234-711-4). (Submission I with focus on potential oral exposure). COSMETICS EUROPE INGREDIENT N° S75. 28 April 2023" pages 37-53/84.

Table 8. Datasets reviewed by study type/endpoint and those achieving moderate or higher weight

Study type	Number of datasets reviewed	Number of datasets achieving moderate or higher weight after WoE assessment
In vitro[7]		
Bacterial reverse mutation (Ames test)	15	0
Mammalian cell gene mutation	16	2
Micronucleus (MN) or chromosomal aberration (CA)	62	12
In vivo[8]		
Gene mutation	9	2
MN or CA	35	13
Comet	51	3
8-hydroxy-deoxyguanosine (8-OHdG) adducts	4	2
Totals	**192**	**34**

Table 9: Summary of moderate, moderate-high or high weight *in vitro* Studies

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
Nano, P25 anatase/rutile (NM- 105), average size 21 nm. When dispersed in PBS and sonicated, mean size distribution increased to 112 nm (with FBS) and 296 nm (without FBS). *NanoTEST dispersion protocol used for hazard studies. NPs suspended in 10% FBS (in PBS) at a concentration of 5 mg/mL probe sonicated for 15 min. and diluted in cell culture medium.	Nano score 10. * Titanium dioxide NPs obtained from JRC Nanomaterial Repository (NM-105) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	**HPRT Mutations** **Cell type**: V79-4 cells **Concentrations**: 3-75 µg/cm^2 for 24 hours.	Negative	Top concentration equivalent to 585 µg/mL. Only slight cytotoxicity. ROS/oxidative stress not investigated. ToxR Klimisch score 2	Kazimirova *et al.*, 2020 in Kirkland *et al.*, 2022
Nano (40 nm). *Lack of detail provided about NP preparation for genotoxicity studies. Stock concentration of NPs suspended in deionised water.	Nano score 1. * No information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. No characteris-ation performed in relevant biological medium.	**TK Mutations** **Cell type:** L5178Y cells **Concentrations**: 4 hours treatment – and + S9, 24 hours treatment -S9; 312- 2000 µg/mL in each case.	Negative	Top concentration induced ca.50-60% reduction in RTG. Followed OECD TG 490 (2016). ROS/oxidative stress not investigated. ToxR Klimisch score 1.	Du *et al.*, 2019 in Kirkland *et al.*, 2022
Ultrafine (called uf-C in Warheit *et al.*, 2007; 140 nm median size).	Not done – not relevant	**CA** **Cell type**: CHO-K1 **Concentrations**: 4+16 hours – S9	Negative	GLP study, complied with OECD TG 473 (1998). >60% mitotic inhibition at	Donner (2006); unpublished study report published

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
		at 750, 1250 & 2500 µg/mL; 4 + 16 hours +S9 at 62.5, 125 & 250 µg/mL; 20+0 hours – S9 at 25, 50 & 100 µg/mL.		top concentration in all parts of study. ROS/oxidative stress not investigated. ToxR Klimisch score 1.	in Warheit *et al.*, 2007 in Kirkland *et al.*, 2022
Nano/bulk not specified but for T 805. *Stock concentration of NPs prepared in ethanol for genotoxicity studies.	Nano score 3. *Limited independent characterisation performed – reliant on information provided by the supplier. No characterisation performed in relevant biological medium.	**CA** **Cell type**: CHO cells **Concentrations**: 88.72; 209.7 and 800 µg/mL (-S9 20-hour treatment); 167.8; 640; 800 µg/mL (+S9 3-hour treatment)	Negative	S9 3-hour treatment performed in separate study. 800 µg/mL is approximately 10 mM. OECD TG 473 (1998). ROS/oxidative stress not investigated. ToxR Klimisch score 2.	Riley (1999) in Kirkland *et al.*, 2022
Assumed to be pigmentary since nano is not mentioned.	Not done – not relevant	**CA** **Cell type**: CHO-K1 cells **Concentrations**: 4+16 hours -S9 (25, 50, 100 µg/mL), 4+16 hours +S9 (25, 50, 75, 100, 150 µg/mL), or 20+0 hours -S9 (25, 50, 75 µg/mL).	Negative	Little or no mitotic inhibition but >50% growth inhibition at top concentration. GLP study complied with OECD TG 473 (1998). ROS/oxidative stress not investigated. ToxR Klimisch score 1.	Glover (2011) in Kirkland *et al.*, 2022
Nano; anatase <25 nm (Sigma Aldrich). *NPs suspended in serum free culture medium (0.1 mg/mL) and probe sonicated for 20 minutes on ice for	Nano score 7. * Information on NP characteristics obtained from the supplier provided. Some independent characterisation	**MN** **Cell type**: Caco-2 cells (from ATCC) **Concentrations**: 1, 2, 3.5, 5, 10 and 20 µg/cm2 (corresponding	Negative	6 hours treatment without cytochalasin B may not be long enough for nanos, but 24+24 hours is robust.	Zijno *et al.*, 2015 in Kirkland *et al.*, 2022

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
genotoxicity studies.	performed. Some characterisation performed in relevant biological medium.	to 6.4–128.0 µg/mL) in culture medium (without FCS.); 6 and 24 hours then adding cytochalasin B for 24 hours.		ROS/oxidative stress not investigated in this study but previously shown ROS induced at these concentrations. ToxR Klimisch score 2	
T-LiteTM SF (Titanium dioxide for Sunscreens), 10 x 50 nm, Rutile, coated with aluminium hydroxide and dimethicone/ methicone copolymer. *For the MN assay NPs were suspended in cell culture medium for genotoxicity studies.	Nano score 8. * Some information on NP characteristics obtained from the supplier provided. Independent characterisation also performed. Characterisation performed in relevant biological medium.	**MN** **Cell type**: V79 cells **Concentrations**: 75 to 300 µg/mL for 4-hours; 18.8 to 75 µg/mL for 24 hours.	Negative	The authors clearly identified that NP can be seen on the slides at 2.5 µg/mL and above. ROS/oxidative stress not investigated. ToxR Klimisch score 1.	Landsiedel *et al.*, 2010 in Kirkland *et al.*, 2022
Nano; AEROXIDE P25, (NM-105 manufactured by Evonik for JRC Ispra); 24 nm, 86% anatase/14% rutile. *NPs were suspended in ultrapure sterile water (10 mg/mL) and probe sonicated (in pulsed mode) for 30 min. Suspensions were vortexed and diluted in cell culture medium for genotoxicity studies.	Nano score 8. * Titanium dioxide NPs obtained from JRC Nanomaterial Repository (NM-105) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	**MN** **Cell type**: A549 cells **Concentrations**: 1 – 50 µg/mL over 2 months with 2 medium changes (containing NPs) per week. MN measured at 24 hours, 1 week, 2 weeks, 1 month and 2 months.	Negative	No cytotoxic effect even after 2 months of treatment with 50 µg/mL. ROS increased and oxidative DNA damage (measured with Fpg modified comet) has been shown. ToxR Klimisch score 1.	Armand 2016 in Kirkland *et al.*, 2022

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
Nano; NM-102 (JRC, Ispra) 21 nm. *Nanogenotox dispersion protocol used: NPs were pre-wetted in 0.5% ethanol and then suspended in 0.05% BSA in MilliQ water (2.56 mg/mL) and probe sonicated for 16 min. on ice. Stock suspension diluted in cell culture medium for genotoxicity studies.	Nano score 9. * Titanium dioxide NPs obtained from JRC Nanomaterial Repository (NM-102) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	**MN** **Cell type**: BEAS-2B cells **Concentrations**: 1, 10 and 20 µg/mL for acute (24 hours) and chronic treatment (1 to 3 weeks); sequential treatment with NPs and cytochalasin B.	Negative	Cytotoxicity not assessed. Oxidative stress investigated but no positive effect for titanium dioxide. ToxR Klimisch score 1.	Vales *et al.*, 2014 in Kirkland *et al.*, 2022
Nano; NM-100 (anatase, 50–150 nm, non-coated), NM-101 (anatase, 5–8 nm, coated) and NM-103 (rutile, 20–28 nm, coated). *NANOoREG dispersion protocol used for hazard studies: NPs were suspended in 0.05% BSA in MilliQ water (2.56 mg/mL) and probe sonicated for 15 min. on ice. Stock suspensions were then diluted in 0.05% BSA to a concentration of 0.1 mg/mL and then diluted in cell culture medium.	Nano score 10. * Titanium dioxide NPs obtained from JRC Nanomaterial Repository (NM-101 and NM-103) which have been extensively characterised and this information is summarised. Additional characterisation performed in relevant biological medium.	**MN** **Cell type**: BEAS-2B cells **Concentrations**: 1–30 µg/mL, 3-, 24- or 48-hour treatments under serum free conditions. MN scored with flow cytometry and manually by the CBMN cytochalasin B assay (added after 20 hours).	Weak positive (<2-fold and inverse dose-response) for NM-103	Authors noted induction of oxidative DNA damage for all three materials & increased necrotic cells particularly for NM-103. ToxR Klimisch score 1.	Di Bucchianico *et al.*, 2017 in Kirkland *et al.*, 2022
Commercial titanium dioxide (84% anatase, 16% brookite crystal phase composition, 8), NP as nanopowder and as colloidal	Nano score 6. * Information on NP characteristics obtained from the supplier provided. Some independent characterisation	**MN** **Cell type** : Balb/3T3 cells **Concentrations**: 10, 20 and 40 µg/cm^2, (corresponding to 32,	Positive for citrate-coated titanium dioxide and P25 (only at lowest concentrat	Oxidised purines & pyrimidines induced by all particles tested. Significant apoptotic & necrotic cells	Stoccoro *et al.*, 2016 in Kirkland *et al.*, 2022

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
nanosuspension (nanosol). Pristine (uncoated), citrate-coated and silica-coated titanium dioxide were tested with Aeroxide® P25 as benchmark material. *No information on NP preparation for genotoxicity studies provided.	also performed. Some characterisation performed in relevant biological medium.	64, and 128 µg/mL); 48 hour treatment	ion), others weakly positive.	induced by citrate-coated & P25. ToxR Klimisch score 1.	
Nano: Anatase 20-60 nm; Rutile 30 x 100 nm rods; Mixture anatase and rutile 45 – 262 nm; Anatase 50 – 270 nm; Rutile 50 – 3000 nm (Sigma-Aldrich, USA). *NPs were suspended in cell culture medium without serum and bath sonicated for 45 min.	Nano score 4. * Reliant on information provided by the supplier. Limited independent characterisation performed. Some characterisation performed in relevant biological medium.	**MN** **Cell type**: Human peripheral blood lymphocytes from 2 healthy male donors (<40 years old) **Concentrations**: 50, 100 and 200 µg/mL, 20 hours.	Negative for all particle types	Authors used 2 protocols: (1)sequential treatment (20 hours NP and then cytochalasin B was added for the next 28 hours); (2) co-treatment (30 min NP alone and then together with cytochalasin B for 28 hours). The results did not differ. Treatments carried out in the dark. Oxidative DNA damage suggested, 8-OHdG induced at highest concentration. 100 and 200 µg/mL. ToxR Klimisch score 1	Andreoli *et al.*, 2018 in Kirkland *et al.*, 2022
AEROXIDE_ P25 (Degussa- Evonik);	Nano score 3 *Reliant on	**MN** **Cell type**: A549, A172,	Negative	Uptake of titanium dioxide was	Brandao *et al.*, 2020 in

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
25 nm, 80% anatase/20% rutile. *NPs were suspended in cell culture medium and probe sonicated for 5 min. on ice (1.5 min. on and 1 min. off twice, and 2 min. on) for genotoxicity studies.	information provided by the supplier. Limited characterisation performed in relevant media. *Whilst limited information on NP characteristics was provided in the manuscript P25 has been extensively characterised in the published literature.	HepG2 & SH-SY5Y cells **Concentrations**: 10, 50, 100 and 200 µg/mL, 3- and 24-hours treatments.		clearly shown for all cell lines. ROS/oxidative stress not investigated. ToxR Klimisch score 1.	Kirkland *et al.*, 2022
Commercial rutile (TiPure R-103). *NPs were suspended in cell culture medium for genotoxicity studies.	Nano score 6. *No information on NP characteristics45 obtained from the supplier provided. Some independent characterisation performed. No Characterisation in relevant biological medium.	**MN** **Cell type**: L-929 mouse fibroblasts **Concentrations**: 15, 30 and 60 ppm, 6- and 24-hour exposures without S9, cytochalasin B then added until harvest at 72 hours. Data given for 24-hour exposures only	Negative	Agglomeration of nanos in culture medium. ROS/oxidative stress not investigated. ToxR Klimisch score 1.	Pittol *et al.*, 2018 in Kirkland *et al.*, 2022

(CE, 2022, 2023; TDMA, 2022; Kirkland *et al.*, 2022)

Table 10. Summary of recently conducted *in vitro* studies on titanium dioxide nano grade

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
Nano: RM09 (purity ≥99%; surface modification: coated with amorphous silica; particle number size distribution: number weighted median x50: 20 nm measured by SEM, Feret min) *NPs were suspended following the Nanogenotox protocol (Jensen *et al.*, 2011); suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication	* Information on NP characteristics obtained from Sponsor. Dispersion stability analysis in relevant biological medium.	**HPRT Mutations** **Cell type**: V79 lung fibroblast cell line **Concentrations**: 0.8-100 µg/mL for 24 hours in the absence of S9 (since test item core and coating are inorganic materials, which are not metabolised by S9 fraction)	Negative	The cellular uptake of RM09 nanoparticles by V79 cells was demonstrated and the test substance was observed exclusively in cytoplasmic vesicles but not in the cell nucleus.	Sokolowski, 2023 in CE, 2023

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
Nano: RM11 (purity ≥99%; surface modification: coated alumina and dimethicone; particle number size distribution: Number weighted median x50: 19 nm measured by SEM, Feret min) * NPs were suspended following the Nanogenotox protocol (Jensen *et al.*, 2011); suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication	* Information on NP characteristics obtained from Sponsor. Dispersion stability analysis in relevant biological medium.	**HPRT Mutations** **Cell type**: V79 lung fibroblast cell line **Concentrations**: 0.8-100 µg/mL for 24 hours in the absence of S9, and for 4 hours in the absence and presence of S9.	Negative	The cellular uptake of RM11 nanoparticles by V79 cells was demonstrated at all concentrations evaluated and the test substance was observed exclusively in cytoplasmic vesicles but not in the cell nucleus.	Sokolowski, 2023 in CE, 2023
Nano: RM09 (purity ≥99%; surface modification: coated with amorphous silica; particle number size distribution: number weighted median x50: 20 nm measured by SEM, Feret min). * NPs were suspended following the Nanogenotox protocol (Jensen *et al.*, 2011);	* Information on NP characteristics obtained from Sponsor. Dispersion stability analysis in relevant biological medium (data from Sokolowski, 2023).	**MN** **Cell type**: Chinese hamster lung fibroblast V79 cell line **Concentration**: 1.1-100 µg/mL for 24 hours in the absence of S9 (since test item core and coating are inorganic materials, which are not metabolised by S9 fraction)	Negative	The cellular uptake of RM09 nanoparticles by V79 cells was demonstrated and the test substance was observed exclusively in cytoplasmic vesicles but not in the cell nucleus (data from Sokolowski, 2023).	Naumann, 2023 in CE, 2023

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication					
Nano: RM11 (purity ≥99%; surface modification: coated with amorphous silica; particle number size distribution: number weighted median x50: 19 nm measured by SEM, Feret min), * NPs were suspended following the Nanogenotox protocol (Jensen *et al.*, 2011); suspended in 0.05% w/v bovine serum albumin (BSA)-water solution containing 0.5% ethanol using ultrasonication	* Information on NP characteristics obtained from Sponsor. Dispersion stability analysis in relevant biological medium (data from Sokolowski, 2023).	**MN** **Cell type**: Chinese hamster lung fibroblast V79 cell line **Concentration**: 1.1-100 µg/mL for 24 hours in the absence of S9, and for 4 hours in the absence and presence of S9 followed by 20 hours recovery	Negative	The cellular uptake of RM11 nanoparticles by V79 cells was demonstrated and the test substance was observed exclusively in cytoplasmic vesicles but not in the cell nucleus (data from Sokolowski, 2023).	Naumann, 2023 in CE, 2023

Table 11: Summary of moderate, moderate-high or high weight *in vivo* studies (adapted from Kirkland *et al.*, 2022)

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
Unitane 220 (comparable to food grade E-171)	Not done – not relevant	**Bone marrow CA** **Species:** Mice **Doses:** Single IP dose of 625, 1250 & 2500 mg/kg; bone marrow sampled 17 & 36 hours later	Negative with some limitations.	Only 50 cells/animal scored for CA. Not clear whether slides coded. No direct measure of bone marrow toxicity, but %PCE reduced in MN study in same paper. IP route not considered physiologically relevant. ROS/oxidative stress not investigated. ToxR Klimisch score 2	Shelby & Witt 1995 in Kirkland *et al.*, 2022
Unitane 220 (comparable to food grade E-171)	Not done – not relevant	**Bone marrow and blood MN** **Species**: Mice **Doses**: 3 IP studies. 3 daily doses, #1: 250, 500 & 1000 mg/kg bw/day, bone marrow 24 hours; #2: "DRF" 500, 1000 &1500 mg/kg bw/day,	Positive, with reproducible, weak increase at 1000 mg/kg bw/day in bone marrow, but at lowest dose in blood so no significant trend.	IP route not considered physiologically relevant. Only 2000 PCE/animal scored for MN. Peripheral blood 52% toxicity seen; minimal bone marrow toxicity ROS/oxidative stress not investigated. ToxR Klimisch score 1.	Shelby & Witt, 1995 & Shelby *et al*. 1993 Kirkland *et al.*,2022

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
		Peripheral blood 48 hours; #3: 500, 1000, 1500 mg/kg, bone marrow 24 hours			
E171 purchased from Bolsjehuset (Denmark). 99.8% anatase, 0.2% rutile. *NPs suspended in saline with 0.25% lecithin and sonicated for 5 min for genotoxicity studies.	Nano score 7. *Some information on NP characteristics obtained from the supplier provided. Some independent characterisation also performed. Characterisation performed in relevant biological medium. *NB The characteristics of the NPs (P25) have been extensively	**8-OHdG adducts in lung cells** **Species**: Rats **Dose:** Single intratracheal instillation of 0.15, 0.3, 0.6 & 1.2 mg. Tissues sampled 90 days later	Negative	Although 30 rats/group were treated, unclear how many were sampled. No oxidative damage found. ToxR Klimisch score 1	Rehn *et al.*, 2003 in Kirkland *et al.*, 2022
Nano (Aeroxide P25). * NP suspended in drinking water and bath sonicated for 15 minutes.	Nano score 6. * Information on NP characteristics obtained from supplier provided and this information is summarised. Limited independent characterisation performed but P25 has been extensively characterised, and citations are provided to relevant literature. Some characterisations in relevant	**Peripheral blood MN** **Species**: Mice **Doses:** Drinking water, 50, 100, 250, and 500 mg/kg total from 5 days dosing. Water consumption ranged 3-7 mL/mouse/day. Average of 5 mL/day for 30g avg. weight mouse was used to calculate total dose.	Positive, 2.1x increase at top dose, but error bars for control and treated measurements overlap, so may not be biologically relevant.	Not clear whether NCE or PCE were scored. Difficult to verify exposure doses from the descriptions, and whether settling out of particles in drinking water was controlled. Oxidative stress indicated since 8-OHdG increased, and evidence of pro- inflammatory response. ToxR Klimisch score 1.	Trouiller *et al*. 2009 in Kirkland *et al.*, 2022

Type of titanium dioxide tested	Nanoparticle characterisation	Endpoint tested/ method	WoE conclusion	Comments	Reference
	biological medium.				
Micro (TDM) and nano simethicone (TDN). *NPs suspended in distilled water for genotoxicity studies.	Nano score 2. *No information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. No characterisation in relevant biological medium.	**Bone marrow, forestomach, colon & testis MN** **Species:** Mice **Doses:** Oral dosing at 40, 200, and 1000 mg/kg bw/day for 7 days. Bone marrow and testis sampled 24 hours after last dose.	TDM induced 2X increase in MN in bone marrow; TDN simethicone was negative. TDM and TDN negative in forestomach, colon & testis.	TDM and TDN induced apoptosis in testis and cytotoxicity in forestomach & colon. Authors conclude genotoxic effects are secondary to inflammation and/or oxidative stress. ToxR Klimisch score 3, unreliable.	Sycheva *et al.*, 2011 in Kirkland *et al.*, 2022
Nano, 10 nm anatase. *NPs suspended in PBS (5 mg/mL) and vigorously mixed and sonicated for genotoxicity studies.	Nano score 7. * NPs synthesized by the researchers. Characterisation of NPs performed. Some characterisation performed in relevant biological medium.	**Peripheral blood reticulocytes MN** **Species**: Mice **Doses**: IV dosing at 0.5, 5.0, and 50 mg/kg bw/day for 3 days. Blood sampled on day 4.	Negative	Target tissue exposure assessed by measuring titanium in bone marrow. ROS/oxidative stress not investigated. ToxR Klimisch score 1	Sadiq *et al.*, 2012 in Kirkland *et al.*, 2022
Nano, anatase (ST-01), 5 nm. *NPs suspended in 2 mg/mL disodium phosphate followed by agitation in a bead mill with 15 µm zirconium oxide beads for 2 hours, centrifuged and the supernatant used for genotoxicity studies.	Nano score 5. *Limited information on NP characteristics obtained from the supplier provided. Limited independent characterisation performed. Some characterisation in relevant biological medium.	**Comet in lung** **Species**: Rats **Doses:** Intratracheal instillation; 1 & 5 mg/kg single dose, 0.2 & 1 mg/kg once per week for 5 weeks.	Negative	Slides not coded. Inflammatory response at 1 & 5 mg/kg. Inflammation induced, oxidative stress discussed, but no DNA damage. ToxR Klimisch score 2.	Naya *et al.*, 2012 in Kirkland *et al.*, 2022

Table 12. Comparison of test response profiles from titanium dioxide to the profile characteristics of confirmed genotoxic carcinogens (adapted from Brusick *et al.*, 2016; based on Bolt *et al.*, 2004 and Petkov *et al.*, 2015)

Characteristic	Carcinogens with a proven genotoxic mode of action	Titanium dioxide
Profile of Test Responses in Genetic assays	Positive effects across multiple key predictive endpoints (i.e., high weight studies such as gene mutation in bacteria or *in vivo*, chromosomal aberrations or micronuclei *in vivo).*	No valid evidence for gene mutation in mammalian cells or *in vivo*; chromosomal damage in rodents only at doses inducing cytotoxicity, inflammation, oxidative stress.
Structure Activity Relationships	Positive for structural alerts associated with genetic activity.	Not done
DNA binding	Agent or breakdown product are typically electrophilic and exhibit direct DNA binding.	No evidence of DNA binding, and no evidence of 8-OHdG adducts in robust *in vivo* studies.
Consistency	Positive test results are highly reproducible both *in vitro* and *in vivo.*	Conflicting and/or non-reproducible responses in the same test or test category both *in vitro* and *in vivo.*
Response Kinetics	Responses are dose dependent over a wide range of exposure levels.	Dose responses in robust, reliable test systems generally not observed.
Susceptibility to Confounding Factors (e.g. Cytotoxicity)	Responses are typically found at nontoxic exposure levels.	Positive responses in robust, reliable test systems typically associated with evidence of apoptosis, necrosis, inflammation, and oxidative stress.

(TDMA, 2022; Kirkland *et al.*, 2022)

The SCCS note: not all references cited in the text were listed by the Applicant in the References section they provided.

Annex U. The SCCS analysis of two *in vitro* study reports submitted by the Applicant, which did not include any genotoxic endpoint

IN VITRO STUDY #1. The alveolar macrophage assay

Materials and methods

Physicochemical characterisation of raw materials

The following TiO_2 raw materials were tested by the Applicant:

Table 1. Specific surface area of test particles

Particle name	BET (m^2/g)
E171-E	10
G1-1b	48
G2-5	302
G3-1	6
G4-19	15
G5-4	14
G6-3	70
G7-5b	57
G8-2b	65, 65.9, 64.0[1)]
G9-5c	8
G9-5d	8
G10-4	80
G16-5	n.d.
G17-5	n.d.

n.d.: not determined

To prepare stock suspensions of all particles for cell culture experiments, all powder materials were retrieved with heat-sterilized spatula from their containers and were dispersed in sterile pyrogen free H20 at a concentration of 2 mg/mL. Suspensions were vortexed and ultrasonicated for 12 s, using a Branson 450D Sonifier, equipped with a 5 mm sonotrode; total ultrasonic energy amounted to 18 J/mL. As shown in the Figure below, all aqueous suspensions prepared this way tended to settle and were re-suspended before each testing round.

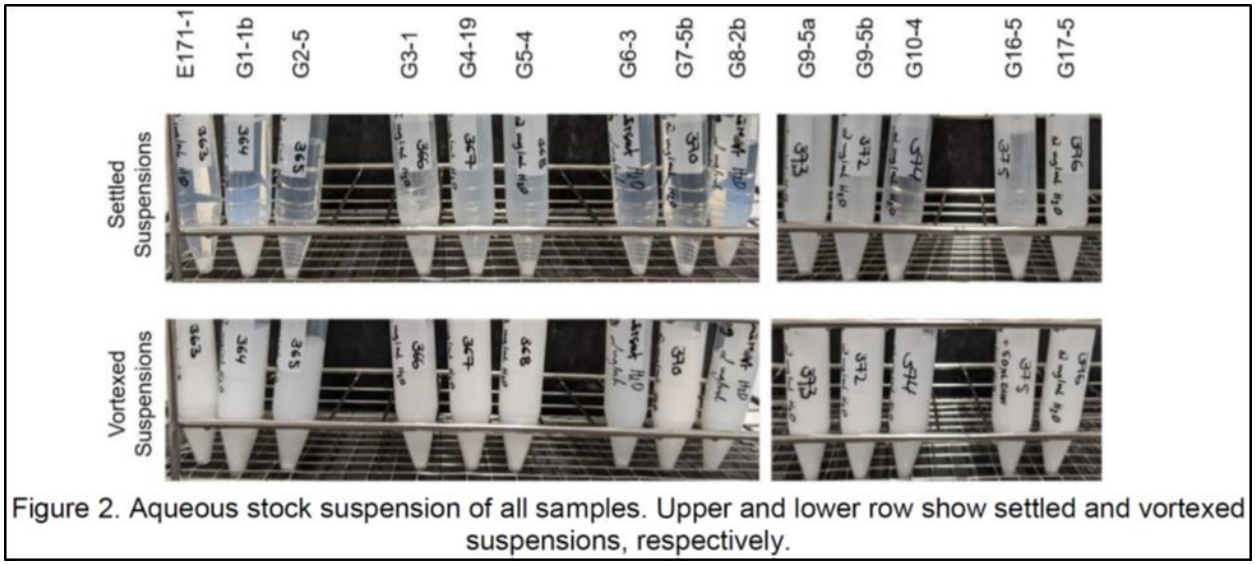


Figure 2. Aqueous stock suspension of all samples. Upper and lower row show settled and vortexed suspensions, respectively.

Two materials, namely G16-5 and G6-3, were found to be too hydrophobic to be dispersed in H_2O and were pre-wetted with a low volume of ethanol (50 µL added to 22 mg of dry powder) thus allowing the subsequent immersion in H_2O. Of note, the final concentrations of ethanol and in the cell assay amounted to less than 0.05 % (v/v), which is without measurable effect on the toxicological assays, as previously reported.
The particle size distribution of the stock suspensions was determined by particle tracking analyses (PTA) which calculated the hydrodynamic particle diameter from recorded particle trajectories.

Biological testing
NR8383 cells, alveolar macrophages that were isolated from the lungs of a normal rat (ATCC, USA; ATCC® Number: CRL-2192TM) were maintained in F-12K cell culture medium supplemented with 15% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% L-glutamine as described by Wiemann *et al.*, 2016 (doi: 10.1186/s12951-016-0164-2). For the assay, cells were seeded into 96-well plates (3 x 105 cells/well) and kept at 37 °C and 5 % CO2. Each well contained 200 µL F-12K cell culture medium in which the concentration of FCS was reduced to 5%. After 24 h, the medium was replaced by serum-free test material preparations: to determine the release of LDH, GLU and TNF from the cells, the test material suspensions were serially diluted to 90, 45, 22.5, and 11.25 µg/mL with serum-free F-12K. To measure release of H_2O_2, the same dilutions were prepared in KRPG buffer (129 mM NaCl, 4.86 mM KCl, 1.22 mM $CaCl_2$, 15.8 mM NaH_2PO_4, 5-10 mM glucose; pH 7.3-7.4).

Assays were carried out as described (Wiemann *et al.*, 2016; doi: 10.1186/s12951-016-0164-2). In brief, H_2O_2 released into the KRPG supernatant was quantified with the Amplex Red® assay measuring the formation of resorufin. Lactate dehydrogenase (LDH) activity was measured photometrically (in triplicates) using 50 µL from each well for the Roche Cytotoxicity Kit and measured according to the manufactures protocol. To measure glucuronidase (GLU) activity, 50 µL of the supernatant (sampled after 16-h test material incubation) were incubated with 100 µL 0.2 M sodium acetate buffer (pH 5) containing 13.3 mM p-nitrophenyl-D-glucuronide and 0.1% Triton X-100. Concentration of tumor necrosis factor α (TNF) was determined with a specific enzyme-linked immosorbent assay (ELISA) for rat TNF (Quantikine ELISA Kit, Bio-Techne GmbH, Wiesbaden-Norderstadt, Germany) according to the manufacturer's protocol.

RESULTS

Physicochemical characterisation of raw materials

Calculated hydrodynamic diameter values (Mean and Mode values, D10, D15 and D90 values are listed in Table below). Mode values ranged from 59.1 (G17-5) to 277.9 (G7-5b) and hardly exceeded 300 nm.

Table 2. Particle size characteristics in aqueous suspension

Particle name	Pestled in a Mortar[1)]	Dispersion Protocol	Conc. for Measureing [µg/mL]	Diluent	Particles/mL[2)]	Hydrodynamic Diameter[3)]				
						Mean ± SEM	Mode ± SEM	D10 ± SEM	D50 ± SEM	D90 ± SEM
E171-E	no	1	18	H_2O	1.16E+09	179.2 ± 2.1	137.9 ± 13.3	87.3 ± 0.9	167.9 ± 4	278.3 ± 4.7
G1-1b	no	1	18	H_2O	1.14E+09	180.4 ± 9.2	169.4 ± 27.5	87.2 ± 14.8	182.6 ± 10	268.7 ± 7
G2-5	no	1	18	H_2O	2.66E+08	204.4 ± 19.3	120.2 ± 45.4	78.1 ± 22.2	208.9 ± 22	311.4 ± 6.8
G3-1	no	1	n.m.	H_2O	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
G4-19	no	1	18	H_2O	1.60E+09	191.7 ± 2.2	140.7 ± 7.6	117.9 ± 4.8	188.5 ± 4.9	272.8 ± 1.6
G5-4	no	1	18	H_2O	1.32E+09	218.5 ± 2.3	214.2 ± 39.1	132.2 ± 2.4	225.5 ± 4.8	296 ± 2.7
G6-3	yes	1	18	H_2O	9.64E+08	214.5 ± 3.8	171.6 ± 5.8	126.8 ± 3.3	204.5 ± 7	312 ± 6.1
G7-5b	no	1	18	H_2O	6.18E+08	252.2 ± 5.7	277.9 ± 12.5	156.8 ± 10.9	261.1 ± 4.9	340.5 ± 4.8
G8-2b	yes	2	18	H_2O	1.31E+09	173.1 ± 4.3	146.5 ± 1.8	104.8 ± 1.1	159.2 ± 1.7	267 ± 10.3
G9-5c	no	1	18	H_2O	2.88E+08	237 ± 4.9	211.4 ± 29	136 ± 4.4	240.6 ± 8.9	321.2 ± 4.1
G9-5d	no	1	18	H_2O	7.04E+08	230.2 ± 2	214.4 ± 51.3	123.1 ± 5.1	239.3 ± 0.8	317.6 ± 4.1
G10-4	no	1	n.m.	H_2O	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
G16-5	no	2	18	H_2O	1.10E+09	202.1 ± 5.3	208.3 ± 9.7	117.6 ± 13	204.6 ± 8.2	284.3 ± 1
G17-5	no	1	18	H_2O	1.13E+08	174.2 ± 4.7	59.1 ± 16.6	62.5 ± 12.1	158.1 ± 12.6	299.7 ± 34.2

Dispersion Protocol 1: Particles were dispersed in H_2O (2.56 mg/ mL) by vortexing followed by ultrasonic treatment with 18 J/mL. Protocol 2: As Protocol 1, but particles were pre-wetted with ethanol.
[1)] Size reduction was carried out for samples with large particle aggregates/agglomerates using a hand-operated pestle in a porcelain mortar for 5 min. [2)] As indicated by the NTA software; not validated by a dilution experiments. [3)] According to NTA software Ver. 3.0 for n=3 measurements.

Also, in the Figure below, no values larger than 400 nm were found. Of note, no nanosized TiO_2 particles were detectable by PTA in cell culture (37oC) medium or KRPG buffer incubated with the particles for 16 h (at or 90 min, respectively). The absence of diffusible TiO_2 (nano)particles under these conditions shows that nanoparticles present in aqueous stock suspensions agglomerate upon transfer into physiological media and are subject to complete gravitational settling.

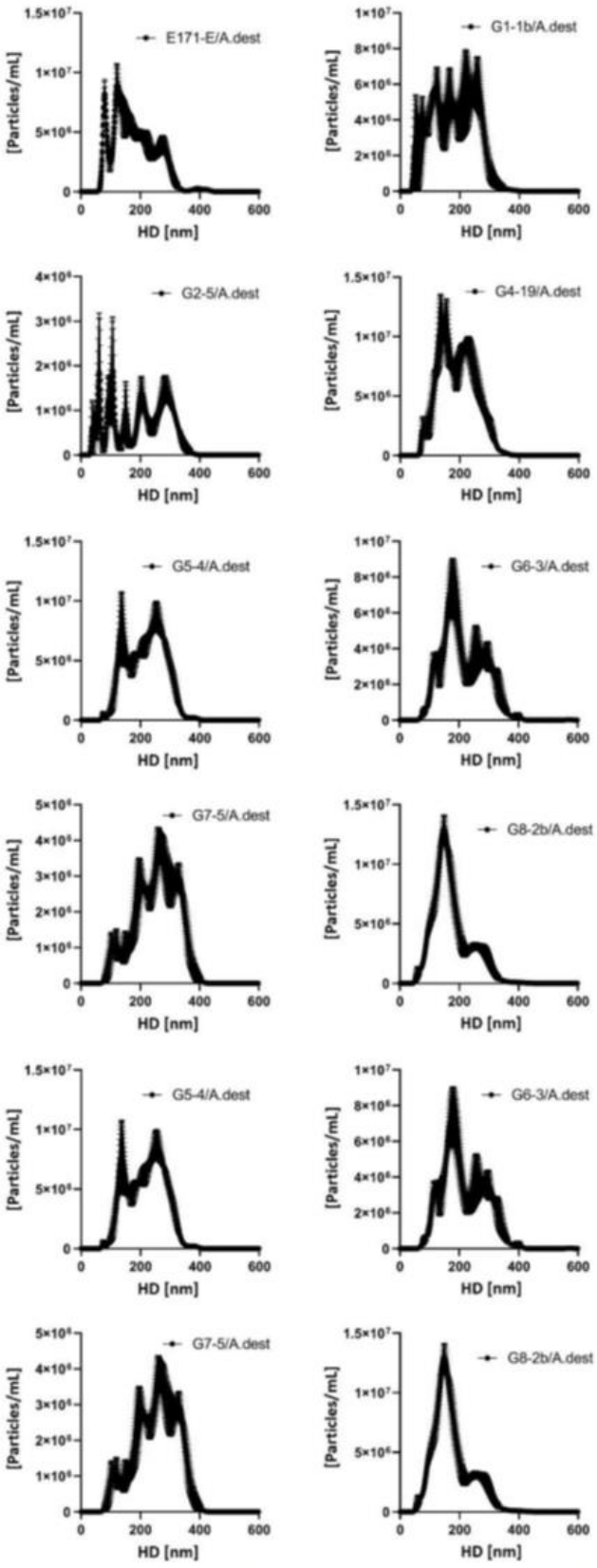


Figure 3. Particle size distribution (hydrodynamic diameter) of 12 TiO_2 materials in dH_2O, as determined with particle tracking analyses.

***In vitro* findings**

In the macrophage assay, all TiO_2 materials were applied at a nominal concentration of 22.5, 45, 90, and 180 µg/mL. Four parameters were tested in the cell culture supernatant after administration of particles. Lactate dehydrogenase (LDH, a cytoplasmic enzyme), glucuronidase (GLU, a (phago)lysosomal enzyme), and tumor necrosis factor α (TNF α) were measured after 16 h. The concentration of H_2O_2 released from the cells was measured in KRPG buffer after 90 min.

TiO_2 samples

In general, the response of the NR8383 alveolar macrophages to all TiO_2 samples were largely uniform (Table below). Most materials elicited moderate dose-dependent increases of LDH and GLU beginning at concentrations of 45-90 µg/mL. Even at the maximum concentration (180 µg/mL) baseline values of the cell control were hardly doubled. Induction of H_2O_2 formation/release was measurable at a low level and significant values were reached at 90-180 µg/mL. Induction of TNF was hardly found except for G8-2b, where an endotoxin contamination was found to be a likely explanation.

Table 3. Results from in vitro tests with the alveolar macrophage model.

	[µg/mL]	LDH [% of pos. CTR] mean ± SD	GLU [% of pos. CTR] mean ± SD	H_2O_2 [µmol/L] mean ± SD	TNFα [pg/mL] mean ± SD
E171-E	0	18.53 ± 0.97	1.38 ± 0.12	1.17 ± 0.48	20.16 ± 4.44
	22.5	21.48 ± 1.11	1.75 ± 0.50	0.39 ± 0.12	18.86 ± 10.31
	45	24.23 ± 0.62 *	2.12 ± 0.21 **	0.34 ± 0.45	23.76 ± 6.15
	90	28.96 ± 2.65 ***	2.24 ± 0.22 **	0.82 ± 0.25	25.81 ± 6.80
	180	31.49 ± 3.59 ***	3.10 ± 0.28 ***	1.54 ± 0.67	24.26 ± 0.38
G1-1b	0	18.53 ± 0.97	1.38 ± 0.12	1.17 ± 0.48	20.16 ± 4.44
	22.5	21.29 ± 0.64	1.78 ± 0.15	0.78 ± 0.32	27.78 ± 14.64
	45	27.32 ± 1.73 ***	1.75 ± 0.21	0.98 ± 0.27	37.68 ± 26.41
	90	39.14 ± 2.59 ***	2.33 ± 0.26 ***	1.41 ± 0.44	32.42 ± 4.52
	180	63.41 ± 4.02 ***	5.16 ± 0.50 ***	2.11 ± 0.31 **	35.98 ± 3.95
G2-5	0	18.53 ± 0.97	1.38 ± 0.12	1.17 ± 0.48	20.16 ± 4.44
	22.5	18.40 ± 0.27	1.85 ± 0.18	0.94 ± 0.14	24.20 ± 10.46
	45	24.57 ± 0.68 **	1.95 ± 0.40	1.75 ± 0.27	34.25 ± 19.74
	90	31.80 ± 0.75 ***	2.68 ± 0.45 ***	2.82 ± 0.42 ***	33.12 ± 4.01
	180	39.21 ± 3.85 ***	3.65 ± 0.44 ***	3.03 ± 0.31 ***	43.97 ± 6.40 *
G3-1	0	18.53 ± 0.97	1.38 ± 0.12	1.17 ± 0.48	20.16 ± 4.44
	22.5	18.54 ± 0.37	1.61 ± 0.37	1.12 ± 0.33	20.57 ± 5.23
	45	22.62 ± 1.44	1.92 ± 0.26	1.14 ± 0.28	22.56 ± 4.93
	90	25.95 ± 2.75 **	2.36 ± 0.18 ***	1.50 ± 0.27	24.15 ± 7.17
	180	34.24 ± 3.55 ***	3.70 ± 0.41 ***	2.07 ± 0.38 **	22.95 ± 3.01
G4-19	0	16.27 ± 1.74	1.20 ± 0.02	1.07 ± 0.28	19.68 ± 5.10
	22.5	18.27 ± 3.36	1.77 ± 0.38 *	0.61 ± 0.25	17.72 ± 2.40
	45	23.33 ± 1.22 **	2.16 ± 0.16 ***	1.18 ± 0.56	21.74 ± 4.91
	90	27.63 ± 0.87 ***	2.41 ± 0.17 ***	1.47 ± 0.49	21.74 ± 4.98
	180	29.52 ± 1.81 ***	2.90 ± 0.30 ***	2.58 ± 0.25 ***	20.04 ± 1.26
G5-4	0	16.27 ± 1.74	1.20 ± 0.02	1.07 ± 0.28	19.68 ± 5.10
	22.5	18.99 ± 2.25	1.72 ± 0.25	0.88 ± 0.35	22.71 ± 9.03
	45	22.98 ± 3.43 **	1.61 ± 0.29	0.85 ± 0.48	22.21 ± 7.76
	90	26.56 ± 2.10 ***	1.94 ± 0.23 **	1.62 ± 0.48	22.16 ± 6.20
	180	28.16 ± 0.75 ***	2.98 ± 0.25 ***	1.43 ± 0.45	22.62 ± 3.50
G6-3	0	16.27 ± 1.74	1.20 ± 0.02	1.07 ± 0.28	19.68 ± 5.10
	22.5	14.83 ± 1.76	1.86 ± 0.31	0.80 ± 0.25	18.23 ± 4.08
	45	21.08 ± 3.09 *	2.31 ± 0.69 **	1.02 ± 0.11	18.35 ± 3.49
	90	25.89 ± 1.60 ***	2.34 ± 0.40 **	1.79 ± 0.14 **	18.94 ± 3.35
	180	27.32 ± 1.30 ***	2.61 ± 0.61 ***	2.69 ± 0.36 ***	18.99 ± 0.54
G7-5b	0	16.27 ± 1.74	1.20 ± 0.02	1.07 ± 0.28	19.68 ± 5.10
	22.5	17.26 ± 3.09	1.34 ± 0.47	1.13 ± 0.27	19.96 ± 8.76
	45	18.95 ± 2.89	1.32 ± 0.33	1.43 ± 0.40	21.22 ± 8.25
	90	23.23 ± 1.14 **	1.70 ± 0.42	1.69 ± 0.18 *	29.79 ± 11.69
	180	30.04 ± 2.50 ***	3.24 ± 0.77 ***	2.17 ± 0.37 ***	32.43 ± 6.67
G8-2b	0	16.81 ± 3.51	1.20 ± 0.16	0.96 ± 0.58	17.93 ± 4.72
	22.5	19.69 ± 1.95	1.81 ± 0.28	0.55 ± 0.32	127.64 ± 42.35
	45	22.88 ± 1.73 *	2.11 ± 0.51 *	0.95 ± 0.32	349.08 ± 85.46 ***
	90	27.71 ± 2.97 ***	1.64 ± 0.48	1.67 ± 0.33	1032.10 ± 187.94 ***
	180	30.67 ± 2.23 ***	1.25 ± 0.82	2.75 ± 0.75 ***	1892.39 ± 103.95 ***
G8-2b [1] (heated)	0	19.84 ± 3.25	1.29 ± 0.20	±	8.40 ± 0.99
	22.5	16.99 ± 3.21	2.65 ± 1.29	±	10.22 ± 0.82
	45	23.26 ± 3.40	2.50 ± 0.64	±	11.89 ± 1.92
	90	27.11 ± 3.68	2.63 ± 0.39	±	12.70 ± 2.12
	180	27.82 ± 1.82	2.73 ± 0.02	±	15.58 ± 3.22
G8-2b [1]	0	19.84 ± 3.25	1.29 ± 0.20	±	8.40 ± 0.99
	22.5	17.99 ± 4.48	2.20 ± 0.02	±	55.69 ± 19.19
	45	22.36 ± 2.80	2.47 ± 0.13	±	101.27 ± 37.20 *
	90	28.95 ± 6.00	2.87 ± 0.50 *	±	353.29 ± 156.83 ***
	180	33.18 ± 1.80 **	2.42 ± 0.21	±	1103.00 ± 402.80 ***

Table 3 (continued). Results from in vitro tests with the alveolar macrophage model.

	[µg/mL]	LDH [% of pos. CTR] mean ± SD	GLU [% of pos. CTR] mean ± SD	H_2O_2 [µmol/L] mean ± SD	TNFα [pg/mL] mean ± SD
G9-5c	0	16.81 ± 3.51	1.20 ± 0.16	0.96 ± 0.58	17.93 ± 4.72
	22.5	18.28 ± 2.45	1.46 ± 0.38	1.03 ± 0.28	20.67 ± 7.37
	45	24.08 ± 1.02 **	1.93 ± 0.41 *	1.06 ± 0.30	22.12 ± 8.82
	90	25.73 ± 2.52 ***	2.11 ± 0.11 **	1.44 ± 0.23	23.15 ± 8.68
	180	29.39 ± 3.29 ***	3.26 ± 0.63 ***	1.76 ± 0.47 *	21.24 ± 3.75
G9-5d	0	16.81 ± 3.51	1.20 ± 0.16	0.96 ± 0.58	17.93 ± 4.72
	22.5	16.07 ± 1.01	1.64 ± 0.24	0.71 ± 0.52	23.04 ± 10.61
	45	21.53 ± 1.24	1.59 ± 0.22	1.33 ± 0.30	23.18 ± 8.90
	90	24.26 ± 2.35 *	1.67 ± 0.34	2.28 ± 0.12 **	23.92 ± 9.27
	180	30.01 ± 5.53 ***	2.41 ± 0.53 ***	2.78 ± 0.75 ***	20.94 ± 3.17
G10-4	0	16.81 ± 3.51	1.20 ± 0.16	0.96 ± 0.58	17.93 ± 4.72
	22.5	16.20 ± 1.55	1.55 ± 0.22	0.99 ± 0.14	22.38 ± 8.77
	45	20.47 ± 1.67	1.70 ± 0.35	1.29 ± 0.21	25.97 ± 11.45
	90	27.48 ± 2.65 ***	1.94 ± 0.24 **	1.60 ± 0.06 *	37.05 ± 19.82
	180	35.51 ± 3.79 ***	2.72 ± 0.35 ***	2.12 ± 0.12 ***	31.28 ± 3.36
G16-5	0	17.89 ± 0.45	1.36 ± 0.21	1.06 ± 0.24	18.00 ± 3.63
	22.5	17.82 ± 2.36	1.98 ± 0.23 *	0.60 ± 0.29	14.09 ± 3.93
	45	24.00 ± 2.86 *	2.04 ± 0.15 **	0.79 ± 0.14	18.85 ± 3.26
	90	28.56 ± 2.26 ***	2.54 ± 0.31 ***	1.10 ± 0.32	19.04 ± 2.25
	180	28.42 ± 2.92 ***	3.38 ± 0.20 ***	2.08 ± 1.23 *	20.69 ± 2.65
G17-5	0	17.89 ± 0.45	1.36 ± 0.21	1.06 ± 0.24	18.00 ± 3.63
	22.5	16.64 ± 1.54	1.86 ± 0.12 *	0.90 ± 0.35	16.43 ± 1.29
	45	21.79 ± 2.62	2.16 ± 0.19 **	0.83 ± 0.25	17.40 ± 1.97
	90	25.88 ± 3.57 **	2.49 ± 0.27 ***	1.00 ± 0.41	19.16 ± 1.64
	180	26.07 ± 2.52 ***	3.42 ± 0.29 ***	1.26 ± 0.25	21.69 ± 3.77
Quartz DQ12	0	17.89 ± 0.45	1.36 ± 0.21	1.06 ± 0.24	18.00 ± 3.63
	22.5	15.38 ± 2.57	1.57 ± 0.11	0.86 ± 0.22	19.88 ± 7.15
	45	22.42 ± 2.54	2.12 ± 0.23	1.09 ± 0.08	31.71 ± 11.98
	90	47.40 ± 1.60 ***	5.57 ± 0.18 ***	1.35 ± 0.14	60.75 ± 10.53 ***
	180	74.49 ± 2.61 ***	13.24 ± 0.32 ***	1.76 ± 0.31	89.69 ± 13.22 ***
Quartz DQ12 [1)]	0	16.98 ± 1.26	1.51 ± 0.36	±	8.40 ± 0.99
	22.5	18.93 ± 0.92	2.00 ± 0.86	±	12.01 ± 2.27
	45	27.78 ± 0.51 *	2.85 ± 0.30	±	22.11 ± 6.10
	90	58.47 ± 1.96 ***	7.41 ± 0.29 ***	±	76.77 ± 31.37 **
	180	89.73 ± 8.82 ***	17.62 ± 1.54 ***	±	119.14 ± 43.96 ***
Zymosan	360			15.54 ± 0.73	
LPS	0.5				454.55 ± 146.70

[1)] Results from a second measurement campaign with heat-treated G8-2b (220oc, 16 h, n=2) to destroy putative endotoxin contamination. LDH: lactate dehydrogenase, GLU: glucuronidase, H_2O_2: hydrogen peroxide, TNFα: tumor necrosis factor α. All Measurements are mean ± stansard deviation of three biological replicas (n=3). Value significantly different from controls are marked by asterisks: *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$.

Discussion (by the Applicant)

Analysis of the *in vitro* bioactivity of 14 TiO_2 materials revealed largely homogeneous responses of the particle-treated NR8383 alveolar macrophages with respect to cytotoxicity which was reflected by a uniform dose-dependent release of both, LDH and GLU. Considering the degree of cytotoxicity, G1-1b and G2-5 were somewhat more bioactive than all other substances. The oxidative response to TiO_2 particles H_2O_2 production) was small and significant elevations were mostly confined to the maximum concentration. Pro-inflammatory effects, reflected by a release of TNFα were found in two cases, one of which could be attributed to a heat-sensitive contamination with endotoxin.

The cellular particle loading could be successfully documented by phase contrast microscopy combined with PTA analysis of the culture medium under cell culture conditions. It became clear that nearly all TiO_2 particles were completely ingested secondary to gravitational settling. Limitations were found for the ground materials G8-2b and G16-5 where some large particles were still present and found to be associated with macrophages, and for G1-1b, G2-5, G6-3 and G7-5 where few small particles remained visible outside the cells at the highest concentration step. Since also in these cases cells were heavily loaded with particles, the contribution of the non-ingested particles fraction to the cellular particle burden is deemed to

be very small. For those TiO_2 samples which were completely ingested the cellular burden may be calculated from the constant cell numbers per well (3x105), and the administered dose (200 µL with 22.5, 45, 90 and 180 µg/mL), thus calculating to a mean cellular dose of 15, 30 60 and 120 pg/cell, respectively. Of note, this cellular burden matches with the cellular burden found for lavaged alveolar macrophages from inhalation experiments with AlOOH which has a slightly lower 17% lower density (Pauluhn 2009; doi: 10.1093/toxsci/kfp046).
The administered concentrations of all materials covered the No Adverse Effect Concentration (NOAEC) and the Low-Adverse-Effect-Concentration (LOAEC) at least for one parameter (LDH). Thereby standard deviations of the biological replicas were surprisingly low, and this partly contributed to the low LOAECs as calculated by ANOVA. Nevertheless, the cells' responses to all TiO_2were widely homogeneous, as shown by the color coded LOAECs in Table below. The Table below also outlines the allocation of the TiO_2 samples to the active/passive categories, which is based on the specific surface area of internalized particles. Following the considerations outlined in Wiemann *et al.*, 2016 (doi: 10.1186/s12951-016-0164-2), a particle is deemed to be active, if 2 out of 4 possible LOAECs underscore a defined threshold. Thus, 12 out of 14 TiO_2 materials may be categorized as active, although their effect on the cells is comparatively low and their BET value may differ up to 50-fold. The specific surface area is generally believed to drive the bioactivity of nano-sized particles and has been used as a well-accepted dose metric. It is therefore surprising that TiO_2 samples with low and high BET surface exhibit a very similar overall reactivity and that the materials with a BET up to 70 m^2/g were classified as active, whereas G2-5 and G10-4 with a BET value of 302 and 80 m^2/g, respectively, were classified as passive. However, we cannot exclude that the effective surface of TiO_2 particles contacting or influencing cellular components becomes reduced by the agglomeration of particles in cell culture medium which may contributes to this finding. At least G2-5 with the largest BET surface was found to induce some TNFα release. Further particle characterization data (such as crystallinity, coating) need to be considered to fully interpret the findings.
Overall, the response to hydrophilic as well as hydrophobic TiO_2 (nano)materials was uniform and dominated by a mild cytotoxicity.

Table 4. Summary of LOECs and active/passive calculation.

Material		LOAEC (µg/mL)				LOAEC x BET (mm^2/mL)					Result
	BET (m^2/g)	LDH	GLU	TNFα	H_2O_2	LDH	GLU	TNFα	H_2O_2	No. of Results < 6000 mm^2/mL	Active (A) Passive (P)
E171-E	10	45	45			450	450			2	A
G1-1b	48	45	90		180	2160	4320		8640	2	A
G2-5	302	45	90	180	90	13590	27180	54360	27180	0	P
G3-1	6	90	90		180	360	360		720	3	A
G4-19	15	45	45		180	675	675		2700	3	A
G5-4	14	45	90			630	1260			2	A
G6-3	70	45	45		90	3150	3150		6300	2	A
G7-5b	57	90	180		90	5130	10260		5130	2	A
G8-2b[1)]	65	45		45	180	2925		2925	11700	2	A
G9-5c	8	45	45		180	360	360		1440	3	A
G9-5d	8	90	180		90	720	1440		720	3	A
G10-4	80	90	90		90	7200	7200		7200	0	P
G16-5	n.m.	45	22.5		180						
G17-5	n.m.	45	22.5								

[1)] Heat-sensitive endotoxin contamination led to high TNFα value

Ref.: Final report – DRAFT. Effects of Fourteen TiO2 Materials on NR8383 Alveolar Macrophages. Prof. Dr. Martin Wiemann (Responsible Scientist). 29th of July 2022

The SCCS comments on the results from study report on Effects of TiO_2 raw materials on NR8383 macrophages

The study results do not include any genotoxicity endpoints.
The raw materials tested induced rather mild cytotoxicity on NR8383 cells measured with LDH and GLU tests. However, the SCCS noted that 16 h of exposure is a relatively short exposure time for other cell impairment/death signs to develop. After prolonged incubation time, cytotoxic effects could be observed at even lower TiO_2 concentrations. Considering high persistence of TiO_2 particles in biological tissues, pulse exposure with prolonged observation time would also be a valuable option. Even if no confirmation of cellular uptake by electron microscopy was provided, the SCCS assumes that all the TiO_2 raw materials could be internalised by the macrophages, as was partially documented by phase contrast imaging. Although ROS generation was measured after 90 minutes with generally low (usually at the 2 highest concentrations tested) or no response from the cells, the SCCS noted that longer incubation times could be applied by the Applicant with possibly greater effects. As was shown by the results, all the raw materials induced no or very slight increase (G2-5) of TNF-α. The proposed calculation of biological activity of TiO_2 raw materials could be interesting for

regulatory purposes, however, in the opinion of the SCCS, the proposal would need further, more stringent validation.
In conclusion, the results indicate rather low cytotoxicity of the TiO_2 raw materials on NR8383 rat macrophages after 16 h of exposure, however longer incubation times with extended panel of cytotoxicity endpoints would be required to gain broader view on potential hazard of the raw materials.

IN VITRO STUDY #2. MucilAir-Rat-RF
The aim of the study was to evaluate and rank the potential toxicity, (1) inflammatory effects, (2) innate immune response, (3) and ciliary function, of a single exposure to TiO_2 materials (nanoparticles) over one week (endpoints at 48, 96 and 168 hours) to correlate these early key events observed in *in vivo* intratracheal rat instillation studies.

Materials and methods

The following TiO_2 raw materials were tested by the Applicant:

Name	Code	Batch	CAS munber	EC number	Purity	Comments
Aeroxide® TiO_2 P 25 (Evonik ; August 8, 2021)	G1-1b	618052498	13463-67-7	236-675-5	>99.5 %	Phase 1a-b
Sachtleben® TR_AA (Venator ; August 8, 2021)	G3-1	G2TMGE0110/ 433009363/ UOC9363	13463-67-7	236-675-5	≥90-≤100 %	Phase 1a-b
Ti-Pure™ Titanium Dioxide Pigment (Chemours ; August 8, 2021)	G7-5	40003782184	13463-67-7	236-675-5	80.7 %	Phase 1a-b
Ti-Pure™ Titanium Dioxide Pigment (Chemours)	G7-5b	3328610025	13463-67-7	236-675-5	85.09 %	Phase 1b

The study was structured into 3 phases:
- Phase 1: Feasibility Test. 3 TiO_2 forms were used (high inflammatory, mid inflammatory and non-inflammatory compounds). This phase is subdivided into two main tasks.

  Phase 1a. Dose range finding study
  Phase 1b: Extended feasibility study including some relevant biomarkers
  Phase 2: Main test (part 1) – conducted only if Phase 1 is successful. 4 TiO_2 forms will be evaluated (5 non-inflammatory and 1 repetition mid-inflammatory compounds).

Exposure to the test materials

Name	Volume applied	Exposure time	Exposure	Concentrations (semi-log scale)	Comments
All products Phase 1a	20 µl	168 hours (at 48 and 96 hours TEER/washing)	1	0.002, 0.01, 0.05, 0.2, 1, 5, 20, 100 µg/cm^2	Apical, in 0.9 % NaCl
All products Phase 1b	20 µl	168 hours (at 48 and 96 hours TEER/washing)	1	1, 5, 20, 50 µg/cm^2	Apical, in 0.9 % NaCl
Vehicle	20 µl		1		Apical 0.9 % NaCl

Exposure parameters in both Phase 1a and 1b; The surface of the epithelium is 0.33 cm^2 and the exposure volume is 20 uL. For 100 ug/cm^2 the quantity needed is 3.3 ug in 20 uL, which corresponds to a solution of 1.65 mg per mL of saline solution.

Assay Model:

MucilAir™-Rat-RF is a reconstituted 3D tissue from rat airways, fully differentiated, pseudostratified *in vitro* epithelium co-cultured with rat fibroblasts. Cultured at the air liquid interface, the model displays high trans-epithelial electrical resistance and cilia beating, demonstrating the full functionality of the epithelial tissue.
The mature MucilAir™-Rat-RF is composed of basal cells, ciliated cells and mucus producing cells.
TiO_2 forms (high inflammatory, mid-inflammatory and noninflammatory compounds – provided by sponsor, G1-1b, G3-1, G7-5).
Number of repeats: 3
Number of concentrations: 8 concentrations (semi-log scale – 0.002; 0.01; 0.05; 0.2; 1; 5; 20; 100 µg/cm^2; N=72)
Negative controls: Untreated cultures (UN) N=3; Vehicle control - Apical treatment (20 uL of 0.9 % NaCl; N=3);
Positive controls (N=3): Triton X-100 (10 %, 50 µL apical; for cytotoxicity); Number of MucilAir™-Rat = 81

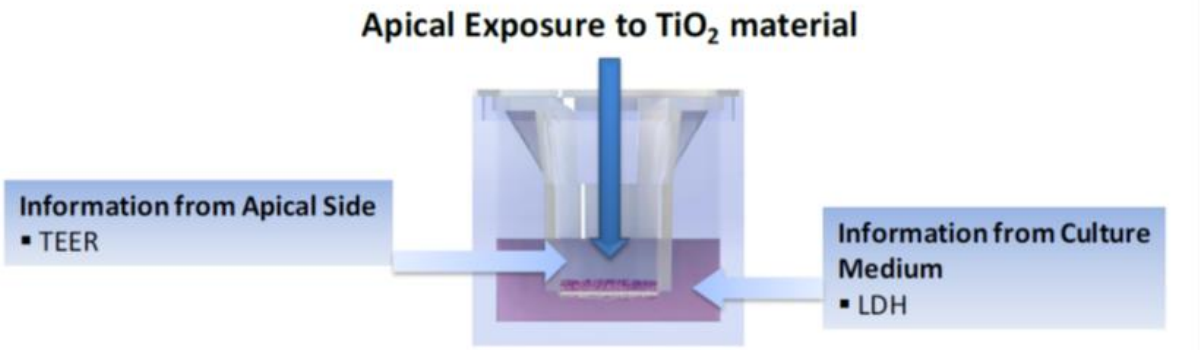


Figure 1: Apical exposure to TiO_2 materials on MucilAir™-Rat-RF. Endpoint measurements were performed at both the apical and basal sides. Measures of transepithelial electrical resistance (TEER) and cytotoxicity (LDH assay) were performed.

Phase 1b
Tested compounds: 3 TiO_2 forms used (high inflammatory, mid-inflammatory and noninflammatory compounds – Samples: G1-1b, G3-1, G7-5b)
Number of repeats: 3
Number of concentrations: 4 concentrations (1, 5, 20, 50 µg/cm^2 – N=36)
Additional concentration: 1 concentration for sample G7-5 (3 repeats) to show comparability with the new sample, G7-5b = 50 µg/ cm^2
Negative controls: Untreated cultures (UN) N=3, Vehicle control - Apical treatment (20 µL of 0.9 % NaCl) N=3
Positive controls: Triton X-100 (10 %, 50 µL apical; for cytotoxicity) N=3, Cytomix - Basal treatment (500 ng/mL TNFα, 0.2 mg/mL LPS, 1 % FCS; for inflammation) N=3

An additional parallel series performed for oxidative stress gene markers (SOD-2, GPx, GST) at 48h including TEER and LDH release measurement.

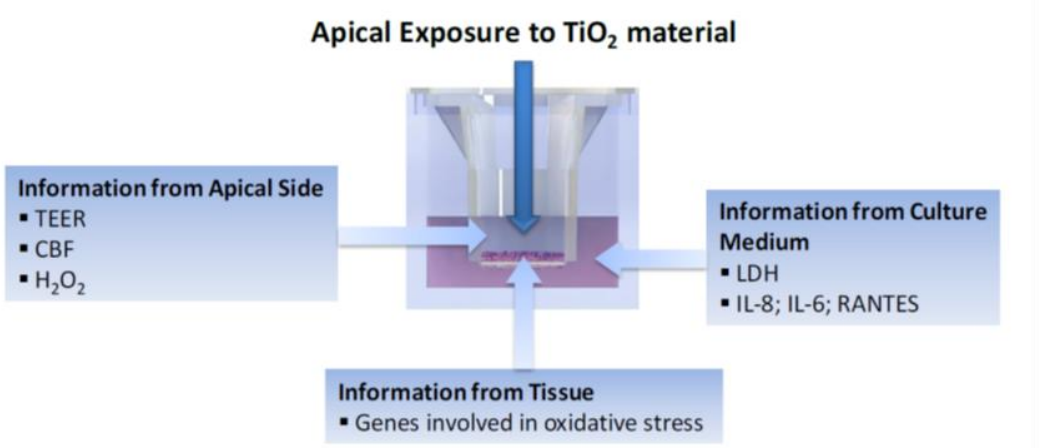


Figure 2: Single apical exposure to TiO_2 materials on MucilAir™-Rat-RF. Endpoint measurements were performed at both the apical and basal sides and from the MucilAir™ tissue. Transepithelial electrical resistance (TEER), cilia beating frequency (CBF) and H_2O_2 were assessed on the apical side. Cytotoxicity (LDH assay) and cytokine release were measured from the basolateral medium. The epithelial tissue was lysed for gene expression analysis.

Methods:
Tissue integrity TEER. (An increase of the TEER value reflects a blockage of the ion channel activities), cytotoxicity (LDH release), cilia beating frequency (CBF), cytokines (release of Interleukin 8 and 6,) and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) by ELISA, Hydrogen peroxide concentration measured fresh (without storage) from the apical wash using OxiSelect™ Hydrogen Peroxide/ Peroxidase Assay Kit; Oxidative stress-related genes *(*SOD-2, GPx, GST) and Quantitative RT-PCR; houskeeping *(*reference) gene GAPDH.
Statistical analysis: one-way or two-way ANOVA with Dunnett's multiple comparison post-tests, Student's t test.

Results:
Phase 1a
Tissue integrity (TEER)
Effect of single apical exposure to G1-1b, G3-1 and G7-5 TiO_2 material on tissue integrity in MucilAir™-Rat-RF.
TEER was measured 2, 4 and 7 days post exposure (n=3 cultures, mean±SEM). Threshold limit is 100 $\Omega.cm^{-2}$.
The untreated and vehicle treated cultures showed TEER values in the normal range of MucilAir™ (200-600 $\Omega.cm^{-2}$). Positive control Triton X-100 (10 %) induced a decrease of TEER below 100 $\Omega.cm^{-2}$.
Apical exposure to G1-1b tended to decrease TEER values in a dose-dependent manner, but the integrity of the tissue was well preserved (> 100 $\Omega.cm^{-2}$) at all concentrations. Apical exposure to G3-1 induced a decrease in TEER at 4 and 7 days after exposure at all concentrations, but the integrity of the tissue was well preserved (> 100 $\Omega.cm^{-2}$) at all concentrations. After apical exposure to G7-5, a dose-dependent decrease of TEER was observed at 48 hours (except for 20 $\mu g/cm^2$) and a general decrease in TEER at 4 and 7 days post exposure, but the integrity of the tissue was well preserved (> 100 $\Omega.cm^{-2}$) at all concentrations. The reason for the outliers at 20 $\mu g/cm^2$ is unknown.

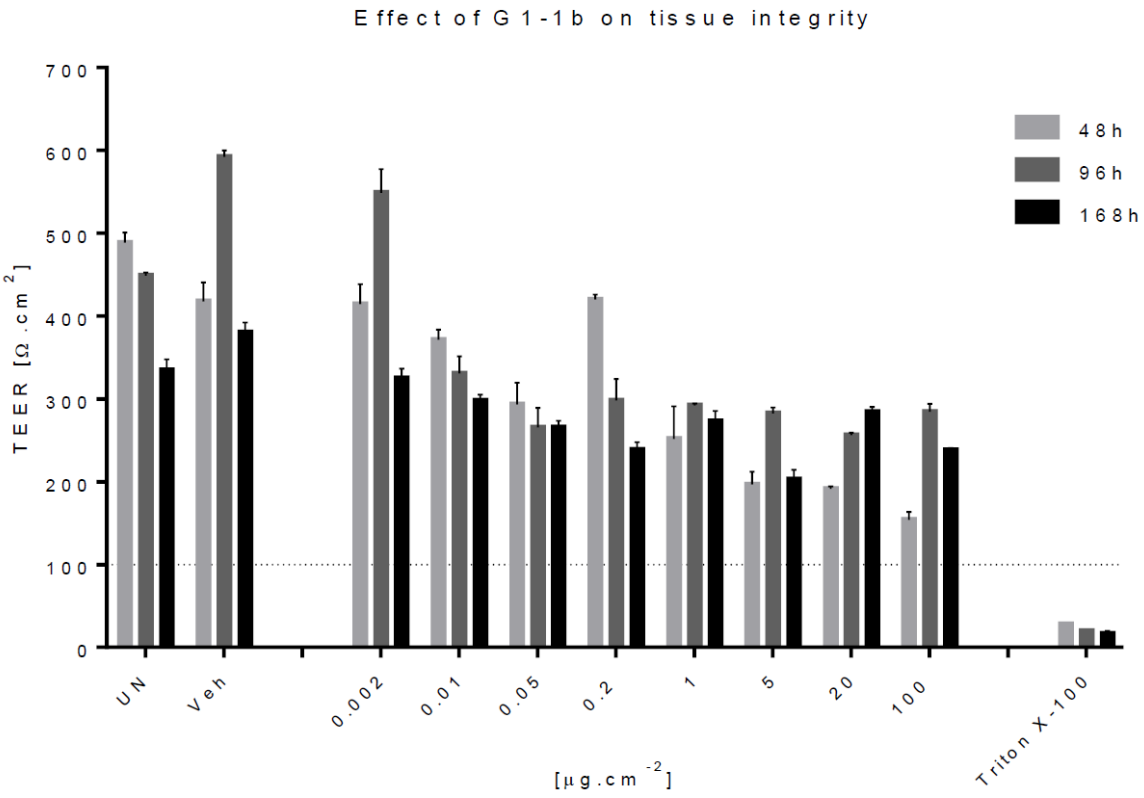


Figure 3: Effect of single apical exposure to G1-1b TiO_2 material on tissue integrity in MucilAir™-Rat-RF. TEER was measured 2, 4 and 7 days post exposure (n=3 cultures, mean±SEM). Threshold limit is 100 Ω.cm-^{2}.

Cytotoxicity (LDH release)

Effect of single apical exposure to three TiO_2 materials on cytotoxicity in MucilAir™-Rat-RF. LDH release was measured at 2, 4 and 7 days post exposure (n=3 cultures, mean±SEM) (48 or 72 hours accumulation). Threshold limit is 5 % cytotoxicity, which corresponds to a physiological LDH release in MucilAir™ (human). No cytotoxicity was detected in negative control. The 10 % Triton X-100 solution induced toxicity was 100 %. No cytotoxicity (< 5 %) was detected for single apical exposure to G1-1b, except for a small cytotoxicity, 10 %, at 0.002 µg/cm^2 at 96 hours. No cytotoxicity (< 5 %) was detected for single apical exposure to G3-1 and G7-5.

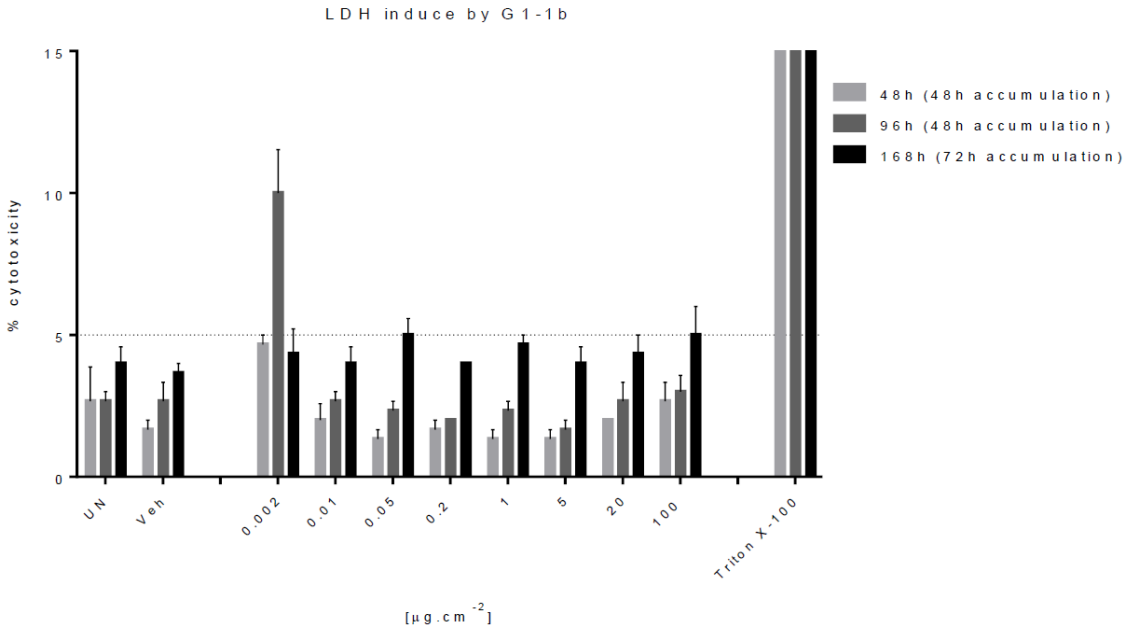


Figure 4: Effect of single apical exposure to G1-1b TiO_2 material on cytotoxicity in MucilAir™-Rat-RF. LDH release was measured at 2, 4 and 7 days post exposure (n=3 cultures, mean±SEM) (48 or 72 hours accumulation). Threshold limit is 5 % cytotoxicity, which corresponds to a physiological LDH release in MucilAir™ (human).

Summary of results of phase 1a:

	Presence of TiO_2 in apical part (after wash)			Tissue integrity	Cytotoxicity
	48h	96h	168h		
G1-1b	[20] and [100] µg/cm² +++	[20] and [100] µg/cm² ++	[20] and [100] µg/cm² ++	Tight	Potentially artefactual increase for 0.002 µg/cm² at 96h
G3-1	[20] and [100] µg/cm² +++	[20] and [100] µg/cm² ++	[20] and [100] µg/cm² ++	Tight	No
G7-5	[20] and [100] µg/cm² +++	[20] and [100] µg/cm² ++	[20] and [100] µg/cm² ++	Tight	No

Phase 1b

Tissue integrity (TEER)

Effect of single apical exposure to TiO_2 materials on tissue integrity in MucilAir™-Rat-RF. TEER was measured 2, 4 and 7 days post exposure (n=6 at 48 hours, n=3 cultures at 96 and 168 hours, mean±SEM). Threshold limit is 100 Ω.cm^{-2}. The untreated and vehicle treated cultures showed TEER values in the normal range of MucilAir™ (200-600 Ω.cm^{-2}). Triton X-100 (10 %) induced a decrease of TEER below 100 Ω.cm^{-2}. Apical exposure to G1-1b, G3-1, G7-5b and G7-5 had no effect on TEER values, the integrity of the tissue was well preserved (> 100 Ω.cm^{-2}) at all concentrations.

Cytotoxicity (LDH release)

Effect of single apical exposure to four TiO_2 materials on cytotoxicity in MucilAir™-Rat-RF (LDH release) was measured at 2, 4 and 7 days post exposure (n=3 cultures, mean±SEM) (48 or

72 hours accumulation). Threshold limit is 5 % cytotoxicity, which corresponds to a physiological LDH release in MucilAir™ (human). No cytotoxicity was detected in negative control. The 10 % Triton X-100 solution induced toxicity was 100 %.
After exposure to G1-1b, the cytotoxicity was between 3.7 and 11.3 %. The increase was moderate compared to vehicle, not at all time points and no dose dependence was observed. Currently no historical data is available to determine the acceptable threshold of cytotoxicity for MucilAir™-Rat-RF. Using threshold of 10 %, an increase of cytotoxicity was found for 5 and 50 µg/cm^2 at 96 hours.
After exposure to G3-1, the cytotoxicity was between 3.7 and 11.3 %. The increase was moderate compared to vehicle, not at all time points and no dose dependence was observed. Currently no historical data is available to determine the acceptable threshold of cytotoxicity for MucilAir™-Rat-RF. Using a threshold of 10 %, an increase of cytotoxicity was found for 50 µg/cm^2 at 96 hours.
After exposure to G7-5b and G7-5, the cytotoxicity was between 2.7 and 10 %. A very slight, dose-dependent increase was observed at 96 hours compared to vehicle. Currently no historical data is available to determine the acceptable threshold of cytotoxicity for MucilAir™-Rat-RF. Using a threshold is 10 %, no cytotoxicity was observed.

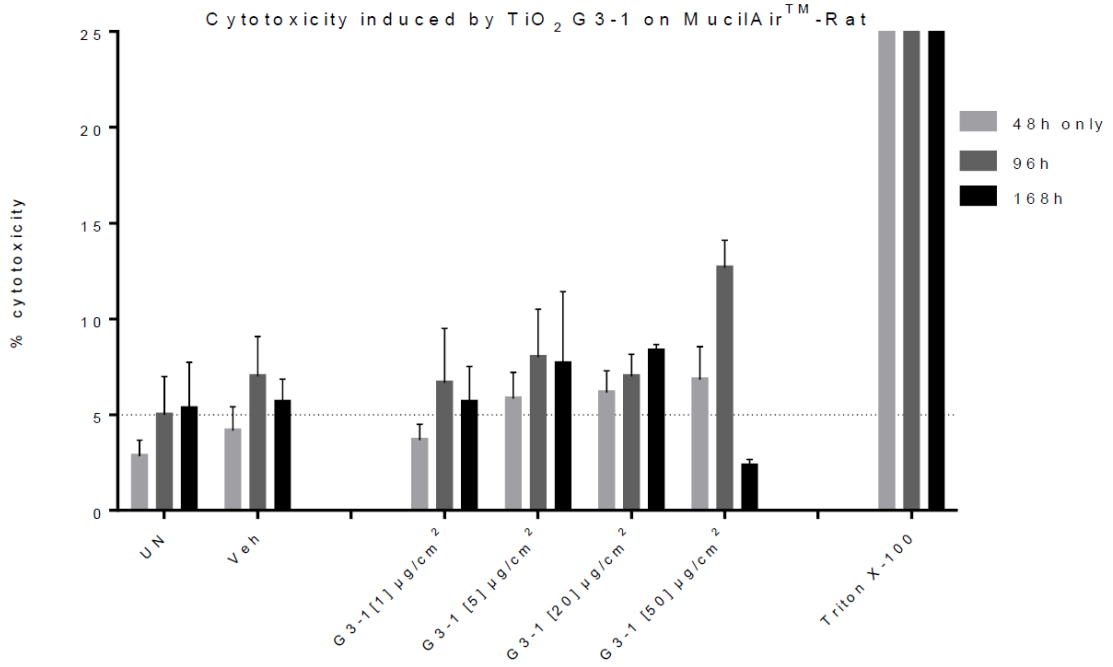


Figure 5: Effect of single apical exposure to G3-1 TiO_2 material on cytotoxicity in MucilAir™-Rat-RF. LDH release was measured at 2, 4 and 7 days post exposure (n=6 at 48 hours, n=3 cultures at 96 and 168 hours, mean±SEM) (48 or 72 hours accumulation). Threshold limit is 5 % cytotoxicity, which corresponds to a physiological LDH release in MucilAir™ (human).

Cilia beating frequency (CBF)
Effect of single apical exposure to four TiO_2 materials on cilia beating frequency in MucilAir™-Rat-RF was measured 7 days post exposure (n=3 cultures, mean±SEM).
The untreated and vehicle treated cultures showed cilia beating frequency of 12.8 and 12.9 Hz at room temperature, which is above the normal range of human MucilAir™ (5-10 Hz) at this temperature. Currently, there is insufficient data available to determine the normal range of rat culture. Apical exposure to TiO_2 did not modify CBF compared to vehicle.

Apical H_2O_2 release
Effect of single apical exposure to TiO_2 materials on apical H_2O_2 release in MucilAir™-Rat-RF. H_2O_2 was measured in the apical wash 2, 4 and 7 days post exposure (n=6 at 48 hours, n=3

cultures at 96 and 168 hours, mean±SEM). The apical wash from the untreated and vehicle treated cultures had surprisingly high, 44 and 53 µM, H_2O_2 concentrations at 48 hours. In contrast, very low level of H_2O_2 was detected at 96 and 168 hours. The apical wash of 48 hours contained materials accumulated during 5 days (-3 days apical wash before experiment, according to Epithelix SOP, and 2 days post exposure) on the surface of epithelia, in contrast to 96 hours (2 days accumulation) and 168 hours (3 days accumulation).
Apical exposure to any of tested TiO_2 did not modify H_2O_2 concentration compared to vehicle.

Basal Interleukin 8 and 6 release
Effect of single apical exposure to TiO_2 materials on basal Interleukin 8 and 6 secretions in MucilAir™-Rat-RF was measured in the basal culture medium 2, 4 and 7 days post exposure. The untreated and vehicle treated cultures had IL-8 concentrations between 444-537 pg/mL (no historical data is available for comparison). Positive control Cytomix induced a 2-3 fold increase in IL-8 secretion, the concentrations were 1588, 1185 and 1326 pg/mL at 48, 96 and 168 hours, respectively. Apical exposure to any of the tested TiO_2 had no effect on IL-8 secretion.
The untreated and vehicle treated cultures had IL-6 concentrations between 104-625 pg/mL (no historical data is available for comparison). Positive control Cytomix induced a huge increase in IL-6 secretion, the concentrations were 5511, 7107 and 6436 pg/mL at 48, 96 and 168 hours, respectively. Apical exposure to G1-1b and G3-1 had no effect on IL-6 secretion. Apical exposure to G7-5b at the highest dose, 50 µg/cm^2, increased IL-6 secretion at 168 hours (1297 pg/mL). The increase was a bit lower for G7-5. (According to the simple unpaired Student t test at 168 hours – G7-5b vs. vehicle or G7-5 vs. vehicle – the differences are not significant).

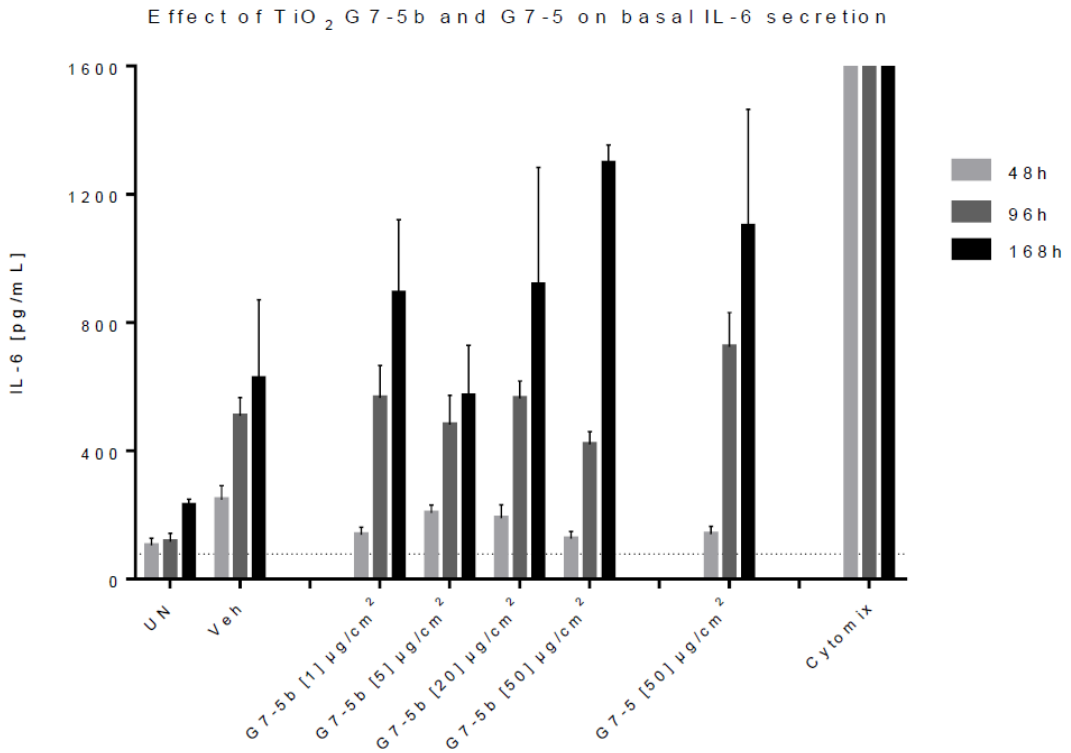


Figure 6: Effect of single apical exposure to G7-5b and G7-5 TiO_2 material on basal Interleukin 6 secretion in MucilAir™-Rat-RF. IL-6 concentrations were measured in the basal culture medium 2, 4 and 7 days post exposure (n=6 at 48 hours, n=3 cultures at 96 and 168 hours, mean±SEM). The dotted line represents the lower limit of the standard curve.

Basal RANTES release

Effect of single apical exposure to TiO_2 materials on basal RANTES secretion in MucilAir™-Rat-RF was measured in the basal culture medium 2, 4 and 7 days post exposure. The untreated and vehicle treated cultures had low RANTES concentrations between 5-55 pg/mL (no historical data is available for comparison). Cytomix induced an increase in RANTES secretion, the concentrations were 446, 313 and 192 pg/mL at 48, 96 and 168 hours, respectively. Apical exposure to any of the tested TiO2 had no effect on RANTES secretion.

Gene expression analysis

Effect of single apical exposure to TiO_2 materials on the expression of three oxidative-stress related genes in MucilAir™-Rat-RF was measured. As a reference gene, GAPDH was used and the expression was presented relative to the vehicle mean, and thus vehicle represents 1. In general, > 2 fold change is considered biologically relevant. Exposure to G1-1b did not modify the expression of Sod2, Gpx2 and Gstp1 at 2 and 7 days post exposure. Exposure to G3-1 induced a dose-dependent increase of Sod2 gene (2.3, 5.2, 7.7 and 16.4-fold change for 1, 5, 20 and 50 µg/cm^2, respectively), while Gpx2 and Gstp1 were not modified at 2 days after exposure. In contrast, at 7 days after exposure Sod2 gene showed downregulation, and Gpx2 and Gstp1 remained unchanged. Exposure to G7-5 and G7-5b at the highest dose, 50 µg/cm^2 decreased the expression of all three genes 2 days after exposure (approximately 0.5 fold-change). Seven days after exposure, only Sod2 gene showed a dose-dependent decrease of expression (0.8, 0.4 and 0.2-fold change for 5, 20 and 50 µg/cm^2).

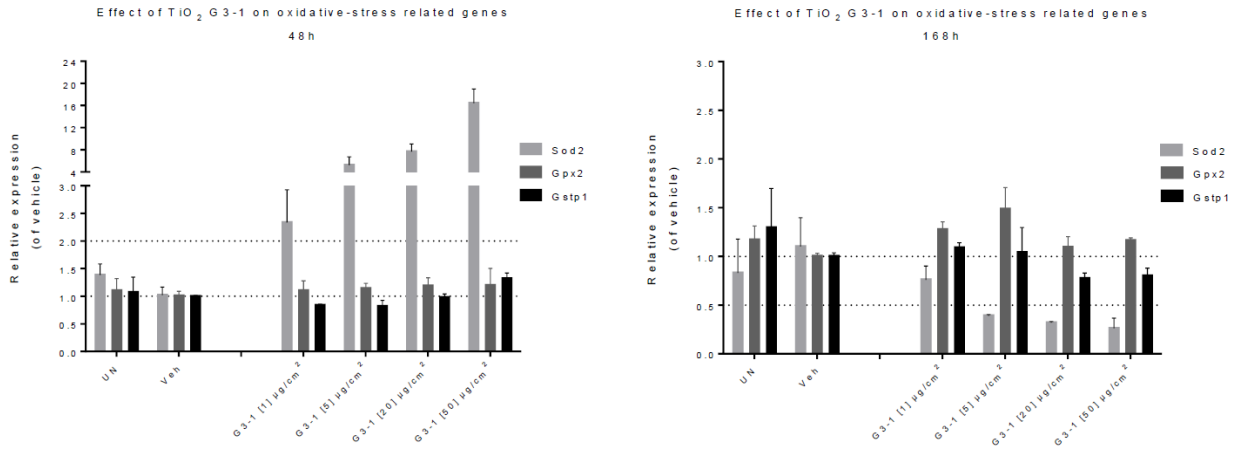


Figure 7: Effect of single apical exposure to G3-1 TiO_2 material on the expression of three oxidative-stress related genes in MucilAir™-Rat-RF. Candidate transcripts were quantified by Taqman RT-PCR 2 and 7 days post exposure (n=3 cultures, mean±SEM).

Summary of results of phase 1b:

	Presence of TiO_2 in apical part (after wash) and microscopic obervation			Toxicity (TEER, LDH, CBF)	Cytokine secretions			Gene expressions		
	48h	96h	168h		RANTES	IL-6	IL-8	Sod2	Gpx2	Gstp1
G1-1b	[50] and [20] µg/cm² +++	[50] and [20] µg/cm² ++	[50] and [20] µg/cm² +	No	No effect	No effect	No effect	No effect	No effect	No effect
	[50] µg/cm² few detached cells	[50] µg/cm² few detached cells	[50] µg/cm² few detached cells							
G3-1	[50] and [20] µg/cm² +++	[50] and [20] µg/cm² ++	[50] and [20] µg/cm² +	No	No effect	No effect	No effect	↗ 48h dose-dependent; ↘ 168h	No effect	No effect
	[50] µg/cm² few detached cells	[50] µg/cm² few detached cells	[50] µg/cm² few detached cells							
G7-5b	[50] and [20] µg/cm² +++	[50] and [20] µg/cm² ++	[50] and [20] µg/cm² +	No	No effect	No effect	No effect	↘ 48h [50] µg/cm²; ↘ 168h dose-dependent	↘ 48h [50] µg/cm²	↘ 48h [50] µg/cm²
	[50] and [20] µg/cm² few detached cells	[50] and [20] µg/cm² few detached cells	[50] and [20] µg/cm² few detached cells							
G7-5	[50] µg/cm² +++	[50] µg/cm² ++	[50] µg/cm² +	No	No effect	No effect	No effect	↘ 48h [50] µg/cm²; ↘ 168h [50] µg/cm²	↘ 48h [50] µg/cm²	↘ 48h [50] µg/cm²
	few detached cells	few detached cells	few detached cells							

Conclusion by the Applicant

No effects on tissue integrity and no cytotoxicity were observed at all 3 time points, for exposure to TiO_2 material G1-1b, G3-1 and G7-5B/G7-5 on the apical surface of the epithelia. Overall morphology of the rat epithelia was good with presence of TiO_2 materials and the cilia beating was visible. However, a few detached, floating cells were observed at the periphery of the inserts at the highest concentration, 50 µg/cm², for G1-1b, G3-1, G7- 5, and at 20, 50 µg/cm² for G7-5b. RANTES, IL-8 and IL-6 secretions were not changed significantly for all TiO_2 materials.

In conclusion, a single apical exposure to TiO_2 materials on MucilAir™-Rat-RF induces changes in the expression of oxidative stress-related genes, and thus this parameter could be the first relevant biomarker for *in vitro* TiO_2 research. This study shows that a single apical exposure to G1-1b does not induce any change in the measured parameters, whereas the TiO_2 materials G3-1 and G7-5 induce both up- and down-regulation of oxidative stress-related genes. The most marked change is the dose dependent upregulation of Sod2 at 48 hours by G3-1 TiO_2 at the measured time points.

Ref.: Single dose testing of TiO_2 materials on MucilAir™-Rat Phase 1a & 1b by and between Epithelix and Titanium Dioxide Manufacturers Association (TDMA). Date: 31 May 2022. Final Report – ST210902 & ST220203, 2022

The SCCS comments to the MucilAir™-Rat-RF study

- The study results do not include any genotoxicity endpoints (such as expression of genes related to DNA damage and repair, cell cycle etc.).
- No information on characterisation of tested TiO_2 nanomaterials in exposure medium (0.9 % NaCl) or in culture medium was provided.
- The MucilAir™-Rat-RF model is a promising 3D model of rat airway epithelium, constituted with primary epithelial cells isolated from trachea and bronchi of rats and co-cultured with primary rat airway fibroblasts, but it is still not validated for endpoints measured and no OECD TG or GD exists.
- No information on the internalisation of nanoparticles or penetration through the multilayers has been provided.
- The Applicant designed the study in three phases but only the first phase was performed, which is incomplete, even if this first phase yielded some positive results (expression of oxidative stress and antioxidant defence genes).
- The study was not conducted under GLP and no quality controls have been provided in addition to the negative and positive controls.
- No historical controls were provided for any of the endpoints.
- For cilia beating frequency, no positive control was included and the Applicant noted that currently, there is insufficient data available to determine the normal range of rat culture. These data have limited value.
- For *"Apical H_2O_2 release no positive control was provided".* The apical wash from the untreated and vehicle treated cultures had surprisingly high, 44 and 53 µM, H_2O_2, concentrations at 48 hours. In contrast, a very low level of H_2O_2 was detected at 96 and 168 hours. The apical wash of 48 hours contained materials accumulated over 5 days (-3 days apical wash before experiment, according to Epithelix SOP, and 2 days post exposure) on the surface of epithelia, in contrast to 96 hours (2 days accumulation) and 168 hours (3 days accumulation)". The data have limited value.
- For gene expression study – no positive control included. Two TiO_2 (G3-1 and G7-5b) affected gene expression of oxidative stress related genes. Data from gene expression are difficult to explain.
- The response in gene expression after exposure to TiO_2 was different for different TiO_2 materials. No response was measured after exposure to G1-1, while higher expression was measured after exposure to G3-1 in 48h and lower expression in 168h. For G7-5 and G7-5b lower expression in each time point was detected.
- While gene expression in antioxidant enzyme was affected, no effect on inflammatory markers was observed.
- SCCS is of opinion that to select only a few genes to study the effect of TiO_2 on gene expression is not meaningful. SCCS agrees with the Applicant that broad selection of relevant genes and a more global approach, such as DNA array, could give more insight into transcriptomic changes after exposure of TiO_2 materials.

Annex V: List of publications on TiO_2 particles genotoxicity analysed by the SCCS

1. Abdel-Wahhab *et al.* (2021): Abdel-Wahhab MA, El-Nekeety AA, Mohammed HE, Elshafey OI, Abdel-Aziem SH, Hassan NS. *Elimination of oxidative stress and genotoxicity of biosynthesized titanium dioxide nanoparticles in rats via supplementation with whey protein-coated thyme essential oil.* Environ Sci Pollut Res Int. 2021 Nov;28(41):57640-57656. doi: 10.1007/s11356-021-14723-7.
2. Armand *et al.* (2016): Armand L, Tarantini A, Beal D, Biola-Clier M, Bobyk L, Sorieul S, Pernet-Gallay K, Marie-Desvergne C, Lynch I, Herlin-Boime N, Carriere M. *Long-term exposure of A549 cells to titanium dioxide nanoparticles induces DNA damage and sensitizes cells towards genotoxic agents*. Nanotoxicology. 2016 Sep;10(7):913-23. doi: 10.3109/17435390.2016.1141338. Epub 2016 Feb 22
3. Asare *et al.* (2012): Asare N, Instanes C, Sandberg WJ, Refsnes M, Schwarze P, Kruszewski M, Brunborg G. *Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells*. Toxicology. 2012 Jan 27;291(1-3):65-72. doi: 10.1016/j.tox.2011.10.022. Epub 2011 Nov 6
4. Bessa *et al.* (2017): Bessa MJ, Costa C, Reinosa J, Pereira C, Fraga S, Fernández J, Bañares MA, Teixeira JP. *Moving into advanced nanomaterials. Toxicity of rutile TiO(2) nanoparticles immobilized in nanokaolin nanocomposites on HepG2 cell line* Toxicol Appl Pharmacol. 2017 Feb 1; 316:114-122. doi: 10.1016/j.taap.2016.12.018. Epub 2016 Dec 28."
5. Bhattacharya *et al.* (2008): Bhattacharya K, Cramer H, Albrecht C, Schins R, Rahman Q, Zimmermann U, Dopp E. *Vanadium pentoxide-coated ultrafine titanium dioxide particles induce cellular damage and micronucleus formation in V79 cells*. J Toxicol Environ Health A. 2008;71(13-14):976-80. doi: 10.1080/15287390801989218.
6. Bischoff *et al.* (2020): Bischoff NS, de Kok TM, Sijm DTHM, van Breda SG, Briedé JJ, Castenmiller JJM, Opperhuizen A, Chirino YI, Dirven H, Gott D, Houdeau E, Oomen AG, Poulsen M, Rogler G, and van Loveren H. *Possible Adverse Effects of Food Additive E171 (Titanium Dioxide) Related to Particle Specific Human Toxicity, Including the Immune System*. Int J Mol Sci. 2020 Dec 28;22(1):207. doi: 10.3390/ijms22010207
7. Botelho *et al.* (2014): Botelho MC, Costa C, Silva S, Costa S, Dhawan A, Oliveira PA, Teixeira JP. *Effects of titanium dioxide nanoparticles in human gastric epithelial cells in vitro.* Biomed Pharmacother. 2014 Feb;68(1):59-64. doi: 10.1016/j.biopha.2013.08.006. Epub 2013 Aug 23.
8. Boyadzhiev *et al.* (2022): Boyadzhiev A, Solorio-Rodriguez SA, Wu D, Avramescu ML, Rasmussen P, Halappanavar S. *The High-Throughput In Vitro CometChip Assay for the Analysis of Metal Oxide Nanomaterial Induced DNA Damage.* Nanomaterials (Basel). 2022 May 27;12(11):1844. doi: 10.3390/nano12111844
9. Catalan *et al.* (2012): Catalan J, Jarventaus H, Vippola M, Savolainen K, Norppa H. *Induction of chromosomal aberrations by carbon nanotubes and titanium dioxide nanoparticles in human lymphocytes in vitro*. Nanotoxicology. 2012 Dec; 6:825-36. doi: 10.3109/17435390.2011.625130. Epub 2011 Oct 13.
10. Chen *et al.* (2022): Chen Z, Shi J, Zhang Y, Han S, Zhang J, Jia G. *DNA Oxidative Damage as a Sensitive Genetic Endpoint to Detect the Genotoxicity Induced by Titanium Dioxide Nanoparticles.* Nanomaterials (Basel). 2022 Jul 29;12(15):2616. doi: 10.3390/nano12152616.
11. Dekanski *et al*. (2018): Dekanski D, Spremo-Potparevic B, Bajic V, Živkovic L, Topalovic D, Sredojevic DN, Lazic V, Nedeljkovic JM. *Acute toxicity study in mice of orally administrated TiO(2) nanoparticles functionalized with caffeic acid*. Food Chem Toxicol. 2018 May; 115:42-48. doi: 10.1016/j.fct.2018.02.064. Epub 2018 Mar 3
12. Dhein *et aL.* (2022): Dhein J, Haller C, Reichl FX, Milz S, Hickel R, Kollmuss M, Högg C. *Intranuclear cell uptake and toxicity of titanium dioxide and zirconia particles as well as bacterial adhesion on dental titanium- and zirconia-implants.* Dent Mater. 2022 Mar;38(3):517-528. doi: 10.1016/j.dental.2021.12.142.
13. Di Giampaolo *et al.* (2021): Di Giampaolo L, Zaccariello G, Benedetti A, Vecchiotti G, Caposano F, Sabbioni E, Groppi F, Manenti S, Niu Q, Poma AMG, Di Gioacchino M, Petrarca C. *Genotoxicity and Immunotoxicity of Titanium Dioxide-Embedded Mesoporous Silica*

Nanoparticles (TiO(2)@MSN) in Primary Peripheral Human Blood Mononuclear Cells (PBMC). Nanomaterials (Basel). 2021 Jan 21;11(2):270. doi: 10.3390/nano11020270

14. Diabaté *et aL* (2020): S Diabaté, L Armand S Murugadoss, M Dilger, S Fritsch-Decker, Ch Schlager, D Béal, M-E Arnal, M Biola-Clier, S Ambrose, S Mülhopt, H-R Paur, I Lynch, E Valsami-Jones, M Carriere, C Weiss. *Air-Liquid Interface Exposure of Lung Epithelial Cells to Low Doses of Nanoparticles to Assess Pulmonary Adverse Effects*. Nanomaterials (Basel). 2020 Dec 29;11(1):65. doi: 10.3390/nano11010065
15. El Yamani *et al.* (2022): El Yamani N., Rubio L., Garcia-Rodriguez A., Kazimirova A., Runded-Pran E., Magdalena B., Marcos R., Dusinska M. *Lack of mutagenicity of TiO_2 nanoparticles in vitro despite cellular and nuclear uptake*. Mutat Res Genet Toxicol Environ Mutagen. 2022 Oct; 882:503545. doi: 10.1016/j.mrgentox.2022.503545. Epub 2022 Sep 5
16. El-Said *et al*. (2014): El-Said KS, Ali EM, Kanehira K, Taniguchi A. *Molecular mechanism of DNA damage induced by titanium dioxide nanoparticles in toll-like receptor 3 or 4 expressing human hepatocarcinoma cell lines.* J Nanobiotechnology. 2014 Dec 2; 12 48. doi: 10.1186/s12951-014-0048-2
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Annex W. Comparison of approaches to evaluation of the genotoxicity of TiO_2 (shaded rows show discrepancies) used by EFSA (2021), Kirkland et al. (2022), and the SCCS

Parameter	EFSA FAF Panel approach 2021	Kirkland et al., 2022	SCCS
LITERATURE SEARCH TERMS			
Search period	The last search on 2020-12-30	The last search on 2021-04-12	The last search on 2023-04-16
TiO2 forms considered	Included: Food grade E171 pigmentary grade microparticles nanoparticles anatase with diameter less than 30 nm was excluded (because less than 1% of primary particles detected in pristine samples of food-grade TiO2 as well as food products with TiO2 on the European market were smaller than 30 nm (Verleysen et al. 2020; 2021)	Included: Food grade E171 pigmentary grade microparticles nanoparticles	Included: **Microsized:** - food-grade (E171, anatase/rutile) - pigment grade: non-coated/coated - other: non-coated/coated **Nanoparticles:** - anatase: non-coated/coated - rutile: non-coated/coated
Studies performed with TiO2 nanoparticles	Included Opinion largely based on studies that used TiO2-NPs (of which many used TiO2-NPs < 30 nm) to characterize the genotoxic potential of TiO2 added to food.	Included	Included
Studies performed only with coated TiO2	Excluded (at TiAb stage)	Included (if endpoint and test system had default "Moderate" or "High" weight)	Included
Studies performed only with TiO2 nanofibres, nanocomposites or nanotubes, titanates (FeTiO3, H4TiO4)	Excluded (at TiAb stage)	Included (if endpoint and test system had default "Moderate" or "High" weight)	Excluded
Studies using sonication of TiO2 particles before exposure	Included	Included	Included
EVALUATION CRITERIA			
Reviews, editorials, letters to the editor etc.	Excluded (at TiAb stage)	Excluded (but if original data included in a review paper was found, this was included and both references cited)	Excluded (but if original data included in a review paper was found, this was included and both references cited)
Abstract only	Excluded (at TiAb stage), unless there was sufficient information provided	Included (if endpoint and test system had default "Moderate" or "High" weight)	Excluded (at TiAb stage)
Scoring for reliability	Klimisch (1997) giving 5 categories	ToxR Tool (Schneider et al., 2009) giving 3 Klimisch categories	Klimisch (1997) giving 5 categories
Nano considerations	4 categories from 1 (highest) to 4 (lowest) based on study design (dispersion and/or confirmation of internal exposure)	Nano score from 0 (lowest) to 10 (highest) identified based on whether characterisation of physico-chemical properties had been performed according to Card & Magnuson (2010)	Physicochemical characterisation of test article (source) and sample preparation considered but no scoring used. General criteria for nanoscale considerations taken from EFSA Opinion 2021 „Appendix E – Advice of the ccWG on Nanotechnology: Nanoscale considerations for the assessment of the study design and study results of TiO_2 toxicity studies".
Relevance categories for endpoints	3 categories (High, Limited or Low)	4 default weights (High, Moderate, Low or Negligible) but final weights could also be Moderate-high or Low-moderate	3 categories (High, Limited or Low)

NON-BIOLOGICAL STUDIES	Excluded (at TiAb stage)	Excluded (only studies with a conventional genotoxic endpoint were reviewed)	Excluded (at TiAb stage)
BIOLOGICAL STUDIES			
Studies on non-mammal species (e.g., fish, *Drosophila*, bees) and plants)	Excluded (at TiAb stage)	Excluded	Excluded (at TiAb stage)
STUDIES ON MAMMALS			
In vitro and *in vivo* studies	Included	Included	Included
Gut microbiota studies	Included	Excluded	Excluded
Toxicokinetic studies	Included	Included (if genotoxicity data in the same publication)	Included (if genotoxicity data in the same publication)
Local effects (e.g., inflammation, immune dysregulation, proliferation)	Included	Included (if genotoxicity data in the same publication)	Included (if genotoxicity data in the same publication)
Apical effects, general toxicity	Included	Included (if genotoxicity data in the same publication)	Included (if genotoxicity data in the same publication)
Mechanisms of action (e.g., oxidative stress)	Included	Included (if genotoxicity data in the same publication)	Included (if genotoxicity data in the same publication)
Test/measured endpoints	Different recognized genotoxicity endpoints	Only those endpoints and test systems with default "Moderate" or "High" weight were included according to the publication by Brusick et al 2016.	Different recognized genotoxicity endpoints
Information on study design (e.g., type of cells/animal species, doses tested, duration of studies etc.)	Included	Included	Included
IN VITRO ENDPOINTS			
Cytotoxicity evaluation *in vitro*	A low weight was given to studies in which no parallel toxicity evaluation was performed or an inappropriate toxicity test had been used.	Both negative and positive studies in which there was no concurrent measure of cytotoxicity, or an inappropriate measure of cytotoxicity was used, were considered unreliable and weight was downgraded.	Cytotoxicity assessed and its influence in each study considered. A low weight was given to studies in which no parallel toxicity evaluation was performed or an inappropriate toxicity test had been used.
Exposure of cells *in vitro*	More weight was given to study designs including observations confirming that cells were exposed to the nanoparticles. Negative results from studies where the cell uptake was not demonstrated were considered as inconclusive (to which only low relevance was assigned).	Negative results in mammalian cells were accepted, even if cellular exposure was not demonstrated, as long as treatment was for at least 1 cell cycle. Relevance (weight) of the study was then determined by other design and quality factors.	Negative results in mammalian cells were accepted only if different design and quality factors were acceptable (e.g. treatment was for at least 1 cell cycle).
Concentrations tested *in vitro*	A low weight was given to studies performed using only excessively high concentrations *i.e.* higher than 100 µg/ml (because of aggregation/agglomeration and precipitation of the tested nanoparticles at high concentration).	The relevance (weight) of the study was not changed just because high concentrations were tested, but agglomeration/aggregation was noted if it was measured and reported. Several studies with testing to concentrations >100 µg/mL retained Moderate weight.	The relevance of the study was not changed just because high concentrations were tested. Agglomeration/aggregation status was considered where possible. Several studies with testing to concentrations >100 µg/mL retained Limited relevance.
Ames test	Bacterial reverse mutation (Ames) assay is not considered suitable for investigation of gene mutations (due to limitations in the penetration of particles through the bacterial cell wall and the lack of	All Ames studies reviewed were given only Low or Low- moderate weight for the reasons given, whereas mammalian cell studies could retain Moderate weight if otherwise well-conducted.	Bacterial reverse mutation (Ames) assay is not considered suitable for investigation of gene mutations (due to limitations in the penetration of particles through the bacterial cell wall and the lack of internalisation in

	internalisation in bacteria), and therefore assigned low relevance.		bacteria), and therefore assigned low relevance and not analysed.
Gene mutations in mammalian cells *in vitro*	High relevance	Moderate default weight	High relevance
In vitro micronucleus test	Higher weight was given to studies with an extended treatment, covering at least one cell cycle. A low weight was given to studies in which cytochalasin B and nanoparticles were simultaneously added. A higher weight was given to studies in which the uptake capability of the selected cell lines was demonstrated. A low weight was given to studies based on cell lines with high background micronuclei frequency (higher than 2%).	Studies with an extended treatment, covering at least one cell cycle (either without cytochalasin B or before cytochalasin B was added) were more likely to retain Moderate weight. Studies with shorter treatments and no demonstration of cellular uptake, or where treatment was done in the presence of cytochalasin B, were considered unreliable and weight was downgraded. The uptake capability of the cells was not considered since there are few comparative data to make such judgements. The final weight was assessed on multiple design and quality factors. The weight of a study was not influenced by whether the background MN frequency was high, but on whether the control MN frequencies were within pre-agreed normal ranges (see Appendix 3 (ii)). The same approach was applied to *in vitro* CA and gene mutation studies (not discussed by EFSA).	Higher weight was given to studies with an extended treatment, covering at least one cell cycle. A low weight was given to studies in which cytochalasin B and nanoparticles were simultaneously added. A higher weight was given to studies in which the uptake capability of the selected cell lines was demonstrated. A low weight was given to studies based on cell lines with high background micronuclei frequency (higher than 2%).
Structural and numerical chromosomal aberrations *in vitro*	High relevance	Moderate default weight	High relevance
In vitro comet assay	High relevance Evaluation of the relevance of the test design included identification of possible interferences (e.g., interaction of nanoparticles with dye and lysis condition) within the comet assay at the applied test conditions.	*In vitro* comet assays were not reviewed (not included) because, as indicator tests (as specified in OECD guidance document; OECD, 2015a), they are less relevant in terms of genotoxic or carcinogenic risk.	High relevance Evaluation of the relevance of the test design included identification of possible interferences (e.g., interaction of nanoparticles with dye and lysis condition) within the comet assay at the applied test conditions.
Other genotoxic endpoints (SCE, UDS, γH2AX, direct DNA binding etc.)	Lower relevance (but included)	Low or Negligible default weight (and therefore excluded)	Lower relevance (but included)
IN VIVO ENDPOINTS			
In vivo studies Routes of exposure	Because TiO2 needs to be assessed as a food additive, administration by **non-oral** routes of exposure was considered of **limited or low relevance**, depending on the reliability of the study and other aspects such as information on the level of dispersion. Included: ingestion (feed, drinking water, gavage) intravenous, intraperitoneal injection Excluded: dermal contact injection (subcutaneous) inhalation dental bone implants	Of the non-oral routes, IP dosing was considered less physiologically relevant. However, IV studies were considered particularly relevant since exposure of the target tissue (e.g., bone marrow, liver) was more likely than by oral dosing. Included: ingestion (feed, drinking water, gavage) intravenous, intraperitoneal injection Excluded: dermal contact injection (subcutaneous) inhalation	Of the non-oral routes, IP dosing was considered less physiologically relevant. However, studies with inhalation, dermal and intravenous exposure were considered relevant. Included: ingestion (feed, drinking water, gavage) intravenous, intraperitoneal injection inhalation dermal contact Excluded: subcutaneous injection dental bone implants
Gene mutations *in vivo*	High relevance	High default weight	High relevance
Structural and numerical	High relevance	High default weight	High relevance

chromosomal aberrations *in vivo*			
Comet assay in vivo	High relevance	Moderate weight	High relevance
FINAL CONCLUSIONS ON GENOTOXICITY	Concerning the genotoxicity studies, combining the available lines of evidence, the Panel concluded that TiO2 particles have the potential to induce DNA strand breaks and chromosomal damage, but not gene mutations. No clear correlation was observed between the physico-chemical properties of TiO2 particles – such as crystalline form, size of constituent particles, shape and agglomeration state – and the outcome of in vitro or in vivo genotoxicity assays. The Panel concluded that several modes of action (MOA) may operate in parallel and the relative contributions of the different molecular mechanisms resulting in the genotoxicity of TiO2 particles are unknown. Based on the available data, no conclusion could be drawn as to whether the genotoxicity of TiO2 particles is mediated by a mode (s) of action with a threshold(s). Therefore, the Panel concluded that a concern for genotoxicity of TiO2 particles cannot be ruled out.	The conclusions from the 34 robust datasets reviewed, that achieved "Moderate" or higher weight, do not support a direct DNA-damaging mechanism for TiO2. However, carefully designed studies of apical endpoints (gene mutation, MN and/or CA, in vitro and in vivo), following OECD recommended methods, performed with well characterised preparations of TiO2, would allow firmer conclusions to be reached.	

TiAb = title and abstract (initial stage of literature screening)
Table adapted according to Kirkland et al., Regulatory Toxicology and Pharmacology 136 (2022) 105263; https://doi.org/10.1016/j.yrtph.2022.105263

Annex X. SCCS and EFSA analysis of studies on TiO_2 genotoxicity

The Annex is presented in three separate documents (pdf), linked to the Scientific Advice.