Scientific Committee on Consumer Safety

SCCS

GUIDANCE ON THE SAFETY ASSESSMENT OF NANOMATERIALS IN COSMETICS

2nd revision

The SCCS adopted this document

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About the Scientific Committees
Two independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) and are made up of scientists appointed in their personal capacity.
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SCCS
The Committee shall provide Opinions on questions concerning all types of 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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The SCCS GUIDANCE ON THE SAFETY ASSESSMENT OF NANOMATERIALS IN COSMETICS is not open for commenting as it remains a living document, which is regularly updated. Any observation may be sent to SCCS mailbox (SANTE-SCCS@ec.europa.eu) for further consideration by the SCCS.

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Applicants are invited to visit the SCCS website:

https://health.ec.europa.eu/publications/checklists-applicants-submitting-dossiers-cosmetic-ingredients-be-evaluated-sccs_en

where they will find a checklist

for submitting a safety dossier of a nanomaterial used in cosmetics.

Applicants are invited to visit the following website for further legislative information:

https://ec.europa.eu/growth/sectors/cosmetics/legislation_en

This Guidance on nanomaterials should be used in conjunction with the general guidance for the submission of safety dossiers of cosmetic ingredients "The SCCS Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation, 12th revision, SCCS/1647/22" or any future revision.

MAIN CHANGES IN THIS REVISION OF THE SCCS GUIDANCE ON THE SAFETY ASSESSMENT OF NANOMATERIALS IN COSMETICS

- New sections have been introduced (solubility and dissolution rate, solubility in non acqueous media, evidence for the absence of nanoparticles, dispersion, aspect ratio, uptake into blood cells, reproductive toxicity, endocrine disruption),
- The new European Commission recommendation for a definition of nanomaterials published in 2022 has been introduced,
- Key aspects triggering safety concerns over a nanomaterial based on SCCS/1618/2020 have been introduced,
- Other sections and Annex 1 have been updated based on literature that has been published since the last update,
- Section on read-across and grouping has been revised.
- A text explaining when historical/existing data can be used has been included.

This Guidance may be subject to future changes based on the evolution of science in the field of safety assessment of nanomaterials.

1. BACKGROUND

Introduction

Developments in the field of nanotechnology have opened up new prospects for innovation in cosmetics. At the same time, the use of very small particles in consumer products has raised concerns over their safety to human health and the environment (Borm *et al.*, 2006; Fadeel *et al.*, 2017; Wu and Tang, 2018). In Europe, the use of nanomaterials in cosmetics is specifically covered under the Cosmetic Regulation (EC) No 1223/2009, which provides a definition of a nanomaterial (NM) and requires premarket notification, safety evaluation, and labelling of cosmetics containing NM ingredients. If the Commission has concerns regarding the safety of an NM, the Commission shall refer it to the SCCS for a scientific opinion.

Until now, the SCCS has assessed several safety dossiers on NMs intended for use in cosmetic products. A list can be found at: https://ec.europa.eu/health/scientific-committees/former-scientific-committees/scientific-committee-consumer-safety-2016-2021/sccs-opinions-2016-2021_en

A number of issues and questions have been identified by the SCCS regarding the types and quality of the information and data that must form part of the safety dossiers on NMs. In view of this, the SCCS published a memorandum (SCCS/1524/13 Revision of 27 March 2014) to highlight the importance of relevance, adequacy and quality of the data provided in a safety dossier on NMs. In 2019, the Scientific Committee on Consumer Safety (SCCS) updated its first Guidance (SCCS/1484/12) on safety assessment of NMs in cosmetics (SCCS, 2019).

As such, this Guidance is an up-to-date revision of the existing Guidance (SCCS, 2019) and is aimed at providing an overview of the key issues and data requirements relating to the safety assessment of NMs in cosmetics. In updating the Guidance, the SCCS has considered information available in published literature as well as other relevant documents; such as those published by the European Food Safety Authority (EFSA, 2021a); the European Chemicals Agency (ECHA, 2012, 2017a, b, c, 2021); the guidance published by the US Food and Drug Administration (FDA, 2014, 2022); a report of the International Cooperation on Cosmetics Regulation (ICCR, 2012); as well as reports from the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2009, 2010, 2015) and the Organisation for Economic Co-operation and Development (OECD, 2009, 2010a, c, 2022a). In addition, the ISO (International Organization for Standardization, Geneva, Switzerland) Technical Committee (TC) 229 "Nanotechnologies" has published a number of documents regarding nanomaterial and nanoparticle characterization and testing that are of interest as well for the evaluation of nanomaterials used as cosmetic ingredient (https://www.iso.org/committee/381983.html).

This Guidance is applicable to any material that meets the criteria for an NM as outlined in the Cosmetic Regulation (EC) No 1223/2009, *i.e.* "An insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm." In addition, in 2022 the Commission adopted a Recommendation (2022/C 229/01) that provides an overarching definition of NM. The Recommendation has proposed a threshold of 50% or more particles of the total number of particles in a material to be in the nanoscale for it to be regarded an NM. This Recommendation has not yet been applied to the definition of NM under Cosmetic Regulation

(EC) No 1223/2009. However, it is recommended that Applicants keep it in mind when assessing safety of the materials used in cosmetics that are comprised of or consist of small particles, or exhibit a size-related change in properties, behaviour, and/or effects compared to the conventional (bulk) ingredients. In view of the EU Chemicals Strategy for Sustainability (COM(2020) 667 final, Brussels, 14.10.2020) it is likely that the definition for a nanomaterial in the Cosmetic Regulation will be aligned (Ares(2021)6011962 - 04/10/2021) with the recently published Commission Recommendation of 10 June 2022 on the definition of nanomaterial (2022/C 229/01).

Since the current definition for NM as outlined in the Cosmetic Regulation (EC) No 1223/2009 explicitly mentions insoluble or biopersistent nanomaterials, it may pose a difficulty in regard to the interpretation of the term 'insoluble'. For example, NMs that only show a partial solubility may be regarded as 'soluble' in relative terms. However, it needs to be considered whether the nanomaterial is present in a cosmetic formulation in particulate form, and if it is present for a specific functionality. When dealing with the question of solubility, as provided in the current definition, it is important to note that any nano-specific risk may change (even diminish) when a nanomaterial is dissolved. But it is the time over which the dissolution happens that determines the considerations for risk assessment based on either particle risk or soluble substance risk. For the current definition in the Cosmetic Regulation, it may be mistaken to claim that materials that show partial dissolution over a long period of time are 'soluble', and therefore not a nanomaterial under the scope of the current definition provided in the Cosmetic Regulation (EC) No 1223/2009.

Conclusions

The Guidance aims to help Applicants byfacilitating the procedure of preparing safety dossiers and to assist risk assessors and risk managers in the implementation of the provisions of article 16 of Cosmetics Regulation (EC) No 1223/2009. The Regulation imposes strict conditions and timelines for notification and assessment of cosmetic products containing NMs on the Responsible Persons, as well as on the SCCS. All the essential elements that would be required in an NM safety dossier are covered in this Guidance, *i.e.* physicochemical characterisation, exposure assessment, toxicological evaluation and risk assessment. As such, this Guidance is complementary to the SCCS general Notes of Guidance for specifically addressing safety aspects of NMs, and therefore must be considered in conjunction with the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision).

The Guidance will be revised and updated by the SCCS when considered appropriate to take account of any new scientific advancements and the new knowledge and experience in this field.

The Cosmetic Regulation (EC) No 1223/2009 specifically covers the risk of nanomaterials (NMs) used in cosmetic products. In case of a concern over the safety of an NM, the European Commission refers it to the SCCS for a scientific opinion. To assist Applicants and risk assessors in preparing and assessing safety dossiers on nanomaterials, the SCCS published a Guidance on safety assessment of NMs in cosmetics in 2012 that was updated in 2019 (SCCS, 2019). More scientific knowledge has since come to light, and several NMs intended for use in cosmetic products have gone through safety evaluation by the SCCS. This Guidance

is therefore intended to provide an up-to-date revision of the 2019 Guidance by taking into account new developments in the area of NM safety research and the experience gained in safety assessment of nanomaterials so far. Irrespective of the foreseen changes in the definition of nanomaterial in the upcoming revision of Cosmetics Regulation, certain aspects of nanomaterials, such as solubility, will remain of paramount importance for the safety assessment.

2. GUIDANCE

In addition to other requirements under relevant regulation, this document is intended to provide specific guidance on the safety evaluation of NMs intended to be used as cosmetic ingredients. NMs may exhibit certain physicochemical properties, biokinetic behaviour, biological interactions, and/or toxicological effects that are different from the conventional or bulk form of the same ingredients. This guidance therefore highlights specific aspects that should be considered when testing and reporting data for NMs. It points out the type of data/information that must be provided by the Applicant to the Commission in support of the safety of the NMs intended for use in cosmetics. For the overall safety assessment of cosmetic ingredients, this guidance should be used in conjunction with the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or or any future revision).

The Guidance is structured in separate sections covering Requirements for Safety Assessment (2.1), Physicochemical Characterisation (3), Exposure Assessment (4), Hazard Identification and Dose-Response Characterisation (5), and Risk Assessment (6) of NMs. A summary and conclusions of the main aspects discussed are provided in Section 7.

It also needs to be emphasised that the guidance provided in this document is based on the currently available knowledge. As the field of NM safety assessment is still evolving, future revisions will be carried out as necessary when new scientific knowledge becomes available.

2.1 Requirements for safety assessment of NM in cosmetics

Introduction

Regulation (EC) No 1223/2009 specifically covers the use of NMs in cosmetic products. It not only provides a definition of NM, but also a mechanism for the notification, labelling, and consumer safety evaluation of NMs used in cosmetic products. It should be noted that the recent proposal for the EU Chemicals Strategy for Sustainability (COM(2020) 667 final Brussels, 14.10.2020) also includes a revision of the Cosmetic Products Regulation as a targeted revision alongside other chemicals regulations including the REACH Regulation and the CLP Regulation (Ares(2021)6011962 - 04/10/2021).

According to Article 13 (1) of Regulation (EC) No 1223/2009 ('Cosmetics Regulation'), the Responsible Person should notify the Commission prior to placing the cosmetic product on the market. For cosmetic products containing NMs, there is a specific deadline for the notifications, *i.e.* they must be notified at least six months prior to being placed on the market (Article 16 (3) of the Cosmetics Regulation). If the Commission has concerns about the safety of an NM, it shall request the SCCS to give an opinion within a period of six months (Article

16 (4)). The SCCS evaluation of NM safety is mainly based on the dossier submitted by the Applicant(s) and notifications received through the CPNP Portal of the Commission by the Notifiers. In addition, the SCCS may also use information gathered from published literature and/or received from other stakeholders as a result of a Commission's call for data. In cases where further data/clarifications are needed, the 6-month clock starts again once the necessary data/information is provided by the Applicant.

Certain categories of cosmetic ingredients - *e.g.* colourants, UV-filters and preservatives, including their nanoforms - can only be used in cosmetic products when 'authorised', *i.e.* listed in Annexes IV-VI, respectively (Article 14 (1) (c)-(e)). These substances are designated to be subjected to SCCS opinions in order to be 'authorised'. NMs belonging to these categories are not assessed under Article 16 (4)[1]. Consequently, the deadline of six months for notification does not apply to products containing NMs that are used as colourants, UV-filters and preservatives. Such products should be notified to the Commission as is the case for any other product, *i.e.* prior to being placed on the market (Article 13).

When a cosmetic ingredient fulfils the criteria defining an NM, as set out in the Cosmetic Regulation (EC) No 1223/2009, Article 2 (1) (k)[2] (or any revisions), safety data with special considerations to the properties of that specific NM will be required for safety assessment. This will apply to any new or already approved ingredient if it fulfils the criteria for definition of an NM; for example, when an approved ingredient is manufactured by a different process and the generated material is comprised of particles in the nano-scale.

Definition of a nanomaterial

In 2022, the Commission adopted a Recommendation on an overarching definition of NM (2022/C 229/01). According to this Recommendation:

'Nanomaterial' means a natural, incidental or manufactured material consisting of solid particles that are present, either on their own or as identifiable constituent particles in aggregates or agglomerates, and where 50 % or more of these particles in the number-based size distribution fulfil at least one of the following conditions:

(a) one or more external dimensions of the particle are in the size range 1 nm to 100 nm;
(b) the particle has an elongated shape, such as a rod, fibre or tube, where two external dimensions are smaller than 1 nm and the other dimension is larger than 100 nm;
(c) the particle has a plate-like shape, where one external dimension is smaller than 1 nm and the other dimensions are larger than 100 nm.

In the determination of the particle number-based size distribution, particles with at least two orthogonal external dimensions larger than 100 µm need not be considered.

However, a material with a specific surface area by volume of < 6 m^2/cm^3 shall not be considered a nanomaterial.

[1] Article 16 (2) states that "The provisions of this Article do not apply to NMs used as colorants, UV-filters or preservatives regulated under Article 14, unless expressly specified."
[2] According to the definition under Article 2(k) of Cosmetic Regulation, 'nanomaterial' means an insoluble or biopersistant and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm.

According to the Recommendation:

- 'particle' means a minute piece of matter with defined physical boundaries, single molecules are not considered 'particles',
- 'aggregate' means a particle comprising of strongly bound or fused particles,
- 'agglomerate' means a collection of weakly-bound particles or aggregates where the resulting overall surface area is similar to the sum of the surface areas of the individual components.

How this new EU definition recommendation differs from the previous one (*i.e*. 2011) was explained in a 2023 publication (Rauscher *et al.*, 2023a). In addition, a JRC guidance to support implementation of the new definition recommendation became available in 2023 (Rauscher *et al.*, 2023b). More detailed and technical information about the definition of an NM is available in the 'questions and answers' section of the European Commission website[3].

This Recommendation suggests excluding non-solid (flexible) complex structures (*e.g.* manufactured from proteins, lipids and/or polymers). However, these structures may have characteristics of particulates and behave like particles. This has to be seen in conjuction with Recital 23 of that Recommendation where it is stated: "It may likewise be considered necessary to develop regulatory requirements for additional materials not falling under the definition of the present Recommendation, in the scope of application of specific Union legislation or legislative provisions targeting nanomaterials. Such legislation should, however, aim to differentiate between a 'nanomaterial' and a member of such subgroup as to maintain consistency with the definition and consequently other legislation".

The 2022 EC Recommendation (2022/C 229/01) has not yet been applied to the definition of a nanomaterial under the Cosmetic Regulation (EC) No 1223/2009. In view of the announced targeted revision of the Cosmetics Regulation (Ares(2021)6011962 - 04/10/2021), however, it is advisable that Applicants take this Recommendation (and any resulting revision of the definition) into consideration when assessing the safety of the cosmetic ingredients that are comprised of or consist small particles. Furthermore, even when the materials in question do not strictly fall under the EU recommendation for a definition of a NM, it may be necessary to considerif particle toxicology is relevant due to the presence of a fraction of small particles in a conventional material. Applicants should consult the EFSA Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particle including nanoparticles (EFSA, 2021b).

In situations where a particulate material has internal nano-structures, or exists in the form of larger agglomerates or aggregates, the use of volume specific surface area (VSSA) (Kreyling *et al.,* 2010; OECD TG 124 (OECD, 2022d), and/or other parameters, such as electron microscopy images (Scanning Transmission Electron Microscopy (STEM); Transmission Electronic Microscopy (TEM); Scanning Electronic Microscopy (SEM)), can provide further information - *e.g.* on the size of primary nanoparticles (NPs), structure and coatings. A decision flow scheme has recently been developed by the NanoDefine project (www.nanodefine.eu) to make it easier to establish whether or not a material should be regarded as an NM according to the previous EC recommended criteria for definition (Recommendation 2011/696/EU), and to identify suitable methods and tools for NM

[3] http://ec.europa.eu/environment/chemicals/nanotech/faq/questions_answers_en.htm

characterisation. It should be noted that a nanomaterial cannot be defined by its size only, as the size of all the particles in a nanomaterial is not uniform, but should also be characterised by size distribution. So, for the identification of a specific nanomaterial the size AND size distribution (in particle number) are critical parameters, as described in OECD TG 125 (OECD, 2022b).

The International Organization for Standardization (ISO, Geneva, Switzerland) has published a series of documents dealing with several aspects of nanotechnology nomenclature, the ISO 80004 series on nanotechnology vocabulary including, for example, ISO/TS 80004-2:2015 (confirmed in 2021, previously ISO/TS 27687:2008) that describes the terms nanoparticle, nanofiber and nanoplate. A fundamental document will be the revision of ISO/TS 80004-1 that will contain nanomaterial terminology including the core terms (currently ISO/TS 80004-1:2015), nano-objects (currently ISO/TS 80004-2:2015), nanostructured materials (currently ISO/TS 80004-4:2011), and nanolayer, nanocoating, nanofilm, and related terms (currently ISO/TS 80004-11:2017).

Safety assessment

As indicated by SCENIHR (2009), NMs, like other substances, may or may not be harmful. In principle, the risk assessment paradigm including exposure assessment, hazard identification, dose response characterisation, and risk characterisation, routinely used for conventional substances, also applies to NMs. However, because of the nano-scale dimensions, and the potential qualitative and quantitative differences in physicochemical properties, biokinetic behaviour, and toxicological effects, there may be additional or different concerns in regard to the safety of NMs to consumer health. As indicated in this Guidance, the testing and subsequent safety assessment of NM ingredients will therefore require certain additional considerations, and/or adaptation of testing methods in view of the nano-scale features and properties of the NMs. These aspects need to be specifically addressed when NM ingredients are used in a cosmetic product. In particular, aspects relating to particle nature and nano-dimensions need to be considered throughout the safety assessment; *i.e.* during material characterisation, hazard identification and characterisation, exposure assessment, and safety evaluation. It is therefore important that relevant data and information on the various testing and production stages are provided by the Applicant for each NM intended for use in cosmetic products (see also SCCS/1588/17 "Checklists for Applicants submitting dossiers on Cosmetic Ingredients to be evaluated by the SCCS").

Irrespective of the presence of NM(s), the existing regulations and the SCCS Notes of Guidance on testing of cosmetic ingredients and their safety evaluation (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision) must be followed.

Cosmetic Regulation (EC) No 1223/2009 provides a definition of NM as well as a mechanism for pre-market notification, safety evaluation and labelling of cosmetic products containing a NM. This Guidance is applicable to cosmetic ingredients that fulfil the criteria defining an NM as set out in the Cosmetic Regulation (EC) No 1223/2009, Article 2(1) (k) (or any future revisions). In view of the targeted revision of the Cosmetics Regulation, it is also advisable to take into account the Commission Recommendation (2022/C 229/01) on the overarching criteria for definition of NM when assessing the safety of a material that is comprised of, or consists small particles. This Guidance might also be used for the risk assessment of

particulate materials that contain small particles (not falling under the EC Recommendation for nanomaterial definition).
Safety assessment of NMs is carried out using the same principles that are routinely used for conventional substances. However, because of the nano-scale dimensions and the potential differences in physicochemical properties, biokinetic behaviour, and toxicological effects, additional aspects need to be considered in testing and safety assessment of NMs. The data and information provided for an NM must be relevant, of high quality and adequate to allow safety assessment (see also SCCS/1524/13 and SCCS/1588/17).
Irrespective of the presence of NM(s), the existing regulations and the SCCS Notes of Guidance on testing of cosmetic ingredients and their safety evaluation (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or or any future revision) must be followed.

The use of nanomaterials in cosmetics is regulated under the EU Cosmetics Regulation so as to provide a high level of protection of human health. This is because nano forms may differ from their conventional (bulk) forms in terms of physicochemical properties, biokinetic behaviour, and/or biological effects. Some materials manufactured at the nano-scale show significant deviations in physicochemical properties, interaction with biological systems, and/or toxicological effects, compared to conventional equivalents. For example, nanoparticles (NPs) in the lower nanometre (nm) range may penetrate biological membrane barriers that normally prevent the entry of (larger) particulate materials into cells and tissues (Jani *et al.,* 1990; Geiser and Kreyling 2010; Landsiedel *et al.,* 2012; Treuel *et al.,* 2013; Hougaard *et al.,* 2015; ECHA, 2017b, c; Nakamura and Watano, 2018). It is therefore possible that, once internalised in the form of NPs, some insoluble or poorly soluble materials may be able to reach those parts of the body that larger sized particles cannot reach. An accumulation of NPs in an organ may also result in a high local concentration of released substance that would not be reached when the (soluble) substance would be equally distributed in the whole body. As particle size at the nanoscale may be accompanied by certain specific changes in physicochemical properties, a detailed characterisation of the NM intended for use in cosmetic products becomes crucially important. Characterisation is not only highly important for proper identification of the NM in terms of chemical composition and physical characteristics, but also in relation to other particle-associated properties that are important for safety assessment (see Section 3 – Physicochemical Characterisation).

Following a mandate by the Commission, the SCCS published a Scientific Advice in 2020 (SCCS/1618/2020), which highlights the key aspects of NMs, the presence of which in an NM should raise safety concerns for a safety assessor. The advice provides the scientific reasoning behind such aspects to help prioritise nanomaterials for further evidence-based safety assessment. In this regard, the SCCS considers that in the absence of any hard and fast rules for identifying safety concerns for NMs, as a general principle, each of the following attributes should add a further degree of concern over the safety of an NM. For example, where:

1. The NM has constituent particles that have sizes in the lower range of the nanoscale.

2. The NM is insoluble, or only partially soluble.

3. The chemical nature of the NM suggests the potential for a toxicological hazard.

4. The NM has certain physical/morphological features (*e.g.* needle shape, rigid long fibres) that point to the potential for harmful effects.

5. The nanomaterial has surface reactivity in terms of catalytic (including photocatalytic) activity, potential for radical formation, or other surface properties (*e.g.* potential allergenicity due to proteinaceous surface).

6. The NM has a different biokinetic behaviour than the conventional equivalent. For example, a surface modification/coating (*e.g.* hydrophobic coating, encapsulation) has been applied to the core NPs to alter their ADME properties, and as a result make them more systemically available, compared to the neat NPs and/or their conventional chemical forms.

7. The NM is used as vehicle to carry other substances that have not been assessed for safety as individual components, and when together in the form of the nano-scale delivery entity.

8. There is a likelihood of systemic exposure of the consumer to NPs through the use of final products. The frequency of use, and/or the amounts of the relevant consumer product are relatively high.

9. There is evidence for persistence/accumulation of NPs in the body.

10. The NPs have other distinctive properties not present in conventional form of the same material, or have a new activity/function (*e.g.* a smart/functional NM).

11. The NM is a novel entity so that it does not have a conventional comparator to allow assessment of changes in properties, behaviour or effects.

12. The NM is used in a product that is inhalable (taken up by inhalation into respiratory tract and lung), and the particles are respirable (can reach respiratory epithelium *i.e.* alveoli).

13. The assessment of genotoxicity is performed inadequately*, e.g. in vitro* studies are without information on stability of the test suspension, or evidence of cell exposure (internalisation).

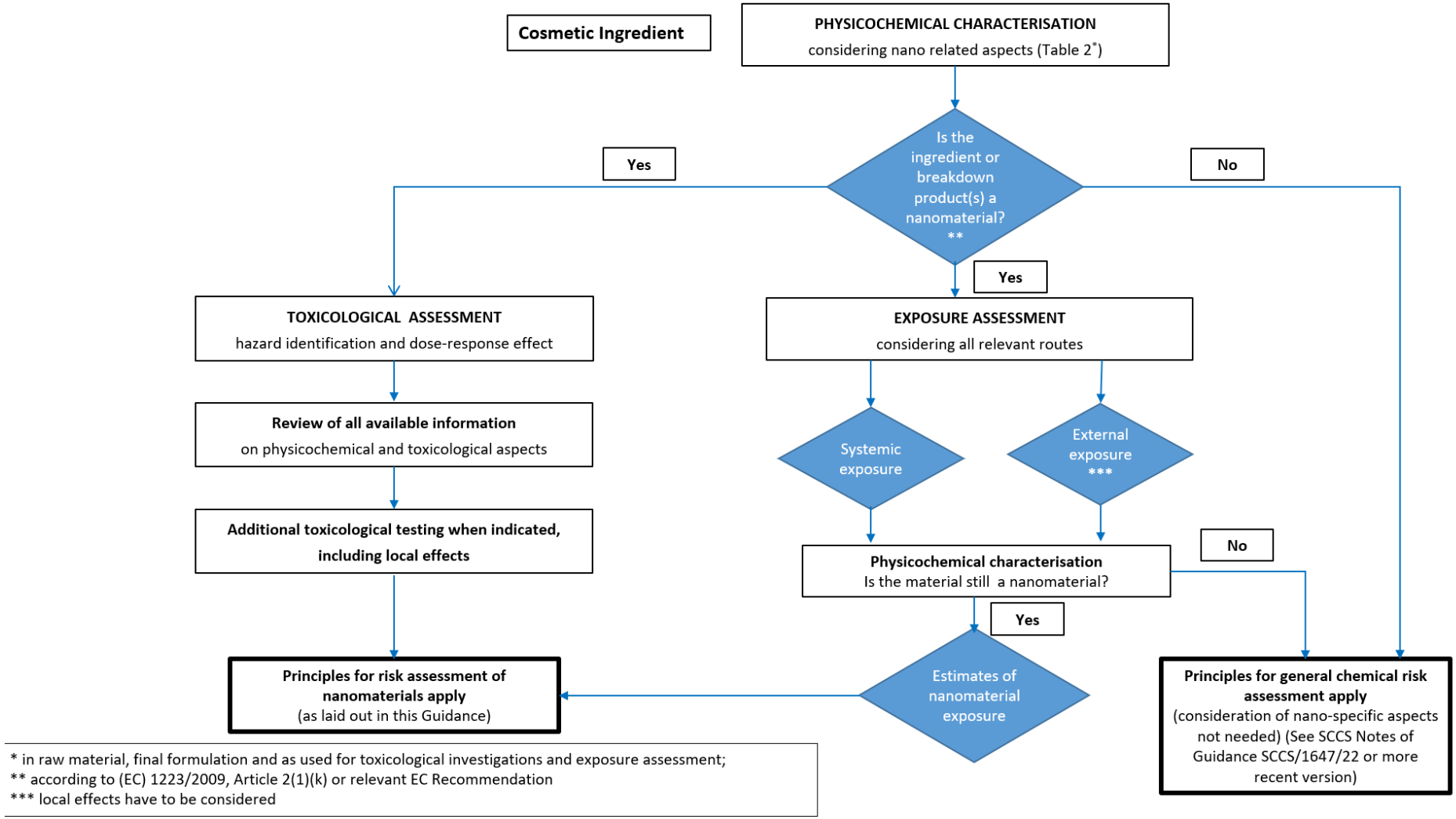
A number of studies and reports investigating possible regulatory gaps have concluded that the current risk assessment paradigm used for conventional bulk materials should in principle be also applicable to NMs (SCENIHR, 2009; OECD, 2009; Chaudhry *et al.,* 2010; EC, 2012). The current hazard identification/dose-response characterisation, which is based on a structured framework for toxicological evaluation of conventional chemicals, should also identify/characterise toxic effects of NMs, provided that nano-related aspects have been duly considered during testing. However, it should be noted that the possibility to evaluate toxicity and dose-response relationship from *in vitro* studies will be limited as a recourse to animal studies will not be available due to the ban on animal testing under the Cosmetic Regulation.

As indicated in Figure 1, any risk assessment needs to start with an extensive literature review considering the NM under investigation. The focus of this review should be based on the characterisation of the NM under investigation. On the basis of the information obtained, dedicated tests may be undertaken to investigate NM biological and/or toxicological activity.

The conventional risk assessment approach for chemicals considers both hazard and exposure – where the absence of one means no risk to the consumer. Keeping this in mind, safety assessment of NM cosmetic ingredients may, in the first instance, be driven by exposure considerations, with attention to any distinctive particle-related characteristics at the nano-scale (see Figure 1 and Table 1 in Chapter 3.1 Key physicochemical parameters). This will inevitably require detailed characterisation of NMs and determination of the likelihood and extent of systemic exposure resulting from potential translocation of nanoparticles across dermal, respiratory, or gastrointestinal barriers depending on the route(s) of product exposure and the possible systemic uptake (see Section 4). In addition, local effects will need

to be considered, irrespective of whether or not the use of a cosmetic product containing NMs can lead to systemic exposure. Even in the absence of systemic availability as an NM, and when no local effects are being observed, it should be assessed whether any chemical constituents have translocated that could cause systemic effects. This means that the safety of the NM also needs to be assessed as a chemical in accordance with the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). The evaluation of photocatalytic activity is also required for dermally applied cosmetic products containing NMs.

Figure 1: Schematic outline for the safety assessment of nanomaterials in cosmetics



* in raw material, final formulation and as used for toxicological investigations and exposure assessment;
** according to (EC) 1223/2009, Article 2(1)(k) or relevant EC Recommendation
*** local effects have to be considered

Exposure considerations

As mentioned before, due to the nano-scale dimensionsand potentially altered uptake and biokinetics, some NMs may pose a health risk to the consumer because of the ability of insoluble or poorly-soluble NPs to penetrate biological membrane barriers and reach those parts of the body that are otherwise protected from exposure to (larger) particles. Although transport of NMs to secondary organs has been observed, it is still not clear if accumulation of those NMs that are considered to be of low toxicity or apparently non-toxic could also lead to a toxicological effect, and/or contribute to a pathological change in organs in the long term (Kermanizadeh *et al.*, 2015). The uptake mechanism of a particular NM can also differ depending on the cell type and the exposure route (dos Santos *et al.*, 2011; Bezhadi *et al.,* 2017). At present, there is insufficient understanding of the nature of interaction of NMs with biological moieties that may take place at or close to the molecular level. Keeping this in mind, where there is evidence for systemic availability of NPs, further investigations into hazard identification and dose-response characterisation will be required in consideration of the nano aspects.

For NMs, determination of ADME (Absorption, Distribution, Metabolism, Excretion) parameters should receive special attention. These aspects have historically been determined through *in vivo* studies. However, Cosmetics Regulation (EC) No 1223/2009 has placed a complete ban on *in vivo* testing and marketing for cosmetic products and their ingredients. The generation of *in vivo* data for cosmetic products and ingredients was forbidden in the EU as of September 2004 and March 2009, respectively, with the exception of skin sensitisation, repeated dose toxicity, toxicokinetics and reproductive toxicity (when carried out outside the EU). Subsequently, the generation of *in vivo* data for all endpoints was forbidden as of March 2013. Thus, only data produced before these timelines can be used in support of safety assessment of cosmetics and their ingredients. A key scientific objective of the EU is to promote the development and validation of alternative methods that adhere to the 3Rs principle (replace, reduce, refine), and to provide a level of safety equivalent to that obtained through animal testing while using fewer animals, causing less suffering, or avoiding any use of animals. In view of the ban, the need for implementing non-animal alternatives is particularly crucial for safety assessment of cosmetic ingredients/products because safety data can only be drawn from alternative methods, meaning that the 3Rs choices are effectively restricted to 1R (*i.e.* Replacement of animal testing). In view of this, the SCCS considers all available scientific data, taking into account the testing and marketing bans in force under Regulation (EC) No 1223/2009. This includes physical and chemical properties of the compounds under investigation, *in silico* data such as the results obtained from (Q)SAR {(Quantitative) Structure Activity Relationship} modelling, chemical categories, grouping, read-across, Physiologically Based Pharmacokinetics (PBPK)/Toxicokinetics (PBTK) modelling, *in vitro* and *ex vivo* experimental results. There may, however, be situations where *in vivo* data are available for an NM from studies carried out before the testing bans, or from studies that had been carried out to fulfil data requirements of a different (non-cosmetic) legislation; *e.g.* for assessment as a medicinal or food ingredient, a pesticide or biocide, or an industrial chemical under REACH-Regulation (EU, 2008). Such data may be accepted for the safety assessment of the NM intended for use as a cosmetic ingredient if evidence is provided that the data had been generated prior to the animal testing bans (*i.e.* before March 2009 or March 2013 depending on the toxicological endpoint), or the *in vivo* data were required for other

regulations for non-cosmetic applications of the NM (see also Factsheet on the Interface between REACH and Cosmetic Regulation https://echa.europa.eu/documents/10162/13628/reach_cosmetics_factsheet_en.pdf/2fbcf6bf-cc78-4a2c-83fa-43ca87cfb314. If such data are available, these should be submitted as part of the safety dossier of a cosmetic ingredient.

Hazard considerations

Despite some refinement and reduction improvements to the existing *in vivo* test guidelines, and development of guidelines for replacement methods, the available **validated replacement** methods only cover **some** of the toxicological endpoints that are needed for safety assessment. Also, the data/information generated by most alternative methods relate to **hazard identification**. The currently available and validated *in vitro* methods for conventional chemicals concern skin corrosion, skin irritation, skin sensitisation, eye irritation, mutagenicity/genotoxicity and phototoxicity. For reproductive toxicity, there are three validated methods (Annex I), but these have not been taken up in the regulatory context because of the lack of specificity. For carcinogenicity, recently validated *in vitro* cell transformation assays (CTAs) are promising tests for predicting NM-induced cell transformation as one of the crucial endpoints of carcinogenicity. Due to a variety of reasons, including the complexity of vertebrate organisms, at present there is no validated *in vitro* method available either for repeated dose toxicity (including reproductive toxicity, developmental toxicity and carcinogenicity), or any proposal currently in place for pre-validation/validation (Worth and Balls, 2002; Rogiers and Pauwels, 2005; Adler *et al.*, 2011; JRC annual status reports on non-animal methods (JRC, 2016a), (JRC, 2017), (JRC, 2019), (JRC, 2020), (JRC, 2021), (JRC, 2022), (JRC, 2023); SCCS 1628/21.

It is also of note that none of the currently available validated alternative methods for conventional chemical substances has so far been validated specifically for NMs (OECD 2018a). Also, apart from testing dermal absorption, the currently available *in vitro* tests are not suited for dose-response characterisation of possible *in vivo* harmful effects (SCCP, 2007; SCCS, 2009; Adler *et al.* 2011; JRC status reports on non-animal methods for 2016 (JRC 2016a), 2017 (JRC, 2017), 2018 (JRC, 2019), 2019 (JRC, 2020), 2020 (JRC, 2021), 2021 (JRC, 2022), 2022 (JRC, 2023). This means that conducting quantitative risk assessments of cosmetic NMs based on alternative methods is challenging at present. However, this situation is not specific to NMs, and equally applies to conventional cosmetic ingredients as well. Notwithstanding such limitations, the use of *in vitro* methods for NMs will require certain additional considerations of the particle nature and other nanoscale aspects, and the testing methods may need certain adaptations or further characterisation and validation. These aspects are discussed in more detail in Section 5.

The ban on animal testing is another reason why the safety assessment of cosmetic NMs may be driven by consideration of exposure scenarios and exposure related aspects (see Figure 1), with a focus on detailed characterisation of the NMs (Table 1), and with nano-related considerations during toxicological evaluations (Section 5 and Annex I). In view of the current lack of alternative methods that have been specifically validated for NMs, the SCCS also considers data obtained from those methods that may not yet have undergone formal validation but can be demonstrated to be scientifically valid. A recent analysis in a report from QSAR LAB (personal communication, to be published on the EUON site in autumn 2023) has shown that currently (June 2023) only a few nano-specific NAMs can be considered acceptable

for regulatory use (1 on phototoxicity, 5 on *in vitro* toxicity and 2 on dissolution in biological media). In addition, OECD has published a Study Report and Preliminary Guidance on the Adaptation of the *In Vitro* micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials (OECD, 2022c).

Regarding toxicological studies, it is important to note that interactions of an NM with biological systems may be different from those expected from conventional forms of the same material. Some of these interactions may bring about further changes in physicochemical characteristics of the NM. A well-known example of the latter is adherence of molecules including proteins to the NM surface, the so-called 'protein corona' (Mortensen *et al.,* 2013; Ke *et al.,* 2017; Garcia-Alvarez *et al.,* 2018; Da Silva *et al.,* 2019; Francia *et al.,* 2019; Breznica *et al.,* 2020; Galdino *et al.*, 2021; Kopac, 2021; Cai *et al.*, 2022; Choi *et al.*, 2022). Therefore, toxicological investigations also need to consider any changes in the physicochemical properties of NMs (see Section 5). The key parameters to consider include nano-scale dimensions (size, morphology, surface area), agglomeration/ aggregation behaviour, surface characteristics of the particles, etc. (Rocks *et al.,* 2008; SCENIHR, 2009; OECD, 2009; Chaudhry *et al.,* 2010; Yu *et al.*, 2017; Miceli *et al.,* 2017). As suggested for nano-TiO_2, the induction of reactive oxygen species (ROS), seems to be a key event in initiating NM toxicity in the lung and gastrointestinal tract (Brand *et al.*, 2020; Braakhuis *et al.*, 2021a).

A schematic outline for the safety assessment of NMs is presented in this section. Detailed physicochemical characterisation of NMs is crucially important for identification of the NM under investigation and in view of the potential changes in material properties at the nanoscale. Any safety assessment needs to start with an extensive literature review considering the NM under investigation.

The current hazard identification/dose-response characterisation strategies used for conventional chemicals should also be applicable to NMs, provided that nano-related aspects have been duly considered during testing. Safety assessment should consider local and systemic exposure to NPs, local and systemic harmful effects, and any health risks to the consumer as a result of the exposure.

In the first instance, safety assessment of NMs may be driven by exposure considerations, with attention to distinctive material characteristics at the nano-scale. Even when there is no systemic absorption of NMs, and/or local effects, safety of the NM as a chemical will need to be assessed according to the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). Where there is evidence for systemic availability of NPs, further investigations into hazard identification and dose-response characterisation will be required in consideration of the nano aspects. For systemically available NMs, determination of ADME parameters should receive special attention.

Cosmetics Regulation (EC) No 1223/2009 placed a complete ban on animal testing of cosmetics and marketing of cosmetic ingredients/products that have been tested in animals from March 2013. Thus, toxicological data need to be derived from validated or scientifically valid alternative means, such as *in vitro* and *ex vivo* methods, *in silico* models, grouping and read-across, physiologically-based pharmacokinetic (PBPK) and toxicokinetic (PBTK) modelling.

In addition, interactions of an NM with biological systems have to be considered. Animal data can be accepted if the testing had been carried out either on a date prior to the animal test

ban, or to meet requirements under a different regulatory framework (*i.e.* not for cosmetics use).

3. PHYSICOCHEMICAL CHARACTERISATION

The properties, behaviour, and biological effects of NMs may be influenced by a number of physicochemical parameters. Detailed data and information on physicochemical characterisation of NMs therefore forms an integral part of the risk assessment. The characterisation data presented in a safety dossier should provide an unambiguous **identification** of the chemical composition of the NMs. They must also be relevant to the NM that is used in the final cosmetic product. Where the data relate to a different NM, or a different form of the NM than that intended for use in the final product, justification should be given, and the scientific basis provided for considering both as 'similar' to allow data read-across between the NMs for safety assessment.

Changes in the manufacturing process may lead to significant differences in the physicochemical and morphological characteristics of different batches of the same NM. They may also introduce new/different impurities and other residual materials. For some materials, fundamentally different production processes are used (*e.g.* for the production of silica via pyrogenic and precipitation processes) which may define the surface characteristics and thus particle properties. It is therefore important to provide a description of the manufacturing process (EFSA, 2021b).

Due to the potential for significant differences in the physicochemical characteristics of the same pristine NM resulting from variations in the manufacturing process, or when produced by different manufacturers, or due to aging (*e.g.* agglomeration/aggregation, sedimentation), it is important that detailed specifications of the NM intended for use in a cosmetic product are provided by the Applicant. The specification should include an acceptable range for each physicochemical parameter in consideration of the batch-to-batch variation, and/or any aging effects. This information will be used by the risk assessors to decide whether or not the batch(es) used in toxicity testing can be considered representative for safety assessment of the NM intended to be used in cosmetic products (EFSA, 2021a).

Different formulations can also affect physicochemical properties of NMs. It is therefore also of utmost importance that the physicochemical status of an NM in the final cosmetic product is determined at different stages, as detailed below.

Each NM has a specific (bio)chemical composition of its core and surface, as well as a physical structure of the surface. The behaviour, interaction, fate and effects of an NM are inevitably influenced both by the nano-dimensions (size, morphology, surface area), the nature of the chemical(s) that make up the NM including surface characteristics, and the structural form (crystalline structure). A NM may pose a hazard to health and/or the environment not only due to inherent chemical composition, but also due to the nano-scale features, including surface composition (*e.g.* coatings), which may modulate the uptake, biokinetics and toxic effects.

In this regard, it is important to note that any nano-related properties are intrinsically linked to the physical integrity of the nano-structure of an NM. Where an NM loses its nano-structure, *e.g.* in a formulation, a test medium, or biological surface/environment, due to solubilisation, breakdown or degradation, or interactions with other substances, it will no longer be expected to behave differently from its non-nano equivalent. It may still pose a toxicological hazard at the local level in case the chemical constituents can cause local effects by themselves. Additionally, systemic toxic effects might occur if, before disintegration, the nanostructure had delivered the chemical constituents to a biological site where the conventional form would have not led to a comparable exposure. Determining stability of the NM under experimental conditions is therefore of prime importance for the interpretation of any test results. Stability may be measured in terms of dissociation constants, dissolution rates, and solubility of an NM in the final cosmetic product and in the media/vehicle(s) used in exposure/hazard evaluations using appropriate characterisation methods. In addition, determining the stability of the NM surface is equally important, because certain reactions, such as oxidation/ hydroxylation, may take place during handling/storage which may alter the interaction of the NM with biological systems. In this regard, surface characterisation should consider both surface modification by substances that are strongly bound to the particle surface or applied as a thin layer of coating that covers the entire surface of a particle and is strongly bound (either chemically or physically) to the surface (EFSA, 2021 a, b).
As the physicochemical parameters may change in various environments, it is recommended that, as a minimum, characterisation of NMs intended for use in a cosmetic product should be determined at three stages:

- as manufactured (pristine state) to identify the basic NM,
- as used for toxicological investigations, and
- after addition to the final cosmetic formulation to identify how consumers are exposed.

In the case of application in spray products, it is also necessary to characterise and to determine the concentration of NM in the spray mist released from the container (see Section 4).

When characterisation of an NM is not feasible at any of these stages, *e.g.* due to the lack of suitable methods or due to degradation of the NM, this should be justified and documented.

It is important to note that environmental impacts of cosmetic ingredients are not considered during safety assessment under the Cosmetic Regulation. They, however, fall under the remit of different regulatory frameworks, such as REACH (EU, 2008).

Physicochemical characterisation of NMs should provide unambiguous identification of the NM that is used in the final cosmetic product and for which test data have been provided. If these are not the same material, justification should be provided for the scientific basis for considering them 'similar'.
A description of the manufacturing process should be provided, along with data on batch-to-batch variation. Where there is a significant variation between batches produced by one manufacturer, or by different manufacturers, it is important that detailed specifications of the NM intended for use in a cosmetic product are provided by the Applicant with indication of the range for each physicochemical parameter.

Due to potential changes in physicochemical characteristics, the status of an NM in the final cosmetic product should be determined at different stages.
Determination of the stability of the core NM as well as surface moieties is important. It is recommended that, as a minimum, characterisation of NMs intended for use in a cosmetic product should be determined at three stages:

- as manufactured (pristine state) to identify the basic NM,
- as used for toxicological investigations, and
- after addition to the final cosmetic formulation to identify how consumers are exposed.

If characterisation of an NM is not feasible at any of these stages, it should be justified and documented.

3.1 Key physicochemical parameters

Selection of the key physicochemical parameters that can adequately describe an NM, and the selection of the characterisation methods that can be used to measure them, will depend on the composition, properties, and intended use(s) of the NM. Due to the current knowledge gaps in regard to the relationship(s) between physicochemical properties and potential adverse health effects of NMs, it is difficult to select a definitive priority list of parameters for characterisation of NMs. This issue has been the subject of discussions by several international expert committees and working groups, the reports of which have been considered in preparation of this Guidance Document. The key reports considered in this regard include those published by the EU's Scientific Committee on Consumer Products (SCCP, 2007), the OECD Working Party on Manufactured Nanomaterials (OECD, 2009; 2010a, c), the EU's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2009), the International Organization for Standardization – ISO 10808:2010 (ISO, 2010), the European Food Safety Authority (EFSA, 2021a, b), the ICCR Working Groups (2011), a publication by DeLoid *et al.* (2017), a publication by Mourdikoudis *et al.* (2018), the ECHA Appendix R.6-1 for nanomaterials applicable to the Guidance on QSARs and Grouping of Chemicals (ECHA, 2019a), the ECHA guidance on the preparation of registration dossiers that cover nanoforms (ECHA, 2021a). The physicochemical parameters identified as important by these expert reports for the safety assessment of NMs have been summarised in Table 1.

In some instances, not all parameters listed in Table 1 will be relevant for a given material as these are determined on the basis of composition, function, purpose and/or intended use. In such cases, justification should be provided for the characteristics that are not determined or provided, or for why they were not deemed applicable for a given NM (EFSA, 2021a). In the case of NMs exhibiting various crystallographic phases (*e.g.* anatase/rutile TiO_2, amorphous/crystalline SiO_2), selected area electronic diffraction (SAED) studies can provide clear information on the identified structures of the compound and on the spatial distribution and localisation (typically core/shell, 3D mixture, multilayers) of the various crystallographic phases from dark field electronic images. For NMs present in multi-component composites, the overall material should also be described along with the individual components. In addition, energy dispersing X-ray analysis (EDX chemical analyses) and chemical cartographies coupled to SEM/TEM may provide clear material identification and information on the size distribution and particle localisation. In the case of an NM consisting of a mixture of different types of particles, each component should be described individually according to

Table 1 and the ratio of all components in the mixture provided. The structure of the particles should also be described as exactly as possible. This includes information on the distribution of individual components in the particle, *e.g.* homogeneous mixture, core/shell and coatings.

It should be noted that the non-exhaustive list provided in Table 1 only includes mainstream methods currently available. It can be expected that other new and improved methods will also become available in due course.

Table 1: Important parameters and methods for identification and characterisation of nanomaterials intended for use in cosmetic products that should be provided

Parameter	Description	Methods *) (non-exhaustive list, see Glossary for abbreviations)
Chemical identity	Information on structural formula(e)/molecular structure(s) of the constituents of NM along with chemical and common names, and CAS and EINECS numbers (where available).	A wide range of analytical methods, including MS, AAS, ICP-MS, FTIR, NMR, Mössbauer spectroscopy, etc.
Chemical composition	Information on full chemical composition of the NM including purity, nature of impurities, coatings or surface moieties, doping material, encapsulating materials, processing chemicals, dispersing agents, and other additives or formulants *e.g.* stabilisers.	A wide range of analytical methods, including UV-Vis, HPLC, GC/LC-MS, AAS, ICP-MS, FTIR, NMR, XRD, Mössbauer spectroscopy etc.
Production process particles	The entire processes used for production/ modification of the NM since they can have a significant effect on the properties of the NM, *e.g.* pyrogenic or precipitated silica, sulfate, chloride or argex process for TiO_2.	
Particle size and size distribution including presence of agglomeration or aggregation state	Data and graphical representation of the size distribution of primary and secondary particles in terms of mean (±SD) and median sizes in nm should be provided, in terms of particle number size distribution, and particle mass size distribution. Material specifications and data on batch-to-batch variation for at least 5 batches should be provided. The use of more than one method (one being electron microscopy-based imaging) has been recommended by OECD, 2010a, b; SCENIHR, 2015 and SCCS, 2019 as well as OECD TG 125 (OECD, 2022b) for the determination of particle size-related parameters. Detailed information should be provided on the characterisation techniques used along with documentation on the validity of the method used. The recent EFSA Guidance on Technical Requirements (2021b) has proposed a step-wise approach to help decide whether safety assessment of a material should consider aspects related to small particles.Example methods indicated for	Descriptive EM and quantitative electron microscopy; CLS; PTA, ultrafiltration coupled with chemical analysis Descriptive EM and quantitative EM methods have been described in detail in the EFSA Guidance on Technical Requirements (EFSA, 2021b); CLS (ISO standards 13318 series); PTA (ISO, 2016a ISO standard 19430:2016); Bresch *et al.*, 2022; Rauscher *et al.*, 2023a, b).

	determining whether a material has <10% of the particles with at least one dimension smaller than 500 nm for waiving off small particle-related safety assessment include centrifugal liquid sedimentation (CLS); particle tracking analysis; descriptive EM; and filtration complemented with chemical analysis. Proper dispersion of the material needs to be ensured. If the material contains >10% of the particles with at least one dimension smaller than 500 nm, then quantitative EM method (or a different method with justification) is recommended to determine the number-based size distribution of the sub-500 nm fraction.	
Morphology / Shape	Information on the physical form and shape (particle-, tube-, rod- or fibre shape, porosity). Aggregation/agglomeration state (primary particulates or agglomerates/ aggregates). Information on the NM preparation (powder, solution, suspension or dispersion). Aspect ratio (for fibre/tube like materials), specially for biopersistent materials with aspect ratio > 3 Appropriate EM images to support the description.	AFM, TEM, SEM, NMR, XRD Aspect ratio to be presented as the Mean and SD from length and diameter both measured on the same individual particle. Number based distribution of the aspect ratio should be presented.
Structure	Description of the structure, including 1D, 2D and or 3D spatial distribution of the components (*e.g.* homogeneous mixture, core-shell, surface coating) (EFSA, 2021a, b). High quality electron microscopy images of non-homogeneous particles.	TEM, SEM, AFM
Crystallographic structure	Description of crystalline form (amorphous, polycrystalline, crystalline including specification of phase and volumic fraction as well as spatial distribution).	XRD, TEM
Surface area	Information on BET specific surface area of the NM, and volume specific surface area (VSSA) (see Kreyling *et al.,* 2010 for calculation of VSSA). At the moment the VSSA is applicable only if the NMs are in powder formulation. Ideally, density of NMs should be used for calculation of VSSA, rather than density of bulk material. OECD published a technical guidline on BET determination (see OECD, 2022d TG 124).	BET
Surface characteristics	Detailed information on NM surface, *e.g.* the components bound to the surface, presence of any functional groups (*e.g.* carboxy, amino, hydroxy). Information on surface charge (zeta potential), morphology / topography,	LDE, SPM, XPS, MS, RS, FTIR, NMR, analytical ultracentrifugation (for surface composition), GE, SPM, LDE, Phase Analysis Light Scattering (for zeta potential), Nano SIMS, SERS, and Mössbauer spectroscopy.

	interfacial tension, reactive sites, as well as any chemical / biochemical modifications or coatings that could change the surface reactivity or add a new functionality. Information on any surface contamination.	
Solubility	Information on solubility of the NM in relevant solvents and partitioning between aqueous and organic phases (*e.g.* log K_{ow} for organic NMs, and surface modified inorganic NMs). Dissolution rates in relevant solvent(s) for soluble and partially soluble NMs (solubility should not be confused with dispersibility of insoluble NMs). For slowly dissolving NMs: data on dissolution rate and the conditions under which the measurements were made. Information on hygroscopicity of powders.	Solubility/ dissolution rate in water and other relevant media/matrix (OECD, 2021b)
Dispersibility	For insoluble dispersible NMs: information on dispersibility in terms of a relative amount of the particles that can be dispersed in a suspending medium (including information on stability of the dispersion in the given media and the conditions applied (EFSA, 2021a, b; OECD, 2021b).	
Catalytic activity	Information on the chemical reactivity of the NM core material or surface coating. Information on photocatalytic activity and radical formation potential of relevant materials.	Kinetic measurements of chemical, biochemical and/or catalysed reactions
Concentration	Information on concentration in terms of particle mass and particle number size distribution per volume for dispersions, and per mass for dry powders.	A wide range of analytical methods, including UV-Vis, HPLC, GC/LC-MS, AAS, ICP-MS, etc.
Dustiness	Information on dustiness of dry powder materials.	Methods described in DIN EN 15051:2017
Density and pour density	Information on density/porosity of granular materials and pour density.	Methods described in ISO 697:1981 and EN ISO 60:1977
Redox potential	Information on oxidation state and redox potential (for inorganic materials) including the conditions under which redox potential was measured	Potentiometric methods, X-ray absorption spectroscopy.
pH	pH of aqueous suspension.	pH in aqueous media
Viscosity	Information on viscosity of liquid dispersions.	Methods described in OECD TG 114 (OECD, 2012c)
Stability	Data on physical and chemical stability/ dissociation constant of the NM and coatings (if applicable) in relevant formulation/ media.	MS, HPLC, DLS, FTIR, NMR
Other aspects	UV absorption (extinction coefficient), light reflection.	UV-Vis

*) All abbreviations of the methods are explained in the glossary.

As mentioned before, a thorough physicochemical characterisation of NMs is critical for supporting the safety assessment and needs to be carried out at different stages (see above).

In general, characterisation of an NM in a cosmetic formulation can be more challenging than in a raw material, and even more so when the NM is contained in a biological matrix. Depending on the concentration of an NM contained in a formulation/ matrix, and the nature of the formulation/ matrix, a suitable characterisation scheme should be developed to include appropriate methods for isolation, purification and concentration (if necessary) before analysis of the NM. Characterisation of an NM in a cosmetic product should also provide information on any changes in the NM characteristics during formulation, *e.g.* in terms of primary/ secondary particle sizes (*e.g.* occurrence of agglomeration/aggregation of the nanoparticles), chemical composition, structural state, surface characteristics, etc. These parameters should also be considered when evaluating stability and shelf life of the NM ingredient in a final product. Similar considerations are needed during toxicological evaluations.

Parameters such as size, aggregation states, crystallographic state, surface charge, coatings and other properties may change in different solvents, test media, and biological environments. Therefore, conditions under which measurements are made should be given a careful consideration and documented at each stage of production and while the material is on the shelf and should be detailed in the dossier.

The sample preparation step for electron microscopy is very important as it is known to influence the results of physicochemical characterisation of NMs (Taurozzi, 2012a).

Where needed, the SCCS may ask for the provision of a detailed description of the production processes, any surface modifications, and the preparatory steps carried out for integrating the NMs in the final cosmetic products as input into the safety assessment process.

Dispersion

Dispersing NPs or their aggregated and/or agglomerated forms in liquids is an important step in the sample preparation for most particle size measurement methods. Information on the protocol used to prepare a sample should be provided, in particular when dispersion was carried out by ultrasonic treatment (Retamal *et al.*, 2017; Taurozzi 2011, 2012a, b). There is no universally applicable protocol for preparing stable dispersions of NMs and specific methods for certain types of particles have been published. A more systematic approach has been proposed in the EU project NanoDefine that has developed optimised dispersion protocols and SOPs for a number of priority NMs (Mech *et al.*, 2020). Examples include $CaCO_3$ (fine grade), $BaSO_4$ (fine and ultrafine grade), kaolin, coated TiO_2 (pigment grade), zeolite powder or MWCNT (highly tangled fibrous carbonaceous material). Specific dispersion requirements need to be met for each of these materials along with the corresponding types of dispersants, stabilisers and sonication conditions. Typical probe sonication dispersion conditions involve applying energies between 600 J/mL and 2,500 J/mL sample volume.

Another dispersion protocol is the NanoGenoTox protocol (Jensen *et al.*, 2011 https://www.anses.fr/en/system/files/nanogenotox_deliverable_6.pdf) that was further developed by the EU NanoREG project (https://www.rivm.nl/en/international-projects/nanoreg) and Hartman *et al.*, 2015.

DeLoid *et al.* (2017) have developed a multi-step *in vitro* dosimetry methodology to quantify the delivered dose metrics as a function of time which consists of three interconnected elements: 1) NM dispersion preparation; 2) NM dispersion characterisation; 3) numerical fate and transport modelling to derive delivered dose metrics.
These SOPs can provide a starting point for developing protocols for other materials on the basis of physicochemical similarities with one of the standard nanomaterials used in developing the SOPs.

The key point to consider in this regard is that the dispersion method used for a nanomaterial should lead to a particle size distribution that contains the smallest dispersible particles. A dispersion protocol can be considered effective if it yields samples that consist as much as possible of non-agglomerated /non-aggregated particles. To monitor the effectiveness of a dispersion protocol, analytical methods should also be capable of reliably distinguishing between constituent particles and agglomerates and aggregates. The methods should include EM techniques, such as SEM or TEM.

Considering that the dispersion procedure used may influence particle size distribution of a nanomaterial, all measurements of particle size distribution should ensure proper dispersion of the samples. Further details on this aspect have been discussed in section 3.2 of the EFSA Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (EFSA, 2021b).

The dispersion also needs to be sufficiently stable, *i.e.* showing a constant size distribution pattern or minimal re-agglomeration, avoiding particle sedimentation over the time. A sufficiently stable dispersion is necessary for particle size measurements in dispersions or during *in vitro* or *in vivo* toxicological tests. The effective stability (in terms of limited re-agglomeration and/or sedimentation) of a prepared sample during the time between sample preparation and end of the measurement should be verified (Rauscher *et al.*, 2019). According to EFSA Guidance (2021b), ISO Guideline (ISO, 2013) and an OECD TG 318 test approach for NMs in simulated environmental media (OECD, 2017c) have addressed the key aspects. As a guide, a minimum required stability time of 30 min has been suggested for the use in various measurement techniques for nanomaterials (Mech *et al.*, 2020).

Aspect ratio

The aspect ratio is a geometrical shape descriptor defined as the length to width ratio of a particle. It is obtained from particle size measurements by measuring the length/lateral dimension (or longest dimension) and the width (or the smallest dimension perpendicular to the length dimension) of individual particles in the nanoform (ECHA, 2022). In case of elongated particles or platelets, the average aspect ratio with an indication of the variation (as a range), as well as the size distribution of both the length/lateral dimension (longest dimension of the particle) and the width/thickness of the particle have to be reported.

Aspect ratio should be given as the Mean and SD values from length and diameter, both measured on the same individual particle. In addition, the number-based distribution of the aspect ratio should be given.

Solubility and dissolution rate

The solubility and dissolution rate of a nanomaterial are amongst the crucial physicochemical properties that can inform a risk assessor whether or not there will be the likelihood of

consumer exposure to nanoparticles through the use of a final cosmetic product. From a safety assessment perspective, any nano-related properties of an NM are intrinsically dependent on physical integrity of its particles in the nanoscale. If and when the NM loses its nanoparticulate structure, due to solubilisation or other physical, chemical or biological process(es), its risk can be expected to be similar to corresponding non-nano chemical form(s).

It should be noted that 'insolubility' is a relative term that cannot be measured directly but is determined by the solubility of a material, which indirectly reflects a material's lack of solubility. For cosmetic ingredients both the classical solubility in water or relevant non-aqueous media or solubility of the nanomaterial as ingredient used in a cosmetic formulation are of importance.

Solubility

Solubility in water

In the current definition of NM outlined in the Cosmetic Regulation (EC) No 1223/2009, insoluble and bio-persistent have been explicitly mentioned as two essential features of an NM. The term 'insoluble' requires some clarification to avoid misinterpretation, as even partially-soluble NMs may be wrongly regarded as 'soluble' in relative terms. It needs to be remembered that when a nanomaterial is used as a cosmetic ingredient, it is used in a (nano)particulate form, and for a specific functionality. Therefore, due consideration must be given when a substance is intended to be used as a cosmetic ingredient in particulate form, and risk assessment must be performed on the particulate form of the material, where applicable. It is also worth noting that 'insolubility' is a relative term that cannot be measured directly but is reflected indirectly as a material's lack of solubility. This inevitably requires measurement of solubility of an NM in appropriate solvent(s) under defined conditions of temperature and pH (relevant to the use and testing conditions). If there is a strong dependence of solubility on pH, it should be reported. From this it is obvious that for the risk assessment of NMs intended to be used in cosmetic products that may result in oral exposure, it is necessary to determine solubility under gastric and intestinal conditions.

In addition to the intrinsic solubility, it is also important to consider the dissolution rate to estimate the time required for a nanomaterial to reach equilibrium solubility. As explained in the EFSA Guidance on Technical Requirements for Regulated Food and Feed Product Applications to Establish the Presence of Small Particles including Nanoparticles (EFSA, 2021b), solubility is a property of the substance defined as the proportion of a solute in a solvent under equilibrium conditions (*i.e.* in a saturated state), whereas dissolution is a process and the dissolution rate refers to the kinetics of dissolution. It is important to note that dissolution for nanomaterials means that the material is solubilised into individual ionic or molecular species in an aqueous medium or biological environment, with the loss of nano features. This needs to be differentiated from dispersion, which is a basically colloidal suspension of the particles. It is also important to consider the form in which a nanomaterial may be solubilised as some materials may solubilise as a result of a chemical transformation, *e.g.* hydrolysis or oxidation under conditions of solvent media.

It is thus important to consider solubility and dissolution rate of an NM in water, or in the intended final formulation, because any nano-specific risk may change, diminish or even dissapear, when a nanomaterial is fully solubilised. This information is crucial for determining whether safety assessment should be based on particle risk or solubilised substance risk.

Solubility values are generally drawn from tests in aqueous media and not cosmetic formulations, whereas solubility is dependent on a number of factors such as the solvent medium, pH, temperature, duration, chemical composition of the NM (including impurities), surface chemistry, as well as aging of NMs. As the 2019 SCCS Nano Guidance (SCCS, 2019) has explained, 'Solubility in the context of this guidance means disintegration of a nanomaterial in an aqueous medium or biological environment into molecular components with the loss of nano features'.

In this regard, the OECD TG 105 (OECD, 1995) method for water solubility as such is not considered suitable for measuring solubility of NMs because the method is designed for conventional (non-nano) substances. Since insoluble and partially soluble NMs are likely to form suspensions of nanoparticles in the solvent media, it is important that any suspended particles are completely removed from the suspension before carrying out chemical analyses to avoid overestimation of the truly solubilised amounts of the NM. For this, ultrafiltration has been recommended by OECD (2021a) and EFSA (2021b) for removing (nano)particles from the solubilised fraction. Other methods reported in scientific literature include ultracentrifugation and dialysis. However, as explained in the EFSA Guidance (2021b), the separation process in these methods may be too slow compared to the dissolution process and for this and other reasons, the use of ultracentrifugation and dialysis are not recommended (OECD, 2021a).

It is of note that, depending on the concentration of an NM in a cosmetic product, the final formulation may contain (nano)particles if the material is not fully solubilised. Nano-specific risk assessment may not be needed only when an NM ingredient in a cosmetic product is in fully solubilised form at the intended use concentration. This means that a nanomaterial that solubilises slowly over a long period of time, and/or solubilises only to a partial degree, cannot be considered as 'soluble' because it will contain an undissolved fraction that is still in (nano)particulate form.

As a guide, the solubility of NMs used as cosmetic ingredients should be seen in the context of internationally agreed categories defining various degrees of solubility, such as those provided by the European and US Pharmacopoeias (European Pharmacopoeia 10th Edition (2019); USP 38 and USP 38 - NF33).

Table 2: Categorisation of solubility of substances as defined by US and European Pharmacopoeias (European Pharmacopoeia 10th Edition (2019); USP38 and USP 38 - NF33).

Term	Parts of Solvent Required for 1 Part of Solute	Solubility defined in g/L
Very soluble	Less than 1 part	>1000
Freely soluble	1 to 10 parts	100-1000
Soluble	10 to 30 parts	33.3-100
Sparingly soluble	30 to 100 parts	10-33.3
Slightly soluble	100 to 1000 parts	1-10
Very slightly soluble	1000 to 10000 parts	0.1-1
Insoluble*	>10000 parts	<0.1

*the European Pharmacopeia terms it as 'practically insoluble'

It should be noted that 'insolubility' is a relative term to explain a material's lack of solubility. Therefore, due consideration must be given when a substance is intended to be used as a cosmetic ingredient in particulate form, and risk assessment must be performed on the particulate form of the material, where applicable. Also, depending on how much material was added to a cosmetic product, the final formulation may still contain (nano)particles even when the material is partially solubilised. Only when the substance is used in fully solubilised form, *i.e.* not used as a nanomaterial, nano-specific risk assessment may not be needed.

For materials undergoing partial dissolution, the first step of the process is to identify possible differences between the particulate and solubilised material. In these cases, the assessment of nanomaterials may require a case-by-case approach considering both particulate and dissolved forms.

It needs to be remembered that 'solubility' and 'insolubility' are two sides of the same coin, and the degree of insolubility can only be measured in terms of measuring solubility. Although detailed explanation of insolubility/solubility may not have been provided in the legislation, a clear understanding exists in the scientific context. Based on the values for different degrees of solubility (Table 1), the SCCS may accept a waiver for nano-specific risk assessment for a material composed/comprised of small particles that has a high solubility (*i.e.* solubility of 33.3 g/L or more). On the other hand, a 'sparingly soluble' material, for example, will, by definition, have virtually most of the material in insoluble particle form. For this reason, the SCCS considers all those nanomaterials within the scope of the NM definition of a solid material that fall under the categories of 'practically insoluble', 'very slightly soluble', 'slightly soluble' or 'sparingly soluble', as presented in Table 1.

Solubility in non-aqueous media

As mentioned above, the measure of water solubility is a useful criterion for consideration for waiving nano-specific safety assessment of a nanomaterial on the basis that it would lose nano-form on solubilisation in aqueous media. This, however, may not be applicable to nano-forms of organic substances as they may not be soluble in water. However, they may fully soluble in a lipophilic medium, and given that such a nano-form may dissolve in oil/lipid, solvent(s) or other lipophilic formulants used in a final cosmetic product, it is pertinent to consider that a case for waiving nano-specific safety assessment may be made by an Applicant for nano-form of a lipophilic substance for which it can be demonstrated that it will fully dissolve at the concentrations used in the final formulation.

In this regard, the measure or estimate of octanol-water partition coefficient (K_{ow}) may provide a useful physicochemical parameter for lipophilicity of a lipophilic substance to indicate its potential to be dissolved in an oil or other lipophilic medium. However, justification based on Kow alone is only appropriate for crystallised/precipitated form of a lipophilic material that is not 'manufactured' into a nano-form, and also not applicable where the nano-form in question is composed of multiple components, consists of impurities/contaminants, or has been subjected to a chemical modification, coating or encapsulation. For such cases, the request for waiving nano-specific safety assessment should be supported by experimental data to clearly demonstrate full dissolution of the nano-form (not to be mistaken for miscibility/dispersion of the particles) in the relevant lipophilic medium, and if applicable, further supported by high Log K_{ow} values for the chemical component(s).

It is also important to emphasise that the considerations for dissolution of a lipophilic material in non-aqueous media will not be applicable to a situation where the same nano-form is used in non-lipophilic formulations.

Evidence for the absence of nanoparticles

The absence of particles is directly related to the solubility of nanomaterials. As stated in the Background, it should be noted that 'insolubility' is a relative term to explain a material's lack of solubility, because it is only the solubility of a substance that can be measured. Therefore, due consideration must be given when a substance is intended to be used as a cosmetic ingredient in particulate form. Also, depending on how much material was added to a cosmetic product, the final formulation may still contain (nano)particles even when the material is partially solubilised. One way to demonstrate the solubility of a nanomaterial is to evaluate the absence of particles in the solution.

The presence of particles may be determined visually by looking for sedimentation of the particles present in a solution. Dynamic light scattering (DLS) or other appropriate techniques for measuring particles may be used for further evaluation. The use of a dispersion protocol and its quality control may also indicate the presence or absence of particles, especially when harmonised protocols are used (Hartmann *et al.*, 2015). For example, a quick and rough qualitative assessment of the particle dispersions can also be made by different optical microscopy analyses (Jensen *et al.*, 2011).

The absence of particles needs to be evaluated by using ultrafiltration techniques (membrane filter with pore size in the range 3–10 kDa - EFSA, 2021b) followed by evaluation of the presence of particles on the filters used for observation by electron microscopy evaluation, including determination of particle chemical composition (*e.g.* by EDX analysis). Also, chemical analysis of the solutions before and after ultrafiltration may provide supportive evidence for the absence of particles.

3.2 Methods for Characterisation

A wide range of analytical methods is available for measuring the physicochemical parameters of conventional chemical substances. Some of these methods can also be used (or adapted) for detection and characterisation of NMs. The most relevant methods for NM characterisation are based on light scattering (*e.g.* DLS), electron microscopy (*e.g.* TEM, SEM), size separation and extraction (*e.g.* (ultra) centrifugation, Field Flow Fractionation (FFF), Hydrodynamic Chromatography (HDC)), and chemical analysis/detection by spectroscopic or mass spectrometric techniques (*e.g.* ICP-MS, UV spectroscopy, AAS), surface area determination (BET), and their different variants and combinations. Methods for *in situ* imaging of NMs, *e.g.* magnetic particle imaging (MPI) and positron emission tomography (PET) are currently under development. Similarly, antibody, binding protein, and enzyme-based methods are also under development for organic or coated-inorganic NMs. Mainstream methods for characterisation that may be used for NMs are listed in Table 1, and additional details have been provided in the ICCR report (2011) and other documents (OECD, 2012a, 2014a; ECHA, 2019a; ISO 2017a - ISO 10993-22:2017; EFSA, 2021b; Rauscher *et al.*, 2023a, b).

A particular challenge in regard to characterisation of NMs is the fact that different analytical methods may yield different measurement values, *e.g.* particle size, because they may be based on different principles for measurement of the same aspect (Domingos *et al.*, 2009). Characterisation of NMs in complex matrices poses a further challenge. Preference should therefore be given to the use of standardised analytical methods. However, it is also important to note that currently there is no single method that can be regarded a 'gold' standard for characterisation of different physicochemical parameters of NMs as such, nor is there one best suited method to fully assess an NM in a cosmetic product. The exact choice of analytical method(s) to measure a parameter will be dependent on the chemical composition and the physical form of individual NMs. However, as pointed out in the recent EFSA Guidance (2021b), a carefully chosen portfolio of established analytical techniques should provide adequate data for the purpose, provided that measurements are carried out properly, and results are backed up by appropriate documentation.

Any analytical method used for physicochemical characterisation of NMs should be fit for purpose and reliable. Ideally, the method should have undergone validation in terms of performance parameters (*e.g.* specificity; selectivity; robustness/ruggedness; recovery/ trueness; repeatability, and reproducibility), and provide detection/quantification limits and measurement uncertainties. Guidance for the validation of methods for the detection and quantification of engineered NMs in food has been published by Linsinger *et al.* (2013). These principles should also be applicable to other matrices.

The EFSA Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles EFSA (2021b), OECD (2010b) and SCCS/1611/19 (SCCS, 2019) have concluded that the determination of NM size parameters (number-based particle size distribution, aspect ratio and percentage of nano fraction) should include the use of an EM method. The SCCS considers that size parameters for nano-scale ingredients intended for use in cosmetic products must be measured by EM (preferably quantitative high-resolution TEM) and at least one other method.

Different aspects including measurement uncertainties relating to TEM, calibration and use of appropriate standards are described by Boyd *et al.*, 2011; Rice *et al.*, 2013; De Temmerman *et al.*, 2014; Dudkiewicz *et al.*, 2015a; EFSA, 2021a, b; Bresch *et al.*, 2022 and Rauscher *et al.*, 2023a, b).

For size measurements, including electron microscopy, several reference materials are available, *e.g.* gold NP developed by the US National Institute of Standards and Technology (NIST) (https://www.nist.gov/programs-projects/chemical-characterization-nanoparticles) as well as certified reference nanomaterials developed or representative industrial nanomaterials characterised by the European JRC (JRC, 2016b) (https://joint-research-centre.ec.europa.eu/scientific-tools-and-databases/jrc-nanomaterials-repository_en), respectively.

The representativeness and reliability of the particle size measurements by EM also need to be seen in conjunction with other methods as the EM results may be influenced by a number of factors. In particular, sample preparation and handling play an important role in the reproducibility of the analytical results. Dudkiewicz *et al.* (2015a) have shown that the number of particles measured constitutes only a minor source of uncertainty in the size measurement of NMs in food using EM, compared to the combined contribution of the uncertainties relating to sampling, sample preparation, and image analysis.

3.3 Performance of Characterisation Methods

With regard to characterisation of NMs, it is important to note that different measurement techniques may yield slightly different results. This is due to the different characteristics of the measurements of the very small dimensions, and/or the low amount of material evaluated in general. Furthermore, these differences may reflect the differences in the aggregation/ agglomeration behaviour of NPs during different sample handling/ preparation procedures, dilutions, or dispersions used in different methods, and/or the different measurement principles applied in individual methods (Domingos *et al.*, 2009). A study by Dudkiewicz *et al.* (2015a) has identified that sampling, sample preparation, and image analysis are the main sources of uncertainty in the analytical results from the measurement of NP size by EM methods. Dudkiewicz *et al.* (2015b) have proposed a uniform measurement expression of a mass equivalent diameter (MED) for cross-method comparison of NP aggregate size distributions. The use of such approaches can bring uniformity and standardisation between results from different analytical methods. This inevitably requires the use of standardised protocols for sample handling and preparation. Dispersion protocols for various NMs have been developed by Masuda and Gotoh (1999); Hartmann *et al.* (2015); Mast and De Temmerman (2016); NIST (2012) (NIST Special Publication 1200-1 to 1200-5); OECD (www.oecd.org/science/nanosafety/); JRC (JRC, 2016b) (https://joint-research-centre.ec.europa.eu/scientific-tools-and-databases/jrc-nanomaterials-repository_en); NanoGenoTox (www.nanogenotox.eu); NanoDefine (www.nanodefine.eu); NANoREG (www.nanoreg.eu). It is therefore important to ensure that sample preparation is carried out in a consistent manner to obtain reproducible results, and to allow a comparison between the results of different samples analysed by a specific analytical method, or by different methods.

In line with the EFSA Guidance Guidance on risk assessment of nanomaterials to be applied in the food and feed chain (EFSA, 2021a), method performance parameters to be determined and documented should include criteria such as specificity, selectivity, recovery, repeatability, reproducibility, and limits of detection/quantification. Where possible, existing guidelines (*e.g.* of the International Union of Pure and Applied Chemistry (IUPAC, 2002) should be taken into account, or adapted from guidelines available for that specific material or product category if no specific guideline is applicable for an NM. The use of a method that differs from internationally agreed protocols should be justified and documented.

Reference materials

Reference materials are essentially needed for the alignment and calibration, as well as validation of the performance of analytical methods used for characterisation of NMs. Guidelines for the alignment and calibration of electron microscope are provided in ISO 29301 (ISO, 2017b).

At present, only a few certified reference nanomaterials are available that have been developed for size or surface area parameters. The European Commission's Joint Research Centre (JRC) provides a few certified reference materials – mainly silica and titanium dioxide (https://crm.jrc.ec.europa.eu/). The JRC has also made available a repository of 33 representative NMs (The JRC Nanomaterials Repository JRC (2016b)), which include representative materials for the nano-forms of TiO_2, ZnO (coated and uncoated), SiO_2, CeO_2, gold, MWCNT, graphene, nano-clay. Although not certified reference materials, these are also useful as NM working standards that can help comparing different studies, and have been

used in different EU-funded projects (*e.g.* MARINA, NanoGenoTox, NANoREG) and the OECD WPMN (Totaro *et al.*, 2016).

The NIST (2021) catalogue for standard reference materials (SRMs) has listed SRMs for TiO_2, polystyrene spheres, single-wall carbon nanotubes, multiwall carbon nanotubes; gold nanoparticles; PVP-coated silver nanoparticles and silicon nanoparticles.

The COMAR database established by the German Federal Institute for Materials Research and Testing (BAM) provides inventories of the currently available NM reference materials from different sources, such as JRC, NIST, BAM, LGC, and others (https://rrr.bam.de/RRR/Navigation/EN/Reference-Materials/COMAR/comar.html).

In the absence of a (certified) reference NM, a self-generated and properly characterised and documented test material may be used provided that the ISO/TS 16195 technical specification for preparation of reference NMs has been taken into consideration (ISO, 2013) and it has the same chemical composition, and closely matches with the test NM in terms of particle size distribution and other physicochemical properties.

3.4 Characterisation of NM for toxicological testing and in biological fluids and tissues

For the toxicological assessment of NMs, it is essential to know in which form the NMs are presented to the test systems. In addition, characterisation of the NMs in the test system is relevant to determine the effect of the test medium/ formulation (and its constituents) on the characteristics and properties of the NM, to determine the validity of the toxicity test outcomes, and to allow for comparison with the NM in the cosmetic product to which exposure takes place. ISO/TR 13014 (ISO, 2012) lists the key properties for engineered NMs to be characterised in the context of toxicological testing. The methodologies to be used are indicated in Table 2.

When performing *in vitro* toxicity studies, it is necessary to characterise NMs directly in the same testing medium. It is advised to use more than one method; some of these methods are described in SOPs developed within FP7 project NANoREG: https://www.rivm.nl/en/international-projects/nanoreg.

The currently available information indicates that special consideration is needed to address the potential batch-to-batch variations and aging effects (*e.g.* agglomeration/aggregation, sedimentation, degradation, slow dissolution).

There may be particular difficulties in measuring the amounts of NM in biological fluids and in establishing the form in which NM are present in the body. NM surface transformations (*e.g.* the dynamics of adherence of proteins and other biomolecules) can have a profound effect on the ADME. For determination of NMs in biological fluids/biological systems it is essential that measuring systems are available for detection of the NM and its elemental composition in biological samples. The available methodologies are indicated in Table 2.

3.5 Dose Metrics

The metrics used for toxicological assessments are normally measured and expressed in weight or volume units (such as mg/Kg, or mg/L) for conventional chemicals. Also, for NMs, mass- or volume-based concentration units are commonly used. However, such metrics may not be appropriate for NMs to define a dose response relationship because of the large surface

areas per particle mass or volume. Until suitable parameters are identified that can describe and predict dose-effect relationships, it is important that tests on NMs are evaluated using different dose-describing metrics, such as weight/volume concentration, particle number concentration, surface area etc. Therefore, the characterisation data on an NM should provide sufficient information to convert doses based on mass into other parameters such as number of particles or surface area. These data for dose conversion should be available for NMs as the preparation of the exposure dose will based on mass (*e.g.* mg or µg/mL).

In regard to *in vitro* testing using cell cultures, the exposure concentration should also be expressed in relation to the area [µg/cm^2], and, if possible, per cell [µg/cell]. Additionally, exposure concentrations can be expressed as number of NPs per ml [NPs/ml], per cm^2 [NPs/cm^2] or per cell [NPs/cell] as well as surface area of NPs per ml [NP cm^2/ml], per cm^2 [NP cm^2/cm^2] or per cell [NP cm^2/cell]. The use of the dose description as exposure concentration per cell has been regarded as particularly appropriate for NP testing (Huk *et al.*, 2015).

Thorough physicochemical characterisation of NMs is critical for safety assessment. A list of important physicochemical parameters is provided in this section. These include chemical identity, chemical composition, production process particles, number-based particle size distribution including presence of agglomeration or aggregation state, morphology/shape (aspect ratio), structure, crystallographic structure, surface area, surface characteristics, solubility, dispersibility, catalytic activity, concentration, dustiness, density and pour density, redox potential, pH, viscosity, stability, and other aspects such as light absorption/reflection. All of these parameters relevant for a given NM should be measured.

Some parameters such as size, aspect ratio, aggregation states, crystal structure, surface charge, coatings and other properties may change in different solvents, test media, and biological environments. Therefore, conditions under which measurements are made should be carefully considered and documented at each stage of the production and when the material is on the shelf, and details should be provided in the dossier.

A wide range of analytical methods is available for measuring the physicochemical parameters of conventional chemical substances, some of which can also be used (or adapted) for detection and characterisation of NMs. The most relevant methods for NM characterisation have been listed in this section. However, the exact choice of analytical method(s) to measure a parameter will be dependent on chemical composition and physical form of the individual NM.

Sample preparation is known to influence physicochemical characteristics of NMs. An appropriate dispersion method must be used in order to ensure it yields samples that consist as much as possible of non-agglomerated particles. Therefore, information on the protocol used for preparing the samples for analysis should be provided.

Measurement of physicochemical characteristics of NMs is compounded by the fact that different analytical methods may yield different results, and characterisation in complex matrices poses a further challenge.

The analytical methods used for physicochemical characterisation of NMs should be fit for purpose and reliable. Ideally, the methods should have undergone validation in terms of performance parameters (*e.g.* specificity, selectivity, robustness/ruggedness, recovery/ trueness, repeatability and reproducibility) and provide detection/quantification limits and measurement uncertainties. Although most of the available analytical methods have not been

specifically validated for NMs, a careful choice of the established techniques should provide adequate data for the purpose, provided that measurements are carried out and documented with due consideration of the nanoscale particulate nature of the materials.
EM techniques provide a very useful visual means for the determination of the particle shape, size and aspect ratio of NMs, as well as chemical composition when linked with spectroscopic or spectrometric methods. It is therefore recommended that size parameters for nano-scale ingredients intended for use in cosmetic products should be measured by at least two methods, one being EM (preferably high-resolution TEM).
Reference NMs or standardised test materials should be used to validate the performance of analytical methods.
ISO/TR 13014 (ISO, 2012) lists the key properties for engineered NMs to be characterised in the context of toxicological testing. For determination of NMs in biological fluids/biological systems, it is essential that a measuring system is able to detect either the NM or its elemental composition in biological samples. Dose metrics used for toxicological assessment of conventional chemicals are normally measured and expressed in weight or volume units. For NMs, it is important to also consider other dose-describing metrics in addition to weight/volume concentration, such as particle number concentration, surface area, etc.

4. EXPOSURE ASSESSMENT

As mentioned before, in view of the animal testing and marketing bans, safety assessment of NM cosmetic ingredients may be driven primarily by exposure considerations. Prior to commencing the detailed safety assessment of the NM, anticipated exposure scenarios from the proposed uses should be outlined. These exposure scenarios will contribute to decisions on the extent of the hazard characterisation and will be the basis for selecting parameter values for the exposure assessment required for the safety assessment. In particular, determining whether or not any systemic exposure to an NM is possible due to the foreseeable use(s) of a cosmetic product is an important consideration in the safety assessment process. This can for example be determined by analysis of the receptor fluid for NPs in *in vitro* dermal absorption studies. Furthermore, systemic exposure can be assessed based on the concentration levels in organs and/or blood *in vivo*, or by considering other information from toxicological studies, if available (for example from studies on toxicokinetics, acute or repeated dose toxicity, etc.) and in the case of *in vivo* animal studies, when performed before the animal testing ban for cosmetic ingredients or performed in compliance with other regulatory requirements. However, the methods used need to be state of the art, and the limit of detection low enough to demonstrate a potential lack of exposure. In this regard, the use of sensitive methods for chemical analysis (Table 2) should generally be sufficient. For example, the use of imaging methods, such as EM, should be sufficiently sensitive to determine whether or not the absorbed material was present in nanoparticle form in receptor fluids and tissue samples.

It should be noted that even in the absence of systemic translocation of the nanoparticles themselves, degradation products or dissolved fractions of the nanoparticles could be translocated and then would need to be assessed according to their chemical properties by following the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision).

Exposure assessment and the identification of potential exposure routes form the first crucial decision point in the overall safety assessment (Figure 1). The exposure assessment for ingredients in cosmetic products as described in the SCCS Notes of Guidance is a general approach that applies to NMs as well. The use of cosmetic products that contain NMs is likely to be similar to the use of other products that contain conventional ingredients. If this is the case, default values in relation to exposure (*e.g.* used amounts of cosmetic products) as provided in the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision) can be used.

Special attention, however, should be paid to any distinctive material characteristics at the nano-scale (see Figure 1 and Table 1). This will require detailed characterisation of NMs and determination of the likelihood and extent of systemic exposure due to potential translocation of NMs across dermal, respiratory, or gastrointestinal barriers, respectively. This assessment needs to be specific for the respective routes, since the behaviour and structural changes and metabolic transformations of the NMs may be different for the different routes of exposure. In addition, local effects will need to be considered, irrespective of whether or not the use of a cosmetic product containing NMs can lead to systemic exposure.

Where there is evidence for systemic absorption, further investigations will be required to confirm whether the absorbed material was in nanoparticle form or in solubilised/ ionic/metabolised form. Where the absorption of NPs cannot be ruled out either by experimental measurements or justified on the basis of solubility/degradation of the NM, the SCCS will apply a default approach and assume that 100% of the absorbed material was in nanoparticle form.

4.1. Functions and uses of cosmetic ingredient

NMs as cosmetic ingredients may serve various functions, *e.g.* as UV-filters (such as Titanium dioxide or Zinc oxide), as pigments (*e.g.* Carbon black), or as antimicrobial agents.

For substances that are evaluated as cosmetic ingredients, the concentration, function and way of achieving that function in marketed cosmetic products should be reported. In particular, if substances are meant to be included in sprays or aerosols, this should be explicitly mentioned since consumer exposure via inhalation is then probable and needs to be taken into consideration in the overall safety assessment.

In addition, other uses of the substance (*e.g.* in consumer products, industrial products) and, wherever possible, the concentrations involved in such uses, should be described.

4.2. Identification of relevant exposure scenarios

In order to assess exposure of the general population, relevant exposure scenarios have to be identified that comprise all the important functions and uses of a cosmetic ingredient as detailed in Section 4.1. These scenarios need to describe 'reasonably foreseeable exposure conditions' under which the cosmetic products should be safe (Cosmetics Regulation (EC) No 1223/2009, Article 16f).

The SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision) include a non-exhaustive list of parameters that are needed to construct an exposure scenario. For NMs, in addition to the weight-based concentration of the NM, the concentration should also be given in terms of particle number concentration and surface

area. Also, changes in the aggregation and/or degradation/dissolution status of the NM during exposure should be accounted for.

4.3. Calculation of external exposure

NM particle characteristics during consumer use (*e.g.* in terms of variable particle size distribution) may be different from NM particle characteristics established in experimental settings (*e.g.* stable particle size-distribution). However, factors such as particle size and size distribution/agglomeration state of NMs are considered to be important in determining the hazard. Therefore, the experimental settings for NMs may need to include a broader range of scenarios than those necessary for non-NMs, in order to allow extrapolation to exposure conditions during consumer use (*e.g.* different particle sizes).

Information on size distribution in particular is necessary as an input for calculating size-dependent uptake and subsequent internal exposure (see Section 4.4). It has been shown that the uptake and subsequent distribution of NPs may depend on the size of the particles (Lankveld *et al.*, 2010; Bachler *et al.*, 2015), so that the respective size distributions of the particles need to be considered.

4.3.1 Dermal exposure

Dermal exposure to NMs can in principle be calculated as outlined in the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). However, since the metric of concern may be particle number, it may be necessary to calculate the exposure based on particle number. Furthermore, since particle uptake depends on the size of the particles, it is necessary to take into account the size distribution of the particles in the cosmetic product to allow calculation of internal exposure from external exposure.

4.3.2 Inhalation exposure

Cosmetic ingredients can enter the human body by inhalation. The inhalable fraction determines lung exposure, and part of this inhalable fraction deposits in the respiratory tract. The exposure or deposition dose may be normalised by an inherent property of the inhaled substance *e.g.* its particle size, mass, surface area, or other characteristics. After deposition, the biological targets affected by the substance may be in the respiratory tract itself (local exposure of various parts of the lung) or elsewhere in the body either after mucociliary clearance into the GI tract, translocation (absorption) across the alveolar barrier, distribution via lymph or blood circulation (Section 4.4.2.2).

Inhaled and deposited particles are continually cleared from the respiratory tract. Inhaled insoluble particles are cleared from the human lung by two different mechanisms, mucociliary clearance and phagocytosis clearance. In addition, free particles may translocate out of the alveolar region of the lung into the lymphatic system or the lung interstitium. Depending on their lipophilicity, hydrophilicity, and/or size, soluble particles may be dissolved prior to physical clearance. It has been observed from animal studies in rats that when NMs are present in high amounts and accumulate in the alveoli, they can no longer be cleared by macrophages due to an excessive presence of the particles, denominated as 'lung overload'. Therefore, chronic irritation, chronic inflammation and cytokine releases may occur, leading to local toxic effects. For example, the carcinogenic hazard of TiO_2 nano (and also for other particulates) has been observed in rats when dust is inhaled in quantities leading to reduction

of normal particle clearance mechanisms in the lung (ECHA, 2017b; Braakhuis *et al.*, 2021a). The relevance of the "lung overload" as observed in the rat model for human risk assessment (*e.g.* lung carcinogenicity) is not yet established and is under scientificdebate.

Particle size determines inhalation exposure, since only particles and droplets smaller than 10 µm can enter the lung via inhalation. Particle deposition in the lung depends on particle size, density, and hygroscopicity (ability of a substance to attract and hold water molecules from the surrounding environment) and is influenced by the local anatomy and airflow as reviewed by Braakhuis *et al.* (2014). They report that NMs with diameters in the range of 10-100 nm preferentially enter the alveolar areas. For particles in the mentioned diameter range (10 – 100 nm), the deposition of NMs is mainly governed by diffusion of the NMs in the inhaled air (Brownian motion) and the density is less relevant. For particles (or agglomerates) larger than 100 nm, diffusion is less likely and also the density increasingly determines the site and extent of deposition.

Similarly, size-distribution is essential for the calculation of internal exposure via inhalation (see Section 4.4.2.2). It is most likely that particle shape also contributes to the rate of deposition of particles in the respiratory tract. Based on size, the British-Adopted European Standard BS EN 481 (1993) distinguishes three different fractions of particles that deposit in different regions of the lung: the inhalable, thoracic and alveolar fraction. NPs fall into an even smaller-sized category within the respirable fraction, which is referred to as ultrafine particles ($PM_{0.1}$), *i.e.* with an aerodynamic diameter d_{ae} of ≤0.1 µm (British Standards Institution, 1993).

Inhalation exposure is relevant for products meant to be applied in spray form (SCCS/1539/14) and for exposure to volatile cosmetic ingredients used in dermally applied products. It can be assessed either by using exposure models or by experiments.

One of the modelling tools available to assess inhalation exposure to NMs is the ConsExpo nano tool (https://www.consexponano.nl). ConsExpo nano is based on the module for spray products in the ConsExpo tool (Delmaar and Bremmer, 2009), which was originally developed for estimating exposure to dissolved substances in spray products. This ConsExpo module was adapted for estimating exposure to NMs and may also be used for other products that contain particles, *e.g.* powder products. The central metrics in ConsExpo nano is the alveolar load. This is based on the finding that the most relevant effect after inhalation exposure to NMs is the induction of inflammation in the alveoli (Braakhuis *et al.*, 2014). The most critical determinants of this effect are both the magnitude and the duration of the alveolar load caused by an NM. In order to estimate the alveolar load arising from the use of NM-containing spray products, ConsExpo nano combines models that estimate the external aerosol concentration in indoor air with models that estimate the deposition in and clearance of inhaled aerosol from the alveolar region. ConsExpo nano provides the mass-based inhalation exposure, and also alternative dose metrics such as total number or total surface area of the NPs inhaled because of the ongoing debate regarding the most appropriate dose metric for NP exposure (Duffin *et al.*, 2007; Schmid *et al.*, 2009; Sayes *et al.*, 2010; Braakhuis *et al.*, 2015).

Another possibility to assess external exposure from spray applications of products containing NMs is to measure the particle size distribution in the aerosol leaving the spraying can. On this basis, a size-specific exposure calculation can be performed. In such a study, careful characterisation is needed of the droplet size and the NM distribution in the droplets (Lorenz

et al., 2011). Determination of the generated droplet size distribution alone is not sufficient and needs to be complemented with the size distribution of the dried residual aerosol particles. Furthermore, the test sprays have to be chosen so that they cover the worst-case aerosol generation (*i.e.* normally the distribution with the largest fraction of very small droplets). For this, it has to be taken into account that spray cans, spray nozzles and spray formulations influence the droplet size distribution of the generated aerosols, and as a consequence, the resulting particle size distribution available for inhalation.

When the droplet size distribution in the spray mist is small enough to reach the lung, the deposition of NPs needs to be calculated. Different models are available to estimate the total and regional lung deposition of aerosol and/or particles. Examples include the Human Respiratory Tract Model (HRTM) (International Commission on Radiological Protection - ICRP, 1994, 2002a, b), the NCRP model (National Council on Radiation Protection and Measurement), the IDEAL model (Inhalation, Deposition and Exhalation of Aerosols in/from the Lung) or the MPPD model (Multiple-Path Particle Dosimetry). For a more detailed description of these models, see Section 4.4.2.2.

Most widely used among these models are the HRTM (ICRP 1994, 2002a, b) and the MPPD model (Asgharian *et al.*, 1995; Asgharian *et al.*, 2001; Cassee *et al.*, 2002). The HRTM model is a semi-empirical model based on experimental data for regional particle deposition in humans under well-controlled conditions (see Section 4.4.2.2). It has been used for example for NPs in sprays by Lorenz *et al.* (2011), who calculated specific external exposures for each region of the respiratory tract based on the particle size distributions of spray mist. Other investigators have developed similar and more user-friendly dosimetry software, *e.g.*, the MPPD model.

The HRTM was developed by the ICRP and can be used for the estimation of deposited doses of inhaled particles in the respiratory tract. It is a semi-empirical model based on experimental data for regional particle deposition in humans under well-controlled conditions. In the model, the respiratory tract is divided into two compartments: the extrathoracic (ET) and the thoracic (TH) airways. The TH regions are bronchial (BB: trachea, bronchi), bronchiolar (bb), the alveolar interstitial region (AI) (*i.e.* gas exchange region, airway generations), and the thoracic lymph nodes. The ET regions are the anterior nasal passage (ET1); the posterior nasal passage, pharynx, and larynx (ET2); and the extrathoracic lymph nodes (see Figure 2).

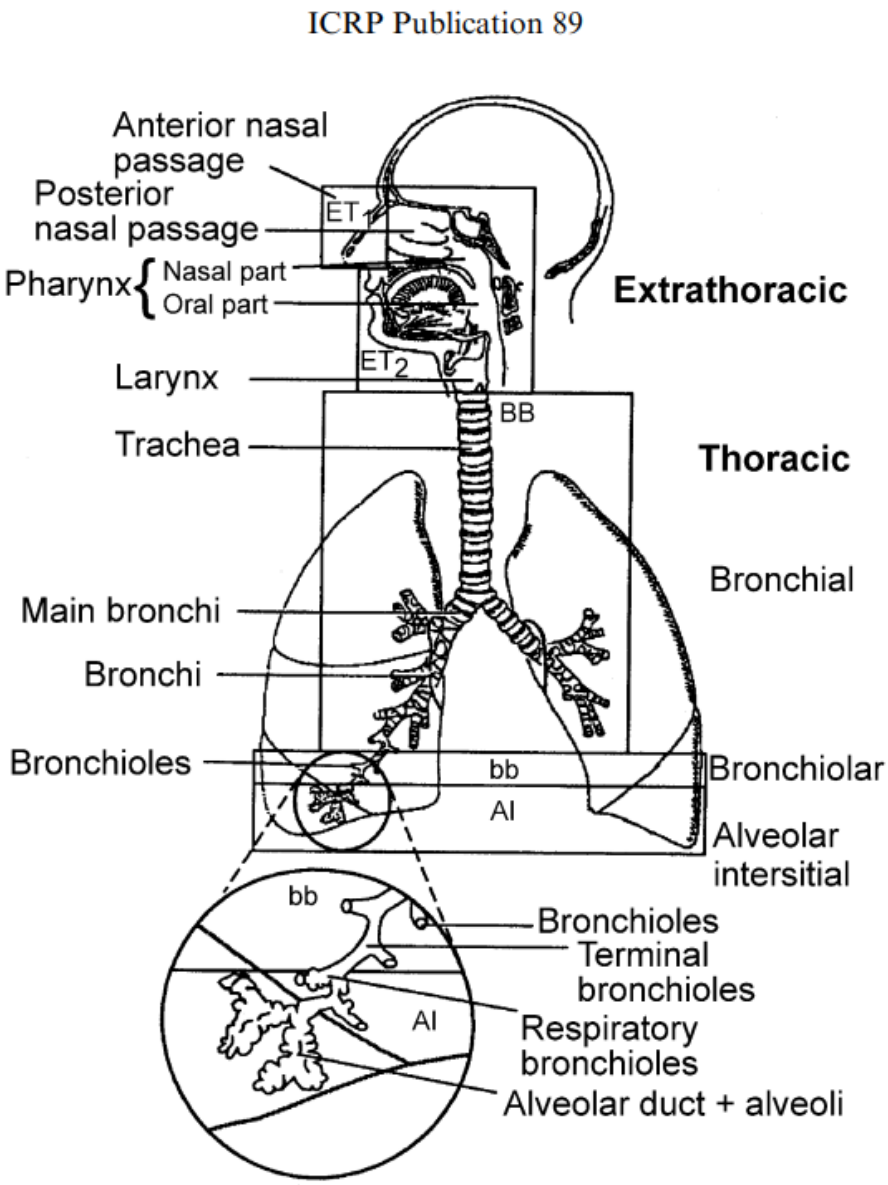


Figure 2: Respiratory tract regions defined in the HRTM.

ET1: extrathoracic region including the anterior nasal passage; ET2: extrathoracic region including posterior nasal passage, pharynx and larynx; BB: bronchial region; bb: bronchiolar region; AI: alveolar interstitial region. Figure taken from ICRP Publication 89 (Fig. 5.1, page 88, 2002b).

The model evaluates fractional deposition of a particle in each region for all particle sizes (0.6 nm–100 mm). For the ET regions, measured deposition efficiencies were related to characteristic parameters of particle size and air flow, and scaled by anatomical dimensions to predict deposition for different anatomical conditions (*e.g.* age, sex). For the TH airways, a theoretical model of particle deposition was used to calculate particle deposition in each of

the BB, bb, and AI regions, and to quantify the effects of the subject's lung size and breathing rate.

The model describes several routes of clearance from the respiratory tract. Some particles deposited in ET1 are removed by extrinsic means such as nose blowing. In other regions, clearance varies between the movement of particles towards the alimentary tract (mucociliary transport) and clearance by the lymphatic system (particle transport to the draining lymph nodes), and the absorption into blood of material from the particles in the respiratory tract, which depends on the physical and chemical form of the deposited particles. In the HRTM, by default, absorption is assumed to occur at the same rate in all regions of the lung (including the lymph nodes), except ET1 for which it is assumed that no absorption takes place. The default values can be changed for a specific assessment.

Another deposition model is the MPPD model. This deterministic model calculates the deposition fraction in humans averaged over the entire lung compartment. In contrast to the ICRP model, it allows the selection of different particle size ranges and exposure conditions, and allows choosing the exposed species among rats, rhesus monkeys, mice, pigs, sheep and humans. This allows simulations of particle deposition for a variety of inhalation scenarios, and takes into account different variables, such as the age of the subjects that are exposed to aerosols.

The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) determine the site of deposition in the respiratory system. Large particles or droplets deposit by impaction in the upper respiratory tree of the lung (oropharyngeal and tracheo-bronchial region), where the air velocity is high and the airflow is turbulent. Particles in the size range of 0.5–5 μm deposit by sedimentation in the terminal bronchioli and alveolar regions. The larger the GSD, the more sites the aerosol will be deposited within the respiratory tract. NPs may reach the alveolar space and deposit in the alveoli, but will also be partly exhaled as they remain dispersed in the inhaled air.

4.3.3 Oral exposure

Oral exposure is relevant for product categories like toothpaste, mouthwash and lipstick, since these may be inadvertently ingested. In principle, for calculating oral exposure the same procedure is followed for NMs as for other cosmetic ingredients. The difference in this case is that the size and agglomeration status of NPs can change due to the low pH in the stomach and the high ionic strength in the whole gastrointestinal tract. NMs may even lose their nano-specific properties, *e.g.* due to breakdown or dissolution. For such NMs, the properties and effects are more likely to be similar to those of the corresponding ions (Oberdörster, 2000; Utembe *et al.*, 2015) so that nano aspects do not need to be considered further once the particles lose their nano-character. According to EFSA (2021a), the characteristics that may indicate a loss of nano-specific properties, and thus reduce the chance of exposure to the NM, include: high degradation rate in water, in the food matrix or in gastrointestinal fluids; (bio)degradability to non-nanosized products; formation of larger aggregates (>100 nm); NPs being fixed or embedded in other matrices (*e.g.* polymer composites used as food contact materials).

It is therefore advised to test first whether the NM or nanosized degradation products remain present as particles under conditions of the gastrointestinal tract. This can be tested *e.g.* through a simulated *in vitro* digestion test (EFSA, 2021a). Also, information on general

biodegradability in other simulated body fluids, such as under lysosomal conditions, may give an indication whether the NM will be stable under the conditions in the gastrointestinal tract (Utembe *et al.*, 2015) so that it may be taken up and potentially accumulate in the body.

Although cosmetic products are not intended to be orally ingested, some limited exposure takes place for oral product categories like toothpaste, mouthwash and lipstick.

However, before ingestion, some exposure of the oral mucosae occurs. This could be of importance in particular when nanomaterials are present. Actually, mucosae do not have the same barrier function as the skin. The latter is protected by the stratum corneum while mucosae only are covered by a mucous layer.

Results from *in vitro* studies in cell lines (Best *et al.,* 2015), 3D buccal mucosa models (Konstantinova *et al.,* 2017), and ex-vivo porcine buccal tissue sections (Teubl *et al.*, 2014, 2015; Vignard *et al*., 2023) indicate that NMs can be internalised by oral mucosa cells and that this cellular uptake can be a relatively rapid process (within a few minutes – according to Teubl *et al*., 2015).

It is known that the oral mucosal epithelium depending on the region of oral cavity has a continuous turn-over of around 14 days (buccal mucosa) to 24 days (hard palate) (Squier and Kremer, 2001). However, considering that some oral products, such as toothpastes and mouthwashes, will be used every day and potentially more than once a day, there is the possibility of a continued local presence of nanoparticles in cells of the oral cavity. This needs to be considered in the safety assessment of NMs intended to be used in oral applications, especially where toxiclogical studies indicate potential harmful effects of a given nanomaterial.

Local effects further down in the gastrointestinal tract are most likely to be limited under realistic conditions due to an anticipated exposure to (very) small amounts of NMs. However, there may be exceptions, such as the reported association between TiO2-NP exposure and colitis (Bouwmeester et al., 2018 and references cited therein).

For the mass-based calculation of systemic exposure from 'external' gastrointestinal exposure, in the absence of information on biodegradation, it should be assumed that all of the NM is available for uptake in the same form as initially added to the product.

4.4. Calculation of systemic exposure

4.4.1 General aspects of toxicokinetics of nanomaterials

The ability of NPs (especially in the lower nm range) to penetrate cellular membrane barriers has added another dimension to the toxicology of particulate materials. Due to the very small size, and certain surface characteristics, insoluble or partially soluble NPs may be able to reach unintended parts of the body that are otherwise protected from exposure to particulate materials by biological membranes. Toxicokinetics of NMs within the entire organism is considered as an important building block of toxicological studies. Small fractions accumulated in secondary organs over short-term exposures may not manifest adverse health effects. However, NM may trigger the production of effect mediators in the primary organ, which are then released into the blood. These mediators may initiate adverse health effects in other organs such as have been observed for the cardiovascular system after inhalation exposure (Miller *et al.,* 2017a, b). In addition, during chronic exposure (*e.g.* via lungs or gut), NM

concentrations in secondary organs may accumulate to an extent large enough to trigger adverse health effects (OECD, 2016d).

Compared to soluble chemicals, the uptake of NPs may considerably differ between various organs. This is because the uptake and bio-kinetics of NMs is governed by processes that are different from (solubilised) molecules. Transport of particles across biological barriers is, unlike most molecules, not based on diffusion gradient-driven partitioning, but on endocytosis or other active (energy-driven) transcellular transport systems. Particles are removed from the blood circulation by cells of the mononuclear phagocytic system (MPS) and mainly end up in organs rich in phagocytic cells like the liver (Kupffer cells) and spleen (macrophages) (*e.g.* Geraets *et al.*, 2014). Non-degradable particles are not expected to be metabolised, but some may undergo (slow) dissolution (*e.g.* Ag-NPs), resulting in the gradual formation of ions and smaller particles. When (slow) dissolution occurs, both the toxicokinetics of the dissolved particle present as soluble substance and the toxicokinetics of the remaining particles should be considered. For the dissolved substance, classical exposure scenarios (and following risk assessment) as described in the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision) on cosmetic ingredients can be used. Before dissolution occurs, the toxicokinetics is governed by the particulate nature of the NPs, thus the location of the possible dissolution of the NPs/material (*e.g.* stomach, small intestines, liver) is important to consider. For possibilities of dissolution, the route of potential exposure is very important. Particle distribution may be carrier-mediated and therefore be affected by corona formation and other transformations. Aggregation and agglomeration of NMs might complicate the transport across biological barriers. Particles are generally removed from the blood rapidly and distributed mainly to the liver and spleen, but may also be distributed to lungs, brain and testes (*e.g.* Geraets *et al.*, 2014). For example, inhalation exposure may result in systemic exposure as reviewed by Hougaard *et al.* (2015) for reproductive effects. Lastly, possible coatings on the nanoparticles also have an effect on their distribution, for example PEG coating is known and used widely in nanomedicine to prolong blood circulation (Lankveld *et al.,* 2011; Hristov *et al.,* 2021). In any case, the kinetics of NMs cannot be extrapolated from the toxicokinetics of the dissolved form and needs to be determined experimentally. For more guidance, see Sections 5.4 and 6.

For non-NMs, OECD TG 417 (OECD, 2010d) addresses the assessment of toxicokinetics. However, as stated in Paragraph 9 of that guideline, it is not intended for the assessment of NMs. An OECD workshop (OECD, 2016d) has also concluded that this guideline was designed primarily for chemicals, for which the kinetics is governed mainly by diffusion/perfusion and metabolic processes, rather than particulates, which behave fundamentally different with respect to absorption, distribution and clearance. The OECD TG 417 (OECD, 2010d) is also considered inadequate for NMs because timeframes recommended for exposure and post-exposure observations are considered inappropriate; there are no considerations with respect to test item preparation and other relevant aspects for the inhalation route; and there is insufficient consideration that relatively small changes in the exposure situation can have significant impact on the kinetic behaviour, in particular for inhalation studies. Furthermore, an overview of NP toxicokinetics developed by ISO TC 229 Nanotechnologies has now been published (ISO, 2019 - ISO/TR 22019:2019 Nanotechnologies - Considerations for performing toxicokinetic studies with NMs). In addition, OECD is currently developing a new test guideline on toxicokinetics to accommodate testing of NPs https://www.oecd.org/env/ehs/testing/work-plan-test-guidelines-programme-july-2021.pdf.

In view of the current animal testing ban, the estimation of systemic exposure relies on the determination of translocation over *in vitro* biological barriers, *i.e.* dermal, oral and inhalation *in vitro* models, and on so-called physiologically based pharmacokinetic (PBPK) models for nanomaterials. A range of physiologically based pharmacokinetic (PBPK) models have been developed for some NMs (*e.g.* Bachler *et al.*, 2013; Lin *et al.*, 2016; Hinderliter *et al.*, 2010) for common metal NMs, and other models available in the literature). However, as further detailed in Section 5.4.1, PBPK models as other *in silico* modelling tools are still at an elementary stage for NMs.

4.4.2 Determination/Estimation of Absorption

Relevant exposure routes for cosmetic ingredients are the dermal, inhalation and oral uptake route. It is important to know whether these uptake routes lead to systemic exposure. Systemic exposure of conventional cosmetic ingredients has previously been assessed by chemical analysis of blood, tissues and excreta in *in vivo* experiments. *In vitro* models can provide information on the potential translocation/absorption over biological barriers. For the assessment of dermal absorption rates, OECD TG 428 (Skin absorption: *in vitro* method, OECD, 2004a) has been validated for conventional chemicals. For the other biological barriers there are no validated guidelines to estimate the respective translocation rates. However, such methods are available in the literature for the GI-tract and the lung.

The uptake of NMs across different barriers can be evaluated by advanced 2D (dimensional) and 3D multicellular co-culture *in vitro* models that are designed to closely mimic the *in vivo* anatomy and functionality of *in vivo* organs/barriers such as lung, alveolar and GI-tract. These models can fill the gap between external and systemic exposure. In addition, *in vitro* models for relevant internal organs and barriers such as the liver, kidney and blood brain barrier can deliver some information about the potential internal distribution, metabolism and excretion. Investigations on dissolution rates or stability in relevant biological fluids may provide clarity on whether or not a substance remains in the nano form after uptake, as this will determine its further distribution.

Where there is evidence for systemic absorption of an NM, further investigations will be required to confirm whether the absorbed material was in particulate form or in solubilised/metabolised form. Where the absorption of NPs cannot be ruled out either by experimental data or justified on the basis of solubility/degradation of the NM, the SCCS will apply a default approach to assume that 100% of the absorbed material was in NP form (see below). This, however, does not imply that the particulate form of a chemical is associated with a greater toxicity potential. Depending on the chemical composition of the NM, certain solubilised/metabolised forms may be more toxic than the corresponding particulate forms. This needs to be considered for the safety assessment.

For each uptake route, a portion of the particle entering the body can be absorbed into the bloodstream and distributed systemically.

There are 'biokinetic models' available, like the HRTM (Human Respiratory Tract Model) and the HATM (Human Alimentary Tract Model) for oral exposure. In combination with PBPK/TK models, such models allow the calculation of systemic exposure, excretion and absorbed tissue doses (ICRP, 2006).

4.4.2.1 Dermal

Dermal absorption of NMs as well as the efficacy of their transport in the human body may depend on the size of the NPs (Bachler *et al.*, 2015). Therefore, in order to calculate internal exposure, the particle size distribution under realistic exposure conditions (external exposure) needs to be related to uptake rates for similar sizes. Therefore, a dermal penetration study should be performed with a formulation containing a typical size distribution of the NM.

In addition to animal skin and human skin available from surgeries, reconstructed human epidermis (RhE) models might be useful for obtaining information on skin translocation. The models have been described in OECD TG 439 (OECD, 2021c) (*In vitro* skin irritation), and for the determination of skin irritation of medical device extracts (De Jong *et al.*, 2018).

For the assessment of dermal absorption, the SCCS basic criteria for *in vitro* assessment of dermal absorption of cosmetic ingredients (SCCS/1358/10) as well as OECD TG 428 (OECD, 2004a) should be followed. However, it is of note that these guidelines have been developed for conventional chemicals. As mentioned before, data from any *in vivo* study will only be accepted if the testing was performed before the animal testing bans, or if data were obtained for the purpose of compliance with other (non-cosmetic) legislations, *e.g.* REACH (EU, 2008). Furthermore, high quality EM images can provide more information on the dermal absorption of NPs.

Measuring uptake and effects of NMs on compromised skin poses a challenge due to the current lack of standardised model(s) that can be used to generate results that are reproducible and can be used to compare studies carried out within a laboratory and between different laboratories. In view of this, OECD (2011) has recommended studies on intact skin. According to OECD TG 428 (OECD, 2004a), *in vitro* skin absorption studies should be conducted using intact healthy skin. This is also reflected in the recommendation to perform skin integrity checks, as described in the current guidelines for *in vitro* skin penetration studies (OECD, 2004a; SCCS, 2010a, 2010b). Where studies on compromised skin are specifically required, the models used should be well characterised to generate reproducible results, and appropriate controls should be included in the studies. In 2020, a critical review on the factors determining dermal absorption of nanomaterials as well as available tools for the assessment of dermal absorption has been published (ECHA, 2020). From this review, it can be concluded that crucial information on particle size in the application medium or dissolution of the particles was missing in many studies. *Ex vivo* studies with human or porcine skin were found to be most relevant for determinating the dermal absorption of NMs, while rodent skin studies were considered less relevant, due to differences in skin and lack of knowledge on penetration mechanisms. Compromised skin integrity and formulations that increase permeability of the skin appear to increase penetration. Some indications were found for larger penetration of smaller particle sizes, lower penetration after agglomeration and an increase of penetration with positive surface charge of NMs. Lack of validated and standardised methods to measure the NMs limits the drawing of further conclusions. It is further recommended to use *ex vivo* studies with human or porcine skin to evaluate dermal absorption of NMs and to perform proper studies with fully characterised NMs and comparable testing protocols to assess influential factors (ECHA, 2020). Further research is needed to develop appropriate test models of compromised skin that can be reliably used to assess possible absorption of cosmetic ingredients, including NMs.

For conventional cosmetic ingredients, in cases where no (adequate) information is available on dermal absorption, the SCCS assumes 50% absorption based on literature analysis for conventional substances. However, this analysis is not valid for NMs. So far, only a very limited or no dermal absorption has been demonstrated for most NMs. On the other hand, the SCCS is aware of specific modifications of NMs that can stimulate dermal penetration. In view of this, dermal absorption of NMs will need to be determined experimentally (see Annex I). Where experimental data are not provided, the SCCS will apply the default value of 50% of the administered dose as determined for conventional substances, or higher if warranted by the composition of a specific NM.

4.4.2.2 Inhalation

Once deposited in the lung, (partially) soluble NMs (partially) dissolve in the lining fluid (mucus layer) of the epithelium where inert NMs may form non-dissolved colloidal suspensions. Local clearance from the airways occurs as macrophages take up the NPs and transport non-dissolved NMs (single and agglomerated but still relatively small NMs) by the mucociliary cascade up to the laryngopharynx (Yang *et al.,* 2008). Soluble NMs that dissolve in the lining fluid of the lung epithelium can be transferred to the blood and distributed to the whole body (Oberdörster *et al.*, 2005). Solubility (rate and extent of dissolution) depends on chemical composition, size, coating, stability and the biological environment (Braakhuis *et al.*, 2014).

Less soluble NMs may be absorbed via cell-mediated active translocation from the site of deposition through the lung epithelium to interstitial sites. From there NMs may be directed to the local lymph nodes, and as lymph nodes are drained by blood, they may ultimately reach the systemic blood circulation. Uptake from the site of deposition into systemic blood may also happen directly by crossing the lung barrier in the alveoli (Borm *et al.*, 2006).

The possibility for uptake via the lung and thus systemic exposure can be evaluated by *in vitro* cellular models that mimic the lung alveolar barrier, the so-called air liquid interface (ALI) models (Bachler *et al.*, 2015). These models are comprised of a membrane that may contain alveolar cells either with or without macrophages added on one apical side of the membrane, and endothelial cells on the distal side of the membrane. The advantage of this model is that it simulates the actual conditions in the lung, where the cells are exposed to air on one side of the lung alveolar barrier, and to liquid on the other side. Application of the studied NPs as spray mist ensures an even and realistic application (Rothen-Rutishauser *et al.*, 2008; Carton *et al.*, 2022). The similarlity of the ALI system with the lung might be improved by including additional cell types. The use of a 3D human bronchial epithelial model with a mucociliary apparatus cultured at the air–liquid interface may allow for an understanding of the key role played by the mucus and cilia in modulating translocation of NPs into the epithelial cells and crossing the barrier (Kuper *et al.*, 2015).

Other authors have modified this model by establishing an ALI by using advanced *in vitro* studies with different combinations of cells (e.g microfluidic platforms, see Tenenbaum-Katan *et al.*, 2018). Particularly interesting are the 3D human lung-on-a-chip models, where a Matrigel layer (simulating the extracellular matrix) separates the two cell monolayers (Zhang *et al.*, 2018). The importance of dynamic conditions mimicking breathing in the functionality of i*n vitro* lung barriers was indicated by Doryab *et al.* (2021); Camassa *et al.* (2022) and Elje *et al.* (2023). A lung bioreactor where alveolar cells were grown at the air–liquid interface adhering on a biomimetic co-polymeric membrane under periodic stretching was developed.

By this dynamic system, it was possible to demonstrate that the cellular uptake of NPs is significantly increased by the physiological stretching, which indicated a possible underestimation of the transbarrier transport of nanoparticles in static models.

Where there is evidence for systemic absorption, further investigations will be required to confirm whether the absorbed material was in a NP form or in solubilised/metabolised form. This may be investigated in experiments or justified on the basis of solubility/degradation of the NM. Ifthe absorption of the particulate form cannot be ruled out, the SCCS may apply a default approach and assume that 100% of the absorbed material was in NP form.

Information on the extent of inhalation absorption should be obtained from experimental studies and/or estimated from physicochemical parameters. However, if no data are presented, the SCCS considers for conventional chemicals that for the calculation of inhalation exposure an absorption of 100% should be used (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). For the absorption of NPs from the lung, a similar default absorption of 100% of the calculated deposition of NPs in the lung will be used, if other information, *e.g.* data on possible inhalation absorption obtained in *in vitro* ALI systems are not available.

4.4.2.3 Oral

Particles initially deposited in the respiratory tract during inhalation are partly transported out of the lungs and the extrathoracic airways to the larynx by mucociliary action and mainly swallowed into the GI tract.

NMs may undergo degradation or dissolution on their way into the body, where internal exposure occurs. For example, after oral exposure, NMs may be completely dissolved in the gastrointestinal tract. In order to demonstrate this, suitable *in vitro* methods should be used. In the absence of respective data, the SCCS will assume that no dissolution occurs.

Proposed approaches to dissolution and/or degradation can be divided into two categories:

(i) static models – fast, simple, however applicable only to limited digestion conditions (DeLoid *et al.*, 2017a; Marucco *et al.*, 2020) and
(ii) dynamic models - more complex but more physiologically relevant and applicable for complex digestion studies (Shi *et al.*, 2020).

There are also models under development simulating digestion consisting of an oral, gastric and small intestinal phase using the fasting food model, the standard food model, which is based on the average American diet, and the high fat food model, composed of approximately 3% protein and 13% fat (Coreas *et al.* 2020). After the digestion procedure, the particles may even be used for determination of their toxic properties in various *in vitro* co-culture intestinal epithelial models (Bazina *et al.*, 2021).

Dissolution and/or degradation studies should be performed before any estimation of systemic uptake from the GI tract. The following parameters may indicate a loss of nano-properties or a low exposure to NPs (adapted from EFSA (2021a)):

1) high dissolution rate (*e.g.* in water, cosmetic matrices or body fluids such as synthetic gastric or lysosomal fluids);

2) high rate of degradability (*e.g.* biological or photocatalytic) to non-nanosized degradation products. According to EFSA (2021a), an NM is considered to have a high degradation rate if

the degradation rate profile in the intestinal phase shows a clear decrease in the presence of particles over time (no plateau), and that 12% or less of the material (mass-based and compared with the particulate concentration at the beginning of the *in vitro* digestion) is present as particles after 30 min of intestinal digestion. This is indicative that the rest of the material should be fully degraded to non-NM (*e.g.* ionic) under gastrointestinal conditions.

3) the presence of/as aggregates rather than agglomerates (*e.g.* determined by conditions of production),

4) fixed, permanent bonding in matrices (*e.g.* stability of matrix, type of bond, end-of-life behaviour) or effective entrapment in food contact materials (FCMs) (*e.g.* polymer nanocomposites).

In the absence of data on degradation and dissolution, the SCCS would assume that 100% of the ingested material remains in particulate form.

Up to now, no *in vitro* model for the absorption of NMs via the oral route has been validated. Available *in vivo* information on oral absorption can be used provided that the testing had been performed before the animal testing bans, or the data were obtained for the purpose of compliance with other (non-cosmetic) legislations, *e.g.* REACH (EU, 2008).

In vitro models indicated in the literature include the use of Caco-2 cells and more complicated multicellular models of cells growing on membranes (Bouwmeester *et al.*, 2011).

ICRP (2006) has developed the Human alimentary tract model (HATM) which may be useful for particle dose calculation for the GI tract. This model depicts the entry of a particle into the oral cavity by ingestion or into the oesophagus after particle transport from the respiratory tract. It describes the sequential transfer through all alimentary tract regions, including the oral cavity, oesophagus, stomach, small intestine, and segments of the colon, followed by emptying in faeces. In this model, the fractional absorption of particles is specified by the alimentary tract transfer factor that describes total absorption from all regions of the alimentary tract, although the default assumption is that all absorption takes place in the small intestine.

For conventional cosmetic ingredients, it is consideredthat no more than 50% of an orally administered dose is systemically available. Thus, in the absence of data, 50% of the administered dose is used as the default oral absorption value for a cosmetic ingredient and the PODsys (see Section 6) is derived from the POD by dividing by 2. If there is information indicating poor oral bioavailability, a default value of 10% oral absorption could be considered (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). For NMs, depending on solubility, oral absorption can be expected to be lower. Therefore, whenever oral absorption data are available, these should be used, also when using other dose descriptors. Also, *in vitro* translocation/migration data, along with any other available kinetic data, should be considered.

When data on dissolution and/or degradation of the NMs are available, the non-dissolved/degraded fraction, when absorption data are not available, could be used as a starting point for default absorption percentage in the risk assessment.

For the exposure assessment of NMs, in principle the same exposure scenarios and assessment methodologies can be applied as for bulk substances. However, during consumer use, the NM characteristics may be different from laboratory conditions (*e.g.* variable versus stable particle size-distribution), so that a larger number of experimental conditions may need

to be investigated. The estimates of exposure should be provided in mass per volume metrics. Additionally, where relevant, other metrics such as particle number and size distribution, and surface area should also be provided.

The uptake and bio-kinetics of NMs are governed by processes that are different from (solubilised) substances as the transport of particles across biological barriers is not based on diffusion gradient-driven partitioning, but on endocytosis or other active transcellular transport mechanisms. The uptake of NMs across different barriers can be evaluated by advanced 2D (dimensional) and 3D multicellular co-culture *in vitro* models that are designed to closely mimic the *in vivo* anatomy and functionality of *in vivo* organs/barriers such as lung, alveolar and GI-tract.

Where there is evidence for systemic absorption, and when absorption of NPs cannot be ruled out either by experimental measurements or justified on the basis of solubility/degradation of the NM, the SCCS will apply a default approach and assume that 100% of the absorbed material was in nanoparticulate form.

4.4.2.4 SCCS default values for absorption

When experimental data are not available, default values will be used by the SCCS for absorption: for dermal absorption, in the absence of experimental data, the SCCS will apply the default value of 50% of the administered dose as currently used for conventional substances. If warranted by the composition of a specific NM, a higher value may be used. For inhalation exposure to products in spray form and for volatile cosmetic ingredients, in the absence of data on absorption, a default absorption percentage of 100% of the calculated deposition of nanoparticles in the lung will be used. For oral absorption, in the absence of data on absorption a default value of 50% of the administered dose is used. If there is information to suggest poor oral bioavailability, a default value of 10% oral absorption could be considered.

For the exposure assessment of NMs, in principle the same exposure scenarios and assessment methodologies can be applied as for bulk substances. However, during consumer use the NM characteristics may be different from laboratory conditions (*e.g.* variable versus stable particle size-distribution), so that a larger number of experimental conditions may need to be investigated. The estimates of exposure should be provided in mass per volume metric. Additionally, where relevant, other metrics such as particle number and size distribution, and surface area should also be provided.

NMs may undergo degradation or dissolution on their way into the body, where internal exposure occurs. For example, after oral exposure, NMs may be completely dissolved in the gastrointestinal tract. In order to demonstrate this, suitable *in vitro* methods should be used. In the absence of respective data, the SCCS will assume no dissolution.

The uptake and bio-kinetics of NMs are governed by processes that are different from (solubilised) substances, as the transport of particles across biological barriers is not based on diffusion gradient-driven partitioning, but on endocytosis or other active transcellular transport mechanisms. The uptake of NMs across different barriers can be evaluated by advanced 2D (dimensional) and 3D multicellular co-culture *in vitro* models that are designed

to closely mimic the *in vivo* anatomy and functionality of *in vivo* organs/barriers such as lung, alveolar and GI-tract.

Where there is evidence for systemic absorption, and absorption of NPs cannot be ruled out either by experimental measurements or justified on the basis of solubility/degradation of the NM, the SCCS will apply a default approach and assume that 100% of the absorbed material was in nanoparticulate form.

Further default values apply for absorption: In the absence of experimental data, the SCCS will apply the default value of 50% dermal absorption of the administered dose as currently used for conventional substances. If warranted by the composition of a specific NM, a higher value may be used. For inhalation exposure to products in spray form and for volatile cosmetic ingredients, in the absence of data on absorption, a default absorption percentage of 100% of the calculated deposition of nanoparticles in the lung will be used. For oral absorption, in the absence of data on absorption a default value of 50% of the administered dose is used. If there is information to suggest poor oral bioavailability, a default value of 10% oral absorption could be considered.

5. HAZARD IDENTIFICATION AND DOSE-RESPONSE CHARACTERISATION

5.1. General Considerations

Safety assessment of a cosmetic ingredient involves evaluation of its potential to pose a health risk to the consumer. This has historically been based on data from a series of *in vivo* studies in animals. However, due to the EU ban on animal testing of cosmetic ingredients and products, safety data from *in vivo* studies can only be used by the applicant if the tests have been performed in accordance with the provisions laid down in Cosmetic Regulation (EC) No 1223/2009. This means that *in vivo* data can only be accepted if testing of ingredients was performed before the animal testing ban deadlines of 11 March 2009 and 11 March 2013 as given under Article 18 of Cosmetic Regulation (EC) No 1223/2009. It is also possible that some ingredients used in cosmetic products are also used in other consumer and industrial sectors, such as pharmaceuticals, food, and industrial chemicals. As such, they may have been tested on animals under the relevant legal frameworks. For example, some ingredients used in cosmetics may also be subject to the requirements of REACH regulation (EU, 2008), and as a last resort testing may have been performed on animals to complete the respective data packages. In addition, some cosmetic ingredients may have been tested on animals in the context of pharmaceutical legislation according to "The rules governing medicinal products in the European Union". Data from such cases, where animal tests have been clearly driven by compliance with a non-cosmetic regulatory framework, may be used for the safety assessment of cosmetics. The submission of a cosmetic ingredient safety assessment including such animal data needs to be accompanied by a justification identifying the respective legislation and its requirements. Apart from such specific situations, all toxicological data for use in cosmetics safety assessment needs to be derived from alternative non-animal means - such as *in vitro* assays and *in silico* modelling/read-across (see also Factsheet on the Interface between REACH and Cosmetic Regulation, ECHA-14-FS-04-EN).

Various reports and reviews published so far have concluded that the existing risk assessment paradigm, in use for conventional chemicals, should, in principle, be applicable to engineered NPs. However, it has also been pointed out that the current testing methods may need certain adaptations to take account of the special features of NPs (SCENIHR, 2007; Rocks *et al.*, 2008; SCENIHR, 2009; OECD, 2009; SCCS, 2012; EC, 2012; ECHA, 2022; EFSA, 2021a, b). Thus, although safety assessment of an NM requires consideration of the same criteria applicable to other (non-nano) cosmetic ingredients, there are certain special aspects that need to be considered for a cosmetic ingredient if it falls within the definition of an NM under the Cosmetics Regulation (EC) No 1223/2009. Regarding the NM definition, the recently published revised recommendation for the definition of a nanomaterial (see EC document 2022/C 229/01) needs to be consulted in view of the proposed revision of the cosmetic regulation in line with the impact assessment of the EU Chemicals Strategy for Sustainability (see document Ares(2021)6011962 – 04/10/2021).

As already mentioned in Section 3, a thorough physicochemical characterisation of the NM is crucially important in planning studies into the potential behaviour and effects. The initial focus of hazard assessment needs be on determining ADME parameters to investigate the potential of the NM for systemic availability through the relevant uptake route(s) (oral, dermal, via inhalation) dependent on product type.

If there is convincing evidence that the NM is not systemically available, information on local toxicity considering the relevant exposure route as well as information on genotoxicity should be provided. Although not a local toxic effect, sensitisation can be initiated after an NM becoming bioavailable in the skin, and therefore needs to be evaluated.

Where the evidence suggests systemic availability of an NM, studies carried out in consideration of nano-specific aspects and addressing a base set of systemic toxicological endpoints will be needed, in addition to local toxicity and genotoxicity. In case where systemic exposure cannot be shown to be insignificant, further information on carcinogenicity and reproductive toxicity may be required. Data on photo-induced toxicity are specifically required when a cosmetic product is expected or intended to be used on sunlight-exposed skin and is able to absorb light. Several *in vitro* methods exist for the identification of toxicological hazards. However, information on dose-response relationships that can be used in the current risk assessment scheme to identify a point of departure (PoD) for risk assessment, *e.g.* NOAELs, LOAELs or BMDLs, has up to now generally been derived from *in vivo* studies and these tests are only accepted under the conditions described at the start of this section. Therefore, instead of a clearly defined quantitative risk assessment, it may be possible to demonstrate safety of the cosmetic ingredient under evaluation on the basis of weight of evidence from data/information from alternative methods.

5.2. Requirements for Dossiers on nanomaterials as cosmetic ingredients

When a safety dossier on a cosmetic ingredient is submitted for evaluation by the SCCS, the Applicant provides the Commission with all available information in regard to physicochemical properties, exposure assessment, and toxicological studies relating to the required endpoints for the specific cosmetic ingredient. The endpoints have been listed in the SCCS Checklists for Applicants submitting dossiers on cosmetic ingredients to be evaluated by the SCCS (SCCS/1588/17) corresponding to Cosmetics Regulation (EC) No 1223/2009, Article 16 d.

More details on the specific requirements for toxicological assessment are provided in Annex 2 to this Guidance. Depending on the type of product, and/or the nature and extent of the exposure, one or more toxicological endpoint(s) may not be regarded relevant for safety assessment of the specific cosmetic ingredient by the Applicant. In such cases, the Applicant must provide a valid justification for not addressing these endpoint(s).

To avoid unnecessary testing, each safety assessment/evaluation of an NM cosmetic ingredient should start with an evaluation of the already available information in the scientific literature. Focused toxicological testing may then need to be performed to fill any data gaps if the existing information is found to be insufficient for safety assessment.

A systematic review of the scientific literature must therefore be provided by the Applicant as an essential part of the safety dossier. This should include the search terms used in the review, the total number of relevant articles found, and the basis for selecting and excluding the articles for drawing conclusions. In particular, scientific reasoning must be provided for not considering any articles that may have been in contradiction with the conclusions drawn by the Applicant.

During preparation of literature review on available toxicological data for NMs the process of assessing data quality according to clear criteria for the quality for NM studies should be used. Examples of such quality assessment approaches for human health risk assessment have been proposed:

1) the two-step process (Card and Magnuson, 2010), DaNa Literature Criteria Checklist (DaNa, 2016), and

2) the GUIDEnano quality assessment approach (Fernandez-Cruz, 2018).

The study results submitted as part of a safety dossier should accompany a declaration that the relevant tests were conducted using a substance that had the same (or justifiably similar) specifications and physicochemical characteristics to that intended for use in the final cosmetic product (SCCNFP/0633/02). For NM cosmetic ingredients, this means that the test substance and the substance in the final cosmetic product both have the same or comparable profiles in relation to chemical composition, particle size distribution, particle surface characteristics, morphological forms, etc. Matching chemical identity and physicochemical characteristics of an NM used in the various toxicity studies with that used as a cosmetic ingredient is therefore essential. Data on an NM that does not fall within the specifications of the NM intended for use as a cosmetic ingredient is not accepted.

The safety of a NM form of a cosmetic ingredient must not be based on the assumption that the bulk form (or another nano form) of the same materials is safe, or vice versa, without specific evidence to support it. Any non-relevant data – for example relating to unrelated materials, or materials with unknown composition/characterisation, is not accepted. If data from other materials are also included along with an NM ingredient (*e.g.* a bulk material as a comparator), it should be clearly defined and segregated, and not presented in a mixed-up manner with the data on NM(s) under evaluation. Unless a close similarity between different NMs can be justified, it is advisable to include a complete set of supporting data for each NM, rather than presenting several different NMs in a single, incomplete or data-poor submission. If more than one NM is to be included in a single safety assessment, the basis for 'close similarity' must also be provided to demonstrate that data read-across between the NMs is scientifically acceptable/valid. This substantiation should relate to the chemical composition

of the core NM, as well as physical/morphological features and other characteristics, such as any surface coatings and/or other surface modifications (SCCS 1524/13).

Information on the stability of the test substance under experimental conditions is of prime importance for the interpretation of any test results (Section 3.1). Data on the stability of the test material should therefore be reported, and data on the dissolution rate and the solubility of the NM in the finished cosmetic product and in the vehicle(s) used in the tests must be provided (if applicable).

Together with the data on relevant experimental investigations, the following information should be provided:

- for *in vivo* studies: the study date (to indicate compliance with the animal testing ban under Cosmetic Regulation (EC No 1223/2009), and/or a justification if testing was done after March 2013 for another regulatory context, this regulatory context and its data requirement should be identified and presented;
- any report on epidemiological and/or observational experiences (*e.g.* cosmetovigilance data);
- an appraisal of all relevant published literature, along with a description of the bibliographical methods used; any information from 'grey material' available. Any other relevant findings by the Applicant and/or other industry/agencies should also be transmitted to the Commission for review;
- only relevant literature and/or data of the same or closely similar NMs (for which sameness needs to be demonstrated) can be used for the safety assessment (SCCS 1524/13).

Conditions for the use of existing studies

The question of acceptance of the evidence to support safety assessment of a nanomaterial generally comes up when the submitted data are partly or solely drawn from existing/historic studies. The conduct of such studies may render some of them unacceptable because they may have been carried out either without or under obsolete guidelines, do not provide sufficient information on physicochemical characterisation and whether or not a method for particle dispersion was used, and whether the testing conditions had taken account of the (nano)particulate nature of the test material. In view of this, the recent EFSA Guidelines (EFSA 2021a, b) have set out some criteria for the acceptance of existing studies for safety assessment of nanomaterials, and other particulate materials that may contain small particles - including nanoparticles.

Whilst detailed guidance is provided in EFSA (2021a, b), Schoonjans *et al.* (2023) have recently summarised the key elements considered in this regard. At the outset, it is important to provide physicochemical characterisation data to show that the material that had been used in an existing study is the same as the material under current safety assessment in terms of the content and characteristics of the fraction of small particles. This means that the available information should demonstrate that the test material included the same qualitative/quantitative profiles in regard to nanoparticles, and that the study selection, study design and the level of dispersion/degree of agglomeration of the test material were suitable for assessing the hazard of small particles including nanoparticles.

For repeated dose oral toxicity studies, the duration of exposure needs to be sufficient to address the potential hazard of small particles. Other considerations in this regard include demonstrating that: 1) sample preparation of the test material had been carried out appropriately. Considering that the doses tested are often much higher than those used in

consumer products, it is imperative to provide information on the level of dispersion/agglomeration as this is more likely to happen at higher concentrations; 2) information on potential local effects should be provided; 3) results from two different mammalian *in vitro* genotoxicity assays will be needed as the bacterial Ames test is not considered a suitable method for particulate materials. Also, to substantiate the validity of a negative genotoxicity result, it is essential that evidence of cellular uptake of the particles is demonstrated; and 4) toxicokinetic information will need to be evaluated following uptake and potential accumulation of the particles in tissues.

According to Schoonjans *et al.* (2023), where a detailed report covering sample preparation according to the OECD guidelines was available, a weight of evidence supported by information on solubility/dispersibility of the material could provide sufficient indications on the coverage of the fraction of small particles. If these issues have not been addressed adequately, evidence will be needed from NAM studies.

Safety assessment of cosmetic ingredients has historically been based on data from *in vivo* studies in animals. Due to the ban on animal testing of cosmetic ingredients and products, *in vivo* data can only be used if the tests were either performed before the ban, or to fulfil other (non-cosmetic) regulatory requirements.

Overall, the safety assessment of NM cosmetic ingredients can follow the existing risk assessment paradigm for conventional chemicals. However, certain testing methods may need to be adapted to take account of the nano-scale particulate aspects.

The essential elements of safety dossiers on NM cosmetic ingredients include a thorough and up-to-date review of the published literature, detailed physicochemical characterisation, exposure assessment, and toxicological studies. The initial focus of hazard assessment should be on ADME parameters to investigate the potential for systemic availability of nanoparticles via all relevant uptake route(s). If there is convincing evidence that the NM is not systemically available, information on local toxicity considering relevant exposure route(s), as well as information on genotoxicity, should be provided. Although not a local toxic effect, sensitisation can be initiated after an NM becoming bioavailable in the skin. As a whole, the safety assessment is exposure driven instead of determined by the potentially identified hazards. Where there is evidence for systemic availability of an NM, studies addressing a base set of systemic toxicological endpoints will be needed, in addition to local toxicity, sensitisation and genotoxicity. Where systemic exposure is possible, further information on carcinogenicity and reproductive toxicity may be required. Data on photo-induced toxicity are specifically required for a cosmetic product intended to be used on sunlight-exposed skin and is able to absorb light.

5.3. Specific Considerations relating to testing of Nanomaterials

5.3.1. Solubility/Dispersion

When testing insoluble or partially soluble NPs, it must be kept in view that they will be present in a dosing or test medium as a nano-dispersion rather than as a solution. Therefore, special attention should be paid to the agglomeration/aggregation behaviour, and the insoluble/partially soluble nature of NMs (SCCP, 2007; Rocks *et al.,* 2008; SCENIHR, 2009;

OECD, 2009; Chaudhry *et al.,* 2010; Gottardo *et al.,* 2017). Possibilities for disagglomeration of NPs under different testing and physiological conditions should also be considered (OECD, 2012a). In this regard, an appropriate dispersion protocol should be followed (see Section 3.1).

During toxicological evaluations, some properties of NMs may change due to interaction with the surrounding media. Thus, a focus of investigations should be on ascertaining that the tested NMs are in exact form/composition as intended for use in a cosmetic formulation and as the formulation is delivered to the end-user. Where toxicological data on a different NM, or a different form of the same NM, is presented in the dossier, justification (*e.g.* data) must also be provided to indicate that the original and dispersed preparations are indeed similar.

Special care is also needed in regard to the applied doses, because the concentration of an NM may decrease during a test due to sedimentation, binding with other moieties in the test medium, or adhesion to glass/plastic ware. It is therefore important to ascertain the stability and uniformity of the NM dispersion in a test medium to ensure that the applied concentration/ dose is maintained for the intended period during the test. Possible interaction of the NM with other components of a test medium/formulation will also need to be determined.

5.3.2. Surface interactions

The interactions of a NM with the surrounding media and biological systems largely take place through its surface. The surface characteristics of particles are determined by the nature of the entities present on the surface due to the inherent (bio)chemical composition of the material itself, or because of other moieties that may have adhered or attached to the surface due to van-der-Waals forces or electrostatic interactions, or may have been deliberately applied as a coating. It is well known that due to high surface energies, NPs tend to stick together to form larger agglomerates and aggregates, and may adsorb or bind various moieties on the surface, including proteins (Cedervall *et al.*, 2007; Šimon and Joner, 2008; Lynch and Dawson, 2008; Monopoli *et al.*, 2012; Moore *et al.*, 2015; Ke *et al.,* 2017; Garcia-Alvarez *et al.,* 2018; Da Silva *et al.,* 2019; Francia *et al.,* 2019; Breznica *et al.,* 2020; Galdino *et al.*, 2021; Kopac, 2021; Cai *et al.*, 2022; and Choi *et al.*, 2022). An NM with different surface characteristics (*e.g.* hydrophilic versus hydrophobic surface) may have profoundly different ADME properties and may interact differently with biological fluids, cell membranes and other biological entities (Mirshafiee *et al.*, 2016). In view of the potential agglomeration/aggregation of particles, it is essential that attention is paid to the process used for dispersing NPs in preparations used in toxicological testing.

It has been shown that composition of protein corona is highly dependent on the initial mixing steps involved (Lundqvist *et al.,* 2011; Jayaram *et al.*, 2018; Simon *et al.*, 2018).

Due to the potential to bind other moieties on surface, and penetrate cellular membrane barriers, NPs may transport other substances into the test systems (the so-called 'Trojan Horse' effect), which may lead to altered (increased or decreased) activity/toxicity. For example, NPs may bind and carry certain immunogens/antigens to the immune cells and impart or trigger an immunological effect.

Such a transport of certain components of the test systems by NPs may also lead to artefacts and false indications of harmful effects. This can be avoided by a thorough characterisation of the NMs, and the use of appropriate controls within the testing scheme. Selection of

controls should also consider possible interaction of the NM with the readout system of the assay as it has been demonstrated for various NMs for tetrazolium salts or other dye-based cytotoxicity assays (Worle-Knirsch *et al.*, 2006; Monteiro-Riviere *et al.*, 2009; Lanone *et al.*, 2009; Wilhelmi *et al.*, 2012; Guadagnini *et al.*, 2015; ECHA, 2017b; Sen *et al.*, 2023). In case of doubt over the validity of the outcome of an assay, the use of an additional independent analytical method may provide more information (ECHA, 2017b). The presence of a light-absorbing/reflecting NM in the assay can itself have an influence on a read-out system, especially if the readout is based on spectroscopy. Similarly, the composition of the culture medium (*e.g.* the presence or absence of serum) in a test system may influence the outcome of the assay (Guadagnini *et al.* 2015). The occurring interferences can be both assay and/or particle specific (Guadagnini *et al.*, 2015). Proper controls should be included in the test assay to determine possible interference (*e.g.* incubation of cells with NP solvent, incubation of the NP with the detection system, incubation of the NP with the chemicals used in the detection system, possibility for light scattering in spectrophotometer).

Special attention should be paid to agglomeration/aggregation behaviour, and the insoluble/partially soluble nature of NMs. Possibilities for de-agglomeration of NPs under different testing and physiological conditions should also be considered. As properties of NMs may change during toxicological evaluations due to interaction with the surrounding media, investigations should also focus on whether the tested NMs are in exactly the same form/ composition as intended for use in a cosmetic formulation delivered to the end-user. The Applicant should also consider any changes in the applied doses of NMs due to sedimentation, binding/adhesion with test medium or glass/plastic ware to ensure that the applied concentration/dose is maintained during the test. The so-called 'Trojan Horse' effect and possible interaction of the NM with the readout system of the assay should also be considered and proper controls for detection of these should be included in the tests performed. Furthermore, proper controls should be in place to evaluate possible interference especially when colorimetric methods are used as readout system.

5.3.3 General criteria to be considered for different coatings on a nanomaterial

Particle surfaces of reactive (*e.g.* photocatalytic) NMs are generally modified, coated, or doped with other materials to 'quench' the reactivity before use in cosmetic products. Surface modification of an NM, however, may also bring about profound changes in the physicochemical properties (*e.g.* hydrophobicity/hydrophilicity), ADME profile and interaction with biological entities. A significant alteration in the properties and biokinetic behaviour may also alter their toxicity due to the potential penetration and accumulation of particles in organs that are not expected to be the target of an unmodified or a differently coated form of the same NM. It is therefore important that not only the NMs, and the materials used for surface modification, are assessed individually, but that they are also assessed for safety together when in the form of a surface-modified/coated NM. In particular, a major change in hydrophobicity of the NP surfaces may affect dermal absorption. This raises the question whether an NM with several different surface modifications/coatings will need to be tested each time.

The SCCS Opinion (SCCS/1580/16) considered the use of different coatings on an NM in the context of titanium dioxide (nano-form). In brief, where a coating material applied to an NM

surface has not already been evaluated for such an application, it will need to be demonstrated to the SCCS to be safe and not affect the particle properties related to behaviour and/or effects. In this regard, a full dataset on the physicochemical properties, biokinetic behaviour and toxicological effects of the NM with each new surface modification/coating would be preferable. However, as a minimum, in addition to safety data on the core NM, the SCCS will require the following:

1. Information/data on each material used for surface modification/coating of the NM to indicate that it is safe for use in the intended cosmetic product - *e.g.* it is an approved cosmetic ingredient, or not a banned or restricted substance under Annex II and III of the Regulation (EC) No 1223/2009.

2. Data on physicochemical properties of the surface-modified/coated NM to show that they have not significantly changed compared to the same material when uncoated or with a different surface modification/coating that has already been assessed as safe by the SCCS. However, when a coating is applied for a specific purpose (*e.g.* reduction of (photo)catalytic activity), the effect of the coating on the intended activity should also be demonstrated.

3. Data on dermal penetration, stability of the surface modification/coating, and (photo)catalytic activity (where final products are intended for use on skin exposed to sunlight) of the NM to indicate that:

    a. the surface modification/coating is stable in final formulation,

    b. the surface modification/coating does not lead to any significant absorption of the nanoparticles through the exposure route(s) anticipated for the intended use,

    c. the (photo)catalytic activity of the surface modified/coated NM is relatively low (*i.e.* not more than 10% compared to the non-coated equivalent).

    d. when testing a combined use of different coating materials, a combination of the individual concentrations that represents 'worst case' in terms of hydrophobicity should be used and justification why a certain combination should be considered as worst case should be given.

The SCCS would consider an NM that has been surface modified or coated with a new substance 'similar' to an already assessed surface variant of the same NM if both compare well in terms of the above criteria. However, a full toxicological dataset would be required for safety evaluation if the new material used for surface modification/coating is not already known to be safe, or brings about a significant change in the physicochemical properties, dermal absorption, and/or (photo)catalytic activity of an NM.

Where a coating material is applied to an NM surface, it will need to be demonstrated to the SCCS to be safe and not to affect the properties relating to particle behaviour and/or effects with exception of the intended modification/purpose. As a minimum, data/information should indicate that: 1) each material used for surface modification/coating is safe for use in the intended cosmetic product; 2) data on physicochemical properties of the surface-modified/ coated NM to show that they have not significantly changed compared to uncoated form of the same material (or with a different surface modification/coating that has already been assessed safe by the SCCS); and 3) data on dermal penetration, stability of the surface modification/coating and (photo)catalytic activity of the NM (for use in products intended for

application on skin exposed to sunlight). Where more than one coating material is applied, data should be provided on a combination of the individual concentrations which represents 'worst case' in terms of hydrophobicity.

5.3.4 Nano-carriers and nano-encapsulated materials

Encapsulation and other forms of formulation have been increasingly used to develop nano-sized carriers or delivery systems for (bioactive) substances (Sabliov *et al.,* 2015). The nanocarriers may be in the form of solid particles (*e.g.* mesoporous silica), or polymer, protein or lipid-based delivery systems (*e.g.* micelles or liposomes). Nano-scale encapsulation is generally intended for use as a delivery vehicle for cosmetic ingredients, or to fulfil a technological function – such as to increase dispersibility and/or bioavailability of the encapsulated ingredients, to alter lipophilic or hydrophilic characteristics, or to protect the ingredients from degradation when exposed to air, solvents, or UV light. It is therefore imperative for nano-encapsulated cosmetic ingredients that their safety is assessed in regard to the individual components (*e.g.* the encapsulating material and the encapsulated contents), as well as of all the components when put together in the form of the nano-sized entity (Chaudhry and Castle, 2015; EFSA, 2021a; Eder *et al.,* 2022).

This is because nano-sizing of substances may impart certain changes in their properties, behaviour and effects compared to corresponding conventional forms, and the data on safety of individual components in conventional forms may not be sufficient for safety assessment when they are assembled together in the form of a nano-encapsulated entity. Therefore, any significant changes in the physicochemical properties and toxicokinetic behaviour of the encapsulated ingredients, as a result of nano-encapsulation, need to be investigated. Any increase in the uptake and bioavailability of the nano-encapsulated entity should be considered in relation to toxicity, in particular, if the data suggest that the materials are absorbed in the encapsulated form via the relevant exposure routes.

It is indicated in the SCCS Opinion on nano-encapsulated substances in (sodium) styrene/acrylate copolymer (SCCS/1595/18) that safety assessment could not be concluded on the basis of data on individual components, and data on the nano-encapsulated entity as a whole would be required. This requires the Applicants to provide a clear description of the nano-encapsulated form in terms of full chemical composition, purity, concentration, physicochemical properties, stability, and dermal penetration of both the individual components and the nano-encapsulated entity.

Safety assessment of such applications will also require consideration of the potential toxicological effects and exposure estimates under foreseeable use conditions both for each individual component, as well as the nano-encapsulated entity as a whole. The intended function and uses of the nano-encapsulated forms should also be clearly described.

For encapsulated NMs, a clear description of the intended function and uses, chemical composition, purity, concentration, as well as physicochemical properties, stability, and dermal penetration of the individual components of the nano-encapsulated entity should be provided. Safety assessment should consider toxicological and exposure aspects under

foreseeable use conditions for each individual component, as well as the nano-encapsulated entity as a whole.

5.3.5. Immunotoxicity

NPs absorbed into the body through different routes of exposure may lead to interaction with the immune system. Some NMs can stimulate and/or suppress the immune responses and their interaction with the immune system is largely determined by their size, shape, composition, surface properties, protein binding and administration routes. Such effects may result from induction of reactive oxygen species, apoptosis, cell cycle inhibition, complement activation, enhanced secretion of cytokines and chemokines, interaction through toll-like receptors, inflammatory responses, induction of autophagy, reduced viability of the major cell types involved in the innate and adaptive immune system (reviewed by WHO, 2019).

Due to the potential for binding other substances on the surface, NPs need special attention because they may carry other substances including proteins to the immune cells and thus act as a 'Trojan horse'. This has also been exploited in the form of NP carriers of various immunogens in the development of vaccines. This carrier effect has been recently reviewed by EFSA (2021a) and different methods for determining immunogenicity/allergenicity and immunotoxicity have been proposed. Immune stimulation more specifically may also result in allergy and auto-immune responses. Inflammation in the lung as major target organ for NM toxicity cannot be considered as immunotoxicity, however, a prolonged stimulation of the various components of the inflammatory system may result in pathological conditions such as asthma that are immunologically driven. NPs can modify immune responses and are known to exacerbate allergic responses in the lung (reviewed by WHO, 2019).

In view of the potential of nanoparticles to interact with various cells of the immune system, for example phagocytes and other cells of the mononuclear phagocytic system, specific attention is needed for the interaction of nanoparticles and their possible effect on the immune system. Currently, there are no regulatory documents specifically dedicated to evaluate immunotoxicity of NMs. Their immunotoxicity assessment is performed based on existing guidelines for conventional substances or medicinal products (Giannakou *et al.*, 2016a, 2020). Research groups involved in developing NMs already use a wide range of *in vitro* assays to screen for essential aspects of the immunosafety profile that are not included in the current regulatory guidances. For example, a number of international projects have produced guidelines for testing strategies and test methods, including *in vitro* assays, for NM safety evaluation (*e.g.*, the FP7 EU projects NANOMMUNE and MARINA and H2020 project REFINE). Dobrovolskaia and McNeil (2016) reported a number of *in vitro* immunoassays that provide results with a good or fair correlation to *in vivo* assay outcomes. Good correlation was indicated for the *in vitro* assays of hemolysis, complement activation, opsonization and phagocytosis, and cytokine secretion assays. Other assays can also be regarded as broadly predictive of the functional alterations of the immune system, including the Colony Forming Unit-Granulocyte Macrophage assay, the leukocyte proliferation test (immunomodulatory assays), and platelet aggregation, leukocyte procoagulant activity, and various plasma coagulation tests (thrombogenicity assays). Detailed protocols of many of these assays have been published (McNeil *et al.*, 2018). A number of these assays are available on the website of the National Cancer Institute, USA (https://www.cancer.gov/search/results?swKeyword=nanoparticle+ITA).

Although the assays were developed for nanomedicines for cancer therapy, some are useful for evaluation of nanoparticle interaction with the immune system. Recently interlaboratory comparisons were reported for inflammasome activation (Vandebriel *et al.,* 2022) and complement activation (David *et al.,* 2023).
An important issue regarding possible activation and/or effects on cells of the immune system is the potential contamination of NM preparations with endotoxin (also indicated with lipopolysaccharide, LPS), part of the cell wall of Gram-negative bacteria. LPS can induce pre-inflammatory cytokines and especially activates macrophages (sterilization of a NM product kills the bacteria (and thus the risk for bacterial infection) but does not remove the LPS that remains as part of the bacterial cell walls). For NM a check on the presence of endotoxins is recommended by ECHA (ECHA, 2021b). The presence of LPS can be demonstrated by the so-called Lymulus Amebocyte Lysate (LAL) Assay (see for example ISO, 2010b – ISO 29701:2010 confirmed in 2021). As interference in the LAL may be an issue, a confirmation by an additional or alternative assay also maybe applied like the identification of specific phospholipids as part of the LPS (Giannakou *et al.,* 2016b, 2019). Also, a validated *in vitro* assay is available for endotoxin determination, the so-called macrophage activation assay (Dobrovolskaia *et al.,* 2014).

When NMs enter the body, the body may react with an inflammatory response. Although the inflammation is mediated by the immune system, such an inflammatory response should not be seen as an immuntoxic effect. However, the extent of such a (local) inflammation could be seen as local adverse effect of the NM.

5.3.6. Skin sensitisation

The sensitisation potential of the NMs used in cosmetics needs to be evaluated as part of the safety assessment. The Adverse Outcome Pathway (AOP) for skin sensitisation is well known and described (Corsini *et al.*, 2018). For the evaluation of potential skin sensitisation of conventional chemicals, some *in vitro* assays are available that mimic Key Events (KE) that are part of the AOP for sensitisation including methods assessing covalent binding to proteins (KE1) (OECD TG 442C, OECD 2022e), keratinocyte activation (KE2) (OECD TG 442D, OECD 2022f), comprising the ARE-Nrf2 luciferase test method (KeratinoSens™) and the ARE-Nrf2 luciferase LuSens test method, and dentritic cell activation (KE3), comprising the h-CLAT, the U-SENS, the IL-8 Luc and the GARD™ (Genomic Allergen Rapid Detection) assays (OECD 442E, OECD 2022g). It has been proposed to combine the NAMs with other information sources (*e.g.*, in silico tools) in an integrated (defined) approach. Three such defined approaches are covered in OECD TG 497 (OECD, 2021d) describing defined approaches for Skin Sensitisation. These assays/approaches may be applicable to nanomaterials as well, provided that the stable dispersion criteria can be fulfilled. The applicability of OECD TG 442D has been investigated for a limited number of NMs (OECD, 2022j). For the h-CLAT and U-SENS assays, a strategy was reported for the testing of engineered nanomaterials and nanotechnology-formulated drug products (Potter *et al.*, 2018). For graphene, the application of the *KeratinoSens*™ assay showed negative results for sensitization (Kim *et al.*, 2021). For medical device extracts of solid sensitiser spiked polymers, the SENSE-IS assay was able to correctly identify the presence of sensitisers in the medical devices extracts. So, materials extraction in a polar and/or non-polar solvent might be an alternative approach for the testing of solid nanomaterials. Furthermore, more specific modifications may also be needed when using nanomaterials in the test system (WHO, 2019).

For systemically or locally available NMs in a cosmetic product, it is important to ascertain that they will not exert an adverse immunological effect. This is particularly important for NMs that are composed of, or contain on the surface, peptides/proteins or other immunogenic/allergenic substance(s). A number of *in vitro* immunoassays can provide results with a good or fair correlation to *in vivo* assay outcomes.
The sensitisation potential of the NMs used in cosmetics needs to be evaluated as part of the safety assessment. Although not yet validated for nanomaterials, several *in vitro* assays based on key events of the Adverse Outcome Pathway (AOP) have been established to address skin sensitisation.

5.3.7. Genotoxicity

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. These changes may involve a single gene or gene segment, a block of genes or chromosomes. The term clastogenicity is used for agents giving rise to structural chromosome aberrations. A clastogen causes breaks in chromosomes that result in the loss or rearrangement of chromosome segments. Aneugenicity (aneuploidy induction) refers to the effects of agents that give rise to a change (gain or loss) in chromosome number in cells, resulting in cells that do not have an exact multiple of the haploid number as described in the REACH regulation (2006/1907/EC).

NMs may induce genotoxic damage by a) primary (direct or indirect) or b) secondary mechanisms: i) directly by interaction with DNA, by disturbing the process of mitosis, or by producing Reactive Oxygen Species (ROS) directly or after alterations of mitochondrial functions or ii) by secondary mechanisms as result of oxidative DNA attack during NM-elicited chronic inflammation caused by activation/recruitment of immune cells, such as macrophages and/or neutrophils (Magdolenova *et al.*, 2014; OECD, 2014b; KEMI, 2016; Evans *et al.*, 2017).

For *in vitro* genotoxicity assessment, both chromosomal damage (clastogenicity and aneugenicity) and gene mutations should be evaluated. The widely used bacterial reverse mutation (Ames) test is not considered appropriate for NM mutagenicity assessment due to size of bacteria and limited or no uptake of NMs by the bacteria (SCCS, 2019). The bacterial cell wall hinders uptake and thus NP internalisation is unlikely to occur to the same extent as observed in mammalian cells, hence sensitivity of the assay is questionable (Doak *et al.*, 2012; Magdolenova *et al.*, 2014; Dusinska *et al.*, 2017; Elespuru *et al.*, 2018, 2022). Therefore, a negative outcome of the Ames test would not be accepted as indication for the absence of genotoxicity.

It is suggested that for NMs, the following *in vitro* genotoxicity tests be conducted:

- Mammalian cell chromosome aberration/clastogenicity tests (either *in vitro* chromosome aberration test or micronucleus test). The micronucleus test can be performed using either the mononucleate or cytokinesis blocked protocols. However, if the cytokinesis blocked micronucleus assay is to be applied then the blocking agent (cytochalasin B) addition must be post-treatment (after the NM exposure period) as described in the OECD Study Report and Preliminary Guidance Document on the Adaptation of In Vitro Mammalian assay (OECD TG 487) for Testing of Manufactured Nanomaterials (OECD, 2022a). Alternatively, a delayed-co-

treatment protocol is also acceptable if a sufficient NM exposure period has been allowed to enable uptake into the cells of the test system. Co-exposure to both cytochalasin B and the test NM for the duration of the experiment should be avoided due to possible interference of NMs with cytochalasin B in terms of the cellular uptake of the NM (Li *et al.*, 2017). Most poorly soluble NMs are not metabolised, and the extracellular metabolic activation system (S9-mix) may interfere with the assay reducing the NM uptake into cells. Organic NMs or some inorganic NMs coated with organic functional groups may, however, exert their genotoxic effects in the presence of the metabolic activation system. The use of S9-mix in the tests should therefore be considered case-by-case.

- An *in vitro* mammalian cell gene mutation test (*e.g.* Hypoxanthine-guanine Phospho Ribosyl Transferase (Hprt), Thymidine Kinase (Tk) or Xanthine-guanine Phospho Ribosyl Transferase gene (Xprt) tests) (Chen *et al.*, 2022).

- Other indicator tests, such as the Comet assay may be included for further weight of evidence. The Comet assay modified with repair enzymes is useful for detection of DNA oxidation damage induced by NMs (Collins *et al.,* 2017, 2023; Cardoso *et al.*, 2022). The Comet assay is especially suitable in a high throughput version (Collins *et al.*, 2017; El Yamani *et al.*, 2017, 2022a), to cope with large numbers of NM samples, concentrations, and incubation times. Another useful test that has already been validated is the cell transformation assay (CTA) (Sakai *et al.* 2010; Sakai *et al.* 2010, Ohmori *et al*., 2022, Colacci *et al*., 2023; Hayrapetyan *et al*., 2023). Besides the Comet assay, several existing genotoxicity testing methods which are amenable to HTS/high content screening (HCS) approaches were identified, *e.g.* the *in vitro* micronucleus assay, the γH2AX assay and the ToxTracker assay (Kohl *et al.*, 2020).

Additional *in vitro* tests that provide mechanistic understanding may be taken into consideration in a weight of evidence approach, *e.g.* Pig-a test, toxicogenomics, recombinant cell models (GreenScreen HC, BlueScreen HC, ToxTracker), γH2AX, epigenetic responses (*e.g.* DNA methylation, non-coding small single-stranded RNAs termed microRNAs (miRNAs) and histone modifications).

Advanced models for in vitro genotoxicity testing

Cells cultured in 3D models resemble the organ structure better, due to their more "*in vivo*-like" behavior for key parameters such as cell viability, proliferation, differentiation, morphology, gene and protein expression and function. For genotoxicity assessment, robust protocols for 3D models have been established for skin, airways and liver tissue equivalents.

Many of the 3D cell culture systems applied in genotoxicity testing of NMs are spheroids, such as liver spheroids constructed from primary hepatocytes, HepG2 hepatocellular carcinoma cells or the HepaRG cell line applied mainly to the Comet assay (Mandon *et al.*, 2019; Štampar *et al.*, 2019; Elje *et al.*, 2019, 2020) and micronucleus assay (Shah *et al.*, 2018; Conway *et al.*, 2020). There are also commercially available human reconstructed 3D airway models (*e.g.* MucilAir™, EpiAirway™, EpiAlveolar™ or other models)[4] as well as air-liquid-interface models under development consisting for example of the bronchial epithelial cell line BEAS2B and the tumour lung epithelial cell line A549 human macrophages THP-1 or human blood

[4] These models are presented as non exhaustive list of examples, and as such do not indicate any endorsement by the SCCS.

monocyte-derived dendritic cells, endothelial cells (Camassa *et al.*, 2022, Elje *et al.*, 2023). For human skin models, two methods are promising for genotoxicity assessment of NMs: the reconstructed skin micronucleus test (Wills *et al.*, 2016) and the reconstructed skin Comet assay (RS comet assay). The 7th International Workshop on Genotoxicity Testing (IWGT) Working Group experts concluded that '3D tissue-based assays provide a more realistic test system to study particulate materials (*e.g.* NMs), compared to 2D test systems' (Pfuhler *et al.*, 2020). The SCCS is the opinion that indeed these 3D models for liver, airways and skin show great promise, but they need further improvement for genotoxicity assessment of NMs. A recent review of selected methods useful for genotoxicity testing of NMs (*e.g.* the Alamar Blue assay, the colony-forming efficiency assay, the expression of anti-oxidative enzymes under the control of the nuclear erythroid 2-related factor 2 (NRF2) transcription factor) is given in the special issue on "Methods and protocols in nanotoxicology" published in Frontiers in Toxicology (2022) (https://www.frontiersin.org/research-topics/18580/methods-and-protocols-in-nanotoxicology).

Secondary genotoxicity mechanisms can only be detected *in vitro* if co-culture models are used, consisting of both immune and epithelial cells (Evans et al., 2019; Vallabani and Karlsson, 2022).

The most recent innovation in microfluidics, called organ-on-a-chip technologies (OOC), aim at modeling *in vitro* the micro-physiological conditions prevalent in the body as closely as possible, thus avoiding the typical disadvantages of conventional cell models.

An IATA for grouping of NMs based on existing genotoxicity methodologies has been proposed (Verdon et al., 2022). Several descriptors for prediction of NMs toxicity were identified by quantitative structure-activity relationship (QSAR) on cytotoxicity and genotoxicity of 17 NMs (El Yamani et al., 2022b).

Requirement for cell uptake testing

Uptake of NMs by cells can take place by active or passive processes. It depends on various factors including NM size, shape, shell structure, surface chemistry and surrounding environment *i.e.* corona formation (Shang *et al.*, 2014; Li et al, 2017; Behzadi *et al.*, 2017; Sabourian *et al.*, 2020, El Yamani *et al.*, 2022a). Cell types and cell lines differ in their uptake; for example, cell lines originating from lymphocytes and lymphoblasts have a lower uptake than THP-1 cells (monocytes) especially when tested at low concentrations (Rubio *et al.*, 2020). Primary lymphocytes seem to lack active uptake (Hannukainen *et al.*, 2009). When selecting a cell type for genotoxicity testing of NMs, serious consideration must be given to the ability of the cells to take up the NMs (SCCS, 2023a). To ensure that the NM actually reaches the DNA during mitosis, a prolonged exposure period (24-48 h) including a complete cell cycle is recommended.

Thus, for *in vitro* genotoxicity studies, it is necessary to demonstrate uptake of the NPs in the cell and preferably the nucleus to demonstrate exposure of cellular target structures (*e.g.* DNA). If such exposure cannot be demonstrated, a negative outcome of such assay might be meaningless, as the target exposure will not be known. In addition, the amount taken up by the cells may be considered for expression of the possible dose response relationship (OECD, 2014b). The uptake of NMs into cells was, for example, considered in the case of hydroxyapatite (nano) (SCCS/1648/22).

Requirement for interference testing

Properties of NMs such as adsorption capacity, optical properties, hydrophobicity, chemical composition, surface charge and surface properties, catalytic activities as well as agglomeration can result in interference with standard toxicity tests (Guadagnini *et al.*, 2015) see also Section 5.3.2 above. Agglomeration of NMs affects their bioavailability to the cell and thus might lead to false positive/negative results. Several cytotoxicity, oxidative stress and genotoxicity assays, such as the Comet assay and the micronucleus test, have been investigated for the possibilities of such interferences and suggestions have been made for a modification of the micronucleus assay to ensure correct genotoxicity assessment (Doak *et al.*, 2009, 2012; Magdolenova *et al.*, 2012) and for inclusion of additional controls for the Comet assay (Magdolenova *et al.*, 2012; Azqueta and Dusinska, 2015; Huk *et al.*, 2015; Bessa *et al.*, 2017; El Yamani *et al.*, 2022a).

For *in vitro* genotoxicity assessment, both chromosomal damage (clastogenicity and aneugenicity) and gene mutations should be evaluated. The widely used bacterial reverse mutation (Ames) test is not considered appropriate for NM mutagenicity assessment and an *in vitro* mammalian cell gene mutation test should instead be carried out. Other indicator tests should also be considered, such as Comet assay modified with repair enzymes, and the cell transformation assay (CTA). Advanced 3D models are promising, especially when co-cultured with immune cells to detect secondary genotoxicxity.

When selecting a cell type for genotoxicity testing of NMs, serious consideration must be given to the ability of the cells to take up the NMs. Demonstration of cellular uptake is crucial when obtaining negative genotoxicity results (SCCS, 2023a). To ensure that the NM actually reaches the DNA during mitosis, a prolonged exposure period (24-48 h) including a complete cell cycle is recommended.

5.3.8. Carcinogenicity

Substances are defined as carcinogenic if they induce tumours (benign or malignant) or increase their incidence, malignancy or shorten the time of tumour occurrence when they are inhaled, ingested, dermally applied or injected (ECB, 2003).

A carcinogenicity study is in general only submitted when already available, *e.g.*, when carried out before the animal testing ban or when generated for compliance under a different (non-cosmetic) legislative framework. The decision on the carcinogenic potential of mutagenic or genotoxic substances may thus be made on the outcome of *in vitro* mutagenicity tests. A positive *in vitro* result in mutagenicity tests is also seen as indicative of the carcinogenic potential of the substance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). There are several ongoing initiatives to develop *in vitro* tests for the indication of carcinogenicity. New *in vitro* approaches, such as cell transformation assays (CTAs) or toxicogenomic approaches may also be useful for the identification of genotoxic as well as non-genotoxic carcinogenic NMs (Ohmori *et al.*, 2022). The latter in combination with transcriptomics provide mechanistic information at the molecular level. Additionally, novel toxicity endpoints such as epigenetic toxicity will have to be considered in the future. Epigenetics refers to heritable changes in gene expression that occur without alterations in DNA sequence. A growing body of evidence indicates that environmentally induced epigenetic

alterations play a role in the onset of several human diseases, including cancer, mental disorders, obesity, and other severe conditions (reviewed by Smolkova *et al.*, 2015, 2017; Marczylo *et al.*, 2016). Several studies show that epigenetic toxicity/activity can be induced by NMs and can occur at sub-cytotoxic and sub-genotoxic concentrations (Ghosh *et al.*, 2017; Zhang *et al.,* 2020).

So far only the *in vitro* CTAs that can detect both genotoxic and non-genotoxic carcinogens have been validated. CTAs are *in vitro* tests measuring the conversion from normal to transformed phenotype of mammalian cells (primary Syrian hamster embryo (SHE), or stable cell lines such as mouse BALB/c-3T3 or C3H/10T1/2 cells when exposed to test compounds. A guidance document on the *in vitro* SHE CTA was adopted in 2015 by the OECD (OECD, 2015a). The OECD Guidance Document on *In vitro* Cell Transformation Assay Based on the Bhas 42 Cell Line was adopted in 2016 (OECD, 2016f).

The CTAs have been used to test NMs (and larger particles and fibres) (Ponti *et al.,* 2009; Ohmori *et al.*, 2013, 2022; Gabelova *et al.*, 2017). SHE and BALB/c 3T3 CTAs have the potential to detect non-genotoxic as well as genotoxic carcinogens in conventional forms. The most frequently used endpoint is morphological transformation. Morphologically transformed cells are characterised by the loss of density-dependent regulation of growth and the formation of colonies with crisscrossed cells or foci of piled-up cells that are not observed in untreated control cultures (Sasaki *et al.*, 2014; Gabelova *et al.*, 2017). CTAs are promising tests for predicting NM-induced cell transformation as one of the crucial carcinogenicity endpoints.

An international Working Group of experts convened by the International Agency for Research on Cancer (IARC) has identified 10 key characteristics (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision) of established human carcinogens that should be taken into account. Representative *in silico* and *in vitro* assays to measure the key characteristics of carcinogens are presented in Smith *et al.*, 2020. That publication, originally prepared to facilitate a systematic and uniform approach to organising the available mechanistic data relevant to carcinogens in a standard form, could also be applied for the assessment of many cases of NMs. High-throughput assay systems, such as the US Environmental Protection Agency's (EPA) Toxicity Forecaster (ToxCast) program (Chiu *et al.*, 2017), which can provide *in vitro* mechanistic data on many of the key characteristics, may be useful in the overall weight of evidence assessment. However, as the ToxCast database is on conventional chemicals, it may be of limited use for NMs.

Novel Adverse Outcome Pathway (AOP) frameworks show great promise for providing a basis for validation and uptake of alternative mechanism-based methods in risk assessment. Recently, a strategy for the generation of testable adverse outcome pathways for NMs (Murugados *et al.*, 2021) and an AOP for lung cancer induced by nanosized foreign matter, anchored to a selection of 18 standardised methods and NAMs for *in silico*- and *in vitro*-based integrated assessment of lung carcinogenicity, have been proposed (Nymark *et al.*, 2021).

Novel Adverse Outcome Pathway (AOP) frameworks show great promise for providing a basis for validation and uptake of alternative mechanism-focused methods in risk assessment. Recently, a strategy for the generation of testable adverse outcome pathways for NMs (Murugadoss *et al.*, 2021) and an AOP for lung carcinogenicity induced by nanosized foreign matter (Nymark *et al.*, 2021; Braakhuis *et al.*, 2021b) and for colon cancer (Braakhuis *et al.*, 2021b; Rolo *et al.*, 2022) have been proposed.

Identifying non-genotoxic carcinogens is a challenge in the absence of recourse to animal testing. Because non-genotoxic compounds can exert carcinogenicity through different mechanisms, it is advisable that a battery of tests (as exemplified above) should be used to exclude the non-genotoxic carcinogenicity potential of the NM.

Although a carcinogenicity study is only submitted when already available due to the EU ban on *in vivo* testing of cosmetic ingredients/products, a positive *in vitro* result in mutagenicity testing should be seen as indicative of the carcinogenic potential of the substance. *In vitro* approaches, such as cell transformation assays or toxicogenomic approaches, may also be useful for the identification of genotoxic as well as non-genotoxic carcinogen NMs.

5.3.9. Developmental and reproductive toxicity of nanomaterials

The database on developmental and reproductive toxicity of NMs following skin exposure or exposure by inhalation is very limited. Indeed, it is only recently that attention has been directed towards the potential reproductive toxicity of NMs (Iavocoli *et al.*, 2013; Hougaard *et al.*, 2015; Brohi *et al.*, 2017; Skovmand, 2018, 2019; Wang *et al.*, 2019). Some NMs have been shown to pass through the blood–testis barrier, placental barrier, and epithelial barrier, which protect reproductive tissues, and then accumulate in reproductive organs. Transplacental particle transport is affected by the particle size, particle material, dose, particle dissolution, and surface modification, as well as the NP administration route and gestational stage of the employed model (Bongaerts *et al.*, 2020). However, only in a few studies was an effect on foetuses noted after particle inhalation (Campagnolo *et al.*, 2017; Bernal-Meléndez *et al.*, 2019).

The literature also provides some limited evidence that some NMs (such as anatase TiO_2 particles) after oral exposure may affect foetal development of the male reproductive system. It has been shown that accumulation of NMs in reproductive organs (testis, epididymis) may cause damage to those organs by destroying Sertoli cells, Leydig cells, and germ cells, causing reproductive organ dysfunction that adversely affects sperm quality, quantity, morphology, and motility (Iavocoli *et al.*, 2013; Winkler *et al.*, 2018; Wang *et al.*, 2019). Some observations on the female reproductive system have also been made, such as changes in sex hormones, changes in regulation of certain proteins (*e.g.* vitellogenin), changes in vaginal opening, reduction in the number of mature oocytes and disruption of primary and secondary follicular development (overview in Iavocoli *et al.*, 2013). There is some evidence to suggest that different NMs can alter the expression of genes encoding proteins involved in steroidogenesis, including ovarian genes crucial to the synthesis of estrogen and/or progesterone (Larson *et al.*, 2014; Brohi *et al.*, 2017). However, reproductive function in female offsprings has hardly been studied and cannot be commented upon. In addition, NMs (such as anatase TiO_2) can disrupt the levels of secreted hormones, causing changes in sexual behaviour. Neurodevelopmental consequences of nano-TiO_2 exposure were suggested by a study in which pregnant Wistar rats were treated by oral gavage with anatase TiO_2 particles (primary size of 10 nm) at 100 mg/kg body weight (Mohammadipour *et al.*, 2014).

The molecular mechanisms involved in NM toxicity to the reproductive system are not clearly understood yet, but possible mechanisms include oxidative stress, apoptosis, inflammation, genotoxicity or endocrine activities. Previous studies have shown that NPs can increase inflammation, oxidative stress, and apoptosis and induce ROS, causing damage at the

molecular and genetic levels, which results in cytotoxicity. It is also plausible that NPs may translocate from the respiratory tract to the placenta and foetus. In addition to effects observed after placental translocation of NMs to the fetus, several mechanisms have been identified contributing to indirect (secondary) developmental effects, amongst them maternal and placental oxidative stress and inflammation, activation of placental toll-like receptors (TLRs), impairment of placental growth and secretion of placental hormones, and vascular factors (Hougaard *et al.*, 2015; Dugershaw *et al.*, 2020).

Effects of NPs used in cosmetic products should be considered for potential reproductive effects including mechanisms and ED mediated mode of action. Due to the animal testing ban, NAMs should be evaluated for potential reproductive toxicity using a weight of evidence approach. In an *in vitro* embryonic stem cell test for silica nanoparticles and coated silver nanoparticles toxic effects were reported affecting cell differentiation and cell cycle arrest (Park *et al.*, 2009; Rajanahalli *et al.*, 2015). Further research and development is required in this area, in particular with regard to the value of *in vitro* testing by the embryonic stem cell test, the micromass embryotoxicity assay, and the whole rat embryoculture.

The database on developmental and reproductive toxicity of NMs following skin exposure or exposure by inhalation is currently very limited. NMs used in cosmetic products should be considered for potential reproductive effects, including endocrine mediated mode of action. Due to the animal test ban, a weight of evidence should be derived from NAMs for potential reproductive toxicity of NMs.

5.3.10. Endocrine Disruption

The definition of Endocrine Disrupters (EDs) endorsed at the European level and as proposed by WHO/IPCS (WHO/IPCS, 2002) and is as follows: "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations".

There are indications that some NMs might act via an endocrine mediated mode of action. Apart from mechanisms mentioned in Section 5.3.8 that might have an effect on reproductive functions or on development (*e.g.* impact on sex hormones), further endocrine-related pathways might be influenced by NPs. So far, changes in thyroid hormones and histological or histomorphological changes in thyroid, ovaries and adrenals have been reported after short-term (5-day) oral exposure of rats to anatase TiO_2 nanoparticles (0, 1, 2 mg/kg body weight per day) (Tassinari *et al.*, 2014). An overview on possible actions of NMs on thyroid function, insulin action and metabolism, neuroendocrine function and other effects is given in Iavocoli *et al.*, 2013.

When assessing potential ED mediated mode of action of NMs, OECD Guidance Document 150 (OECD, 2018b) and the joint EFSA/ECHA/JRC guidance document (EFSA, ECHA and JRC, 2018) should be consulted.

There are indications that some NMs might act via an endocrine mediated mode of action. Therefore, investigating particle-related endocrine effects is also important to consider for

safety assessment of NMs. A variety of endocrine-related pathways (associated i.a. reproductive function, development, thyroid or adrenals) might be targeted by NPs.

5.4. Considerations for the replacement of *in vivo* testing by *in vitro* testing

Among the available alternatives, *in vitro* and *ex vivo* assays, and *in silico* modelling approaches take a prominent place. Generally, these methods aim to reduce, refine, or replace the use of experimental animals. However, there is no stand-alone *in vitro* or *ex vivo* test at present that can replace a standardised *in vivo* method for toxicological assessment of NMs (Shatkin and Ong, 2016; Burden *et al.*, 2017). A combination of assays based on an AOP might be used to identify certain hazards as demonstrated for sensitisation for which several AOP based assays are now available that, in combination, may accurately predict the sensitisation possibility of a substance (Casati *et al.*, 2018; OECD, 2021d, e; Gilmour *et al.*, 2022).

A tiered approach based on non-testing and *in vitro* methods has therefore been proposed for the prediction of realistic biological outcomes (Oberdörster *et al.*, 2005; SCENIHR, 2007; Stone *et al.*, 2009; Hirsch *et al.*, 2011; Dekkers *et al.,* 2016) when used in a weight of evidence (WoE) approach (SCHEER, 2018). The proposed approach involves thorough physicochemical characterisation of NMs, *in vitro* screening tests including '-omics', the use of non-testing approaches (*in silico* models, read across) and the use of OECD and EURL ECVAM validated/approved *in vitro* methods. A model for tiered nanotoxicity screening has been proposed for risk assessment of NMs (Oberdörster *et al.,* 2005; SCENIHR, 2007; Stone *et al.*, 2009; Hirsch *et al.,* 2011; Dekkers *et al.,* 2016; EFSA, 2021a). The latest summary on availability of alternative methods for toxicity testing has been published by EURL-ECVAM (JRC, 2023). A comprehensive review of useful New Approach Methodologies (NAMs) in nanomaterials risk assessment has been published by Nymark *et al.* (2020).

For cosmetic purposes, only data from **validated replacement** methods are accepted. However, in the absence of alternative methods that have been specifically validated for NMs, the SCCS also takes into consideration such methods that may have not yet undergone formal validation but can be demonstrated to be scientifically valid.

Validated replacement methods are methods that have passed the various steps of the modular validation process established at EURL-ECVAM and are considered by its Scientific Advisory Committee (ESAC) to comply with the process. Other organisations that evaluate the validation of alternative methods are the ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods, National Institute of Environmental Health Sciences, USA) and the JaCVAM (Japanese Center for the Validation of Alternative Methods, National Institute of Health Science, Japan). Equally so, methods considered by EURL-ECVAM of having the status equivalent to validation, or alternative methods accepted by OECD, are recognised as validated methods in the EU. At present, various *in vitro* guidelines are being adapted and validated to accommodate test protocols for NMs at OECD level, as well as in other initiatives such as the Malta or NanoHARMONY project (*e.g.* citations in Shatkin and Ong, 2016; https://nanoharmony.eu). In addition, the international organisation for standardisation (ISO TC 229; https://www.iso.org/fr/committee/381983.html) has been developing guidelines for NMs. As discussed before, the suitability of *in vitro* methods for NMs can be affected by specific

nano-related properties due to *e.g.* aggregation/agglomeration and subsequent sedimentation, floating and other changes. Furthermore, as already stated in Sections 5.3.2 and 5.3.6, it is well known that NMs might interfere with commonly used assays by influencing readout parameters such as absorbance or fluorescence (see overview in Guadagnini *et al.,* 2015 or ECHA, 2017b). As a consequence, the outcome of an *in vitro* assay for NMs is often difficult to interpret. Work is ongoing to develop suitable protocols for dispersion, analysis of cellular doses and quality criteria for NPs (Gottardo *et al.*, 2017; EFSA 2021a). Understanding the relation between external exposure concentration and the internal cellular dose is also critical for risk assessments of NMs, but presently effective methods/techniques to measure these different doses are not always available (OECD, 2022). An evaluation of various OECD Technical Guidelines for *in vitro* assays revealed many inconsistencies and omissions in the various nanomaterial dossiers evaluated (OECD, 2018a).

OECD (OECD, 2016c) published a state-of-the-art report on 'alternative testing strategies in risk assessment of manufactured NMs and concluded: while stand-alone alternative testing methods may contribute to basic mechanistic or toxicity knowledge, they will not be sufficient for use in quantitative risk assessment; rather, a battery of alternative testing methods will likely be used in a Weight-of-Evidence (WoE) approach (*e.g.*, Nel *et al.,* 2013). Strategically incorporating multiple alternative testing methods into alternative testing strategies will allow for an understanding of human and environmental behaviour and toxicity of NMs across endpoints, receptors and material groups, as reviewed by Drasler *et al.* (2017).

A number of issues need to be considered when applying *in vitro* alternative methods:

- Research must ensure that alternative tests are representative of *in vivo* eukaryotic conditions; for example, the OECD concluded that the commonly used Ames test, a bacterial mutagenicity assay, may not be suitable for detecting potential human genotoxicity induced by manufactured NMs because of the lack of particle uptake and limited NM diffusion across the bacterial cell wall (OECD, 2014b).

- *In vitro* models are becoming increasingly sophisticated and better at simulating human-relevant conditions (*e.g.* 3D cell co-cultures, spheroids, organoids, (micro)fluidic models, organ/tissue-on-chips) (Rothen-Rutishauser *et al.*, 2005; Kostadinova *et al.*, 2013; Astashkina and Grainger, 2014; Roth and Singer, 2014; Chortarea *et al.,* 2015; Horváth *et al.,* 2015, JRC 2021, 2022, 2023; Leung *et al.,* 2022; Baka *et al.*, 2023, Elje *et al.*, 2019, 2020, 2023; Camassa *et al.*, 2022).

- A lack of availability of quality data that can address the issues related to categorisation and grouping of NMs based on their physicochemical properties, mode of action or relevant exposure also hinders the development of *in silico* methods (Tantra *et al.,* 2015; ECHA, 2019a).

- Existing data can be harnessed to develop an adverse outcome pathway (AOP), which starts from a molecular initiating event (MIE), which links to key events (KEs) at different levels of biological organisation (*e.g.*, cellular or organ response), eventually leading to an adverse outcome at an organism or population level (Ankley *et al.,* 2010; OECD, 2013). It has become clear that direct correlations between the physicochemical properties of a single NM and *in vivo* outcomes are not possible; AOPs instead focus on groupings based on both the chemical activity and the consequent biological processes (OECD, 2013). The AOP concept has been applied to a number of human-relevant toxicological endpoints including skin

sensitisation (OECD 2021d; Gilmour *et al.*, 2022) and an AOP for lung carcinogenicity of TiO_2 NPs including KEs was proposed (Braakhuis *et al.,* 2021b).

More recently, as part of the OECD WPMN project on 'Advancing Adverse Outcome Pathway (AOP) Development for Nanomaterial Risk Assessment and Categorisation', a systematic process for searching and mining the toxicity literature was established to identify key events (KEs) and adverse outcomes of relevance to NMs. This project also established a database called NanoAOP to enable gathering of biological plausibility or weight of evidence specifically for assessing tissue inflammation and tissue injury KEs induced by NMs (Halappanavar *et al.*, 2021; Murugadoss *et al.*, 2021). These studies also demonstrated the challenges associated with using the existing toxicology data derived from *in vitro* and *in silico* methods as these methodologies are not formally validated for MNs.

In the absence of *in vivo* and other information on a cosmetic ingredient in nano-form, the following elements should be considered in safety evaluation: chemical structure, physicochemical properties, non-testing information (read across, *in silico* modelling, PBPK modelling) and information from *in vitro* and other alternative methods.

Currently, there is no stand-alone *in vitro* or *ex vivo* test to replace a standardised *in vivo* method for toxicological assessment of NMs. A tiered approach based on non-testing and *in vitro* methods has therefore been proposed that involves physicochemical characterisation, *in vitro* screening tests including '-omics', and the use of non-testing approaches (*in silico* models, read across). Progress has been made since the publication of 'alternative testing strategies in risk assessment of manufactured nanomaterials' by the OECD (OECD, 2016c). For cosmetic purposes, only data from validated replacement methods are accepted. However, in the absence of alternative methods that have been specifically validated for NMs, the SCCS also takes into consideration such methods that are not yet formally validated but can be demonstrated to be scientifically valid.

5.4.1 *In silico* modelling, grouping and read-across

Depending on the need for different end-uses, nano-forms of a cosmetic ingredient may be developed in many different particle sizes/shapes, crystalline forms, surface modifications/coatings, etc. Often adequate data on physicochemical and/or toxicological characterisation for each of the variants of a given nanomaterial are not available. This poses a major difficulty for safety assessment because it requires data and case-by-case assessment of each nanomaterial, as well as each individual variant of a nanomaterial. The need for robust and reliable *in silico* models, and data and tools for grouping and read-across between different NMs or their variants has been highlighted in a number of publications (*e.g.* National Research Council, 2012; Oksel *et al.*, 2015; Tantra *et al.*, 2015; Walser and Studer, 2015).

In this regard, it is notable that developments in the *in silico* modelling field have greatly advanced in the past decades to allow estimation of the toxicity of conventional chemical substances. This is because an enormous database is available for chemical substances, which has been accumulated over a century and provides a basis for deriving the rules and algorithms that define relationship(s) between a chemical structure and biological activity. For nanomaterials, such a database is currently inadequate and patchy, and this has limited the development of robust *in silico* models and tools that can reliably predict the toxicological

effects of nanomaterials. A handful of *in silico* QSAR models is currently available for nanomaterials (Toropov *et al.*, 2006, 2007a, 2007b, 2008; Sayes and Ivanov, 2010; Burello and Worth, 2011). However, as they are largely based on a few physicochemical parameters and limited toxicological datasets, and have not undergone rigorous testing/validation, their applicability to widely diverse nanomaterials has therefore not yet been established. Further developments in this field may lead to *in silico* models in the future as a means for deriving reliable toxicological estimates for safety assessment of nanomaterials.

In regard to grouping/read-across, a number of frameworks have been proposed for NMs (Arts *et al.*, 2014, 2015, 2016; Landsiedel 2014; ECHA/JRC/RIVM, 2016; OECD, 2016a, b; Oomen *et al.*, 2015; ECHA, 2017a; Stone *et al.*, 2020).

The DF4NanoGrouping project proposed a framework incorporating a tiered approach to group nanomaterials. Tier 1 includes collection of data on the intrinsic physicochemical properties and identification of soluble nanomaterials. Tier 2 is based on 3 grouping options (passive, biopersistent fibres and active materials) through a comparison of properties such as agglomeration, reactivity and dissolution. Tier 3 involves toxicological information from short term *in vivo* studies via inhalation exposure to assign the nanomaterial to a group (or a subgroup). The framework was assessed using 24 materials (carbon materials, metal oxides and sulphates, amorphous silica, organic pigments) and its suitability was assessed on the basis of no observed adverse effect concentrations (NOAECs) (Arts *et al.*, 2016).

A reference paper by ECHA, JRC and RIVM (2016), which has resulted in recommendations for nanomaterials applicable to the Guidance on QSARs and Grouping of Chemicals (ECHA, 2017a), proposed an outline for grouping and read-across of nanomaterials on the basis of physicochemical properties, toxicokinetic considerations, and hazard considerations. The use of such methods would need to be scientifically justified, and on a case-by-case basis (ECHA, 2017a; Gottardo *et al.*, 2017). In consideration of the current major data gaps, it is likely that experimental data would be needed in most cases to substantiate and justify the use of a grouping/read-across approach for nanomaterials.

A framework discussed in a report from the OECD expert meeting (OECD, 2016b) is based on intrinsic physicochemical properties of the materials as a starting point, to which other extrinsic aspects (mainly toxicokinetic aspects) may be added.

The nanoGRAVUR framework (Wohlleben *et al.*, 2019) has proposed further data requirements for grouping of nanomaterial-containing products for ecological and consumer risk in a lifecycle perspective.

Stone *et al.* (2020) have recently reviewed the various frameworks for grouping and read-across of nanomaterials and proposed the GRACIOUS framework that aims to facilitate the application of grouping of nanomaterials or nanoforms, in a regulatory context and to support innovation. The framework sets out to initially collect basic information to select an appropriate pre-defined grouping hypothesis and a tailored Integrated Approach to IATA. The GRACIOUS framework identified more than 35 pre-defined grouping hypotheses. The thresholds assign nano-forms to predefined groups based on considerations of "what they are", "where they go", and "what they do". For example, if the aspect ratio, inflammation potential and dissolution rate in specific media are below predefined thresholds for all nano-forms of the same substance, they can be grouped regarding their low acute inhalation hazard, but potential bioaccumulation can be selected. If the predicted or measured information via the IATA suggests that a pre-defined hypothesis is not applicable, then a new

user-defined hypothesis can be generated via use of the hypothesis template. For grouping, the GRACIOUS hypothesis relates to whether nano-forms (and non-nano forms) are sufficiently similar with respect to a specific endpoint (*e.g.* dissolution) to be considered as members of a group. A read-across can be performed, *e.g.* when a very similar or smaller amount of the target material is observed to reach the target site, in combination with a similar or lower hazard potential than that of the source material(s).

El Yamani *et al.* (2022b) identified several descriptors for prediction of 17 NMs' toxicity by applying QSAR analysis. Cheminformatics modeling identified electron properties and overall chemical reactivity as important descriptors for cytotoxic potential. Energy parameters, ionisation potential, pristine size for the NMs and presence of surface coating were found important descriptor for induction of DNA oxidized base lesions (El Yamani *et al.* 2022b).

The SCCS considers that the different proposed frameworks essentially derive from each other and revolve around more or less the same conceptual considerations. For example, structural similarity for particulate materials has been suggested to cover intrinsic physicochemical identity (termed as "what they are"), extrinsic physicochemical properties ("where they go") and reactivity ("what they do"). As such, the SCCS considers that these frameworks may not be directly useful for safety assessment of cosmetic ingredients because:

- They are mainly focused on occupational settings and exposure via the inhalation route, or through the environment, which may make them useful for certain regulatory frameworks (*e.g.* REACH), but not for cosmetic ingredients, where dermal exposure is the main element for risk assessment, and any exposure via oral and inhalation routes is only incidental depending on the type and use of the final product. For example, the proposed framework by Arts *et al.* (2015) has largely discounted the exposure to nanomaterials via the oral route, whereas their use in certain types of cosmetic products (*e.g.* toothpaste, mouthwash, lipstick) would give, albeit unintended, oral exposure to the consumer.
- Toxicological investigations proposed in the higher tiers involve short-term *in vivo* studies. This is out of the scope for nanomaterials used exclusively or primarily in cosmetic products due to the EU ban on animal testing under the Cosmetics Regulation (1223/2009).
- Due importance has not been placed on certain key parameters that can make the nanomaterials 'different' from conventional equivalents. For example, surface characteristics/coatings on nanoparticles have been considered not important in the proposed framework. In this regard, the frameworks regard a nanomaterial 'active' or 'passive' on the basis of intrinsic material properties alone (presumably of the conventional chemical form), and do not take into account any new/additional activity that may be generated at the nano-scale. A typical example is that of certain metals (*e.g.* gold, titanium dioxide), the conventional forms of which are inert but the nano-forms can be reactive, catalytic/photocatalytic.
- It is more likely that a combination of intrinsic properties and surface characteristics leads to deviations in the extrinsic properties (reactivity, biokinetics and toxic effects) of nanoparticles, and the SCCS is of the view that, in addition to intrinsic properties of the materials, other important aspects also need to be considered. For example, hydrophobicity, and surface characteristics including coatings that may alter toxicokinetic behaviour of a nanomaterial. This is in line with the amended REACH

Annexes (Commission Regulation (EU) 2018/1881 of 3 December 2018), which specify that nano-forms should in principle be addressed separately, and that molecular structural similarities alone cannot serve as a justification for grouping. ECHA nano-specific guidance for grouping (ECHA, 2019b), also considers inclusion of other parameters (*e.g.* aspect ratio, particle size, shape, or solubility), in addition to chemical composition, to support the grouping, and highlights the importance of toxicokinetic studies for grouping, read-across, and *in vitro* to *in vivo* extrapolations.

- Certain assumptions in the proposed frameworks are, however, not supported by sufficient scientific data. For example, Arts *et al.* (2015) concluded that 'most nanomaterials do not penetrate the *stratum corneum* of the skin and only minimal amounts enter the systemic circulation from the lung and gastrointestinal tract'. A similar assumption is made in the pre-defined hypotheses proposed in the GRACIOUS framework for non-flexible nano forms >5 nm in size as 'Following dermal application will not penetrate (in their particle form) to viable layers of the skin above 1% of the applied dose'. Whilst this may be true for some nanomaterials, especially when in agglomerated/aggregated forms, more dermal absorption data are needed to see whether this holds true for all nanomaterials in general, including those that have dispersible/dispersed nanoparticles, and/or differ in terms of surface chemistry/coating (Filon *et al.*, 2015).
- It is also not clear what minimum supporting evidence would be required to demonstrate a 'similarity' between different particle properties/behaviour to justify a read-across. Further case studies are therefore needed to demonstrate that the framework works for diverse types of nanomaterials, and whether there are boundaries/limitation of the types of nanomaterials covered by the framework, and also where the use of a case-by case approach instead would be more relevant.

The *in silico* modelling tools and read-across approaches are still at an elementary stage for nanomaterials. A number of frameworks have been proposed for grouping and read-across of nanomaterials based on physicochemical properties, toxicokinetic considerations, and hazard considerations. The use of such methods would need to be justified on strong scientific grounds on a case-by-case basis.

5.4.2 *In vitro* and other non-animal methods

Assessment of overt toxicity and local effects on the port of entry including genotoxicity

The test design needs to be oriented on the relevant exposure scenario (oral, dermal, inhalation) using adequate (context-specific) doses. In the first instance, *in vitro* testing can be targeted to assess overt toxicity that might be exerted even at the port of entry (*e.g.* cytotoxicity, production of ROS, inflammation, cytokine induction, genotoxicity). Such tests might also be able to give an insight to possible mechanisms of toxicity. Assays determining cytotoxicity might reveal damage of the plasma membrane, mitochondria or lysosomes. As NMs have been shown to interfere with certain *in vitro* assays or read-out systems, it has been recommended to use more than one assay for one specific endpoint/parameter to circumvent any limitations of the individual assay (Shatkin and Ong, 2016; OECD, 2017a). In

addition, each assay should include appropriate controls to identify (background) interference of the NMs within the assay. An overview on possible assays for determination of basic cytotoxicity *in vitro* (*i.e.* on cell viability, production of reactive oxygen and nitrogen species, inflammatory response and cytokine induction) is given in Annex 1.

For the assessment of local damage to the skin (skin corrosivity and skin irritation) and the eyes (serious eye damage and eye irritation), a variety of non-animal methods is available that might be used for NMs if nano-specific aspects are taken into consideration (see Annex 1).

Information on *in vitro* assessment of genotoxicity and mutagenicity is given in Section 5.3.6 and also in Annex 1.

The mechanisms involved in skin sensitisation have been described by the OECD in the AOP Covalent Protein binding leading to Skin Sensitisation (OECD, 2012b; https://aopwiki.org/wiki/index.php/Aop:40). The molecular initiating event (MIE) of this AOP is covalent binding of the chemical to skin proteins, leading to an immunogenic hapten-carrier complex. The MIE triggers KE2, keratinocyte activation, and KE3, dendritic cell activation. Subsequently, the activated and differentiated dendritic cells migrate to the draining lymph nodes and present their small peptides of the hapten-carrier complex to the T cells. This leads to KE4: T cell activation and proliferation creating a pool of memory T cells, ultimately leading to skin sensitisation (adverse outcome). For these key events, *in vitro* assays have been validated for conventional chemicals (see Annex 1). Recent work aimed at identification of potential molecular initiation events and/or key events relevant for pathologies induced by ENMs with the decision which of *in vitro* assays can be assigned to test those events (Murugadoss *et al.*, 2021).

In addition, further information on databases and SOPs regarding physicochemical characterisation and *in vitro* testing of NMs is given in Annex I. However, although the SOPs are scientifically valid, they have not gone through formal validation processes yet.

Potential for systemic uptake via the relevant uptake route(s)

Next step to determining local toxicity should be to assess whether an NM is taken up systemically via the exposure route of interest. Investigation of the solubility behaviour in adequate biofluids might give information whether and to what extent an NM remains intact in a particle form, for example after oral and/or systemic uptake (see also Sections 3.1. and 5.3.1. for dispersion). The assessment of potential systemic uptake should also consider any changes in the physicochemical properties of the NM.

In vitro models that simulate different biological barriers have also been developed to determine absorption via different uptake routes. These include *in vitro* models simulating the gastrointestinal, pulmonary or oral mucosal barrier (overviews in Dekkers *et al.*, 2016 and Gottardo *et al.,* 2017). A validated OECD test guideline exists for determining dermal uptake (OECD, 2004a). However, such *in vitro* models have not yet been validated for NMs. As mentioned before, unlike the diffusion gradient driven absorption of conventional chemicals, the translocation of NPs across biological membranes involves endocytosis and/or active transcellular transport mechanisms. In addition to *in vitro* methods, *ex-vivo* methods might also provide some insight to the uptake of NMs.

Local Effects

Studies showed that several NPs (*e.g.* ZnO, Ag, TiO_2, and CeO_2 NPs) do not lead to local irritation after evaluation in a reconstructed human epidermis (RhE) model (Kim *et al.*, 2016; Vinardell and Mitjans, 2017; Miyani and Hughes, 2017). In this model the NMs can be applied in both a watery and lipid solution on top of the epidermal construct that has similar tissue layers as normal human skin. As of June 2021, seven RhE models were validated and accepted for determination of *in vitro* skin irritation of chemicals in OECD TG 439 (OECD, 2021c). For conventional chemicals, recently a Defined Approach (DA) was adopted at the OECD level (OECD TG 467, OECD 2022h) to provide information on potential eye hazard effects on the whole range of classifications required by the UN GHS i.e., Cat. 1, Cat. 2 and No Cat, thus a stand-alone test.

Systemic effects

If there is potential for systemic uptake of the NM, systemic toxicity has to be investigated. In the absence of a recourse to *in vivo* testing, it is very difficult to predict the distribution of NMs in the human body. However, based on past experience with *in vivo* models, it can be assumed that poorly-soluble systemically available NMs are mainly distributed to tissues that are rich in phagocytic cells belonging to the mononuclear phagocytic system (MPS), *e.g.* liver and spleen (Dekkers *et al.*, 2016; OECD, 2016d; ISO, 2019 - ISO/TR 22019). In addition, *in vitro* barrier models, *e.g.* on blood-brain or placental barrier, might give further insight to the distribution of systemically available NMs.

For *in vitro* tests addressing systemic effects, kinetic aspects (*e.g.* absorption via the relevant uptake route, dissolution rate in relevant body fluids, protein binding and protein corona formation, distribution) should be taken into consideration to enable *in vitro* to *in vivo* extrapolation (IVIVE) (Jagiello and Ciura, 2022).

For the investigation of systemic effects in tissues, 3D cell co-culture models and microfluidic models (organ-on-a-chip technology) have been described (see Dekkers *et al.*, 2016; Fitzpatrick and Sprando, 2019; Kang *et al.*, 2021). In addition, *ex-vivo* models and methods, such as precision-cut lung slices, might enable further understanding of the systemic toxicity of NMs. However, the latter are still in early phases of development.

Two Guidance documents (OECD Guidance Documents No. 214 and 231) have been adopted on the CTA that provide partial information on the multi-step processes that lead to cancer (OECD 2015a, 2016f). The assay has already been applied to a variety of NMs (Gabelova *et al.*, 2017; see also Section 5.3.6).

In summary, a number of standalone alternative testing methods may contribute to basic mechanistic or toxicity knowledge, but they will not be sufficient for use in quantitative risk assessment. Instead, the use of a battery of alternative testing methods will be more useful in a WoE approach (Nel *et al.*, 2013; OECD, 2016c; EFSA, 2017; SCHEER, 2018). Strategically incorporating multiple alternative testing methods into an alternative testing scheme will allow for an understanding of the behaviour and toxicity of NMs across human and environmental endpoints, receptors and material groups.

This Guidance provides a list of non-animal methods that could be used for NMs while taking nano-specific aspects into consideration (Table in the Annex 1). The test design needs to be

oriented on the relevant exposure scenario (oral, dermal, inhalation) using adequate context-specific doses. In the first instance, *in vitro* testing can be targeted to assess overt toxicity that might be exerted even at the port of entry (*e.g.* cytotoxicity, production of ROS, inflammation, cytokine induction, local genotoxicity). It is recommended to use more than one assay for one specific endpoint/parameter to circumvent any limitations of the individual assay, with appropriate controls to identify (background) interference of the NMs in the assay. The assessment of potential systemic uptake should also consider any changes in the physicochemical properties of the NM. Investigation of the solubility behaviour in relevant biofluids might give information whether and to what extent an NM may remain intact in particle form for example after oral and/or systemic uptake.
If there is a potential for systemic uptake of the NM, systemic toxicity will need to be investigated. For *in vitro* tests addressing systemic effects, kinetic aspects (*e.g.* absorption via the relevant uptake route, dissolution rate in relevant body fluids, protein binding and protein corona formation, distribution) should be taken into consideration to enable *in vitro* to *in vivo* extrapolation (IVIVE). For the investigation of systemic effects in tissues, 3D cell co-culture models and microfluidic models have been described, and the use of *ex-vivo* models may provide further understanding of the systemic toxicity of NMs. In this regard, the use of a battery of alternative testing methods will be more useful when results are used together in a WoE approach.

6. RISK ASSESSMENT

Risk assessment of NMs follows a similar procedure to that for conventional chemical ingredients. The safety of an NM in a cosmetic application is assessed by considering exposure and toxicological effects. These include local effects as well as systemic effects where there is systemic uptake via the relevant exposure route.

Historically, safety assessment of a cosmetic ingredient has been based on a measured toxicological point of departure (POD) in terms of BMDL or NOAEL from *in vivo* animal studies, along with an estimate of the internal exposure in terms of systemic exposure dose (SED). The latter is usually derived from the dermal route (*e.g.* from the intended daily application of a cosmetic ingredient on the skin). The calculation of the SED is described in Section 3-3.5.4 of the SCCS/1647/22 (12th revision of the SCCS Notes of Guidance or any future revision).

For systemic, threshold effects, the Margin of Safety (MoS) of ingredients in a finished cosmetic product is calculated, which is the ratio between a systemic POD (POD_{sys}) and an estimate of the exposure.

MoS = POD_{sys} / SED (systemic exposure dose)

Where POD_{sys} is a Benchmark Dose Lower Limit (BMDL) or, alternatively, a NOAEL or a LOAEL, if BMDL cannot be calculated. The POD_{sys} is calculated from the external POD by use of the proportion of the substance systemically absorbed (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision).

In the past, a systemic toxicological point of departure (POD_{sys}) for use in safety assessment was derived from animal studies. After the ban on animal testing under the Cosmetic Regulation, this is no longer possible for a new cosmetic ingredient, and such data can only

be accepted if studies had been carried out prior to the animal testing bans (*i.e.* before March 2009 or March 2013 depending on the toxicological endpoint), or if the data were generated to meet a different regulatory requirement (*i.e.* for a non-cosmetic use). This means that, whilst it may be possible to calculate an acceptable risk in relation to local effects, this may not be possible for systemic effects due to the absence of data to derive POD_{sys} for a new cosmetic ingredient. For such cases, the Applicant will need to assemble the relevant information/data from different NAMs and integrate the data to build an overall WoE to support demonstration of the safety of the cosmetic ingredient. Because of the current lack of standardised frameworks for a generalised approach for safety assessment to be based entirely on data from alternative methods, this will need to be carried out on a case-by-case basis. Frameworks for assembling the WoE for scientific assessments have been published by the European Food Safety Authority (EFSA 2017, 2021a) and SCHEER (2018) that can provide guidance in this regard.

In general, a substance for which MoS is ≥ 100 is considered to pose a negligible risk to human health. Depending on the quality and relevance of the available datasets, additional safety factors may, however, be used (*e.g.* when using LO(A)EL instead of NO(A)EL, or when specific toxicological information, *e.g.* on certain endpoints, is missing). It needs to be noted that the assessment factor of 100 (plus any additional uncertainty factor if appropriate) has been developed for conventional ingredients and not specifically for NMs (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). However, this assessment factor has been considered adequate to address aspects of extrapolation and uncertainty and therefore is at present considered to be also applicable and appropriate for NMs (REACH RIP-oN 3, ECHA, 2011).

As stated in the SCCS Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation (SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision), the systemic availability of a cosmetic ingredient is estimated by taking into account the daily amount of a finished cosmetic product applied, the frequency of application, the concentration and systemic absorption of the ingredient, and a mean value for human body weight. As such, the amount of ingredient per kg body weight that would become available daily in the human circulatory system is calculated.

For conventional cosmetic ingredients, in the majority of MoS calculations, the dermal exposure is compared to an oral POD (route to route extrapolation). The oral POD usually corresponds to an amount that has been administered orally, though this may not necessarily be the actual systemically available amount. In many calculations of the MoS for conventional substances, where oral absorption data were not available, the oral bioavailability of a substance had been assumed to be 100%. However, in view of the generally low oral absorption of substances evaluated so far, the SCCS has considered it more appropriate to assume that not more than 50% of an orally administered dose becomes systemically available (see also Section 4.4.2.3 and SCCS/1647/22). Although this value of 50% is an arbitrary choice, it recognises that the GI tract is designed to favour the absorption of ingested substances into the body but that, in most cases, not all of the ingested material will be bioavailable. Thus, in the absence of measured data, the assumption can be made that the effects seen following oral administration have been caused by a fraction of the administered dose, and not the entire dose. Furthermore, if there is evidence to suggest poor oral bioavailability, for example, of a poorly soluble particulate substance, it may be more appropriate to assume that only 10% of the administered dose is systemically available

(IGHRC, 2006; SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). Therefore, any available oral absorption data should be included in the calculations (*e.g.* SCCP/0851/05). In the case of oral-to-inhalation extrapolation, it was proposed that, in the absence of route-specific bioavailability information, a default factor of 2 (*i.e.* the absorption percentage for the oral route is half that of the inhalation route) might be appropriate. The inclusion of this factor 2 means, for example, that 50% (instead of 100%) absorption is assumed for oral absorption, and 100% for inhalation.

Route-to-route extrapolation, however, requires experimental data on absorption for both dermal and oral exposures. Any route-to-route extrapolation also needs to be performed case-by-case, and based on expert judgment of the scientific information, including the available toxicokinetic information. It can only be performed if data are available on systemic toxicity, the degree of absorption and possible metabolic transformation.

If safety assessment is to be based mostly (or entirely) on *in vitro* test results, the *in vitro* concentrations have to be related to external *in vivo* doses (*in vitro- in vivo* extrapolation (IVIVE) as the *in vitro* assays do not take into account the kinetics inside the body. Thus, *in vitro* test results must be complemented with kinetic data.

Extrapolation of *in vitro* to *in vivo* (IVIVE) for toxicokinetic assessment is still under development; even if some methods and guidance exist (*e.g.* orally bioavailable fraction of the dose can be predicted by informatics tool, dermal absorption can be predicted by *in vitro* studies), it should be noted that cellular studies alone cannot mimic the entire organism. For NMs used as cosmetic ingredients, IVIVE is a challenge because; 1) animal *in vivo* data cannot be used to establish and validate toxicokinetic models, and 2) in addition to conventional chemicals, further aspects as stated elsewhere in this document have to be considered for NMs (*e.g.* aggregation/agglomeration, surface interaction, altered kinetics).

Sparse but relevant nano-specific kinetic data may already be available in various databases from the JRC, US EPA, pharmaceutical industry, but most of these are from pilot projects. ISO/TR 22019:2019 provides an overview of the current knowledge on (toxico)kinetics of NMs indicating that most systemically available NMs end up in organs of the MPS (mononuclear phagocytic system), notably the liver and spleen. However, more studies and *in silico* modelling are needed for a realistic estimation of the biokinetics of an NM.

Safety assessment of NMs is carried out in the same way as for conventional chemical ingredients in terms of consideration of the exposure and toxicological effects. For systemic effects, the Margin of Safety (MoS) of ingredients in a finished cosmetic product is calculated, which is the ratio between a systemic point of departure (POD_{sys}) and an estimate of the exposure.

MoS = POD_{sys} / SED (systemic exposure dose)

Historically the toxicological point of departure (POD) has been measured in terms of NOAEL, along with an estimate of the internal exposure in terms of systemic exposure dose (SED). In cases where *in vivo* data, compliant with the provisions of Cosmetic Regulation, are available on repeated dose toxicity, the margin of safety (MoS) can be calculated as a ratio of a POD_{sys} and SED. POD_{sys} is BMDL or, alternatively, NOAEL or LOAEL, where BMDL cannot be calculated. For such cases, a substance for which MoS is ≥ 100 is considered to pose a negligible risk to human health. Depending on the quality and relevance of the available datasets, additional safety factors may also be used (e.g when using LO(A)EL instead of

NO(A)EL, or when specific toxicological information is missing). Although the assessment factors have been developed for conventional ingredients, they have been considered adequate to address aspects of extrapolation and uncertainty, and therefore also applicable to nanomaterials.

With the EU ban on animal testing of cosmetic ingredients/products, derivation of POD_{sys} for systemic adverse effects of a new cosmetic ingredient may not be possible. For such cases, the Applicant will need to assemble the relevant information/data from alternative (non-animal) methods and integrate the data to build an overall weight of evidence (WoE) to support safety of the cosmetic ingredient. Because of the current lack of standardised frameworks for a generalised approach for safety assessment to be based entirely on data from alternative methods, this will need to be carried out on a case-by-case basis. A framework for assembling WoE for scientific assessments published by the European Food Safety Authority (EFSA, 2017, 2021a) and SCHEER (SCHEER, 2018) may provide guidance in this regard.

If risk assessment is to be based mostly (or entirely) on *in vitro* test results, extrapolation of *in vitro* to *in vivo* (IVIVE) data will be required. The *in vitro* test results must be complemented with kinetic data that can be derived from nano-specific kinetic models to enable IVIVE. This approach is valid for non-nano (chemical) substances and should also be valid for nanomaterials.

7. SUMMARY AND CONCLUSIONS

The use of NMs as cosmetic ingredients requires thorough safety evaluation because of the potential for size-related changes in physicochemical properties, biokinetic behaviour, and/or toxicological effects of materials at the nano-scale. Exposure to NMs through the use of cosmetic products may pose a risk of harmful effects from insoluble and persistent nanoparticles that may reach unintended sites in the body and interact with biological entities close to the molecular level.

This Guidance is an up-to-date revision of the existing SCCS Guidances on the Safety Assessment of NMs in Cosmetics (SCCS/1484/12; SCCS/1611/19) and on the safety assessment of NMs in cosmetic products. It covers the main elements of safety assessment, *i.e.* general considerations (Section 2), material characterisation (Section 3), exposure assessment (Section 4), hazard identification and dose-response characterisation (section 5), and risk assessment (Section 6). Due to the evolving nature of NM safety research, the guidance may be revised in the future to take account of any new scientific knowledge. The key recommendations for safety assessment of NMs intended for use in cosmetics are summarised below:

Definition: The regulatory definition of NM is provided in the Cosmetic Regulation (EC) No 1223/2009, under Article 2 (1) (k). It is further advisable that, when assessing the safety of a material consisting of small particles, Applicants should also take into account the Commission Recommendation (2022/C 229/01) (see Section 2.1). In view of the EU Chemicals Strategy for Sustainability (Ref. Ares(2021)6011962 - 04/10/2021) it is likely that the definition for a nanomaterial in the Cosmetic Regulation will be aligned with this recommendation. Material specifications such as particle size distribution, solubility, and

persistence should provide a basis for deciding whether or not a cosmetic ingredient has to be considered an NM. In situations where a particulate material has internal nano-structures, or exists as larger agglomerates or aggregates, the use of volume specific surface area (VSSA) for powders, (quantitative) imaging by EM and/or other parameters may provide further clarity. Where a new or an already-approved cosmetic ingredient fulfils the criteria for defining it as NM, it will be subject to safety assessment based on the data relevant to nano-scale properties.

Material characterisation: In view of the potential changes in properties, behaviour, and effects of NMs, unambiguous identification and detailed characterisation of NMs is an essential requirement for safety assessment. The characterisation data must provide information on the identity of the material(s) in accordance with Cosmetics Regulation (EC) No 1223/2009, Article 16 a) 'identification of the NM…'. As a minimum, characterisation data must be provided on all the parameters listed in Table 1 that are relevant to a given NM. The information should correspond to Cosmetics Regulation (EC) No 1223/2009, Article 16 b) 'specification of the NM…'. It is important that the measurements are carried out using generally accepted techniques in consideration of nano-aspects, and detailed documentation is provided. Primary particle size, being the common denominator for all NMs, must be measured by more than one method - one of which must be high-resolution EM (SEM or TEM). The NM characterisation needs to be carried out at the raw material stage, in the cosmetic formulation, and during exposure for toxicological evaluations. A detailed description of the production processes, any surface modifications, and the preparatory steps carried out for integrating the NMs in the final cosmetic products may be asked for by the SCCS as input into the safety assessment process.

Exposure Assessment: Safety assessment of NMs follows the same procedure as for non-nano ingredients, but with special considerations of the nano-aspects. Safety assessment of NMs may, in the first instance, be driven by considerations of exposure (Figure 1). For this, the likelihood and extent of local and systemic exposure will need to be estimated or determined in relation to dermal, oral and inhalation exposure routes. The focus should be on determining the potential translocation of NPs across skin, lung, or gastrointestinal barriers (as appropriate) whilst mimicking the actual use scenarios. The SCCS is of the view that the method for calculating dermal and oral exposure to NMs (detailed in SCCS/1647/22 and Section 5) will not be substantially different from the calculation of exposure to conventional cosmetic ingredients. Calculation of exposure to aerosols containing NM may however be more challenging.

Potential systemic exposure can be estimated for the dermal route through analysis of the receptor fluid for NPs in *in vitro* dermal absorption studies and, for all possible uptake routes and where available, through analysis of the data on occurrence in organs and/or blood from toxicokinetic or toxicological investigations. The methods used for this purpose, however, need to be mainstream, state of the art, and the limit of detection low enough to demonstrate the lack of systemic exposure.

ADME parameters should be investigated to determine the extent of systemic exposure via the relevant uptake route, to determine the fate and behaviour of the NM (*in vitro*, *ex vivo,* or IVIVE) and to identify the likely target organs.

Irrespective of whether or not systemic exposure is possible, local exposure and local effects along with skin sensitisation and genotoxicity need to be addressed. Where systemic exposure

is indicated by chemical analysis, further investigations (and confirmation by EM) should be carried out to confirm whether the absorbed material was in particle form or in a solubilised/metabolised form. The method for calculating dermal and oral exposure to cosmetic ingredients are provided in the SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision) and are specified for NMs in Section 4 of this Guidance. It is very important to characterise NMs under exposure conditions to ascertain that characteristics have not changed when used in the finished cosmetic product.

For those conventional cosmetic ingredients for which no (adequate) information is available on dermal absorption, the SCCS assumes 50% absorption based on literature analysis for conventional substances. It is acknowledged that this value has not been derived for NPs and that very limited or no dermal absorption has so far been demonstrated for NMs. However, the SCCS is aware of specific modifications of NMs to specifically design them for improved dermal penetration. In view of this, dermal absorption of NMs will need to be determined experimentally (see Annex 2). Where no experimental data are provided, the SCCS will apply the default value of 50% of the administered dose for dermal absorption as determined for conventional substances, or higher if warranted by the composition of a specific NM.

Calculation of inhalation exposure to NM containing aerosols is more challenging and will need determining the generated droplet size distribution as well as size distribution of the dried residual aerosol particles. For the lung the SCCS considers 100% of the lung deposited dose as the default absorption amount. For oral exposure, the SCCS assumes 50% of the administered dose for NP absorption, similar to conventional cosmetic ingredients, and 10% if poor oral bioavailability can be demonstrated.

Concerning the amount of absorbed particles when there is no data on the particle nature of the absorbed NM (*e.g.* by solubility/degradation data of the NM), the SCCS will apply a default assumption that **100%** of the absorbed material is in particle form.

Hazard identification/dose response characterisation: Data from toxicological studies for local toxicity, skin sensitisation and genotoxicity, and - in case of systemic absorption - systemic effects will be required (as per SCCS Notes of Guidance (see SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision and Annex 2). Testing of NMs for hazard identification/dose response characterisation must be carried out in consideration of the nano-related aspects. These include consideration of insoluble or partially-soluble particulate forms, aggregation and agglomeration behaviour of the particles, potential penetration of NPs through biological membranes, possible interaction with biological entities at local and systemic levels, surface adsorption/binding of other substances, surface catalysed reactions, persistence, etc. Testing conditions used should also be documented in the dossier.

The prohibition on animal testing and marketing of animal-tested cosmetic ingredients/products under Cosmetics Regulation (EC) No 1223/2009 must be followed in any toxicological testing. In this regard, the SCCS takes into account any toxicological data derived from alternative means, such as *in vitro* and *ex vivo* methods, *in silico* models, grouping and read-across, physiologically-based pharmacokinetics (PBPK) or toxicokinetics (PBTK) modelling (SCCS/1647/22 - SCCS Notes of Guidance 12th revision or any future revision). Since validated alternative methods that can be used in place of animal tests are not yet available for NMs, the SCCS can accept results from the methods that may not have been formally validated for NMs, but can be demonstrated to be scientifically valid for hazard identification of NMs, provided that they are carried out with due consideration of the nano-

related aspects and appropriate controls In such cases, characterisation of NMs during the tests will be needed as an essential part of the evidence to ensure validity of the results. The *in silico* modelling tools and read-across approaches are currently at an elementary stage for NMs and the use of such methods would need justifying on strong scientific grounds on a case-by-case basis.

For *in vitro* genotoxicity assessment, both chromosomal damage (clastogenicity and aneugenicity) and gene mutations should be evaluated. The widely used bacterial reverse mutation (Ames) test is not considered appropriate for NM mutagenicity assessment and an *in vitro* mammalian cell gene mutation test should instead be carried out. Other indicator tests should also be considered, such as the Comet assay modified with repair enzymes, and the cell transformation assay (CTA). It is imperative that assessment of cellular and, if possible, nuclear uptake is also carried out to demonstrate target exposure during the *in vitro* genotoxicity studies.

Safety Assessment: Historically, calculation of margin of safety (MoS) of a cosmetic ingredient has been based on a measured toxicological point of departure (POD), along with an estimate of internal exposure in terms of systemic exposure dose (SED). With the EU ban on animal testing of cosmetic ingredients/products, derivation of POD_{sys} for systemic adverse effects of a new cosmetic ingredient may not be possible, or only possible in exceptional cases. However, data obtained to comply with other non-cosmetic regulations should be used and submitted when available. For other cases, the Applicant will need to assemble relevant information/data from different alternative (non-animal) methods and integrate the data to build an overall **weight of evidence** to support safety of the cosmetic ingredient. Because of the current lack of standardised frameworks for a generalised approach for safety assessment to be based entirely on data from alternative methods, this will need to be carried out on a case-by-case basis.

Where safety assessment is to be based mostly or entirely on *in vitro* test results, extrapolation of *in vitro* to *in vivo* (IVIVE) data will be required. The *in vitro* test results must be complemented with kinetic data that may be derived from nano-specific kinetic models to enable IVIVE.

Where data have been derived from validated tests, or from relevant and justified tests, and uncertainties are not high, there are no scientific reasons for applying additional margins of safety to an NM than a conventional material. However, where this is not the case, and data provided are either insufficient or from inadequate tests, the risk assessor may consider applying additional uncertainty factors for safety assessment.

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ANNEXES

ANNEX 1: Available replacement methods for the toxicological evaluation of nanomaterials intended for use in cosmetics

The hazard endpoints listed below should be considered for nano-ingredients used in cosmetics. These endpoints are similar to those generally required for non-nano cosmetic ingredients. As the validated NAMs available for non-nano cosmetic ingredients are described in the 12th revision of the SCCS Notes of Guidance (SCCS/1647/22), the different NAMs are only summed up here. For more details, refer to SCCS/1647/22. It should be noted that none of the NAMs have been validated for nanomaterials. For *in vitro* tests, the OECD Guidance Document 286 on Good *In Vitro* Method Practices (GIVIMP) (OECD, 2018c) should be considered.

Endpoint	Nano-related considerations
Cytotoxicity Cytotoxicity testing is concerned with cell death evaluation as well as physiological and biochemical changes leading to cell mortality or to cell cycle arrest. An experimental approach can include basic cellular morphology visualisation or more elaborated assessments (metabolic activity, ATP content, membrane integrity/ permeability). The cell cultures used can be sophisticated and consist of multiple cell types. General classification of basic cytotoxicity assays based upon: **(i) cell viability:** 1) structural cell damage leading to membrane damage/leakage or cell death 2) cell growth (*e.g.* Colony Forming Efficacy, CFE) 3) cellular metabolism	None of these tests have been validated specifically for NMs, but may still be valuable for hazard identification if further (nano-related) aspects are taken into consideration, *e.g.*: - Solubility/dispersion - Adsorption of substances - Cell internalisation - Nanoparticle size (Kad *et al.*, 2022) - Concentration (Kad *et al.*, 2022) - Exposure time (Kad *et al.*, 2022) - Viability/vitality state of the cell lines used (Kad *et al.*, 2022) - Biological target (Kus-Liskiewicz *et al.*, 2021) - Role of oxidative stress (Min *et al.*, 2023) When a dispersant is used to disperse an NM in a toxicological test medium, it should be ascertained that it does not modify the physicochemical properties of the NM (including agglomeration or aggregation state and dynamics), and/or does not adsorb on the NM surface and as such affect toxicity. Similarly, consideration should be given to binding of other moieties (such as proteins from serum, dyes, or other media components) on the NM surface as this might alter ADME properties and/or effects and generate erroneous results. The stability of an NM suspension should ideally be monitored throughout the exposure period as the concentration of the NM to which the test system is being exposed may vary with time (due to agglomeration, precipitation). An adequate number of positive and negative controls should be included in the tests to verify the role of the vehicle. This may also require additional material characterisation in the specific dispersant (*e.g.* in terms of size, size distribution, point of zero charge, etc). Validated positive control (reference) NMs for apoptosis, cytotoxicity, ROS, etc. are not available yet. In many

(ii) the type of measurement 1) Colorimetric assays (MTT, MTS, XTT, WST1-1, Alamar Blue assay, LDH, SRB, NRU and crystal violet assays) 2) Dye exclusion assays (trypan blue, eosin, Congo red, erythrosine B assays) 3) Fluorimetric assays (Alamar Blue assay, CFDA-AM assay, GF-AFC assay) 4) Luminometric assays (ATP, caspases, dead-cell proteases assays and real-time viability assay) 5) label free and real-time cell electrical impedance analysis (*e.g.* xCELLigence system) 6) visual inspection of the cells **(iii) the mode of action** This can be achieved by assessing the ability of NM to: 1) produce reactive oxygen and nitrogen species - (by *e.g.* H2DCF-DA assay, TBA assay for malondialdehyde, GSH/GSSG ratio) 2) trigger an inflammatory response (by *e.g.* CFU-GM and CFU-E, whole blood cultures, hemolysis test, thrombogenicity assay (activated partial thromboplastic time assay, thrombin generation	publications, however, NH2-PS NPs (*i.e.*, positively charged amino modified-polystyrene NP) are used, as they were shown to be toxic to many different cell types and do not release dissolved ions which may cause toxicity as is the case *e.g.* for metallic oxide NPs). Exemplary control settings deduced from the cause-and-effect analysis and implemented into a 96-well plate are described by Elliot *et al.* (2017). NMs can interfere with readout systems. Examples of such specific interference include, but are not limited to, the following (Thorne *et al.,* 2010; Guadagnini *et al.* 2015): - (i) Fluorescence/absorbance-based methods: disturbance by NMs that are fluorescent or absorb light at the wavelength of measurement, or that quench fluorescence, or light scattering. Some of these problems might be overcome by either adding appropriate controls or modifying existing protocols, *e.g.* removal of NMs via centrifugation before reading the assay can reduce data variation (SCENIHR, 2015). Another way is to subtract NM absorbance as background (Ciappellano *et al.,* 2016) - (ii) Luciferase based methods: non-specific activation or inhibition of the luciferase signal that can occur in a concentration-dependent manner. - (iii) Enzymatic assays: alteration of enzyme function, of co-factor, or of other limiting reagents by NM; display of enzymatic activity (or chemical reactivity) by the NM itself; removal of NM before performing the assay may be helpful (Ciapellano *et al.*, 2016). - (iv) Resazurin or MTT reduction: strongly reducing NMs may directly reduce resazurin or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) non-enzymatically. Compounds that trigger the release of superoxide can trigger reduction of resazurin by superoxide. This may result in erroneous cytotoxicity data. When NMs are not sufficiently pure, interference with the test may come from impurities or from ingredients of the formulation. In general, NMs are not soluble in the culture media, and therefore it should be ensured that the highest concentrations used do not produce excessive precipitates or hamper visual inspection of the growing cells. The sterility of the NM suspension has to be assured, as the presence of biological contamination (bacteria, LPS) may induce strong inflammatory reactions in some cell types. For all of the above reasons, multiple assays for cytotoxicity should be employed in order to reduce false negative/positive results (Drasler *et al.*, 2017). As there are no commonly accepted and validated methodologies, care should be taken to consider possible interferences and to avoid misinterpretation of data (*e.g.* Elsabahy and Wooley, 2013).

assay, blood clotting time assay, calibrated thrombin generation assay), phagocytosis assay, DC maturation 3) induction of genotoxicity including cell arrest (by *e.g.* Comet assay, micronuclei presence, TUNEL assay, γ-H2AX, Pig-A test) (Farcal *et.al*., 2015; Drasler *et al.,* 2017; Lewinski *et al.,* 2008; Marrocco *et al.,* 2017; Kohl, 2020). Available cell culture models: To measure cytotoxicity different cell models can be used. Besides the use of standard 2D-cell cultures, more advanced culture systems became available such as co-cultures, 3D-cell cultures, multicellular spheroids, air-liquid interphase (ALI), organ-on-a-chip systems. Cells are preferentially of human origin. - **co-culture**: used to mimic the communication between different cell types *e.g.* for lung epithelial cells, macrophages, endothelial or dendritic cells may be combined. Co-culture models allow high-throughput testing and in-depth monitoring of effects of xenobiotics on cell–cell interactions. Models have been developed exposing cells to aerosols of ENMs at the air-liquid interphase to accurately mimic the cell-particle interactions occurring in lungs (Paur *et al.*, 2011; Diabaté *et al.*, 2020; Wang *et al.*, 2020). - **3D-cell cultures**: cells are cultured within micro-assembled devices supported by a 3D-structure	However, at the moment, the colony forming efficacy (CFE) is considered as one of the most promising tests for NMs (Dusinska *et al.*, 2015). The assay could be included in a testing battery as an early screening method. It may well be used in combination with other *in vitro* assays (*e.g.* genotoxicity *in vitro* assays, such as the *in vitro* micronucleus assay (OECD TG 487 (OECD, 2016h)) to define subtoxic doses *in vitro*. It has to be noted that this assay cannot be used for cell suspensions or cells not forming colonies (Kinsner-Ovaskainen and Ponti, 2014). Useful information can also be found in ISO documents on nanomaterials: - ISO, 2018d - ISO 19007:2018 Nanotechnologies. *In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles Beside the above-mentioned standards, also additional guidance documents are available: - ISO, 2016b - ISO/TS 19006:2016 (confirmed in 2020). Nanotechnologies. 5-(and 6)-Chloromethyl-2',7' Dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) assay for evaluating nanoparticle-induced intracellular reactive oxygen species (ROS) production in RAW 264.7 macrophage cell line - ISO, 2017c - ISO/TR 19601:2017. Nanotechnologies. Aerosol generation for air exposure studies of nano-objects and their aggregates and agglomerates (NOAA) - ISO, 2016c - ISO/TS 19337:2016 (confirmed in 2019). Nanotechnologies. Characteristics of working suspensions of nano-objects for *in vitro* assays to evaluate inherent nano-object toxicity - ISO, 2020 - ISO/TR 21624:2020. Nanotechnologies. Considerations for *in vitro* studies of airborne nano-objects and their aggregates and agglomerates (NOAA) - ISO, 2021 - ISO/TS 21633:2021. Label-free impedance technology to assess the toxicity of nanomaterials *in vitro* Nanomaterial and non-nanomaterial specific information useful in designing physico-chemical characterisation or *in vitro* studies can also be found in databases, *e.g.*: e-NanoMapper (http://www.enanomapper.net), ToxCast EPA (https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data), Adverse Outcome Pathway-knowledge base (AOP-KB, https://aopkb.oecd.org/background.html). Also cloud platforms are available with lists of SOPs developed in nanomaterial specific EU projects, *e.g.*:

mimicking the *in vivo* tissue and the organ-specific microarchitecture. 3D-cell co-cultures and (micro)fluidic models are emerging techniques, which create more realistic exposure conditions by simulating the morphology and physiology of natural tissue (Ozcelikkale *et al.*, 2017). The most recent advancement in this area is the development of integrated organ-on-chip microsystems that reproduce key structural, functional, biochemical, and mechanical features of living organs in a single device. - **Air Liquid Interphase** systems – MucilAir™, Epithelix™, Mattek™ - **multicellular spheroids:** many cell types can be grown in spheroids and cells often behave as seen *in vivo*. These spheroids are composed of a necrotic core with quiescent intermediate and proliferating periphery regions. Such 3D-spheroids offer a simple and highly reproducible model that exhibits many characteristics of natural tissues, such as the production of extracellular matrix and cell–cell interactions.	• Gracious https://mailchi.mp/db7641855282/find-out-more-about-our-project-updates-in-our-newsletter-10584516?e=53488f0001, • NanoDefine http://www.nanodefine.eu/index.php/nanodefine-publications/nanodefine-methods-manual, • Nanopartikel https://nanopartikel.info/en/knowledge/operating-instructions • NanoReg https://www.rivm.nl/en/about-rivm/mission-and-strategy/international-affairs/international-projects/nanoreg/work-package • NanoSolveIT https://nanosolveit.eu • PATROLS https://www.patrols-h2020.eu/publications/sops/index.php • RiskGONE https://riskgone.eu/home-riskgone-project/resources/project-resources • REFINE http://refine-nanomed.eu/project/ Although the SOPs are scientifically valid, they have not gone through formal validation processes yet.
Acute toxicity: Data on acute toxicity is not mandatory. A WoE approach may be sufficiently derived from *in silico*, *in vitro* and *in vivo* studies (when available).	

Skin Corrosivity and irritation **Skin corrosion:** a) Rat Skin Transcutaneous Electrical Resistance (TER) test [OECD TG 430 (OECD, 2015d)] b) EpiSkin™ [EC B.40bis, OECD TG 431 (OECD, 2004b)] c) EpiDerm™ SCT (EPI-200) [EC B.40bis, OECD, TG 431 (OECD, 2004b)] d) SkinEthic™ Reconstructed Human Epidermis (RHE) [EC B.40bis, OECD TG 431 (OECD, 2004b)] e) epiCS® (former Epidermal Skin Test-1000) [EC B.40bis, OECD TG 431 (OECD, 2004b)] f) The *In vitro* Membrane Barrier Test Method [OECD TG 435 (OECD, 2015f)] currently only includes the Corrositex™ test. **Skin irritation**: OECD 439 (OECD, 2021c) a) EpiSkin™ b) EpiDerm™ SIT (EPI-200) c) SkinEthic™ RHE d) LabCyte EPI-MODEL24 SIT e) epiCS® f) Skin+® g) KeraSkin™ SIT OECD TG 439 (OECD, 2021c) is stand-alone replacement test within a WoE approach [EC B.46]. OECD Guidance Document on an Integrated Approach on Testing and Assessment (IATA) for Skin Corrosion and Irritation (OECD, 2017).	The alternative tests proposed for skin corrosion and irritation are based on colorimetric assays (such as sulforhodamine B dye, MTT assay). These techniques may not be suitable for certain NMs because of possible interactions (see endpoint "cytotoxicity" above and Section 5.3.2). Thus, additional controls need to be included to avoid possible interference of NMs with the detection system. Some NMs may themselves disperse/absorb light and therefore interfere with colorimetric measurements. These aspects need to be considered when spectrophotometric methods are applied (Guadagnini *et al.,* 2015; ECHA, 2017b). The measurement of cytokines and chemokines in the test system may provide additional information (*e.g.* IL-1α, tumour necrosis factor α (TNF-a); IL-8, interferon). However, they may bind/adsorb on NM surfaces, and this may lead to false negative results. In OECD (2018a), it was concluded that the guideline might need to be amended in view of application to NMs.

Serious eye damage and eye irritation -As a first step (historic) dermal irritancy or corrosivity data should be considered, [OECD TG 439 (OECD, 2021c)] - Several *in vitro* test guidelines are available to address eye irritation or serious eye damage: a) Bovine Cornea Opacity Permeability (BCOP) test method [OECD TG 437 OECD, 2020a] b) Isolated Chicken Eye (ICE) test method [OECD TG 438 OECD, 2018d] c) Short Time Exposure (STE) test method [OECD TG 491 OECD, 2020b] d) Fluorescein Leakage (FL) test [OECD TG 460 OECD, 2017b] e) Reconstructed Human Cornea-like Epithelium (RhCE) test method [OECD TG 492 OECD, 2019a] f) Vitrigel® - Eye Irritancy Test [OECD TG 494 OECD, 2021f] g) *In vitro* Macromolecular Test [OECD TG 496 OECD, 2019b] h) Defined Approaches for Serious Eye Damage and Eye Irritation [OECD TG 467 OECD, 2022h]	A specific protocol for solid substances exists for the BCOP and ICE tests. Solid substances are mostly tested at 20% (w/w) as a suspension in 0.9% sodium chloride (including in some instances a dispersant). Although no specific validation has been performed for NMs, there is no clear scientific basis against the application of these methods for NMs. It should, however, be kept in mind that: - NMs can aggregate/agglomerate in the suspension or can adsorb the dispersant (see 5.3.2). These aspects should be verified. - opacity measurements may be affected by the presence of NMs. To allow consistent interpretation of the results, this should be kept in view. - for the methods measuring leakage of fluorescein, possible artefacts due to absorption/adsorption of the fluorescent dye by NMs should be verified, and if present, eliminated. The Ocular Irritection® (OI) assay (OECD TG 496) is an acellular biochemical assay that evaluates the ocular hazard effects of test chemicals based on the premise that eye irritation and corneal opacity after exposure to irritating substances is the result of perturbation or denaturation of corneal proteins. The OI assay is recommended as part of a tiered testing strategy for solid and liquid chemicals under certain circumstances and with specific limitations (*i.e.* applicable to solid and liquid chemicals whose 10% solution dispersion (v/v or w/v as appropriate) has a pH in the range $4 \leq pH \leq 9$. Other in-house models could also be used if they have been properly validated against the models mentioned above. The defined approach described in OECD TG 467 is a stand-alone NAM-based methodology to classify eye irritants the whole range of classifications required by the UN GHS i.e., Cat. 1, Cat. 2 and No Cat.
Skin sensitisation: Validated available tests are:	The *in vitro* skin sensitisation methods have not been validated for NMs. Their applicability is therefore limited to soluble test chemicals or substances forming a stable dispersion. The application domain of these tests for NMs still has to be established.

In chemico skin sensitisation: Key Event-Based Test Guideline for in chemico skin sensitisation assays addressing the Adverse Outcome Pathway Key Event on Covalent Binding to Proteins [OECD TG 442C (OECD, 2022e)] describing - The Direct Peptide Reactivity Assay (DPRA) - The Amino acid Derivative Assay (ADRA) - The kinetic Direct Peptide Reactivity Assay (kDPRA) *In vitro* activation of keratinocytes: ARE-Nrf2 Luciferase Test Method OECD TG 442D (OECD, 2022f) describing - ARE-Nrf2 luciferase KeratinoSens™ test method - ARE-Nrf2 luciferase LuSens test method *In vitro* Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation OECD TG 442E (OECD, 2022g) describing the: - Human Cell Line Activation test (h-CLAT) - U937 cell line activation Test (U-SENS™) - Interleukin-8 Reporter Gene Assay (IL-8 Luc assay) - Genomic Allergen Rapid Detection (GARD™) for assessment of skin sensitisers (GARD™skin)	OECD recently published a report on the Applicability of the key event-based Test Guideline 442D for *in vitro* skin sensitisation testing of nanomaterials (OECD, 2022) based on a limited number of relevant nanomaterials for testing within this project as well as limited availability of *in vivo* skin sensitisation data. The report is intended to be a starting point for interested parties carrying out further work related to nanomaterials in the area of skin sensitisation.

- Guideline for Defined Approaches for skin sensitisation [OECD TG 497 OECD, 2021d] - Guidance document to the OECD Guideline 497 on Defined Approaches for Skin Sensitisation. [OECD GD 336 (OECD, 2021e)].	
Dermal/ percutaneous absorption: Dermal absorption of cosmetic ingredients is usually assessed be the *in vitro* skin absorption method [OECD TG 428 (OECD, 2004a)]. A multiplicity of factors play a key role in the determination of the dermal/ percutaneous absorption of a compound and the SCCS considers its own "Basic Criteria" as essential for dermal absorption studies [SCCS, 2010a; SCCS/1647/22].	For any tests on NMs, the dose, volume, and contact time with the skin have to mimic the in-use conditions (also taking the consideration of dispersion – see Section 5.3.2). Appropriate analytical techniques and sampling methods should be used to determine the possible adsorption of substances on NM surfaces – see section 5.3.2). Dermal absorption of NMs needs to be determined experimentally. However, if no experimental data are provided, the SCCS will apply the default value of 50% as determined for conventional substances, or higher if warranted by the composition of a specific NM (see Section 4.4.2.1). If case *in vitro* absorption tests indicate potential systemic absorption, the integrity of the nano structure needs to be confirmed. When absorption of NPs cannot be excluded by experimental data, or justified on the basis of solubility/ degradation of the NM, the SCCS will apply a default approach and assume that 100% of the absorbed material was in nano form. The standard *in vitro* diffusion cell chamber, used for non-nano ingredients, may not be ideal for testing NMs because mechanical factors may interfere. New or optimised methodologies are required (SCCP, 2007). This is in line with OECD (2018a), where it is stated that OECD 428 should be adapted for testing on manufactured NMs. However, several critical points in the protocol may not be adequate for these, including observation time, sampling time, influence of the mechanical process on particles translocation, solubility in and compatibility with the receptor fluid.

<u>Repeated dose toxicity</u>: Currently no validated or generally accepted alternative method is available to replace animal testing. This endpoint is important as effects, which require a long latency period, or which are cumulative, become manifested in this test.	Information generated by *in vitro* testing might be considered within an integrated strategy (*i.e.* combining different pieces of information) in order to draw conclusions for an NM. Of particular interest are local target organ effects, and/or tests to clarify the mechanisms of action (*e.g.* cell viability, oxidative stress, inflammation, etc.).
Mutagenicity/genotoxicity: Base level testing consists of the following *in vitro* 2-test battery: 1. Bacterial reverse mutation test [OECD TG 471 OECD, 1997] for gene mutation testing 2. *In vitro* Micronucleus test [OECD TG 487 OECD, 2016h] for both structural (clastogenicity) and numerical (aneugenicity) chromosome aberrations testing Other *in vitro* genotoxicity test methods: – *In vitro* mammalian cell gene mutation tests using the Hprt and xprt genes [OECD TG 476 OECD, 2016j] – *In vitro* mammalian cell gene mutation tests using the thymidine kinase gene [OECD TG 490 OECD, 2016g] – *In vitro* mammalian chromosome aberration test [OECD TG 473 OECD, 2016i]	The SCCS recommends the following tests for NM genotoxicity testing *in vitro*: • Mammalian cell chromosome aberrations/clastogenicity assay (*in vitro* chromosome aberration test or *in vitro* micronucleus test). The micronucleus test can be performed using either the mononucleate or cytokinesis blocked protocols. However, if the cytokinesis blocked micronucleus assay is to be applied, then cytochalasin B addition must be post-treatment/exposure (after the NM exposure period) or a delayed-co-treatment protocol which is acceptable if a sufficient NM exposure period has been allowed to enable uptake into the test system cells. Co-exposure to both cytochalasin B and the test NM for the duration of the experiment should be avoided due to possible interference of cytochalasin B with the uptake of NMs. • An *in vitro* mammalian cell gene mutation test (*e.g.* Hprt, Tk or Xprt tests). • Other indicator tests, such as the Comet assay, may be included as further weight of evidence. *In vitro* genotoxicity studies for nanomaterials should be always accompanied by an assessment of cellular and preferably nuclear uptake to demonstrate target exposure. The bacterial Ames test is not recommended as a representative test for genotoxicity of NMs because, unlike mammalian cells, bacterial cells have limited or no uptake of NMs through endocytosis. The bacterial cell wall hinders uptake and particle internalisation is unlikely to occur to the same extent as observed in mammalian cells. Therefore, the sensitivity of the assay for NM genotoxicity has been questioned. In addition, some NMs have bactericidal activity, making this test unsuitable for testing NMs (EFSA, 2011). In addition, the use of a metabolic activation system for NMs is questionable. Although not investigated in detail (Szalay *et al.,* 2011), most insoluble NMs (*e.g.* some metals) are not

Supportive tests in overall WoE approach (mechanistic understanding): - Pig-a test *in vitro* (mutation of glycosylphosphatidylinositol (GPI) anchor proteins on the cell surface) - *in vitro* Comet assay for detection of strand breaks; - *in vitro* Comet assay modified with lesion specific repair enzyme for detection of oxidation lesions (oxidised purines and pyrimidines) - toxicogenomics (genes involved in DNA instability) - recombinant cell models (GreenScreen HC, BlueScreen HC, ToxTracker) - γH2AX - epigenetic responses (*e.g.* DNA methylation, non-coding small single-stranded RNAs termed microRNAs (miRNAs) and histone modifications)	metabolised. Instead, the proteins present in a metabolic activation system may interfere with nanomaterials (Kumar *et al.,* 2011), alter their bioavailability, and thus reduce the sensitivity of the assay. Notwithstanding this, it should be verified whether some NMs could be metabolised (*e.g.* organic nanomaterials, some inorganic NMs coated with organic substances or their surface modified with organic functional groups). Caution is also needed when applying an *in vitro* micronucleus test. Cytochalasin B, often used to inhibit cytokinesis, may inhibit endocytosis and may lead to false negative outcomes when particles are present (Landsiedel *et al.,* 2009). Thus, cytochalasin B needs to be applied after the NMs have been taken up by the cells (usually 2 hr after treatment) (Magdolenova *et al.,* 2012, OECD, 2022a, c). For several types of NPs (*e.g.* titanium dioxide, multi-walled carbon nanotubes), microscopic evaluation of the cytokinesis-blocked proliferation index and micronucleus identification was found to be inappropriate at high testing concentrations due to an overload of agglomerates (Corradi *et al.,* 2012). Although not investigated so far, similar problems may be anticipated for other microscopy-based *in vitro* mutagenicity tests (*e.g.* chromosome aberration test). Some of the shortcomings of genotoxicity tests for NM testing may be addressed by a weight of evidence approach based on additional alternative methods, including those methods that have not yet been validated. They could be relevant and scientifically valid, such as a micronucleus test or a Comet assay in reconstructed human skin. These alternatives together with the γH2AX assay will become available in the near future for high-throughput screening (HTS) and high-content analysis (HCA). To add more weight to the evidence, mechanistic information at the molecular level can also be obtained through '-omics' technology (Ates *et al.*, 2018). OECD (2018a) considered the *in vitro* mammalian cell gene mutation tests (OECD TG 476 OECD, 2016j) as an alternative to the bacterial reverse mutation test, as no specific limitations were observed when testing NMs. The *in vitro* Comet assay is often used to test genotoxicity of NMs and, although it is an indicative test, it may help elucidating the mechanism of genotoxicity (Dusinska *et al.,* 2015; Collins *et al.,* 2016, 2023; El Yamani *et al.,* 2017, 2022a). Several *in vitro* genotoxicity tests have been tested for potential interference with NMs and recommendations for assay modification have been published (Magdolenova *et al.,* 2012; Karlsson *et al.,* 2015; El Yamani *et al.*, 2022a). However, in view of the current limitations of *in vitro* tests and the potential introduction of artefacts with specific types of NMs (see also 5.3.2), the SCCS is of the opinion that with the *in vivo* testing ban for cosmetic ingredients, the safety of potential new cosmetic ingredients may not be adequately

	assessed until the assays are validated for NMs. This is in line with OECD (2018a) in which it is stated that results from the Comet Assay for environmental chemicals can only provide an indication of potential genotoxicity. A study report and Preliminary Guidance Document on the Adaptation of *In vitro* Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials has been published by the OECD (OECD No. 359 OECD, 2022c).
Carcinogenicity: The decision on the carcinogenic potential of mutagenic or genotoxic substances may be made based on the outcome of *in vitro* mutagenicity tests. A positive *in vitro* result in mutagenicity testing is seen as indicative for the carcinogenic potential of substances (SCCS/1647/22). When a structural alert for carcinogenicity is present, or positive results are obtained in an *in vitro* mutagenicity test, the following cell tests may be needed: -an *in vitro* Syrian Hamster Embryo (SHE) Transformation Test [OECD Guidance Document 214 (OECD, 2015a)] (CTA). -an *in vitro* Bhas 42 assay [OECD Guidance Document 231 (OECD 2016f)] (CTA) In addition, some information on the carcinogenicity potential can be inferred from mechanistic studies, *e.g.* on cell proliferation, altered gap junction intercellular communication (GJIC) (Spannbrucker *et al.*, 2018), hormone- or other receptor binding, immunosuppressive activity (Huaux, 2018), ability to inhibit or induce apoptosis, or ability to stimulate angiogenesis or	There is currently no validated alternative method to test carcinogenicity. The recently adopted guidance for the CTA (see Sections 5.3.6 and 5.3.7) that measures cell transformation (as one step in the multistep cancer process), has been applied for several NMs (Ponti *et al.,* 2009; Ohmori *et al.,* 2013, 2022; Gabelova *et al.,* 2017). Representative *in silico* and *in vitro* assays to measure the key characteristics of carcinogens are presented in the publication by Smith *et al.,* 2020. Jacobs *et al.*, 2020 describe an OECD work program on IATA on non-genotoxic carcinogens proposing a methodology for evaluation and prioritization of the NGTxC-relevant endpoint (*in vitro*) assays. Selected assays will be reviewed and suggested for a final Guidance document to be used within this IATA. The CTAs can detect both genotoxic and non-genotoxic carcinogens (enabling the phenotypic detection of oncotransformation).

the secretion of angiogenesis factors (Medina-Reyes *et al.*, 2019).	
Reproductive toxicity: No validated alternative method is available. The assessment of reproductive toxicity is complex, and it is expected that the various stages cannot be mimicked using a single alternative method. For embryotoxicity, three alternative methods have been validated, but not regulatory accepted. They were not specific enough to show embryotoxicity: a) The Whole Embryo Culture test (WEC) b) The MicroMass test (MM) c) The Embryonic Stem Cell Test (EST) [ESAC 2001].	The three alternative methods for embryotoxicity could be applicable to NMs, provided that typical nano-related aspects such as dispersion/aggregation, absorption, stability and distribution into the tissue are taken into account. In the EST for nanosilica, inhibition of differentiation into contracting myocardiocytes has been observed (Park *et al.,* 2009). OECD is coordinating a number of ongoing and future projects that aim to foster the development of new developmental neutrotoxiciy (DNT) *in vitro* assays and to advance acceptance of the DNT-*in vitro* battery for regulatory use (https://www.oecd.org/env/ehs/testing/developmental-neurotoxicity.htm).
Endocrine disruption (ED) activity: The assessment of potential ED activity can be done in a stepwise approach using data generated outside the cosmetic field or for a new cosmetic ingredient using NAMs (*in silico* models including read across, *in vitro* assays, other mechanistic techniques such as '-omics'). The currently available *in vitro* methods are: • Estrogen [OECD TG 493 (OECD, 2015c), US EPA TG OPPTS 890.1250] or androgen receptor binding affinity (US EPA TG OPPTS 890.1150, 2009) • Estrogen receptor transactivation [OECD TG 455, (OECD, 2021g), US EPA TG OPPTS 890.1300], human cell-based reporter gene assay (ISO, 2018c 19040-3:2018), yeast	None of the methods to detect potential ED activity is currently validated for NMs. However, if carried out with due caution to nano-aspects, these tests may provide relevant information.

estrogen screen (ISO, 2018b 19040-1, 19040-2:2018) • Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (OECD TG 458 OECD, 2020c) • Steroidogenesis *in vitro* (OECD TG 456 OECD, 2022i; US EPA TG OPPTS 890.1550, 2009) • Aromatase Assay (US EPA TG OPPTS 890.1200) • Thyroid disruption assays (*e.g.* thyroperoxidase inhibition, transthyretin binding). A project on validation of selected *in vitro* methods within EU-NETVAL activity is on-going. • Retinoid receptor transactivation assays • Other hormone receptors assays as appropriate • High-Throughput Screens, See OECD GD No. 211 Describing Non-Guideline *In vitro* (OECD 2014c)	
<u>Toxicokinetic studies (ADME)</u>: Skin absorption *in vitro* [OECD TG 428 OECD, 2004a].	Following systemic absorption, the distribution and fate of an NM is mainly governed by its chemical nature, particle size, surface characteristics, aggregation state, etc. Special considerations relating to exogenous moieties (*e.g.* surfactants, serum, or other media components) that may change surface characteristics (see 5.3.2). Potential toxicity of metabolites and degradation products could be a factor of variability, but less important for insoluble NMs. It should, however, be considered if and when NMs, or their surface coatings, dissolve or degrade. Therefore, where applicable, *in vitro* biotransformation studies may be necessary to ascertain the likelihood of adverse effects due to metabolites/degradation products. OECD TG 417 (OECD, 2010d) is considered inadequate for nanomaterials. There are ongoing initiatives at OECD level addressing toxicokinetics of nanomaterials. A technical report has been published by ISO, 2019 (ISO/TR 22019:2019) describing considerations for performing toxicokinetic studies for nanomaterials.

Photo-induced toxicity: *1) Photo-toxicity (photo-irritation) and photo-sensitisation (photo-allergy)* - UV-VIS spectra of the compound along with the MEC Optional pre-screening: Reactive Oxygen Species Assay for Photoreactivity [OECD TG 495 OECD, 2019c) - The In vitro 3T3 Neutral Red Uptake Photo-toxicity Test (3T3 NRU PT) is a validated method (OECD TG 432 OECD, 2019d). - *In vitro* phototoxicity: reconstructed human epidermis phototoxicity test method (OECD TG 498 OECD, 2021h) *2) Photo-mutagenicity / Photo-genotoxicity* Although the validity of photomutagenicity / photogenotoxicity testing is being questioned, in specific cases when the structure of a molecule, its light absorbing potential or its potential to be photo-activated may indicate a photomutagenic/photogenotoxic hazard, then photomutagenicity tests should be provided, including gene mutations and clastogenicity/aneugenicity endpoints; especially when the substance is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. Additionally, available alternative methods, for example scientifically validated comet assay for detection of oxidized DNA lesions, or *in silico* methods, can be considered.	The reliability and relevance of the *in vitro* 3T3 NRU Test has not been specifically validated for NMs. It should be noted, however, that in some instances, neutral red may interfere with NMs (Lanone *et al.,* 2009; Guadagini *et al.,* 2015) (also see 5.3.2) Some nanoparticles have photocatalytic properties that may lead to phototoxicity in humans. General recommendations regarding the experimental conduct of tests for photo-genotoxicity should be considered.

No photomutagenicity tests are needed when the phototoxicity tests are negative. There is no requirement for a photomutagenicity test if the test material only absorbs at wavelengths lower than 313 nm and if there is insufficient absorption at longer wavelengths.	
Human data: Human data is very valuable. Sources could be: post-marketing surveillance data, results from biomonitoring programs case reports, occupational surveillance data and occupational disease registries, poison centre information, clinical studies, epidemiological studies, tests with human volunteers, etc. Tests with human volunteers confirm that there are no harmful effects, but these can only be envisaged when the toxicological profiles of the components are available and no concern is raised. Finished cosmetic products are usually tested in a small group of human volunteers. Human studies might also become necessary to build up and validate PBPK models.	The same methodology as described for non-NMs are applied, taking into consideration the ethical restrictions as described in the 12th revision of the SCCS Notes of Guidance (SCCS/1647/22).

ANNEX 2: Checklist for Hazard Identification (Toxicological Data) to be provided for safety evaluation of nanomaterials intended to be used in cosmetic products

Information required	**Reference**	**Provided?**
Likelihood and extent of internal exposure via skin, lung or oral route considering the use type	Section 3-3.5 of SCCS/1647/22	
Dermal absorption – for dermally applied products	SCCS/1358/10 and section 3-3.5.1 of SCCS/1647/22	
Biokinetic behaviour, aggregation/ agglomeration considered during tests?	Section 3-3.5 of SCCS/1647/22	
Acute Toxicity	Section 3-4.4 of SCCS/1647/22	
Irritation and Corrosivity	Section 3-4.5 of SCCS/1647/22	
Skin Sensitisation	Section 3-4.7 of SCCS/1647/22	
Mutagenicity / Genotoxicity[(a)]	Section 3-4.10 of SCCS/1647/22	
Repeated dose toxicity	Section 3-4.8 of SCCS/1647/22	
Photo-induced toxicity - for products intended for use in sunlight-exposed skin	Section 3-4.12 of SCCS/1647/22	
Reproductive Toxicity [(b)]	Section 3-4.9 of SCCS/1647/22	
Carcinogenicity [(c)]	Section 3-4.11 of SCCS/1647/22	
Human data (where available)	Section 3-4.13 of SCCS/1647/22 and SCCNFP/0633/02	
Other relevant information		

[a] The Ames test is not considered appropriate for NM mutagenicity assessment. The following scheme based on *in vitro* assays is proposed (SCCS/1647/22).

1. An *in vitro* mammalian cell gene mutation test (*e.g.* Hprt, Tk or Xprt tests).
2. Mammalian cell chromosome aberration/clastogenicity – determined either by *in vitro* chromosome aberration test or micronucleus test. The micronucleus test can be performed by the mononucleate or cytokinesis blocked protocols. In the cytokinesis blocked micronucleus assay, co-exposure to both cytochalasin B and the test NM for the duration of the experiment is not considered acceptable. Additionally, other alternative tests, such as the Comet assay, may be included as further weight of evidence. New *in vitro* approaches such as cell transformation assays or toxicogenomic approaches may also be useful for identification of genotoxic as well as non-genotoxic carcinogen NMs.
3. *In vitro* genotoxicity studies should be accompanied by an assessment of cellular and nuclear uptake to demonstrate target exposure.

[b] Where points 1 and 2 of the above table indicate significant systemic uptake

[c] Where points 1 and 2 of the above table indicate significant systemic uptake and/or bioaccumulation

ABBREVIATIONS AND GLOSSARY OF TERMS

2D	Two-dimensional
3D	Three-dimensional
3R	Refinement, Reduction, Replacement
3T3 NRU PT	3T3 Neutral Red Uptake Phototoxicity Test
AAS	Atomic Absorption Spectroscopy
ADME	Absorption, distribution, metabolism, excretion
Adverse	An adverse response is defined as any treatment-related response that results in change in the morphology, physiology, growth, development or life span of an organism, which results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other environmental influences (WHO, 2004).
AFM	Atomic Force Microscopy
AhR	Aryl hydrocarbon receptor
AI	Alveolar Interstitial Region
ALI	Air liquid interphase
Alternative methods	All those procedures that can completely replace the need for animal experiments, which can reduce the number of animals required, or which can reduce the amount of pain and stress to which the animal is subjected in order to meet the essential requirements for use in human or animal risk assessment (Rogiers *et al.*, 2000; Russell *et al.*, 1992).
AOP	Adverse outcome pathway
ARE-Nrf2	Antioxidant-responsive element-nuclear factor (erythroid-derived 2)-like 2
Art.	Article
ATP	Adenosine Triphosphate
ATP	Adaptation to Technical and Scientific Progress
BAL	Bronchoalveolar lavage
BAM	Bundesanstalt für Materialforschung und Materialprüfung
BB	Bronchial Region
bb	Bronchiolar Region
BCOP	Bovine Corneal Opacity and Permeability
BET	Brunauer Emmett and Teller method
BMD	The Benchmark Dose (BMD) is proposed as an alternative for the classical NOAEL and LOAEL values. The BMD is based on a mathematical model being fitted to the experimental data within the observable range and estimates the dose that causes a low but measurable response (the benchmark response BMR) typically chosen at a 5 or 10% deviation (above or below) of the non treated or control treated animals.
BMDL	The BMD lower limit (BMDL) refers to the corresponding lower limits of a one-sided 95% confidence interval on the BMD.
BrdU	5-bromo-2-deoxy-uridine
CAS n°	Chemical Abstracts Service registry number
CEN	European Committee for Standardization
CFDA-AM	5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester
CLS	Centrifugal Liquid Sedimentation
Colipa	Cosmetics Europe (formerly the European Cosmetic Toiletry and Perfumery Association)

Compatibility test	A test intended to confirm that there are no harmful effects when applying a cosmetic product for the first time to the human skin or mucous membrane; the test must involve exposure (normal or slightly exaggerated) which closely mimics typical consumer use of the product (based on SCCNFP/0068/98).
Cosmetic ingredient	Any chemical substance or mixture of synthetic or natural origin, used in the formulation of cosmetic products. A cosmetic ingredient may be: 1. a chemically well-defined single substance with a molecular and structural formula, 2. a complex mixture, requiring a clear definition and often corresponding to a mixture of substances of unknown or variable composition and biological nature, 3. a mixture of 1 and 2, used in the formulation of a finished cosmetic product. (based on Art. 5a of 93/35/EEC and 2009/1223/EC).
Cosmetic product	Any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours (2009/1223/EC).
Cosmetics Europe	The Personal Care Association (formerly Colipa)
CPNP	Cosmetic Products Notification Portal
CTA	Cell Transformation Assay
DC	Dentritic Cell
Da	Dalton
d_{ae}	Aerodynamic diameter
DC	Dentritic Cell
DG	Directorate-General
DG ENV	Directorate-General for Environment
DG GROW	Directorate-General for Internal Market, Industry, Entrepreneurship and SMEs
DG SANTE	Directorate-General Health and Food Safety
Dir.	Directive
DLS	Dynamic Light Scattering
DMA	Differential Mobility Analyzer
DNA	DeoxyriboNucleic Acid
Doc.	Document
Dose	Total amount of an agent administered to, taken up by, or absorbed by an organism, system, or (sub)population (WHO, 2004). Dose is expressed as weight (grams or milligrams) or as weight of test substance per unit of weight of test animal (*e.g.* milligrams per kilogram body weight), or per skin surface unit (*e.g.* milligrams per square centimetre of skin), or as constant dietary concentrations (parts per million or milligrams per kilogram of food) (based on EC B.26).
Dose descriptor	Dose descriptor is used to designate the exposure level (dose or concentration) that corresponds to a quantified level of risk of a health effect in a specific study such as NOAEL, LOAEL, BMD, T25 etc. (ECHA, 2012).
DPRA	Direct Peptide Reactivity Assay
EC	European Community

EC Number	EC number, meaning either EINECS number, ELINCS number, NLP number or EC Number appointed by the European Commission under REACH Regulation. The European Community number (EC Number) is a unique seven-digit identifier that was assigned to substances for regulatory purposes within the European Union by the European Commission. The so-called EC Inventory comprises three individual inventories, EINECS, ELINCS and the NLP list (1). (ECHA) also applies the EC number format to what it calls 'List number'[6] The number are assigned under the REACH Regulation without being legally recognised. Hence, they are not official because they have not been published in the Official Journal of the European Union. List numbers are administrative tools only and shall not be used for any official purposes.
ECB	The European Chemicals Bureau
ECETOC	An industry-funded expert not-for-profit think tank whose sole purpose is to enhance the quality of chemicals risk assessment so that chemicals management decisions are informed, reliable and safe.
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
ED	Endocrine Disruptor
EDX	Energy Dispersing X-Ray
EEC	European Economic Community
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing commercial Chemical Substances
ELINCS	European List of Notified Chemical Substances
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microsopy
ENM	Engineered Nanomaterial
(US) EPA	(United States) Environmental Protection Agency
EPR	Electron Paramagnetic Resonance
ESAC	ECVAM Scientific Advisory Committee
ESR	Electron Spin Resonance
EST	Embryonic Stem cell Test
ET	Extrathoracic region
Ex vivo	Relates to experiments or measurements done in the laboratory (outside the organism) on a biological substrate (organs, cells, tissues), directly after isolation from a living organism, without modification to the intrinsic properties of the substrate.
EU	European Union
EURL-ECVAM	European Union Reference Laboratory - European Centre for the Validation of Alternative Methods
FCA	Food contact Material
FDA	Food and Drug Administration (federal agency of the United States Department of Health and Human Services)
FFF	Field Flow Fractionation
Finished cosmetic product	The cosmetic product in its final formulation, as placed on the market and made available to the end user, or its prototype (2009/1223/EC)
FL	Fluorescein Leakage test
FTIR	Fourier Transform Infrared Spectroscopy
GARD	Genomic Allergen Rapid Detection

GC/LC-MS	Gas Chromatography/ Liquid Chromatography coupled with Mass Spectrometry
GC-MS	Gas Chromatography–Mass Spectrometry
GE	Gel Electrophoresis
GF-AFC	Glycylphenylalanyl-Aminofluorocoumarin
GI	Gastro-Intestinal
GJIC	Gap Junction Intercellular Communication
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GPMT	Guinea Pig Maximisation Test
GSD	Geometric Standard Deviation
GSH	Glutathione
GSSH	Oxidised Glutathione
GUM	Gesellschaft für Umweltmutationsforschung
H2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
HATM	Human Alimentary Tract Model
HCA	High Content Analysis
HDC	Hydrodynamic Chromatography
HET-CAM	Hen's Egg Test-Chorio Allantoic Membrane
HPLC	High-Performance Liquid Chromatography
HPRT	Hypoxanthine-guanine Phospho Ribosyl Transferase
HRP	Horseradish Peroxidase
HRTM	Human Respiratory Tract Model
HTS	High Throughput Screening
IARC	International Agency for Research on Cancer
IATA	Integrated Approaches to Testing and Assessment
ICCR	International Cooperation on Cosmetics Regulation
ICE	Isolated Chicken Eye
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ICRP	International Commission on Radiological Protection
IDEAL	Inhalation, Deposition and Exhalation of Aerosols in/from the Lung
IL-8 Luc	Interleukin-8 luciferase
***In silico* methods**	Computational approaches that use (quantitative) structure-activity relationship modelling and read-across between substances on the basis of structural or functional similarities (ICCR, 2014).
***In vitro* test method**	Biological method: using organs, tissue sections and tissue cultures, isolated cells and their cultures, cell lines and subcellular fractions. Non-biological method: such as computer modelling, chemical interaction studies, receptor binding studies etc. (based on Rogiers *et al.*, 2000)
***In vivo* test method**	Test method using living (experimental) animals (Rogiers *et al.,* 2000)
IL-1α	Interleukin-1α
IR	Infrared Spectroscopy
IRE	Isolated Rabbit Eye
ISO	International Organization for Standardisation
IUPAC	International Union of Pure and Applied Chemistry

IVIVE	*In vitro-in vivo* extrapolation
JRC	Joint Research Centre
KeratinoSens™	Activation of keratinocytes skin sensitisation assay
KE	Key event
LC50	Median Lethal Concentration 50%: a time dependent, statistically derived estimate of a test article concentration that can be expected to cause death during exposure or within a fixed time after exposure in 50% of animals exposed for a specified time {expressed as mass of test article per unit volume of air (mg/L, mg/m3) or as a unit volume of test article per unit volume of air (ppm, ppb)}.
LC-MS	Liquid Chromatography–Mass Spectrometry
LD50	Median Lethal Dose 50%: a statistically derived single dose of a substance that can be expected to cause death in 50% of the dosed animals (expressed in mg/kg body weight) (EC B.1 bis).
LDE	Laser Doppler Electrophoresis
LDH	Lactate Dehydrogenase
LED	Lowest Effective Dose, *e.g.* LED10
LGC	Laboratory of the Gouvernement Chemist
LLNA	Local Lymph Node Assay
LO(A)EL	The Lowest Observed (Adverse) Effect Level is the outcome of repeat-dose long-term toxicity studies, such as 28-day or 90-day tests with rats, mice, rabbits or dogs, chronic toxicity tests, carcinogenicity tests, teratogenicity tests, reproduction toxicity tests, etc. It is the lowest dose where (adverse) effects can be observed. In the calculation of the MoS, the lowest obtained LOAEL value may be used when a NOAEL is not available. The LOAEL should be expressed as mg/kg bw/d. (ECB, 2003).
Local Effects	A local effect refers to an adverse health effect that takes place at the point or area of contact. The site may be skin, mucous membranes, the respiratory tract, gastrointestinal system, eyes, etc. Absorption does not necessarily occur.
LOD	Level of detection
LOQ	Level of quantification
LPS	Lipopolysaccharides
MEC	Molar Extinction Coefficient
MED	Mass Equivalent Diameter
MIE	Molecular Initiating Event
MM	MicroMass
MMAD	Mass Median Aerodynamic Diameter
MNM	Manufactured Nanomaterials
MoE	Margin of Exposure
MoS	Margin of Safety
MPI	Magnetic Particle Inspection
MPPD	Multiple Path Particle Dosimetry
MPS	Mononuclear Phagocyte System
MS	Mass Spectrometry
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTT	3-(4,5)-dimethyl-2-thiazolyl-2,5-dimethyl-2H-tetrazolium bromide
MW	Molecular Weight

MWCNT	Multi-Walled Carbon Nano Tubes
NAMs	New Approach Methodology
NH2-PS	Positively Charged Amino-Modified-Polystyrene
Nanomaterial	An insoluble or bio-persistent an intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm. (2009/1223/EC). Deviating definitions in other regulatory fields may also exist.
Nanoparticle	Nano-object with all external dimensions in the nanoscale [ISO/TS 80004-2:2015 (CEN ISO/TS 80004-2:2017), Nanotechnologies-Vocabulary-Part 2: Nano-objects]. For the purpose of this document the term 'nanoparticle' is used to also include other forms of nano-object, such as nano-rods, nano-tubes, etc.
Nanoscale	Length range approximately from 1 nm to 100 nm [CEN ISO/TS 80004-1:2015, Nanotechnologies-Vocabulary-Part 1: Core terms]
NanoSIMs	An ultra-high resolution chemical imaging technique
NIST	National Institute of Standards and Technology
NM	Nanomaterial
NMR	Nuclear Magnetic Resonance
NOAEC	No observable adverse effect concentration
NO(A)EL, NO(A)ELsys	The No Observed (Adverse) Effect Level is the outcome of repeated dose toxicity studies, such as 28-day or 90-day tests with rats, mice, rabbits or dogs, chronic toxicity tests, carcinogenicity tests, teratogenicity tests, reproduction toxicity tests, etc. It is the highest dose for which no (adverse) effects can be observed (based on EC B.26). The NOAEL should be expressed as mg/kg bw/d. In the calculation of the MoS, the lowest obtained NOAEL value is used, in order to take into account the most sensitive species, as well as the relevant effect occurring at the lowest dose possible. Whereas the NOAEL is a dose descriptor for an external dose, the NOAELsys is a dose descriptor of the systemic exposure to a substance and is calculated from the NOAEL by use of the proportion of the substance systemically absorbed.
NP	Nanoparticle
NRU	Neutral Red Uptake
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
OPPTS	Test Guidelines on Pesticides and Toxic Substances
P50, P90	50th, 90th percentile
PALS	Phase Analysis Light Scattering
PBPK	Physiologically based pharmacokinetics
PBPK modelling	Physiologically based pharmacokinetic modelling
PBTK	Physiologically based toxicokinetics
PBTK modelling	Physiologically based toxicokinetic modelling
Personal care products	Consumer products used: for beautification (make up products) and in personal hygiene (shower gel, skin cream, shampoo, feminine hygiene products, diapers, toilet paper etc.)
PET	Positron Emission Tomography
PIF	Product Information File
POD	Point of Departure

POD_{sys}	The POD_{sys} is a dose descriptor for the systemic exposure to a substance and is calculated from the oral POD by use of the proportion of the substance systemically absorbed.
Pow	n-octanol / water partition coefficient
PPAR	Peroxisome proliferator-activated receptor
ppm	Parts per million (*e.g.* mg/kg)
PPRA	Peroxidase Peptide Reactivity Assay
PTA/NTA	Particle Tracking Analysis/Nanoparticle Tracking Analysis
QNAR	Quantitative Nanostructure Activity Relationship
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
Reference material	Material sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process (ISO, 2008).
RhCE	Reconstructed human Cornea-like Epithelium test method
RhE	Reconstructed Human Epidermis
RIP-oNs	The REACH Implementation Projects on Nanomaterials (RIP-oNs) – aimed at providing scientific and technical advice on key aspects of the implementation of REACH in regard to nanomaterials
RIVM	Rijks Instituut voor Volksgezondheid en Milieu
rLLNA	Reduced Local Lymph Node Assay
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RS	Raman Spectroscopy
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAED	Selected Area Electronic Diffraction
SAR	Structure-activity relationship
SC	Stratum Corneum
SCC	Scientific Committee on Cosmetology
SCCNFP	Scientific Committee on Cosmetic products and Non-Food Products intended for consumers
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SCHEER	Scientific Committee on Health, Environmental and Emerging Risks
SCs	Scientific Committees
SED	The Systemic Exposure Dose of a cosmetic ingredient is the amount expected to enter the blood stream (and therefore be systemically available) per kg body weight and per day. It is expressed in mg/kg body weight/day. For this definition a mean human body weight of 60 kg is commonly accepted. Since the majority of cosmetic products are applied topically, systemic availability will strongly depend on the dermal absorption of the compound. This can be determined according to the tests described in Section 3-4.1.1. Nevertheless, the results of these tests can be interpreted in two different ways (see Section 3-12.2: dermal absorption issues).

SD	Standard Deviation of the mean
SEM	Scanning Electron Microscopy
SENS-IS®	an *in vitro* model that measures keratinocyte activation using the human skin model Episkin™ RhE
SERS	Surface Enhanced Raman Spectroscopy or Surface Enhanced Raman Scattering
SHE	Syrian Hamster Embryo
SIT	Skin Irritation Test
SMPS	Scanning Mobility Particle Sizer
SPM	Scanning Probe Microscopy
Spray, sprayable cosmetic product	A formulation is either dispensed by the use of propellant gas as defined in Directive 75/324 (propellant spray), or by a spray bottle with a pump dispenser that forces a liquid through a nozzle generating a spray stream or a mist of a liquid (pump spray) (SCCS/1539/14).
SRB	Sulforhodamine B
SSA	Specific Surface Area
S9	Fraction (supernatant) containing cytosol and microsomes of cells after centrifugation at 9000g
STEM	Scanning Transmission Electron Microscopy
Substance	A chemical element and its compounds in the natural state or obtained by any manufacturing process, including any additive necessary to preserve its stability and any impurity deriving from the process used but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (2009/1223/EC).
Systemic effects	Systemic effect refers to an adverse health effect that takes place at a location distant from the body's initial point of contact and presupposes absorption has taken place.
TBA	Thiobarbituric Acid
TEM	Transmission Electron Microscopy
TG	Test Guideline
TH	Thoracic
Tk	Thymidine Kinase
Toxicodynamics	Cover the process of interaction of chemical substances with target sites and the subsequent reactions leading to adverse effects (ECB, 2003).
Toxicokinetics	Describe the time-dependent fate of a substance within the body and include absorption, distribution, biotransformation and/or excretion (ADME) (ECB, 2003)
TTC	Threshold of Toxicological Concern
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
Undesirable effect	An adverse reaction for human health attributable to the normal or reasonably foreseeable use of a cosmetic
VSSA	Volume Specific Surface Area