INTERNATIONAL COOPERATION ON COSMETICS REGULATION

Report of the ICCR Working Group:

SAFETY APPROACHES TO NANOMATERIALS IN COSMETICS

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EXECUTIVE SUMMARY

- An ICCR Joint Regulator-Industry Working Group (WG) carried out a review of the existing safety approaches in regard to the use of nanomaterials in cosmetics, to identify any specific aspects relevant to consumer safety.
- The WG considered various relevant scientific publications, reports, opinions, guidance documents, etc, and compiled Experts' views on the key safety aspects that should be considered in relation to the use of nanomaterials in cosmetic products. Irrespective of the presence or absence of nanomaterials in a cosmetic product, general safety considerations for testing of ingredients and their safety evaluation as provided by the ICCR report Principles of Cosmetic Product Safety Assessment1, and/or other requirements under specific regulatory frameworks should be followed.
- The existing risk assessment paradigm (based on exposure assessment, hazard identification and hazard characterization, followed by risk characterization), in use for conventional chemicals, is also applicable to nanomaterials.
- In general, the methods used for toxicological investigation of conventional materials are also applicable to nanomaterials. However, some methods may need adaptations in view of the distinctive physicochemical characteristics, agglomeration/aggregation behavior, uptake and biokinetics of nanoparticles, and in regard to sample preparation and dosimetry considerations.
- Detailed characterization is crucial for safety assessment of nanomaterials. The WG identified those physicochemical parameters that should be measured for nanomaterials at the raw material stage, in a cosmetic formulation, and as delivered to the end-user. Characterization should also ascertain that the nanomaterial is the same (or reasonably similar) to that intended for use in the final product.
- Any nano-related properties of a material are intrinsically linked to the physical integrity of the nano-structure. Where a nanomaterial loses its nano-structure, whether in a formulation, test media, or biological environment, it will no longer be expected to behave any differently from the non-nano equivalent.
- Determination of systemic exposure and investigations into local effects, carried out in consideration of the nano-related aspects, are among the most crucial elements of an exposure-driven safety assessment. The assessment should also consider the foreseeable uses of a cosmetic product, and the possible routes of exposure (dermal, respiratory, oral).
- Where the evidence shows systemic absorption, further investigations should be carried out to confirm whether the absorbed material was in particle form or in a solubilized/ metabolized form. Further investigations should focus on ADME (absorption, distribution, metabolism, excretion) to investigate fate and behavior of the nanoparticles in the body, and identify the likely target organs.
- In case of (very) low absorption of a nanomaterial, processes such as accumulation should also be considered.
- Testing of nanomaterials for exposure assessment or hazard identification/characterization should consider certain nano-related aspects, such as insoluble or partially-soluble particle nature, agglomeration and aggregation behavior in test media and biological environment, potential to penetrate biological membranes, possible interactions with biological entities, surface adsorption/ binding of other moieties, surface catalyzed reactions, stability and persistence, etc.

[1] ICCR (2011a) Principles of cosmetic product safety assessment, A Report prepared for ICCR, available at http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/iccr5_safety_en.pdf

- Nanoparticles may stick together, and/or to other materials, and form larger agglomerates or aggregates[2]. This can pose a challenge to maintaining a uniform concentration in a test medium for the duration of a test.
- Nanoparticles may adsorb or bind different moieties on surfaces. With the possibility of crossing cellular barriers, they may transport some unwanted substances from the test medium to the exposed test systems. This may cause artefacts and may give a false indication of harmful effects.
- Possible formulation effects should also be considered as certain formulations may enhance bioavailability and toxicological effects of active ingredients.
- The use of mass based dose metric alone may not be sufficient for nanomaterials, and other metrics, e.g. weight/volume concentration, particle number concentration, surface area, should also be considered.
- Currently, toxicological testing is carried out mainly in animals. However, the EU Cosmetics Regulation ((EC) No 1223/2009) establishes a prohibition on testing finished cosmetic products and cosmetic ingredients on animals (testing ban), and a prohibition on marketing in the European Community, finished cosmetic products and ingredients included in cosmetic products that were tested on animals (marketing ban)[3]. This makes the safety assessment of new nanomaterial cosmetic ingredients more difficult.
- A number of validated alternative methods can be used in place of animal tests for conventional substances. Although, none of the methods is yet validated for nanomaterials, they may still be relevant for hazard identification, and provide additional supporting evidence to the results of in vivo studies.
- Due to the current insufficient level of scientific understanding of the possible changes in properties, behavior, and effects of nanomaterials compared to conventional forms, the use of a read-across or categorization approach based on inter- or intra- nanomaterial extrapolation may not be feasible for safety assessment. However, on the basis of similar toxicity profiles in short-term toxicity studies, together with the outcome of genotoxicity and ADME, the extrapolation of toxicity data between selected non-nano and nano forms, or between different nano-forms of the same nanomaterial, may be justified (bridging toxicity approach).
- The overall risk characterization of a nanomaterial will not be any different from a conventional cosmetic ingredient. Where a given nanomaterial in a cosmetic product is well-characterized, both from a qualitative and quantitative points of view, and an adequate toxicological dataset is available, there should not be a reason to consider that risk characterization of the nanomaterial containing product is associated with an intrinsically higher uncertainty than that containing conventional ingredients. However, where this is not the case, a risk assessor may consider applying additional safety/uncertainty factors, similar to conventional chemicals.
- There is a need for research into the development and validation of characterization methods for nanomaterials as such, in final formulations, and during local and systemic exposures for toxicological evaluations. Research is also needed into the development of in vitro models that can mimic in vivo situation more closely, and well designed studies to generate high quality data for in silico modeling to identify the key parameters, derive rules, and develop predictive models to estimate physicochemical properties, biokinetic behavior, and toxicological effects of nanomaterials.

[2] In agglomerated form, the primary particles are held together by weak van der Waals forces that may disagglomerate under certain conditions, e.g. a change in pH. Compared to this, aggregates are usually formed during high energy manufacturing processes in which nanoparticles become fused together by metallic bonds or strongly bound by covalent bonds and are therefore unlikely to disaggregate under normal conditions.

[3] Cfr. Communication from the Commission to the European Parliament and the Council on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics (COM(2013) 135 final).

1.0 Introduction

1.1 Purpose

Discussions at the 4th annual meeting of International Cooperation on Cosmetics Regulation (ICCR-4) on cosmetics and cosmetic-like drug/quasi-drug products (hereinafter collectively referred to as cosmetics) in Canada in July 2010 led to the formation of a Joint Industry/Regulator Working Group (WG) for nanomaterial safety. The purpose of this Joint WG is to examine existing safety approaches for applicability to nanomaterials in use by, or relevant to, activities within the cosmetic industry.

1.2 Scope

The main task of this Joint WG was to carry out a review of existing safety approaches, identify any specific aspects relevant to consumer safety that should be taken into consideration when assessing nanomaterials in cosmetics, and produce a draft report for discussion by the ICCR members.

1.3 Approach

To achieve the objectives set out by this Joint WG, the members met regularly via audio-conferences and discussed the main issues in order to reach a consensus. In preparing this report, the members considered and discussed a number of key reports, opinions, guidance documents, and other documents, including those developed by other ICCR WGs, as well as relevant publications in the regulatory and scientific literature. The Joint WG produced this report with the aim to provide information to those intending to use or assess the safety of nanomaterials in a cosmetic product. This report expresses the Experts' views on the key safety aspects that need due consideration when assessing nanomaterials in cosmetic products. It is of note that the Joint WG did not focus exclusively on regulatory (mandatory) safety testing or on developing a strict a protocol for such safety assessments. However, for the sake of completeness, the Joint WG took the current requirements for safety assessment under the different regulatory frameworks within the ICCR jurisdictions into account in developing its approach.

The WG in their approach:

- used the ICCR report *Principles of Cosmetic Product Safety Assessment*[4] and other relevant information[5], and identified the important considerations for engineered nanomaterials in regard to physicochemical properties, possible routes and likelihood of exposure, potential toxicological effects, and assessment of safety when used in cosmetic products;
- identified the critical stages where a special consideration in relation to nano-related aspects may be necessary;
- identified the need for special considerations in regard to the use of currently available methods for safety testing of nanomaterials (including *in vitro* assays);
- highlighted physicochemical and/or toxicokinetic properties of nanomaterials that may trigger special (nano-related) safety considerations compared to conventional equivalents;
- investigated the possible basis for extrapolation of physicochemical and/or safety data between different morphological forms of a nanomaterial – or between different types of nanomaterials;

[4] ICCR (2011a) Principles of cosmetic product safety assessment, A report prepared for ICCR, available at http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/iccr5_safety_en.pdf

[5] Derived from reports, opinions, and guidance documents from Cosmetics Industry, OECD, ISO, SCCP, SCCS, EFSA, JRC, SCENIHR, EPA, as well as peer-reviewed articles in scientific journals.

- explored the possible use of short exploratory toxicological studies as a bridging approach to determine whether or not detailed investigations need to be undertaken.

1.4 Definitions

In preparing this report, the WG used the following criteria developed by the ICCR Ad Hoc Working Group on Nanotechnology in 2010[6].

"For purposes of the International Cooperation on Cosmetic Regulation, a nano-ingredient is an insoluble particle, intentionally manufactured, with one or more dimensions on the order of 1 to 100 nanometers in the final formulation and should be sufficiently stable and persistent in biological media to allow for the potential of interaction with biological systems".

These criteria provide a basis for deciding whether or not a cosmetic ingredient would fall under the definition of a nanomaterial, and hence need safety assessment with certain special considerations of nano-related characteristics.

1.5 Safety considerations

1.5.1 General Considerations

Cosmetics and personal care sectors represent an area with current interest regarding nanomaterial applications. The main reasons nanomaterials are used in cosmetics include improved dispersibility of ingredients, antimicrobial or antioxidant properties, visual clarity of sunscreen formulations, etc. A number of nanomaterial-containing cosmetic products are available on the global market[7,8]. These include skin lotions and creams, lipsticks and balms, toothpastes, shampoos etc. The largest category amongst these is sunscreen products containing nanomaterials as UV filters. Typical examples of the nanomaterials used in cosmetic products include inorganic materials, such as titanium dioxide (TiO_2), zinc oxide (ZnO), gold (Au), silica (SiO_2); carbon materials such as fullerene (C60); and organic nanomaterials7,8. The nanomaterials used in cosmetic products include uncoated, coated, or doped materials. Despite the different material types, a common denominator amongst all nanomaterials is the very small size, which is also the most important point of consideration to reflect on when assessing the potential risks associated with their use in cosmetic products. It is understood from the scientific literature that some of the conventional physicochemical rules may not fully apply at the nanoscale, and some nanomaterials may show a change in physicochemical properties, behaviour, and/or effects, compared to conventional equivalent that can impact the risk assessment of these materials[9]. For example, the small particle size has the potential to alter the distribution and biological availability in a number of ways compared to the equivalent bulk size particles. Nanoscale forms may cross membranes more readily and gain access to biological compartments normally not accessible by particulates. The smaller size also means increased surface area relative to the mass of the particle, which could result in increased biological interactions, and in some cases faster rates of solubility. Even detection by macrophages and other cells in the reticular endothelial system, which are normally effective in clearing particulates, may not be as effective in detecting and clearing particles in the nanoscale. All of these factors can contribute to changes in availability of nanoscale materials compared to bulk forms and can subsequently impact the exposure portion of the risk assessment process.

[6] www.fda.gov/downloads/InternationalPrograms/HarmonizationInitiatives/UCM235485.pdf

[7] Woodrow Wilson International Centre for Scholars (2010) The Nanotechnology Consumer Inventory Available at: www.nanotechproject.org/inventories/consumer/, accessed 28 August 2012.

[8] ICCR Report on the 2011 Associations Survey of Nanomaterials Used in Cosmetic Products, June 3, 2011 http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/iccr5_nano_en.pdf

[9] In this report, the term 'conventional' materials has been used for all non-nano forms of a material – i.e. a material comprised of larger particles, as well as atomic, ionic, molecular, gaseous or dissolved forms of the same chemical substance as that of a nanomaterial.

Like other substances, the assessment of nanomaterial safety will require data and information on both exposure and hazard, and a lack of either exposure or hazard (or both) will be construed as no risk to the consumer. Thus, on hypothetical grounds, a health risk to the consumer from the use of a nanomaterial-containing cosmetic product should only arise if:

1. the use of the product could lead to a systemic exposure to nanoparticles; and
2. such an exposure could lead to harmful effect(s) at the local and/or systemic levels.

This means that where the use of a cosmetic product containing nanomaterial(s) does not give rise to systemic exposure to nanoparticles, or local effects, it may be regarded of no risk to the consumer. Unlike conventional chemicals, toxicological testing of nanomaterials using available methods may not be so straightforward due to their certain distinctive properties and behaviour (see Section 1.5.2). The exact role of different physicochemical parameters in driving the properties and/or effects of nanomaterials is currently not fully understood. It has, ,not been possible so far to derive a meaningful extrapolation from the enormous amount of existing safety data on conventional chemicals to predict the properties and/or effects of nanomaterials. It is also unlikely that detailed toxicological data on new nano cosmetic ingredients will be available in the short term. A possible way forward in the interim is to consider exposure in the assessment of nanomaterial safety. A scheme for approaching the safety assessment of nanomaterials in cosmetic products is proposed in Figure 1. This approach proposes a few key questions that should be asked to guide such safety assessment and/or testing where most appropriate. These include:

1. Is an ingredient intended for use in a cosmetic product a nanomaterial (on the basis of ICCR criteria and characterization data)?
2. Could the use of the nanomaterial in a cosmetic product give rise to systemic exposure to nanoparticles (considering all possible routes)?
3. Could such exposure lead to toxicological effect(s) at the local and/or systemic levels?
4. Could the use of such a product pose a health risk to the consumer?

Thus, a key consideration in the safety assessment of nanomaterials is whether or not systemic exposure and/or local effects are possible during the foreseeable use(s) of a nanomaterial-containing cosmetic product. As outlined in Figure 1, safety testing may start with a detailed characterization of nanomaterials, followed by determination of the exposure both at local and systemic levels considering the likely routes. Appropriate evaluations should be carried out to determine the possible translocation of nanoparticles across dermal, respiratory, or gastrointestinal barriers (as appropriate for the specific use(s) of a given product). In addition, an important aspect to investigate at this stage is whether there are any local effects. These should be determined in consideration of the likely routes, e.g. on skin after dermal application, or respiratory tract after a spray application.

The data on systemic absorption of nanoparticles, and on the possible manifestation of local effects, should inform the need for further steps towards a detailed nano-related safety assessment. Where investigations show the possibility of systemic exposure to nanomaterials from the use of a cosmetic product, and/or a localized effect, further investigations into hazard identification and characterization with consideration to nano-related aspects should be carried out (see Sections 2, 3 and 4).

Besides the very small size, other physicochemical properties are also likely to influence the distinctive properties, behaviour, and/or effects of a nanomaterial. Therefore, detailed characterization of the nanomaterial should be a crucial part of any corresponding safety assessment. This includes proper identification of the constituents that make up a nanomaterial, as well as characteristics and properties of the nanostructure itself. The key parameters to consider in this regard should include chemical composition, particle nature, morphological form, agglomeration/aggregation behaviour, surface characteristics, persistence behaviour, etc (Rocks et al., 2008; SCENIHR, 2009; OECD, 2009; Chaudhry et al., 2010). Certain surface modifications,

and/or coatings[10], may play a very important role in that they may enable nanoparticles to penetrate membrane barriers more easily, or protect the 'core' nanomaterial from degradation processes during translocation. Similarly, because of the high surface energy, nanomaterials may bind certain unwanted substances on the surface (Šimon and Joner, 2008), and transport them across biological membrane barriers. It is important that physicochemical and morphological properties of nanomaterials be determined using appropriate characterization methods at different stages of product development (see Section 2).

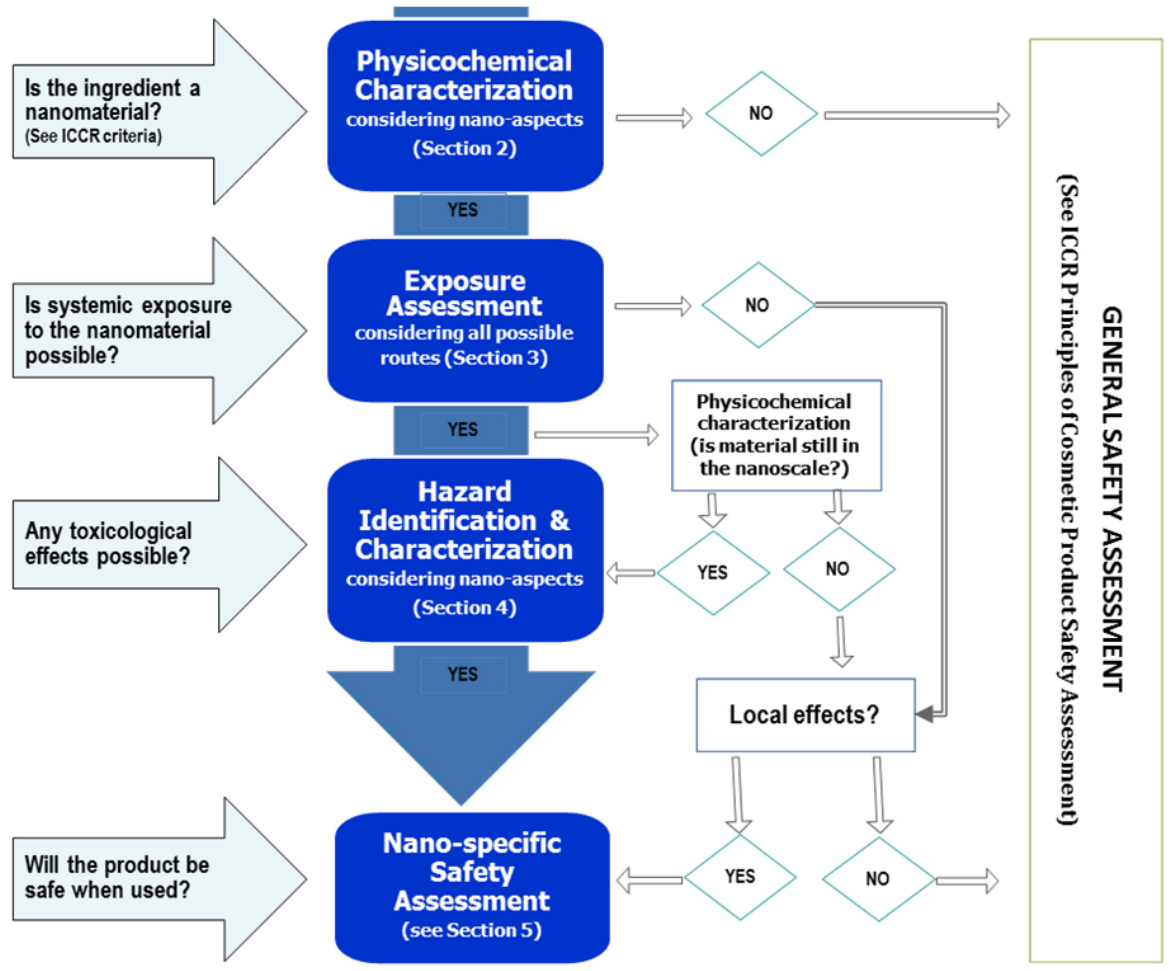

Figure 1: A schematic outline for safety assessment of nanomaterials in cosmetics

It should be noted that interactions of a nanomaterial with the surrounding media in a formulation or test system may also bring about certain changes in the physicochemical properties of the nanomaterial, such as the binding of chemicals or biomolecules onto nanomaterial surfaces (Cedervall et al., 2007, Šimon and Joner 2008, Lynch and Dawson 2008). Consideration should be given to not only the distinctive properties of nanomaterials, but also to any possible changes in relative properties during nanomaterial risk characterization (see Section 5). In view of the possible

[10] Functionalisation would refer to an alteration of the nanomaterial surface by a chemical reaction, creating a *de facto* new nanomaterial, whereas a coating would be an envelope around the nanomaterial that is not bound and could be removed in the body exposing the tissue to the bare material.

changes in nanomaterial properties, it is recommended that characterization is carried out at the following three stages:

1. in the raw materials as manufactured, i.e. before adding to a cosmetic formulation
2. after addition to the final cosmetic formulation
3. during exposures at the local and systemic levels.

In the first instance the characterization should establish identity of the nanomaterial to ascertain that the tested nanomaterial is in the exact (or comparable) form/composition as the material intended for use in a cosmetic formulation. Where toxicological data do not relate to a nanomaterial intended for use in a cosmetic formulation, justification may be necessary to indicate that there is sufficient similarity to another nanomaterial to allow for "read-across" using toxicological data..

1.5.2 Specific considerations relating to Nanomaterials

Most aspects of the safety assessment of nanomaterials, including material characterization, exposure assessment, toxicological testing and overall safety evaluation, are carried out much the same way as for the non-nano forms. Nevertheless, certain nanoscale properties, agglomeration/aggregation behaviour, and potentially altered uptake and biokinetics may pose additional challenges to safety testing of nanomaterials. A number of reports and reviews have concluded that the existing risk assessment paradigm, used for conventional chemicals, should in principle be also applicable to nanomaterials. At the same time, the need for nano-related considerations and possible adaptations in the current testing methods, to take account of the special features of nanomaterials, have also been highlighted (Rocks et al. 2008, SCENIHR 2009, OECD 2009). More specifically, these aspects include the following key considerations.

1.5.2.1 Physicochemical characteristics

The properties, behaviour, and biological effects of nanomaterials are influenced by various physicochemical parameters. The safety evaluation of a nanomaterial must include measurement of some important parameters (see Section 2) for the same, or a justifiably comparable[11], nanomaterial that is intended for use in the final cosmetic product.

Toxicologists and safety assessors will need to keep in mind that nano-related properties are intrinsically linked to the physical integrity of the nano-structure of a nanomaterial. Where a nanomaterial loses nano-structure, whether in a formulation, in test media, or in biological environment due to solubilization, breakdown or degradation, it will not be expected to behave any differently from its non-nano equivalent. On the other hand, if a nanomaterial maintains its structure it may display different distribution characteristics than the larger-sized particles of the same chemical composition. In this situation, the potential for effects in other targets would have to be considered. Determining stability of nano-structure in a nanomaterial under experimental conditions is of prime importance for the interpretation of any test results. Stability may be measured in terms of dissociation constant, dissolution rate, and solubility of a nanomaterial in the final cosmetic product and in the media/vehicle(s) used in exposure/hazard evaluations. Consideration should be given to avoid any batch variations in terms of physicochemical and morphological characteristics between the nanomaterials that are tested for safety, and those added to the final product. The presence of certain impurities or contaminants can also have an effect on the interpretation of all studies regardless of the test material, but this possibility takes on a particular significance with nanomaterials because of the lack of historical data that can be employed to help interpret findings. For example, carryover of certain impurities/contaminants from a low-efficiency manufacturing process could lead to erroneous conclusions pertaining to the toxicity of nanomaterials, whereas the toxicity may in fact be due to the impurities/contaminants. This has been shown in the case of carbon nanotubes where removal of metal contaminants led to a loss of the toxic effects that were observed for the material in an unpurified state (Pulskamp et al., 2007).

[11] On the basis of chemical purity/impurity profile and physicochemical characteristics

Although a wide range of analytical techniques is available for measurement of physicochemical properties of nanomaterials (e.g. see CDER, 2010[12]; EFSA, 2011; ICCR, 2011a), many of these have not yet been validated for nanomaterials. However, as indicated by EFSA guidance (2011), a careful choice of the mainstream method(s) should provide sufficient data for the purpose of adequately characterizing a nanomaterial (Section 2). Where characterization of a nanomaterial at any stage is not feasible, for example, due to lack of methods, or degradation of the nanomaterial, it should be justified and documented.

1.5.2.2 Factors affecting distribution and elimination

Translocation, Bioavailability and Biokinetics

The potential for nanoparticles (especially in the lower nm range) to cross cellular and sub-cellular membrane barriers has added another dimension to the evaluation of particulate materials. This may have an impact on the distribution of these particles. As mentioned previously, some nanomaterials, particularly those in the lower nanometer (nm) range, may be able to cross biological membrane barriers that normally prevent the entry of (larger) particulate materials into systemic circulation (e.g. Jani et al., 1990, Geiser and Kreyling 2010). It is possible that, if internalised, some insoluble or partially-soluble nanoparticles may reach those parts of the body that are otherwise protected from entry of (larger) particles, such as brain, liver, kidney, etc. Where the translocating nanoparticles are insoluble, partially-soluble, and/or persistent in nature, their exposure may lead to biological effects due to the potential interaction with organ and cellular compartments different from what is accessed by bulk-sized forms of the same material.

It should also be remembered that the toxico-kinetics/dynamics of particulate materials may be different from conventional chemicals. Because of this, there may be a toxic effect which is not expected from the conventional form of the same material. This may also vary depending on the exposure route (oral, inhalation, dermal), and/or the quality of the biological barriers (e.g. compromised skin versus healthy intact skin). Although the current hazard identification/characterization framework should pick up biological effects of nanomaterials, it is not certain whether the endpoints identified under the current testing schemes will be sufficient to identify and characterize all the potential hazards that may be associated with the use of nanomaterials. Thus, it is logical that, in the first instance, safety assessment of nanomaterials be driven by considerations of exposure. Initial focus of safety considerations should be based on determining the likelihood and extent of translocation of nanomaterials across skin, lung, or gastrointestinal barriers (as appropriate for a given product use). Where there is evidence for systemic translocation of nanoparticles, further investigations into ADME (absorption, distribution, metabolism and excretion) parameters should take special importance. Emphasis should be placed on toxicological tests that are carried out over prolonged periods with repeated doses and followed up by histopathological investigations.

Solubility/dispersion:

With regard to toxicological testing methods, special attention should be paid to agglomeration/ aggregation behaviour, and the insoluble or partially-soluble particle nature of nanomaterials (Rocks et al., 2008; SCENIHR, 2009; OECD, 2009; Chaudhry et al., 2010). Safety testing of insoluble or partially-soluble nanomaterials should take into account that they will be present in a test medium as a nano-dispersion rather than a solution. Because of high surface forces, nanoparticles also tend to stick to each other to form larger agglomerates or aggregates. In agglomerated form, the primary particles are held together by weak van der Waals forces that may disagglomerate under certain conditions, e.g. a change in pH. Compared to this, aggregates are usually formed during high energy manufacturing processes in which nanoparticles become fused together by metallic bonds or are strongly bound by covalent bonds and are unlikely to disaggregate under normal conditions. Depending on the proportion of free nanoparticles and agglomerate/aggregates, a nanomaterial dispersion may or may not have uniform consistency in terms of concentration. This will pose a major challenge to safety testing in terms of maintaining a uniform concentration of the nanomaterial in a test medium for the duration of the test. The applied concentration of a nanomaterial may also drop during a test due to sedimentation, binding with

[12] CDER (2010) Center for Drug Evaluation and Research, Office of Pharmaceutical Science, Reporting Format for Nanotechnology-Related Information in CMC Review, Manual of Policies and Procedures - Mapp 5015.9.

other moieties, or sticking to sides of the glass/plastic ware. It is important to ascertain the stability and uniformity of a nanomaterial in a given test medium and to ensure that the applied concentration is uniformly maintained during the test. Possibilities for disagglomeration of nanoparticles should also be considered.

For (partially) soluble nanomaterials, the toxicity may be governed at least, in part, by the soluble species released from the nanomaterial. For low solubility or slow release nanomaterial, the particulate nature of the substance may be relevant with regard to tissue distribution and local release of toxic species, which should be considered in the risk assessment of such nanomaterials. The dissolution rate of partially-soluble nanomaterials is an important criterion for consideration in nanomaterial safety assessments.

1.5.2.3 Other factors impacting risk assessment

Surface adsorption/binding

Nanomaterials may change properties in biological testing systems. As a result of high surface energies, nanoparticles may adsorb or bind different substances on surfaces, including proteins (Cedervall et al., 2007, Šimon and Joner 2008, Lynch and Dawson 2008). With the possibility of crossing cellular barriers, they may transport certain unwanted substances from the test medium to the exposed test systems. This may lead to artifacts and false indication of harmful effects. For example, the usual way of administering a test substance to a biological system is in the form of a dry powder or a suspension in aqueous or other media. When nanoparticles contact a biological fluid they may become coated with different biomolecules and media components, such as albumin or lung lining fluid phospholipids used for dispersion of proteins. Other substances present in the media, such as dyes, may also bind to the nanomaterial surface and be transported into cells and tissues. Whilst they may interfere with the assay, some others such as polyoxyethylene sorbitan monooleate and Tween used for dispersion, may themselves be toxic (SCCS, 2009). Although many of the possible artifacts can be eliminated by a careful use of controls within the testing scheme, it is advisable that a thorough characterization of nanomaterials is carried out in the test medium to determine any changes in nanomaterial surface characteristics during a toxicological evaluation.

Formulation effects

Nanomaterials may be soluble and/or biodegradable (e.g. nanovesicles, nanoemulsions) or insoluble and/or biopersistent (e.g. some metals, metal oxides). It has not been shown that nanovesicles and/or nanoemulsions can penetrate intact skin. These nanosized structures generally disintegrate into their molecular components and lose nano characteristics. However, certain constituents in the formulation can modify the bioavailability and toxicological behaviour of dispersed active ingredients. For example, lipids or surfactants may act as penetration enhancers, where nanomaterials may be able to penetrate individually into the stratum corneum (after particle disruption on skin surface) and subsequently alter the intercellular lipid lamellae within this skin layer (SCCP 2007).

Metrics for toxicological measurements

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 chemical substances. However, these conventional metrics may not be appropriate for nanomaterials 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 nanomaterials are evaluated using appropriate dose-describing metrics in addition to mass, such as weight/volume concentration, particle number concentration, surface area etc. Scientific data suggest that the total surface area of nanomaterials is a reasonable metric to describe toxicological responses in biological systems (SCENIHR, 2009). According to OECD WPMN (2010)[13], dosimetry should always report mass concentration, but for nanomaterials, the results may be better expressed as a function of surface area or particle number because particle size and total surface area may play a major role in determining the toxicity of nanomaterials. Similarly, recent documents issued in the context of the

[13] ENV/JM/MONO(2010)25. Available online: www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2010)25&doclanguage=en

EU REACH regulation (Reach RIP-ON-2 and Reach RIP-ON-3) concluded that "There are currently no definitive conclusions on the best dose metric for exposure assessment" and that "the recommended practice at this time is that measurement should at least encompass mass, but where possible also number and/or surface area concentration."

Characterization data on a nanomaterial should provide sufficient information to allow conversion of doses based on mass into other metrics, such as number of particles and/or surface area.

2.0 Physicochemical characterization

Characterization of physicochemical properties forms a crucial part of the safety assessment of chemical substances in general. For characterization of nanomaterials, some of these parameters may need special nano-related considerations because they may differ significantly relative to conventional equivalents. Some physicochemical properties may be considered less relevant for safety assessments of traditional chemicals but may be more relevant for nanomaterials. Characterization is also important to demonstrate that the safety data relates to the same (or reasonably comparable) nanomaterials used in cosmetic products.

2.1 Important physicochemical parameters

In view of the importance of characterization in the safety assessment of nanomaterials, it is important that information on physicochemical parameters is available for consideration in the overall safety assessment. Due to the current gaps in knowledge with regard to a relationship between different physicochemical parameters and effects of nanomaterials, it has so far proved difficult to identify a shortlist of key parameters that can adequately describe a nanomaterial, and the characterization methods that can be used to measure them. Furthermore, the choice of parameters and characterization methods is also dependent on the composition, properties, and intended use(s) of individual nanomaterials. This has been the subject of discussions by several international expert committees and working groups. Their reports have regarded a number of physicochemical parameters important for consideration in the safety assessment of nanomaterials. Of these, this WG considered the key reports published by The OECD Working Party on Manufactured Nanomaterials (WPMN, 2009 and WPMN, 2010), the International Organization for Standardization (ISO 10808:2010), the EU's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2009), the EU's Scientific Committee on Consumer Products (SCCP, 2007), the European Food Safety Authority (EFSA, 2011), the EU's guidance documents on REACH (RIP-oNs), and relevant report of other ICCR Working Groups (2011a, 2011b). In addition, a document providing the Cosmetic Industry's perspective on specific characteristics of the safety assessment of nanomaterials used in cosmetic products (2011) was also considered. The important physicochemical parameters identified in these various expert reports are discussed and summarised below in Table 1.

2.1.1 OECD WPMN (2009)

A list of endpoints describing basic characterization, fate, and toxicity information on nanomaterials has been proposed by the OECD's Working Party on Manufactured Nanomaterials (2009)[14]. The document also lists possible methods to measure the physicochemical parameters. These parameters include particle size distribution, agglomeration/ aggregation, specific surface area, zeta potential, water solubility/ dispersibility, surface chemistry, crystalline phase, crystallite size, radical formation potential, photocatalytic activity, dustiness, pour density, porosity, representative electron microscopy (TEM) picture(s), octanol-water partition coefficient, redox potential.

2.1.2 OECD WPMN (2010)

In 2010, the OECD released preliminary guidance notes on sample preparation and dosimetry for the safety testing of manufactured nanomaterials[15]. It called special attention to using test

[14] ENV/JM/MONO(2009)20/REV. Available online: www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2009)20/REV&docLanguage=En

[15] ENV/JM/MONO(2010)25. Available online: www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2010)25&doclanguage=en

guidelines when considering the unique chemical and physical characteristics of nanomaterials. The guidance identified characteristics requiring determination to include, but not limited to, particle size, size distribution, aggregation, agglomeration state, shape, chemical composition, surface area, surface chemistry, dissociation constant, crystal structure, surface charge, zeta potential, Hamaker constant (van der Waal's forces), interfacial tension, and porosity.

2.1.3 ISO (2010)

The International Organization for Standardization published the first standard (ISO 10808:2010) to ensure that the results of inhalation toxicity tests of airborne nanoparticles are reliable and harmonised worldwide. It specifies requirements for, and provides guidance on, the characterization of airborne nanoparticles in inhalation exposure chambers, including a few essential physicochemical properties that should be taken into account when assessing the inhalation toxicity potential of nanomaterials. The main physicochemical properties that were deemed necessary for characterising nanomaterials include composition, morphology, particle size, size-distribution, particle number and mass concentrations, surface area, shape and dispersion, surface chemistry, hygroscopicity, and electrical charge.

With regard to inhalation toxicity testing, characterization of two measurements was considered essential - number-based particle size distribution, and total particle mass concentration. Particle size distribution measurement is deemed essential because the knowledge of particle size is crucial for interpreting and evaluating toxicity testing results, and mass concentration is critical as the dosimetric parameter for inhalation toxicity tests.

2.1.4 ICCR WG (2011b)

The ICCR WG report "Criteria and Methods of Detection" lists key properties for physicochemical characterization of nanomaterials[16]. These include chemical composition, size and size distribution, agglomeration/aggregation, mass concentration, particle number, shape, surface chemistry, surface charge, surface area, solubility/dispersibility, and stability. The report also discusses possible methods that can be used to measure the physicochemical parameters, and provides a useful information resource in regard to characterization of nanomaterials in cosmetic formulations.

2.1.5 SCENIHR (2009)

The EU's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) published their opinion on risk assessment of products of nanotechnologies in 2009. The opinion (SCENIHR, 2009) referred to relevant OECD and ISO work and identified a number of important criteria for consideration in risk assessment of products of nanotechnologies. The main physicochemical parameters of interest with respect to nanoparticle safety were identified as:

- *Physical properties:* size, shape, specific surface area, aspect ratio, agglomeration/aggregation state, size distribution, surface morphology/topography, structure (including crystallinity and defect structure), solubility.
- *Chemical properties:* structural formula/molecular structure, composition of nanomaterial (including degree of purity, known impurities or additives), phase identity, surface chemistry (which is meant to include composition, charge, tension, reactive sites, physical structure, photocatalytic properties, zeta potential), hydrophilicity/ lipophilicity

Of these specific properties, SCENIHR (2009) established that the key parameters that need to be characterized from a risk assessment point of view include size and size distribution of free particles and fibres/rods/tubes, specific surface area, stability in relevant media (including the ability to agglomerate and disagglomerate), surface adsorption properties, and water solubility.

[16] ICCR (2011b) Currently available methods for characterization of nanomaterials, ICCR Report of the Joint Regulator - Industry Ad Hoc Working Group, available at http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/iccr5_char_nano_en.pdf

2.1.6 SCCP (2007)

The EU's Scientific Committee on Consumer Products (SCCP) proposed parameters for physicochemical characterization of nanomaterials in cosmetic products (SCCP, 2007). These include:

Physical properties: size, shape (e.g. spherical or fibrous), surface area, surface charge, surface morphology, rheology, porosity, crystallinity and amorphicity, primary nanoparticles, agglomerates and/or aggregates.

Chemical properties: chemical composition, surface chemistry, oxidative capacity, catalytic activity, stoichiometry (may change for large surface to volume ratios), dissolution kinetics and solubility, hydrophilicity or hydrophobicity, surface coating, impurities (foreign elements, chemical by-products or degradation products etc), intentional or unintentional surface adsorbents, (both of which determine reactivity).

2.1.7 EFSA (2011)

The European Food Safety Authority (EFSA)'s Scientific Opinion on risk assessment of nanotechnologiy for food and feed applications (FSA, 2011) emphasised the need for adequate characterization of nanomaterials in terms of establishing identity and physicochemical forms both in food/feed products and under testing conditions. The EFSA opinion has a direct relevance to nanomaterial applications for cosmetics, because it also highlights and discusses the fact that nanomaterials present in the final products will be incorporated in complex matrices. As physicochemical parameters may change in various environments, the EFSA opinion recommends characterization of a nanomaterial to be ideally determined in five stages,

- as manufactured (pristine state, raw ingredients/additives)
- as delivered for use in food/ feed products
- as present in the food/ feed matrix
- as used in toxicity testing
- as present in biological fluids and tissues.

The important parameters identified in the EFSA opinion include chemical composition/ identity, particle size (primary/secondary), physical form and morphology, particle and mass concentration, specific surface area, surface chemistry, surface charge, redox potential, solubility and partition properties, pH, viscosity, density and pour density, chemical reactivity/catalytic activity, and photocatalytic activity.

2.1.8 REACH RIPoN3

In Europe, REACH (Registration, Evaluation, Authorisation of Chemicals - Regulation (EC) No 1907/2006) is the main chemicals regulation in the EU, which requires registration of all substances produced and/or marketed in the EU in quantities above 1 tonne/ year. To facilitate the implementation of REACH, the European Commission launched a comprehensive REACH Implementation Project on nanomaterials (RIPoN) in 2009. The RIPoN is aimed at providing advice on key aspects of the implementation of REACH with regard to nanomaterials concerning Information Requirements and Chemical Safety Assessment. The objective of one task within the project was to provide a summary analysis report pertaining to the characterization of the hazards and risk of nanomaterials to humans. In addition to size- and surface-related properties, the REACH RIPoN3 (2011) identified a number of other factors that are likely to be important in determining the possible interaction of nanomaterials and cells. These include surface energy; the balance between surface hydrophilicity and hydrophobicity; chemical structure and functional groups; type and density of surface charges; radical formation potential; surface topography and roughness, which vary according to the type of nanomaterial.

2.1.9 Cosmetic Industry's Perspective (2010)

The Cosmetics Industry provided a document detailing the industry's perspective on specific characteristics within the safety assessment framework of nanomaterials used as cosmetic

ingredients[17]. The document determined that the potential biological effects of nanomaterials are influenced by the particle number concentration, surface area and size (i.e. dosimetry). Further, it recognises that certain physicochemical properties of a nanomaterial may change during the time spent in various biological compartments, and during changes in the environment that can affect solubility, agglomeration and disagglomeration. The detection and characterization of nanomaterials is considered crucial for identifying and utilising appropriate safety evaluation methods for nanomaterials. However due to the broad scope of nanomaterial use by the cosmetic industry, and the fact that a consistent relationship has not yet been established between particle size and toxicity, it is recommended that the choice of parameters/methods used should be determined on a case-by-case basis.

2.2 Methods for nanomaterial characterization

A number of methods are currently available that can be employed to determine the physicochemical parameters for a given nanomaterial. These are based on a wide range of approaches that include microscopy, chromatography, electrophoresis, spectroscopy, spectrometry, centrifugation, particle counting/sizing and imaging, and their different variants and combinations. A recent ICCR WG report (2011a) provides an introduction to the most relevant methods for the characterization of nanomaterials in cosmetic products. Other reports, such as CDER (2010)[18], EFSA (2011), and the OECD's Working Party on Manufactured Nanomaterials report (2009) [19] also provide a list of appropriate methods that can be used for the detection/characterization of nanomaterials. The reproducibility and accuracy of any of the methods used for nanomaterial characterization will be largely dependent on sample preparation and calibration of the analytical tools against appropriate standards. The ICCR WG report (2011a) emphasised that 'no single method can, in and of itself, fully describe a nanomaterial. Sample preparation, conditions of use, or formulation milieu will all affect the state of the nanomaterial. Thus, great care must be taken in the reporting and interpretation of results. Indeed a material that may have one dimension in the nanoscale and be considered a nanomaterial based on one set of definitions may in fact present no nanoparticles under 'actual' conditions of use.

A careful choice and use of existing method(s) should provide sufficient data for such characterization. More confidence in measurements can be added by the use of more than one method. In this regard, EFSA guidance (2011) has recommended that the nanomaterial size parameter in food samples should be measured by at least two methods - one being electron microscopy. An ICCR WG on Characterization of Nanomaterials II is currently preparing a report on the methods to characterize solubility; stability, and persistence of nanomaterials in biological media; and measurement of size in the realm of 1 to 100 nm in final formulations. A list of the main characterization methods is provided in Annex Table A.

It is important to note that results of different measurement techniques may not be directly comparable. This is dependent on how different techniques measure a given parameter. Some techniques measure individual primary particles, while others measure aggregates or agglomerates. Some techniques require samples to be dispersed, and/or diluted. It is important to ensure a consistency between sample preparations to avoid artifacts and to allow comparable results from a given method. As discussed in the ICCR WG report (2011a), some of physicochemical parameters may differ when the nanoparticles are in different environments – e.g. a dry form, liquid suspension, or aerosol. Parameters such as size, aggregation states, surface charge, and other properties may change in different solvents, test media and biological environments (Sayes and Warheit, 2009). As different measurements may provide valuable information on different aspects of the physical form and behaviour of a nanoparticle, conditions under which measurements are made need a careful consideration and documentation.

[17] Cosmetic Industry Perspective on specific Characteristics of the Safety Assessment of nanomaterials used in Cosmetic Products (2011)

[18] CDER (2010) Center for Drug Evaluation and Research, Office of Pharmaceutical Science, Reporting Format for Nanotechnology-Related Information in CMC Review, Manual of Policies and Procedures - Mapp 5015.9.

[19] ENV/JM/MONO(2009)20/REV. Available online: www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2009)20/REV&docLanguage=En

Characterization of nanomaterials is generally more difficult in final products than in raw materials, because of the presence of complex matrices. The interaction of nanomaterial with matrix may form a dynamic "corona" surrounding the nanomaterial which may affect the behaviour of both the nanomaterials and the components of the matrix. It may be necessary to use a combination of methods for detection and characterization of a nanomaterial in final formulations that have complex matrices. Various methods and instruments for nanomaterial characterization in complex matrices are currently under development (e.g. the EU FP7 project Nanolyse - www.nanolyse.eu).

2.3 Summary

The reports discussed above list a number of physicochemical properties and parameters which have been considered important in relation to safety assessment of nanomaterials. The most relevant parameters are listed in Table 1.

Table 1

Parameter	Description
Chemical identity	chemical and common name(s), structural formula, molecular structure, CAS number (where available)
Chemical composition	purity, impurities, degradation products, additives, mass and particle number concentration
Size	Primary and secondary particle sizes, size distribution in terms of particle number and mass concentration, batch to batch variation
Morphology	shape, crystalline structure, agglomeration/ aggregation state
Surface characteristics	surface chemistry, morphology/topography, interfacial tension, surface charge (zeta potential), any chemical/ biochemical modifications or coatings, reactive sites
Solubility	aqueous solubility, dissolution rate, hydrophilicity/ lipophilicity
Surface area	(specific) surface area, and volume specific surface area (VSSA)
Catalytic activity	chemical/ biochemical reactivity, radical formation potential, photocatalytic activity
Stability	Stability/ dissociation constant in relevant media

Depending on composition of a nanomaterial, other parameters may also be important in relation to assessment of safety. These include

- dustiness of dry powders[20]
- density and pour density of granular materials[21]
- redox potential of inorganic materials[22]
- hygroscopicity for powders,
- pH in aqueous media
- viscosity of liquid dispersions[23]

As previously noted, a thorough physicochemical characterization of nanomaterials is of utmost importance in supporting safety assessments, and needs to be carried out at different stages (see above).

In general, characterization of nanomaterials in a cosmetic formulation is more difficult compared to characterization in a raw material, and even more challenging when the nanomaterial is in a biological system or has been released to the ecosphere. Depending on concentration of a nanomaterial, and nature of the formulation/ matrix, characterization may need isolation, purification and concentration steps before analysis. Characterization in a cosmetic product should also provide information on any changes in the nanomaterial characteristics during formulation, e.g. in terms of primary/secondary particle sizes, chemical composition, surface characteristics, etc. These parameters should also be considered when evaluating stability and shelf life of a nanomaterial in the final product. Similar care is needed during toxicological evaluations as certain parameters, such as size, aggregation states, surface charge, coatings and other properties, may change in different solvents, test media, and biological environments.

In brief, it is important that any nanomaterial intended for use in a cosmetic product is characterized as thoroughly as possible in raw materials as manufactured, in the final formulation, and during exposures for toxicological investigations at the local and systemic levels.

[20] using methods such as EN 15051:2006, DIN 33897-2)
[21] using methods such as DIN ISO 697, EN/ISO 60
[22] using potentiometric methods
[23] using methods such as OECD TG 114

Characterization should include determination of chemical identity, purity/impurity profile, size and morphological parameters, agglomeration/aggregation state, solubility/ dissolution rate, surface characteristics, and other parameters outlined in Table 1. More information on the parameters and the methods to measure them is provided in Annex Table A.

3.0 Exposure assessment

Estimating exposure of cosmetic ingredients is an essential element of the safety assessment of a cosmetic product. Exposure assessment is carried out in consideration of the likely route(s) of exposure in realistic usage scenario(s) of the products. Most aspects of the safety assessment process, including exposure assessments, are conducted much the same way they are conducted for non-nano forms. However, there are significant challenges in estimating exposure for nanomaterials that deserve additional consideration. As described in the previous section, studies to estimate exposure to nanomaterials should consider not only the chemical characteristics of the nanomaterial but also the physical form since this can have a significant effect on absorption, distribution, and elimination kinetics due to the small size of the materials and the tendency to form aggregates/ agglomerates. Even the metrics used to quantify exposure need to be carefully considered since particle mass may not always provide the best prediction of potential biological effects (see Section 2). In the following sections, these specific considerations are discussed.

3.1 General considerations for estimates of exposure to nanomaterials

Since the goal of the exposure assessment component of a safety assessment of a cosmetic product is to provide a justifiable estimate of exposure from product use, it is important to be aware of, and report on, those properties that can affect exposure and dosimetry.

Many of these properties were identified in a recent opinion issued under the European REACH program RIP-oN3 (2011). These included discrimination from background nanoparticles, measurement of size distribution, maximum relevant particle size, consideration of high aspect ratio nanomaterials, application of exposure models, choice of exposure metric, and instrument measurement strategy.

3.1.1 Size

Mechanical and biological barriers designed to control absorption of particles or remove particles once absorbed, may not be as effective with nanomaterials because of the small size of these materials. This could result in different patterns of absorption, distribution, and elimination. It should be kept in mind that even the relatively smaller sizes of nanoparticles are still much larger than many organic materials and polymers commonly used in cosmetics. In addition to size, other physicochemical factors that can be important in predicting exposure include surface charge, and the presence of surface coatings, including artifacts that can adsorb to the surface of the particles (see section 1.5).

3.1.2 High Surface Area

Nanoscale particles have high ratios of surface area to mass compared to their larger sized forms. Depending on the chemical composition, larger surface area may result in increased rates of solubility of some nanomaterials which can affect bioavailability compared to larger scale forms of the material. A larger surface area may also lead to an increased reactivity of the nanoparticles, and this is an important consideration in the investigations for possible toxicological effects. In addition, the larger surface area to mass ratio could also lead to changes in surface chemistry characteristics that could impact biological effects.

3.1.3 Shape

Most definitions consider particles with one dimension in the nano-scale range to be nanomaterials but that ignores the potential effect of the other dimensions on exposure. Thus, length and shape can also play a role in determining exposure, and potential biological effects. High ratios of diameter to length can affect the interaction of the particle with mechanical barriers such as the epidermis, pulmonary alveolar surface or recognition by biological defences, such as macrophages

or other elements of the reticuloendothelial system. Therefore particle shape should be included in test material characterization and considered in the assessment of exposure.

3.1.4 Aggregates/Agglomerates

As mentioned before (section 1.5.2.2), nanomaterials tend to form clusters, either as aggregates or agglomerates. The latter being held together by weak forces that may disagglomerate under certain conditions. As a result, estimates of exposure to nanomaterials will almost always be a mix of primary particles and cluster forms, and should also consider the possible dis-agglomeration of the clusters. This distribution should be considered in estimating exposure and included in description of the exposure estimates.

The high surface area of the aggregate should also be considered in the risk assessment process, particularly for those biological effects mediated by surface chemistry effects.

3.1.5 Dose metrics

The toxicological hazards of nearly all chemical substances are measured and expressed in weight or volume units (such as mg/kg, or mg/l). For nanomaterials there is an additional consideration that warrants attention. In general, a decreased particle size results in an increase in total surface area and particle number for any given mass. Although there is evidence that increased surface area may be associated with an increase in biological activity or toxicity, this is not yet a general assumption that can be applied across all forms of nanomaterials (Warheit, 2007). In consideration of this, an OECD Working Group (OECD 2010) concluded that "dosimetry should always report mass concentration" and that "*empirical results will continue to be reported in terms of mass based units* ". However, some results may be further expressed as a function of surface area or particle number because these may provide more relevant estimates of the delivered dose (or relative exposures). Sufficient characterization of a material is important to allow results to be expressed in different metrics (see Section 2).

3.2 Routes of exposure relevant to cosmetic use of nanomaterials

Because the definition of cosmetic differs in many jurisdictions, relevant routes of exposure will be taken into consideration. For example, in the United States and Canada, the definition used for cosmetics excludes sunscreens and fluoride-containing toothpastes under their Federal statutes, while in the European Union sunscreens and toothpastes are considered cosmetics under the Cosmetics Directive. In Japan, under the Pharmaceutical Affairs Law, sunscreens and toothpastes are considered cosmetics. They may be considered quasi-drug products if they contain any chemical with pharmaceutical application. For the majority of products sold as cosmetics around the world, skin is the intended site of application and dermal exposure is therefore the primary relevant route.

Dermal exposure has two fundamental considerations – the skin as a target organ and skin as a portal of entry for systemic exposure. Both are discussed below.

3.2.1 Skin as a target organ

When considering skin as a target organ for nanomaterial exposure the basic approach is the same as for non-nano forms and both irritation/corrosion and allergenic contact dermatitis are relevant endpoints to be evaluated as discussed in section 4. For both of these endpoints, the exposure metric is generally normalized on the basis of surface area exposed. This approach acknowledges that since skin is the target, it is the ratio of skin to nanomaterial contact that is important for the assessment of exposure.

The challenge presented with nanomaterials is in adequately characterizing the form of the material that comes into contact with the skin. As discussed earlier, nanomaterials form aggregates and agglomerates which can confound the estimate of exposure. Presumably the nanomaterial will be in a product matrix and this matrix will largely determine the distribution of primary particles, aggregates and agglomerates. That characterization will most often be adequate for estimating exposure to the nanomaterial.

3.2.2 Skin as a portal of entry

Skin, the largest organ of the body, is a complex dynamic organ that has several functions, the primary one being to act as a barrier to the external environment (Rice and Mauro 2008). Evaluations of dermal exposures for the purposes of estimating systemic exposures follow the same general process as for non-nano scale materials. It is important to determine the amount of material in contact with the skin and the time course of such exposure. Because of differences in product use, each estimate of exposure needs to consider product use and account for differences in habits and practices of use. When available, reference to published practices is encouraged. Some sources include, but are not limited to, the EPA exposure handbook (US EPA 2011) trade association publications such as the study conducted by the European Cosmetic Trade Association - COLIPA (Hall, et al, 2007). While none of these specifically address exposure to nanomaterials, they can be a good source of information of product use.

3.2.3 Inhalation

A secondary route of exposure that is common for cosmetics is inhalation. This is most often the result of spray products such as hair sprays when droplet size is in the respirable range (generally considered as < 10μm). Even when the target is skin application, inhalation may become relevant as a secondary source of exposure from aerosols generated during typical cleansing routines and other types of cosmetic product use. Most of these secondary sources are minor but nevertheless have a place for consideration in an exposure assessment program.

From an exposure perspective the biggest challenge for nanomaterials is, once again, analytical characterization. In addition, the time course of exposure may be prolonged for nanomaterials since the smaller aerodynamic diameters means the particles can stay suspended longer once airborne and, hence, increase duration of availability in the breathing zone.

3.2.4 Oral and buccal exposure

Oral exposure can also be a relevant route for some applications such as toothpastes or lipsticks and glosses. For toothpastes, there is an inherent assumption that a small portion of the toothpaste that is applied will be swallowed during brushing. Similarly, buccal absorption is also possible and is often considered along with oral. Yet another potential incidental oral exposure scenario is hand to mouth from residual cosmetic product remaining on the hands after application. This exposure scenario would likewise be expected to be limited. While oral and buccal exposures are normally limited with typical cosmetic uses, they may deserve more attention when considering nanomaterials because of the potential for increased rates of absorption relative to their larger sized counterparts.

3.2.5 Injection

Intradermal or intraperitoneal routes of exposure are generally not relevant for cosmetic applications. If systemic exposure is predicted by the other routes described above, these injection exposures may become relevant as investigational tools to help determine distribution and kinetics. If injections are used for studying nanomaterials, their potential for altered distribution patterns compared to larger sized materials should be recognized In addition, some pharmaceutical applications with cosmetic benefits would need to be considered when these types of applications fall under the regulatory definition of cosmetic applications in a regulatory setting.

3.3 Exposure and dose metrics in toxicology study designs

The same physicochemical factors that are important for estimating exposure to nanomaterials resulting from product use are also important to consider in the exposures generated during toxicological studies. The key challenge continues to be the adequate characterization of both the exposure and the delivered dose.

3.3.1 Dermal irritation, sensitization, and photoxicity studies

When designing studies to evaluate these endpoints, adequate characterization of the test substance is critical for interpretation of results and for comparison with other studies. The distribution of particle sizes, surface coatings, contaminants or impurities from the manufacturing processes, in addition to concentrations, are important in the study design and reporting.

3.3.2 Dermal penetration studies

Most studies on the skin penetration potential of cosmetic ingredients are done using *ex vivo/in vitro* models of excised skin. These models also appear to be appropriate for the evaluation of nanomaterials.

The specific challenge when using these methods for nanomaterials is analytical detection since both the chemical species and the physical form are important. For example, nanoscale zinc oxide is used in some cosmetics and sunscreens. Since zinc is a natural constituent of the skin there will be background levels present. In order to determine penetration of the nano form of zinc oxide, the analytical methods will need to be able to determine the physical form. Simply measuring the presence of zinc will not be adequate to determine the dermal penetration of nanoscale zinc oxide.

An additional approach to address this analytical challenge is to include appropriate non-nanoscale control groups, if available, which could help sort out what fraction of the detected material is due to the nanoscale form and what is due to other factors such as solubility.

To illustrate this point, nanoscale forms of titanium dioxide, in addition to zinc oxide mentioned above, have been widely studied for dermal penetration (Nanoderm, 2007). In these evaluations, the investigators relied on both characterization of penetration by following the chemical signals using a variety of sensitive analytical methods as well as methods that visualized the presence of the nano-particles. In these investigations, the conclusion was that penetration of these materials was limited to the upper few layers of the stratum corneum and there was no significant dermal penetration to systemic circulation. However, these results do not exclude the possibility that other nanoscale materials may penetrate into or through the living skin and separate testing should be considered for each nanomaterial.

3.3.3 Studies in Compromised Skin Models

One additional consideration that deserves comment when considering dermal exposure is the issue of testing compromised skin. Evaluating the effects of materials on compromised skin presents significant challenges regardless of whether the test substance is a nanomaterial, larger-sized particle, or a more traditional-sized cosmetic ingredient. Those challenges lie primarily in the model itself. By international scientific convention, safety studies should be performed under standardized conditions to ensure reproducibility and comparison of results across studies and between laboratories. Compromised skin models generally do not provide the kind of reproducible conditions necessary to meet those standards. Instead, studies on intact, healthy skin are recommended (OECD, 2011). Absorption studies should be conducted using healthy animals (OECD 2004a) or intact healthy skin in vitro (OECD 2004b). The latter requirement is reflected by the recommendation to perform skin integrity checks, as described in current guidelines for the conduct of in vitro skin penetration studies (OECD 2004a, SCCS, 2010a+b).

In addition, a recent review shows limited differences in dermal permeability when compromised skin is compared to healthy skin (Gatu and Maibach, 2011). When observed, differences in dermal penetration are modest and within the degree of variability already accounted for in the uncertainty factors addressing intra-species variability.

If compromised skin models are considered, they should be well-characterized, reproducible between studies, and across laboratories, include appropriate controls, including controls for size, and the model should be relevant for the intended use of the material. Such models are not currently available and need to be developed.

3.3.4 Inhalation

For evaluation of nanomaterial exposure, most of the factors that should be considered for evaluation of non-nano forms of an ingredient also apply to nanomaterials. The biggest challenge with the evaluation of nanomaterials is, once again, analytical. The form of the delivered material should be adequately characterized for concentration and size distribution. The relevance of the form available for inhalation should be considered and described. The additional challenge for inhalation is the influence of the manipulation of the dosing preparation necessary to create the atmospheres for inhalation. Many of the common treatments, such as mechanical grinding or scraping to create suspended dusts can significantly change the physical form of the test material

and make them fundamentally different from the inhalation exposures generated by product use. The International Organization for Standardization's standard on exposure chambers for inhalation toxicity testing (ISO 10808:2010) has recommended the measurement of total particle mass concentration as the dose metric parameter, and number-based particle size distribution in the exposure chambers for evaluation of the toxicity results. Also, for spray application of products with nanomaterial, a careful characterization is needed of the droplet size and the nanomaterial distribution in the droplets. Determination of the generated droplet size distribution alone will not provide sufficient information on the size distribution of nanoparticles. Therefore, consideration should also be given to determining the size distribution of dried residual aerosol particles.

3.3.5 *In vitro* methods

The primary challenge with *in vitro* methods for testing nanomaterials is the appropriate delivery of the test material (Hartung, 2011). Since nanomaterials tend to aggregate/ agglomerate, the delivered dose within an *in vitro* system may be fundamentally different from *in vivo* exposures. Questions to be addressed include what is the physical form of the delivered dose? Did the material reach the target in a relevant form and in a relevant concentration? What is the appropriate time course for exposure? Most of these questions are relevant for any material tested in an *in vitro* system but are particularly challenging when the test substance is a nanomaterial.

In addition to the characterization of the dose and delivery of the dose, there is the potential for artifacts from nanomaterials that can introduce confounding factors for interpretation. For instance, due to the large surface area to mass ratio of nanomaterials, many critical components of cell culture may bind to the nanomaterials tested (also see Section 1.5). This can result in two basic sources of potential artifacts – 1) a decrease in nutrient value of the media, and 2) coating the nanomaterials and thus altering its surface characteristics. It is important to take account of the special considerations relating to nanomaterials described in Section 1.5.

4.0 Hazard identification/ characterization

The safety assessment of cosmetic products, regardless of whether they contain nanomaterials or not, should include consideration of the physicochemical properties of constituent ingredients, assessment of exposure at local and systemic levels, and toxicological data collated by valid *in vitro* and *in vivo* methods. Where studies have shown evidence for systemic absorption of a nano cosmetic ingredient via skin, lung or gastrointestinal tract, further characterization should be carried out to establish whether the absorbed species were in a particle form or in a conventional (e.g. solubilised, molecular or ionic) form (Figure 1). If the characterization data show translocation of the ingredient in a nanoparticle form, detailed investigations into hazard identification and characterization should be carried out in consideration of nano aspects (section 1.5), particularly in relation to designing or assessing/evaluating study protocols, test method selection, dosimetry, and study interpretation and reporting.

The need for toxicological investigations depends on the potential biological effects of an ingredient or formulation, its intended use(s), and the likely routes of exposure. The testing schemes include *in vitro* and *in vivo* tests, and computational modelling. However, it is of note that *in vivo* tests on cosmetic ingredients are banned in Europe under the Cosmetics Regulation (Regulation (EC) No 1223/2009); whereas, validated alternative methods (both *in vitro* tests and predictive computational models) are not yet available for nanomaterials (section 4.16).

Detailed guidelines for hazard identification/characterization of nanomaterials in cosmetic products have not been published yet in any of the ICCR jurisdictions. FDA, in its recent draft "Guidance for Industry - Safety of Nanomaterials in Cosmetic Products", has stated that the current general framework for safety assessment which includes hazard identification, dose-response assessment, exposure assessment, and risk characterization is generally robust and flexible enough to be considered appropriate for nanomaterials, even though nanomaterials can have properties that may be different from conventional ones. The safety of a cosmetic product should be evaluated by analyzing the physicochemical properties and the relevant toxicological endpoints of each

ingredient in relation to the expected exposure levels resulting from the intended use of the finished product[24].

In Europe, guidance on risk assessment of nanomaterials in cosmetic applications is currently being prepared by the SCCS, and is expected to be published soon.

Other guidelines on testing of nanomaterials in general have also been developed, e.g. by the OECD Working Party on Manufactured Nanomaterials (2010). The EFSA (2011) has also recently published guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. While these guidelines are not specific for cosmetic applications, they do provide overarching considerations for safety evaluation of nanomaterials. For example, the OECD (2010) preliminary report has identified important endpoints that may need considering in relation to safety evaluation of nanomaterials. More detail on key endpoints and available OECD test methods has been provided in Table B in the Appendix.

In brief, toxicological investigations should aim to address both relevant short term (acute) and long term (chronic) endpoints, such as toxicokinetics (ADME), acute toxicity, irritation and corrosivity, skin sensitization, dermal/ percutaneous absorption, repeated dose toxicity, and mutagenicity/ genotoxicity. Depending on the extent of the internal exposure, further investigations into carcinogenicity and/or reproductive toxicity may also be necessary. Further specific genotoxicity and/or mutagenicity data may be required on the basis of the results of initial investigations. Photo-induced toxicity studies may also be necessary where a cosmetic product is expected (or intended) to be used on sunlight-exposed skin. Human data should be considered whenever available. In some cases, confirmatory testing of product compatibility and acceptability may be conducted with human volunteers (with due ethical considerations). The investigations should also include any localized effects considering the relevant exposure route(s).

4.1 Considerations regarding toxicological parameters

Depending on the route(s) of exposure and toxicological profile, evaluation of safety of nanomaterials in a cosmetic product may need testing for different toxicity endpoints. For nanomaterials, traditional toxicity testing may need certain modifications or adaptation with respect to such factors as appropriate solvents and dosing formulations, methods to prevent agglomeration of particles, purity and stability, and other testing conditions (see Section.1.5).

Moreover, as discussed before, nanoparticles may change their properties in biological test systems, or may penetrate membrane barriers and become bioavailable (Section 1.5.2.3). Following systemic uptake, the toxicokinetcs and toxicodynamics of particulate material are likely to be different from those of conventional equivalents. Thus, both the exposures and the delivered doses should be adequately characterized while conducting toxicological studies (see Section 3.3). Some of the key points that need to be adapted in the testing methods for nanomaterials due to their distinct properties are discussed below.

4.2 Dermal penetration:

Dermal penetration studies make up an important element of exposure assessment for cosmetics. The studies should be conducted using healthy animals, or intact healthy skin *in vitro* (Table B). No *in silico* models exist yet for nanoparticle uptake (SCCP 2007). In specific cases, *in vivo* or *in vitro* biotransformation studies (such as phase I and phase II biotransformation enzyme activity) may be needed to prove or to exclude certain adverse effects.

In general dermal penetration, testing should be conducted with intact healthy skin for all materials, including nanomaterials. In some cases it may be desirable to also conduct studies with impaired skin (e.g. sunburned, atopic, eczematous, psoriatic skin) to address the possibility of changes in the rate of penetration and systemic availability. However, there are several limitations in using impaired skin models for conducting dermal penetration studies as discussed in section 3.3.3. The inclusion of a non-nanomaterial group in the study may also be helpful to evaluate whether the skin penetration potential of the “nano’ form differs from its conventional counterpart.

[24] http://www.fda.gov/Cosmetics/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ucm300886.htm

There is already a large and growing body of evidence on dermal penetration of nanomaterials, which shows that skin acts as a very good barrier to particulate materials, including the manufactured nanomaterials that have been tested so far (e.g. Filipe et al., 2009). There are a few published studies that suggest a possible passage of nanoparticles, such as surface modified fullerenes and CdSe quantum dots, across pig skin (Xin-Rui et al. 2006, Ryman–Rasmussen 2006). However, the model nanoparticles in these studies are not used in cosmetics, and a recent study has indicated that pig skin is more permeable than human skin to CdSe nanomaterials (Prow et al., 2012). As mentioned before, an important consideration when evaluating the dermal penetration potential of a material is characterization of the physical form of the translocating species. Without this information, it is difficult to duly treat the dermal penetration data in the overall safety assessment. For example, a study in Australia reported that radiolabelled Zn from ZnO particles (primary particle size 19 nm) in sunscreens was absorbed through healthy human skin when exposed to sunlight, and was detected in blood and urine. However, it is not clear whether the absorbed species were ZnO nanoparticles or a solubilised (ionic) form of zinc. Considering the solubility of ZnO, in this case, it is more likely that the trace amounts of Zn detected in blood/urine were that of ionic zinc rather than nanoparticle form (Gulson et al, 2010).

For conventional cosmetic ingredients, the systemic exposure is generally determined by chemical analysis of the receptor fluid, relevant tissues/organs, and/or blood. The analytical methods used for this purpose need to be both state of the art, and have low enough limit of detection to demonstrate the lack of exposure. However, in most cases chemical analysis alone may not provide information on the particle nature of the absorbed material. Therefore, if chemical analysis indicates systemic absorption, further investigations should be carried out to confirm whether the absorbed material was in a particle form or in a solubilised/metabolised form. For ubiquitous substances, such as zinc, chemical analysis may not distinguish between the low levels of absorbed and the natural levels of the same substance already present in the body. In such cases, the use of other techniques such as radiotracer or stable isotope analysis may also be needed. The use of sensitive methods, such as electron microscopy, should provide information on whether the absorbed material was in nanoparticle form.

4.3 ADME profiles and toxicokinetics

The safety testing of nanomaterial containing cosmetic products should consider the uptake and absorption, bioavailability, and other parameters that may affect the safety of the product according to its intended use. An important set of parameters is the absorption, distribution, metabolism and excretion (ADME) that are likely to be influenced by both the chemical composition of the nanomaterial as well as physicochemical properties (e.g. size, shape, solubility, surface charge, surface coating and surface reactivity, etc). As discussed before (section 1.5.2.2), altered ADME properties of nanoparticles, combined with nano-dimensions, may enable them to penetrate membrane barriers, which normally prevent (larger) particulate materials from entering the vital organs, such as brain, liver, kidneys, etc. Thus investigating any major shift in ADME profile of a nanomaterial is very important when there is evidence for internal (systemic) exposure from its use in a cosmetic product.

In specific cases, *in vivo* or *in vitro* biotransformation studies may also be needed to prove or to exclude the possibility of certain adverse effects.

4.4 Inflammatory reactions

Inflammatory reaction is a key event that may occur following exposure to a solid material, including nanomaterials. For several nanomaterials, *in vitro* induction of inflammatory cytokines has been demonstrated (Carlson et al. 2008, Kim et al. 2003, Kocbach et al. 2008, Zhang et al. 2008). These inflammatory cytokines can also bind to nanomaterials (Kim et al. 2003), and this may have implications when *in vitro* assays are used for evaluation of the inflammatory properties of nanomaterials (SCCS, 2009).

4.5 Acute toxicity, irritation/corrosion, skin sensitization

Appropriate tests should be used for the investigation of acute toxicity, irritation/corrosion, and skin sensitisation endpoints (see Table B), with special attention to physicochemical characterization, sample preparation, and dosimetry (see section 1.5.2).

4.6 Ocular effects

When assessing nanomaterials in cosmetic products, the safety assessment process should take into consideration the potential for exposure to the eyes since indirect exposure to nanomaterials may occur by cosmetics intended for use in the vicinity of the eye, or from certain types of cosmetic products e.g. sprays. There are a few published studies which suggest that exposure of eyes to certain nanomaterials may have potential safety issues (Alany et al. 2006). Appropriate irritation/corrosion tests should be conducted where the foreseeable use(s) of a nanomaterial-containing cosmetic product may lead to exposure of the eyes.

4.7 Repeated dose toxicity

In view of the likelihood of different physicochemical properties and kinetic behaviour of nanomaterials compared to conventional forms, a major emphasis should be placed on toxicological tests that are carried out over prolonged periods with repeated doses and followed up by histopathological investigations to detect possible lesions and susceptible organs.

In this regard, appropriate repeated dose tests should be conducted in rodents to obtain information on target organs and systemic toxicity (Table B). For nanomaterials used in food products, EFSA (2011) has recommended considering extended endpoints (e.g. endocrine activity and immuno- and reproductive toxicity) for repeat oral dose toxicity.

4.8 Carcinogenicity

If nano ingredients are found to penetrate the skin or gain systemic bioavailability through other relevant routes of exposure, such as inhalation or oral intake, additional studies to evaluate carcinogenicity may be conducted.

4.9 Mutagenicity/genotoxicity

The genotoxic effects of conventional particles are driven by two mechanisms – direct genotoxicity and indirect (inflammation-mediated) genotoxicity. Nanoparticles may act via either of these pathways since they cause inflammation and can also enter cells and cause oxidative stress. Due to their small size, it is possible that nanomaterials may penetrate into sub-cellular compartments like the mitochondria and the nucleus, as shown by *in vitro* studies by Chen and von Mikecz (2005) and Sun et al. (2011). The presence of nanomaterials in mitochondria and the nucleus opens the possibility for oxidative stress mediated genotoxicity, and direct interaction with DNA, respectively. For some manufactured nanomaterials genotoxic activity has been reported, mainly associated to ROS generation, while for others contradictory results were obtained (SCCS, 2009).

If the genotoxic effects of nanoparticles are linked to inflammation, simple *in vitro* assays may not be adequate for determining genotoxic potential.

Most of the available *in vitro*/*in vivo* genotoxicity studies reported in the open literature have been performed at high particle concentrations. In *in vivo* situations, this may be associated with marked inflammatory and proliferative responses, and hence may obscure and/or modify genotoxicity and even carcinogenicity readouts (SCCS, 2009).

A suitable battery of *in vitro* genotoxicity tests covers the endpoints of gene mutation, and structural and numerical chromosome aberrations. Since nanoparticles may not be able to penetrate the cell wall and because bacterial cells do not have the ability to endocytose particles like mammalian cells, the use of a bacterial reverse mutation test for detection of genotoxicity of nanomaterials may not be appropriate (Landsiedel, 2009; EFSA, 2011).

4.10 Phototoxicity

Photo-induced toxicity testing may be necessary if a cosmetic product is intended or expected for use on sunlight-exposed skin. The OECD TG 432 *in-vitro* 3T3 Neutral Red Uptake (NRU) phototoxicity test is generally used in evaluating phototoxicity of chemicals. It should, however, be noted that the reliability and relevance of the test has not been specifically validated for nanomaterials (Spielmann et al. 1998). Because of other possible issues associated with this test (e.g. nanomaterials may absorb Neutral Red or interfere with color detection), its application in phototoxicity evaluations of nanomaterials will need careful consideration.

4.11 Reproductive and developmental toxicity

The term "reproductive toxicity" is used to describe the adverse effects induced (by a substance) on any aspect of mammalian reproduction. It covers all phases of the reproductive cycle, including impairment of male or female reproductive function or capacity and the induction of non-heritable adverse effects in the progeny such as death, growth retardation, structural and functional effects [ECB 2003].

Information on both exposure and hazard of a nanomaterial, and any available toxicity data on its non-nano form should be taken into consideration for determining the need for a reproductive and developmental toxicity study for the nanomaterial. Need for a developmental toxicity study should also be considered where there is evidence that a nano substance may cross the placenta and thereafter behave in a different way from the non-nanoform.

4.12 Inhalation toxicity

Cosmetic products are primarily intended for use on skin, hair or oral mucosa. Exposure via inhalation may need to be considered for sprayable cosmetic products (Rothe et al, 2011). The deposition of nanomaterials in the respiratory system depends on their aerodynamic and physicochemical properties. The soluble nanomaterials may be dissolved, metabolized and transported to other organs and blood whereas the insoluble nanomaterials may be retained in the airways or swallowed by coughing. Hence, there is the possibility of adverse effects in the respiratory system. In conducting inhalation studies involving nanoparticles, care needs to be taken to ensure that the stability and accuracy of particle size distribution and concentration is maintained throughout the study (section 3.2.3).

4.13 The “bridging toxicity” concept

The safety assessment of nanomaterials poses a considerable challenge because some nanomaterials may be produced in a variety of different particle sizes, shapes, crystalline forms, or surface characteristics. Taking into account the number of potential toxicological endpoints, the number of toxicity studies required to evaluate all nano-forms of a single substance may be very large and possibly prohibitive. This also contravenes ethical constraints that limit the use of animals in laboratory investigations. The number of toxicity studies may, however, be significantly reduced if the concept of “toxicological bridging” is applied.

The toxicological bridging approach allows for the reduction of required safety studies on individual members of a group of related substances, and is complementary to the concept of “read-across” (i.e. extrapolation of results from a reference chemical to closely-related substances). In brief, the approach is based on comparing the results obtained in a relevant short term toxicity test on a substance under evaluation with those obtained on one or several closely related chemical material(s) that have been more thoroughly evaluated. Where the results of short term toxicity tests are found to be consistent across the tested materials, a toxicological equivalence of these materials may be assumed with a reasonable degree of confidence, i.e. results of long-term toxicity studies obtained on one representative chemical will also be applicable to the entire group. In such cases, testing a single, representative substance may be considered to be sufficient from a scientific as well as an ethical point of view. On the other hand, when the safety profiles of related chemicals are significantly different in short-term toxicity studies, it may be necessary to evaluate the different materials in longer-term toxicity studies.

The bridging approach takes its origins from the qualification of new drug substances with changing purity/impurity profiles over the course of development. Often the manufacturing process (scale and synthesis) of a new drug substance is modified throughout the course of drug development, which may result in an alteration of the purity/impurity profile relative to the drug substance originally used in preclinical toxicity studies. In order to avoid the repetition of long-term toxicity studies at the end of a drug substance's development, ICH Guidelines recommend that a bridging toxicity program should be performed. The program consists of a side-by-side comparison of the toxicity of the original substance with that of the new substance of different purity/ impurity profile, and generally comprises *in vitro* genetic toxicity and short-term (e.g. 14-day) general toxicity studies (ICH, 2006). When the bridging toxicity profile of one or several forms with different purity/impurity profiles is similar, long-term toxicity studies on the new drug substance are considered to be unnecessary and are waived.

The bridging approach has also been recommended by the US FDA for non-clinical evaluation of drug or biological combinations (US FDA, 2006). Similarly, the EU EMEA recommended that a bridging approach should be considered in the safety evaluation of a pure chiral drug substance substituting a racemic substance, i.e. chiral drug substances should be toxicologically bridged with their respective racemates (EMEA, 1994). The bridging concept has also been recommended by EMEA for biotechnology-derived products such as proteins or other biological drug substances after a change in the manufacturing process (EMEA, 2006).

More recently, a similar approach has been recommended for the safety assessment of nanomaterials in Europe by EFSA in its "Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain" (EFSA, 2011). Specifically, EFSA recommended that when evaluating the safety of a nano-form of a known and well-characterized bulk substance, a comparison of the safety/ADME profiles of the nano- and bulk forms (i.e. ADME, genotoxicity and 90-day toxicity studies) can provide a comparative basis for deciding whether long-term toxicity testing of a nanomaterial may be needed. Given that the toxicity package needed for cosmetic ingredients is generally less extensive on the basis of exposure considerations, an appropriate "bridging toxicity" package for cosmetic ingredients could, for example, comprise the evaluation of their genotoxicity package as well as acute/subacute toxicity potential. Such a package would be similar to that required for the comparison of toxicity profiles of drug substances with different impurity profiles (see above).

An example of bridging the toxicological profile of nano-sized TiO2 materials with that of micron-sized materials has been published by Warheit et al. (2007). In that program the acute toxicity, irritation, sensitization, pulmonary toxicity, genetic toxicity and ecotoxicological properties of two TiO2-based nanoparticle materials were compared with those of a pigmentary grade TiO2 material (particle size: about 400 nm). Given that results of all tested materials suggested identical toxicological properties, it was concluded by the authors that these materials were toxicologically equivalent and that further toxicity testing of the nano-sized material was not necessary. Another example is a large study on the genotoxicity and photo-genotoxicity of a series of micron- and nano-sized TiO2 particles with different crystalline and surface characteristics (Theogaraj et al., 2007). Again the results showed no differences in the hazard profile of the test substances, the study concluded that further *in vitro* or *in vivo* genotoxicity studies specifically on TiO2 nanoparticles were not necessary.

These examples show that the bridging concept may also be considered in the context of nanomaterials used in cosmetic products, where different forms/shapes of a given material may exist. In addition, the bridging approach may also be used as a selective screen in order to identify those nanomaterials that may pose potential new health risks and to distinguish them from those nanomaterials that share a similar or identical profile in terms of toxicological properties to their corresponding bulk materials.

4.16 Alternative testing methods

Although the toxicological evaluation of cosmetic ingredients involves *in vivo* testing, there has been an increasing emphasis in recent years on the use of alternative (non-animal) methods in all of the ICCR jurisdictions. In the European Community, testing safety of cosmetic ingredients on

animals is already banned under relevant regulation. The EU's Cosmetics Regulation ((EC) No 1223/2009) establishes a prohibition to test finished cosmetic products and cosmetic ingredients on animals (testing ban), and a prohibition to market in the European Community, finished cosmetic products and ingredients included in cosmetic products that were tested on animals (marketing ban). The testing ban on finished cosmetic products has been in place since 11 September 2004; whereas, the testing ban on ingredients or combination of ingredients came into effect 11 March 2009, irrespective of the availability of alternative non-animal tests, and also applies, since 11 March 2009, to cosmetic products containing ingredients tested on animals[25]. This makes the safety assessment of cosmetic ingredients and products in general, and those containing nanomaterials in particular, more difficult.

The main available alternative methods for toxicological characterization include *in vitro* assays and *in silico* modelling approaches. These methods aim to reduce, refine, or replace the use of animals in laboratory investigations (the "3Rs" principle). However, whilst *in silico* modelling tools are well advanced for conventional chemicals, a relationship between physicochemical properties and toxicological effects of nanomaterials has not yet been established. As a result, only a few elementary *in silico* models have so far been published, but these are unlikely to be of much relevance to the toxicological evaluation of nanomaterials. Similarly, whilst a number of *in vitro* methods have been developed, validated, and used for conventional chemical substances, none of them has so far been validated for nanomaterials.

Despite the lack of validated *in vitro* methods, many of the currently available tests are likely to be relevant and valid for nanomaterials, provided that nano-related aspects have been taken into consideration during the conduct of the tests (Section 1.5.2). Information from such tests may provide a valuable additional input to the overall weight of evidence in the safety evaluation of nanomaterials. .Apart from use in hazard characterization, *in vitro* methods can be useful in providing information on the possible mode of toxic action of a nanomaterial, which can inform further toxicological investigations (SCENIHR, 2009). For example, *in vitro* tests may indicate the likelihood of generation of reactive oxygen species which may provide indication of potential toxic effects and direct further studies.

Although testing nanomaterials poses a particular challenge, many of the considerations regarding *in vitro* tests would apply to any material. Particular considerations for nanomaterials relate to the insoluble or partially-soluble particulate nature of nanomaterials, agglomeration/ aggregation behaviour, surface characteristics, etc. (see Section 1.5). Thus, characterization of nanomaterials during the tests is an essential element for validity of the *in vitro* results.

Hartung and Sabbioni, (2011) reviewed different *in vitro* tests for applicability to nanomaterials. These included skin corrosion, phototoxicity, dermal absorption and penetration, skin and eye irritation, genotoxicity, acute oral toxicity, carcinogenicity, sensitization, exotoxicity, and pyrogenicity. Their findings showed that alternative methods can be useful for hazard characterization but will need separate optimisation for each nanomaterial evaluated. They concluded that extensive physicochemical characterization of the test material will be essential to make comparisons of the results across studies. Extrapolation from *in vitro* studies for risk assessment purposes should require a careful characterization of the delivered dose and relevant contact of the test material with the target.

Thus, one of the main challenges in regard to the use of *in vitro* methods is to maintain a uniform concentration of nanomaterials throughout the course of an exposure, and to ensure that it reaches the target in the relevant form and concentration (see Section 1.5.2). Special attention is needed to investigate possible interaction of the nanomaterial with components of the test media (e.g. nutrients, dyes and markers, growth factors, proteins, etc). Such interactions may alter subsequent interaction and uptake of the nanomaterial with cellular targets. If these interactions result in binding or coating of media components on the nanomaterial surface it may also introduce artifacts by altering the surface characteristics, or by delivering unintended substances inside the cellular targets. For example, *in vitro* tests for skin corrosion and skin irritation are based on colorimetric

[25] Cfr. Communication from the Commission to the European Parliament and the Council on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics (COM(2013) 135 final).

assays (such as sulforohdamine B dye, MTT assay) and may not be suitable for nanomaterials because of the possible interaction between reagents and nanomaterials. Moreover, some nanomaterials may themselves disperse/ absorb light and interfere with the measurements in colorimetric assays.

The measurement of cytokines and chemokines in the test system may provide additional information (e.g. IL-1α, tumor necrosis factor α (TNF-a) IL-8, interferon), but they may themselves bind/ adsorb to nanomaterial surfaces and lead to false negative results. Similarly, 3T3 neutral red uptake test for phototoxicity has not been specifically validated for nanomaterials (Spielmann et al. 1998), but it should be noted that in some instances neutral red may interfere with nanomaterials (Ianone et al., 2009). With regard to mutagenicity/ genotoxicity tests, although some published reports have shown positive bacterial reverse mutation results, there are doubts if the conventional Ames test is an accurate predictor of the genotoxic potential of nanomaterials. This is because, unlike mammalian cells, bacterial cells lack the uptake of nanomaterials via endocytosis, and also because some nanomaterials may have bactericidal activity. Therefore this test has not been considered suitable for testing nanomaterials (EFSA, 2011). Furthermore, although not yet investigated for nanomaterials, it is possible that some insoluble particulate materials can be mechanically irritating – an effect that is difficult to be picked up by *in vitro* systems.

The need for validated *in vitro* methods for safety evaluation of cosmetic ingredients has also been highlighted by SCENIHR (Opinion, 2009) and in the SCCS Notes of Guidance (SCCS/1416/11, 2010). Currently, nanomaterials are not used as reference compounds for the validation of alternative methods. SCENIHR and SCCS pointed out that current (i.e., *in vitro*) testing methods may need certain adaptations to take account of the special features of nanoparticles. This refers in particular to the tendency of nanomaterials to stick together because of high surface energy, the insolubility of certain nanomaterials (resulting in the presence of a suspension rather than a solution), the potential ability of nanomaterials to bind substances on their surface, their ability to penetrate membrane barriers and the general unsuitability of the conventional mass metrics for nanomaterials.

The Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIHR) published an opinion in 2007 on "The appropriateness of the risk assessment methodology in accordance with the Technical Guidance Documents for new and existing substances for assessing the risks of nanomaterials". It provides observations on the applicability of *in vitro* test procedures, and makes a series of recommendations for improved methodologies and areas urgently requiring additional data and scientific knowledge. One of the conclusions is that there is a clear need for validated *in vitro* assays for nanoparticle evaluation. According to SCENIHR, *in vitro* tests should address key endpoints, such as genotoxicity, biopersistence, free radical generation, cellular toxicity, cell activation and other generic endpoints. They should also target cell specific endpoints, such as effects on the action potential of nerve cells or the phagocytic capacity of macrophages. To address the need for validated *in vitro* methods, the OECD's Sponsorship Programme under the WPMN project "Safety Testing of a Representative Set of Manufactured Nanomaterials" is testing a set of manufactured nanomaterials using appropriate test methods, which include OECD Test Guidelines or other internationally agreed methods[26]. There are also a number of developments currently in R&D pipeline in this area, for example, *in vitro* models for repeated dose toxicity to assess dermal penetration, including those of nanoparticles and dermal metabolism[27]. Similarly, organotypic lung models have been reported for the study of a possible inflammatory response[28].

[26] OECD (2009). Preliminary review of OECD Test Guidelines for their applicability to manufactured Nanomaterials. OECD Environment, Health and Safety Publications, Series on the Safety of Manufactured Nanomaterials No. 15, ENV/JM/MONO(2009)21.
OECD (2010). Report of the Workshop on Risk Assessment of manufactured nanomaterials in a regulatory context. OECD Environment, Health and Safety Publications, Series on the Safety of Manufactured Nanomaterials No. 21, ENV/JM/MONO(2010)10 ENV/JM/MONO(2011)12, and OECD (2010). Preliminary guidance notes on sample preparation and dosimetry for the safety testing of manufactured nanomaterials.

[27] [Arch Toxicol (2011) 85:367–485] [Jäckh C, Blatz V, Guth K, Reisinger K, Fabian E, van Ravenzwaay B, Landsiedel R (2010) ALTEX 27(suppl 2/10):60; Landsiedel R, Fabian E, Gamer A, Kolle S, Ma-Hock L, Schulz M, Wiench K, Wohlleben W, van Ravenzwaay B (2010). ALTEX 27 (suppl 2/10):78].

[28] [Brandenberger C, Rothen Rutishauser B, Mühlfeld C, Schmid O, Ferron GA, Maier KL, Gehr P, Lenz AG (2010) Toxicol Appl Pharmacol 242(1):56–65]

Cell lines are also available with characteristics that mimic different cell types of the respiratory tract, and co-cultures are also being developed. Epithelial airway cells can be cultured at the air–liquid interface. Also, devices are being developed that represent the *in vivo* respiratory air compartment, and which allow exposure of the cells to gases, liquid aerosols, complex mixtures, nanoparticles and fibres[29]. This implies that, once developed and validated, these methods will provide a means for cosmetics to be applied to potential target cells in realistic product use conditions, and accurate monitoring of the exposure.

A model for tiered nanotoxicity screening has been proposed for risk assessment of nanomaterials (Hirsch et al., 2011, Stone et al., 2009). The proposed approach includes thorough physicochemical characterization of nanomaterials, *in vitro* screening tests, and the use of OECD and ECVAM validated/ approved *in vitro* methods. However, the lack of validated *in vitro* tests or a testing battery means that a category based approach to safety assessment of nanomaterials may not be feasible at present, and assessment of nanomaterials may need to be carried out on a case by case basis. However, *in vitro* methods may be considered as supporting tools to evaluate relative toxicity of nanomaterials in hazard identification and for providing additional information on possible mechanisms of action. Similarly, *in vitro* digestion studies may provide information on dissolution/degradation of nanomaterials. However, *in vitro* test methods should be used with caution to ensure that the exposure and interpretation of effects can be adequately translated to *in vivo* exposures and effects (see Section 4.16). In view of the current state-of-the-art in regard to alternative methods, the ban on the use of *in vivo* tests for cosmetic ingredients in Europe will pose a major challenge to the evaluation of safety of new cosmetic ingredients and products in general, and those containing nanomaterials in particular.

5.0 Risk characterization

Numerous research programs, such as OECD's Sponsorship Programme for the Testing of Manufactured Nanomaterials, have been undertaken to gain an understanding of the potential human health and environmental effects of manufactured nanomaterials. These programs aim at ensuring that the approaches to hazard identification, exposure evaluation and risk assessment of nanomaterials are appropriate and of a high, science-based, and internationally harmonized standard.

Though most of these programs have not delivered yet, the OECD recently organized an expert workshop to identify best approaches for risk assessment of nanomaterials, on the basis of the current state of knowledge (OECD, 2010a). A draft guidance document relevant to cosmetic applications of nanomaterials has recently been published by the FDA (see footnote 22), whilst another guidance document is currently under discussion by the SCCS in Europe. Similarly, the European Food Safety Agency (EFSA, 2011) and the International Standard Organization (ISO, 2011) have recently issued guidance documents on the safety assessment of nanomaterials. Although the latter initiatives did not specifically target cosmetic uses of nanomaterials, their conclusions are also of high relevance to the risk assessment of nanomaterials used in cosmetic products. In particular, the recommendations made in these different guidance documents reach a similar and important conclusion that the existing risk assessment paradigm in use for conventional chemicals (hazard identification and hazard characterization, exposure assessment, and risk characterization) should in principle be applicable to nanomaterials.

In the conventional risk assessment paradigm, risk characterization *per se* is obtained by comparing exposure levels. In a nutshell, exposure level estimates in humans resulting from typical uses of the compound/product considered are directly compared with those obtained in an experimental setting at a dose associated either with no effects (NO(A)EL approach) or with well-characterized quantitative effects (Benchmark Dose approach). Taking into account that uncertainty factors (UFs) are then applied, the question may be asked whether the current uncertainty factors would be sufficient for nanomaterials, since these specific factors have been derived from toxicity studies on conventional (soluble) substances. Chemical-specific UFs might be

[29] [Deschl U, Vogel J, Aufderheide M (2010) Exp Toxicol Pathol. doi:10.1016/j.etp.2010.04.013; Gminski R, Tang T, Mersch-Sundermann V (2010) Toxicol Lett 196(1):33–41]

used, but in practice default UFs are used most of the time. The default UFs that account for possible inter-species differences as well as inter-individual (human intraspecies) variability in relation to toxicokinetics and toxicodynamics are summarized in Figure 1 below.

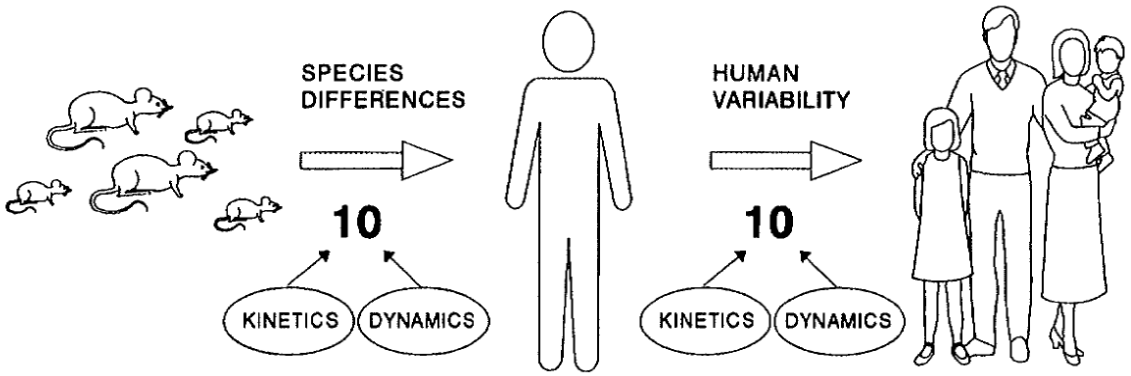

Figure 1: the use of uncertainty factors (from Renwick, 1998)

It is traditionally considered that the margin of safety between exposure levels in humans/consumers and those obtained in the experimental setting should be of at least 100 for systemic toxicity effects in order to consider the use(s) of the chemical as safe. When assessing whether "nano-specific" UFs should be applied, it should be borne in mind that these empirical considerations have been applied for decades in the practice of risk assessment of conventional chemicals before being scientifically substantiated. It is indeed only recently that the analysis of extensive databases that quantify interspecies differences and human variability in metabolism and excretion processes permitted to substantiate and refine these empirical approaches for conventional chemicals (Renwick and Lazarus, 1998; Dorne and Renwick, 2005).

Similarly, ongoing research initiatives will yield toxicity results on a wide variety of nanomaterials, and these results will be used to possibly refine current risk assessment approaches, including uncertainty factors. However, it has recently been recognized that, on the basis of the safety data sets of data already available on traditionally-used nanomaterials that have been traditionally used, "there does not appear to be a scientific rational to justify employing a nano-specific risk assessment uncertainty factor" (OECD, 2010a).

When performing the risk assessment of a conventional chemical, it is generally recommended that the uncertainty associated with the evaluation should also be evaluated. Accordingly, additional safety/uncertainty factors may only be applied where dataset on toxicity is considered to be insufficient. These considerations will also apply to the safety evaluation of nanomaterials used in cosmetic products.

6.0 Summary and conclusions

This ICCR WG report is aimed at providing information to those who intend to assess safety of nano-scale cosmetic ingredients. The report builds upon a number of relevant opinions, guidance documents, and reports from various international bodies, as well as scientific literature. It covers the main elements of safety assessment in relation to the use of nanomaterials as cosmetic ingredients, i.e. general safety considerations (Section 1), physicochemical characterization (Section 2), exposure assessment (Section 3), hazard identification and characterization (Section 4), and risk characterization (Section 5).

The use of nanomaterials as cosmetic ingredients, such as UV filters in sunscreens, offers certain benefits to the consumer. However, the same nano-size that gives a cosmetic product useful properties, may also pose a health risk to the consumer. Nanoscale materials may show a different or novel property, behaviour, and/or effect, compared to the equivalent conventional form. The ability of nanoparticles, especially in the lower nanometre size range, to penetrate biological membrane barriers has added another dimension to the toxicology of particulate materials. This poses a possible risk of systemic exposure to insoluble or partially-soluble nanoparticles that may

be able to penetrate biological membrane barriers and reach certain organs that are otherwise protected from particulate materials. This may lead to certain harmful effects due to the potential interaction of the particle surfaces with biological processes and moieties close to the molecular level. The use of a nanomaterial as a cosmetic ingredient requires a thorough safety evaluation, in the same way as other ingredients, but with special considerations to the nano-features.

The information provided in this report is relevant to the safety assessment of a new or already approved cosmetic ingredient, if the latter fulfils the criteria for definition of a nanomaterial. For example, it would apply to an already approved ingredient if it was manufactured by a new or different process which generated a significant fraction of the material in the nano scale.

The key features to consider for safety assessment of nanomaterials intended for use in cosmetics are given below:

- Irrespective of the presence or absence of nanomaterials in a cosmetic product, general safety considerations for the testing of cosmetic ingredients and their safety evaluation, as provided by the ICCR report *Principles of Cosmetic Product Safety Assessment*[30]*,* and/or required under specific regulatory frameworks, should be followed.
- Detailed characterization of a nanomaterial intended for use as a cosmetic ingredient is of primary importance to safety assessment. In this regard, all physicochemical parameters listed in Table 1, which are relevant to a given type of nanomaterial, should be determined. This should also include ascertaining that the characterized nanomaterial is the same (or reasonably similar to) that intended for use in the final product.
- The existing risk assessment paradigm (exposure assessment, hazard identification and hazard characterization followed by risk characterization), in use for conventional chemicals, is also applicable to nanomaterials.
- Testing of nanomaterials for exposure assessment or hazard identification/characterization should consider certain nano-related aspects, such as insoluble or partially-soluble particle nature, agglomeration and aggregation behaviour in test media and biological environment, potential to penetrate biological membranes, possible interactions with biological entities, surface adsorption/ binding of other moieties, surface catalysed reactions, stability and persistence, etc.
- Determination of systemic exposure and investigations into local effects, carried out in consideration of nano-related aspects, are among the most crucial elements of an exposure driven safety assessment (Figure 1).
- Exposure assessment should consider the foreseeable uses of a cosmetic product, and the possible routes of exposure (dermal, respiratory, oral).
- Where the evidence shows a lack of systemic absorption following application of a nanomaterial containing cosmetic product, local effects should be investigated.
- Where the evidence shows systemic absorption, further investigations should be carried out to confirm whether the absorbed material was in particle form or in solubilised/ metabolised form. Further toxicological investigations with nano-related considerations will be necessary where there is evidence for systemic absorption of a material in nano form. In the first instance, focus should be on ADME parameters to investigate the fate and behaviour of the nanoparticles in the body, and to identify the likely target organs.
- In the case of (very) low absorption of a nanomaterial, processes such as accumulation should also be considered.
- In general the methods used for toxicological investigation of conventional materials are also applicable to nanomaterials. However, some methods may need adaptations in view of the distinctive physicochemical characteristics, sample preparation, and with regard to dosimetry considerations (sections 2 and 3).

[30] ICCR (2011b) Principles of cosmetic product safety assessment, A Report prepared for ICCR, available at http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/iccr5_safety_en.pdf

- Currently, toxicological testing is carried out mainly in animals. However, the EU Cosmetics Regulation ((EC) No 1223/2009) establishes a prohibition on testing finished cosmetic products and cosmetic ingredients on animals (testing ban), and a prohibition on marketing in the European Community, for new finished cosmetic products and ingredients included in cosmetic products that were tested on animals (marketing ban). Current exceptions are tests for repeated dose toxicity, reproductive toxicity, and toxicokinetics, but these will also be banned in 2013. This will pose a major obstacle to safety assessment of new nanomaterial cosmetic ingredients.
- A number of validated alternative methods are available that can be used in place of animal tests for conventional substances. However, none of the methods is yet validated for nanomaterials. They may still be relevant for hazard identification, and may also provide additional supporting evidence to the results of *in vivo* studies, provided that they are carried out with due consideration of the nano-related aspects (section 4).
- Due to the current insufficient level of scientific understanding of the possible changes in properties, behaviour, and effects of nanomaterials compared to conventional equivalents, the use of a read-across or categorisation approach based on inter- or intra- nanomaterial extrapolation may not be feasible for safety assessment of every nanomaterial. However, on the basis of similar toxicity profiles in short-term toxicity studies together with the outcome of genotoxicity and ADME, the extrapolation of toxicity data between selected non-nano and nano forms or between different nano-forms of the same nanomaterial may be justified (bridging toxicity approach).
- The overall risk characterization of a nanomaterial will not be any different from a conventional cosmetic ingredient. Where a given nanomaterial in a cosmetic product is well-characterized, both from a qualitative and quantitative point of view, and an adequate toxicological dataset is available, there should not be a reason to consider that risk characterization of the nanomaterial containing product is associated with an intrinsically higher uncertainty than that of a cosmetic product containing conventional ingredients. In view of this, the use of any additional “nano-specific” safety/uncertainty factors in risk assessment will not be appropriate. However, where this is not the case, and insufficient data, or data from inadequate tests, is available, and uncertainties are higher, a risk assessor may consider applying additional safety/uncertainty factors, similar to what is done for conventional chemicals.
- There is a need for research into the development and validation of characterization methods for nanomaterials as such, in final formulations, and during local and systemic exposures for toxicological evaluations. Research is also needed into the development of *in vitro* models – especially those based on co-culture and or tissue culture systems that can mimic *in vivo* situation more closely – to enable safety assessment in the ICCR jurisdiction where testing on animals is restricted or banned. Well designed studies are also needed to generate high quality data for *in silico* modelling to identify the key parameters, derive rules, and develop predictive models to estimate physicochemical properties, biokinetic behaviour, and toxicological effects of nanomaterials.

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8.0 Annexes

Annex 1: Acronyms and Definitions

3R	Refinement, Reduction, Replacement
AAS	Atomic Absorption Spectroscopy
Alternative methods	All those procedures which 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 needs of humans and other animals [Rogiers et al. 2000]
AUC	Analytical Ultracentrifugation
BET	Brunauer Emmett Teller method for calculating surface area based on the adsorption of a gas, typically nitrogen to the surface of a particle.
CE	Capillary Electrophoresis
Colipa	European Cosmetics Association (formerly the European Cosmetic Toiletry and Perfumery Association)
CPS	Centrifugal Particle Sedimentation
DLS	Dynamic Light Scattering
ECVAM	European Centre for the Validation of Alternative Methods
EDS	Energy Dispersive Spectroscopy
EDX	Energy Dispersive X-ray Spectroscopy
EELS	Electron Energy Loss Spectroscopy
EFSA	European Food Safety Authority
ESI	Electrospray Ionisation
FFF	Field Flow Fractionation
FTIR	Fourier Transform Infra-red
GC	Gas chromatography
HDC	Hydrodynamic chromatography
HPLC	High performance liquid chromatography
ICCR	International Cooperation on Cosmetic Regulation
ICP-MS	Inductively coupled plasma Mass spectroscopy
In silico method	Computational approaches that use (quantitative) structure-activity relationship modelling, and read-across between substances on the basis of structural or functional similarities.
In vitro test method	Biological method that uses organs, tissue sections and tissue cultures, isolated cells and their cultures, cell lines and subcellular fractions, or non-biological method that uses chemical interaction studies, receptor binding studies, etc [Rogiers et al. 2000]
JRC	Joint Research Centre
LDE	Laser Doppler Electrophoresis
MALDI	Matrix-Assisted Laser Desorption/Ionization

NMR	Nuclear Magnetic Resonance
OES	Optical Emission Spectrometry
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
SAXS	Small Angel X-ray Scattering
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SMPS	Scanning Mobility Particle Size
SPM	Scanning Probe Microscopy
STEM	Scanning Transmission Electron Microscopy
TEM	Transmission Electron Microscopy
TOF-MS	Time of flight mass spec
Valid method	A technique that has not necessarily gone through the complete validation process, but for which sufficient scientific data exist demonstrating its relevance and reliability [based on Rogiers 2003]
Validated method	A method for which the relevance and reliability are established for a particular purpose (in most cases according to the criteria established by ECVAM, the OECD, and other international validation bodies, such as ICCVAM, taking into account that a prediction model needs to be present from the start of the validation procedure) [based on Balls et al. 1997 and Worth et al. 2001] These methods are taken up in Regulation (EC) No 440/2008 and/or published as OECD Technical Guidelines.
VSSA	Volume Specific Surface Area
WAXS	Wide-angle X-ray scattering
WDX	Wavelength-Dispersive X-ray spectrometry
XDC	X-Ray Disc Centrifuge
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction
XRF	X-Ray Fluorescence analysis

Annex 2: Regulatory considerations relating to nanomaterials in cosmetics

Like certain other cosmetic ingredients, the use of nanomaterials in cosmetic products may have to go through pre-market evaluation of safety in different ICCR jurisdictions. The main safety concerns are related to the potential exposure of the consumer to nanomaterials through different routes – e.g. inhalation from spray formulations, dermal application from creams and lotions applied to the skin, or ingestion from application of lipsticks and toothpastes). A brief account of relevant regulatory frameworks for safety evaluation of chemical substances in cosmetics within the ICCR jurisdictions is provided below.

- The US legislative instrument of most relevance is the Federal Food, Drug and Cosmetic Act (the FD&C Act), which is administered by the Food and Drug Administration (FDA). The FD&C Act does not require cosmetic products and ingredients to be approved by FDA before they go on the market. As such, a pre-market evaluation is generally not required by the FDA, and there are no specific testing requirements for cosmetic ingredients/ products. The industry is responsible for due diligence and safety evaluation for their ingredients and products. Cosmetics manufactured using nanotechnology are subject to the same legal requirements as any other cosmetic. The FDA has not to date established a regulatory definition of nanotechnology or related terms. In June 2011, FDA has issued a draft guidance "Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology" in which they have proposed certain points that industry should consider when attempting to identify applications of nanotechnology in FDA-regulated products (see section 1.4). A draft guidance for industry on safety assessment of nanomaterials has recently been released for public consultation (See Section 4.0 and footnote 21).

- In Canada, the Acts and Regulations administered by Health Canada have no explicit reference to nanomaterial. To address this gap Health Canada has adopted the 'Policy Statement on Health Canada's Working Definition for Nanomaterials'. This policy statement is an important step toward establishing a transparent working means of identifying nanomaterials. It also provides Health Canada with a consistent set of approaches across the department and a trigger to request information. Substances used in cosmetics are subject to the New Substances Notification Regulations (NSNR) under the Canadian Environmental Protection Act. Generally, notification is required for any new substance manufactured or imported in Canada in quantities exceeding 100 kg per calendar year. Currently, there are no nano specific data requirements under NSNR. Active ingredients in sunscreens are regulated under other acts as drugs or natural health products. Currently data are being collated on nano-TiO_2 and nano-zinc oxide used as UV filter in sunscreen products.

- In Japan, nanomaterials are dealt with under the current regulations. As such, there is no specific route for safety evaluation of nanomaterials, but chemical substances go through evaluation as a new chemical (trigger quantities 1 tonne or more). Under the Japanese Cosmetics Regulation, both positive and negative lists of cosmetic ingredients are maintained on the basis of integrated information about the past assessed chemicals on health concerns since 2001. Formulations of positive compounds are regulated on the basis of application scenario. Other medicinal compounds may also be allowed for use with restricted concentrations. Chemicals/ substances that are not on the positives/negatives list can be used under the company's own responsibility over safety assessment. Any new addition to the positives list requires a dossier based report that is assessed by the Pharmaceutical Council[31].

    i) A present, the EU's Cosmetics Regulation (Regulation (EC) No 1223/2009) is the only framework which specifically covers the use of nanomaterials in cosmetics. The Regulation requires cosmetic products containing nanomaterials to be notified to the Commission six months prior to being placed on the market, and nanoscale ingredients

[31] The required endpoints include: Chemical structure; Physicochemical properties; Single dose toxicity; Repeated dose toxicity; Reproductive/developmental toxicity; Skin irritation; Skin sensitization; Photo toxicity; Photo sensitization; Eye irritation; Genotoxicity; Human patch tests; ADME.

to be labelled (name of nano ingredient, followed by 'nano' in brackets). If there are concerns over safety of a nanomaterial, the EC will refer it to the Scientific Committee on Consumer Safety (SCCS) for opinion. The SCCS assesses dossier based evaluations of safety of non-food consumer products - including certain cosmetic ingredients - under the Cosmetic Regulation. The Committee has adopted an Opinion on a nano-scale organic UV filter ETH50 (1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl-)[32], and published another Opinion on nano zinc oxide which is currently undergoing public consultation[33].

[32] SCCS/1429/11, SCCS (Scientific Committee on Consumer Safety), Opinion on 1,3,5-triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl-, 20 September 2011 http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_070.pdf

[33] SCCS/1489/12, SCCS (Scientific Committee on Consumer Safety), Opinion on zinc oxide (nano form) Colipa S76 , 18 September 2012, http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_103.pdf.

***Table A*: Chemical and Physical Parameters important for safety assessment of nanomaterials in cosmetics[34]**

Parameter	Description	Potentially Useful Method
Chemical identity	All known names by which the material may be accurately or commonly described. Examples include CAS Name, International Union of Pure and Applied Chemistry (IUPAC) Name, trivial name and common trade names if widely used (OECD, 2010). Other descriptors are empirical formula and molecular structure.	
Physical form (crystallinity / amorphous status)	A high level description of the morphological nature of the intended nanomaterial - for example, is the material amorphous or crystalline? Determination of crystalline phase - are the particles spherical, rods, plates (OECD, 2010).	Crystallinity: WAXS, electron diffraction, TEM, SEM, Raman Crystalline phase: WAXS, electron diffraction, TEM, Raman Particle Shape: TEM, SEM, SPM
Molecular weight	Molecular weight of the nanomaterial or an estimate provided when the actual molecular weight is not available such as for polymers.	Light scattering methods (polymers/proteins), TOF-MS methods (polymers/proteins)
Purity, composition and substance codes	Composition of nanomaterial being tested (including degree of purity, known impurities or additives). This is important because there may be nanomaterials that are described by the same name, but have key differences in composition or purity that could affect the biological activity and hence make comparison between seemingly similar materials difficult or impossible. The purity should be expressed as the percentage of the intended nanomaterial present (e.g., 95% pure). The balance should also be described as completely as possible using percentages (OECD, 2010).	A wide range of analytical methods, including Elemental analysis: OES, AAS, XPS, EDX, XRF, NMR, Mass Spectrometry (MS) in particular ICP-MS, TXFX, etc. Molecular composition: Mass spectrometry (ToF, QqQ) using suited ionisation techniques (e.g. MALDI, ESI), coupled with separation methods (e.g. HPLC, GC, CE etc),TOF-SIMS, NMR, FT-IR techniques Shell/core composition (for encapsulates, micelles): by a suitable method after disintegration of the particles and separation of the components by a suitable method (e.g. HPLC, SEC, CE, HDC etc) (EFSA, 2011)
Impurities / accompanying contaminants	Impurities and/or accompanying contaminants (e.g. catalysts from manufacturing or processing aids) including their concentrations.	Elemental analysis: OES, AAS, XPS, EDX, XRF, NMR, Mass Spectrometry (MS) in particular ICP-MS, TXFX, etc.

[34] These parameters are similar to those that would be useful for larger-sized particles as well but are included here for completeness.

Parameter	Description	Potentially Useful Method
		Molecular composition: Mass spectrometry (ToF, QqQ) using suited ionisation techniques (e.g. MALDI, ESI), coupled with separation methods (e.g. HPLC, GC, CE etc),TOF-SIMS, NMR, FT-IR techniques
Solubility	Water solubility/ dispersibility. This refers to the mass proportion of a given sample of nanomaterial which is held in water solution or as a colloidal suspension in water as a function of time or where the sample of nanomaterial loses its particulate character as it changes from a particle form to a molecular form. Note that solubility and dispersibility are not identical though the distinction can be difficult to recognise with nanomaterial (OECD, 2010). For partially-soluble nanomaterials, dissolution rates in relevant solvent should also be determined. For organic and surface modified inorganic nanomaterials, partitioning between aqueous and organic phase (e.g. log Kow) should be determined.	Standard tests for water solubility (e.g. OECD 105), and log kow (OECD 107, 117) can be used. For sparingly/ partially soluble nanomaterials, dissolution rate constants should be measured.
Partition coefficient (Log Pow) – where applicable	Ratio of distribution of material between hydrophobic and hydrophilic solvents, most commonly octanol and water.	OECD TG107 Partition Coefficient (n-octanol/water): Shake Flask Method, TG123 Partition Coefficient (1-Octanol / Water): Slow-Stirring Method
Particle size distribution in terms of mass and particle number concentration	Few preparations are available as monodispersed forms. As a result, characterization of a preparation of nanomaterials is most often presented as a distribution of size expressed as mass numbers (most common) or by particle number.	Spectroscopic methods, e.g. DLS, XRD, SAXS. Chromatographic methods, e.g. FFF, SEC. Microscopic methods, e.g. SEM, TEM, SPM. Other methods, e.g. XDC, CPS, AUC. Specific Surface Area: BET (size can be calculated if particle shape & density are known)
Particle shape	Shape is useful in assessing potential biological activity or distribution parameters.	Spectroscopic methods, e.g. SAXS Microscopic methods, e.g. SEM, TEM, SPM
Specific surface area	The specific surface area can be used to work out Volume Specific Surface Area (VSSA) as described by Kreyling et al., 2010.	BET

Parameter	Description	Potentially Useful Method
Agglomeration/ aggregation state	Characterization of the degree of agglomeration and aggregation. Could also be described in terms of % free primary particles.	Spectroscopic methods, e.g. DLS, SAXS Microscopic methods, e.g. SEM, TEM, SPM Other methods, such as XDC, SMPS, LDE
UV absorption	Data on UV absorption profile of the nanomaterial.	Spectral methods
Zeta potential	Zeta potential of nanomaterial is calculated from electrophoretic mobility. Preferably this should be measured in water to avoid discrepancies between tests in different solvents and pH/ ionic conditions (EFSA, 2011)	Electophoretic methods
Redox potential	Used to help predict potential for size-dependent step changes in surface chemistry.	Electrochemical measurements with electrode and potentiometer
Surface coating/ doping materials	Chemical characterization (identity) and amount of coating on particles. The expression of this information will depend on the particle and coating but should enable an estimate of the ratio of particle mass and mass of the coating.	Will depend on the nanomaterials and the coating. For fluid coatings this could include a solvent extraction and analysis. For solid-state coatings, a mixed ratio estimate may be possible utlizing a variety of techniques.
Homogeneity and stability (including photostability)	Stability of nanomaterials in the ingredient matrix. This will be dependent on the material and matrix but should provide information describing the stability to settling and light.	Various methods
Function and uses	A description of function and intended use(s) of the nanomaterials.	Will depend on end-use of the material in a formulation.

***Table B*: Methods for toxicological evaluation**

Parameter	Description	*In vivo*	*In vitro*
Toxicokinetics	The design of toxicokinetic studies for chemicals is described in OECD guideline 417.	OECD TG 417 Toxicokinetics	Not available.
Dermal / percutaneous absorption	Often used to determine the likelihood of dermal penetration resulting in systemic exposure. This decision can impact the design of the overall safety program.	OECD TG 427 – Skin absorption in vivo test.	OECD TG 428 skin absorption *in vitro* test.
Acute toxicity	Used to assess comparative potency of acute effects, including lethality. Not often used with nanomaterials.	OECD TG420 Acute Oral toxicity – Acute Toxic Class Method, TG423 Acute Oral Toxicity – Acute Toxic Class Method TG425 Acute Oral Toxicity: Up-and-Down Procedure TG402 Acute Dermal Toxicity TG403 Acute Inhalation Toxicity, Additional short term inhalation test. 5-day Inhalation study with 28/90 day post-exposure monitoring and periodic BAL.	OECD GD 129. While not suitably predictive for determining LD50s, this test has been used to predict starting doses for rodent acute oral toxicity testing. Its usefulness with nanomaterials has yet to be established.
Irritation and corrosivity	Used to determine potential for dermal effects of topically applied preparations.	Skin Irritation OECD TG404 Acute Dermal Irritation/ Corrosion.	(Near - OECD validated assays - accepted by EC) *In vitro* Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method Under evaluation by OECD WNT; accepted in the EU: Test Method Regulation (EC) B46
Eye irritation and	Used to determine potential for effects on the cornea and related elements of the eye.	Acute Eye Irritation	OECD TG 437. The Bovine Corneal Opacity and

Parameter	Description	*In vivo*	*In vitro*
corrosion		OECD 405 (Acute Eye Irritation/Corrosion) should be used only if the nanomaterial has not shown evidence of skin corrosivity.	Permeability (BCOP) Test Method for Identifying Ocular Corrosives and Severe Irritants. OECD TG 438. The Isolated Chicken Eye (ICE) Test Method for Identifying Ocular Corrosives and Severe Irritants
Skin sensitisation	Used to determine the potential for a material to cause delayed contact hypersensitivity.	Skin sensitisation Methods have been established and validated for a number of chemicals using the guinea pig skin sensitisation model (OECD TG406 Skin Sensitisation) and the murine local lymph node assay (OECD TG429 Skin Sensitisation: Local Lymph Node Assay)	Numerous methods under development.
Photo-induced toxicity	This toxicity occurs when a material interacts with light and generates a new product or catalyzes the formation of other products that are toxic.	No OECD method available. Numerous protocols available from other sources.	OECD TG 432 In Vitro 3T3 NRU Phototoxicity Test

Parameter	Description	*In vivo*	*In vitro*
Repeated dose toxicity	Repeated dose toxicity studies allow an exploration of cumulative effects of materials on biological systems (OECD, 2010). For orally ingested nanomaterials, EFSA, (2011) has recommended the minimum requirement of a repeated dose 90-day oral toxicity study in rodents (OECD guideline 408), modified to include assessment of some additional parameters described in the more recent guideline on repeated-dose 28-day oral toxicity study in rodents (OECD guideline 407). The results from the repeated dose 90-day oral toxicity can be used to identify a Benchmark Dose lower confidence bound (BMDL) or a No-Observed-Adverse-Effect-Level (NOAEL).	Oral: OECD TG407 Repeated Dose 28-Day Oral Toxicity Study in Rodents OECD TG409 Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents Dermal: OECD TG410 *Repeated Dose Dermal Toxicity: 90-Day* in rodents and in non-rodents. Inhalation: OECD TG411 *Subchronic Inhalation Toxicity: 90-Day,* OECD TG412 *Repeated Dose Inhalation Toxicity: 28/14-Day*, OECD TG413 *Subchronic Inhalation Toxicity: 90-Day*	Not available.
Chronic toxicity (including carcinogenicity)	Chronic toxicity and carcinogenicity study is described in OECD test guideline 453.	TG 453 – Combined Chronic Toxicity\Carcinogenicity Studies	Not available.
Mutagenicity / Genotoxicity	Used to determine the potential for genotoxicity.	OECD TG475 *Mammalian Bone Marrow Chromosomal Aberration Test*, OECD TG474 *Mammalian Erythrocyte Micronucleus Test*), OECD *TG486 Unscheduled DNA* *Synthesis (UDS) Test with Mammalian Liver Cells in*	OECD TG471 *Bacterial Reverse Mutation Test)*, mammalian cell chromosome aberrations (OECD TG473). *Caution should be applied when using this or any other bacterial test with particles including nanoparticles.*

Parameter	Description	*In vivo*	*In vitro*
		vivo.	*Exposure to target organelles is limited.* *In vitro* Mammalian Chromosomal Aberration Test), and mammalian cell gene mutations (mouse lymphoma cells; OECD TG476 *In vitro Mammalian Cell Gene Mutation Test*).
Reproductive toxicity	One-generation reproductive toxicity study which involves exposure of the male and female rodents for a specified length of time prior to mating. Dosing of the pregnant females is continued through parturition and until weaning of the offspring. These studies are typically conducted with oral administration of test article; however, other routes of exposure could be used.	OECD TG415 *One-Generation* *Reproduction Toxicity* A modification of the one-generation reproductive toxicity test is exposure of the dam only to the test article from conception to birth (OECD TG414 *Prenatal Developmental Toxicity Study*). A two-year (or additional generations) reproductive toxicity test (OECD TG416 *Two-generation* *Reproduction Toxicity Study*) extends the exposure of the offspring from the one-generation test through maturation, mating and production of a second generation of offspring (F2). The same considerations as mentioned above should be included in the dossier regarding a two- (or multiple) generation reproductive toxicity test.	Not available.
Photocatalytic activity	If a nanomaterial has catalytic properties, it may catalyse a redox or other reaction which may perpetuate resulting in a much larger biological response even with small amounts	ISO TC 206/WG37, Fine ceramics – Test methods for photocatalytic material, has a number of work items on this subject (OECD, 2010).	Not available

Parameter	Description	*In vivo*	*In vitro*
	of the catalytically active nanomaterial. Thus, compared to a conventional biochemical reaction which uses up the substrate, nanomaterial reaction centres may perpetuate catalytic reactions (EFSA, 2011). Photoreactivity of ZnO has been measured under constant focused photon flux from a 500W medium pressure mercury arc lamp. The photoactivity index is measured as the zero order rate of the photocatalytic oxidation of liquid propan-2-ol to propanone under oxygenated conditions. Photoreactivities are expressed in terms of moles converted per gram of particulate per hour of irradiation (Source OECD, 2010).		
Human Skin Sensitization	Delayed contact skin sensitization (Type IV allergy) can result from repeated exposure to a formulation/ingredient with the potential to induce contact allergy. Clinical testing for dermal sensitization can be utilized with nano-containing formulations provided it is justified by the risk assessment.	Human Repeat Insult Patch Test (HRIPT) used for confirming lack of sensitization potential of a formulation/product. Induction phase consists of nine 24-hour patch applications to skin followed by a 2 week rest. Challenge phase consists of a single 24-hour patch application to a naïve skin site followed by grading 24/48/96 hours post-patch removal. Conducted in compliance with ICH (International Conference on Harmonization) Guidelines for Good Clinical Practice, Sections 4.1, 4.2, 4.4 (when applicable), 4.5, 4.6, 4.7, 4.8, 4.9, 4.11 and 4.12, and any applicable Federal, state or local regulatory requirements or standards (Shelanski and Shelanski, 1953).	Not available.
Human Skin Irritation	Dermal irritation can result from exposure to a formulation/ingredient that may cause irritation under normal use or misuse	Cumulative Irritation Patch Test used for assessing the irritation potential of a product by consecutive daily patch applications to the skin.	Not available.

Parameter	Description	*In vivo*	*In vitro*
	conditions. Clinical testing for dermal irritation can be utilized with nano-containing formulations.	Patches applied for 7-21 consecutive days to determine potential to cause erythema and dryness. Duration of study based on irritation potential of product. Duration of study and patch type (occlusive or semi-occlusive) can simulate exaggerated conditions, if desired. Conducted in compliance with ICH (International Conference on Harmonization) Guidelines for Good Clinical Practice, Sections 4.1, 4.2, 4.4 (when applicable), 4.5, 4.6, 4.7, 4.8, 4.9, 4.11 and 4.12, and any applicable Federal, state or local regulatory requirements or standards (Phillips, et al, 1972).	
Human Safety-In-Use	Dermal irritation may result from exposure to a product under normal use conditions. Clinical testing for dermal irritation can be utilized with nano-containing formulations	Safety-In-Use testing assesses the potential of products to cause erythema and/or dryness when used as intended under normal conditions for a period of 4 weeks. Conducted in compliance with Federal, state and local government regulations, guidelines and standards applicable to such studies including, but not limited to, those relating to Good Clinical Practices and Informed Consent.	

References to the methods in this Table are provided in the list of References (section 7.0)