6.3 Considerations for testing nanomaterials

6.3.1 General Considerations

Each risk assessment/ evaluation of a nanomaterial to be considered as cosmetic ingredient should start with an evaluation of relevant studies available in the scientific literature. Study results submitted as part of a safety dossier should accompany a declaration that the relevant tests were conducted using a substance with a comparable chemical purity/impurity profile, and physicochemical characteristics to that intended for inclusion in the finished cosmetic product [SCCNFP/0633/02]. Considering nanomaterials, this means that the test substance, and the substance in the finished cosmetic product, both have the same or a comparable profile, in relation to chemical composition, size and size distribution, surface properties, morphological form, etc. Proper characterisation/ identification of the nanomaterial used in the various toxicity studies and as used for cosmetic ingredient is therefore essential.

Information on the stability of the test substance under experimental conditions is of prime importance for the interpretation of any test results (Section 4.1). Data on the stability of the test material should therefore be reported, and data on the dissolution rate and the solubility of the nanomaterial in the finished cosmetic product and in the vehicle(s) used in the tests must be provided (if applicable).

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

- all relevant published scientific literature accompanied by a description of the bibliographical methods used;
- any report on epidemiological and/or observational experiences;
- any useful finding to the applicant's best ability;
- any "grey material/literature" available elsewhere.
- any new information acquired by industry, academia and/or agencies should be submitted to the Commission for review (SCCNFP/0461/01).

6.3.2 Specific considerations relating to Nanomaterials

For transparency in the use of a manufacturer’s raw material in potentially different cosmetic formulation types, a detailed description of the production of the nanomaterial, any surface modifications, and the preparatory steps for integrating it in the final cosmetic product, must be fully described in the safety dossier. This information would facilitate a more effective, time-saving and comprehensive risk assessment by the SCCS.

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has adopted two opinions on the appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies (SCENIHR (2007), and SCENIHR (2009). Furthermore, the SCCP (2007) has published an opinion on the safety of nanomaterials specifically in cosmetic products. These reports and other reviews have concluded that the existing risk assessment paradigm, in use for conventional chemicals, should in principle be applicable to engineered nanoparticles. However, it has also been pointed out that the current testing methods may need certain adaptations to take account of the special features of nanoparticles (Rocks et al. 2008, SCENIHR 2009, OECD 2009, SCCP 2007). These aspects are discussed below:

6.3.2.1 Solubility/dispersion:
When testing nanomaterials, it should be noted that some *in vivo* test methods may only be suitable for substances that are soluble at more than 1 mg/l (e.g. carcinogenicity test OECD TG451; reproductive toxicity tests OECD TG415 and 416; Mutagenicity test OECD TG478,

etc) (Rocks et al., 2008). Testing of insoluble or partially-soluble nanoparticles using *in vivo* or *in vitro* methods must also take into account that they will be present in a dosing or test medium as a nano-dispersion rather than in solution. Therefore, any toxicity testing using *in vivo and in vitro* methods should pay special attention to the agglomeration/ aggregation behaviour, and the insoluble/ partially-soluble nature of nanomaterials (SCCP 2007, Rocks et al., 2008; SCENIHR, 2009; OECD, 2009; Chaudhry et al., 2010). Possibilities for disagglomeration of nanoparticles should also be considered.

During toxicological evaluations, some properties of nanomaterials may change due to interaction with the surrounding media. Thus, a focus of investigations should be on ascertaining that the tested nanomaterials are in exact form/ composition as intended for use in a cosmetic formulation, and as the formulation is delivered to the end-user. Where toxicological data on a different nanomaterial, or a different form of the same nanomaterial, is presented in the dossier, justification must be provided to indicate that the two are justifiably comparable.

Special care is also needed in regard to the applied doses, as concentration of a nanomaterial may decrease during a test due to sedimentation, binding with other moieties in the test medium, or adhesion to glass/plastic ware. It is therefore important to ascertain the stability and uniformity of the nanomaterial in a test medium to ensure that the applied concentration/ dose is maintained for the intended period during the test. This will also need determining the possible interaction of the nanomaterial with other component of a test medium/ formulation.

6.3.2.2 Surface interactions

Due to the very high surface energy, nanoparticles are known to adsorb or bind different substances on surfaces, including proteins (Cedervall et al., 2007, Šimon and Joner 2008, Lynch and Dawson 2008). They may bind and transport various substances to the targets in the test system resulting in an altered (increased or decreased) activity/toxicity. Also an interaction of the nanomaterials with components of the test systems may lead to possible artifacts and a false indication of harmful effects. This can be avoided by a thorough characterisation of nanomaterials, and the use of appropriate controls in the testing scheme. One of these controls should consider the possible interaction of the nanomaterial with the read out system of the assay as demonstrated for various nanomaterials and tetrazolium salts or other dye-based cytotoxicity assays (Worle-Knirsch et al., 2006, Monteiro-Riviere et al., 2009; Lanone et al., 2009; Wilhelmi et al., 2012). In case of a doubt over the validity of the outcome of an assay, the use of an additional independent analytical method may provide more information. The presence of a light-absorbing/reflecting nanomaterial itself can have an influence on a read out system, especially if the system is based on spectroscopy. Similarly the composition of the culture medium (e.g. the presence or absence of serum) in a test system may influence the outcome of the assay.

6.3.2.3 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 chemicals. However, such metrics may not be appropriate for nanomaterials because of the large surface areas per particle mass or volume. Until suitable parameters are identified, that are describing and predicting dose-effect relationships, it is important that tests on nanomaterials are evaluated using different dose-describing metrics, such as weight/volume concentration, particle number concentration, surface area etc. Therefore the characterisation data of a nanomaterial should provide sufficient information to converse doses based on mass into other parameters such as number of particles or surface area.

6.3.2.4 Bioavailibility – toxicokinetics

The ability of nanoparticles (especially in the lower nm range) to penetrate cellular membrane barriers has added another dimension to the toxicology of particulate materials.

Due to the very small size, and certain surface characteristics, insoluble or partially-soluble nanoparticles may be able to reach unintended parts of the body that are otherwise protected from exposure to particulate materials by biological membrane barriers. Currently, it is not certain whether the endpoints identified under the current testing schemes will be sufficient to identify and characterise all the hazards that may be associated with a nanomaterial. In view of this, the risk assessment may in the first instance be driven by considerations of exposure, and the initial focus of safety considerations may be on determining the likelihood and extent of translocation of nanomaterials across skin, lung, or gastrointestinal barriers (as appropriate, depending on the nature of 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. For hazard identification, emphasis should be on toxicological tests over prolonged periods with repeated doses that are followed up by histopathological investigations.

6.4 Considerations for the replacement of *in vivo* testing by *in vitro* testing

Any conduct of animal studies must be in compliance with the testing and marketing bans in place under the European cosmetics legislation.

The Directive 76/768/EEC, and as of 11 July 2013 the Cosmetics Regulation ((EC) No 1223/2009) [4], prohibits the testing of finished cosmetic products and cosmetic ingredients on animals (testing ban), and prohibits the marketing in the European Community, of finished cosmetic products and ingredients included in cosmetic products that were tested on animals (marketing ban). The testing ban on finished cosmetic products has applied since 11 September 2004, whereas the testing ban on ingredients or combination of ingredients has applied since 11 March 2009, irrespective of the availability of alternative non-animal tests. The marketing ban also applies since 11 March 2009 for cosmetic products containing ingredients tested on animals. Exceptions are tests for repeated dose toxicity, reproductive toxicity, and toxicokinetics. For these specific tests, the deadline of 11 March 2013 is foreseen, irrespective of the availability of alternative non-animal tests.

Besides the Cosmetics legislation, Article 7 of the Council Directive 86/609/EEC provides for the protection of animals used for experimental and other scientific purposes '*an animal study shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonable and practically available*'. Directive 86/609/EEC will be repealed as off 1 January 2013 and replaced by Directive 2010/63/EU on the protection of animals used for scientific purposes, which contains the principles of replacement, reduction and refinement in its Article 4.

In complying with the ban on testing of cosmetic ingredients in animals, there are only a few choices of alternative methods and these are at present only suited for toxicological hazard identification. Among the main available methods are *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, only data from **validated** methods are accepted for assessment of cosmetic ingredients and products in Europe. These are the methods that have passed various steps of the modular validation process established at the European Centre for the Validation of Alternative Methods (ECVAM), and are considered by its Scientific Advisory Committee (ESAC) to comply with the process. Other methods may also be considered by ESAC to be equivalent with such an approach. *In*

[4] These provisions were introduced by the 7th Amendment of Directive 76/768/EEC, Directive 2003/15/EC

vitro methods that are accepted by the OECD, and other international validation bodies, such as ICCVAM, are also considered validated.

Whilst *in silico* modelling approaches are advancing for conventional chemicals, a relationship between the various physicochemical properties and toxicological effects of nanomaterials has not yet been established to allow development of reliable models for nanomaterials. As a result, only a few rudimentary *in silico* models are currently available for nanomaterials (Toropov et al., 2006; 2007a; 2007b; 2008; Sayes and Ivanov, 2010; Burello and Worth, 2011). However, they are unlikely to be useful in the foreseeable future for the assessment of relevant toxicological endpoints that are needed for risk assessment.

Hartung and Sabbioni, (2011) have recently reviewed different *in vitro* tests for applicability to nanomaterials. These included skin corrosion, phototoxicity, dermal penetration, skin and eye irritation, genotoxicity, acute oral toxicity, carcinogenicity, sensitisation, ecotoxicity, and pyrogenicity. Their finding showed that alternative methods can be useful for hazard identification of nanomaterials but will need optimising for each of the nanomaterials evaluated. For extrapolation of *in vitro* data to *in vivo* situations, they regarded the determination of kinetic (ADME) parameters of nanoparticles versus corresponding microparticles as well as the released metal ions at the cellular level to be a key point. They also highlighted the importance of extensive physicochemical characterisation of the test material, the delivered dose, and consideration of the relevant contact of the test material with the target in hazard identification/characterisation. However, as mentioned before, it seems unlikely that data derived from *in vitro* assays alone will be sufficient for risk assessment of nanoparticles at present (Park et al., 2009a) and in the foreseeable future. In the context of the EU cosmetic legislation, a review of the actual status of alternatives has been carried out by the SCCP (2007), by the SCCS (2009) (Memorandum on Alternative Test Methods, SCCS/1294/10) and by a group of experts under the co-ordination of the European Centre for the Validation of Alternative Methods (ECVAM), hosted by the Institute for Health and Consumer Protection of the European Commission's Joint Research Centre (Adler et al. 2011). **They concluded that considerable scientific challenges would have to be overcome before a full replacement of animal tests could be possible**. Whereas substantial progress over the past years was noted, they predicted that, for five specific areas (toxicokinetics, repeated dose toxicity, carcinogenicity, skin sensitisation, and reproductive toxicity), alternative methods to fully replace animal tests would not be available by 2013. However, the experts noted that significant contributions to reduce, refine and partially replace animal testing had been made.
The conclusions of the SCCS memorandum are also in line with those of Adler et al. (2011). For the acute and local endpoints, the SCCS concluded that the following endpoints are not affected by the EU testing or marketing ban: skin corrosivity, skin irritation, dermal absorption, mutagenicity/ genotoxicity and phototoxicity. However, these conclusions refer to conventional cosmetic ingredients only, and **not** to nanomaterials. Although not validated against nanomaterials, some of the available validated *in vitro* tests might be relevant for hazard identification of nanomaterials and may provide additional supporting evidence to *in vivo* studies, provided that they are carried out with due consideration to the nano-related aspects. Furthermore, it should be realised that, due to the particle nature of nanomaterials, the toxicokinetic profiles will differ considerably from those of conventional chemicals, e.g., as discussed before, most nanomaterials may end up in the reticulo endothelial system (RES) which removes particles from the circulation. A more detailed analysis of the nano-related considerations in relation to toxicological testing of nanomaterials is provided in Table 3.

A model for tiered nanotoxicity screening has been proposed for risk assessment of nanomaterials (SCENIHR, 2007; Oberdörster et al., 2005; Hirsch et al., 2011; Stone et al., 2009). The proposed approach involves thorough physicochemical characterisation of nanomaterials, *in vitro* screening tests, and the use of OECD and ECVAM validated/ approved *in vitro* methods. In order to mimic the *in vivo* situation more closely, the use of *in vitro* co-culture systems has been suggested to evaluate the possible interaction of

nanomaterials with organs likely to be exposed (Clift et al., 2011). However, some *in vitro* systems may also yield invalid results due to interaction of the nanomaterial with the test systems (Worle-Knirsch et al., 2006, Monteiro-Riviere et al., 2009; Wilhelmi et al., 2012). In view of the limitations, the SCCS considers that, in the absence of validated stand-alone *in vitro* tests, or a testing battery, the tiered approach using only *in vitro* and *ex vivo* assays are too premature to be applied for risk assessment at present. However, at the same time, it is important to emphasise that, besides the use of *in vitro* systems in hazard characterisation, they can provide very useful information on relative toxicity, and the possible mode(s) of toxic action and mechanisms of nanomaterials. This can give pointers for further toxicological investigations. For example, *in vitro* tests may indicate the likelihood of generation of reactive oxygen species, which may provide an alert for potential toxic effects via the induction of oxidative stress and activation of inflammatory and proliferative pathways (Unfried et al., 2007).

7. RISK ASSESSMENT

The risk of a nanomaterial is assessed by calculation of the Margin of Safety (MoS). The (MoS) of ingredients in a finished cosmetic product is calculated as follows:

MoS = NO(A)EL* / SED (systemic exposure dosage)
*or LO(A)EL where NO(A)EL is not available

The MoS is determined in order to identify a potential risk for systemic (adverse) health effects. In general, a MoS of >100 is considered acceptable. Depending on the dataset available, additional safety factors may be used (e.g when using LO(A)EL instead of NO(A)EL, or when specific toxicological information, e.g. on certain endpoints, is missing). The assessment factor of 100 (plus additional uncertainty factors if required) has been developed for conventional ingredients and not specifically for nanomaterials (SCCS Notes of Guidance, SCCS/1416/11). However, the assessment factors address aspects of extrapolation and uncertainty and therefore are at present considered to be applicable and appropriate for nanomaterials as well (REACH RIPoN3).

Apart from systemic effects, also local effects (e.g. on skin after dermal application and respiratory tract after spray application) will need to be considered.

In the Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation (SCCS/1416/11), it is stated that the systemic availability of a cosmetic ingredient is estimated by taking into account the daily amount of finished cosmetic product applied (frequency of application), the concentration of the ingredient under study, the dermal absorption of that particular ingredient, and a mean human body weight value. As such, the amount of ingredient per kg body weight that would become available daily in the human circulatory system is calculated.

For conventional ingredients, in the majority of MoS calculations, the **dermal** exposure is compared to an **oral** NO(A)EL value (route to route extrapolation). The oral NO(A)EL value usually corresponds to an amount that has been administered orally, though not necessarily to the actual systemic availability of the compound after oral administration. In many conventional calculations of the MoS, the oral bioavailability of a substance is assumed to be 100% in case oral absorption data are unavailable. However, the SCCS considers it appropriate to assume that not more than 50% of an orally administered dose is systemically available. The value of 50% is an arbitrary choice that recognises that the gastrointestinal tract is designed to favour the absorption of ingested substances into the body but that, in most cases, not all of the ingested material will be bioavailable. Thus, in the absence of data, the assumption is being made that effects seen following oral administration have been caused by a fraction of the administered dose and not the entire amount administered. If there is evidence to suggest poor oral bioavailability, for example

the substance is a poorly soluble particulate, it may be more appropriate to assume that only 10% of the administered dose is systemically available [IGHRC 2006]. Whenever oral absorption data are available, these should be included in the calculations [e.g. SCCP/0851/04]. In the case of oral-to-inhalation extrapolation, it was proposed that, in the absence of route-specific bioavailability information, a default factor of 2 (i.e. the absorption percentage for the starting route is half that of the end route) might be appropriate. The inclusion of this factor 2 means for example that 50% (instead of 100%) absorption is assumed for oral absorption, and 100% for inhalation.

For route-to-route extrapolation experimental data on absorption will be required both on dermal and oral exposure. Any route-to-route extrapolation needs to be performed case-by-case, based on expert judgment of scientific information, including the available toxicokinetic information. It can, however, only be performed if there is systemic toxicity, considering the degree of absorption and also possible metabolic transformation.

For nanomaterials, the calculation of the MoS, especially in the case of (very) low absorption via oral, dermal, and/or pulmonary routes of exposure, can be challenging. In case of (very) low absorption, the validity of NOAELs in toxicological studies may be questionable, and for substances that are hardly absorbed, no toxic effects may be noted. However, in such a case, processes such as translocation and accumulation will need to be accurately studied before a decision on the safe use can be taken.

8. SUMMARY AND CONCLUSIONS

The use of nanomaterials as cosmetic ingredients, such as UV filters in sunscreens, may bring certain benefits to the consumer. However, the same nanomaterial that gives a cosmetic product useful properties, can also pose a risk to the consumer. At the nano-scale, materials may show a change in, or have novel, physicochemical properties, behaviour, and/or effects, compared to conventional equivalents. The ability of nanomaterials, especially nanoparticles in the lower nanometre size range, to penetrate biological membrane barriers adds a further dimension to the toxicology of particulate materials. Due to the very small dimensions, and certain surface characteristics, some insoluble or partially-soluble nanomaterial may be able to penetrate biological membrane barriers and reach certain organs that are otherwise protected from (larger) particulate substances. Where the systemically-available nanomaterials are insoluble or partially-soluble, and persistent, such exposure may lead to harmful effects due to the potential interaction of the particle surfaces with biological processes and moieties close to the molecular level. This requires a thorough safety evaluation of any nanomaterial that is intended for use as a cosmetic ingredient, in the same way as other ingredients, but with special considerations to nano-features.

This Guidance is aimed at providing information to help compliance with the requirements for safety assessment of nanomaterials intended for use in cosmetic products. It highlights the need for special considerations in relation to the safety of nanomaterials, in view of the possible distinct properties, interactions, and/or effects that may differ from conventional form of the same materials. The Guidance builds upon a number of relevant opinions, guidance documents, and reports from various European and international bodies, as well as scientific literature. It covers the main elements of risk assessment of nanomaterials in relation to possible use as cosmetic ingredients, i.e. general safety considerations (section 3), material characterisation (section 4), exposure assessment (section 5), hazard identification and dose-response characterisation (section 6), and risk assessment (section 7).

It needs to be emphasised that the field of nanomaterial risk assessment is still evolving, and the guidance provided in this document is based on the currently available knowledge. The guidance may therefore be revised in the light of new scientific knowledge in the future

The key recommendations for risk assessment of nanomaterials intended for use in cosmetics are summarised below:

1. Definition: Definition of nanomaterial is provided in the Cosmetic Regulation (EC) No 1223/2009, under Article 2 (1) (k). This definition may be adapted in the light of the European Commission's Recommendation (2011/696/EU) on an overarching definition of nanomaterial.

    a. Information on relevant material specifications of a manufactured cosmetic ingredient - in terms of particle size distribution, solubility, persistence - should be sufficient to provide a basis for deciding whether or not it is a nanomaterial in accordance with the definition under the relevant regulation.

    b. In situations where a particulate material has internal nano-structures, or exists in the form of larger agglomerates or aggregates, the use of volume specific surface area (VSSA) for powders, and/or other parameters, such as electron microscopy images, can provide further information.

    c. Where a cosmetic ingredient fulfils the criteria defining a nanomaterial set up in the Cosmetic Regulation (EC) No 1223/2009, Article 2 (1) (k)), safety data with special considerations to the properties of that specific nanomaterial will be required for risk assessment. This will apply to any new or already approved ingredient if it fulfils the criteria for a nanomaterial.

2. Material characterisation: In view of the specific properties, behaviour, and effects of nanomaterials, detailed characterisation and identification of nanomaterials is an essential requirement of risk assessment:

    a. The characterisation data presented in a safety dossier must provide information on the identity of the core material(s), relating to the same (or justifiably comparable) nanomaterial that is intended for use in the final product. The information should correspond to Cosmetics Regulation (EC) No 1223/2009, Article 16 a) "identification of the nanomaterial…").

    b. The characterisation must also include measurement of important physicochemical parameters. As a minimum, the SCCS requires data on all of the parameters listed in Table 1 that are relevant to the given type of nanomaterial. Corresponding to Cosmetics Regulation (EC) No 1223/2009, Article 16 b) "specification of the nanomaterial..."

    c. It is important that the measurements are carried out using mainstream techniques with due consideration to nano-aspects, and results are backed up by appropriate documentation.

    d. Size is the common denominator for all nanomaterials. Hence data on size related parameters must be obtained by more than one method. One of these should be electron microscopy (preferably in the form of high resolution TEM images).

    e. The characterisation needs to be carried out on the nanomaterial at the raw material stage, in the cosmetic formulation, and during exposure for toxicological evaluations. If characterisation at any of these stages is not feasible, e.g. due to lack of methods, or degradation of the nanomaterial, it should be justified and documented.

    f. Where needed, the SCCS may ask for provision of a detailed description of the production processes, any surface modifications, and the preparatory steps carried out for integrating the nanomaterials in the final cosmetic products to facilitate risk assessment.

3. Exposure Assessment: As proposed in this guidance, the risk assessment of cosmetic nanomaterials may in the first instance be driven by considerations of exposure. Data on

exposure assessment will therefore enable the first crucial decision in the overall risk assessment (Figure 1).

a. The initial focus may be on determining the likelihood and extent of translocation of nanomaterials across skin, lung, or gastrointestinal barriers (as appropriate) whilst mimicking the actual use scenarios, with due considerations to nano-aspects.

b. The determination of systemic absorption of conventional cosmetic ingredients is generally carried out by chemical analysis of the receptor fluid or of blood/tissues. However, chemical analysis does not always provide information on the particle nature of the absorbed material. Thus, if chemical analysis indicates systemic absorption, further investigations will be required to confirm whether the absorbed material was in a particle form or in solubilised/metabolised form.

c. The use of imaging methods, such as electron microscopy, should be sufficiently sensitive to determine whether the absorbed material was in nanoparticle form by analysing receptor fluids and tissue samples.

d. The SCCS is of the view that the method for calculating dermal and oral exposure to nanomaterials will not be very different from the calculation of exposure to conventional cosmetic ingredients. These methods are provided in the SCCS Notes of Guidance (SCCS/1416/11 or more recent version) and are detailed in Section 5.

e. Certain assumptions are used for estimation of dermal absorption of conventional chemical ingredients (section 5.1.1). These assumptions are not applicable to nanomaterials. Dermal absorption of nanomaterials will therefore need to be determined experimentally.

f. Calculation of exposure to nanomaterial containing aerosols is likely to be more challenging and will need determination of the generated droplet size distribution as well as size distribution of the dried residual aerosol particles (section 5.1.3).

g. Where there is evidence of systemic absorption, further investigations will be required to confirm whether the absorbed material was in a particle form or in solubilised/metabolised form (Figure 1). Where the absorption of particles cannot be excluded either by experimental data, or justified on the basis of solubility/degradation of the nanomaterial, the SCCS may apply a default approach and assume that 100% of the absorbed material was in particle form.

h. It is very important to characterise the nanomaterial under exposure conditions to ascertain that its characteristics are unchanged when used in the finished cosmetic product.

i. An important question in regard to risk assessment (Figure 1) is whether any systemic exposure to nanomaterial is possible. This can be assessed by analysis of the receptor fluid for nanoparticles, as well as determination of the levels in organs and/or blood in studies, for example on dermal absorption, toxicokinetics, acute or repeated dose toxicity, etc. The methods used for this purpose, however, need to be state of the art, and the limit of detection low enough to demonstrate the lack of exposure. In this regard, the use of sensitive methods for chemical analysis (Table 1) should generally be sufficient. However, where chemical analysis cannot distinguish between the absorbed and the natural levels of a substance in the body (e.g. zinc), the use of other techniques such as radiotracer or stable isotope analysis may be needed.

j. In addition to the assessment of systemic exposure, any local effects will also need considering.

k. Even in the absence of systemic translocation of nanomaterials, and/or local effects, safety assessment will still be required as per SCCS Notes of Guidance (SCCS/1416/11 or more recent version), with consideration of any nano-related aspects.

4. Hazard identification/ dose response characterisation: Where application of a nanomaterial containing cosmetic product can lead to systemic exposure, data on toxicological evaluation will be required. Information on the possible local effects will also be required.

   a. The current hazard identification/ characterisation schemes used for conventional chemical substances are also broadly applicable to nanomaterials. However, because of the possible deviations in physicochemical properties, toxicokinetic behaviour, and interactions with biological entities, it is currently not certain whether the endpoints identified under the current testing schemes will be sufficient to identify and characterise all the hazards that may be associated with a nanomaterial. In view of this, the risk assessment may in the first instance be driven by considerations of exposure, and the initial focus of safety considerations may be on determining the likelihood and extent of systemic exposure due to translocation of nanomaterials across skin, lung, or gastrointestinal barriers (as appropriate, depending on the nature of product use).

   b. Any testing of nanomaterials for hazard identification/ dose response characterisation must be carried out in consideration of the nano-related aspects (section 6.3). These include particulate form, insoluble or partially-soluble nature, aggregation and agglomeration behaviour, potential to penetrate biological membranes, possible interaction with biological entities, surface adsorption/ binding of different substances, surface catalysed reactions, persistence, etc (section 6.3). Details on testing conditions should also be documented and provided in the dossier.

   c. Where there is evidence of systemic exposure, initial focus should be on ADME (absorption, distribution, metabolism and excretion) parameters to investigate the fate and behaviour of the nanomaterial in the body (*in vivo* or *ex vivo*) and to identify the likely target organs.

   d. Like other cosmetic ingredients, data on a base set of toxicological endpoints will be required (Table 2). These include dermal/ percutaneous absorption, acute toxicity; irritation and corrosivity, skin sensitisation, repeated dose toxicity, and mutagenicity/ genotoxicity. Depending on the outcome of the tests, further information on carcinogenicity, reproductive toxicity may also be required. The emphasis should be on toxicological tests over prolonged periods with repeated doses, followed up by histopathological investigations.

   e. The Cosmetics Directive 76/768/EEC, and as of 11 July 2013 the 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). Current exceptions are tests for repeated dose toxicity, reproductive toxicity, and toxicokinetics, but the legislation foresees the full implementation of the marketing ban also for these tests by 11 March 2013.

   f. At present, validated alternative methods that can be used in place of animal tests are only available for conventional substances, and not for nanomaterials. This poses an insurmountable obstacle to safety assessment of cosmetic nanomaterials, and further research work is needed in this area.

   g. Although not validated for nanomaterials, the available validated *in vitro* tests may 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 6.4 and Table 3). Characterisation/ identification of nanomaterials during the tests will be an essential part of the evidence to ensure validity of the results (Section 4).

h. In view of the current lack of alternative methods that are specifically validated for nanomaterials, the SCCS is of the opinion that the complete ban on *in vivo* testing of cosmetic ingredients and products in 2013 poses an obstacle to the risk assessment of cosmetic ingredients in general, and ingredients in nanomaterial form in particular.

i. In the absence of a sufficient knowledgebase on nanomaterial properties, behaviour, and effects that can allow a read-across, the SCCS considers that a category approach to risk assessment is currently not feasible for nanomaterials, and risk assessment of each nanomaterial needs to be carried out on a case-by-case basis. It is, however, inevitable that the ongoing research and development in this area will increase understanding of the key parameters that drive the properties, biological interactions and toxicological effects of nanomaterials. With the availability of the new knowledge, it will be possible to derive the underlying rules that allow a read-across, and mathematical models that enable a category approach to risk assessment of nanomaterials in the future.

5. Risk Assessment: Once necessary data and information on local and systemic exposure and hazard are available, the overall risk assessment of a nanomaterial might not be different from other conventional ingredients in terms of working out Margins of Safety (MoS).

   a. Where data have been derived from validated tests, or from relevant and justified tests, and uncertainties are not higher, there may not be a scientific reason for applying higher margins of safety to a nanomaterial than a conventional material. However, where this is not the case, and insufficient data, or data from inadequate tests, have been provided, the risk assessor may consider applying additional uncertainty factors for a nanomaterial.

   b. In view of the current limitations in regard the availability of validated stand-alone *in vitro* tests, or a testing battery, the SCCS considers that an approach using *in vitro* assays only is too premature to be applied for risk assessment of nanomaterials at present.

   c. For nanomaterials, the calculation of the MoS, especially in the case of (very) low absorption via oral, dermal, and/or pulmonary routes of exposure, can be challenging. In case of (very) low absorption, the validity of NOAELs in toxicological studies may be questionable, and for substances that are hardly absorbed, no toxic effects may be noted. However, in such a case, processes such as translocation and accumulation will need to be accurately studied before a decision on the safe use can be taken.

8.1. Terms of Reference:

The following terms of reference have been asked to the SCCS for the development of this Guidance:

1) *The essential elements that must form part of safety dossiers for the assessment of nanomaterials in cosmetic products, based on the data requirements for the pre-market notification listed in article 16 of Regulation (EC) No 1223/2009, i.e. taking into account points 3a to 3f of article 16 (identification of the nanomaterial; specification; quantity; toxicological profile; safety data and exposure).*

The scientific rationale for special considerations in relation to risk assessment of

nanomaterials has been described in the above sections. These include aspects that should be considered in relation to characterisation of nanomaterials, assessment of exposure, identification of hazard, dose response characterisation, and risk assessment.

General considerations:

- This Guidance will apply to any new or already approved ingredient if it fulfils the criteria for definition of a nanomaterial as in the Cosmetics Regulation, e.g. an approved ingredient that has been manufactured by a different process which has generated a component in the nano scale.

- Irrespective of the presence of nanomaterials, the existing regulations and SCCS Guidance on Testing of Cosmetic Ingredients and their Safety Evaluation must be followed (SCCS/1416/11 or more recent version).

Characterisation considerations:

- Detailed characterisation data is the primary requirement for safety assessment of a nanomaterial intended for use in a cosmetic product. The characterisation data presented in a safety dossier must provide information on the identity of the core material(s), relating to the same nanomaterial that is intended for use in the final product. Where the data relate to a different nanomaterial, or a different form of the same nanomaterial, justification should be provided to show that there is sufficient similarity between the nanomaterials to consider the data for risk assessment. The information should correspond to Cosmetics Regulation (EC) No 1223/2009, Article 16 a) "identification of the nanomaterial…").
- The characterisation must also include measurement of important physicochemical parameters. As a minimum, the SCCS requires data on all of the parameters listed in Table 1 that are relevant to the given type of a nanomaterial. Corresponding to Cosmetics Regulation (EC) No 1223/2009, Article 16 b) "specification of the nanomaterial..."
- The characterisation data need to be derived from appropriate mainstream methods. Data on size parameters must be provided from more than one method, one of which should be electron microscopy (preferably in the form of high resolution TEM images). It is important that measurements are carried out with due considerations to the nano-aspects, and results are backed up by appropriate documentation.

- The characterisation data need to be provided on the raw nanomaterial as manufactured, as in the cosmetic formulation, and as during exposure for toxicological investigations. If characterisation at any of these stages is not feasible, e.g. due to lack of methods, or degradation of the nanomaterial, it should be justified and documented.

- For spray application of products containing nanomaterial, a careful characterisation will be needed to measure droplet size and the nanomaterial distribution in the droplets. Determination of the generated droplet size distribution alone will not be sufficient, and will need to be complemented by the size distribution of the dried residual aerosol particles. It is also very important to characterise the nanomaterial under exposure conditions to ascertain that its characteristics have not changed compared to the material intended for use in the cosmetic product.

Exposure considerations:

- Data on exposure assessment forms a crucial decision point in the overall risk assessment of a nano ingredient (Figure 1), and therefore needs to be assessed with due consideration to nano-aspects, possible routes of exposure, whist mimicking the actual use scenarios. In this respect, the exposure dose needs to be carefully addressed, particularly when a non-physiological administration is chosen; e.g. intratracheal instillation as a surrogate for inhalation; or gavage as a surrogate for ingestion.

Unfortunately, so far doses have frequently been chosen in the open literature that are orders of magnitude too high, and which are likely to be unsuitable for risk assessment because criteria do not exist for extrapolation to low realistic nanomaterial doses. These studies may only be useful for gaining insight to the toxicity mechanisms.

- The SCCS is of the view that the method for calculating dermal and oral exposure to nanomaterials (detailed in the SCCS Notes of Guidance, 2011, and Section 5) will not be substantially different from the calculation of exposure to conventional cosmetic ingredients. Calculation of exposure to aerosols containing nanomaterial may, however, be more challenging, since the existing model(s) have not yet been demonstrated to be suitable for nanomaterials.

- For dermal absorption of conventional cosmetic ingredients, the SCCS considers that when results are derived from an inadequate *in vitro* study, 100% dermal absorption will be assumed. In cases where molecular weight of the ingredient is >500 Da and log Pow <-1 or >4, a value of 10% dermal absorption is considered. These rules are, however, not likely to be relevant for most nanomaterials and therefore the 10% default absorption will not be applicable. In view of this, dermal absorption of nanomaterials will need to be determined experimentally.
- Where the experimental evidence shows a lack of systemic absorption following application of a nanomaterial containing cosmetic product, local effects (e.g. on skin after dermal application, and respiratory tract after spray application) should be investigated.
- Where the experimental evidence shows 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. Where absorption of particles cannot be excluded either by experimental data, or justified on the basis of solubility/degradation of the nanomaterial, the SCCS may apply a precautionary approach and assume that 100% the absorbed material was in particle form.

Hazard considerations:

- Where there is evidence for systemic absorption, data on toxicological evaluation will be required. In the first instance, focus should be on toxicokinetics (ADME) parameters to investigate the fate and behaviour of the nanoparticles in the body, and to identify the likely target organs. Like other cosmetic ingredients, data on a base set of toxicological endpoints will also be required. These include dermal/ percutaneous absorption, acute toxicity; irritation and corrosivity, skin sensitisation, repeated dose toxicity, and mutagenicity/ genotoxicity (Table 2). Depending on the outcome of the tests, further information on carcinogenicity, reproductive toxicity may also be required. The emphasis should be on toxicological tests over prolonged periods with repeated doses, followed up by histopathological investigations.

- Currently much of the available toxicological data in open literature relates to acute studies whereas long-term effects studies are scarce. In view of the continuous use of consumer products containing nanomaterial over years, and in some cases decades, demands carefully designed long-term exposure and toxicological effect studies to inform appropriate risk assessment.

- Currently, toxicological testing is carried out mainly in animals. However, the existing ban in Europe on testing cosmetic ingredients and products in animals, and the imminent ban on marketing cosmetic products containing ingredients tested on animals, will pose an obstacle to safety assessment of nanomaterials in cosmetic products.

- The available alternative testing methods based on *in vitro* assays have not yet been validated for nanomaterials. Although not validated against nanomaterials, the available validated *in vitro* tests may be relevant for hazard identification of nanomaterials and

may also provide additional supporting evidence to *in vivo* studies, provided that they are carried out with due consideration to the nano-related aspects (section 6.3 and Table 3).

2) *The possibility to develop criteria and conditions that would allow the safety assessment of nanomaterials on a category based approach rather than on a case-by-case basis.*

At present, sufficient information on the hazard and/or exposure is not available to enable adequate safety evaluation of the different nanomaterials that may be used as cosmetic ingredients. As a basis for further in-depth evaluation, a nanomaterial of concern will have to be assessed with respect to possible known toxic profiles of the constituents/ components. In this assessment, the lifetime of the particles during exposure, possible uptake, and toxicokinetic/toxicodynamic profiles are the important parameters. This situation is not specific to nanomaterials, and is often applicable also to chemical substances. However, there is more information available on analogous chemicals to allow a read across, or the use of categorisation approach, in risk assessment, than for nano-specific properties of a nanomaterial. This large body of knowledge on chemical substances has been accumulated over the decades. For nanomaterials, such a knowledgebase is currently lacking to provide a similar level of confidence, and a basis for category-based risk assessment. It has been suggested that efforts are underway to address this gap through evolving scientific knowledge that will become available in due course for the safety assessment of new nanomaterials.

In view of the current insufficient level of scientific understanding, and the high level of uncertainties over the potential deviations in the properties, behaviour, and effects of nanomaterials compared to conventional equivalents, the SCCS is of the view that the use of a read-across or categorisation approach based on inter- or intra- nanomaterial extrapolation for risk assessment of nanomaterials is currently not possible. This means that risk assessment shall be carried out on a case-by-case basis, using a precautionary approach where necessary – in terms of requirement for further testing, or by taking a conservative approach in the application of assessment factors. A staged approach, as described by SCENIHR (2007), may be used to identify the various procedures and testing that need to be performed for the risk assessment of cosmetic ingredients. Other approaches based on expert judgment-based decision models are also currently under development (Flari et al., 2011). The ongoing research and development in this area will inevitably increase understanding of the key parameters that drive the properties, biological interactions and toxicological effects of nanomaterials. With the availability of the new knowledge, it will be possible to derive the underlying rules that allow a read-across, and mathematical models that enable a category approach to risk assessment of nanomaterials in the future.

3) The suitability of alternative methods already validated for the assessment of conventional chemical substances for the assessment of nanomaterials in light of the current (as of 2009) ban on animal testing in the EU.

- None of the available validated alternative methods for conventional chemical substances has yet been validated specifically for nanomaterials. Although not validated for nanomaterials, some of the available validated *in vitro* tests may be relevant for hazard identification of nanomaterials and may provide additional supporting evidence to *in vivo* studies, provided that they are carried out with due consideration to the nano-related aspects, e.g. solubility/ dispersion, agglomeration/ aggregation, adsorption/ binding of various moieties on nanomaterial surfaces, and proper controls (see Section 6.3 and Table 3).
- Appropriate characterisation of nanomaterials during the tests will form an essential part of the evidence to support validity of the results (Section 4). More details on nano-related considerations in toxicological testing of nanomaterials are provided in Table 3.

- It should be noted that there may be additional considerations for certain alternative tests. For example, the *in vitro* tests proposed for skin corrosion and skin irritation are based on colorimetric assays (such as sulforhodamine B dye, MTT assay). These assays may not be suitable for those nanomaterials that can interact with the reagents, and/or absorb/ disperse light themselves and thus interfere with measurements in the colorimetric assays. Similarly, there are doubts over whether the results of Ames test will provide an accurate representation of genotoxicity potential of a nanomaterial. This is because, unlike mammalian cells, bacterial cells lack the uptake of particles via endocytosis, and also that some nanomaterials may have bactericidal activity.

- Despite the current limitations, the SCCS recommends the use of *in vitro* assays as supporting tools to evaluate relative toxicity of nanomaterials in hazard identification, and to provide additional information on the possible mechanism(s) of toxic action of nanomaterials.

4) The set of attributes unique to manufactured nanomaterials that will need to be addressed by newly developed and/or newly validated alternative methods for the testing of toxicological end points for which there will be a ban on the testing on animals after March 2013.

The issues addressed at item 3) above, are also important in regard to any newly developed, and/or newly validated, alternative methods for the testing of toxicological endpoints for which there will be a ban on testing in animals after March 2013. Other aspects need considering in the development and validation of new alternative methods should include:

- Appropriate scheme for characterisation of nanomaterials to determine any changes during the tests in the physicochemical properties, such as surface characteristics, agglomeration/aggregation state, solubility, etc.

- Appropriate methods/ reagents for dispersion of nanomaterials in the test medium to ensure contact with the tests systems.

- Careful choice of media components and assay reagents to avoid artifacts due to interaction with nanomaterials.

- Use of appropriate controls for media components/ reagents to eliminate possible artifacts. Also, the use of (larger) particle and conventional forms of the nanomaterial as controls to investigate any nano-specific effects.

- Sufficient replication of the tests to draw a statistical significance of the results.

- A careful consideration of the potential local toxicity, especially in the respiratory tract.

- Design of toxicological assessments in regard to relevant routes, and sensitivity of the detection methods in consideration of the expected poor bioavailability of nanomaterials.

- Careful selection of the tested doses of nanoparticles that are in accordance with realistic exposures. However, overload exposure to particle materials should be avoided.

- Testing of adverse health effects in view of the possible long-term effects which may appear only after long-term use of a nanomaterial-containing product by the consumer over years and possibly decades.

- Relevant tools for extrapolation of results obtained from alternative testing to the health risk of consumers using nanomaterial containing consumer products.

- More emphasis on the use of *in vitro* models based on co-culture, 3D-culture and/ or tissue culture systems that mimic the *in vivo* situation more closely as they are likely to provide more relevant information on a toxicological hazard. Also the use of human-based *in vitro* systems is preferred.

- More emphasis on systematically designed studies that generate high quality data for modelling, and efforts in *in silico* modelling and data-mining to make use of the existing (and growing) databases on nanomaterials to derive basic rules and models to identify the key parameters that underpin the distinctive properties, behaviour, and effects of nanomaterials.

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10. ANNEXES

Table 3: Available methods of toxicological evaluation of nanomaterials

Endpoints/ available methods	Nano-related considerations
Acute toxicity: The term "acute toxicity" is used to describe the adverse effects on health, which may result from a single exposure to a substance via the oral, dermal, or inhalation route [ECB 2003] The following methods are used to assess acute toxicity: *1) Acute oral toxicity* The original test method [EC B.1, OECD 401] has been superseded [2001/59/EC] and replaced by: - The fixed dose method [EC B.1 bis, OECD 420] - The acute toxic class method [EC B.1 tris, OECD 423] - The up-and-down procedure [OECD 425] *2) Acute inhalation toxicity* - The acute toxic class method by the inhalation route [OECD 436]. OECD 433 is a draft guideline of the fixed concentration procedure by inhalation. - RIP-oN2 proposed the use of BAL as a standard in acute toxicity inhalation tests. *3) Acute dermal toxicity* - *In vivo* acute dermal toxicity assay [EC B.3, OECD 402]. A draft OECD 434 is also available for the fixed dose procedure. - No *in vitro* alternative method to the *in vivo* acute dermal toxicity is currently available. An integrated project Acute-Tox (www.acutetox.org) under the EU Research Programme (Framework	None of the (alternative) procedures to determine acute toxicity has been validated specifically for nano-substances, but these tests may still be valuable for hazard identification if certain nano-related aspects are taken into consideration, e.g.: - Solubility/dispersion (section *6.3.2.1*) - Adsorption of substances (section *6.3.*2.2*)* When using a dispersant to disperse nanomaterial in a toxicological test medium, it should be considered that it does not modify physicochemical properties of the nanomaterial (including agglomeration or aggregation state and dynamics), and/or does not adsorb on nanomaterial surface and thus influence toxicity. Similarly, consideration should be given to binding of other moieties (such as proteins from serum, dyes, or other media components) on nanomaterial surface as this may also alter ADME properties and/or effects, and generate erroneous results. An adequate number of positive and negative controls should be included in the tests to verify the role of the vehicle. This may also require additional material characterisation in the specific dispersant (e.g. in terms of size, size distribution, point of zero charge, etc).

Programme 6) is aiming to develop a replacement alternative for oral acute toxicity testing. The results are likely to be available in 2012, but the tests are not related to acute dermal or inhalation toxicities that are also important for cosmetic substances.	
Corrosivity and irritation: Steps required before the *in vivo* study [EC B.5, OECD 405]: - evaluation of existing human and animal data; - analysis of structure activity relationships; - evaluation of the available data with comparable bulk materials (any differences in dissolution, *in vitro* toxicity?); - study of physicochemical properties and chemical reactivity (e.g. substances with a pH ≤2.0 or ≥11.5 will be considered as corrosive without *in vivo* testing); - looking at available dermal toxicity data; - taking into account the results from *in vitro* and *ex vivo* tests [EC B.4, OECD 404]. *1) Skin corrosivity and skin irritation* Skin irritation or dermal irritation is defined as reversible damage of the skin following the application of a test substance for up to 4 hours. For skin corrosion, the following five validated *in vitro* alternatives are available (Regulation (EC) No 440/2008 [2008/440/EC]): a) TER test (rat skin transcutaneous electrical resistance test) [EC B.40, OECD 430] b) EpiSkin™ [EC B.40bis, OECD 431] c) EpiDerm™ [EC B.40bis, OECD 431] d) SkinEthic™ [EC B.40bis, OECD 431] e) EST-1000 (epidermal skin test-1000) [EC B.40bis, OECD 431] The Corrositex™ test, which uses penetration of test substances through a hydrogenated collagen matrix (biobarrier) and supporting filter membrane, represents another corrosivity test. It is described in OECD Guideline 435 [OECD 435], which provides a generic description of the components and procedures of an artificial membrane barrier test method for corrosivity assessment. Although the CorrositexTM test passed the ECVAM, it has not yet been taken up by ESAC in the EU legislation. It was considered to be	These steps will also apply to nanomaterials. Although not yet investigated for nanomaterials, it is also possible that some insoluble particulate materials can mechanically interfere with the tissue or the cell. The alternative tests for proposed skin corrosion and skin irritation are based on colorimetric assays (such as sulforhodamine B dye, MTT assay). These techniques may not be suitable for certain nanomaterials because of possible interaction between reagents and these nanomaterials (see section 6.3.2.2). Moreover some nanomaterials may themselves disperse/ absorb light and therefore interfere with the measurements in colorimetric assays. These aspects need to be considered when using colorimetric methods. 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). However, they may bind/ adsorb on nanomaterial surfaces, and this may lead to false negative results. This type of nonspecific absorption of biomarkers should also be verified (see section 6.3.2.2). A specific protocol for solid substances exists for the BCOP and IRE test. Solid substances are mostly tested at 20% (w/w) in a suspension in 0.9%

only useful for acids and bases [ESAC 2000].

For skin irritation:

a) EpiSkin™
b) Modified Epiderm™Skin Irritation Test (SIT)
c) SkinEthic™Reconstructed Human Epidermis (RHE)

The three *in vitro* test methods, based on reconstructed human epidermisare, have been included in OECD 439 and endorsed by ESAC. The recently published EC B.46 counterpart mentions that the test results, depending on information requirements, may allow determining skin irritancy of substances as a stand-alone replacement test within a testing strategy that uses a weight of evidence approach [EC B.46].

2) Mucous membrane irritation

Eye irritation tests have been developed to assess the production of changes in the eye following application of a test substance to interior surface of the eye, which are fully reversible within 21 days of application. Eye corrosion is tissue damage in the eye, or serious deterioration of vision, following application of a test substance to the interior surface of the eye, which is not fully reversible within 21 days of application.

a) the assessment of existing *in vivo* dermal irritancy or corrosivity data on the substance [EC B.5, OECD 405].

There are presently no fully validated alternative methods replacing the classical Draize *in vivo* eye irritation test. The alternative methods for eye irritation/corrosion currently consist of a screening battery of two assays:

- the Bovine Cornea Opacity Permeability (BCOP) [OECD 437], and
- the Isolated Chicken Eye (ICE) [OECD 438].

Together with the Isolated Rabbit Eye (IRE) and the Hen's Egg Test-Chorio Allantoic Membrane (HET-CAM), they provide only supportive evidence for cosmetic ingredient safety Assessment [SCCS/1294/10]. They can be used in the process of hazard identification (not risk assessment) to eliminate severe eye irritants, but fail to distinguish mild from non-irritants. Cytotoxicity / cell function-based assays for water soluble substances Cytosensor Microphysiometer and Fluorescein Leakage assays have been validated by ECVAM in 2009, and a draft OECD guideline is in progress. sodium chloride (and some instances a dispersant). No specific validation has been performed for nanomaterials, although there is no clear scientific basis against the use of the method for nanomaterials. It should, however, be kept in mind that:

- nanomaterials can aggregate/agglomerate in the suspension (see *6.3.2.1)* or can absorb dispersant (see 6.3.2.2). These aspects should be verified.
- Some nanomaterials present in opacity measurements may affect the result, and these should be avoided to allow consistent interpretation of results.
- Both methods measure the leakage of fluorescein. Possible artifacts due to absorption of the dye to nanomaterials should be verified and eliminated.

* in house models can also be used if properly validated against the models mentioned above.

For certain aspects, ISO 10993 series of standards may be used as these deal with the safety testing of solid materials (e.g. intracutaneous irritation test as described in ISO 10993-10:2010). This is an *in vivo* test, which could be used as an indication of irritancy of nanomaterials.

Skin sensitisation: A skin sensitiser is an agent that is able to cause an allergic response in susceptible individuals. The consequence of this is that following subsequent exposure via the skin, the characteristic adverse health effects of allergic contact dermatitis may be provoked [ECB 2003]. The Local Lymph Node Assay (LLNA) [EC B.42, OECD 429]. Work at the OECD level on the acceptance of LLNA using a non-radioactive methodology include Daicel-ATP, which is a modified LLNA method using adenosine triphosphate (ATP) as an endpoint [OECD 442A], and Cell proliferation ELISA (Enzyme-Linked Immunosorbent Assay) BrdU (5-bromo-2-deoxy-uridine) [OECD 442B]. a) The Magnusson Kligman Guinea Pig Maximisation Test (GPMT) [EC B.6, OECD 406] b) The Buehler test [EC B.6, OECD 406] Currently, no validated *in vitro* alternative methods are available. Currently, a peptide reactivity assay, a keratinocyte culture system, two methods employing a 3D reconstructed skin model (one combined with dendritic cells) and a dendritic cell activation assay are in the prevalidation stage at ECVAM. An extensive review of the actual status of *in vitro* testing in this field can be found in a JRC report [Adler et al. 2011].	The standard tests have not been specifically tested for insoluble nanomaterials. A significant difference exists between the LLNA that will involve application of nanomaterials on the surface of the skin, and the GPMT that will involve intradermal application. The LLNA has been used to verify sensitisation of nanomaterials, but no positive response has been found (Lee et al., 2011). In addition, the LLNA has been used to verify whether nanomaterials can potentiate the level of sensitisation of known sensitizers (Lee et al., 2011). The value of both tests has been challenged since dermal penetration was not assessed. Currently no experimental data is available on nanomaterials tested using GPMT. However, negative results have been reported for ZnO from the use of a modified GPMT with topical application on a FCA treated skin (Yanagi et al., 2001). Based on the current knowledge, it is not possible to advice the use of one specific test method. The use of LLNA will probably not result in sensitisation due to possible low skin penetration of nanomaterials. Other tests using intradermal application are not yet available.
Dermal/ percutaneous absorption: The dermal/ percutaneous absorption process is a global term which describes the passage of compounds across the skin. This process can be divided into three steps: - penetration is the entry of a substance into a particular layer or structure such as the entrance of a compound into the stratum corneum; - permeation is the penetration through one layer into another, which is both functionally and structurally different from the first layer; - resorption is the uptake of a substance into the vascular system (lymph and/or blood vessel), which acts as the central compartment. A number of factors play a key role in dermal/ percutaneous absorption,	For any tests on nanomaterials, the dose, volume, and contact time with the skin, have to mimic the in-use conditions (also taking the consideration of dispersion – see 6.3.2.1). Appropriate analytical techniques and sampling methods should be used to determine the possible adsorption of substances on nanomaterial surfaces – see 6.3.2.2). It is also important that dermal absorption tests using *in vitro* skin models or *ex vivo* skin are carried out on viable cells. For conventional cosmetic ingredients, the SCCS considers that when results are derived from an inadequate *in vitro* study, 100% dermal absorption will be assumed. In cases where molecular weight is >500 Da and log Pow <-1 or >4, a value of 10% dermal absorption is considered. These rules are not likely to be relevant for most nanomaterials and

including the lipophilicity of the compounds, the thickness and composition of the SC (body site), the duration of exposure, the amount of topically applied product, the concentration of target compounds, occlusion, etc. For a review of this subject, see E. Howes et al., 1996) At present, the *in vitro* diffusion cell chamber is the standard device for estimating percutaneous absorption. However, because mechanical factors may be important in potential penetration/absorption of nanoparticles, this standard model may not be ideal. Therefore, modified or new optimized methodologies to assess percutaneous penetration pathways are required (SCCP, 2007). The SCC(NF)P/SCCS consider a combination of the EU/ OECD Guidelines, and its own "Basic criteria" as essential for dermal/ percutaneous absorption studies. The test substance should correspond to the substance that is intended to be used and vehicle/ formulation should be representative for the intended cosmetic product. Both *in vivo* and *in vitro* testing protocols form part of the lists of official EU and OECD test methods [EC B.44, 45; OECD 427, 428], accompanied by more detailed guidance on their performance [DG SANCO 2004, OECD 2004]. The SCCNFP adopted its first set of basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients in 1999 [SCCNFP/0167/99]. This opinion, most recently updated in 2010 [SCCS/1358/10], focuses on the *in vitro* testing of cosmetic ingredients, whereas the general EU and OECD Guidance [DG SANCO 2004, OECD 2004] addresses percutaneous absorption from a much broader point of view by mentioning *in vivo* methods besides *in vitro* testing, and by providing specifications for agricultural products and industrial chemicals as well as cosmetics.	therefore, the 10% default absorption will not be applicable. In view of this, dermal absorption of nanomaterials will need to be determined experimentally. If the tests indicate systemic absorption, the integrity of the nano structure will need to be confirmed. Where absorption of nanoparticles has not been excluded by experimental data, or justified on the basis of solubility/ degradation of the nanomaterial, the SCCS may apply a precautionary approach and assume that 100% the absorbed material was in particle form.
Repeated dose toxicity: Repeated dose toxicity comprises the adverse general toxicological effects (excluding reproductive, genotoxic and carcinogenic effects) occurring as a result of repeated daily dosing with, or exposure to, a substance for a specific part of the expected lifespan of the test species [ECB 2003]. In these tests, effects which require a long latency period or which are cumulative, become manifested. The following *in vivo* repeated dose toxicity tests are available: 1) - Repeated dose (28 days) toxicity (oral)[EC B.7, OECD 407] - Repeated dose (28 days) toxicity (dermal)[EC B.9, OECD 410]	None of the currently available test procedures has been specifically validated for nanomaterials. Taking into consideration the dispersion/ aggregation behaviour of nanomaterials, and adsorption of molecules on the surface of nanomaterials, the current test procedures can be applied for nanomaterials. Additional useful information could be available from *in vitro* tests, e.g. on cell viability/ cytogenicity, oxidative stress, inflammation, etc. An alternative inhalation test "5-day inhalation study" has been proposed. Although this has proven to be useful e.g. in dose setting experiments, but its validity is not yet certain and hence is not

- Repeated dose (28 days) toxicity (inhalation)[EC B.8, OECD 412] 2) - Sub-chronic oral toxicity test: repeated dose 90-day oral toxicity study in rodents [EC B.26, OECD 408] - Sub-chronic oral toxicity test: repeated dose 90-day oral toxicity study in non-rodents [EC B.27, OECD 409] - Sub-chronic dermal toxicity study: repeated dose 90-day dermal toxicity study using rodent species [EC B.28, OECD 411] - Sub-chronic inhalation toxicity study: repeated dose 90-day inhalation toxicity study using rodent species [EC B.29, OECD 413] 3) - Chronic toxicity test [EC B.30, OECD 452] For repeated-dose toxicity, there is currently no validated or generally accepted alternative method available to replace animal testing.	acceptable as an alternative for chronic tests.
Mutagenicity/ genotoxicity: Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. Genotoxicity is a broader term and refers to processes which alter the structure, information content, or segregation of DNA, and are not necessarily associated with mutagenicity. In principle, the SCCS recommends three assays for the base level testing of cosmetic ingredients, represented by the following test systems: 1. Tests for gene mutation: i) Bacterial reverse mutation test [EC B.13/14, OECD 471] ii) *In vitro* Mammalian cell gene mutation test [EC B.17, OECD 476] 2. Tests for clastogenicity and aneugenicity i) *In vitro* Micronucleus test [OECD 487] or ii) *In vitro* Mammalian chromosome aberration test [EC B.10, OECD 473]	It should be noted in regard to testing mutagenicity of nanomaterials that, although reports can be found on positive bacterial reverse mutation test, there are doubts if the Ames test is an accurate representative test for genotoxicity. This is because, unlike mammalian cells, bacterial cells lack uptake of nanomaterials through endocytosis, and also that some nanomaterials have bactericidal activity. Therefore this test has not been regarded suitable for testing nanomaterials (EFSA, 2011). In addition, the use of metabolic activation system for nano-substances is questionable. This has not been investigated in any detail (Szalay et al., 2011) but most insoluble nanomaterials (e.g. some metals) are not metabolised. Instead, proteins in the metabolic activation system may interfere with the nanomaterial (Kumar et al., 2011), alter bioavailability of the nanomaterial, and thus reduce sensitivity of the assay. Notwithstanding this it should be verified whether some nanomaterials can be metabolised, e.g. organic nanomaterials, or some inorganic nanomaterials may become coated with organic substances, or surface modified with organic functional groups. Caution is also needed with the Micronucleus Test. Cytochalasin B, which is often used in to inhibit cytokinesis may inhibit endocytosis, and hence has

It should be noted however, that the existing *in vitro* tests yield a relatively high rate of false positive results for non-carcinogens. Under the testing/ marketing ban of the 7th amendment of the Cosmetics Directive [2003/15/EC] on cosmetic ingredients, further *in vivo* testing to confirm or, predominantly, to overrule the positive *in vitro* findings is no longer possible. However, at present no validated methods are available either that allow a follow-up of any positive results from the standard *in vitro* assays [SCCP/1212/09].	been suggested to lead to false negative outcomes with particles (Landsiedel et al., 2009). Moreover, for several types of nanoparticles (e.g. titanium dioxide, multi-walled carbon nanotubes), the microscopic evaluation of cytokinesis-block proliferation index and micronucleus identification was found to be inappropriate at high testing concentrations due to the overload of agglomerates (Corradi et al., 2011). Although not investigated so far, similar problems may be anticipated for other microscopy based *in vitro* mutagenicity tests (e.g. Chromosome Aberration Test). Some of these shortcomings may be addressed by weight of evidence approach based on several alternative methods, including those that have not yet been validated but are relevant. For example: - Micronucleus test in reconstructed human skin - Comet assay in reconstructed human skin However, in view of the current limitations of *in vitro* tests and the potential introduction of artifacts with specific types of nanomaterials (see also 6.3.2.2), the SCCS is of the opinion that with the *in vivo* testing ban for cosmetic ingredients, the safety of many potential new cosmetic ingredients may not be adequately assessed until the assays are validated specifically for nanomaterials. This refers in particular to the modified toxicokinetics, and is also critical for the interpretation of data which are available from *in vivo* mutagenicity tests. For example, the *in vivo* micronucleus test (OECD 475) if applied orally is considered inappropriate if there is evidence that the test substance, or a reactive metabolite, will not reach the target. Therefore, the applied method/route of administration (e.g. topical, intraperitoneal, intravenous, etc) should be considered alongside all available information on the kinetics (see 6.3.2.4) of the tested nanomaterial.
Carcinogenicity: Substances are defined as carcinogenic if they induce tumours (benign or malignant) or increase their incidence, malignancy or shorten the time of tumour occurrence when they are inhaled, ingested, dermal applied or injected [ECB 2003]. Most common carcinogenicity tests *in vivo* are: a) Carcinogenicity test [EC B.32, OECD 451] b) Combined chronic toxicity/ carcinogenicity test [EC B.33, OECD 453] Where there is a structural alert for carcinogenicity, or a positive results in	It is not clear whether the available *in vitro* tests are applicable to nanomaterials because they have not yet been validated for nanomaterials.

in vitro mutagenicity tests, an *in vitro* Syrian Hamster Embryo (SHE) Transformation Test may be needed. The *in vitro* Cell Transformation Assays (CTA's) may detect both genotoxic and non-genotoxic carcinogens. These tests are currently under ECVAM validation (Farmer 2002, Hayashi et al. 2008). Further updates on the assays can be obtained from EUR ECVAM website[5].	
Reproductive 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]. The following *in vivo* tests are generally considered: a) Two-generation reproduction toxicity test [EC B.35, OECD 416] b) Teratogenicity test - rodent and non-rodent [EC B.31, OECD 414] c) Combined Repeated Dose Toxicity Study with the Reproduction / Developmental Toxicity Screening Test 422 Recently, the extended one-generation reproductive toxicity study has been taken up by the OECD [OECD 443]. Although several *in vitro* methodologies have been developed, there is currently no alternative method available in this area. The assessment of reproductive toxicity is complex, and it is expected that the various stages cannot be mimicked using a single alternative method. In the embryotoxicity area, three alternative methods have been developed: a) The Whole Embryo Culture test (WEC) b) The MicroMass test (MM) c) The Embryonic Stem cell Test (EST) [ESAC 2001].	Although none of the tests is specifically validated for nanomaterials, the three alternative methods for embryotoxicity are likely to be applicable to nanomaterials, provided that typical nanomaterial related issues such as dispersion/ aggregation, adsorption, stability and distribution into the tissue are taken into consideration. Nevertheless, more information and research is needed before regulatory acceptance for the alternative methods can be envisaged for nanomaterials. For nanosilica, in the EST inhibition of differentiation into contracting myocardiocytes was observed (Park et al., 2009b)
Toxicokinetic studies The term "toxicokinetic studies" is in the context of chemical substances,	Following systemic absorption, the distribution and fate of a nanomaterial is mainly governed by its chemical nature, size, surface characteristics,

[5] http://ihcp.jrc.ec.europa.eu/our_activities/alt-animal-testing/eurl-ecvam-recommendations

such as cosmetic ingredients, used to describe the time-dependent fate of a substance within the body. This includes absorption, distribution, biotransformation and/or excretion [EC B.36, OECD 417]. In the context of the EU's cosmetic legislation, a review of the actual status of alternatives to studying toxicokinetics in animals has recently been carried out (Adler et al. 2010) which concluded that there are some important gaps in this regard. As toxicokinetic data are important in extrapolating both *in vitro* and *in vivo* animal data to man, more research is needed in this area.	aggregation state, etc (see Table 1*)*. Special considerations relating to nanomaterials therefore should include whether they can absorb/ adsorb endogenous/ exogenous moieties (e.g. surfactants, serum, or other media components) that may change surface characteristics (see 6.3.2.2). For chemicals, consideration of potential toxicity of metabolites and degradation products is also important. This may be less important for insoluble nanomaterials, but should be considered where nanomaterials, or their surface coatings, may dissolve or degrade. Therefore, where applicable, *in vivo* or *in vitro* biotransformation studies may be necessary to ascertain the likelihood of adverse effects due to metabolites/ degradation products.
Photo-induced toxicity: Due to the wavelength of light, phototoxicity may also depend on the size distribution of a particulate material. This is likely to be more relevant to inorganic materials than for organic substances, such as dyes. The main tests include: *1) Phototoxicity (photoirritation) and photosensitization* The "3T3 Neutral Red Uptake Phototoxicity Test (3T3 NRU PT)" is an *in vitro* method based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of UV/visible light. The 3T3 NRU PT has been validated and taken up in Annex V to Directive 67/548/EEC, making its use mandatory for testing for phototoxic potential. Its use is now mandatory since adoption under Regulation (EC) No 440/2008 [EC B.41, OECD 432]. It needs to be noted that the 3T3 NRU PT is not designed to predict other adverse effects that may arise from combined actions of a chemical and light, e.g. it does not address photoclastogenicity/ photomutagenicity, photoallergy or photocarcinogenicity. At present, there is no *in vitro* method available for detection of photosensitisation. However, it is expected that chemicals showing photoallergic properties, are also likely to give positive reactions in the 3T3 NRU PT test [EC B.41]. *2) Photomutagenicity / Photoclastogenicity* For the detection of photochemical clastogenicity/ mutagenicity, several assays have been adapted to testing of chemicals in the presence of UV-Vis	The reliability and relevance of the *in vitro* 3T3 NRU Test has not been specifically validated for nanomaterials (Spielmann et al. 1998). It should be noted that in some instances neutral red may interfere with nanomaterials (Lanone et al., 2009) (also see 6.3.2.2.) The SCCS will take the GUM Task Force results into consideration and evaluate the individual photomutagenicity/ photogenotoxicity tests and their scientific merits on a case-by-case basis. Also, see comments under mutagenicity/ genotoxicity.

light including: - bacterial and yeast mutation assays (Dean et al. 1991; Chetelat et al. 1993a and Averbech et al. 1979); - tests for detecting clastogenicity (Gocke et al. 1998 and Chetelat et al. 1993b); - tests for detecting gene mutations in mammalian cells (Pflaum et al. 1998; Chetelat et al. 1996); - tests for detecting aneugenicity in mammalian cells *in vitro* (Kersten et al. 2002). The SCCNFP had recommended that the test protocols used by Colipa be the subject of a validation study. However, no validation study has yet been undertaken in the absence of *in vivo* reference data. A report of the "Gesellschaft für Umweltmutationsforschung" (GUM) Task Force on photochemical genotoxicity has concluded that in many cases, the concurrent use of irradiation while performing a classical mutagenicity/ genotoxicity study, does not significantly alter the existing OECD protocol without irradiation. Therefore they considered majority of the described photomutagenicity/ photogenotoxicity tests as valid (Brendler-Schwaab 2004).	
Human data: It is known that many tests based on animals and alternative methods are of limited predictive value with respect to the human health. However, it is inconceivable that there would be sufficient testing in human volunteers to replace animal tests.	Apart from epidemiological evidence, or data from clinical patients, any experimental data on human volunteers can only be generated where toxicological profiles of the ingredients, based on animal testing, and/or the use of alternative methods, are already available and no safety concerns have been raised. The general ethical and practical aspects related to human volunteer compatibility studies on finished cosmetic products, are described in SCCNFP/0068/98 and SCCNFP/0245/99. However, as such trials require a high degree of safety, and it is not advisable to expose humans to nanomaterials in view of the current uncertainties over the potential hazards.

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