Scientific Committee on Consumer Safety

SCCS

GUIDANCE ON THE SAFETY ASSESSMENT OF NANOMATERIALS IN COSMETICS

The SCCS adopted this opinion at its 15th plenary meeting

of 26 – 27 June 2012

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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

SCCS

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

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ABBREVIATIONS AND GLOSSARY OF TERMS

3R	Refinement, Reduction, Replacement
3T3 NRU PT	3T3 Neutral Red Uptake Phototoxicity Test
AAS	Atomic absorption spectroscopy
ADME	Absorption, Distribution, Metabolism, Excretion
AFM	Atomic force microscopy
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 and Beken, 2000]
AUC	Analytical ultracentrifugation
BCOP	Bovine Corneal Opacity and Permeability
BET	Brunauer Emmett and Teller method
CAS	A chemical registry system established by the Chemical Abstracts Service (CAS)
CLS	Centrifugal Liquid Sedimentation
Colipa	European Cosmetics Association (formerly the European Cosmetic Toiletry and Perfumery Association). Now Cosmetics Europe.
DLS	Dynamic light scattering
DMA	Differential mobility analyzer
ECVAM	European Centre for the Validation of Alternative Methods
EFSA	European Food Safety Authority
EINECS	European Inventory of Existing Commercial chemical Substances
FFF	Field flow fractionation
FTIR	Fourier transform infrared spectroscopy
GC/LC-MS	Gas Chromatography/ Liquid Chromatography coupled with Mass Spectrometry
GE	Gel electrophoresis
HDC	hydrodynamic chromatography
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ICCR	International Cooperation on Cosmetic Regulation

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 and Beken 2000]
ISO	International Organization for Standardization
IUPAC	A system of chemical nomenclature established by the International Union of Pure and Applied Chemistry (IUPAC)
LDE	Laser doppler electrophoresis
Local effects	A Local effect refers to an adverse health effect that takes place at the point or area of contact. The site may be skin, mucous membranes, the respiratory tract, gastrointestinal system, eyes, etc. Absorption does not necessarily occur.
MPI	Magnetic Particle Inspection
MS	Mass spectrometry
Nanomaterial	An insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm [Regulation (EC) No 1223/2009]
Nanoparticle	A nano-object with all three external dimensions in the nanoscale [ISO/TS 27687:2008, *Nanotechnologies -- Terminology and definitions for nano*]. For the purpose of this document the term 'nanoparticle' is used to also include other forms of nano-object, such as nano-rods, nano-tubes, etc.
Nanoscale	Size range from approximately 1 nm to 100 nm [ISO/TS 80004-1:2010, *Nanotechnologies -- Vocabulary*]
Nano SIMs	An ultra high resolution chemical imaging technique
NMR	Nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
PALS	Phase analysis light scattering
PET	Positron Emission Spectroscopy
REACH	Registration, Evaluation, Authorisation and restriction of Chemicals
RIP-ONs	The REACH Implementation Projects on Nanomaterials (RIP-oNs) – aimed at providing scientific and technical advice on key aspects of the implementation of REACH in regard to nanomaterials
RS	Raman Spectroscopy

SCCNFP	Scientific Committee on Cosmetic products and Non-Food Products intended for consumers
SCCP	Scientific Committee on Consumer Products
SCCS	Scientific Committee on Consumer Safety
SED	Systemic Exposure Dosage
SEM	Scanning electron microscopy
SERS	Surface enhanced Raman spectroscopy or surface enhanced Raman scattering
SMPS	Scanning mobility particle sizer
Solubility	The terms 'solubility' and 'persistence' are often used to describe the rate of "degradation". As such there are a number of definitions of solubility (see SCENIHR Opinion 'Scientific Basis for the Definition of the Term "Nanomaterial", 8 December 2010). Solubility in the context of this guidance means disintegration of a nanomaterial in an aqueous medium or biological environment into molecular components with the loss of nano features.
SPM	Scanning Probe Microscopy
SSA	Skin Surface Area (SSA) referred to in section 5.1.1. – should not be mistaken for specific surface area.
Systemic effects	Systemic effect refers to an adverse health effect that takes place at a location distant from the body's initial point of contact and presupposes absorption has taken place.
TEM	Transmission electron microscopy
UV-Vis	Ultraviolet-visible spectrophotometry
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, 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 (see Kreyling et al., 2010)
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray diffraction

TABLE OF CONTENTS

ACKNOWLEDGMENTS 3

1. BACKGROUND 8

2. TERMS OF REFERENCE 8

3. GUIDANCE 9

4. PHYSICOCHEMICAL CHARACTERISATION 15

5. EXPOSURE ASSESSMENT 19

6. HAZARD IDENTIFICATION AND DOSE-RESPONSE CHARACTERISATION 23

7. RISK ASSESSMENT 30

8. SUMMARY AND CONCLUSIONS 31

10. ANNEXES 50

1. BACKGROUND

Nanotechnologies open new perspectives for useful innovation in cosmetics. A number of documents provide general guidance on the health risk assessment of manufactured nanomaterials (SCENIHR opinions 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 nanomaterial, 2007; Risk Assessment of Products of Nanotechnologies, 2009*). Yet, experience with the assessment of specific substances is limited. The ongoing risk assessments being carried out by the European Commission Scientific Committee on Consumer Safety (SCCS) on three specific manufactured nanomaterials for their inclusion in Annex VII (ultraviolet (UV) filters) of the Cosmetics Directive (76/768/EEC), are the first instances in the EU and worldwide with regulatory implications.

This work has made possible the identification of a number of issues and questions regarding the types of information and data unique to nanomaterials that must form part of future submissions of safety dossiers. It has also highlighted the need for developing specific guidance for the development of similar, consistent and, to the extent possible standardised, safety evaluation dossiers of manufactured nanomaterials.

This will not only facilitate the submission of safety dossiers at present, but will also assist in the implementation of the provisions of article 16 of the Cosmetics Regulation (EC) No 1223/2009 which will impose strict conditions and timelines for the notification and the assessment of cosmetic products containing nanomaterials on the responsible persons and the SCCS respectively, starting on January 2013.

On the basis of the evolving knowledge based on the health risk assessment of specific manufactured nanomaterials, the Commission considers appropriate to request the SCCS to develop guidance on the essential elements that would be required in a manufactured nanomaterial safety dossier i.e. physicochemical characterisation; toxicological evaluation, exposure assessment etc.

This guidance should be revised and updated as considered appropriate by the SCCS, taking into consideration scientific advances and growing experience on this matter.

2. TERMS OF REFERENCE

On the basis of the present experience, the SCCS is requested to develop guidance on:

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).

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.

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.

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.

In elaborating this guidance, and taking into account the growing experience on the matter the SCCS is asked to consider all available documentation on the subject such as the SCCP scientific opinion[1] on safety of nanomaterials in cosmetic products; the documents issued by the OECD Working Party on Manufactured Nanomaterials[2]; the EFSA scientific opinion on guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain[3].

3. GUIDANCE

In addition to the requirements under relevant regulation, this document is intended to provide guidance for the safety evaluation of nanomaterials to be used as cosmetic ingredients, and as per the Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation (SCCS/1416/11 or more recent version). As nanomaterials may have certain properties, interactions with biological systems, and/or effects that are different from conventional or bulk ingredients, this guidance has highlighted them for consideration when testing and reporting data for nanomaterials. In this sense, it addresses what information must be provided by industry to the Commission in order to perform the risk assessment of nanomaterials intended for use in cosmetics.

This guidance builds upon the 2007 Opinion on safety of nanomaterials of the SCCP (SCCP/1147/07). It also refers to other key reports, such as the Risk assessment of products of nanotechnologies of SCENIHR (2009), the OECD Working Party on Manufactured Nanomaterials (2009 and 2010), the International Organization for Standardization (ISO 10808:2010), the REACH RIP-oNs, the Potential risks arising from nanoscience and nanotechnologies on food and feed safety of EFSA (2009), the EFSA guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain (EFSA 2011), and relevant ICCR reports, such as on the currently available methods for characterization of nanomaterials (ICCR, 2011a), and principles of cosmetic product safety assessment (ICCR, 2011b). In addition, a document providing Cosmetic Industry's perspective on specific characteristics of the safety assessment of nanomaterials used in cosmetic products (2010) was also considered.

The document is structured in separate sections that discuss Requirements for Safety Assessment (3.1), Physicochemical Characterisation (4), Exposure Assessment (5), Hazard Identification and Dose-Response Characterisation (6), and Risk Assessment (7). A summary and conclusions of the main aspects discussed are provided in section 8.

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.

3.1 Requirements for safety assessment of nanomaterial in cosmetics

The Cosmetics Directive (76/768/EEC) contains provisions for certain ingredients of cosmetics that require an authorisation based on a scientific risk assessment through their

[1] Scientific Committee on Consumer Products (2007) SCCP - Opinion on the safety of nanomaterials in cosmetic products adopted by the SCCP after the public consultation on the 14th plenary of 18 December 2007, SCCP/1147/07. http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_123.pdf

[2] OECD (2010) OECD Environment, Health and Safety Publications Series on the Safety of Manufactured Nanomaterials: Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's sponsorship programme; first revision, 02 June 2010, ENV/JM/MON.

[3] EFSA Scientific Committee; Scientific Opinion on Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Journal 2011;9(5):2140 [36 pp.]. Available online: www.efsa.europa.eu/efsajournal.htm

inclusion in Annexes IV, VI and VII to the Directive. Some of these substances may be particles at the nanoscale.

The EU Cosmetics Regulation (Regulation (EC) No 1223/2009), specifically covers the use of nanomaterials in cosmetic products. The Regulation provides a definition of nanomaterial, as well as a mechanism for notification, labelling, and safety evaluation of cosmetic products containing nanomaterials.

In this Regulation (EC) No 1223/2009, Article 2 (1) (k), "**nanomaterial" means an insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm**".

In view of the definition, **the Regulation intends to cover mainly those nanomaterials that are intentionally made, and are insoluble/ partially-soluble or biopersistent** (e.g. metals, metal oxides, carbon materials, etc), and not those that are soluble or degradable/non-persistent in biological systems (e.g. liposomes, emulsions, etc). There is also a provision in the Regulation under Article 2 (3) that provides for a possible future adaptation of the definition to technical and scientific progress and to definitions subsequently agreed at international level. Article 16 of this Regulation requires any cosmetic product containing nanomaterials to be notified to the Commission six months prior to being placed on the market, and Article 19 (1) requires the nano-scale ingredients to be labelled (name of the ingredient, followed by 'nano' in brackets). If there are concerns over the safety of a nanomaterial, the Commission will refer it to the Scientific Committee on Consumer Safety (SCCS) for evaluation.

The SCCS provides opinion to the Commission on the safety of non-food consumer products, including cosmetics and personal-care products, by assessing dossiers based evaluations submitted by the manufacturers. The Committee has already published general guidance on safety assessment of cosmetic products (SCCS/1416/11 or more recent version), and an Opinion on Safety of Nanomaterials in Cosmetic Products (SCCP/1147/07).

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; for example, if an approved ingredient has been manufactured by a different process that has also generated a component in the nano scale.

In 2011 the Commission adopted a Recommendation on an overarching definition of nanomaterial. According to this Recommendation (2011/696/EU):

- "Nanomaterial" means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.
- In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.
- By derogation from the above, fullerenes, graphene flakes and single wall carbon nanotubes with one or more external dimensions below 1 nm should be considered as nanomaterials."

This Recommendation has not yet been applied to the definition of nanomaterial under Cosmetic Regulation (EC) No 1223/2009. Should the Cosmetics Regulation definition be aligned to the Recommendation, it will provide further information on whether

or not a material falls under the definition of a nanomaterial; for example, a larger sized particulate material that contains a certain percentage of particles in the nano-scale.

In situations where a particulate material has internal nano-structures, or exists in the form of larger agglomerates or aggregates, the use of volume specific surface area (VSSA) (Kreyling et al., 2010), and/or other parameters, such as electron microscopy images, can provide further information.

Irrespective of the presence of nanomaterial(s), the existing regulations and SCCS guidance on testing of cosmetic ingredients and their safety evaluation must be followed (Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation (SCCS/1416/11) or more recent version).

As indicated by SCENIHR (2009), nanomaterials, like other substances, may or may not be toxic. In principle, the risk assessment paradigm including exposure assessment, hazard identification, dose response characterisation, and risk characterisation, routinely used for conventional substances, will also apply to nanomaterials. However, because of the nano-scale dimensions of nanomaterials, and the possible qualitative and quantitative differences in physicochemical characteristics, there may be additional concerns over safety. The testing requirements and the subsequent risk assessment of nanomaterial ingredients will therefore require certain additional considerations as indicated in this document. These aspects need to be specifically addressed in the risk assessment for nanomaterial ingredients in a cosmetic product. Especially, the aspects relating to particle nature in the nano-dimension need to be considered throughout the risk assessment; i.e. during material characterisation, hazard identification and characterisation, exposure assessment, and safety evaluation. Therefore, relevant data and information on the various testing and production stages must be provided for each nanomaterial intended for use in cosmetic products by the manufacturer.

3.2 Safety Considerations Relating to Nanomaterials

It has emerged from extensive studies that some materials manufactured at nano-scale may show significant deviations in physicochemical properties, interaction with biological systems, and/or effects, compared to conventional equivalents. For example, nanoparticles in the lower nanometre (nm) range may penetrate biological membrane barriers that normally prevent the entry of (larger) particulate materials (Jani et al., 1990, Geiser and Kreyling 2010). It is therefore possible that, if internalised in the form of nanoparticles, some insoluble or partially-soluble materials may be able to reach certain parts of the body that could not be reached by larger particles. As the size at the nanoscale may be accompanied by certain specific physicochemical properties, detailed characterisation of the nanomaterial submitted for risk assessment becomes crucially important. Characterisation is also important for proper identification of the nanomaterial. Thus, in addition to the chemical identification, specific information relating to the characteristics and properties of the nanomaterial will also need to be provided (see Section 4).

The main safety concerns in relation to the use of nanomaterials in cosmetics relate to whether such products could lead to:

1. local and systemic exposure to nanoparticles;
2. harmful effects as a result of the exposure; and
3. a potential risk to the consumer.

A number of reports have concluded that there is no evidence that the current risk assessment paradigm used for conventional bulk materials should not be applicable to nanomaterials (SCENIHR 2009). The current hazard identification/ dose-response characterisation regime, which is based on structured toxicological evaluations of conventional chemicals, should also pick up toxicity of nanomaterials, provided that certain nano-related aspects are duly considered during the evaluations.

The conventional risk assessment approach considers both hazard and exposure – where the absence of one means no risk. Thus, risk assessment of nanomaterial cosmetic ingredients may, in the first instance, be driven by exposure considerations, with attention to any distinctive material characteristics at the nano-scale (see Figure 1 and Table 1). This will require detailed characterisation of nanomaterials and determination of the likelihood and extent of systemic exposure due to potential translocation of nanomaterials across dermal, respiratory, or gastrointestinal barriers depending on the possible routes of exposure (see Section 5). In addition, local effects will need considering, irrespective of whether or not the use of a cosmetic product containing nanomaterials can lead to systemic exposure. Also, 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 nano-related aspects.

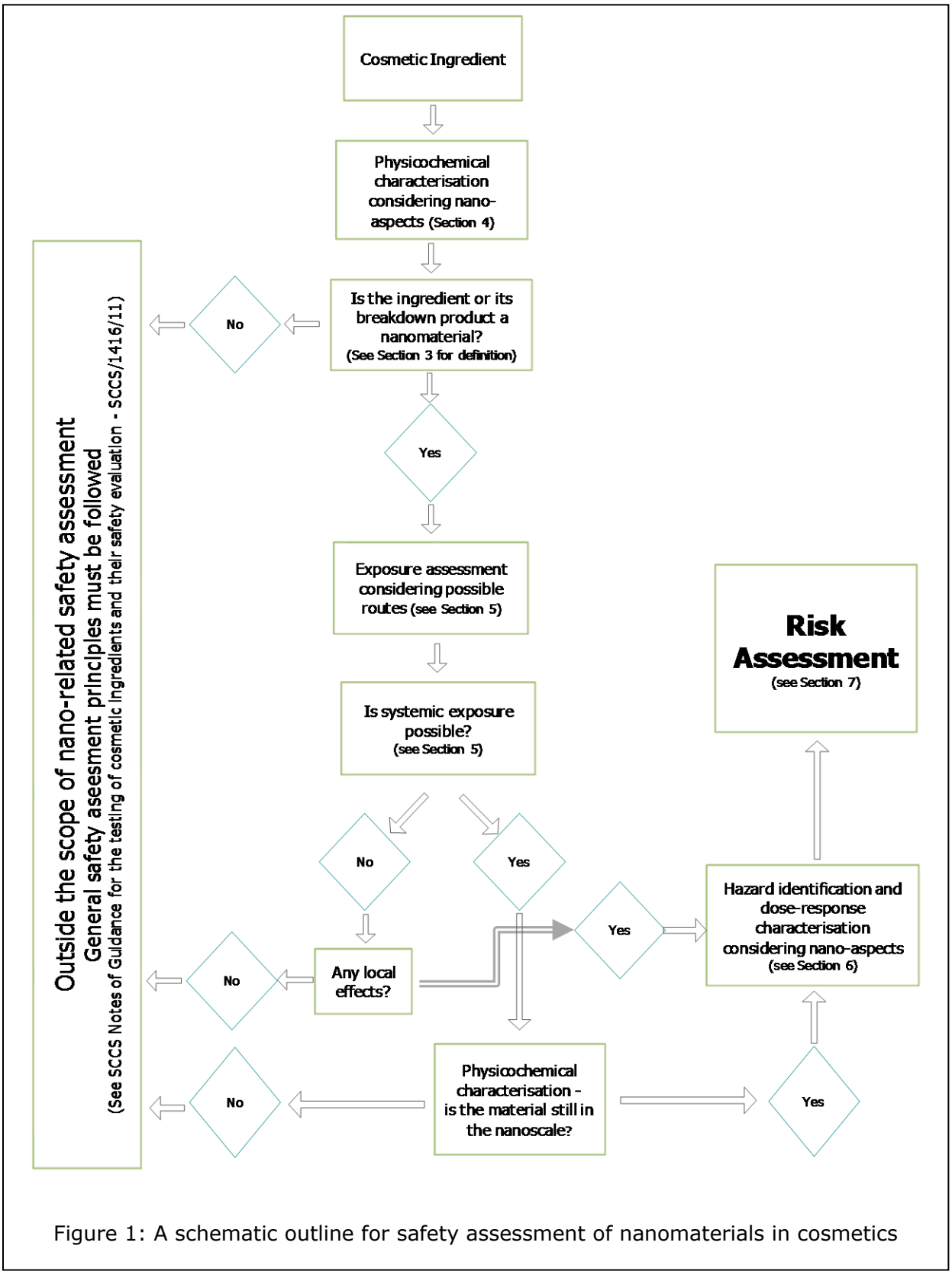

Figure 1: A schematic outline for safety assessment of nanomaterials in cosmetics

Due to the nano-dimensions, and altered uptake and biokinetics, some nanomaterials may pose a risk to the consumer because of the ability of insoluble or partially soluble particles to penetrate biological membrane barriers and reach those parts of the body that are otherwise protected from (larger) particles. There is an increasing but still insufficient understanding of the interaction of nanomaterials with biological systems at the molecular level.

Thus, where there is evidence for systemic translocation of nanoparticles, further investigations into hazard identification and dose-response characterisation will be required in consideration of nano aspects. In such a case, ADME parameters should take special importance. These data are normally obtained from *in vivo* studies. It should also be noted that interactions of a nanomaterial with biological systems may be different from those taking place between the conventional form of the same material, and such interactions may bring about further changes in the physicochemical characteristics of the nanomaterial. The most obvious example of the latter is adherence of proteins on the nanomaterial surface (Cedervall et al., 2007, Šimon and Joner 2008, Lynch and Dawson 2008), and therefore consideration should be given to any changes in the physicochemical properties of nanomaterials during toxicological investigations (see Section 6). The key parameters to consider in this regard include nano-dimensions (size, morphology, surface area) of the particles, agglomeration/ aggregation behaviour, surface characteristics etc (Rocks et al., 2008; SCENIHR, 2009; OECD, 2009; Chaudhry et al., 2010).

In regard to toxicological investigations, there is currently a ban in place in Europe under the Cosmetics Directive 76/768/EEC and the same provisions are included in the Cosmetics Regulation ((EC) No 1223/2009) on testing cosmetic ingredients and finished cosmetic products on animals, and a marketing ban on finished cosmetic products where either the final formulation or an ingredient or combination of ingredients has been subject to animal testing to meet the requirements of the legislation. 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. This will inevitably require the use of alternative testing methods (mainly *in vitro* assays and systems) in hazard identification/ dose-response characterisation.

One of the scientific objectives of the EU is the development and validation of 3R-alternative methods that can provide an equivalent level of information as current animal tests, but which use fewer animals, cause less suffering, or avoid the use of animals completely. In this respect, some refinement and reduction improvements have been made to existing *in vivo* guidelines, and a number of replacement guidelines have been developed. However, at present, the available **validated** alternative methods only cover **some** of the toxicological endpoints of **hazard identification** required for the toxicological data needed for risk assessment. These include *in vitro* methods relating to skin corrosion, skin irritation, mutagenicity, photomutagenicity, phototoxicity, and dermal absorption (Table 3). Due to a variety of reasons, including the complexity of the vertebrate organism, there are at present neither any validated *in vitro* method for repeated dose animal toxicity studies (including reproductive and developmental toxicity) available, nor any proposals in place for prevalidation/validation (Worth et al. 2002, Rogiers and Pauwels 2005, , Adler et al. 2011).

It is of note that none of the validated alternative methods currently available for conventional chemical substances has been validated specifically for nanomaterials. Also, apart from testing dermal absorption, the currently available *in vitro* tests are not suited for dose-response characterisation (SCCP 2007, SCCS 2009, Adler et al. 2011), which means quantitative risk assessment of cosmetic nanomaterials based on alternative methods is at present not possible. However, this is not specific to nanomaterials and applies to conventional cosmetic ingredients as well. Any possible use of *in vitro* methods for nanomaterials will require additional consideration of a number of nano-aspects that are different from those considered in the assessment of conventional chemicals and therefore may need certain adaptations of the current testing methods or further complementary characterisation and finally validation. These aspects are discussed in more detail in Section 6. However, given that a complete ban on animal testing will come in force in 2013, at

present, the validated alternative methods available only cover some toxicological endpoints of hazard identification required for the toxicological data needed for risk assessment. Apart from testing dermal absorption, they are not suited for dose-response characterisation, which would mean an insurmountable obstacle for the risk assessment of cosmetic nanomaterials as well as for their conventional equivalents. For these reasons, risk assessment of cosmetic nanomaterials may, in the first instance, be driven by exposure considerations (see Figure 1), with a focus on detailed characterisation of the nanomaterials (Table 1), and nano-related considerations during toxicological evaluations (Section 6 and Table 3).

4. PHYSICOCHEMICAL CHARACTERISATION

4.1 Important Physicochemical Parameters

The properties, behaviour, and biological effects of nanomaterials may be influenced by a number of physicochemical parameters. Thorough characterisation of nanomaterials therefore forms an integral part of the risk assessment. In the first place, the characterisation data presented in a safety dossier must demonstrate that it relates to the same (or justifiably comparable) nanomaterial that is intended for use in a cosmetic product. The characterisation is considered a very important aspect in relation to the **identification** of the nanomaterial tested. Therefore, a clear statement should be provided to indicate that the data relate to the same nanomaterial which is intended for use in the final product. Therefore, 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.

It is also of utmost importance that the physicochemical status of a nanomaterial in a cosmetic product is determined at different stages.

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

It is also important to note that any nano-related properties are intrinsically linked to the physical integrity of the nano-structure of a nanomaterial. Where a nanomaterial loses the nano-structure – e.g. in a formulation, a test medium, or biological surface/environment, due to solubilisation, breakdown or degradation, it will no longer be expected to behave any differently from its non-nano equivalent. It may still pose a toxicological hazard at the local level because of its chemical constituents, or at systemic level if before disintegration the nanostructure delivered the chemical constituents to a biological site where such a concentration of the conventional form would have not reached. Determining stability of the nanomaterial under experimental conditions is therefore of prime importance for the interpretation of any test results. Stability may be measured in terms of dissociation constants, dissolution rates, and solubilities of a nanomaterial in the final cosmetic product and in the media/ vehicle(s) used in exposure/ hazard evaluations and should be determined by appropriate physicochemical methods. In addition, determining stability of the nanomaterial surface is also important, as certain reactions, e.g. oxidation/ hydroxylation, may take place during handling/storage which may modulate the interactions of the nanomaterial with biological systems.

As the physicochemical parameters may change in various environments, the EFSA Guidance (2011) recommends that characterisation of nanomaterials should ideally be determined in five stages i.e. as manufactured (pristine state), as delivered for use in food products, as present in the food matrices, as used in toxicity testing, and as present in biological fluids and tissues during testing. The SCCS recommends that, as a minimum, characterisation of nanomaterials intended for use in a cosmetic product should include description of the pristine nanoparticles as produced, as added to the cosmetic product, and as present during exposure for toxicological assessment, i.e.:

1. in the raw material form as manufactured
2. after addition to a final cosmetic formulation
3. during toxicological investigations

If characterisation of nanomaterial at any of these stages is not feasible, for example, due to the lack of methods, or due to degradation of the nanomaterial, it should be justified and documented.

The selection of key physicochemical parameters that can adequately describe a nanomaterial, and selection of characterisation methods that can be used to measure them, will depend on the composition, properties, and intended use(s) of the nanomaterial. Due to the current gaps in knowledge in regard to possible relationship(s) between physicochemical properties and adverse health effects of nanomaterials, it is difficult to identify a shortlist of priority parameters for characterisation of nanomaterials. However, this has been the subject of discussions by several international expert committees and working groups, the reports of which have been considered in preparation of this Guidance Document. The key reports considered in this regard include those published by the 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 the ICCR Working Groups (2011a). In addition, a document providing Cosmetic Industry's perspective on specific characteristics of the safety assessment of nanomaterials used in cosmetic products (2010) was also considered. These expert reports have identified certain physicochemical parameters as important in relation to safety assessment of nanomaterials. These are summarised in Table 1.

Table 1: Important parameters for identification and characterisation of nanomaterials intended for use in cosmetic products

Parameter	**Description**	**Methods***
Chemical identity	Information on structural formula(e)/ molecular structure(s) of the constituents of nanomaterial must be provided, along with chemical and common names, and CAS and EINECS numbers (where available).	A wide range of analytical methods, including MS, AAS, ICP-MS, FTIR, NMR, etc.
Chemical composition	Information on full chemical composition of the nanomaterial must be provided. This should include purity, nature of impurities, coatings or surface moieties, doping material, encapsulating materials, processing chemicals,dispersing agents, and other additives or formulants e.g. stabilisers.	A wide range of analytical methods, including UV-Vis, HPLC, GC/LC-MS, AAS, ICP-MS, FTIR, NMR, XRD etc.
Size	Information on primary and secondary particle size, particle number size distribution and particle mass size distribution must be provided. Product	FFF, HDC, HPLC, AUC, CLS disc centrifugation, TEM, SEM, AFM, DLS,

	specification and any batch to batch variation during manufacturing must be indicated. The use of more than one method (one being electron microscopy based imaging) for determination of size parameters has been recommended by EFSA (2011) and OECD (2010). This must also be a prerequisite for nano-scale cosmetic ingredients.	DMA
Morphology	Information on the physical form and crystalline phase/shape must be provided. The information should indicate whether the nanomaterial is present in a particle-, tube-, rod- shape, crystal or amorphous form. Also, whether the nanomaterial is in the form of primary particulates or agglomerates/ aggregates. Information should also indicate whether the nanomaterial preparation is in the form of a powder, solution, suspension or dispersion. Aspect ratio of nanomaterial should be calculated and provided (for fibre/tube like material).	AFM, TEM, SEM, NMR, XRD
Surface characteristics	Detailed information on nanomaterial surface must be provided. This should include information on surface charge (zeta potential), morphology/topography, interfacial tension, reactive sites, as well as any chemical/ biochemical modifications or coatings that could change the surface reactivity, or add a new functionality.	LDE, SPM, XPS, MS, RS, FTIR, NMR, AUC (for surface composition), GE, SPM, LDE, PALS (for zeta potential), Nano SIMS, SERS
Solubility	Information on solubility of the nanomaterial in relevant solvents and partitioning between aqueous and organic phase (e.g. log Kow for organic nanomaterials, and surface modified inorganic nanomaterials) must be provided. Dissolution rates in relevant solvent for soluble and partially-soluble nanomaterials should also be provided. Information on hygroscopicity of powders should also be provided.	Solubility/ dissolution rate in water and other solvents
Surface area	Information on BET specific surface area of the nanomaterial, and volume specific surface area (VSSA) must be provided (see Kreyling et al., 2010 for calculation of VSSA). At the moment the VSSA is only applicable to nanomaterials in powder formulation.	BET
Catalytic activity	Information on the chemical reactivity of the nanomaterial core material or surface coating must be provided. Information on photocatalytic activity, and radical formation potential of relevant materials must also be provided.	Kinetic measurements of chemical, biochemical and/or catalysed reactions
Concentration	Information on concentration in terms of particle mass and particle number per volume must be provided for dispersions and per mass for dry powders.	A wide range of analytical methods, including UV-Vis, HPLC, GC/LC-MS, AAS, ICP-MS, etc.
Dustiness	Information on dustiness of dry powder products must be provided.	EN 15051:2006, DIN 33897-2.
Density and pour density	Information on density/porosity of granular materials and pour density must be provided.	DIN ISO 697, EN/ISO 60
Redox potential	Information on oxidation state and redox potential (for inorganic materials) must be provided, including the conditions under which redox potential was measured should be documented.	Potentiometric methods, X-ray absorption spectroscopy
pH	pH of aqueous suspension must be provided.	pH in aqueous media
Viscosity	Information on viscosity of liquid dispersions must be provided.	OECD TG 114
Stability	Data on stability/ dissociation constant of the	MS, HPLC, DLS, FTIR,

	nanomaterial in relevant formulation/ media must be provided.	NMR
Other aspects	UV absorption (extinction coefficient), light reflection	UV-Vis

*The list includes mainstream methods but is not exhaustive.

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

In general, characterisation of a nanomaterial in a cosmetic formulation is more difficult compared to characterisation in a raw material, and even more challenging when the nanomaterial is contained in a biological matrix or has been released to the ecosphere. Depending on the concentration of nanomaterial contained in a formulation/matrix, and the nature of the formulation/ matrix, a suitable characterisation scheme may be needed that includes isolation, purification and concentration steps (if necessary) before analysis of the nanomaterial. Characterisation 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 ingredient in a final product. Similar care is needed during toxicological evaluations. Parameters such as size, aggregation states, surface charge, coatings and other properties may change in different solvents, test media, and biological environments. Therefore, conditions under which measurements are made should be given a careful consideration, and documented at every stage of production and on the shelf, and should be provided in the dossier.

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.

4.2 Methods for Characterisation

A wide range of analytical methods is available for measuring the physicochemical parameters of conventional chemical substances. Some of these methods can also be used (or adapted) for detection and characterisation of nanomaterials. The most relevant methods for nanomaterial characterisation are based on light scattering (e.g. DLS), microscopy (e.g. TEM, SEM), size separation and extraction (e.g. (ultra) centrifugation, FFF, HDC), and chemical analysis/ detection by spectroscopic or mass spectrometric techniques (e.g. ICP-MS, UV spectroscopy, AAS); surface area determination (BET), and their different variants and combinations. Methods for *in situ* imaging of nanomaterials, e.g. magnetic particle imaging (MPI) and positron emission tomography (PET), are currently under development. Similarly, antibody, binding protein, and enzyme based methods are also under development for organic or coated-inorganic nanomaterials. Main methods for characterisation of nanomaterials have been listed in Table 1, and additional details have been provided in the recent ICCR WG report (2011a), and other documents (EFSA 2011).

It is important to note that currently there is no single method that can be regarded a 'gold' standard for characterisation of the different physicochemical parameters of nanomaterial as such, nor is there one suited to fully assess a nanomaterial in a cosmetic product. The exact choice of analytical method(s) to measure a parameter will be dependent on the chemical composition and the physical form of individual nanomaterials. However, as pointed out in a recent EFSA Guidance (2011), a carefully chosen portfolio of established analytical techniques, should provide adequate data for the purpose, provided that measurements are carried out properly, and results are backed up by appropriate documentation.

In this regard, electron microscopy techniques provide a very useful visual means for the determination of the particle shape and size, of which size is the common denominator of all nanomaterials. Electron microscopy can also be linked with spectroscopic or spectrometric methods to provide more information on both particle size/shape as well as chemical composition of nanomaterials. The EFSA Guidance (2011) and OECD (2010), recommend that the determination of nanomaterial size parameters should include the use of an electron microscopy method. The SCCS also recommends that size parameters for nano-scale ingredients intended for use in cosmetic products should be measured by at least two methods, one being electron microscopy (preferably high resolution TEM).

4.3 Performance of Characterisation Methods

With regard to characterisation of nanomaterials, it is important to note that different measurement techniques may yield slightly different results. This is due to the different characteristics of the measurements of the very small dimensions, and/or the low amount of material. Furthermore, these differences reflect the differences in the aggregation/ agglomeration behaviour of nanomaterials during different sample handling/ preparation procedures, dilutions, or dispersions used in different methods, and/or the different measurement principles applied in individual methods (Domingos et al., 2009). It is therefore important to ensure that sample preparation is carried out in a consistent manner to obtain reproducible results, and to allow a comparison between the results of different samples analysed by a specific analytical method, or by different methods.

In line with the EFSA Guidance (2011), method performance parameters to be determined and documented should include criteria such as specificity, selectivity, recovery, repeatability, reproducibility, and limits of detection/ quantification. Where possible, existing guidelines (e.g. IUPAC, 2002) should be taken into account, or adapted from guidelines available for that specific material or product category if no specific guideline is applicable for a nanomaterial. The use of a method that differs from internationally agreed protocols should be justified and documented.

At present certified reference nanomaterials are only available for size determinations (gold, silica). It is preferable to use certified reference materials as internal standards in the analyses. However, where certified reference materials are not available, self-generated standards may be used instead, provided that details are documented.

5. EXPOSURE ASSESSMENT

Exposure assessment and the identification of potential exposure routes form the first crucial decision point in the overall risk assessment (Figure 1). The exposure assessment for ingredients in cosmetic products as described in the SCCS Notes of Guidance is a general approach that applies to nanomaterials as well. There is currently no indication that the use of consumer/cosmetic products that contain nanomaterials is likely to be any different from the use of other products that contain conventional ingredients. This means that default values in relation to exposure e.g. used amounts, will be the same to those considered for cosmetic products as provided in the Notes of Guidance.
If the systemic exposure is estimated by using in vitro or in vivo experiments, 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. 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.

Corresponding to *Cosmetics Regulation (EC) No 1223/2009, Article 16 f) "reasonably foreseeable exposure conditions" need to be taken into account.* The following factors are important for an exposure assessment:

- class of cosmetic product(s) in which the ingredient may be used,
- method of application: rubbed-on, sprayed, applied and washed off, etc.,
- concentration of the ingredient in the finished cosmetic product,
- quantity of the product used at each application,
- frequency of use,
- total area of skin contact,
- duration of exposure
- foreseeable misuse which may increase exposure,
- consumer target groups (e.g., children, people with sensitive, damaged or compromised skin) where specifically required
- quantity likely to enter the body (fraction absorbed),
- application on skin areas exposed to sunlight,
- use area (indoors/outdoors) and ventilation
- all routes of exposure (dermal, oral and inhalation exposure) should be considered in view of the intended use of the product

Measuring the effects of nanomaterials on compromised skin poses a challenge due to the current lack of standardised model(s) that can be used to generate results that are reproducible and can be used to compare studies carried out within a laboratory and between different laboratories. In view of this, OECD (2011) has recommended studies on intact, healthy skin. According to OECD (2004a), absorption studies should be conducted using healthy animals or intact healthy skin *in vitro* (OECD 2004b). This is also reflected by the recommendation to perform skin integrity checks, as described in the current guidelines for *in vitro* skin penetration studies (OECD 2004a, SCCS, 2010a, 2010b). Where studies on compromised skin are specifically required, the models used should be well characterised to generate reproducible results, and appropriate controls should be included in the studies. Urgent research is needed to develop appropriate test models of compromised skin that can be reliably used to assess possible absorption of cosmetic ingredients, including nanoparticulate materials.

The aim of the exposure assessment is to determine the Systemic Exposure Dosage (SED), which is an important parameter for calculating the Margin of Safety (MoS) of ingredients in a finished cosmetic product (see Section 7).

MoS = NO(A)EL* / SED
*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. Depending on the data set available, additional safety factors may be used (e.g. when using LO(A)EL instead of NO(A)EL).

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

The systemic exposure dosage (SED in mg/kg bw /day) after dermal application can be calculated on the basis of the dermal absorption expressed in microg/cm² or as a percentage of the amount of substance applied.

5.1 Calculation of systemic exposure

5.1.1. Dermal exposure

In regard to potential health effects, as mentioned in Section 6.3.2.3, it is currently not clear which metric is the best dose descriptor for nanomaterials - mass, particle numbers,

or surface area – that should be used in exposure assessment and subsequent risk assessment. For practical reasons mass based exposure is generally used at present. It is therefore important that tests on nanomaterials are evaluated using different dose-describing metrics, e.g. weight/volume concentration, particle number concentration, surface area etc. The characterisation of the nanomaterial ingredient (see Section 4) should provide sufficient information that the various dose metrics can be derived from the characterisation data.

For conventional cosmetic ingredients, in cases where no (adequate) information is available on dermal absorption, the SCCS assumes 100% absorption. 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 (see annex).

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 penetrated 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. 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 default approach and assume that 100% of the absorbed material was in particle form. This, however, does not imply that the particulate form of a chemical is always associated with a greater toxicity potential. Depending on the chemical composition of the nanomaterial, certain solubilised/metabolised forms may be more toxic than the corresponding particulate forms, which needs to be taken into account for the safety assessment.

Dependent on whether the dermal absorption is reported in µg/cm² or as a percentage of the substance applied, different exposure parameters must be known in order to calculate the actual SED:

1) Dermal absorption of test substance reported in µg/cm²:

$$SED = \frac{\mathbf{DA_a}\ (\mu g/cm^2) \times 10^{-3} (\text{conversion factor mg/µg}) \times \mathbf{SSA}\ (cm^2) \times \mathbf{F}\ (day^{-1})}{60\ kg}$$

With: SED (mg/kg bw/day) =	Systemic Exposure Dosage
DA_a (µg/cm²) =	Dermal Absorption reported as amount/cm_2, resulting from an assay under in-use mimicking conditions
SSA (cm²) =	Skin Surface Area expected to be treated with the finished cosmetic product (see section 4-2 of the SCCS Notes of Guidance for SSA values per product type)
F (day^{-1}) =	Frequency of application of the finished product
60 kg =	default human body weight

The use of this expression implies that the **skin surface area (SSA)** envisaged to be treated with the finished cosmetic product containing the ingredient under study, has to be known, as well as the **frequency of application (F)** of the finished product.

2) Dermal absorption reported as a percentage of the amount of substance applied:

SED = **A** (mg/kg bw/day) x **C** (%)/100 x $\mathbf{DA_p}$ (%)/100

With: SED (mg/kg bw/day) = Systemic Exposure Dosage

A (mg/kg bw/day) =	Estimated daily exposure to a cosmetic product per kg body weight, based upon the amount applied and the frequency of application:
C (%) =	the Concentration of the ingredient under study in the finished cosmetic product on the application site
DA_p (%) =	Dermal Absorption expressed as a percentage of the test dose assumed to be applied in real-life conditions

In this case it is key to know the **daily exposure of formulation applied per kg body weight (A)** under intended use conditions (IGHRC, 2006).

5.1.2 Oral exposure

For the oral exposure, the equation is comparable to the equation for dermal exposure.

SED = **A** (mg/kg bw/day) x **C** (%)/100 x $\mathbf{D_{oral}}$ (%)/100

SED (mg/kg bw/day) =	Systemic Exposure Dosage
A (mg/kg bw/day) =	Estimated daily exposure to a cosmetic product per kg body weight, based upon the amount applied and the frequency of application:
C (%) =	Concentration of the ingredient under study in the finished cosmetic product on the application site
D_{oral} (%) =	Oral Absorption expressed as a percentage of the test dose assumed to be applied in real-life conditions. The amount used is multiplied with the use frequency and the concentration of the ingredient. In some cases, a retention factor is used to account for the fact that not the whole amount used is actually ingested (e.g. in the case of toothpaste).

5.1.3 Inhalation exposure:

Exposure assessment is required for products in spray form, and for exposure to solvents. In such cases, exposure models can be used to estimate exposure. One of the tools that could be used to assess exposure to solvents or exposure to aerosols generated after the use of spray applications is the ConsExpo model (www.consexpo.nl). This tool comprises two modules for inhalation: 1) exposure to vapour and 2) exposure to sprays. For nanomaterial containing sprayable products, only the second module is of relevance (see definition of nanomaterials).

The spray module calculates the exposure based on the inhalable fraction of the generated aerosols. For conventional substances it is assumed that these are homogeneously distributed over the generated aerosols, on a mass basis. For that reason, in the experiments carried out for the calibration of the model, aerosols with a size <1 µm are not be taken into account. It should be noted that the mass of aerosol droplets <1µm is negligible compared to the aerosols present in the inhalable fraction of 1-20 µm. Key parameters in the calculation of the inhalation exposure are: room volume, spray duration, ventilation rate, exposure duration and product specific parameters, such as mass generation rate, airborne fraction, aerosol size distribution, and weight fraction of the ingredient.

At present the applicability of ConsExpo spray module to nanoparticles has not yet been determined. Therefore, for spray application of products with nanomaterial, a careful characterisation is needed of the droplet size and the nanomaterial distribution in the

droplets. Determination of the generated droplet size distribution is not sufficient, but needs to be complemented by the size distribution of the dried residual aerosol particles.

Exposure patterns during consumer use (e.g. in terms of variable particle size distribution) might be different from exposure patterns in experimental settings (e.g. stable particle size-distribution). However, factors such as particle size and size distribution/ agglomeration state of nanomaterials are known to be important in determining the hazard.

As highlighted in the outline scheme in Figure 1, the following questions need to be asked in relation to the risk assessment of nanomaterials in cosmetics:

- Is any of the cosmetic ingredients a nanomaterial (based on the available definition)?
- Would the use of a cosmetic product containing the nanomaterial give rise to:
    - systemic exposure (considering all possible routes)?
    - a toxicological effect at the local and/or systemic levels?
    - a risk to the consumer?

Thus determining whether or not any systemic exposure to nanomaterial is possible during the foreseeable use(s) of a cosmetic product would be an important consideration in the risk assessment process. This can be determined 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. However, the methods used 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. 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.

6. HAZARD IDENTIFICATION AND DOSE-RESPONSE CHARACTERISATION

6.1 Introduction

The hazard or toxic potential of a cosmetic ingredient is assessed through a series of studies which include, *in silico, in vitro,* and *in vivo* evaluations. Several *in vitro* methods exist for the identification of certain hazards. However, information on dose response relationships that can be used in the current risk assessment scheme, e.g. NOAELs, LOAELs or BMDLs, is generally derived from *in vivo* studies. The toxicological studies need to be conducted in accordance with the guidelines provided in Regulation (EC) No 440/2008 [2008/440/EC], and complying with the principles of Good Laboratory Practice (Directive 87/18/EEC), or by means of adequate and acceptable scientific methods. It must be stressed that the SCCS is of the opinion that *in vivo* testing methods are at present indispensable for the derivation of the above dose descriptors (Adler et al. 2011, SCCP 2007, SCCS 2009).

A limited number of *in vitro* test methods have been developed and validated for conventional chemicals to assess various toxicological endpoints. Of these, methods relevant to ingredients in cosmetic products include methods for assessing skin corrosion, skin irritation, mutagenicity/genotoxicity, photomutagenicity, phototoxicity, and dermal absorption. These *in vitro* methods are used as validated alternative methods for testing of hazard identification of conventional cosmetic ingredients, but **none of the methods has yet been validated for nanomaterials**. Nanomaterials pose many challenges when tested using *in vitro* methods (Section 6.3.2). Unlike solubilised chemicals, nanomaterials generally exist in an assay system as a suspension of insoluble or partially-soluble nanoparticles, and/or larger agglomerates and aggregates, which in addition to surface properties, may affect the *in vitro* assay methodologies.

It should be noted that nanomaterials, due to their particulate nature, are likely to have a different toxicokinetic profile when compared to conventional chemicals. Nanomaterials tend to end up in the reticulo-endothelial-system (RES). For example, after intravenous administration, many nanomaterials have been found to end up in liver and spleen, the two major organs of the RES ((Semmler-Behnke et al. 2007; Semmler-Behnke et al. 2008; De Jong et al., 2008; Hirn et al., 2011; Lankveld et al., 2010). Furthermore nanomaterials have also been found to accumulate in other organs such as kidneys (Hirn et al. 2011), soft tissue (muscle, connective tissue, fat, skin), the bone marrow (Rinderknecht et al., 2008; Kreyling et al., 2009) and also the foetus (Semmler-Behnke et al. 2007). Even, with intratracheal inhalation, bypassing the nasal passages, nanoparticles were found in the brain which had been translocated from the lungs via blood (Kreyling et al., 2009)

Surface modifications, e.g. protein adsorption/coatings, may considerably affect the toxicokinetics of a nanomaterial. This may result in unexpected toxicity, not seen with conventional chemicals, due to accumulation of nanomaterials in certain organs because a different toxicokinetics can be anticipated from the same particulate material if coated differently. Such differences in toxicokinetics are dependent on the uptake and systemic availability of the nanomaterials that may vary with the exposure route (oral, inhalatory, dermal). Also the quality of the barrier (e.g. compromised skin versus healthy intact skin) that separates the cosmetic ingredient and the circulation system is of importance. For spray applications, it should be considered that, for several nanomaterials, transport into the brain has been demonstrated after inhalation. It was suggested to most likely take place via the olfactory nerve (Oberdorster 2009).

The SCCS emphasizes that some of the specific hazards related to nanomaterials remain difficult to assess by conventional *in vivo* studies according to accepted guidelines. Currently available *in vitro* or other alternative methods can only be used as supportive tools in this respect (Adler et al. 2011, SCCP 2007, SCCS 2009).

6.2 General requirements

When a cosmetic ingredient dossier is submitted for evaluation by the SCCS, the manufacturer provides the Commission with information on a number of toxicological endpoints. These have been listed in Table 2, corresponding to *Cosmetics Regulation (EC) No 1223/2009, Article 16 d) "toxicological profile of the nanomaterial"*.

Table 2: Main toxicological endpoints assessed for safety evaluation of cosmetic ingredients, which also need to be determined for nanomaterials in cosmetic products

1. Dermal/ percutaneous absorption 2. Toxicokinetics	Where tests on oral, inhalation or dermal/ percutaneous absorption show evidence for systemic absorption of nanoparticles, initial focus of toxicological investigations should be on determining ADME parameters to understand the fate and behaviour of nanoparticles in the body, and to identify the likely target organs. The investigations should determine whether there are any changes in physicochemical characteristics of the nanoparticles, in terms of surface binding of proteins or other moieties, that may have altered interaction with biological systems, and whether there are any changes in the integrity of the nano-structure, or agglomeration/ aggregation behaviour.

3. Acute toxicity (if available); 4. Irritation and corrosivity; 5. Skin sensitisation; 6. Mutagenicity/ genotoxicity; 7. Repeated dose toxicity*;	In general, these endpoints, together with dermal/ percutaneous absorption, are considered the minimal base set requirement for conventional cosmetic ingredients, and must also be assessed for any nanomaterial intended for use in cosmetic products. Mutagenicity/genotoxicity testing is initially performed using *in vitro* assays. *In vivo* assays to demonstrate non mutagenicity may be necessary when positive results are noted *in vitro*.
8. Carcinogenicity; 9. Reproductive toxicity;	In cases where a considerable oral intake is expected, or when the data on dermal/ percutaneous absorption indicate a considerable penetration of the ingredients through the skin (taking into account the toxicological profile of the substance and chemical structure), further toxicological investigations (8 and 9) may become necessary, together with specific additional genotoxicity, and/or mutagenicity data submitted under 7.
10. Photo-induced toxicity;	Photo-induced toxicity data are specifically required when the cosmetic product is expected or intended for use on sunlight-exposed skin and is able to absorb light at a certain wavelength. As a consequence of light absorption, a substance may undergo certain transformations in configuration that may lead to a change in chemical reactivity. Hence there may be a need for further investigation into specific phototoxic effects, such as photoirritancy, photosensitisation and photomutagenicity. Studies on phototoxic potential of a cosmetic ingredient must be performed by applying relevant UV light wavelengths derived from the absorption spectrum of the ingredient [SCCNFP/0633/02], and photostability data under conditions of use should be provided.
11. Human data.	In general, the SCCS considers human data as extremely useful and should be included whenever available. Nevertheless, in case of volunteer studies considering nanomaterials a potential risk for the volunteers cannot be excluded since there is still a lack of information on the severity and frequency of adverse effects and is therefore subject to ethical concerns. [SCCNFP/0633/02].

** considering the various routes of exposure (oral, dermal and inhalation)*

More details on the specific requirements for toxicological assessment have been provided in Annex I (Table 3).

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.

9. REFERENCES

Acute-Tox. www.acutetox.org

Adler S., Basketter D., Creton S. et al. (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects – 2010. Archives of Toxicology. 85(5) 367-485.

Balls M. and Fentem J.H. Progress toward the validation of alternative tests. ATLA 25,33-43,1997

Burello E., Worth A.P. (2011) QSAR modeling of nanomaterials, Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 3: 298–306.

Cedervall T., Lynch I., Lindman S., Berggard T., Thulin E., Nilsson H., Dawson K.A., Linse S. (2007) Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. PNAS 104, 2050-2055.

Chaudhry Q., Bouwmeester H. and Hertel R.F. (2010) The Current Risk Assessment Paradigm in Relation to Regulation of Nanotechnologies, In. G.A. Hodge, D.M. Bowman and A.D. Maynard (eds), International Handbook on Regulating Nanotechnologies. Cheltenham: Edward Elgar, 124-143.

Clift M.J.D., Gehr P., Rothen-Rutishauser B. (2011) Nanotoxicology: a perspective and discussion of whether or not in vitro testing is a valid alternative. Arch Toxicol 85, 723-731.

ConsExpo www.consexpo.nl

Corradi S., Gonzalez L., Thomassen L.C., Bilaničová D., Birkedal R.K., Pojana G., Marcomini A., Jensen K.A., Leyns L., Kirsch-Volders M. (2011) Influence of serum on in situ proliferation and genotoxicity in A549 human lung cells exposed to nanomaterials. Mutat Res. 2011 Oct 19. [Epub ahead of print].

Cosmetic Industry Perspective on specific Characteristics of the Safety Assessment of Nanomaterials used in Cosmetic Products (2011).

De Jong W.H., Hagens W.I., Krystek P., Burger M.C., Sips A.J.A.M., Geertsma R.E. Particle size dependent organ distribution of gold nanoparticles after intervenous administration. Biomaterials 29, 1912-1919, 2008

Domingos RF, Baalousha MA, Ju-Nam Y, Reid MM, Tufenkji N, Lead JR, Leppard GG and Wilkinson KJ, (2009). Characterizing manufactured nanoparticles in the environment: multimethod determination of particle sizes. Environ Sci Technol., 43(19): 7277-84.

EFSA (European Food Safety Authority) (2009) The potential risks arising from nanoscience and nanotechnologies on food and feed safety, Scientific Opinion of the Scientific Committee, The EFSA Journal (2009) 958, 1-39.

EFSA (European Food and Safety Authority) (2011) Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain, EFSA Journal 9(5): 2140, 36 pp, available at: www.efsa.europa.eu/en/efsajournal/pub/2140.htm

EU (European Union) (1967) Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Official Journal L196, 16.8.1967, p. 1

EU (European Union) (1976) Council Directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products. (76/768/EEC). Official Journal L 262, 27.9.1976, p. 169

EU (European Union) (1986). Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). Official Journal L 358, 18.12.1986, p. 1.

EU (European Union) (1987). Council Directive 87/18/EEC of 18 December 1986 on the harmonization of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances. Official Journal L15, 17.1.1987, p. 29

EU (European Union) (2003) Directive 2003/15/EC of the European Parliament and of the Council of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. Official Journal L 66, 11.3.2003, p 26

EU (European Union (2008). Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Official Journal L 142, 30.5.2008, p. 1

EU (European Union) (2009) Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast). Official Journal L 342, 22.12.2009, p. 59

EU (European Union) (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal L 276, 20.10.2010, p. 33

EU (European Union) (2011) Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU). Official Journal L 275, 20.10.2011, p. 38

Flari V., Chaudhry Q., Neslo R. and Cooke R. (2011) Possible use of expert judgment based multi criteria decision model to overcome uncertainties in risk assessment of nanotechnology-enabled food products, Journal of Nanoparticle Research: Volume 13(5): 1813-1831.

Geiser M., Kreyling W.G. (2010) Deposition and biokinetics of inhaled nanoparticles. Particle Fiber Toxicol 2010:7:2.

Hartung T. and Sabbioni E. (2011) Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 3(6): 545-573.

Hirn S., Semmler-Behnke M., Schleh C., Wenk A., Lipka J., Schaffler M., Takenaka S., Moller W., Schmid G., Simon U. and Kreyling W.G. (2011). Particle size-dependent and surface charge-dependent biodistribution of gold nanoparticles after intravenous administration. Eur. J. Pharm. Biopharm. 77, 407-16, 10.1016/j.ejpb.2010.12.029.

Hirsch C., Roesslein M., Krug H.F., Wick P. (2011) Nanomaterial cell interactions: are current in vitro tests reliable? Nanomedicine 6, 837-847.

ICCR (2011a) 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

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

IGHRC 2006. Guidelines on route-to-route extrapolation of toxicity data when assessing health risks of chemicals. The Interdepartmental Group on Health Risks from Chemicals, http://www.silsoe.cranfield.ac.uk/ieh/ighrc/ighrc.html

ISO (International Organisation for standardisation) ISO/TS 27687:2008. Nanotechnologies – terminology and definitions for nano-objects – nanoparticle, nanofibre and nanoplate.

ISO (International Organisation for standardisation) ISO 10808: 2010: Nanotechnologies – Characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing.

ISO (International Organisation for standardisation) ISO 10993-10:2010. Biological evaluation of medical devices -- Part 10: Tests for irritation and skin sensitization

ISO (International Organisation for standardisation) ISO/TS 80004-1:2010. Nanotechnologies – vocabulary – part 1: core terms

IUPAC (2002) Harmonized guidelines for single-laboratory validation of methods of analysis, Pure and Applied Chemistry 74 (5), 835 - 855 (2002)), 2002/EC/657.

Jani P., Halbert G.W., Langridge J., Florence A.T. (1989) The uptake and translocation of latex nanospheres and microspheres after oral administration to rats. J Pharm Pharmacol 41(12):809-812.

Jani P., Halbert G.W., Langridge J., Florence A.T. (1990). Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J Pharm Phamacol 42, 821-826.

Kreyling W.G., Semmler-Behnke M., Seitz J., Scymczak W., Wenk A., Mayer P., Takenaka S. and Oberdorster G. (2009). Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. Inhal. Toxicol. 21, 55-60.

Kreyling W.G., Semmler-Behnke M. and Chaudhry Q. (2010) A complementary definition of nanomaterial, Nano Today 5:165-168.

Kumar A., Pandey A.K., Singh S.S., Shanker R., Dhawan A.A. (2011) flow cytometric method to assess nanoparticle uptake in bacteria. Cytometry A 79:707-712.

Landsiedel R., Kapp M.D., Schulz M., Wiench K., Oesch F. (2009) Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations--many questions, some answers. Mutat Res 681: 241-258.

Lankveld D.P.K., Oomen A., Krystek P., Troost-De Jong A., Noorlander C.W., Van Eijkeren J.C.H., Geertsma R.E., De Jong W.H. The kinetics of the tissue distribution of silver nanoparticles of differents sizes. Biomaterials 31, 8350-8361, 2010.

Lanone S., Rogerieux F., Geys J., Dupont A., Maillot-Marechal E., Boczkowski J., Lacroix G., Hoet P. (2009) Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. Part Fibre Toxicol. 6:14.

Lynch I. and Dawson K.A.(2008), 'Protein- nanoparticle interactions', Nano Today, 3, 40–47.

Monteiro-Riviere N.A., Inman A.O., Zhang L.W. (2009) Limitations and relative utility of screening assays to assess engineered nanoparticle toxicity in a human cell line. Toxicol Appl Pharmacol 234, 222-235.

Oberdörster G., Elder A., Rinderknecht A. (2009) Nanoparticles and the brain: cause for concern? J Nanosci Nanotechnol. 9: 4996-5007.

Oberdörster G., Maynard A., Donaldson K., Castranova V., Fitzpatrick J., Ausman K., Carter J., Karn B., Kreyling W., Lai D., Olin S., Monteiro-Riviere N.A., Warheit D.B. and Yang H. (2005). Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part. Fibre Toxicol. 2, 8-35.

OECD (1981a). OECD Guideline for the Testing of Chemicals (OECD TG 114), viscosity of liquids, adopted 12 May 1981

OECD (1981b). OECD Guideline for the Testing of Chemicals (OECD TG 401), Acute oral toxicity. Following the OECD Council decision, the test 401 '*Acute Oral Toxicity*' was deleted on 17 December 2002

OECD (1981c). OECD Guideline for the Testing of Chemicals (OECD TG 410), Repeated dose dermal toxicity: 21/28-day study, adopted 12 May 1981

OECD (1981d). OECD Guideline for the Testing of Chemicals (OECD TG 411), Subchronic dermal toxicity: 90-day study, adopted 12 May 1981

OECD (1981e). OECD Guideline for the Testing of Chemicals (OECD TG 412), Repeated dose inhalation toxicity: 28-day or 14-day study, adopted 12 May 1981

OECD (1981f). OECD Guideline for the Testing of Chemicals (OECD TG 413), Subchronic inhalation toxicity: 90-day study, adopted 12 May 1981

OECD (1981g). OECD Guideline for the Testing of Chemicals (OECD TG 451), carcinogenicity studies, adopted 12 may 1981

OECD (1981h). OECD Guideline for the Testing of Chemicals (OECD TG 452), chronic toxicity studies, adopted 12 may 1981

OECD (1981i). OECD Guideline for the Testing of Chemicals (OECD TG 453), Combined chronic toxicity/carcinogenicity studies, adopted 12 may 1981

OECD (1983). OECD Guideline for the Testing of Chemicals (OECD TG 415), One-generation reproduction toxicity testing, adopted 26 May 1983

OECD (1984a). OECD Guideline for the Testing of Chemicals (OECD TG 417), Toxicokinetics , adopted 4 April 1984

OECD (1984b). OECD Guideline for the Testing of Chemicals (OECD TG 478), Genetic toxicology: rodent dominant lethal test, adopted 4 April 1984

OECD (1987). OECD Guideline for the Testing of Chemicals (OECD TG 402), Acute dermal toxicity, adopted 24 February 1987

OECD (1992). OECD Guideline for the Testing of Chemicals (OECD TG 406), Skin sensitisation, adopted 17 July 1992

OECD (1995). OECD Guideline for the Testing of Chemicals (OECD TG 407), Repeated dose 28-day oral toxicity study in rodents, adopted 27 July 1995

OECD (1996). OECD Guideline for the Testing of Chemicals (OECD TG 422), Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test, adopted 22 March 1996

OECD (1997a). OECD Guideline for the Testing of Chemicals (OECD TG 471), Bacterial reverse mutation test, adopted 21 July 1997

OECD (1997b). OECD Guideline for the Testing of Chemicals (OECD TG 473), In vitro mammalian chromosome aberration test, adopted 21 July 1997

OECD (1997c). OECD Guideline for the Testing of Chemicals (OECD TG 475), Mammalian bone marrow chromosome aberration test, adopted 21 July 1997

OECD (1997d). OECD Guideline for the Testing of Chemicals (OECD TG 476), In vitro mammalian cell gene mutation test, adopted 21 July 1997

OECD (1998a). OECD Guideline for the Testing of Chemicals (OECD TG 408), Repeated dose 90-day oral toxicity study in rodents, adopted 21 September 1998

OECD (1998b). OECD Guideline for the Testing of Chemicals (OECD TG 409), Repeated dose 90-day oral toxicity study in non-rodents, adopted 21 September 1998

OECD (2001a). OECD Guideline for the Testing of Chemicals (OECD TG 414), Prenatal developmental toxicity study, adopted 22 January 2001

OECD (2001b). OECD Guideline for the Testing of Chemicals (OECD TG 416), Two-generation reproduction toxicity testing, adopted 22 January 2001

OECD (2001c). OECD Guideline for the Testing of Chemicals (OECD TG 420), Acute oral toxicity - fixed dose procedure, adopted 17 December 2001

OECD (2001d). OECD Guideline for the Testing of Chemicals (OECD TG 423), Acute oral toxicity – acute toxic class method, adopted 17 December 2001

OECD (2001e). OECD Guideline for the Testing of Chemicals (OECD TG 425), Acute oral toxicity – up-and-down procedure, adopted 17 December 2001

OECD (2002a). OECD Guideline for the Testing of Chemicals (OECD TG 404), Acute dermal irritation/corrosion, adopted 24 April 2002

OECD (2002b). OECD Guideline for the Testing of Chemicals (OECD TG 405), Acute eye irritation/corrosion, adopted 24 April 2002

OECD (2002c). OECD Guideline for the Testing of Chemicals (OECD TG 429), Skin sensitisation – Local Lymph Node Assay, adopted 24 April 2002

OECD (2004a). OECD Guideline for the Testing of Chemicals (OECD TG 427), Skin absorption: in vivo method, adopted 13 April 2004

OECD (2004b). OECD Guideline for the Testing of Chemicals (OECD TG 428), Skin absorption: in vitro method, adopted 13 April 2004

OECD (2004c). OECD Guideline for the Testing of Chemicals (OECD TG 430), In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test (TER), adopted 13 April 2004

OECD (2004d). OECD Guideline for the Testing of Chemicals (OECD TG 431), In Vitro Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method, adopted 13 April 2004

OECD (2004e). OECD Guideline for the Testing of Chemicals (OECD TG 432), In vitro 3T3 NRU Phototoxicity test, adopted 13 April 2004

OECD (2004f). OECD Guideline for the Testing of Chemicals (OECD TG 433), Acute inhalation toxicity - fixed concentration procedure, draft 8 June 2004

OECD (2004g). OECD Guideline for the Testing of Chemicals (OECD TG 434), Acute dermal toxicity - fixed dose procedure, draft 14 May 2004

OECD (2006). OECD Guideline for the Testing of Chemicals (OECD TG 435), In Vitro Membrane Barrier Test Method for Skin Corrosion, adopted 19 July 2006

OECD (2009a). OECD Guideline for the Testing of Chemicals (OECD TG 436), Acute inhalation toxicity – acute toxic class (ATC) method, adopted 7 September 2009

OECD (2009b). OECD Guideline for the Testing of Chemicals (OECD TG 437), Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants, adopted 7 September 2009

OECD (2009c). OECD Guideline for the Testing of Chemicals (OECD TG 438), Isolated Chicken Eye Test Method for Identifying Ocular Corrosives and Severe Irritants, adopted 7 September 2009

OECD (2009d) Working Party on Manufactured Nanomaterials Preliminary, Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials, ENV/CHEM/NANO(2009)6/REV1, Environment Directorate, OECD, Paris.

OECD (2010). Preliminary Guidance Notes on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials, ENV/JM/MONO(2010)25, Environment Directorate, OECD, Paris.

OECD (2010). Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme; First Revision, ENV/JM/MONO(2009)20/REV, Environment Directorate, OECD, Paris.

OECD (2010a). OECD Guideline for the Testing of Chemicals (OECD TG 439), In vitro skin irritation - Reconstructed Human Epidermis Test Method, adopted 22 July 2010

OECD (2010b). OECD Guideline for the Testing of Chemicals (OECD TG 442a), Skin sensitisation – Local Lymph Node Assay: DA, adopted 22 July 2010

OECD (2010c). OECD Guideline for the Testing of Chemicals (OECD TG 442b), Skin sensitisation – Local Lymph Node Assay: BrdU-ELISA, adopted 22 July 2010

OECD (2010d). OECD Guideline for the Testing of Chemicals (OECD TG 487), In vitro mammalian cell micronucleus test, adopted 22 July 2010

OECD (2011a). OECD Guideline for the Testing of Chemicals (OECD TG 443), Extended one-generation reproductive toxicity study, adopted 28 July 2011

OECD (2011b) OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

Park M.V.D.Z., Lankveld D.P.K, Van Loveren H., De Jong W.H. (2009a) The status of in vitro toxicity studies in the risk assessment of nanomaterials. Nanomedicine 4, 669-685, 2009

Park M.V.D.Z., Annema W., Salvati A., Lesniak A., Elsaesser A., Barnes C., McKerr G., Howard C.V., Lynch I., Dawson K.A., Piersma A.H., de Jong W.H. (2009b) In vitro developmental toxicity test detects inhibition of stem cell differentiation by silica nanoparticles, Toxicology and Applied Pharmacology 240: 108-116.

REACH, Specific Advice on Exposure Assessment and Hazard/Risk Characterisation for Nanomaterials under REACH (RIP-oN 3) RNC/RIP-oN3/FPR/1/FINAL, 07 July 2011

REACH, Specific Advice on Fulfilling Information Requirements for Nanomaterials under REACH (RIP-oN 2) RNC/RIP-oN2/FPR/1/FINAL 01 July 2011

Rinderknecht A., Prud'homme R., Poreda R., Gelein R., Corson N., Pidruczny A., Finkelstein J., Oberdörster G., and Elder A. 2008. Biokinetics of AU nanoparticles relative to size surface coating and portal of entry. Presented at the 47th Annual Society of Toxicology Meeting, March 2008, Seattle WA. Toxicologist 102:abstr. 1404.

Rocks S., Pollard S., Dorey R., Levy L., Harrison P. and Handy R. (2008), Comparison of risk assessment approaches for manufactured nanomaterials, Defra, London.

Rogiers V. and Beken S. (Editors and authors) Alternative Methods to Animal experiments. Actual status, development and approach in Belgium, VUB Press, Brussels. ISBN 90-5487-264-0 (2000).

Rogiers V. "Validated" and "valid" alternative methods available today for testing of cosmetic products and their ingredients. Safety Assessment of Cosmetics in the EU. Training Course Vrije Universiteit Brussel, 7-12 April 2003, part 2, p1

Rogiers V. and Pauwels M. Good science must be the key factor in the development and use of alternative methods for safety assessment of cosmetics ALTEX 22,Special Issue, 259, 2005

Sayes C., Ivanov I. (2010) Comparative study of predictive computational models for nanoparticle-induced cytotoxicity, Risk Analysis 30 (11): 1723–1734.

SCCNFP (Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers) (1998) Opinion concerning guidelines on the use of human volunteers in compatibility testing of finished cosmetic products, adopted on 23 June 1999. doc n° SCCNFP/0068/98. http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/sccnfp_opinions_97_04/sccp_out87_en.htm

SCCNFP (Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers) (1999) Opinion concerning basic criteria for the in vitro assessment of

percutaneous absorption of cosmetic ingredients, adopted on 23 June 1999. doc n° SCCNFP/0167/99. http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/sccnfp_opinions_97_04/sccp_out86_en.htm

SCCNFP (Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers) (1999) Opinion concerning basic criteria of the protocols for the skin compatibility testing of potentially cutaneous irritant cosmetic ingredients or mixtures of ingredients on human volunteers, adopted on 8 December 1999, doc n° SCCNFP/0245/99. http://ec.europa.eu/health/archive/ph_risk/committees/sccp/documents/out101_en.pdf

SCCNFP (Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers) (2001) Memorandum on Scientific Evaluations and Opinions, adopted on 12 June 2001. doc n° SCCNFP/0461/01. http://ec.europa.eu/health/scientific_committees/consumer_safety/opinions/sccnfp_opinions_97_04/sccp_out142_en.htm

SCCNFP (Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers) (2002) Updated basic requirements for toxicological dossiers to be evaluated by the SCCNFP. Revision 17 December 2002. doc n° SCCNFP/0633/02. http://ec.europa.eu/health/archive/ph_risk/committees/sccp/documents/out185_en.pdf

SCCP (Scientific Committee on Consumer Products) (2005) Opinion on Triclocarban For other uses than as a preservative. COLIPA n° P29. Adopted on 1 June 2005. doc n° SCCP/0851/04. http://ec.europa.eu/health/archive/ph_risk/committees/04_sccp/docs/sccp_o_016.pdf

SCCP (Scientific Committee on Consumer Products) (2007a) Memorandum on the actual status of alternative methods on the use of experimental animals in the safety assessment of cosmetic ingredients in the European Union, SCCP/1111/07 http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_s_06.pdf

SCCP (Scientific Committee on Consumer Products) (2007b) Safety of nanomaterials in cosmetic products. Adopted on 18 December 2007. doc n° SCCP/1147/07. http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_123.pdf

SCCP (Scientific Committee on Consumer Products) (2007c) Position statement on genotoxicity / mutagenicity testing of cosmetic ingredients without animal experiments. Adopted on 21 January 2009. doc n° SCCP/1212/09. http://ec.europa.eu/health/archive/ph_risk/committees/04_sccp/docs/sccp_s_08.pdf

SCCS (Scientific Committee on Consumer Safety) (2009) Memorandum on "Alternative test methods in human health safety assessment of cosmetic ingredients in the EU", SCCS/1294/10. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_s_001.pdf

SCCS (Scientific Committee on Consumer Safety) (2010a). – Opinion on Basic Criteria for the in vitro assessment of dermal absorption of cosmetic ingredients, SCCS/1358/10, adopted on 22 June 2010. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_s_002.pdf

SCCS (Scientific Committee on Consumer Safety) (2010b). The SCCS's Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation. 7th revision, SCCS/1416/11, adopted on 14 December 2010 http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_s_004.pdf

SCENIHR (Scientific Committee on Emerging and Newly-Identified Health Risks) (2007), Opinion 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, 21-22 June 2007. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_010.pdf

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks) (2009), Risk assessment of products of nanotechnologies, 19 January 2009. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_023.pdf

SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks) (2010), Scientific Basis for the Definition of the Term "nanomaterial", adopted 8 December 2010. http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_032.pdf

Semmler-Behnke M., Fertsch S., Schmid O., Wenk A., Kreyling W.G. (2007): Uptake of 1.4 mm versus 18mm Gold particles by secondary target organs is size dependent in control and pregnants rats aft er intratracheal or intravenous application. Proceedings of Euro Nanoforum - Nanotechnology in Industrial Applications pp102-104 (2007) www.euronanoforum2007.de/download/Proceedings%20ENF2007.pdf

Semmler-Behnke M., Kreyling W.G., Lipka J., Fertsch S., Wenk A., Takenaka S., Schmid G. and Brandau W. (2008). Biodistribution of 1.4- and 18-nm gold particles in rats. Small 4, 2108-11.

Šimon P. and Joner E. (2008), 'Conceivable interactions of biopersistent nanoparticles with food matrix and living systems following from their physicochemical properties', Journal of Food and Nutrition Research, 47, 51–9.

Stone V., Johnston H. and Schins R.P.F. (2009) Development of in vitro systems for nanotoxicology: methodological considerations, Critical Reviews in Toxicology, 39(7): 613–626.

Szalay B., Tátrai E., Nyírő G., Vezér T., Dura G. (2011) Potential toxic effects of iron oxide nanoparticles in in vivo and in vitro experiments, J Appl Toxicol. 2011 Dec 7. doi: 10.1002/jat.1779.

Toropov A.A., Rasulev B.F., Leszczynska D. and Leszczynskiet J. (2008) Multiplicative SMILES-based optimal descriptors: QSPR modeling of fullerene C60 solubility in organic solvents, Chemical Physics Letters 457: 332-33 6.

Toropov, A.A., Leszczynska, D., Leszczynski, J. (2007a) Predicting thermal conductivity of nanomaterials by correlation weighting technological attributes codes, Materials Letters, 61 (26), pp. 4777-4780.

Toropov A.A., Leszczynska D., Leszczynski J. (2007b) Predicting water solubility and octanol water partition coefficient for carbon nanotubes based on the chiral vector, Computational Biology and Chemistry, 31 (2), pp. 127-128.

Toropov A.A., Leszczynski J. (2006) A new approach to the characterization of nanomaterials: Predicting Young's modulus by correlation weighting of nanomaterials codes, Chemical Physics Letters, 433 (1-3), pp. 125-129.

Unfried K., Albrecht C., Klotz .LO., Von Mikecz A., Grether-Beck S., Schins R.P.F. (2007) Cellular responses to nanoparticles: Target structures and mechanisms. Nanotoxicology 1: 52-71.

Wilhelmi V., Fischer U., van Berlo D., Schulze-Osthoff K., Schins R.P.F., Albrecht C. (2012) Evaluation of apoptosis induced by nanoparticles and fine particles in RAW 264.7 macrophages: facts and artefacts. Toxicol In Vitro 26(2):323-334.

Worle-Knirsch J.M., Pulskamp K., Krug H.F. (2006) Oops They Did It Again! Carbon Nanotubes Hoax Scientists in Viability Assays. NanoLetters 6, 1261-1268, 2006

Worth A.P. and Balls M. The importance of the prediction model in the development and validation of alternative tests. ATLA 29,135-143, 2001

Worth A.P. and Balls M. Alternative (Non-animal) methods for chemicals testing:current status and future prospects. A report prepared by ECVAM and the ECVAM Working Group on Chemicals, ATLA 30, Suppl. 1,1-125, 2002

Yanagi M., Hoya M., Mori M. and Katsumura Y. (2001) Modified short-term guinea pig sensitization tests for detecting contact allergens as an alternative to the conventional test, Contact Dermatitis 44(3): 140–145.

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.

REFERENCE:

Adler S., Basketter D., Creton S. et al. (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects – 2010. Archives of Toxicology. 85(5) 367-485.

Averbeck D., Moustacchi E. Genetic effect of 3-Carbethoxypsoralen angelicin, psoralen and 8-methoxypsoralen plus 365 nm irradiation in Saccharomyces cerevisiae: induction of reversions, mitotic crossing-over, gene conversions and cytoplasmic “petite” mutations. Mutation Research 68, 133-148 (1979).

Brendler-Schwaab S., Czich A., Epe B., Gocke E., Kaina B., Müller L., Pollet D., Utesch D. Photochemical genotoxicity: principles and test methods - Report of a GUM task force. Mutation Research 566(1), 65-91 (2004).

Chetelat A., Albertini S., Dresp I.H., Strobel R., Gocke E. Photomutagenesis test development. Part I. 8-Methoxypsoralen, chloropromazine and sunscreen compounds in bacterial and yeast assays. Mutation Research 292, 241-250 (1993a).

Chetelat A., Dresp J.H., Gocke E. Photomutagenesis test development. Part II. 8-Methoxypsoralen, chlorpromazine and sunscreen compounds in chromosomal aberration assays using CHO cells. Mutation Research 292, 251-258 (1993b).

Chetelat A., Albertini S., Gocke E. The Photomutagenicity of fluoroquinolones in tests for gene mutation, chromosomal aberration, gene conversion and DNA breakage (Comet assay). Mutagenesis 11(5), 497-504 (1996).

Dean S.W., Lane M., Dunmore R.H., Ruddock S.P., Martin C.N., Kirkland D.J., Loprieno N. Development of assays for the detection of photogenotoxicity of hemicals during exposure to UV light. Part 1. Assay development. Mutagenesis 6, 335-341 (1991).

DG SANCO, SANCO/222/2000: Guidance Document on Dermal Absorption. European Commission, Health and Consumer Protection Directorate General, Doc.Sanco222/2000 revision 7 of 19 March 2004.

ECB Technical Guidance Document on Risk Assessment in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation N° 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Doc.EUR 20418 EN/1, European Communities 2003

ESAC Statement on the application of the Corrositex™ assay for skin corrosivity testing. Adopted by the ECVAM Scientific Advisory Committee (ESAC) at its 15th Meeting of 5-6 December 2000 at ECVAM, Ispra, Italy (2000).

ESAC Statement on the scientific validity of the Embryonic Stem Cell Test (EST), the Micromass Test and the Postimplantation Rat Whole-Embryo Culture Assay - in vitro tests for embryotoxicity. Adopted by ESAC at its 17th Meeting of 16-17 October 2001,at ECVAM, Ispra, Italy, 2001

Farmer P. Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment ILSI/HESI Research Programme on Alternative Cancer Models: Results of Syrian Hamster Embryo Cell Transformation Assay, toxicologic pathology, vol 30, no 4, pp 536–538, 2002

Gocke E., Albertini S., Chetelat A., Kirchner S., Muster W. The photomutagenicity of fluoroquinolones and other dyes. Toxicology Letters 102/103, 375-381 (1998) Howes D.,Guy R.,Hadgraft. Et al. Methods for assessing percutaneous absorption, ECVAM Workshop Report N°13, ATLA 24,81-106,1996

Kersten B., Kasper P., Brendler-Schwaab S.Y., Muller L. Use of the photo-micronucleus assay in Chinese hamster V79 cells to study photochemical genotoxicity. Mutation Research 519, 49-66 (2002).

Lee, S., Yun, H.S., Kim, S.H. (2011) The comparative effects of mesoporous silica nanoparticles and colloidal silica on inflammation and apoptosis, Biomaterials 32(35): 9434-9443.

OECD Environmental Health and Safety Publications, Series on Testing and Assessment N° 28, Paris, 5 March 2004

Pflaum M., Kielbassa C., Garmyn M., Epe B.G. Oxidative DNA damage induced by visible light in mammalian cells; extent, inhibition by antioxidants and genotoxic effects. Mutation Research 408, 137-144 (1998).

1 Spielmann H., Balls M.,Dupuis J., Pape W.J.W., De Silva O.,Holzhutter H.G., Gerberick F., Liebsch M., Lowell W.W., and Pfannenbecker V. A study on UV filter
2 chemicals from Annex VII of the European Union Directive 76/768/EEC in the in vitro NRU Phototoxicity test; ATLA 26, 679-708, 1998