Scientific Committee on Consumer Products

SCCP

OPINION ON

SAFETY OF NANOMATERIALS IN COSMETIC PRODUCTS

Adopted by the SCCP after the public consultation
on the 14th plenary of 18 December 2007

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the sound 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 Products (SCCP), 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 Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCP

Questions concerning the safety of consumer products (non-food products intended for the consumer). In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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ABSTRACT

A nanoparticle is a particle with one or more dimensions at the nanoscale (at least one dimension <100nm). A nanomaterial is a material with one or more external dimensions, or an internal structure, on the nanoscale, which could exhibit novel characteristics compared to the same material without nanoscale features. Nanoparticles can be divided into two groups: i) soluble and/or biodegradable nanoparticles which disintegrate upon application to skin into their molecular components (e.g. liposomes, microemulsions, nanoemulsions), and ii) insoluble particles (e.g. TiO_2, fullerenes, quantum dots).

For the soluble and/or biodegradable group, conventional risk assessment methodologies based on mass metrics *may* be adequate, whereas for the insoluble particles other metrics, such as the number of particles, and their surface area as well as their distribution are also required.

It is crucial when assessing possible risks associated with nanoparticles to consider their uptake. It is primarily for the insoluble particles that health concerns related to possible uptake may arise. Should they become systemically available, translocation/ transportation and eventual accumulation in secondary target organs may occur.

At present, there is inadequate information on: i) hazard identification, ii) exposure assessment, iii) uptake (including physiologically normal and compromised human skin), iv) the role of physico-chemical parameters of nanoparticles determining absorption and transport across membranes in the gut and lungs, v) the role of physico-chemical parameters of nanoparticles in systemic circulation determining biokinetics and accumulation in secondary target organs, vi) possible health effects (including susceptible individuals), vii) translocation of nanoparticles via the placenta to the foetus.

For the safety assessment of cosmetics, the 7th Amendment imposes animal testing and marketing bans, which prohibit *in vivo* testing of finished cosmetics now and their ingredients in the near future. Only validated *in vitro* methods may be used but at present no methodology has been validated for nanomaterials.

Review of the safety of the insoluble nanomaterials presently used in sunscreens is required.

Keywords: SCCP, opinion, nanomaterials, skin, nanoparticle, cosmetics, safety, sunscreen, toxicology, alternative methods, skin penetration

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EXECUTIVE SUMMARY

In response to the growing importance of nanotechnology, the Royal Society and the Royal Academy of Engineering issued a report on nanoscience and nanotechnologies in which one of the key suggestions was that nanomaterials should be treated as new chemicals from a risk-point of view and evaluation of skin absorption should be considered in both normal and abnormal (diseased) skins. Additionally, the increased surface area of nanomaterials may lead to greater toxicity per unit mass; assessing exposure on a mass basis may not be appropriate for nanomaterials. The report also queried whether suitable non-animal models will be available for testing nanomaterials.

In view of the above, the SCCP was requested to address the safety evaluation of nanomaterials for use in cosmetic products and to consider the implications on animal testing and whether the previous opinions on nanomaterials currently used in sunscreen products would need to be revised.

A nanoparticle is a particle with one or more dimensions at the nanoscale and is defined as a particle with at least one dimension <100nm. A nanomaterial is a material with one or more external dimensions, or an internal structure, on the nanoscale, which could exhibit novel characteristics compared to the same material without nanoscale features. Two principal factors cause the properties of nanomaterials to differ significantly from bulk materials: increased relative surface area, and quantum effects.

Nanoparticles can be divided into two groups: i) soluble and/or biodegradable nanoparticles which disintegrate upon application to skin into their molecular components (e.g. liposomes, microemulsions, nanoemulsions), and ii) insoluble and/or biopersistent particles (e.g. TiO_2, fullerenes, quantum dots).

For the first group, conventional risk assessment methodologies based on mass metrics *may* be adequate, whereas for the insoluble particles other metrics, such as the number of particles, and their surface area as well as their distribution are also required. It is crucial when assessing possible risks associated with nanoparticles to consider their uptake. For topical applications, the route of exposure is essentially transdermal including follicular and other transadnexal pathways; exposure via inhalation, ingestion, conjunctival and mucosal surfaces may sometimes be relevant.

It is primarily for the insoluble particles that health concerns related to possible uptake arise. Should they become systemically available, translocation/ transportation and eventual accumulation in secondary target organs may occur. This could become important with repeated application of cosmetic products. Inevitably, insoluble nanoparticles do represent a burden for the environment and a complete life cycle analysis is required.

At present, there is concern about insufficient information in the following areas:

- Hazard identification
- Exposure assessment
- Uptake (including physiologically compromised human skin)
- The role of physico-chemical parameters of nanoparticles determining absorption and transport across membranes in the gut and lungs
- The role of physico-chemical parameters of nanoparticles in systemic circulation determining biokinetics and accumulation in secondary target organs
- Possible health effects (including susceptible individuals)
- Translocation of nanoparticles via the placenta to the foetus.

In traditional risk assessment, skin penetration studies are carried out using healthy and/or intact skin. Possible enhanced uptake in case of impaired skin is considered to be covered in

the Margin of Safety (MoS). However, in the case of nanomaterials the conventional MoS may not give an adequate expression of the safety. If there is systemic absorption to vital tissues it may lead to rapid clearance from skin to systemic circulation. It may be anticipated that any systemic absorption is more likely to occur in conditions of abnormal skin e.g. sunburnt, atopic, eczematous, psoriatic skin. There is evidence that physical, in particular mechanical and/or chemical action on the skin may have an effect on nanoparticles penetration.

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, the standard model may not be ideal. Therefore, new methodologies to assess percutaneous penetration pathways are urgently needed.

There are large data gaps in the risk assessment methodologies regarding data on nanoparticles in cosmetic products via inhalation and ingestion.

The biodistribution (toxicokinetics) of nanomaterials has not been studied in detail.

Although the basic requirements of testing the mutagenicity/genotoxicity of nanoparticles are similar to those of other particulate materials, the specific characteristics of nanoparticles may require further considerations. The present validated *in vivo* genotoxicity tests, however, do not cover the expected target organs of nanoparticles (particularly the respiratory tract) and have not been validated with reference substances including nanomaterials of relevance for cosmetics.

All *in vivo* and *in vitro* risk assessment methods for nanomaterials are still in the research phase. Although some validated *in vitro* methods do exist they have never been validated with nanomaterials as reference compounds.

Although animal testing can be largely reduced for skin penetration studies, they are essential for translocation and accumulation studies as well as for chronic toxicity studies.

The SCCP emphasizes that for the safety assessment of cosmetics, the 7^{th} Amendment imposes animal testing and marketing bans, which prohibits *in vivo* testing of cosmetics now and their ingredients in the near future. Only validated *in vitro* methods may be used but at present none of the methodologies mentioned above have been validated for nanomaterials.

For the nanomaterials presently used in sunscreen products, a safety dossier on nanosized ZnO was requested by SCCNFP in its opinion on ZnO in 2003 (SCCNFP/0649/03). An opinion on the safety of such material will be dependent on an adequate dossier. Since the SCCNFP opinion on TiO_2 (SCCNFP/0005/98), much new scientific data on nanosized particles, including TiO_2, have emerged. Therefore, the SCCP considers it necessary to review the safety of nanosized TiO_2 in the light of recent information and to consider the influence of physiologically abnormal skin and the possible impact of mechanical action on skin penetration.

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1. BACKGROUND

In response to the growing importance of nanotechnology, the Royal Society and the Royal Academy of Engineering have issued a report on nanoscience and nanotechnologies ("the report"). One of the key findings in the report is that nanoparticles should be treated as new chemicals from a risk-point of view (cf., pp. 43, 73, and 83 of the report).

In particular the issue of skin absorption leading to a higher resorption of nanomaterials may have to be addressed explicitly in order to assess their risk. Particular regard should be held to the state of the skin, which may be injured, sunburnt or damaged by diseases, and the size of the particles (cf., p. 44 of the report).

Secondly, issues of exposure might have to be re-assessed as far as nanomaterials are concerned. The increased surface area of nanomaterials can lead to greater toxicity per unit mass. Thus, assessing exposure on a mass basis may not be appropriate for nanomaterials (cf., p. 82 of the report).

The report asked also – albeit in an auxiliary manner- the question whether suitable non-animal models will be available for testing nanoparticles (cf. p. 44, 73 of the report).

The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) was requested to deliver an opinion on "the appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies" (SCENIHR 2006).

These concerns raised the need to consider whether a specific chapter on the safety evaluation procedures for nanomaterials should be added to the SCCNFP's Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation (SCCP/1005/06).

The SCCNFP delivered an opinion on titanium dioxide in 2000 and on zinc oxide in 2003 for their use as cosmetic ingredients. In the light of potential new findings on the safety of nanoparticles in general, it may be necessary to review these opinions.

In order to obtain all the most relevant safety information, two calls for information on nanocosmetics were launched in August 2005 and November 2006. The contributions received from both research and industry have been used for the compilation of the opinion.

2. TERMS OF REFERENCE

The SCCP is requested:

1. *In view of the concerns recently raised about the use of nanomaterials in cosmetics the SCCP is requested to review and, if appropriate, to amend its notes of guidance for the testing of cosmetic ingredients and their safety evaluation as concern cosmetic ingredients in the form of nanomaterials, including nanoparticles and nanoliposomes, and in particular as regards skin absorption and resorption of these substances. In assessing this, regard should be made to differing skin conditions, different sizes of particles and to question whether mass unit is the appropriate basis for regulating the exposure to nanomaterials. Possible implications on animal testing of nanoparticles and nanoliposomes should be addressed.*

2. *In the light of the findings under (1), does the SCCP consider it is necessary to review existing opinions on nanosized TiO_2 and ZnO as cosmetic ingredients and if appropriate to identify which additional elements are required for the submission of a*

safety file?

3. SCIENTIFIC RATIONALE

Nanomaterial is the term given to the materials having dimensions in the nanometer scale. They can be utilised in numerous applications because of some of their novel physical, chemical and biological characteristics. New materials having different properties than the classical materials, at their original scale, are opening new fields in technology including cosmetic products and their ingredients. In the cosmetic domain, nanomaterials can be manufactured for their own properties (TiO_2, ZnO), they can also be developed for their capacity to carry specific molecules at a defined level of the skin (liposomes, nanoparticles).

3.1. Glossary

The opinion published, 10 March 2006, by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) entitled: "The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies", used the terms of nanotechnology according to a report of the British Standards Institution (2005) "Publicly Available Specification on the Vocabulary for Nanoparticles". In this report, the definitions below for the major general terms were proposed and these definitions are used in the present opinion in the absence of internationally agreed definitions. The BSI definitions are indicative of descriptions of nanomaterials without being exhaustive. There is a need for harmonised definitions of nanomaterials for risk assessment.

Nanotechnology: *the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale.*
Nanotechnology is a sub-classification of technology in colloidal science, biology, physics, chemistry involving the study of phenomena and manipulation of material at the nanoscale level. Two main approaches are used in nanotechnology: one is a "bottom-up" approach where materials and devices are built up atom by atom, the other a "top-down" approach where they are synthesized or constructed by removing existing material from larger entities. Nanotechnology is also used as an umbrella term to describe emerging or novel technological developments associated with nanoscale dimensions.

Nanoscale: *having one or more dimensions of the order of 100 nm or less.*
Nanotechnology components are ranging in size between 1 nanometer (nm) and 100 nanometers.

Nanoparticle: *particle with one or more dimensions at the nanoscale*.
A nanoparticle is defined as a particle with at least one dimension <100nm. Solid, semi-solid and soft nanoparticles have been manufactured. A prototype nanoparticle of semi-solid nature used for cosmetic formulation is the liposome. Suspensions of nanoparticles are possible because the interaction of the particle surface with the solvent is strong enough to overcome differences in density. Nanoparticles often have unexpected visible properties because they are small enough to scatter visible light rather than absorb it.

Nanomaterial: *material with one or more external dimensions, or an internal structure, on the nanoscale, which could exhibit novel characteristics compared to the same material without nanoscale features.*
As a particle decreases in size, a greater proportion of atoms are found at the surface compared to those inside. Thus nanomaterials have a much greater surface area per unit mass compared with larger particles so, they have novel characteristics that might include increased strength, chemical reactivity, conductivity or electrical characteristics. Porous materials such as e.g. zeolites have large internal surfaces and play a role in many applications, e.g. in catalysis but as their physical outer dimension usually considerably exceeds 100 nm they usually are not called nanomaterials.

Percutaneous/dermal absorption: 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.

The glossary of other relevant terms is provided in Annex I.

3.2. Importance of surface area

Bulk materials may show novel properties when they become nanosized and there is increasing exploitation of these novel properties. Two principal factors cause the properties of nanomaterials to differ significantly from bulk materials: increased relative surface area, and quantum effects.

With constant mass, a decreased particle size results in increased total surface area. (Nel et al. 2006, Oberdörster et al. 2005a and 2005b, Hoet et al. 2004, Kreyling 2006a) (see table 1). The resultant larger surface area causes surface chemistry to become increasingly important, hence smaller particles may exhibit greater biological activity per given mass compared with larger particles. In other words, the vast amount of a reactive molecule species located only on the surface of insoluble particles and of particle cores (remaining after dissolution of the soluble components) may be the ultimate metric determining adverse outcomes, although this molecule may only add a small fraction to particle mass.

Previously, the most common metric used was 'particle mass concentration'. With closer insights into particle-lung interactions, other measures such as the concentration of particle numbers and surface area need to be taken into account. However, exposure metrics may be inadequate, since it may be that the number of deposited particles per unit surface area of airways, bifurcations (crest at airway division) and alveoli, or dose to a specific cell such as the macrophage, determine responses for specific regions. Therefore, the use of a metric depends on the specific questions posed, requiring specifically defined measures.

Table 1: Particle size and Surface Area

Particle diameter	**Particle Number**	**Total Particle Surface Area**
nm	**N per g**	**cm^2/g**
1000	$1.9*10^{12}$	$6*10^{4}$
100	$1.9*10^{15}$	$6*10^{5}$
10	$1.9*10^{18}$	$6*10^{6}$

Although a correlation between increasing surface area and biological effects is shown in many cases, there are also research reports in which this relationship between size, surface area and toxicity is not straightforward or even in reverse. Therefore, it is not always possible to predict effects on the basis of size or surface area alone (Warheit et al. 2006, Yin et al. 2005). In addition, modifications of the surface may change the interactions of nanoparticles with biological systems affecting their biocompatibility and toxicity (Warheit et al 2007a and 2007b, Sayes et al. 2007b). Strictly speaking, the surface area does not cause biological effects itself but the number of reactive sites does (e.g. surface atoms and molecules and their physical structures being catalytically and chemically reactive centers on the surface). When surface per mass increases with decreasing nanoparticle size, the number of reactive sites will increase. This in turn will cause an increased biological response. Consequently, with a better understanding of the nature of the reactive sites there will be possibilities to modify those sites and thereby reduce the biological responses to nanoparticles.

The size range of nanoparticles enables them to interact with cells, subcellular structures and macromolecules including proteins, and some materials are even designed to do so (Sakamoto et al. 2005, Lohbach et al. 2006). Some of these interactions may have deleterious effects (Hoet et al. 2001, Yin et al. 2005, Lynch et al. 2006, Barrett et al. 1999).

Nanoparticles may agglomerate and/or aggregate resulting in larger particles. The surface area of an aggregate and/or agglomerate generally depends on the object in contact, i.e. there are "outer" and "inner" surfaces, as illustrated by the following two examples.

1) Case of "outer surface":
   Larger particles with dimensions in the range of 20-50 µm can be recognised more readily by e.g. alveolar macrophages and, therefore, stimulate these cells to phagocytose the agglomerated particles, while isolated nanosized materials will not activate alveolar macrophages to phagocytosis, but rather inhibit phagocytosis (Renwick et al. 2001).

2) Case of "inner and outer surface":
   As biological fluids can enter into pores as small as 1-2 nm it is likely that the entire surface area of nanoparticles is of relevance. Very early studies on the intracellular dissolution of porous metal oxide particles in alveolar macrophages showed that the entire surface area, including the inner surface area, needs to be considered (Kreyling et al. 1990). In addition, the determination of the surface area of aggregated or agglomerated powders using nitrogen absorption covering the entire surface area with nitrogen molecules including nanometer-sized pores is relevant. Several studies have shown a clear relation between the total surface area and biological effects (Kreyling et al. 2006a, Hoet et al. 2004), e.g. via oxidative mechanisms (Brown et al. 2001).

3.3. Functions and uses

3.3.1. Cosmetic formulations and their ingredients as nanomaterials

Ordinary cosmetic emulsions have droplet sizes between 100 and 100,000 nm. Nanoemulsions contain the same type of ingredients as the former but their droplet dimensions may be as low as 10 nm. Given this small droplet size, nanoemulsions are transparent and have particular rheological properties that so far have not been obtained by other formulation methods. Because of these properties, nanoemulsions are used in a number of cosmetics. Generally, when applied to skin or hair, nanoemulsions, are not stable and break down into their constituent ingredients.

To manufacture nanospheres, nanocapsules, oleosomes and liposomes, containing active ingredients such as vitamins or anti-oxidants, the same ingredients as found in common cosmetic formulations can be used. The production techniques involved, such as coacervation or phase separation, are important because they may improve the stability of the cosmetic formulation and the 'active ingredients' involved.

3.3.2. Mineral-based cosmetic ingredients with nanosized dimensions

Some cosmetic products use mineral-based materials and their performance depends on particle size. In sunscreen products, mineral nanoparticles (e.g. titanium dioxide and zinc oxide with particle size in the order of 20 nm) are efficient UV-filters. They transmit, reflect and scatter the visible part of the solar radiation while they strongly absorb in the UV region. These mineral UV- filters consist of micron-sized aggregates, which are composed of nanosized primary particles. The surface of these nanoparticles may be treated with an inert coating to improve their dispersion in sunscreen formulations and to prevent photocatalytic activity (SCCNFP/0005/98). The advantage of mineral UV -filters is that they provide broad UV-protection and usually do not cause cutaneous adverse health effects such as contact allergies.

Coatings may contain Al_2O_3 and SiO_2 but also organic materials such as cyclomethicone, dimethicone, stearic acid and hydrophobic silanized versions of, for example, ZnO also exist.

3.3.3. Other nanosized materials as cosmetic ingredients

It is unclear which other types of nanomaterials are currently present in cosmetic products. A report by Friends of the Earth (http://www.foe.org/camps/comm/nanotech/) and information on the web (http://ewg.org/issues/cosmetics/20061010/table2.php) provide an extensive list of cosmetic products claiming to contain nanomaterials. Manufacturers claim that their products contain registered (™) descriptions of materials but no indication is given of their physico-chemical identity. Fullerenes (C_{60}) are reported to be used in a number of cosmetic products including face creams. It is unknown as to what extent quantum dots, nanotubes or other nanomaterials have found use in marketed cosmetic products, but patents for such products exist[2] and products containing such nanomaterials may appear in the market.

Hitherto, in response to the Calls for Information by the SCCP in 2005 and 2006, only interest in mineral based nanomaterials has been expressed.

Table 2: Examples of nanocosmetic products in the market
Source: Comments to U.S. Food and Drug Administration
Docket: FDA Regulated Products Containing Nanotechnology Materials Docket number: 2006N-0107

After sun products VITAMIN NANOCAPSULES
Anti aging FULLERENES Firming Anti-Oxidant Serum FULLERENES Aging Skin Resuscitating Serum MICRONIZED GLUCONOLACTATE Anti Aging Finishing Powder MICRONIZED INGREDIENTS Vitamin A and C Serum MICRONIZED LIPOSOMES Serum MICRONIZED ZINC OXIDE, MICRONIZED TITANIUM DIOXIDE NANOENCAPSULATED INGREDIENTS RETINOL NANOCAPSULES VITAMIN NANOSOMES OF SODIUM LACTATE, NANOSOMES OF CALENDULA, NANOSOMES OF WITCH HAZEL, NANOSOMES OF GINSENG, NANOSOMES OF UREA, NANOSOMES OF VITAMIN A AND E, NANOSOMES OF PRO-VITAMIN B5, NANOSOMES OF ALPHA-BISABOLOL AND GERMAL II NANOSOMES OF VITAMIN A
Anti-itch / rash cream MICRONIZED ZINC OXIDE NANOENCAPSULATED INGREDIENTS
Around-eye cream FULLERENES LYPHAZOME NANOSPHERES MICROSOME Eye Gel MICRONIZED LIPOSOMES
Blush MICRONIZED INGREDIENTS MICRONIZED POWDER BRUSHES MICRONIZED TITANIUM DIOXIDE (COATED or not WITH DIMETHICONE) MICRONIZED ZINC OXIDE
Body firming lotion NANO DELIVERY SYSTEM Reduction Anti-Cellulite NANOSOMES OF CENTELLA ASIATICA

2 http://v3.espacenet.com/results?EC=Y01NandAB=cosmeticsandsf=aandDB=EPODOCandPGS=10andCY=epandLG=enandST=advanced

Body wash /cleanser NANOSOMES OF VITAMIN A
Bronzer/highlighter MICRONIZED ITALIAN TALC POWDER MICRONIZED ROSE QUARTZ POWDER, MICRONIZED TOPAZ POWDER MICRONIZED ZINC OXIDE NANO-VITAMINS
Camouflage makeup MICRONIZED GLUCONOLACTATE
Concealed MICRONIZED POWDER MICRONIZED TITANIUM DIOXIDE, MICRONIZED ZINC OXIDE NANOSPHERES OF HYALURONIC ACID AND FULVIC ACID
Conditioner MICRONIZED TITANIUM DIOXIDE
Diaper cream MICRONIZED ZINC OXIDE
Exfoliant/scrub MICRONIZED PEARL
Eye liner MICRONIZED TITANIUM DIOXIDE
Eye shadow MICRONIZED TITANIUM DIOXIDE (COATED or not WITH DIMETHICONE) MICRONIZED ZINC OXIDE
Facial cleanser MICRONIZED SPHERICAL DIAMOND DUST NANOTECHNOLOGY INGREDIENTS
Facial moisturizer/treatment MICRONIZED CORNFLOUR MICRONIZED LIPOSOMES MICRONIZED PEARLIZERS MICRONIZED TITANIUM DIOXIDE MICRONIZED TOPAZ POWDER, MICRONIZED ROSE QUARTZ POWDER NANO-PARTICLE DELIVERY SYSTEM
Foundation MICRONIZED MINERALS MICRONIZED TITANIUM DIOXIDE (COATED or not WITH DIMETHICONE) MICRONIZED ZINC OXIDE
Glitter MICRONIZED POWDER
Hair-loss treatment NANOSOMES
Lip balm/treatment NANO ZINC OXIDE
Lip gloss MICRONIZED TOPAZ POWDER, MICRONIZED ROSE QUARTZ POWDER
Lip liner MICRONIZED TITANIUM DIOXIDE
Lipstick MICRONIZED TOPAZ POWDER, MICRONIZED ROSE QUARTZ
Mask MICRONIZED QUARTZ SILICA
Moisturizer LYPHAZOME NANOSPHERES
Nail treatment LYPHAZOME NANOSPHERES MICRONIZED POLYCARBONATED RESIN
Powder MICRONIZED FORMULA MICRONIZED GLUCONOLACTATE MICRONIZED PIGMENTS MICRONIZED TITANIUM DIOXIDE, MICRONIZED ZINC OXIDE
Skin fading/lightener MICRONIZED TITANIUM DIOXIDE NANO LOTION NANO-RETINYL
Styling gel/lotion MICRONIZED INGREDIENTS Skin Lightening Gel

Sunscreen/tanning oil
MICRONIZED INGREDIENTS FORMULA MICRONIZED PIGMENTS
NANOPARTICLES
MICRONIZED PARTICLES
NANOTECHNOLOGY INGREDIENTS
VITAMIN NANOCAPSULES
MICRONIZED TITANIUM DIOXIDE
MICRONIZED ZINC OXIDE

3.4. Formulation effects

It has not been shown that nanovesicles and/or nanoemulsions can penetrate intact skin. These nanosized structures generally disintegrate into their molecular components and loose their typical characteristics related to nanosize. However, they can modify the bioavailability and toxicological behaviour of dispersed active ingredients. This should be taken into account in the safety assessment of nanomaterials.

3.4.1. Nanovesicles

Vesicular systems, including liposomes, have been incorporated into many cosmetic formulations. Liposomes are colloidal particles, typically consisting of phospholipids and cholesterol, with other possible ingredients. The lipid molecules form bilayers surrounding an aqueous core. Both the bilayer and the core can be used to entrap and present ingredients to the skin. Most studies describe a non-specific targeting effect whereby vesicles allow accumulation of ingredients in the stratum corneum or other upper skin layers. A number of studies have reported enhanced delivery of a variety of pharmaceutical substances, including triamcinolone, methotrexate, hydrocortisone, tretinoin, tacrolimus, rhodamine, ciclosporin and antiandrogens into and through the skin using liposomal formulations (Mezei and Gulasekharam 1982, Patel 1985, Wohlrab and Lasch 1987, 1989, Wohlrab et al. 1989, Lasch and Wohlrab 1986, Kim et al. 1997, Masini et al. 1993, Kitagawa and Kasamaki 2006, Erdogan et al. 2002, Scheinfeld 2004). Reports on cosmetic 'actives', however, are less available.

Despite these observations, other groups have shown that there is no change in the delivery of ingredients compared to classical preparations (Ganesan et al. 1984, Ho et al. 1985, Komatsu et al. 1986a, 1986b). Nevertheless, transdermal delivery using liposomes as carriers has been illustrated in a few cases where the entrapped drug was able to cross all skin layers (Kato et al. 1987, Nishihata et al. 1987, Jacobs et al. 1988, Kimura et al. 1989).

A number of studies have demonstrated that the vesicle composition (e.g. inclusion of skin lipids (Fresta et al. 1996), positively charged lipids (Kitagawa and Kasamaki 2006), presence of surfactants in the bilayer (Hofland et al. 1994, Cevc et al. 2002) may have an effect on substance permeation. The state of the lipid bilayers of the vesicles, namely the liquid crystal phase or gel phase, also affects dermal and transdermal delivery: liquid crystal state vesicles may be more effective (Ogiso et al. 1996). Such results have been confirmed *in vivo* (Ogiso et al. 1996, Perez-Cullell et al. 2000). Other physico-chemical properties, such as particle charge (Kitagawa and Kasamaki 2006), particle size (du Plessis et al. 1994) and lamellarity (Fresta et al. 1996), might also influence the degree of substance transport.

Vesicles other than liposomes have been devised with the aim of improving transdermal and topical delivery of substances. Examples of these include vesicles made of non-ionic surfactants (niosomes™) (Schreier et al. 1994 , Waranuch et al. 1998, Manconi et al. 2006) vesicles containing a high percentage of ethanol (ethosomes) (Touitou et al. 2000a and 2000b) and ultraflexible vesicles (transfersomes) (Planas et al. 1992, Paul et al. 1995, Cevc 1996, Cevc et al. 1998, Cevc 2003), etc.

3.4.2. Nanoemulsions and microemulsions

Nanoemulsions consist of very fine emulsions, with a droplet diameter smaller than 100 nm. Unlike microemulsions, nanoemulsions are metastable systems, whose structure depends on the process used to prepare them. (Sonneville-Aubrun et al. 2004). Nanoemulsions are used to improve solubility and to protect ingredients from degradation, hydrolysis and oxidation. A few studies have looked at the dermal effect of applying nanoemulsions to the skin. A recent study of a nanoemulsion containing the drug paclitaxel, has shown that the active ingredient became localised in the deeper skin layers, but with minimal systemic uptake (Khandavilli et al. 2007).

Microemulsion formulations have also been shown to be superior for both transdermal and dermal delivery of mostly lipophilic compounds compared to emulsions and liposomes (Kreilgaard 2001 and 2002). In a human experiment, using a pharmacodynamic assessment method (skin blanching), cutaneous delivery of hydrocortisone in either water (w)- or an oil (o)-continuous microemulsion have been compared to an amphiphilic cream (Lehmann et al. 2001). Both microemulsion formulations appeared to increase hydrocortisone penetration into the skin to a greater extent than the cream. The pharmacokinetics of lidocaine were evaluated in eight subjects with the microdialysis technique. A substantial increase in absorption of lidocaine was found, when applied in the microemulsion vehicle compared to the o/w emulsion. The pharmacokinetic study demonstrated a four times larger total amount of lidocaine absorbed into the skin from the microemulsion relative to the o/w emulsion during a 4-h application period. The improved absorption was primarily shown to be attributable to a 3-fold increase in penetration rate into the dermis, but also the mean lag time was significantly reduced from 110 to 87 min (Kreilgaard et al. 2001 and 2002). In a recent article, skin penetration of a tetracaine formulated in a series of micro- (droplet size: 1.0, 1.8 or 3.5 µm) and nanoemulsions (droplet size: 18, 68 or 88 nm) was compared. The results showed no increase in skin absorption of the drug, including when surfactant concentrations were kept constant, indicating that there is no effect of emulsion droplet size on the skin penetration (Izquierdo et al. 2007). Enhancement of skin penetration by emulsions might be a complex phenomenon that depends on their composition. Upon disintegration at the surface of the skin some surfactants used in the formulation might penetrate into the skin and change barrier properties (Kogan and Garti 2006).

3.4.3. Nanocapsules and nanospheres

Nanocapsules are submicronic particles that are made of a polymeric capsule surrounding an aqueous or oily core, whereas nanospheres are matrix systems where the matrix is made of solid polymer or solid lipids (called solid lipid nanoparticles).

Experiments using nanocapsules were undertaken with systems made of poly(alkylcyano-acrylate) or poly-ε(caprolactone). Permeation of indomethacin entrapped in poly(butyl-cyanoacrylate) was determined by measuring drug plasma levels in rats after topical administration. Higher drug plasma levels over 6 hours were obtained with indomethacin poly(isobutylcyanoacrylate) nanocapsules (Miyazaki et al. 2003). When poly-ε(caprolactone) nanocapsules were used to modify the transport of chlorhexidine, it was shown in an *ex vivo* model that the drug encapsulation reduced the percutaneous absorption through stripped skin. The slow delivery of the drug might be responsible for reducing the substance level in the skin (Lboutounne et al. 2002 and 2004).

The transport of the UV-filter octyl methoxycinnamate by nanocapsules has been compared to other carriers. The use of nanocapsules decreased the penetration of octyl methoxycinnamate in pig skin when compared with conventional emulsions (Jimenez et al. 2004) or with nanoemulsions (Olvera-Martinez et al. 2005, Calderilla-Fajardo et al. 2006). However, encapsulation of octyl methoxycinnamate increased its delivery to the stratum corneum illustrating the role of the carrier in the delivery profiles (Alvarez-Roman et al.

2004a).

A series of independent studies have concluded that the vesicles themselves used by the cosmetic industry do not penetrate beyond the most superficial layers of the stratum corneum, but break down (Ganesan et al. 1984, Schreier et al. 1994, Van den Bergh et al. 1999, Alvarez-Roman et al. 2004b, Honeywell-Nguyen et al. 2005).

3.4.4. Summary

It should be emphasized that the results described above and obtained with nanosized delivery systems are not consistent. It is difficult to establish how the delivery systems behave individually once applied on the skin. It is, however, possible to consider a list of potential properties:

(a) Nanomaterial constituents (such as lipids or surfactants) may act as penetration enhancers by penetrating individually into the stratum corneum (after particle disintegration on skin surface) and subsequently altering the intercellular lipid lamellae within this skin layer;

(b) Nanomaterials may serve as a depot for sustained release of dermally active compounds;

(c) Nanomaterials may serve as a rate-limiting membrane barrier for the modulation of systemic absorption, hence providing a controlled transdermal delivery system;

(d) Although validated *in vitro* methodologies exist, their applicability for nanomaterials has not been demonstrated

3.5. Methods for characterisation and effects assessment of nanomaterials

3.5.1. Introduction

The opinions of the Scientific Committee on Emerging and Newly Identified Risks (SCENIHR) emphasize the need for adequate characterisation of nanomaterials (SCENIHR 2006, SCENIHR 2007). It is considered imperative that samples of particulates are representative of the substance and that the particle size and shape characteristics are measured in the most relevant dispersal state and mimicking conditions of potential human and environmental exposure. Mass is probably not the most relevant measure for nanoparticles. Other parameters may be of importance, including size and surface area. The methods for characterisation are discussed in these opinions.

A variety of techniques have been used to study and quantify skin penetration of chemicals (Sekkat and Guy, 2001). However, the use of topical formulations containing nanomaterials has raised concerns about the applicability of traditional assessment methods.

Hazard identification studies are carried out *in vivo* and are indispensable for information on translocation, biodistribution, accumulation and clearance of nanomaterials. According to the 7th Amendment of Cosmetic Directive, (2003/15/EC), the use of animals for safety testing of cosmetic ingredients, including nanomaterials will not be permitted after 2009 for acute and local toxicity testing and after 2013 for repeated dose toxicity and developmental toxicity and toxicokinetics. Efforts are required to provide validated *in vitro* methods, including optimisation for nanomaterials. The development of surrogates for human skin e.g. Episkin™, Epiderm™, Skinethic™ and other examples of reconstructed human skin could provide tools for safety assessment of cosmetic ingredients and finished products provided that the validation is carried out using nanomaterials of relevance to cosmetics. As mentioned under 3.5.4.2.(i), *in vivo* dermal absorption for nanomaterials forms a basic test in safety assessment (OECD 427), but needs to be replaced by the *in vitro* test (OECD 428) in the case of cosmetic ingredients.

The specific characteristic of nanomaterials will necessitate new test strategies to determine the mechanisms of potential injury that they may cause (Nel et al. 2006).

3.5.2. Mathematical modelling

Considerable attention has been devoted to the development of predictive models for skin penetration, and a diverse spectrum of approaches has been reported in the literature (Roberts et al 2002). The models range from simple, empirical algorithms to complex mathematical equations which sometimes require knowledge and estimation of experimentally-inaccessible parameters. In the most practical cases, the roles of molecular size and penetrant lipophilicity have been quite clear and some confidence in these approaches has evolved (Geinoz at al. 2004). However, in none of these strategies has data relating to macromolecular compounds or particle structures been included, meaning that the models available cannot be confidently used to predict what might happen when such entities contact the skin, especially in non-equilibrium situations. For 'steady state' situations, extrapolations even to modest molecular weights in the order of 10^3-10^4 Daltons result in the prediction of negligible penetration in the timescale of the turnover of the stratum corneum.

3.5.3. Microscopic techniques

More useful information from the *in vitro* approach described above (or even from certain *in vivo* procedures) can be obtained by microscopic examination of the skin post-treatment. While absolute quantification may not be possible, visualization of the tissue to which an active ingredient in a vector has been applied can provide valuable insight.

Laser scanning confocal microscopy (LSCM) has been applied to this challenge and has provided both optical "slices" of the treated skin and cross-sectional images (Alvarez-Román et al. 2004a). The advantage of this method is that 3-dimensional views of the skin can be obtained from relatively thick tissue samples with little or no tissue artefacts. On the other hand, the method depends upon suitable fluorophores being available, ideally to allow both active and vector to be tracked independently (an objective not yet attained). Nevertheless, it has been possible with this approach to clearly demonstrate the impact of a nanoparticle formulation on the delivery of a model active (Alvarez-Román et al. 2004a), and to visualize the affinity of particulate vectors for follicular openings (Alvarez-Román et al. 2004b).

High resolution transmission electron microscopy is an alternative approach which can allow visualization of individual particles in ultra-thin sections of tissue. Further, it is then possible to use X-ray analysis to identify the chemical composition of the visualized vector. The drawbacks of this method are (i) the limited field of view (and hence a question mark over the representativeness of a particular image), and (ii) the potential for artefacts given the number of steps involved in the sample preparation procedure. Artifacts inherent in current electron microscopic methods have to be considered.

A series of ion-beam techniques are also being applied to the field, including particle induced X-ray emission (PIXE) which produces elemental maps, Rutherford back-scattering spectrometry, and scanning transmission ion microscopy. The positive features of these methods are their large fields of view, easy sample preparation and facile elimination of artefacts; they cannot, however, visualize individual particles.

A final approach uses radiolabelling with the positron emitter ^{48}V (half-life = 16 days). This autoradiographic method uses thin sections and nuclear microemulsions. The technique is ultra-sensitive, relatively easy to use, provides a large field of view and shows individual positron tracks, but cannot visualize particles *per se*. It appears to be useful for localizing particles to specific structures in/on the skin, such as hair follicles and skin 'furrows'.

3.5.4. In vitro methods

3.5.4.1. Validated *in vitro* methods

In vitro toxicology has developed significantly in recent years, the principle in Europe being the 3Rs strategy (refinement, reduction, replacement) of Russell and Burch (1959). Whereas an important number of alternative methods and technologies exist for studying the molecular mechanisms involved in the biological activity of compounds, only a limited number of alternative methods exist that are applicable for regulatory purposes, namely for risk assessment of substances. For cosmetic ingredients and products, only validated methods are permitted. These are methods that have passed the various steps of the modular validation process established at the European Centre for the Validation of Alternative Methods (ECVAM) and that are considered by its Scientific Advisory Committee (ESAC) to comply with his process. Methods can also be considered by ESAC to be equivalent with such an approach.

The existing validated 3R-methods, in use today for cosmetics, are given in Annex V, Part B of the Dangerous Substances Directive and its relevant adaptations to technical progress (67/548/EEC) and/or OECD guidelines. An overview was provided in the 6th Revision of the Notes of Guidance of the SCCP for Testing of Cosmetic Ingredients and their Safety Evaluation (SCCP/1005/06). The latest adaptations to progress were taken up in a memorandum on *in vitro* methods by the SCCP (SCCP/1111/07).

Although these *in vitro* methods have been validated for the safety assessment of traditional cosmetic ingredients, optimal test conditions for nanomaterials have not been studied. Thus as required by the Cosmetic Directive (78/768/EC), validated methods must be used when safety assessment of cosmetic ingredients is required. Among the validated methods are, in particular, methods that can be used for measuring acute and short-term toxicity but not yet repeated-dose toxicity or long-term toxicity. Of the existing validated methods only a limited number are replacement methods:

- skin irritation testing via Episkin©, which is a reconstructed human skin model (adopted by ESAC April 2007) that uses 3-(4,5)-dimethyl-2-thiazolyl-2,5-dimethyl-2H-bromide reduction as an endpoint. A higher sensitivity is obtained, without losing specificity, by measuring as a second endpoint IL-1α (interleukine-1 α);
- skin corrosion testing via TER (transcutaneous electrical resistance), Episkin™ or Epiderm™ (two reconstructed human skin models);
- phototoxicity testing via the 3T3 NRPT (3T3 fibroblasts neutral red uptake phototoxicity test), applicable to UV absorbing substances;
- dermal absorption measurements on human/pig skin in a Franz cell with the specifications as recently revised by the SCCP (SCCP/0970/06);
- genotoxicity/mutagenicity testing via a set of 3 recommended tests: bacterial reverse mutation test, *in vitro* mammalian cell gene mutation test, *in vitro* micronucleus test or *in vitro* mammalian chromosome aberration test;
- embryotoxicity testing via 3 tests EST (embryonal stem cell test), MM (micromass assay) and WEC (whole embryo culture)*

Although these *in vitro* replacement methods have been validated for the safety assessment of traditional cosmetic ingredients, optimal testing conditions for nanomaterials have not been studied.

Also the question may be raised whether *in vitro* technology, validated or not, is suitable for safety assessment of nanomaterials. Some doubts have been raised as a low correlation was seen for example between *in vivo* and *in vitro* measurements for pulmonary toxicity of nanoparticles (Sayes et al. 2007a and 2007b).

3.5.4.2. Non-validated *in vitro* approaches

A number of non-validated approaches exist. These are, in particular, concerned with hazard identification of chemicals within REACH (Regulation (EC) No 1907/2006) but are not suitable for quantitative risk assessment. Among these is a non-validated test that is of interest for cosmetics, namely the screening of eye irritation.

A number of *in vitro* tests and strategies are under development in EC funded (FP6 Research) projects such as Predictomics, ReProTect, Sensit-iv, AcuTox, Liintop, Carcinogenomics and others, but optimisation for nanoparticles is not included and deserves further study.

Thus, validated *in vitro* methods, specifically developed or optimised for cosmetic ingredient application of nanomaterials are needed.

However, from the literature it is evident that considerable efforts are going into the development of *in vitro* testing strategies that potentially could be used for the toxicological evaluation of nanomaterials. Relevant toxicological endpoints important for nanomaterials are the following (Report prepared by NRCG Task Force 3, August 2006):

(i) penetration through physiological barriers;
(ii) uptake and translocation;
(iii) cell damage or cytotoxicity;
(iv) induction of cellular stress with emphasis on oxidative stress and inflammation;
(v) mutagenicity/genotoxicity.

(i) Penetration through physiological barriers

Skin: The stratum corneum of human skin is an excellent barrier. When cosmetics are applied on the skin, penetration of cosmetic ingredients may occur. Animal skins are generally not suitable for testing cosmetic products. It is recognized that the skin from furry rodents results in overestimation of human skin penetration. Hairless species are more useful, but any effects of skin penetration enhancers in the formulation may be exaggerated in these models. The large differences in follicular density in haired species compared to man may influence tests of systems containing nanomaterials. When hairy skin is shaved or depilated before treatment, there is an additional risk of damage to barrier function exacerbating further the problem of reliably assessing nanoparticle absorption. Pig skin reasonably approximates absorption in man and its usefulness *ex vivo* has been demonstrated in some applications, nevertheless, the question remains as to whether follicle properties in this model are reasonably similar to those of human skin.

OECD Guideline 427 (OECD 2004a) describes *in vivo* dermal absorption and, therefore, will not be permitted in future for cosmetic ingredients. Dermal absorption can also be measured *in vitro* using excised human or pig skin in a diffusion cell (Franz cell). The technique is described in OECD Guideline 428 and may be used for traditional cosmetic ingredients (SCCNFP/0750/03; SCCP/0970/06), but is not optimised for nanoparticles and requires further study.

In OECD guideline 428, skin separates a donor compartment, containing the formulation of the active, from a receptor phase, typically comprising a physiological buffer (OECD 2004b). The permeation of the ingredient can be assessed by sampling the receiver chamber as a function of time; alternatively, the experiment can be stopped at a specified time, and the tissue removed and sectioned before analyzing the ingredient in different compartments. The approach, however, is not well-adapted for monitoring the relationship between ingredient and carrier. It may be possible to resolve whether the carrier alters the transfer of the ingredient, but the mechanism for this cannot be deduced by analyzing the ingredient in the skin or the receptor phase. Equally, the probability that a nanoparticle carrier can be quantified in the receiver medium is extremely small. Furthermore, the integrity of the skin must be rigorously assessed through TER (Transcutaneous Electrical Resistence) or TEWL (Trans Epidermal Water Loss) measurements.

Lung: Bronchial and alveolar epithelia constitute a major barrier for entry. A number of human cell lines have been proposed for bronchial epithelium. They may not adequately represent the *in vivo* situation (Forbes and Ehrhardt 2005). The same is true for cell lines mimicking alveolar epithelium. The use of isolated primary bronchial and alveolar cells in monolayers may be superior to single cell cultures, but specific validation of these and other systems for nanomaterials has not yet been done.

Until today no good predictability of *in vivo* effects of nanomaterials can be obtained from *in vitro* systems (Sayes et al 2007a). When using *in vitro* testing, there is some evidence that multiple cell culture systems could be superior to single cell cultures (Geiser et al 2005, Rothen-Rudishauser et al 2006). None of these are yet validated or optimised for nanomaterials.

Oral route: The importance of the oral route for cosmetics is limited to those ingredients and products that can enter the gastro-intestinal system such as those present in lipstick, toothpaste, etc, possibly including nanomaterials. Immortalised cell lines are mainly used to investigate the penetration across intestinal epithelium. In particular, Caco-2 cell lines derived from human colon adenocarcinoma are used in culture. The standardisation of the different types of cell lines, however, is a problem and is a topic under investigation in the FP6 project, LIINTOP, but this type of in *vitro* system does not really reflect the real *in vivo* situation. It must be emphasised that these tests apply to conventional substances, not necessarily to nanomaterials. Once having been developed, they still require validation and optimisation for nanomaterials.

(ii) Uptake and translocation

It is important to consider the uptake of nanomaterials by macrophages, in relation to size, chemical composition and surface reactivity of the nanomaterial. Moller et al. (2005) showed that ultrafine carbon black particles impaired phagosome transport and caused cytoskeletal dysfunctions with a transient increase of intracellular calcium. The specific surface area of the particles seemed to play an important role in these effects. A method using the cumulative projected area of the particles involved in macrophage uptake was developed by Moss and Wong (2006). It has been suggested that nanoparticles cross cellular membranes by non-endocytotic mechanisms (Geiser et al. 2005, Rothen-Rutishauser et al 2006). The sub-cellular distribution can be different from one particle type to another e.g. endolysosomal, cytoplasmic, nuclear, mitochondrial (Li et al. 2003, Rothen-Rutishauser et al. 2006). An *in vitro* system to measure the translocation of nanoparticles over a layer of pulmonary cells has been developed recently (Geys et al. 2007). For skin penetration studies, different *in vitro* models have been used including reconstructed epidermis and excised human or pig skin (Chen et al. 2006). Few *in vitro* studies are available on translocation across other types of barriers. Lu et al. (2006) established a co-culture model with brain capillary endothelial cells and astrocytes of rat to evaluate nanoparticle blood brain barrier (BBB) transcytosis and toxicity at the endothelial tight junction.

Although several *in vivo* studies have shown that inhaled ultrafine particles can pass into the circulation (Nemmar et al. 2002b and 2002a, Oberdörster et al. 2002, Kreyling et al. 2002), the mechanisms and kinetics of particle translocation are not known. When studying nanoparticles translocation in humans no detectable translocation from lungs and secondary target organs could be observed, but the authors emphasize that the detection limit was only 1% (15 ng) that was deposited in lungs (Wiebert et al. 2006a and 2006b, Möller et al. 2007). After a single one hour inhalation of nanoparticles in a rat model a significant amount of nanoparticles was retained in the lung and in secondary target organs after six months (Semmler et al. 2004 and 2007).

(iii) Cell damage and cytotoxicity

Cytotoxicity can be measured *in vitro* using a variety of cell lines and primary cells in culture, depending on the target tissue under investigation. It must, however, be emphasized that the current methodology has not been validated for nanomaterials and is not necessarily suitable for them. Measurements are usually based on the quantification of membrane damage, intracellular metabolic changes or apoptosis. Some current possibilities are listed below:

- ***Membrane damage*** measurements are based on:

  - passive dye uptake, e.g. trypan blue, by damaged and dead cells and light microscopic counting;
  - cell lysis with intracellular enzyme release e.g. LDH (lactate dehydrogenase) release in the culture medium from damaged and dead cells and spectrophotometric quantification (LDH kits are commercially available);
  - active dye uptake, e.g. neutral red, by living cells and spectrophotometric quantification.

- ***Intracellular metabolic changes*** can be quantified by measuring:
  - phase I and phase II biotransformation enzyme activity by spectrophotometric, or mass spectrometric techniques, radioactivity, GC, HPLC or other methodologies;
  - impaired mitochondrial reductive activity e.g. by the reduction of tetrazolium salts to purple coloured formazan products (MTT assay). The *in vitro* test may yield conflicting results due to interference with the assays. These data suggest that a single cytotoxicity assay should not be relied upon in assessing nanomaterial toxicity (Wörle-Knirsch et al 2006, Monteiro-Riviere et al 2005 and 2006, Beck-Speier et al. 2005, Hussein et al. 2005, Blanchet et al. 2004);
  - the ATP content of cells and energy failure in dead cells (enzymatic assay).

- ***Apoptosis*** (controlled cell death) provokes a number of biochemical and morphological changes that can be quantified routinely by determining::
  - caspase activity (in particular caspase 3);
  - expression of Apaf-1, pro-apoptotic Bcl-2 proteins Bax and Bid, tumour suppressor p53;
  - annexin V-labelling of phosphatidyl serine of plasma membranes;
  - TUNEL assay: DNA fragmentation analysis by gel electrophoresis and labelling of DNA ends.

Several examples applied to nanomaterials can be found in the recent scientific literature. It is out of scope to mention all these here. Hussain et al. (2005) proposed the use of BRL 3A immortalized rat liver cells (ATCC, CRL-1442) to evaluate the acute toxic effects of different sizes of metal/metal oxide nanomaterials. For toxicity evaluations, morphology, mitochondrial function (MTT assay), membrane leakage (LDH assay) and reduced glutathione (GSH) levels, reactive oxygen species (ROS) and mitochondrial membrane potential (MPP) were assessed under control and exposed conditions. Nanoparticles led to morphological modifications, LDH leakage and mitochondrial dysfunction. Different metals displayed different toxicity levels, e.g. silver presented a specific toxicity related to oxidative stress with significant depletion of GSH level, reduced mitochondrial membrane potential and increase in ROS level (Hussain et al. 2005).

Also data exist that are contrary and claim that cytotoxicity does not depend on particle size in the case of biological inert ceramic or metallic materials (Yamamoto et al. 2004). Other examples can be found (Hussain et al. 2005, Choi et al. 2005).

Other cytotoxicity studies on nanomaterials have been carried out in human lung cancer cell lines focusing on oxidative stress as an endpoint for cytotoxicity (Lin et al. 2006a, b). Also human dermal fibroblasts in culture, human hepatoma (HepG2) cells and human neuronal astrocytes have been used successfully (Sayes et al. 2005).

From cytotoxicity studies with industrial nanomaterials it became clear that nanoparticle dissolution is a key factor affecting cytotoxic responses (Brunner et al. 2006) making this *in vitro* methodology only suitable as a screening method but not a replacement for *in vivo* studies.

The behaviour of nanoparticles in suspension needs consideration with regard to cytotoxicity. Nanoparticles may diffuse, settle and agglomerate in cell culture media as a function of the environment (media, ionic strength, pH and viscosity) and particle properties (size, shape and density) (Teeguarden et al. 2006). Cellular dose is therefore affected as the factors highlighted determine the delivery rate to the cultured cells. Nanoparticle kinetics in cell culture systems must be studied as simple use of the concentration of the nanomaterial in the culture medium can cause significant misinterpretation of response and uptake data observed *in vitro* (Teeguarden et al. 2006).

(iv) Induction of cellular stress

In vitro study of oxidative stress

Many studies have demonstrated the reactivity of nanoparticles and their capacity to produce reactive oxygen species (ROS), inducing oxidative stress. Oxidative stress has, therefore, been suggested as a suitable endpoint (Oberdörster et al. 2005b) for multiple tissue types and can be studied in cell-free (Brown et al. 2001, Beck-Speier et al. 2005, Xia et al. 2006) as well as in cellular systems. Examples are: macrophages (Beck-Speier 2005,) lung cells (Xia et al. 2006), bronchial epithelial cells (Gurr et al. 2005) and brain microglia (Long et al. 2006). In most of these studies, the mechanisms of oxidative stress were analyzed. Particles differed with respect to cellular uptake, subcellular localisation and ability to catalyze the production of reactive oxygen species under biotic and abiotic conditions. Specific assays have been performed to compare the abilities of environmental and manufactured nanoparticles to induce oxidative stress (Xia et al. 2006, Nel et al. 2006). Quantifying ROS can be performed in several ways (Tarpey et al. 2004). Currently used techniques quantifying ROS include measurement of:

- the ratio of reduced glutathione (GSH) versus its oxidized form (GSSG);
- free radical formation through the colorimeter thiobarbituric acid method and more specific by spin trapping agents and electron spin resonance measurements of the stable adducts formed;
- adduct formation of hydroxyl radicals with 8-OH-deoxyguanosine.

In vitro study of inflammation

Monitoring the release of pro-inflammatory mediators such as cytokines, chemokines, nitric oxide, up-regulation of transcription factors such as nuclear factor kappaB (NF-κB) and activator protein 1, (all known to be important in initiating inflammatory responses), is possible in different cellular sources. Cellular targets of nanoparticles can be either the epithelium cells of entry routes and resident macrophages and neutrophils, or cells belonging to target organs such as liver, kidney, nervous system, etc. Primary cells of different species can be used but interspecies differences may occur in comparison to the human response. Cell lines derived from non-cancerous tissue are easier to work with, but again comparability with the *in vivo* situation can be a problem.

For nanoparticles, an association between surface area, ROS-generating capacity and pro-inflammatory effects of nanoparticles has been established when other parameters have been kept constant (Nel et al. 2006, Oberdörster et al. 2005b, Donaldson et al. 2004). Most of the mechanistic studies, however, have been performed with diesel particles and particulate matter of 2.5 µm size (Bonvallot et al. 2003, Baulig et al. 2003). These studies have demonstrated that the activation of nuclear factor NF-κB depends on oxidative stress associated with the transcription of pro-inflammatory cytokines such as IL8, IL1β, GM-CSF (Granulocyte-Macrophage-Colony Stimulating Factor). Ultrafine carbon black particles have been shown to cause increased expression of NF-κB related genes in lung cells (Shukla et al. 2000). These particles not only induced cytotoxic injury and inflammation but also inhibition of cell growth of vascular endothelial cells (Yamawaki and Iwai 2006). Comparable results were obtained previously using human dermal endothelial cells (Peters et al. 2004).

(v) Mutagenicity/genotoxicity

In principle, the genotoxic potential of nanoparticles can be assessed using *in vitro* assays in mammalian cells. However, the timing of the tests may have to be adjusted to allow the nanomaterial to reach the nucleus during mitosis. In the *in vitro* chromosome aberration test, it may be necessary to examine the second post-treatment metaphase in addition to the first one. In the cytokinesis-block micronucleus test *in vitro*, the exposure could also occur for one cell cycle without cytochalasin B (Cyt-B), followed by another cycle in the presence of Cyt-B, to examine the cells after the 2nd post-treatment mitosis. As with other particulate materials, bacterial genotoxicity assays are not expected to be useful.

3D-human skin models are currently under investigation for their usefulness in genotoxicity testing of cosmetic ingredients. The technique seems promising but again once established for traditional cosmetics ingredients, optimisation for nanomaterials will be necessary. It is unclear whether *in vitro* tests are suitable for insoluble particles or nanomaterials with a very limited solubility.

If the genotoxic effects of nanoparticles are related to inflammation, simple *in vitro* assays may not be adequate in showing the genotoxic potential. In general, the relationship between inflammation and genotoxic effects is poorly understood, and this question is also unclear for many types of poorly soluble larger (non-nano) particles. Depending on the endpoint studied, demonstration of an association between inflammation and genotoxic effects may require relatively long-term experiments, and it is unclear whether such studies are practicable in genotoxicity assessment. In rats, intratracheal instillation of nanosized carbon black, but also of fine titanium dioxide (anatase) and fine α-quartz resulted in an increase of *hprt* mutations in alveolar type II cells 15 months after the treatment (Driscoll et al. 1997). It appears that fundamental studies on the association between inflammation and genotoxicity are required before conclusions can be drawn on the importance of studying inflammation-related genotoxicity of nanoparticles.

In vivo, actively dividing cells are expected to be the primary targets of genotoxic effects associated with carcinogenesis, as mutations can be fixed only when the cell divides. Cells that produce new lung cells (e.g. type II pneumocytes) are of interest. In the skin, the basal layer of the epidermis is of primary interest. The same concerns cells of the gastro-intestinal tract. The *in vivo* genotoxicity tests widely used, the bone marrow micronucleus test and the liver UDS (Unscheduled DNA Synthesis) test, detect genotoxic agents that reach the bone marrow and the liver, respectively, or that have systemic genotoxic effects. There are presently no validated standard methods for assessing genotoxic effects in the expected target tissues *in vivo*, but techniques such as the comet assay, micronucleus test, and gene mutation analysis in transgenic animals could probably be applied.

3.5.5. *In vivo* methods

The use of sequential tape-stripping of the stratum corneum post-treatment with a formulation provides information on the penetration of materials in the upper layers of the skin (Alvarez-Román et al. 2004, Herkenne et al. 2007a and 2007b). However, residual formulation in skin 'furrows' and/or hair follicles can compromise estimations although recently it was shown for TiO_2 nanoparticles that these were not systematically available (Mavon et al. 2007). Also Cross et al (2007) showed that ZnO particles did not penetrate through human skin. Additionally, defects in the stratum corneum barrier may permit entry of particles (Gontier et al. 2007).

Although stratum corneum tape-stripping is an established tool and provides information on substances present in the stratum corneum, it does not provide sufficient information for nanoparticle translocation to the deeper layers of the skin, which requires better visualisation techniques such as confocal and high-resolution electron microscopy, ion beam technologies and autoradiography. The ability to individually label in a stable manner and detect both carrier particles and active ingredients are required.

The EC and the OECD guidelines have been developed for conventional chemicals, and as optimised conditions for nanomaterials are not yet available for nanomaterials, it is unknown whether the current regulatory toxicological tests are adequate or relevant for testing of nanomaterials or whether specific adaptations will be required and new methods developed. Therefore, further research must be performed and the current hazard identification of nanomaterials needs to be assessed on a case-by-case basis.

Instillation of particles into rat lungs has often been used in particle toxicology. The advantage of instillation is that it involves the administration of a more precise nanoparticle dose. As this mode of exposure is not physiological, inhalation studies are recommended because of the physiological route of intake. However, inhalation studies are prone to artifacts and have to be designed and performed carefully. The method of instillation is not suitable for risk characterization, but is useful for hazard identification.

3.5.6. Summary

- Classic skin permeability techniques do not take into account possible mechanical effects that may be relevant for the penetration of insoluble nanoparticles or nanomaterials.

- No *in silico* models exist yet for nanoparticle uptake.

- As for all substances, current *in vitro* methodologies (validated and non-validated) are not available to show nanoparticle transport in living organisms. Animal models remain indispensable for biodistribution, translocation, accumulation and clearance studies.

- Although the basic requirements of testing the mutagenicity/genotoxicity of nano-particles are similar to those of other particulate materials, the specific characteristics of nanoparticles may require further considerations. The genotoxic *potential* of nanoparticles could probably be assessed in mammalian cells *in vitro*. The present validated *in vivo* genotoxicity tests do not cover the expected target organs of nanoparticles (particularly the respiratory tract), and have not been validated with reference substances including nanomaterials of relevance for cosmetics.

- For nanoparticles, not only the dose of the intake organ needs to be considered, but also the dose in secondary target organs.

- Nanomaterials may target more cell types than larger particles do because of endocytotic and non-endocytotic pathways and different efficacies.

- Mass is probably not the most relevant measure for nanoparticles. Other parameters may be of importance including size and surface area.

In general, it can be stated that it is prudent to apply more than one technique for the characterisation and effect assessment of nanomaterials to ensure the generation of consistent results. It is, however, clear that all techniques mentioned are still in research phase. This has implications for safety assessment of cosmetics, since validated *in vitro* methods for nanomaterials are not available and are still in early development phase.

3.6. Routes of exposures

3.6.1. Introduction

The exposure sites of nanosized structures are the skin, respiratory tract, intestinal tract and the eyes. In the use of microscopic images, the observation of a concentration gradient is a strong indicator of diffusive transport of nanoparticles. However, in the absence of concentration gradients, the results are difficult to interpret. In the present opinion only dermal absorption will be discussed in detail.

3.6.2. Dermal absorption

3.6.2.1. Pathways of permeation across the skin

Three pathways of penetration across the skin have been identified: intercellular, transfollicular and transcellular. The importance of the inter-corneocyte lipid domains is well-established and clearly implicates these structures as crucial to barrier function of the skin. The appendageal contribution to the passive diffusion of low molecular weight chemicals is believed to be minor (although the first molecules to cross the barrier probably do so via follicular 'shunts'); on the other hand, particles may have an affinity for these structures (see below). The transcellular pathway is favoured in terms of surface area, and the ability of the corneocytes to take up permeating chemicals has been shown. However, whether the roles of these routes are those of conduits or reservoirs is, as yet, not entirely clear.

3.6.2.2. How can nanoparticles penetrate the skin barrier?

The passive transport of nanoparticles through intact stratum corneum is considered highly unlikely because of the matrix of corneocytes, lipid bilayers within the intercellular spaces and the physiological environment below the stratum corneum containing high levels of proteins. If the skin is damaged, and the normal barrier disrupted, then the probability of entry of particles may be substantially increased.

Follicular openings are compatible with particulate dimensions. Therefore, it is not unreasonable to anticipate a size-dependent phenomenon whereby particles lodged within the appendageal openings may allow increased drug delivery. Additionally, nanoparticles may have increased substantivity in skin "furrows", and may not be efficiently removed by standard cleansing procedures.

The passage of fullerenes across dermatomed porcine skin has been determined by stripping (Xin-Rui et al. 2006) and also penetration to vital tissue has been observed with quantum dots (CdSe) (Ryman–Rasmussen 2006). It has been demonstrated that spherical and elliptical quantum dots penetrate the stratum corneum and localize within the epidermal and dermal layers (Ryman–Rasmussen 2006).

The breaching by dextran particles (500 nm) (Tinkle et al. 2003) and primary and aggregated functionalised buckyballs (3.5 nm) following flexing the skin (Rouse et al. 2007) has been described. In both studies, nanoparticles were found in deeper dermal layers when the skin was flexed.

Current investigations of nanoparticles penetration into the skin using static imaging technology do not detect small quantities of nanoparticles which may reach the vascular bed of the dermis and then be removed from the blood. If the skin is exposed to large nanoparticle doses even small fractions may become important to accumulating secondary target organs. For example, skin exposure to TiO_2 through suncreen products may amount to several grams of TiO_2 during a summer season. If only as little as 10^{-4} of TiO_2 nanoparticles topically applied are absorbed and made systemically available through the blood circulation and lymphatic drainage, the translocated fraction would be in the order of 100-500 µg which either could be excreted or accumulated in all or particular organs.

Currently there is no information available about the associated risk to the various organs. One of the possible secondary organs is the lung, on which most data are available. A significant inflammatory response has been observed at a very low dose, e.g. when 500 µg of ultrafine TiO_2 was administered to the lungs of rats, a neutrophilic infiltrate was present 24 hours after application (Oberdörster 2001). Observations of this type indicate the need to quantitate the biodistribution of nanoparticle TiO_2 in view of wide consumer exposure.

3.6.2.3. *The problem of abnormal skin*

Although cosmetic products are meant to be used on normal skin (76/768/EEC), it is known that they also are applied on non-healthy and/or physiologically compromised skin. A large proportion of the European population is atopic and 2% has psoriasis. In such groups the barrier properties of the skin may be impaired. Within the EC funded NANODERM-project, the potential penetration of formulations containing TiO_2 nanoparticles in some individuals with psoriatic skin was investigated.

There is not yet published information available on the potential penetration of nanomaterials through atopic or sunburnt human skin.

3.6.3. Respiratory tract

3.6.3.1. Possible uptake of nanosized materials from cosmetics via inhalation

In studying the health effects of inhaled particle matter most attention has been paid to alveolar macrophages and type II alveolar epithelial cells (type II cells or pneumocytes). The alveolar macrophages reside as free cells within the alveolar air spaces, from where they may migrate to the bronchioles and then, via the mucociliary escalator, to the lumen of the conducting airways.

The alveolar macrophage plays an important role in the response of the lung to inhaled dusts and in the development of inflammatory lung disorders. Their essential function is phagocytosis and clearance of particulates and micro-organisms. The type II cell is a secretory cell (producing surfactant) and is considered to be the progenitor cell for type I cells. Type II cells have been shown to contain significant quantities of biotransformation enzymes, particularly cytochrome P450-dependent mono-oxygenases. In addition, these cells carry out several other metabolic functions, such as the active uptake of endogenous and exogenous compounds, permeability functions, immunologic functions, etc.

3.6.3.2. Fate of nanosized particles in the respiratory system

Deposition of nanosized particles (< 100 nm) in the respiratory tract is determined predominantly by diffusional motion of particles < 0.5 µm (thermodynamic diameter) as a result of thermal (Brownian) motion of air molecules. Diffusional nanosized particle deposition works through three important components (aerosol properties and physiology) during breathing:

(a) particle dynamics, including the size and shape, and its possible dynamic change;
(b) geometry of the branching airways and the alveolar structures;
(c) breathing pattern determining the airflow velocity and the residence time in the respiratory tract, (including nose and mouth breathing) (Kreyling et al. 2006b, Oberdörster et al. 2005a). A proportion of nanoparticles are retained long-term in the airways (Kreyling et al. 2006b, Kreyling et al. 2004.)

3.6.3.3. Fate of nanosized particles in the lungs

On the walls (epithelium) of the respiratory tract, particles first come into contact with the mucous or serous lining fluid and its surfactant layer. Therefore, the fate of particle compounds that are soluble in this lining fluid needs to be distinguished from that of slower-dissolving or even insoluble compounds (Kreyling et al. 2006a).

Soluble particle compounds will be dissolved and often metabolised in the lining fluid, and will eventually be transferred to the blood, undergoing further metabolism. In this way they may have the potential to reach any organ and to produce toxic effects far from their site of entry into the lungs.

Much of the slower-dissolving and insoluble nanoparticles deposited <u>on the airway wall</u> will be moved by the action of ciliated cells (mucociliary escalator) or by coughing and are swallowed. However, there is evidence that a significant fraction of nanoparticles is retained in the airways.

Slower-dissolving and insoluble particles deposited <u>in the alveolar region</u> will be taken up by macrophages in the alveoli under normal physiological conditions. However, macrophages are less able to take up nanoparticles, which may penetrate into the interstitium. Macrophage-mediated particle removal may be impaired especially in young old people and individuals with diseased lungs.

As noted above, nanoparticles are less effectively taken up by macrophages, but may interact to a greater extent with lining cells than larger particulates. The combined surface area of nanoparticles may be large and is the reactive interface with cells. Depending on the molecular surface these particles may have a greater capacity to induce or mediate adverse effects than larger particles, not only in the respiratory system, but also in the heart and blood vessels, the central nervous system and the immune system (Oberdörster et al. 2005b).

3.6.4. Intestinal tract

Little information is available about the intake of nanoparticles via the intestinal tract. Particulate uptake occurs not only in the gut-associated lymphoid tissue (GALT), but also in the normal intestinal enterocytes. There have been a number of reviews on the subject of intestinal uptake of particles (Jani et al. 1989, Jani et al. 1990), but these do not deal with cosmetic ingredients. In a human screening study from the UK, Lomer et al (2004) found that about 4.5 mg of micron-sized TiO_2 (with an unknown ultrafine fraction) per day was ingested from various consumer products (toothpaste, food etc) and pharmaceuticals. Crystalline TiO_2 (rutile, particle size 0.5 μm) was shown to have limited uptake in the gastrointestinal tract (Bockman et al 2000, Eldridge et al 1990). Translocation through the intestinal barrier of particles made from different materials (biodegradable and non-biodegradable) was extensively demonstrated. Particle uptake and translocation towards circulation was shown to depend strongly on size (in an inverse fashion) and on surface charges as well as surface ligand modification (Florence et al. 1995, 2001, Eldridge et al. 1990, Shakweh et al. 2005 and 2004)

3.6.5. Eye

The eye provides only a small surface area for potential exposure but indirect exposure to nanomaterials may occur through it by cosmetics intended for use in the vicinity of the eye or from other types of cosmetic products e.g. sprays.

3.6.6. Summary

Nanoparticles may enter the human body via several routes but evaluation of exposure is limited. The probability of penetration depends on size and surface properties of particles and on the anatomical structure of the specific sites of the exposure routes. Penetration via the skin is less evident although it is possible that some particles can penetrate through the skin by mechanical effects. Nanomaterials may concentrate inside the pilosebaceous follicles and release ingredients locally. Following penetration, the distribution of particles in the body is a function of the physico-chemical characteristics of the nanoparticles.

3.7. Toxic effects of exposure to nanomaterials

The toxicological profiles of nanoparticles have been investigated in recent years. The studies have mainly focussed on effects on the respiratory tract and the cardiovascular system. In the early 1990s, toxicological studies began to seek biological plausibility for the epidemiological findings of the association of health effects and ambient ultrafine particle concentrations (Dockery et al. 1993). An early key study demonstrated that ultrafine TiO_2 caused more inflammation in rat lungs than exposure to the same mass concentration of fine TiO_2 (Ferin et al. 1992, Oberdörster et al. 1994). If the dose of nanoparticles is very large, as is the case for TiO_2 in sunscreens or dietary exposure etc., even absorbed fractions as small as 10^{-4} of applied dose may cause adverse health effects should accumulation occur.

Issues related to possible impact of contaminants in the toxicology of nanomaterials (e.g. Ni, Co, Fe, Cd, Mn) need to be addressed (Pulskamp et al 2007, Kagana et al 2006).

3.7.1. Skin

Concerns whether nanosized mineral UV-filters in sunscreen products could penetrate into or through healthy human skin were raised by the SCCNFP in 1998. Subsequent studies on TiO_2 concluded that TiO_2, used as a mineral UV-filter in sunscreen cosmetic products, does not penetrate through the stratum corneum of healthy skin and poses no local or systemic risk to human health from cutaneous exposure (SCCNFP/0005/98, Borm et al. 2006, Gamer et al. 2007, Lademann et al. 1999).

Little information is available concerning other nanoparticles. In the light of recent reports on penetration into to the epidermis and dermis of functionalized fullerenes (Rouse et al 2007) and quantum dots (Ryman-Rasmussen et al 2006) –until now not used in consumer products - no generalization should be made. Moreover, mechanical flexing appears to enhance nanomaterial penetration (Tinkle et al 2003, Rouse et al 2007). In the case of nanomaterial penetration, the dose administered to the intake organs, as well as the uptake dose in the secondary target organs as a result of biokinetics, should be considered. In addition, nanoparticles may affect more cell types than larger particles because of endocytotic and non-endocytotic pathways. It should be noted, however, that if the administered dose of nanoparticles is very large, as for instance could be the case for TiO_2 in sunscreens, a possible minute uptake fraction of nanoparticles may be of relevance should accumulation occur and especially if such exposure is repeated over a long period. It should be kept in mind that long-term exposure studies are missing and each analytical technique has minimum detection limits.

3.7.2. Respiratory tract

Apart from the direct chemical toxicity of inhaled nanoparticles (intrinsic chemical toxicity), the physical characteristics of the nanoparticles, including their surface properties, also have to be considered. With decreasing size of the nanoparticle, the surface area increases relatively. As a result there is an increased ability to generate reactive oxygen species, which could result in augmented inflammation (Nel et al. 2006, Oberdörster 2005, Donaldson 2003, Koike 2006). In addition, other factors such as surface treatment and coating, agglomeration and/or aggregation state of the materials and the release of chemicals (dissolution) to the lumen also play a role in the nanospecific responses (Kreyling et al. 2006a).

In the respiratory tract, nanoparticles may escape phagocytosis by macrophages to some extent and in a number of studies the uptake of nanoparticles has been described in non-phagocytotic cells (Donaldson 2002 and 2003, Oberdörster 2005, Kreyling et al. 2006a). Furthermore, phagocytosis of nanomaterials inhibits the phagocytosis of micron-sized particles and, therefore, the clearance of the material from the lung (Renwick et al. 2004).

Two different pathways have been identified in the extra-pulmonary effects after inhalation:

1. Entry into the circulation via the lung with direct and/or indirect effects on extra-pulmonary targets (Chen 2006, Kreyling et al. 2002, Nemmar et al. 2002, Semmler et al. 2004, Takenaka et al. 2006, Mills et al. 2006, Wiebert et al. 2006a and 2006b).
2. Pulmonary oxidative stress with consequent inflammatory reactions causing distal inflammation even without the translocation of the nanoparticles from the lung. This is due to changes in platelet activity (Khandoga et al. 2004, Nemmar et al. 2006, Bai et al. 2007)

The surface charge of nanoparticles may play a role in the systemic response (Xia et al. 2006). There is evidence that following nasal administration some nanoparticles may reach the brain (Oberdörster 2004, Elder et al. 2006). In addition, iridium nanoparticles circulating in blood can cross the blood-brain barrier (Kreyling et al. 2006a, Semmler et al. 2004). The toxicological importance of these observations is of concern (Lewis 2005).

Limbach et al (2007) have reported recently that inhaled nano-metal oxides can act as 'Trojan horses', adsorbing heavy metals and carrying them into lung epithelial cells where they provoke oxidative stress up to eight times higher than reference cultures exposed to aqueous solutions of the same metal. Nanomaterials in cosmetic products are not in contact with heavy metals and thus this should not occur.

3.7.3. Intestinal tract

Little information is available and the uptake of insoluble nanoparticles has not been appropriately studied. Ashwood et al (2007) found that 200nm particles of titanium dioxide join together with bacterial lipopolysaccharides to mimic invasive pathogens. The result is that bacterial components are 'smuggled' into human intestinal tissue where they provoke inflammation.

Many particles, man-made and/or environmental will absorb organic compounds. The presence of lipopolysaccharides on any material is very common and this may result in inflammatory responses. It has not been shown that nanomaterials will absorb more or less lipopolysaccharides compared to larger ones (e.g. Osornio-Vargas et al. 2003).

3.7.4. Eye

It is considered that some nanoparticles may cause damage to the cornea (Alany et al. 2006). The use of nanosized (colloid) carriers for drug delivery is described and particles have been found months after dosing in the eye (Shedova et al. 2003, Jani et al. 1989).

3.7.5. Mutagenity and genotoxicity

Genotoxicity is used as a short-term measure of potential carcinogenicity. Genotoxicity assays also reflect potential for inducing heritable mutations in germ cells, and genotoxic agents often have pronounced reproductive toxicity. As regards the possible genotoxicity of nanoparticles, direct reactions with DNA and other macromolecular structures (microtubules, kinetochores, centrioles, etc) and especially indirect effects related to potential oxidative stress and inflammatory activity are of interest.

Those nanoparticles that readily pass cellular membranes can be expected to reach the nucleus and DNA, which is important when genotoxic effects are considered. For instance, functionalized single-walled carbon nanotubes were reported to enter the cell nucleus (Pantarotto et al. 2004). Even if nanoparticles did not go through the nuclear envelope, they would eventually have access to the nucleus in dividing cells, because the nuclear envelope disappears in cell division.

Various particulate materials that are also available in the nano-range are known or suspected to be carcinogenic as bulk material. For example, crystalline silica is a human lung carcinogen (IARC, 1997), and titanium dioxide (all forms) and carbon black were recently classified by IARC as possibly carcinogenic to humans (class 2B) on the basis of animal carcinogenicity data (IARC in press, Mohr et al 2006). Although genotoxicity may play a role in the carcinogenesis of such materials, the process appears to be complex, and may involve, depending on the type of the particles, inflammation, lipid peroxidation, generation of reactive oxygen and nitrogen species, overloading of macrophage clearance, accumulation in tissues due to poor clearance and so on. Many of the specific questions in genotoxicity testing of nanoparticles (possible indirect mechanisms, lungs as target organ *in vivo*, requirement for material characterisation, etc) also concern larger particles and fibres for which standard genotoxicity tests may not be optimal (Speit 2002, Muhle and Mangelsdorf 2003, Schins 2002). In addition, the unique characteristics of nanoparticles (small size, high surface area, etc) may result in effects that differ from those of the bulk material.

For most types of nanoparticles, it is unknown whether they interact and at which size and charge, with DNA or the mitotic spindle, to give rise to gene mutations or structural and numerical chromosome damage. However, there are indications that certain types of nanoparticles could be capable of such effects. Cationic functionalized carbon nanotubes are able to condense DNA. Nanotube surface area and charge density are considered to be critical in determining electrostatic complex formation with DNA (Singh et al. 2005). Cell death of several cancer cell types was thought to be initiated by binding of 1.4 nm gold nanoparticles into the major grove of the DNA helix while smaller and larger gold nanoparticles did not show this effect (Pan et al. 2007).

The composition and coating of nanoparticles are probably key issues in determining their genotoxic effects. Nanotubes coated with a positively charged polyelectrolyte, functioning as a counterpart for negatively-charged DNA, have been wrapped with DNA to produce DNA sensors (He and Bayachou, 2005), and the property of various types of nanoparticles to complex with DNA has been utilized for cellular and nuclear delivery of DNA and oligonucleotides (Tan et al. 2002, Corsi et al. 2003, Tondelli et al. 2003, Ravi Kumaret al. 2004, Ramesh et al. 2004, Bejjani et al. 2005, Gemeinhart et al. 2005, Liu et al. 2005). Single-walled nanotubes bind double- and single stranded DNA and peptide nucleic acid (PNA) (Zheng et al. 2003, Rajendra et al. 2004, Rajendra and Rodger 2005) and specially engineered protein nanotubes bind DNA (Audette et al. 2004). Water-soluble semiconductor quantum dots (cadmium selenide capped with a shell of zinc sulfide, with biotin surface functionality) can nick DNA due to photogenerated and surface-oxide-generated free radicals (Green and Howman 2005).

Some genotoxicity information exists on fullerenes. The mutagenicity of fullerenes in *Salmonella typhimurium* (Ames test) was observed to depend on molecular groups associated with the fullerene and the solvent used (Babynin et al. 2002). In general, cationic chains in water-soluble fullerene C_{60} derivatives were observed to induce significant toxic effects, unlike neutral or anionic moieties (Bosi et al. 2004). In rat microsomes exposed to UV and visible light, fullerene (as a cyclodextrin complex) induced time- and concentration dependent oxidative damage seen as lipid peroxidation and protein damage (Kamat et al. 1998). Fullerene C_{60} gave negative results in the SOS chromotest (bacterial genotoxicity test) (Guillardet et al. 1982) in *Escherichia coli* (indicating DNA damage), while a slight genotoxic effect was seen in the somatic mutation and recombination test (SMART) in *Drosophila melanogaster* at the highest concentration of fullerene tested (Zakharenko et al. 1997).

Fullerenes are phototoxic; under photoirradiation fullerenes induced DNA cleavage, mutations, cancer initiation, and cell toxicity (Miyata et al. 2000, Yamakoshi et al. 2003). Fullerene dissolved in polyvinylpyrrolidone was mutagenic to *Salmonella* TA102, TA104, and YG3003 in the presence of rat liver microsomes when irradiated by visible light, possibly through oxidized phospholipids (linoleate). Treatment of 2´-deoxyguanosine plus microsomes or linoleate with C_{60} highly elevated 8-hydroxy-deoxyguanosine formation (Sera et al. 1996). Under laser irradiation, sugar-pendant C_{60} fullerenes produced singlet oxygen and were pro-phototoxic to HeLa cells (Mikata et al. 2003). The photo-induced bioactivities of fullerene were suggested to be caused by reduced oxygen species (superoxide radical and OH^-) generated by electron transfer reaction of C_{60} with molecular oxygen (Miyata et al. 2000, Yamakoshi et al. 2003).

On the other hand, $C_{60}(OH)_{22}$ fullerol was found to be a potent hydroxyl radical scavenger in human breast cancer cell lines (Bogadanović et al. 2004). This type of water-soluble fullerols had excellent anti-oxidant capacity in cultured cortical neurons (Dugan et al. 1996) and prevented hydrogen peroxide- and cumene hydroperoxide-elicited changes in rat hippocampus *in vitro* (Tsai et al. 1997). Fullerol was not genotoxic in the SOS chromotest or in *Drosophila* (SMART) (Zakharenko et al. 1997). Carboxyfullerene, when infused together with ferrous citrate, prevented iron-induced oxidative injury in rats (Lin et al. 1999).

No published genotoxicity data are available on carbon nanotubes or other fibrous carbon nanomaterials. Carbon nanotubes, especially single-walled nanotubes were, however, strongly cytotoxic to alveolar macrophages at dose levels where C_{60} fullerene did not exhibit cytotoxicity (Jia et al. 2005). This may suggest that carbon nanotubes have stronger biological activity than fullerene; such a difference may also concern genotoxicity. Experience from fullerenes has shown that relatively minor alterations in fullerene structure can alter the lethal cytotoxic dose by over 7 orders of magnitude, an aggregated form of C_{60} being substantially more toxic than highly soluble derivatives (Sayes et al. 2004). Cell death was associated with oxidative damage in cell membranes, which was assumed to relate to observed generation of superoxide anions or other oxygen radicals from fullerenes in water. Further studies supported the idea that lipid peroxidation -generated ROS are responsible for such effects (Sayes et al. 2005). Also single-walled nanotubes induced oxidative stress, exemplified by the formation of free radicals, accumulation of peroxidative products, antioxidant depletion, and cytotoxicity in human keratinocytes (Shedova et al. 2003). Carbon nanotubes and fibres often contain traces of metals (e.g. Ni, Co, Fe, Cd, Mn) that may contribute to the possible (geno)toxic effects observed (Pulskamp et al 2007, Kogana et al 2006).

Intratracheal instillation of carbon black nanoparticles (median diameter 15 nm), non-nanosized α-quartz (median diameter 900 nm) or non-nanosized titanium dioxide (anatase, median diameter 180 nm) to rats was associated *in vivo* with an increase in gene mutations in the *hprt* locus of rat alveolar type II cells, 15 months after intratracheal instillation (Driscoll et al. 1997). Furthermore, the inflammatory cells collected from the lungs by bronchoalveolar lavage (BAL), 15 months after the intratracheal instillation of carbon black nanoparticles or non-nanosized α-quartz, induced *hprt* mutations in rat RLE-6TN epithelial cells *in vitro* (Driscoll et al. 1997). Both macrophage and neutrophil enriched BAL cell populations were mutagenic, although neutrophils showed a higher mutagenic activity than macrophages. Addition of catalase to the co-cultures of BAL cells and RLE-6TN cells inhibited the mutation induction. The study suggested that poorly soluble particles (nano- or non-nano) producing marked neutrophilic inflammation increase mutations in rat alveolar type II cells. It may be mentioned that clearance of particles from the lungs of rats, as compared with some other species, is less efficient, and rats are, therefore, considered to be more susceptible to the carcinogenic effects of particles (Oberdörster 1995, Elder et al. 2005).

Ultrafine titanium dioxide (diameter ≤ 20 nm) induced micronuclei in Syrian hamster embryo cells *in vitro* when fine titanium dioxide (diameter ≥200 nm) did not (Rahman et al. 2002). Ultrafine titanium dioxide also increased micronuclei, nucleoplasmic bridges, DNA damage (comet assay), and *HPRT* mutations in human B-cell lymphoblastoid WIL2-NS cells *in vitro* (Wang et al. 2007a). Nanosized (10 and 20 nm) anatase induced oxidative DNA damage, micronuclei, lipid peroxidation, and the formation of hydrogen peroxide and nitric oxide in human bronchial epithelial BEAS 2B cells, while anatase of larger particle size (200 nm and >200 nm) did not (Gurr et al.al. 2005) but 200 nm -sized rutile did produce oxidative DNA damage and H_2O_2. Results on the genotoxicity of non-nanosized titanium dioxide are somewhat contradictory. No induction of chromosomal aberrations, sister chromatid exchanges (SCEs), or micronuclei by ordinary titanium dioxide was observed in Chinese hamster ovary CHO cells *in vitro* (Ivett et al. 1989, Miller et al. 1995). However, Lu et al. (1989) saw a dose-dependent increase in both micronuclei and SCEs in Chinese hamster ovary CHO-K1 cells.

Instillation of a commercially available nanosized TiO_2 (hydrophilic surface) and trimethoxyoctylsilane-coated TiO_2 (hydrophobic), both with primary particle diameters of approximately 20 nm, did not induce damage in lung cell DNA of rats *in vivo* (Rehn et al. 2003). DNA damage was assessed by immunohistochemical detection of 8-oxoguanine (8-oxoGua) in lung sections of the rats; in α-quartz-exposed animals (positive control) a strong and significant increase in the amount of 8-oxoGua in DNA of lung cells was detected.

When coupled with UV irradiation, anatase TiO_2 (hydrophilic, circa 20 nm) was clearly more photogenotoxic than TiO_2 (anatase and rutile, both 255 nm) in mouse lymphoma L5178Y cells, as measured by the comet assay (Nakagawa et al. 1997). Rutile of larger particle size (420 nm) was not photogenotoxic. The nanosized anatase TiO_2 was also photogenotoxic in Chinese hamster lung CHL/IU cells, when assessed by chromosome aberration induction, but not in *Salmonella typhimurium* or in mouse lymphoma L5178Y tk+/- cells, when studied for mutation induction (Nakagawa et al. 1997). Furthermore, this nanosized TiO_2 (hydrophilic surface) only induced DNA damage, chromosome aberrations and mutations with UV radiation.

Ultrafine crystalline silica (SiO_2) nanoparticles induced a dose-dependent induction of micronucleated binucleate cells and an increase in *HPRT* mutations in human WIL2-NS cells *in vitro.* However, no increase was seen in DNA damage measured by the comet assay (Wang et al. 2007b).

Polybutylcyanoacrylate nanoparticles were not mutagenic to *Salmonella* and did not induce micronuclei in mouse bone marrow or mouse foetal liver (Blagoeva et al. 1992). A nanocomplex root filling material (HA-PA66) was reported not to induce micronuclei in Chinese hamster V79 cells *in vitro* (Ye et al. 2004).

Maghemite nanoparticles (nano-γFe_2O_3; mean coherent diameter 6 nm) coated with meso-

2,3-dimercaptosuccinic acid (DMSA) did not significantly increase DNA damage in human dermal fibroblasts *in vitro*, as measured by the comet assay (Auffan et al. 2006).

3.7.6. Margin of Safety (Mos)

In risk characterisation, the last phase in the safety evaluation of a cosmetic ingredient, an uncertainty factor applies (SCCP/1005/06). For cosmetics, this factor is called the MoS. It is generally accepted that the MoS of a substance can be calculated by dividing its lowest NO(A)EL (No Observed (Adverse) Effect Level) value by its possible Systemic Exposure Dosage (SED). This MoS value is used to extrapolate from a group of test animals to an average human being, and subsequently from average humans to sensitive subpopulations. It is generally accepted that the MoS should at least be 100 to declare a substance safe for use. This value consists of a factor 10 for the extrapolation from animal to man and another factor 10 taking into account the interindividual variations within the human population. In the case of nanomaterials, the conventional MoS may not give an adequate expression of the safety.

3.7.7. Summary

For soluble nanomaterials, standard, mass-based risk assessment methodologies should be adequate if disintegration of the particles occurs. For insoluble nanomaterials, other metrics are required, including toxicokinetics and toxicodynamics with relevance to different exposure routes.

a) Only a few long term inhalation studies (apart from SiO_2, carbon black and TiO_2) are available, but none for the other routes of intake. Until there is better understanding of underlying mechanisms, a case by case approach for risk assessment is required. If reproducible generation of nanoparticles is guaranteed, no batch by batch evaluation is required.

b) Nanoparticles may exhibit a potential oxidative capacity associated with their particulate state. This is more pronounced in nanoparticles than in larger particles because of their larger surface area and their specific physico-chemical properties. Hence, nanoparticles may induce local (lungs, gut, and skin) oxidative stress and subsequent adverse health effects.

c) Current investigations of nanoparticle penetration into the skin using static imaging technology are unable to detect small fractions of nanoparticles reaching the dermis, vascular bed of the dermis, and hence, the blood stream. However, if the dose of nanoparticles is very large, as is the case for TiO_2 in sunscreens, even absorbed fractions as small as 10^{-4} may cause adverse health effects should accumulation occur. Therefore, it appears necessary to quantitate biodistribution to estimate risks associated with the increasing exposure to nanosize TiO_2, an example of a nanoparticle widely used both in cosmetics and other materials.

d) *In vitro* studies provide mechanistic insights, but their relevance needs to be demonstrated in *in vivo* studies.

e) The available information, mostly limited to fullerenes and TiO_2, indicates that certain nanoparticles may be genotoxic and photogenotoxic. Some evidence exists to suggest that nanosized TiO_2 is more genotoxic than TiO_2 of larger particle size.

4. OPINION

Cosmetic products[3] are primarily intended for use on skin, hair or oral mucosa (76/786/EEC). Products may contain particulate matter with dimension(s) below 100 nm, henceforth called nanoparticles. The nanoparticles are claimed to serve various purposes: enhancing the formulation properties and acceptability, a direct effect on skin and hair, e.g. moisturizing or anti-aging formulations, make-ups and hair-conditioners or protect the skin e.g. UV-filters in sunscreens.

A crucial factor in assessing possible risks associated with nanoparticles is their possible uptake. For topical applications, the route of exposure is essentially transdermal including follicular and other transadnexal pathways. In addition to this route, exposure via inhalation, ingestion, conjunctival and mucosal surfaces may have to be considered, e.g. nanoparticles from hairsprays, lipsticks, or toothpastes. Although pigmentary grades of TiO_2 are usually considered to consist of micron sized particles, particles below 100 nm may be present in such grades. Information on the particle size distribution is required for all micro- and/ or nanosized materials.

Nanoparticles can be divided into two groups: i) soluble and/or biodegradable nanoparticles which disintegrate upon application to skin into molecular species (e.g. liposomes, microemulsions, nanoemulsions), and ii) insoluble and/or biopersistent particles (e.g. TiO_2, fullerenes, quantum dots). For the first group, conventional risk assessment methodologies based on mass metrics *may* be adequate, whereas for the latter other metrics such as the number of particles, and their surface area as well as their size distribution are also required.

It is primarily for the insoluble particles that health concerns related to possible uptake arise. Should they become systemically available, translocation/transportation and eventual accumulation in secondary target organs may occur. This feature does become important with repeated application of cosmetic products. Inevitably, insoluble nanoparticles do represent a burden for the environment and a complete life cycle analysis is required. These analyses would need to be made on a case by case basis. If the technical specifications of nanomaterials are maintained from batch to batch, no toxicological assessment of different batches is required.

When characterising nanoparticles for risk assessment, in general the following properties need to be considered:

physical characteristics:

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

[3] (76/768/EEC) Article 1: A ‘cosmetic product’ shall mean any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition.

chemical characteristics**:**

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

In other words, the characterisation of the properties of nanoparticles themselves is insufficient. The interactions of the nanoparticles within a given environment must be examined including solubility/insolubility, free radical formation etc.

There are major data gaps in the assessment of the exposure and the uptake of nanoparticles via dermal absorption, inhalation, oral ingestion and eye contact.

The actual situation for dermal exposure is as follows:

1) There is evidence of some skin penetration into viable tissues (mainly into the *stratum spinosum* in the epidermal layer, but eventually also into the dermis) for very small particles (less than 10 nm), such as functionalised fullerenes and quantum dots.

2) When using accepted skin penetration protocols (intact skin), there is no conclusive evidence for skin penetration into viable tissue for particles of about 20 nm and larger primary particle size as used in sunscreens with physical UV-filters.

3) The above statements on skin penetration apply to healthy skin (human, porcine). There is an absence of appropriate information for skin with impaired barrier function, e.g. atopic skin or sunburned skin. A few data are available on psoriatic skin.

4) There is evidence that some mechanical effects (e.g. flexing) on skin may have an effect on nanoparticle penetration.

5) There is no information on the transadnexal penetration for particles under 20 nm. Nanoparticles of 20 nm and above penetrate deeply into hair follicles, but no penetration into viable tissue has been observed.

The situation for inhalation of nanoparticles can be summarized as follows:

1) During inhalation nanoparticles in a size range of 10 -100 nm deposit predominantly in the alveolar region of the respiratory tract and also in small bronchioles with deposition probabilities of 0.2 – 0.6. In contrast, nanoparticles < 10 nm will deposit predominantly in upper airways.

2) Nanoparticles will cross the epithelial barrier into interstitial spaces.

3) Translocation studies after inhalation and instillation were performed which show that nanoparticles translocate to various organs and are able to cross the blood-brain-barrier.

4) Inhalation of ultrafine particles has been linked to thrombotic (blood clotting) effects,

due to direct effect or via pulmonary inflammation.

5) Clearance from the lung can be slow, because of
   a. lower phagocytic activity of macrophages;
   b. poor clearance from the alveoli;
   c. retention in the interstitium.

   and therefore nanomaterials may persist in the lung.

6) Possible local effects in the lung are
   d. Oxidative stress, induced by the particle itself and/or by the activation of phagocytotic and epithelial cells;
   e. Inflammatory response ;
   f. Mutagenity/genotoxicity: whilst there is evidence of such effects in cultured lung cells, there are no data on the genotoxicity of nanomaterials after inhalation exposure;
   g. Cytotoxicity.

The situation for intestinal and eye exposure to nanoparticles may be summarized as follows:

- There is absence of information.

The situation for cellular studies may be summarised as follows:

1. Various skin cells have been studied *in vitro* for their cellular responses to nanoparticles. The observed effects have been diverse, ranging from internalization via endocytotic and non-endocytotic mechanisms, increased calcium and reactive oxygen species concentration within cells, and an effect on cell proliferation and cell viability.

2. Although *in vitro* tests may be useful for hazard identification, no alternative methods, validated or optimized by using nanomaterials exist. Nanoparticles diffuse, settle and agglomerate in cell culture media as a function of the environment (media, ionic strength, acidity, and viscosity) and particle properties (size, shape and density). Consideration of these properties and effects would significantly improve the basis for nanoparticle toxicity testing.

3. *In vitro* assays (validated and non-validated) are at present unavailable for appropriate risk assessments.

The overall situation may be summarised as follows:

At present, there is concern about insufficient knowledge in the following areas:

- Hazard identification;
- Exposure assessment;
- Uptake (including physiologically compromised human skin);
- The role of physico-chemical parameters of nanoparticles determining absorption and transport across membranes in the gut and lungs;
- The role of physico-chemical parameters of nanoparticles in systemic circulation determining biokinetics and accumulation in secondary target organs;
- Possible health effects (including susceptible individuals);
- Translocation of nanoparticles via the placenta to the foetus;
- *In vitro* and *in vivo* test methods validated or optimized for nanomaterials.

Responses to the questions in the Terms of Reference

Question 1

In view of the concerns recently raised about the use of nanomaterials in cosmetics the SCCP is requested to review and, if appropriate, to amend its notes of guidance for the testing of cosmetic ingredients and their safety evaluation as concerns cosmetic ingredients in the form of nanomaterials, including nanoparticles and nanoliposomes, and in particular as regards skin absorption and resorption of these substances. In assessing this, regards should be made to differing skin conditions, different sizes of particles and to question whether mass unit is the appropriate basis for regulating the exposure to nanomaterials. Possible implications on animal testing of nanoparticles and nanoliposomes should be addressed.

In the safety evaluation of nanomaterials, actual or intended marketed nanomaterials should be used for material characterisation and hazard identification. Furthermore, distinction should be made between soluble and/or biodegradable versus insoluble and/or biopersistent nanomaterials. Nanoparticles which disintegrate into molecular species upon application have to be distinguished from insoluble particles. For the former, conventional risk assessment methodologies based on mass metrics *may* be adequate for their use in cosmetic products, whereas for the latter (e.g. TiO_2, ZnO, fullerenes, carbon nanotubes, and quantum dots) other metrics are needed. A complete characterisation of physico-chemical characteristics and properties is required for these nanomaterials. Particle size, particle number, shape and surface characteristics are considered essential additional metrics. Consideration should also be given to certain moieties (e.g. surface modifications) on nanomaterials which could possibly enhance or reduce potential adverse health effects.

In traditional risk assessment, skin penetration studies are carried out using healthy or intact skin. Possible enhanced uptake in the case of impaired skin is considered to be covered in the Margin of Safety (MoS) approach. However, in the case of nanomaterials the conventional MoS may not give an adequate expression of the safety. If there is any penetration into the vital layers of the skin there may be a transfer to the systemic circulation. It may be anticipated that any systemic absorption will be more likely in conditions of abnormal skin e.g. sunburnt, atopic, eczematous, psoriatic skin. There is evidence that physical, in particular mechanical, and/or chemical action on the skin may have an effect on nanoparticle penetration.

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.

There are large data gaps in risk assessment methodologies with respect to nanoparticles in cosmetic products. To evaluate possible pulmonary effects (and the linked systemic effects), simple *in vitro* systems exist, e.g. to study cytotoxicity, pro-inflammatory effects. However, these are not suitable for studying effects that reflect the complexicity of the lung. *In vitro* models for systemic and (sub-)chronic toxicity do not yet exist and need to be developed, in particular for translocation, biodistribution, accumulation and clearance studies. Therefore, *in vivo* studies on potentially toxic nanomaterials are still necessary.

Size dependence of the deposition probability of inhaled nanoparticles is reasonably understood in the respiratory tract of healthy subjects; however, for individuals with respiratory disorders, predictions for nanoparticle deposition probability are limited.

The biodistribution (toxicokinetics) of nanomaterials has not been studied in detail. Therefore, it is impossible to model, *in silico*, hazard characterization and the distribution of nanomaterials. In particular, there is limited information on the role of physico-chemical parameters of nanoparticles determining their absorption and transport across barriers, e.g. skin, gut, lungs and eye, and their subsequent uptake in the systemic circulation, metabolism, potential accumulation in secondary target organs and excretion.

Mutagenicity/genotoxicity testing is required in general for cosmetic ingredients, including nanomaterials, but the specific characteristics of nanoparticles may require further consideration. The mutagenic/genotoxic *potential* of nanoparticles could probably be assessed in mammalian cells *in vitro*. The presently validated *in vivo* genotoxicity tests, however, do not cover the expected target organs of nanoparticles (particularly the respiratory tract) and have not been validated or optimized for reference substances including nanomaterials for cosmetics.

All *in vivo* and *in vitro* risk assessment methods for nanomaterials are still under development. Although some validated *in vitro* methods do exist they have not yet been validated and/or optimized with nanoparticles as reference compounds. This implies that for safety assessment of cosmetic ingredients, there are no validated *in vitro* methods available for nanoparticles.

Although animal testing can be largely reduced for skin penetration studies, it remains essential for translocation and accumulation studies as well as for chronic toxicity studies. Finally, the SCCP emphasizes that for the safety assessment of cosmetics, the 7th Amendment of the Cosmetic Directive (76/678/EEC) imposes animal testing and marketing bans, which will soon prohibit *in vivo* testing of cosmetic ingredients. Only validated *in vitro* methods are to be used for risk assessment.

Each safety dossier concerning nanomaterials needs to be evaluated on a case by case basis.

Question 2

In the light of the findings under question 1, does the SCCP consider it is necessary to review existing opinions on nanosized TiO_2 and ZnO as cosmetic ingredients and if appropriate to identify which additional elements are required for the submission of a safety file?

A complete safety dossier on micronsized and nanosized ZnO was requested by SCCNFP in its opinion on ZnO in 2003 (SCCNFP/0649/03). An opinion on the safety of such materials will be dependent on the availability of on an adequate dossier.

The SCCNFP opinion from 2000 (SCCNFP/0005/98) is on micro-crystalline preparations of TiO_2 and preparations of coarse particles. However, since this opinion new scientific data on nanosized particles, including TiO_2 has become available. Therefore, the SCCP considers it necessary to review the safety of nanosized TiO_2 in the light of recent information. Also, a safety assessment of nanosized TiO_2, taking into account abnormal skin conditions and the possible impact of mechanical effects on skin penetration need to be undertaken.

5. MINORITY OPINION

None

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APPENDIX 1 GLOSSARY OF TERMS USED IN THE OPINION

The glossary is intended to be helpful and indicative of the current nomenclature and understanding, but is not meant to be exhaustive. It uses the various terms of nanotechnology mainly according to the recently published "Publicly Available Specification on the Vocabulary for Nanoparticles" of the British Standards Institution (BSI 2005) with explanatory text from the point of view of cosmetics (the original BSI terms are given in Italics).

Agglomerate: group of particles held together by relatively weak physical forces, including van der Waals forces, electrostatic forces and surface tension.
Aggregate: heterogeneous particle in which the various components are not easily broken apart.
Secondary particles are formed through agglomeration or aggregation of primary particles (smallest identifiable subdivision in a particulate system). Attention is drawn to the inconsistent definitions in the literature of agglomerate and aggregate, which reflect the uses of these terms according to the industry context. It is recommended that when the term agglomerate is used that it be specified whether the bonding is strong or weak. Aggregate refers to strongly bonded associated particles that cannot easily be re-dispersed by mechanical means.
Carbon Nanotubes: *nanotubes consisting of one or several graphene sheets rolled up into a seamless tube, forming a single- or multi-walled tube.*
A carbon nanotube is an allotropic form of carbon (i.e. different molecular configurations). Carbon nanotubes can be either single-walled comprising a single layer of carbon atoms arranged in a cylinder, or multi-walled comprising multiple concentric tubes with diameters significantly greater. A carbon nanotube is a. member of the fullerene structural family.
Cellular dose: The quantity of nanomaterial adsorbed and/or internalized into the cell**.**
Coacervation: *separation, by addition of a third component, of an aqueous solution of a macromolecule colloid (polymer) into two liquid phases, one of which is colloid-rich (the coacervate) and the other an aqueous solution of the coacervating agent (the equilibrium liquid).*
A coacervate is a spherical aggregation (size 1 to 100 micrometers) of lipid molecules making up a colloidal inclusion which is held together by hydrophobic forces. Coacervates possess osmotic properties and form spontaneously from certain weak organic solutions.
Colloid: substance consisting of particles not exceeding 1 µm dispersed in a fluid
Colloidal nanoparticles: Colloidal suspension
Colloidal Suspension: particles suspended in a liquid that are too fine to settle out under the effect of gravity and are not readily filtered.
Cosmetic product: *any substance or preparation intended to be placed in contact with the various parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition* (Art. 1 of 93/35/EEC)
Ferrofluid*: colloidal suspension of ultramicroscopic magnetic particles in a carrier liquid*.
A ferrofluid is a liquid that becomes strongly polarized in the presence of a magnetic field. It is composed of nanoscale ferromagnetic particles suspended in a carrier fluid, usually an organic solvent or water. The magnetic nano-particles are coated with a surfactant to prevent their agglomeration (due to van der Waals and magnetic forces). There are a number of magnetic states of which ferromagnetism and superparamagnetism are the most prominent ones within ferrofluids. The ferromagnetic order in particles breaks down even below the Curie temperature if the particle size falls short of a certain value, usually in the order of 1-10 nm when the particle becomes superparamagnetic.
Fullerene*: any closed-cage structure having more than twenty carbon atoms consisting entirely of three-coordinate carbon atoms.*
The best known and most stable fullerene, buckminsterfullerene (C60, nicknamed

"buckyball"), has 60 carbon atoms arranged like a standard soccer ball. A fullerene allows other molecules to penetrate into it like a reservoir which may then permit a controlled release and delivery of the substance from fullerene. Ions can also be implanted by high energy implantation. Inorganic fullerene-like material exists, they are nanoparticles with a layered fullerene-like structure but composed of non-carbon atoms. Fullerene functionality and toxicity depends on the C-C distance and added functionalities.

Liposome*: artificial microscopic vesicle consisting of an aqueous core enclosed in one or more phospholipid layers.*

Phospholipids consist of a nonanoparticlesolar (hydrophobic) structure with a polar (hydrophilic) structure at one end. When dispersed in water, they spontaneously form bilayer membranes, which are composed of at least two monolayer layer sheets of lipid molecules with their nonanoparticlesolar surfaces facing each other and their polar surfaces facing the aqueous medium.

Nanocapsule: *submicronic particles made of a polymeric capsule surrounding an aqueous or oily core.*

Nanoencapsulation has evolved from and can be considered to be the miniaturisation of microencapsulation. The basic reason for nanoencapsulation is to protect the core material and to then release it when it is required. Applications can be: chemical/drug targeted delivery systems that release the ingredient when arrived at the site in the body where it is required, chemical/drug timed release delivery where the nanoencapsulated material slowly allows the ingredient to be released into the body, increased shelf life and stability of fragile chemicals.

Nanocluster*: group of atoms or molecules whose largest overall dimension is typically in the range of a few nanometers*.

This is a subset of nanoparticles

Nanocomposite: *composite in which at least one of the phases has at least one dimension on the nanoscale.*

Nanocrystal*: nanoscale solid formed with a periodic lattice of atoms*.

A nanocrystal is a crystalline material with dimensions measured in nanometers or a nanoparticle with a structure that is mostly crystalline. Many of their electrical and thermodynamic properties show strong size dependence.

Nanofibre: *nanoparticle with two dimensions at the nanoscale and an aspect ratio of greater than 3:1.*

Types of nanofibre include: nanowhiskers, nanorods and nanowire.

Nanomaterial: *material with one or more external dimensions, or an internal structure, on the nanoscale, which could exhibit novel characteristics compared to the same material without nanoscale features.*

As a particle decreases in size, a greater proportion of atoms are found at the surface compared to those inside. Thus nanomaterials have a much greater surface area per unit mass compared with larger particles so, they have novel characteristics that might include increased strength, chemical reactivity, conductivity or electrical characteristics. Porous materials such as e.g. zeolites have large internal surfaces and play a role in many applications, e.g. in catalysis. However, they usually are not called nanomaterials because their physical outer dimension usually exceeds 100 nm by far.

Nanoparticle: particle with one or more dimensions at the nanoscale.

A nanoparticle is defined as a particle with at least one dimension <100nm. Solid, semi-solid and soft nanoparticles have been manufactured. A prototype nanoparticle of semi-solid nature used for cosmetic formulation is the liposome. Suspensions of nanoparticles are possible because the interaction of the particle surface with the solvent is strong enough to overcome differences in density. Nanoparticles often have unexpected visible properties because they are small enough to scatter visible light rather than absorb it.

Nanopolymer: *nanostructured polymers (the repetition of units of atoms – monomers - in their chains, polymers are including substances from proteins to high-strength kevlar fibres). Nanopolymers may be of different shape (e.g., platelets, fibers, spheroids) but at least one dimension must be ca. 1 to 50 nm.*

Nanorod: *straight solid nanofibre* with an aspect ration smaller than 2:1.

Nanoscale: *having one or more dimensions of the order of 100 nm or less.*

Nanotechnology components are ranging in size between 1 nanometer (nm) and 100 nanometers.
Nanoscience: the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales, where properties differ significantly from those at a larger scale.
Nanospheres: *matrix systems made of solid polymer or solid lipids (called solid lipid nanoparticles).*
Nanostructured materials: *having a structure at the nanoscale*
Nanotechnology: *the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale.*
Nanotechnology is a sub classification of technology in colloidal science, biology, physics, chemistry involving the study of phenomena and manipulation of material at the nanoscale level. Two main approaches are used in nanotechnology: one is a "bottom-up" approach where materials and devices are built up atom by atom, the other a "top-down" approach where they are synthesized or constructed by removing existing material from larger entities. Nanotechnology is also used as an umbrella term to describe emerging or novel technological developments associated with microscopic dimensions.
Nanotubes: *hollow nanofibre*
Nanotubes are consisting of one or several graphene sheets (organic carbon nanotube) or non-carbon atoms like boron nitride (inorganic nanotube) rolled forming a single- or multi-walled tube.
Niosome: *Liposome made of synthetic non-ionic lipids instead of phospholipids.*
Particle dissolution*: the process of a particle going into solution*.
The substance causing it to dissolve, i.e. the dissolving medium, is called the solvent. Dissolution corresponds to a change from a solid to a fluid state (solution) by heat or moisture (liquefaction, melting).
Particle solubility*: a measure of the ability of a given substance to dissolve in a liquid.*
The amount of substance that can be dissolved in a liquid under specified conditions characterises the solubility. Usually it is measured by the mass (weight) of substance to dissolve per unit volume of solvent (water or another liquid). Solubility may be expressed as a ratio or may be described using words such as insoluble, very soluble or miscible. Aqueous solubility is the maximum concentration of a chemical that will dissolve in pure water at a reference temperature. In biological systems the dissolution kinetics is particularly important.
Percutaneous/dermal absorption: global term which describes the passage of compounds across the skin. This process can be divided into three steps:

1. penetration is the entry of a substance into a particular layer or structure such as the entrance of a compound into the stratum corneum,
2. permeation is the penetration through one layer into another, which is both functionally and structurally different from the first layer,
3. resorption is the uptake of a substance into the vascular system (lymph and/or blood vessel), which acts as the central compartment.

Phase separation: transformation of a homogenous system in two (or more). Phase separation occurs with partially miscible solvents and induces the crystallization of a solid from a solution.
Polymersome*: bilayered membranes of amphiphilic synthetic polymers which are similar to liposomes.*
Polymersomes exhibit increased stability and reduced permeability than liposomes. Furthermore, the use of synthetic polymers enables the controlled manipulation of the characteristics of these capsules,
Primary or secondary target organ*:* organ of $_{\text{nanoparticles}}$ intake are primary target organs (e.g. respiratory tract, gastro-intestinal tract, skin, eyes, etc.) in contrast $_{\text{nanoparticles}}$ are transported by biokinetic pathways to secondary target organs $_{\text{nanoparticles}}$ in which they may accumulate.
Quantum dot: *nanoscale particle that exhibits size dependent electronic and optical properties due to quantum confinement.*
A quantum dot contains a small number (of the order of 1-100) of conduction band

electrons, valence band holes, or excitons (pairs of conduction band electrons and valence band holes). Therefore a quantum dot has a discrete quantized energy spectrum. One of the optical features of quantum dots is colour. However, coloration is also shown by metallic nanoparticles like Au nanoparticles due to surface plasmons.

Rheological properties: *properties of materials (liquids, semi solids, solids) that describe their ability to deform and flow as a function of temperature, pressure, and chemical conditions.*

Rheology is part of mechanics which deals, when a mechanical force is exerted on a material, with the relation between force and deformation in material bodies.

Specific surface area: ratio of the surface area to the mass of a nanopowder

Ultrafine particle: *term traditionally used by the aerosol research, occupational and environmental health communities to describe airborne particles smaller than 100 nm in diameter.* Although no formal distinction exists between "ultrafine particles" and "nanoparticles", the term "ultrafine" is frequently used in the context of nanometer-diameter particles that have not been intentionally produced but are the incidental products of processes involving combustion, welding, or diesel engines. As a result, the two terms are sometimes used to differentiate between engineered (nanoparticle) and incidental (ultrafine) nanoscale particles. "Nanoparticle" and "ultrafine" are not rigid definitions. For example, since the term "ultrafine" has been in existence longer, some intentionally-produced particles with primary particle sizes in the nanosize range (e.g., TiO_2) are often called "ultrafine" in the literature (NIOSH 2006).

APPENDIX 2 - STRUCTURE OF SKIN

Macroscopically, skin comprises three main layers: the epidermis, the dermis (~0.1 and 1 mm in thickness, respectively) and the hypodermis. The dermo-epidermal junction is highly convoluted. Other anatomical features of the skin of interest are the appendageal structures: the hair follicles and sweat glands. The epidermis is a stratified, squamous, keratinising epithelium. The epidermis *per se* can be divided into five distinct strata which correspond to the consecutive steps of keratinocyte differentiation. The ultimate result of this differentiation process is formation of the functional barrier layer, the stratum corneum (~0.01 mm). The stratum basale or basal layer is responsible for the continual renewal of the epidermis (a process which normally takes 20-30 days). Proliferation of the stem cells in this layer creates new keratinocytes which then push existing cells towards the surface. During this upward transit, the keratinocytes begin to differentiate, finally achieving terminal differentiation in the stratum corneum. The epidermis is avascular and as such must receive all nutrition by passive diffusion from the microcirculation in the upper dermis.

The stratum corneum is usefully thought of as a "brick wall", with the fully-differentiated corneocytes comprising the 'bricks', embedded in the 'mortar' created by the intercellular lipids. The corneocytes are flat, functionally dead cells, the cytoplasmic space of which is predominantly keratin. Filling the intercellular spaces are various lipids, organized into extremely well-ordered, multilamellar, bilayer sheets. A layer of lipid covalently-bound to the cornified envelope of the corneocyte is also believed to contribute uniquely to this exquisite organisation. The intercellular lipids of the stratum corneum are composed of an approximately equimolar mixture of ceramides, cholesterol and free fatty acids. These non-polar and somewhat rigid components of the stratum corneum's 'cement' play a critical role in barrier function.

The dermis, the inner and larger (90%) skin layer, comprises primarily connective tissue and provides support to the epidermis. The dermis incorporates blood and lymphatic vessels and nerve endings. The extensive microvasculature network found in the dermis represents the site of resorption for drugs absorbed across the epidermis; it is at this point that transdermally absorbed molecules gain entry to the systemic circulation and access to their central targets.

The dermis also supports skin's appendageal structures, specifically hair follicles, sweat, sebaceous and apocrine glands. The pilosebaceous unit comprises the hair follicle, the hair shaft and the sebaceous gland. The hair follicle is an invagination of the epidermis that extends deeper into the dermis. The lining of the lower portion of the hair follicle is not keratinised and presumably offers a lesser barrier to diffusion than the normal stratum corneum. Under the dermis is the hypodermis or subcutaneous fat layer, which has mainly a protective role.

The total surface area of the skin of an adult person is approximately 1.5 – 2 m^2. In cosmetic products, the skin is the usual target organ for exposure because many products are for direct application to the skin.

With respect to percutaneous penetration, interest in these structures has centered upon the possibility that they may provide "shunt" pathways across the skin, circumventing the need to cross the full stratum corneum. While this is plausible, the practical significance is generally small because the follicles occupy a relatively insignificant fraction of the total surface area available for transport (~0.1%). As noted later, however, appendageal transport may assume a much more important role when specialised technologies are used to improve (trans)dermal delivery.

Stratum corneum

On average, there are about 20 cell layers in the stratum corneum, each of which is ~0.5μm in thickness. However, the architecture of the layer is such that this very thin structure limits, under normal conditions, the passive loss of water across the entire skin surface to only about 250 mL per day, a volume easily replaced in order to maintain homeostasis. This remarkable fact is achieved despite the large area across which transport can occur (1.5 to 2 m^2 in adults) and despite the significant water concentration gradient between the inner and outer surfaces of the stratum corneum. The critical barrier function of this layer can be illustrated simply by measurements of transepidermal water loss as the stratum corneum is progressively removed by adhesive tape-stripping.

The link between skin barrier function and stratum corneum lipid composition and structure has been clearly established. For example, changes in intercellular lipid composition and/or organisation typically results in a defective and more permeable barrier. Lipid extraction with organic solvents provokes such an effect. Skin permeability at different body sites has been correlated with local variations in lipid content. And, most convincingly, the conformational order of the intercellular lipids of the stratum corneum is correlated directly with the layer's permeability to water.