National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Interaction of inorganic nanoparticles with the skin barrier

Relevant studies for human dermal risk assessment of nanomaterials in consumer products

RIVM Letter report 340370006/2012

Colofon

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This investigation has been performed by order and for the account of the Dutch Food and Consumer Products Safety Authority, within the framework of Risks of nanomaterials in non-food products (9.1.6)

Rapport in het kort

Interactie van inorganische nanodeeltjes met de huid

Relevantie voor de humane risicobeoordeling van nanomaterialen in consumentenproducten

Consumentenproducten die op de markt komen, en hun ingrediënten, moeten veilig zijn. Dit geldt ook voor de groeiende markt aan consumentenproducten waarin nanomaterialen verwerkt zijn, zoals cosmetica en textiel. Het is op dit moment nog moeilijk om de veiligheid van consumentenproducten met nanomaterialen te beoordelen omdat nog weinig over het gedrag en de schadelijkheid van nanomaterialen bekend is. Aan de andere kant is ook onduidelijk of de testen waarmee blootstelling, gedrag en schadelijkheid getest worden voor een veiligheidsbeoordeling, wel toepasbaar zijn voor nanomaterialen.

In Europa heeft het Wetenschappelijke Comité voor Consumenten Veiligheid (WCCV) de taak om stoffen in consumentenproducten (voornamelijk cosmetica) te beoordelen op hun veiligheid. Een belangrijke route waardoor consumenten in contact komen met chemische stoffen in cosmetica is de dermale route (via de huid). Om te kunnen bepalen of een product dat op de huid wordt toegepast, veilig is voor de consument, is het van belang om te bepalen of en hoeveel van een stof door de huid heen kan dringen en in het menselijk lichaam terecht kan komen. Hiervoor zijn een aantal standaard testen beschikbaar (zgn. huid-penetratie testen).
De vraag die in dit rapport aan de orde komt is enerzijds of nanomaterialen door de huid heen kunnen dringen. Hiervoor is een literatuurstudie uitgevoerd naar recente wetenschappelijke publicaties. Anderzijds is, aan de hand van opinies en documenten van de WCCV, onderzocht of de testen die gebruikt zijn voor de huid penetratie geschikt zijn voor nanomaterialen. Kan er op basis van deze testen een conclusie worden getrokken over de veiligheid van cosmetica die nanomaterialen bevatten?

Trefwoorden:
dermale penetratie van chemicalien, nanomaterialen in consumentenproducten, toepasbaarheid van penetratietesten voor nanomaterialen, humane dermale risicobeoordeling

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Summary

Consumers are exposed to chemicals via the use of consumer products containing a variety of chemical ingredients (including nanomaterials). These chemical substances are not allowed to be a concern for human health. One of the most predominant exposure routes from the use of consumer products is the dermal route, especially in the case of personal care products and cosmetics. The Scientific Committee on Consumer Safety (SCCS) of the European Commission provides opinions on health and safety risks of consumer products (e.g. cosmetic products and their ingredients). In the past few years, SCCS has published relevant documents on various aspects of risk assessment of cosmetic ingredients as well as on the *in vitro* assessment of dermal absorption.
In a dermal penetration test, the absorption or penetration of a substance through the skin barrier (stratum corneum, SC) and into the skin is measured. Standard test protocols for dermal absorption are the OECD test guidelines 427 (*in vivo*) and 428 (*in vitro*). Since *in vivo* testing will not be permitted in future for cosmetic ingredients, this report is focussed on the *in vitro* test and its basic criteria. Several determinants can affect dermal absorption, and consequently, a number of factors may affect dermal absorption when performing *in vitro* dermal absorption studies.

With the increasing number of consumer products containing nanomaterials, including cosmetics, there are a couple of questions with respect to dermal exposure. First, what is the state of knowledge of dermal penetration of nanoparticles? How do inorganic nanoparticles (NPs) in the size range <100nm, as well as submicron particles, interact with the skin barrier? Do they have the ability to penetrate the SC into the viable deeper skin layers? If so, what is the possible mechanism of skin penetration and what are the factors contributing to skin penetration? Are these mechanisms and factors different for nanomaterials when compared to the non-nano form of the same substances? What is the effect of a coating?

In the current report, an overview of recent data on dermal penetration of nanoparticles is given. From these data, it becomes clear that there is still a strong debate on the ability of inorganic NPs to penetrate the skin. From all studies reported in recent years, about 50% points to penetration of NPs and 50% points to an absence of penetration. The size range of the particles, which has been shown to be the primary determinant of skin penetration, is largely overlapping for particles reported to penetrate the SC (4 nm – 1.5 µm) and particles that could not (4 nm – few microns). In addition, no firm conclusions can be drawn on penetration of one specific nanomaterial. This is because there is a set of critical determinants of NP skin penetration, which are either general (not specific nano) factors like skin factors (skin model or skin treatment) and experimental factors (concentration of dispersion, exposure time, diffusion cell). On the other hand, particle characteristics are playing a role in skin penetration, like size, shape and surface charge of the particles.
One of the most critical issues in measurement of skin penetration of nanoparticles is detection/characterisation of nanoparticles in the skin. There is only a sparse fraction of NPs able to penetrate the skin (detection limit) and the integrity of the particulate nature is difficult to analyse. Techniques available are either qualitative microscopic techniques or more quantitative techniques that cannot detect particles themselves (only elemental composition).

In the last part of this report, several SCCS documents are used to determine data gaps for dermal exposure to nanomaterials and to assess the appropriateness of dermal penetration tests for nanomaterials.
In general, the standard OECD *in vitro* test is appropriate for nanomaterials, however, one of the specific concerns of the SCCS is that the probability that a nanoparticle can be quantified in the receptor fluid is extremely small. The recently published SCCS guidance on safety assessment of nanomaterials in cosmetics is used to analyse the elements that are required to be reported in a manufactured nanomaterials safety dossier. The exposure assessment for nano ingredients in cosmetic products should be performed according to the general principles as described in the SCCS Notes of Guidance. The skin penetration test with nanomaterials should be performed on healthy skin, measuring the effects of nanomaterials on compromised skin poses a challenge due to the current lack of standardised model(s) that can be used. Therefore, urgent research is needed to develop appropriate test models of compromised skin.
With regard to the risk assessment, the Margin of Safety (MoS) calculation for nanomaterials is the same as for conventional chemicals (MoS = NO(A)EL/ Systemic Exposure Dosage (SED)). In general, a MoS of > 100 is considered acceptable, the assessment factor of 100 is not specific for conventional chemicals but considered to be applicable and appropriate for nanomaterials as well. However, the classical methodology of comparing a dermal exposure to an oral NO(A)EL is challenging because of the low absorption of nanomaterials via the oral and dermal route of exposure, and the oral absorption value is normally not provided. Therefore, the calculation of the MoS for nanomaterials using route to route extrapolation is difficult to perform.
These (and other) issues on human dermal risk assessment of nanomaterials in cosmetic products are further illustrated based on recent opinions of nanomaterials as UV filters in sunscreens (ETH50 and ZnO).

1 Introduction

Consumers are frequently exposed to chemical substances in or released from everyday consumer products like paint, cosmetics, deodorant, cleaning products etc. In the past 10 years, an increase in the use of nanomaterials in consumer products has been observed. Nanomaterials are developed and used because of their new specific physico-chemical properties compared to the conventional material of the same chemical composition, which can lead to an improved functionality of the used material and the product in which it is incorporated.

Chemical substances in consumer products (including nanomaterials) are not allowed to be a concern for human health. To assess the risk of chemicals in a consumer product, it is very important to know the exposure to these chemicals. The exposure during use of the consumer product is mainly determined by the way the product is used (exposure scenario), the concentration of the ingredient in the product and the release of the substance from the product during use. Focusing on the human (specifically the consumer) exposure assessment as a part of the risk assessment, exposure can be via the oral, inhalation, and dermal route. Exposure to chemicals from the use of consumer products is predominantly via the two latter, i.e. the inhalation and dermal route.

This report is focused on the dermal risk assessment of chemicals in consumer products, with specific attention to nanomaterials and cosmetic products. What are the standard tests for dermal penetration and are these tests applicable for nanomaterials? What are the differences in the risk assessment of nanomaterials when compared to the risk assessment of the bulk chemical? Which other risk assessment issues have to be considered when focusing on nanomaterials in cosmetic products?

In chapter 2 of this report, background information is given on the dermal exposure to (non-nano) chemicals in consumer products. Also the penetration of chemicals through the skin and standard tests for dermal absorption are illustrated in this chapter. Chapter 3 describes the assessment of *in vitro* studies on dermal penetration. General test principles and determinants that influence skin penetration are described.
Chapter 4 is focused on the dermal absorption of nanomaterials. First, the potential dermal exposure to nanomaterials via the use of consumer products is illustrated. Second, the available studies on skin penetration of inorganic nanomaterials are described, including the mechanism of penetration of NP and the nano-specific factors that are influencing this penetration. In chapter 5, the dermal risk assement of nanomaterials is further discussed, based on recent SCCS opinions on nanomaterials in cosmetics.

2 Dermal absorption of chemicals

2.1 Dermal exposure to chemicals

Consumers are exposed to chemicals via the use of consumer products that contain a variety of chemical ingredients. These chemical substances in consumer products (including nanomaterials) may not be a concern for human health. Human chemical risk assessment involves the characterization of the chemical exposure compared to the intrinsic toxicity or hazard of that chemical to determine whether it is likely to result in adverse health effects in the exposed subjects (WHO 2006). Focusing on the human (specifically the consumer) exposure assessment as a part of the risk assessment, exposure can be via the oral, inhalation, and dermal route. Exposure to chemicals from the use of non-food consumer products and within occupational settings is predominantly via the two latter, i.e. the inhalation and dermal route (WHO 2006). For the dermal route of exposure, textiles, personal care products and cosmetics are important product categories.

2.1.1 Safety of cosmetic products in Europe and the SCCS

The Scientific Committee on Consumer Safety (SCCS, formerly known as SCCNFP and SCCP) of the European Commission, provides opinions on health and safety risks (chemical, biological, mechanical and other physical risks) of non-food consumer products (e.g. cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products) and services (e.g. tattooing, artificial sun tanning). Between its establishment in 1997 and its disbandment in 2004, the Scientific Committee on Consumer Non-Food Products (SCCNFP) provided opinions on more than 400 chemical substances and/or their mixtures and the Scientific Committee on Consumer Products (SCCP) has added more than 150 opinions to that list. The majority of these opinions have been adopted into Cosmetic Legislation as modifications of the Annexes to Directive 76/768/EEC (Art. 8.2 and Art. 10 of Directive 76/768/EEC).
In the past few years, SCCP and SCCS have published relevant documents on various aspects of risk assessment of cosmetic ingredients (Notes of Guidance, 2010b) as well as on the *in vitro* assessment of dermal absorption (basic criteria of in vitro assessment of dermal absorption, 2010a). These documents are used for discussion in the current report, where relevant.

2.2 Dermal absorption of chemicals

One of the primary roles of the skin is to form a barrier to protect humans from substances contacted in the environment such as chemicals in consumer products. The structure of the skin is depicted below (Figure 1).

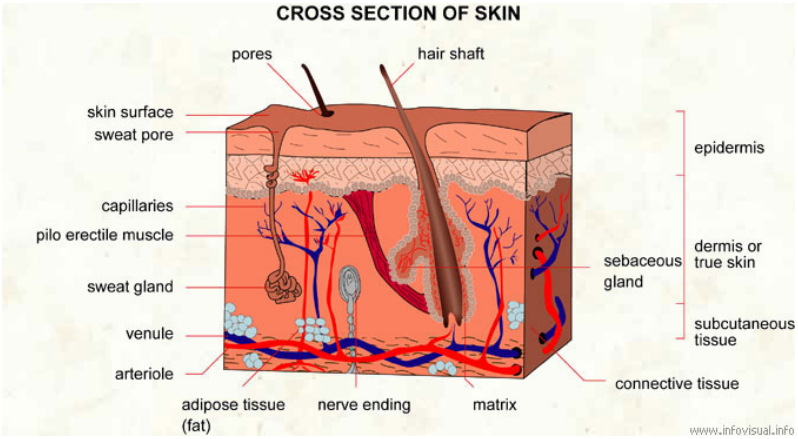

Figure 1. Cross section of skin - Visual Dictionary - Copyright © 2005-2008 - All rights reserved

The dermal absorption process describes the passage of compounds across the skin into the systemic compartment of the human body.

This process can be divided into three different steps (WHO, 2005):

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

The stratum corneum (SC) is the outermost layer of the skin. The quality of this top layer of the epidermis typically determines the rate of dermal penetration (see also chapter 4). Because the SC is the principal *in vivo* barrier against penetration and uptake of chemicals into the body, the use of *in vitro* dermal absorption studies on isolated skin is justified (SCCS, 2010a).

2.3 Dermal penetration testing (standard tests)

Testing of dermal penetration measures the absorption or penetration of a substance "through the skin barrier and into the skin" (Organisation for Economic Co-operation and Development, OECD, 2004a). Dermal penetration studies are conducted to determine how much of a chemical penetrates/ permeates the skin, and thereby whether it has the potential to be absorbed into the systemic circulation. Dermal penetration is considered to occur by passive diffusion; however, biotransformation of the test substance within the skin (metabolism) prior to systemic absorption can also occur (OECD, 2004a).
The OECD Guidelines for the Testing of Chemicals (Test Guidelines) are a collection of the most relevant internationally agreed testing methods used by government, industry and independent laboratories to assess the safety of chemical products. Within these guidelines, both in vivo and in vitro methods are available for determining the dermal penetration of a substance.

2.3.1 Skin Absorption: In Vivo Method (Test No. 427)

The *in vivo* percutaneous absorption study set out in Test Guideline 427 provides the linkage necessary to extrapolate from oral studies when making safety assessments following dermal exposure. The method allows the determination of

the penetration of the test substance through the skin into the systemic compartment.
The test substance, preferably radiolabelled, is applied, for a fixed period, to the clipped skin of animals at one or more appropriate dose levels in the form of a representative in-use preparation. The rat is the most commonly used species. At least four animals of one sex should be used for each test preparation and each scheduled termination time. A known amount of the test preparation is evenly applied to the site. This amount should normally mimic potential human exposure, typically 1-5 mg/cm² for a solid or up to 10 µl/cm² for liquids. A relevant exposure period (typically 6 or 24 hours) should be used, based on the expected human exposure duration. The animals should be observed for signs of toxicity/abnormal reactions at intervals for the entire duration of the study. This study includes daily measurements (excreta), regular detailed observations, as well as sacrifice at the scheduled time and blood collected for analysis (OECD, 2004b).
This guideline described *in vivo* dermal absorption and therefore, will not be permitted in future for cosmetic ingredients.

2.3.2 Skin Absorption: In Vitro Method (Test No. 428)

This test method has been designed to provide information on absorption of a test substance, (ideally radiolabelled), applied to the surface of a skin sample separating the two chambers (a donor chamber and a receptor chamber) of a diffusion cell. Static and flow-through diffusion cells are both acceptable.
Skin from human or animal sources can be used. Although viable skin is preferred, non-viable skin can also be used The use of viable skin is preferred in certain circumstances. Non-viable human or animal skin can be used; however, the ability to assess skin metabolism of the test substance will be lost. Viable skin has been shown to have the capability to metabolise some chemicals during percutaneous absorption. In this case, metabolites of the test chemical may be analysed by appropriate methods. Normally more than one concentration of the test substance is used in typical formulations, spanning the realistic range of potential human exposures. The application should mimic human exposure, normally 1-5 mg/cm^2 of skin for a solid and up to 10 µl/cm^2 for liquids. The temperature must be constant because it affects the passive diffusion of chemicals. The absorption of a test substance during a given time period (normally 24h) is measured by analysis of the receptor fluid, and the distribution of the test substance chemical in the test system and the absorption profile with time should be presented (OECD, 2004c).

For the specific application of this Test guideline in the safety testing of cosmetic ingredients, SCCS published a document- “Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients”- (SCCS, 2010a), in which an more detailed guidance is provided for the specific testing of cosmetic ingredients (chapter 3).

3 Basic criteria for the *in vitro* testing of dermal absorption of cosmetic (non-nano) ingredients

3.1 General principles and principle of the *in vitro* dermal absorption test

As already mentioned in chapter 2, *in vitro* studies on isolated skin are justified for the testing of dermal absorption of cosmetic ingredients. However, the following issues should be included in the protocol (SCCS, 2010a). Note that these considerations are general considerations for chemicals. Specific issues for dermal absorption of nanoparticles are further discussed in the next chapter.

- Studies should be performed on appropriate standardized skin preparations. The respective choice should be justified in the protocol. The WHO recommends human skin as the gold standard.
- At the end of the experiment, a full mass balance should be performed
- When considerable cutaneous metabolism of the test compound occurs *in vivo*, further studies may be necessary. For example, frozen skin preparations may lack the enzyme systems for biotransformation of the test compound and may not provide an accurate picture of the formation of metabolites and their dermal absorption.
- Sometimes an irreversible binding of an ingredient to the epidermis may occur, followed by elimination through *in vivo* desquamation of the skin surface. When this mechanism is assumed, it must be documented by separate experiments.

In principle, for dermal absorption studies, OECD guideline 428 should be followed as close as possible with skin preparations of natural origin. The test substance should be applied in an appropriate formulation on the skin sample, which is placed in a diffusion cell. The skin is positioned between the upper and lower chambers of the cell.

Diffusion cells may be of static or flow-through design. The integrity of the barrier should be checked by an appropriate method. The test sample should remain in contact with the skin on the donor side for a defined period of time, corresponding to the typical use of the cosmetic end product, such as leave-on or rinse-off conditions. The receptor fluid should be sampled at an early time point (e.g. after 30 minutes), at the end of the experiment and at appropriate time points in between in order to obtain an absorption-time profile. The skin and/or fluid samples should be analysed by appropriate and validated analytical methods, such as liquid scintillation counting, HPLC, GC or other suitable methods. Information on the sensitivity and repeatability / time-different intermediate precision of the analytical method(s) should be provided.

3.2 Methodology and factors affecting dermal absorption

Several determinants can affect dermal absorption (reviewed in SCCS, 2010a):

- physical and chemical properties of the substance
- type and composition of the formulation
- occlusion
- concentration of the substance in the formulation
- exposure pattern
- skin site of the body
- technical aspects of the respective *in vitro* test

Consequently, a number of factors may affect dermal absorption when performing *in vitro* dermal absorption studies; these are described below (see for more details, SCCS, 2010a).

3.2.1 Diffusion cell design

The diffusion cell consists of an upper donor and a lower receptor chamber, separated by the skin preparation under investigation. The stratum corneum faces the donor chamber. Diffusion cells should consist of inert non-adsorbing material. Temperature control of the receptor fluid is crucial throughout the experiment.

3.2.2 Receptor fluid

The composition of the receptor fluid is chosen so that it does not limit the extent of diffusion of the test substance, i.e. the solubility and the stability in the receptor fluid of the chemical under investigation have to be guaranteed. In addition, the receptor fluid should have a physiological pH. The receptor fluid, preferably degassed in order to avoid formation of air bubbles during the experiment, should be thoroughly stirred (static cells) or continuously replaced (flowthrough cells) during the entire experiment.

3.2.3 Skin preparations

The skin preparations used in the *in vitro* study are a very important determinant. Human skin is the best choice but is not always readily available. Alternatively, pig skin may be used because it shares essential permeation characteristics with human skin. Rat skin is not recommended because it is 2 to 10 times more permeable than human skin.
The following information of the skin samples is important in case of assessment of a dermal absorption study:
Origin of skin samples used, species, skin location, gender and age, fresh or frozen skin, details on preservation and storage conditions of the skin and numbers of skin samples and donors.

Skin samples that may be used are split-thickness (200-500 µm, preferred in case of human skin) or full-thickness (500-1000 µm, preferred for pig skin) skin preparations. Dermatomed skin is often used. Skin thickness should be measured by an appropriate method, which should be described in the report. When epidermal membranes are used for the *in vitro* dermal absorption study, the reason for this should be justified. The minimum skin area to be covered is 0.64 cm^2.

3.2.4 Skin integrity

Barrier integrity is crucial for the experiment, and must therefore be measured and reported. This is achieved by either measuring the penetration of a marker molecule or by physical methods.

3.2.5 Skin temperature

Because the rate and extent of skin absorption is temperature-dependent, the skin disc temperature should be maintained constant (32 ± 1°C, corresponding to the normal human skin surface temperature).

3.2.6 Test substance

The relevant physical and chemical data of the test substance should be given.

The purity of the test substance should be described and should be comparable to that of the substance in marketed products.

3.2.7 Preparation of the dose and vehicle/ formulation

The dose and vehicle / formulation should be representative for the in use condition(s) of the finished cosmetic product. The quantitative composition of every formulation used during the experiment should be given.

3.2.8 Dose and volume of the test substance

The dose of the test formulation as well as its contact time (exposure) with the skin should resemble use conditions. The amount of the formulation to be applied should be adapted to the consumer use/technical conditions.

3.2.9 Study period and sampling

The exposure time and sampling period(s) should be defined in the protocol. The normal exposure time is 24 hours with regular sampling intervals. The frequency of sampling should be chosen adequately to allow the determination of the extent/rate of absorption and the absorption profile. In order to estimate absorption kinetics, samples should be obtained from at least 6 post-application time points, including one early time point (30 minutes).

3.2.10 Analytical methods

Appropriate analytical techniques, e.g. liquid scintillation counting, HPLC or GC, should be used.

3.2.11 Data collection

The test compound must be determined in the following compartments:
- Product excess on the skin (dislodgeable dose)
- Stratum corneum (e.g. adhesive tape strips)
- Living epidermis (without stratum corneum)
- Dermis
- Receptor fluid

To calculate the mass balance correctly, it is also necessary to measure the amounts of test substance adsorbed to the equipment (included in rinsing solutions and/or compartments).

3.2.12 Mass balance analysis/ recovery

The mass balance of the applied dose must be determined. The overall recovery of test substance (including metabolites) should be within the range of 85-115%. Lower or higher recovery rates should be investigated and/or explained.

3.2.13 Variability/ validity/ reproducibility

The technical ability of the performing laboratory and the validity of the method used should be assessed at regular intervals. Factors that affect the variability in the dermal absorption of a test substance are:

- Inter-individual and intra-individual characteristics of the stratum corneum barrier,
- The variation in various parameters, such as skin temperature, skin thickness, vehicle, concentration of applied substance, amount of applied formulation, and exposure duration
- The use of static or flow through cell system
- The uncertainty of the measurement of the test substance.

For a reliable dermal absorption study, 8 skin samples from at least 4

donors should be used. When studies correspond to all of the basic requirements of the SCCS, the *mean + 1SD* will be used for the calculation of the MoS. The reason for not using the mean *per se* is the frequently observed high variability in the *in vitro* dermal absorption assays. Moreover, the method was validated based upon practical experience only and did not go through the elaborated validation process as we know it today.

The conclusion of the SCCS is that for (non-nano) cosmetic ingredients, the dermal absorption can be expressed as an absolute amount (ug/cm^2 of skin surface) and/ or as a percentage of the amount of test substance contained in the intended dose applied per square centimetre of skin surface. Furthermore, in a classical in vitro absorption setting, the amount of penetrated substance(s) found in the receptor fluid is considered to be systemically available. Both the epidermis (except for the stratum corneum) and dermis are considered as a sink, for which the amounts found in these tissues are considered as absorbed and are added to those found in the receptor fluid. The amounts that are retained by the stratum corneum at the time of sampling are not considered to be dermally absorbed, and thus they are not expected to contribute to the systemic dose.

The absorption rate and mass balance should be calculated separately for each diffusion cell. Considering skin samples as the main contributor of the variability in results of a dermal absorption study, the mean and SD of the dermal absorption rate should be calculated from at least 8 evaluable results representative of skin from at least 4 donors. All measurements, statistical processing and obtained kinetic curves should be provided.
The SCCS considers the following criteria as critical for the decision if a study fulfils the requirements:

- the availability of 8 evaluable samples originating from 4 donors (for studies performed previous to this decision, 3 donors may be accepted);
- a mass balance of the applied dose which shows to be ≥ 85%;
- a clear mention of the relative standard deviation (RSD or CV) of the measured dermal absorption rate;
- thorough consideration of all the factors described in the previous

When studies correspond to all of the basic requirements of the SCCS, the *mean + 1SD* will be used for the calculation of the MoS. In case of significant deviations from the protocol and/or very high variability, the *mean + 2SD* will be used as dermal absorption for the calculation of the margin of safety.
In case the results are derived from an inadequate *in vitro* study, 100% dermal absorption is used. However, in case MW > 500 Da and log P_{ow} is smaller than -1 or higher than 4, the value of 10% dermal absorption is considered [ECHA 2008].

4 Dermal absorption of nanomaterials

4.1 Dermal exposure to nanomaterials in consumer products

The market of consumer products claiming to contain nanomaterials is increasing very fast. Since the main product categories as described by Woodrow Wilson database (Figure 2) are “Personal care products”, “Textiles such as clothing”, “Cosmetics”, and “Sunscreens”, a very likely exposure route via which consumers are exposed to nanomaterials is the dermal route (via skin, hair, lips).

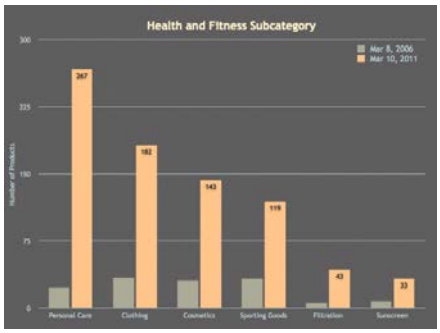

Figure 2. Subcategories of consumer products containing nanomaterials (source: http://www.nanotechproject.org/inventories/consumer/analysis_draft/)

Also in the recent RIVM inventory of Wijnhoven et al (2010), important product categories were “Personal care products and cosmetics”, “Textile” and “Home furnishing and household products”. These categories were having the largest number of newly identified products between 2007 and 2010, indicating that dermal exposure of consumers to nanomaterials via the use of consumer products is still increasing. In Table 1, product categories with potential dermal exposure as described above are further specified in subcategories and the corresponding number of new products are depicted (adopted from Wijnhoven et al, 2010).

Table 1. Number or products per product (sub) category of consumer products claiming to contain nanomaterials (as described earlier in Wijnhoven et al, 2010).

Personal care products and cosmetics	**304**
Care-Sun cosmetics	26
Care-Baby care products	17
Care-Hair care (shampoo, gel, hair dyes, etc.)	84
Care-Skin care (shower gel, creams, deodorant, foot care, shaving soap, etc)	155
Care-Make-up and nail care (lipstick, eye shadow, etc.)	8
Textile	**81**
Text-Clothing	55
Text-Other textiles (sheets, etc.)	8
Text-Coating	18
Home furnishing and household products	**108**
Home-Cleaning products	52
Home-Cooking utensils	3
Home-Construction materials	8
Home-Coating	45

There is a small set of inorganic materials explicitly referenced in consumer products claiming to contain nanomaterials. In the latest update of the Woodrow Wilson database, the most common material mentioned in the product descriptions is silver (313 products). Carbon, which includes fullerenes, is the second most referenced (91), followed by titanium (including titanium dioxide) (59), silica (43), zinc (including zinc oxide) (31), and gold (28) (http://www.nanotechproject.org/inventories/consumer/analysis_draft/). Apart from the use of nanomaterials in consumer products, also other sources for exposure to nanomaterials are increasing constantly, such as nanomaterials in the environment (water, food and air), but also people handling nanomaterials in research and industry.

4.2 Studies on dermal penetration of inorganic nanoparticles

The potential for applying nanomaterials in an increasing number of consumer products such as cosmetics has triggered the investigation of the interaction between nanomaterials, especially nanoparticles (NP) and the various biological barriers. The skin is an excellent biological barrier and has been addressed in several recent studies regarding NP penetration. The overview of penetration studies presented in this section is based on the recent review of Labouta and Schneider (2012).

There are two main reasons why skin penetration of inorganic particles is the subject of many recent studies.

1. Design of potential topical and transdermal nanocariers and biomedical diagnostic agents
2. Health risk analysis

The focus of this report is health risk analysis.

In the review of Labouta and Schneider (2012), recent research on the interaction of inorganic NPs with the skin barrier is discussed and analysed, in an attempt to answer the following questions:

- How do inorganic NPs in the size range <100nm, as well as submicron particles interact with the skin barrier?
- Do they have the ability to penetrate the SC into the viable deeper skin layers (DSLs)? If so,
- What is the possible mechanism of skin penetration?
- What are the factors contributing to skin penetration?

In this respect, it is important to note the difference between skin penetration (transport of the NP across the SC into the DSLs, see figure 3 for possibilities) and skin permeation (transport across the entire skin barrier into the receptor solution *in vitro* or systemic circulation *in vivo*).

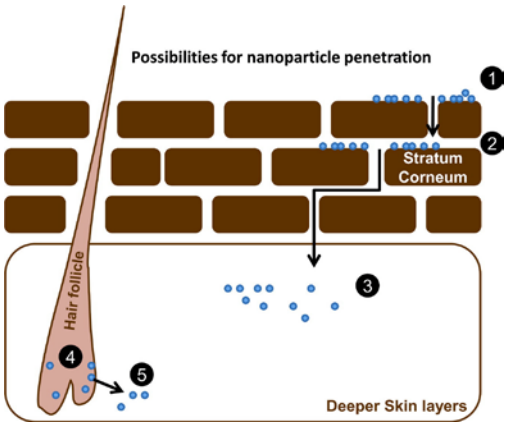

Figure 2. Possibilities for nanoparticle penetration through the skin (Labouta and Schneider, 2012)

Nanoparticles could remain on the skin surface (1), penetrate into the skin through intercellular pathways and localize in the stratum corneum (2) or even permeate the whole stratum corneum into deeper skin layers (3). On the other hand, hair follicles could act as a depot for particles (4) from where particles could further penetrate into deeper skin layers (5).

4.2.1 *Current dilemma in the status of skin penetration of NPs*

Although the ability and the possible mechanism of particle penetration through skin is an area of great interest by researchers since 2004, the ability of inorganic NPs to penetrate the skin is still a subject of strong debate.
Since 2004, a total number of 40 research articles have been published describing more than 125 penetration/ permeation experiments with a variety of NPs. Different outcomes were reported for particle penetration (reviewed by Labouta and Schneider, 2012); these are illustrated in detail in Appendix 1 of this report.

In short, 49% of all experiments were reported to result in particle penetration with five studies even showing particle permeation through the entire skin thickness either *in vitro* (2 studies) or *in vivo* (3). The other 51% of the experiments did not show any particle penetration. In the period 2008-2010, however, an increasing amount of positive penetration results were reported. This could be due to more approaches enhancing the penetration or better measurement techniques during the experiments.
The dilemma is further demonstrated by the fact that the size range of the particles, which has been shown to be the primary determinant of skin penetration, is largely overlapping for particles reported to penetrate the SC (4 nm – 1.5 µm) and particles that could not (4 nm – few microns).

4.2.2 *Results on penetration of relevant nanoparticles in consumer products*

As mentioned already earlier in this chapter, a small set of nanomaterials is referenced frequently in consumer products: silver, carbon, titanium (including titanium dioxide), silica, zinc (including zinc oxide), and gold.

In table 2, a short simplistic summary of the results of penetration studies for these nanomaterials is given, based on the overview of Labouta and Schneider (2012). Studies on relevant nanomaterials of various shapes and sizes were selected, and only studies without any additional enhancement of penetration in the experiment are described. Study results have been divided in experiments using human and animal skin. For further details, see the overview in Appendix 1.

Table 2. Summary of penetration study results per nanomaterial

Nanomaterial (various forms and sizes)	Studies with human skin (n)	Penetration without enhancement yes/ no	Studies with animal skin (n)	Penetration without enhancement yes/ no
Au	7	4 x yes, 3 x no	2 (mouse and rat)	2 x yes
Ag	1	1 x yes but low	1 (porcine)	1 x no
TiO2	10	1 x yes, 9 x no	7 (porcine, mouse)	2 x yes, 5 x no
ZnO	4	4 x no	2 (porcine, mouse)	2 x no

From this table no firm conclusions or trends regarding penetration of one specific nanomaterial can be drawn. This is not surprising since it is a collection of experiments with nanomaterials of various sizes and shapes as well as different experimental set ups. Another important factor that could be the cause of the discrepancy in penetration is the dose (ml/ cm^2) that is used in the studies, which is not described in detail in the table in Appendix 1.
To understand the variation of the outcomes of the various skin penetration studies, the question is raised what the mechanism of skin penetration is and what the critical determinants of NP skin penetration are.

4.3 Mechanism of dermal penetration of NPs and critical factors for penetration

4.3.1 Mechanism of skin penetration of NPs

A possible explanation of skin penetration of NPs could be found in the skin architecture. The skin comprises three main layers: the epidermis, the dermis and the hypodermis (see also Figure 1) of which the SC in the epidermis is the main barrier for penetration of chemicals.
The SC consists of intercellular lipids, these are arranged in a head-to-head and tail-to-tail manner. This leads to both lipophilic pores (tail-to-tail) and hydrophilic pores (head-to-head) providing possible routes for NP penetration (Baroli, 2010). The question rises whether the NPs penetrate the SC via this intercellular pathway, as has been demonstrated in a study with PEG-coated quantum dots (QDs) (Zhang et al, 2008). Labouta et al (2011a) also found these intercellular lipids to be the main barrier for skin penetration of NPs, but it could also be the whole microstructure of the SC with its tortuous intercellular aqueous and lipidic channels. Another possibility is the combination of the intercellular and intracellular pathway as has been demonstrated for iron oxide by Lee et al (2010). In this study, particles were found to be distributed in both inter- and intracellular spaces of the viable epidermis. Finally, the follicular pathway could also be a possible mechanism for skin penetration of inorganic NPs. However, the skin follicles occupy only a very small fraction (1/1000) of the entire skin surface. For more details on skin structure and more explanations on possible mechanisms of skin penetration see also Appendix 2 (adopted from SCCP, 2007).

4.3.2 Factors affecting skin penetration of inorganic NPs

Labouta and Schneider (2012) describe in their review the following three groups of determinants that can influence skin penetration of NPs; skin factors,

experimental factors and particle factors. The first two groups of factors (skin factors and experimental factors) are general determinants for dermal penetration, not specifically applicable to nanomaterials. These factors are already discussed in more detail in the previous chapter for the assessment of dermal penetration studies for cosmetic ingredients. However, in the section below, the determinants will be discussed in light of dermal penetration studies of NPs.

1. Skin factors

a. Skin model
   i. Human skin is the gold standard (recommended by WHO)
   ii. Animal skin is structurally different from human skin
   iii. Pig skin is used for testing follicular penetration

b. Skin treatment
   i. Non-intentional
      Hair removal affects the skin barrier
      Formulation ingredients may affect the skin barrier and should be addressed
   ii. Intentional
      Most of the employed approaches are physical methods

Ad 1. Skin factors: Animal skin versus human skin.
For human dermal risk assessment, excised human skin is the gold standard for in vitro skin penetration studies. However, in the review of Labouta and Schneider (2012), it was only used in 51% of the experimental set ups (35% in vitro and 16% in vivo). Due to the limited availability of human skin, 47% of the skin penetration studies of inorganic NPs were conducted on animal skin (pig, mouse and rat skin) in vitro ((31%) or in vivo (16%). There are structural and morphological differences between animal and human skin, especially in density of hair follicles, thickness of SC and total skin, and the amount of skin lipids.

Furthermore, hairy animals are used and often hair removal is necessary to be able to apply a formulation. It is known that hair removal can have an effect on the barrier function of the skin used and consequently, particle penetration might also be affected. In addition to this, SCCP described already in their first opinion on the safety of nanomaterials in cosmetic products in 2007 that the large differences in follicular density in haired species compared to man may influence the outcome of the tests for 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 to a reasonable extent 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 (SCCP, 2007).

This implies that the dermatomed skin of thickness 200-400 µm, recommended by OECD guideline 428, could possibly overestimate the skin penetration of NPs. This is especially the case for particles likely to accumulate in the hair follicles because the hair follicle is cut on splitting the dermis, and the NPS can then diffuse into the dermis/ receptor solution (Senzui et al, 2010).

Ad 1. Skin factors: Skin treatment approaches

Different skin treatment approaches, both physical and chemical, were adopted for inducing or enhancing skin penetration of NPs. This is in addition to other factors like formulation factors (surface coatings and vehicle) which differ greatly from one study to another.

2. **Experimental factors**
    a. Concentration of the nano-dispersion
        i. A concentration series should be tested
    b. Skin exposure time
        i. A minimum of 6 hrs or better 24 hrs
    c. Other factors
        i. Diffusion cell (static versus flow through cells, but expected to have minimal effect on particle penetration)
        ii. Volume of dispersion/ diffusion area

Ad 2. Experimental factors: Skin exposure time
Skin exposure time also differed greatly among studies. Penetration of particles has been tracked over a period of a few hours (1 hour, 3 hours) up to several days (60 days). For particles, it is not practically feasible to generate appropriate pharmacokinetic parameters as is usually done for penetration of drug molecules. This further limits the ability to analyse and combine data of different studies raising an analytical problem in determination of the amount of NPs present in the skin in typical penetration/ permeation experiments.

Ad 2. Experimental factors: Diffusion cell
Different results were also observed on using flow-through diffusion cells versus static Franz diffusion cells. However, this is expected to have a small effect on the results.

Ad 2. Experimental factors: Application dose and volume and diffusion area
The application dose and volume and the diffusion area are very important factors that would significantly affect results of any penetration experiment. This is illustrated by the following example. According to Labouta et al, AuNPs are not able to cross the SC of human skin (Labouta et al, 2011 a,b). However, Sonavane did show permeation of AuNps prepared by the same method and almost the same size (Sonavane et al, 2008). The difference between the experiments is the volume of donor solution per square centimetre of skin surface. The permeation of AuNPS occurs in the experiment that has a 2.2 times larger volume of donor solution (0.6 ml/ cm^2 versus 0.28 ml/ cm^2). → ref to appendix
Therefore it is inappropriate to draw generalized overestimated conclusions on the safety of NPs on topical application based on experiments with one single concentration.

3. **Particle Factors**
    a. Particle size, shape and surface charge
    b. Nanodispersion stability and skin contact
    c. Vehicle nature

Ad 3. Particle factors: Physico-chemical attributes of the NPs and formulation factors
The physicochemical characteristics of NPs and the nature of the dispersing vehicle are key factors governing their skin penetration. Also the effect of particle surface charge and shape on penetration through skin has been demonstrated. Aside from the surface charge, also the hydrophobicity of the

particle surface has recently been shown to favour skin penetration of AuNPs through human skin.
Furthermore, tracking of the physical state of the particles during or after contact with the skin barrier is also required for better interpretation of the of skin penetration experiments. Aggregation of applied particles over time limits the availability of the individual particles that would have a higher probability of penetrating the barrier.
Besides the physicochemical parameters, the vehicle nature could affect the barrier state of the skin or the physical state of the particles, and thus penetration.

The exact contribution of the relevant parameters for potential skin penetration of inorganic NPs is still unknown.

4.4 What is the nano effect in skin penetration?

In dermal penetration studies with NPs, the size of the nanoparticle is the most prominent parameter (Labouta and Schneider, 2012). However, it was mentioned already before that in the available studies on NP penetration (see table 1, Appendix 1), major overlap exists for particles reported to penetrate the SC (4 nm – 1.5 µm) and particles that could not (4 nm – few microns). This overlap could be due to diversity in experimental set up of the studies.

To further investigate the actual importance of the particle size in skin penetration, the reported data in Labouta and Schneider (2012) were analysed in an attempt to select on real-case scenarios for human dermal risk assessment.
For this, 22 different studies on excised human skin were selected. This is regarded as the “gold standard” since human in vivo studies on volunteers are very difficult to perform (reviewed in Labouta and Schneider, 2012). Of these 22 studies, 14 studies showed no particle penetration into the DSLs using either *in vitro* or *in vivo* human skin, as well as human skin grafted in immune deficient mice. The eight remaining studies were very difficult to interpret because either the penetration was induced after skin treatment, or the applied nanodispersion included ingredients that could have influence on the skin integrity, thus favouring particle penetration. In conclusion, it is difficult to dissect the effect of nanosize on dermal penetration, since it appears to be an interplay of multivariate factors including the physicochemical characteristics of the NPs as well as the formulation, environmental and mechanical factors.

4.5 Detection of inorganic NPs in the skin

Currently, monitoring and accurately quantifying the amount of drugs present in the skin after penetration/ permeation experiments is feasible for chemicals because of the rapid development of sensitive analytical techniques in the past decades. High Pressure Liquid Chromatography (HPLC) is a convenient method with a suitable limit of detection. However, for detection of NPs, the available techniques are limited or a combination of techniques is needed for two reasons.

1. there is only a sparse concentration of NPs able to penetrate the skin (detection limit)
2. the integrity of the particulate nature is difficult to analyse

The currently available techniques for analysis of NPs are qualitative microscopic techniques such as light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), fluorescence microscopy, confocal laser

scanning microscopy, multiphoton laser scanning microscopy, multi-photon microscopy fluorescence-lifetime imaging microscopy (MPM-FLIM), and nuclear imaging. With the development of confocal and multiphoton laser scanning microscopy, three dimensional information on the distribution of NPs has been made possible. Other analytical techniques are focused on a more quantitative approach of detecting NPs. However, the techniques available so far, ICP-optical emission spectrometry, ICP-MS and atomic absorption spectroscopy (AAS), are not detecting the particles themselves but their elemental composition. So, there will be always the question whether the analysed atoms or ions are from the NPs themselves or from raw salts or chemical ingredients used in particle synthesis. This is in addition to possible interference from trace elements in biological materials (skin) such as zinc. All techniques, together with their advantages and disadvantages are described in Appendix 3.

Despite the recent developments in techniques as described in Laboute and Schneider 2012, there is still a need for an in vivo non-invasive quantitation for any analytical technique.

5 Human dermal risk assessment of nanomaterials

5.1 Safety of nanomaterials in cosmetics

One important product category of consumer products claiming to contain nanomaterials with potential dermal exposure are cosmetics. The SCCS recently published a series of documents that are related to the safety assessment of cosmetic products containing nanomaterials; an opinion on the safety of nanomaterials in cosmetic products (2007), a guidance document on the safety assesment of nanomaterials in cosmetics (2012a) and two recent opinions on UV filters in nanoform (ETH50, 2011 and ZnO, 2012b). These documents pinpoint the issues on human dermal risk assessment of nanomaterials.

5.1.1 Opinion on the safety of nanomaterials in cosmetic products

In 2007, the first 'Opinion on the Safety of Nanomaterials in Cosmetic Products' has been published in which data gaps for dermal exposure to nanomaterials have been identified (SCCP, 2007). At that time, the situation for dermal exposure was described 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 recent dermal penetration studies with nanomaterials as described by Laboute and Schneider confirmed the SCCS conclusions above. However, it must be realized here that the scientific studies as described in public literature are not standardized according to the SCCS criteria. A large variation between studies exists, this can be due to the different experimental conditions, e.g. variation in the dose applied.

5.1.2 Applicability of OECD dermal absorption guidelines for nanomaterials

In the first report of SCCS on safety of nanomaterials in cosmetic products (SCCS, 2007) the conclusion on the applicability of the OECD dermal absorption test guideline for nanomaterials was that OECD Guideline 427 (OECD 2004a) describes *in vivo* dermal absorption and, therefore, will not be permitted in future for cosmetic ingredients.

Dermal absorption *in vitro* using excised human or pig skin in a diffusion cell (Franz cell) is described in OECD Guideline 428 and is not optimised for nanoparticles, thus requires further study.
One of the specific concerns of the SCCS is that the probability that a nanoparticle can be quantified in the receiver medium is extremely small (see also the section on detection of nanomaterials in skin in the previous chapter). Furthermore, the integrity of the skin must be rigorously assessed through TER (Transcutaneous Electrical Resistence) or TEWL (Trans Epidermal Water Loss) measurements because the penetration of nanoparticles could be much easier through compromised skin.
This is in line with the conclusion of the OECD's Working Party on Manufactured Nanomaterials (WPMN) in their project to review the published OECD Test Guidelines to assess whether or not they are suitable for manufactured nanomaterials. For the absorption/ penetration studies as mentioned above the following comments were made by WPMN (OECD, 2009):

"There is general agreement that absorption/distribution studies are of key importance with regard to investigating the likely toxicity of nanomaterials. However, studies will probably need to be designed on a case by case basis rather than using a specific guideline. In addition to the need for well-characterised material and measurement of actual exposures, there will be the added analytical difficulty of tracking distribution in vivo at realistic exposure scenarios. Labelled nanoparticles are likely to be needed for such studies.
This is also a specific concern regarding the in-vitro TG for skin absorption (TG 428). Knowledge of skin absorption is a key factor for nanomaterials applied to the skin. This assay has the potential to provide such information using an in-vitro method".

When performing a dermal penetration study with nanomaterials, mass may not be the most appropriate dose metric, number of particles or particle surface area of the nanomaterial may be better. Therefore, the number of particles per mass or particle surface area per mass should be reported.
Dermal absorption is expressed as ug/cm^2, or as a percentage of the amount of substance applied (see below). This should also be expressed as number of particles per cm^2 or particle surface area per cm^2.

5.2 Guidance on the safety assessment of nanomaterials in cosmetics

Furthermore, a guidance has been published in 2012, on the safety assessment of nanomaterials in cosmetics. In this guidance, essential elements that are required to be reported in a manufactured nanomaterials safety dossier are described, i.e. physicochemical characterisation; toxicological evaluation, exposure assessment etc (SCCS, 2012a). Especially the elements describing skin penetration and dermal absorption of nanomaterials as well as the risk assessment of nano-sized ingredients are interesting for the current report and are described below.

5.2.1 Exposure assessment

SCCS states that the exposure assessment for ingredients in cosmetic products as described in the SCCS Notes of Guidance (2010b) is a general approach that applies to nanomaterials as well. There is currently no indication that the use of consumer/cosmetic products that contain nanomaterials is likely to be any different from the use of other products that contain conventional ingredients. This means that default values in relation to exposure e.g. used amounts, will be

the same to those considered for cosmetic products as provided in the Notes of Guidance.

Corresponding to *Cosmetics Regulation (EC) No 1223/2009, Article 16 f) "reasonably foreseeable exposure conditions" need to be taken into account.* The following factors are important for an exposure assessment:
- class of cosmetic product(s) in which the ingredient may be used,
- method of application: rubbed-on, sprayed, applied and washed off, etc.,
- concentration of the ingredient in the finished cosmetic product,
- quantity of the product used at each application,
- frequency of use,
- total area of skin contact,
- duration of exposure
- foreseeable misuse which may increase exposure,
- consumer target groups (e.g., children, people with sensitive, damaged or compromised skin) where specifically required
- quantity likely to enter the body (fraction absorbed),
- application on skin areas exposed to sunlight,
- use area (indoors/outdoors) and ventilation
- all routes of exposure (dermal, oral and inhalation exposure) should be considered in view of the intended use of the product.

Measuring the effects of nanomaterials on compromised skin poses a challenge due to the current lack of standardised model(s) that can be used to generate results that are reproducible and can be used to compare studies carried out within a laboratory and between different laboratories. Where studies on compromised skin are specifically required, the models used should be well characterised to generate reproducible results, and appropriate controls should be included in the studies. Urgent research is needed to develop appropriate test models of compromised skin that can be reliably used to assess possible absorption of cosmetic ingredients, including nanoparticulate materials.

5.2.2 Risk assessment

The aim of the exposure assessment is to determine the Systemic Exposure Dosage (SED), which is an important parameter for calculating the Margin of Safety (MoS) of ingredients in a finished cosmetic product.
MoS = NO(A)EL* / SED
*or LO(A)EL where NO(A)EL is not available

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

Apart from systemic effects, also local effects (e.g. on skin after dermal application and respiratory tract after spray application) need to be considered, but on a qualitative basis.
The systemic exposure dosage (SED in mg/kg bw/day) after dermal application can be calculated on the basis of the dermal absorption expressed in µg/cm² or as a percentage of the amount of substance applied.
For conventional ingredients, in the majority of MoS calculations, the dermal exposure is compared to an oral NO(A)EL value (route to route extrapolation). The oral NO(A)EL value usually corresponds to an amount that has been administered orally, though not necessarily to the actual systemic availability of the compound after oral administration. In the past, the oral bioavailability of a substance was assumed 100% in case oral absorption data are unavailable. However, the SCCS considers it appropriate to assume as default that not more than 50% of an orally administered dose is systemically available. The value of 50% is an arbitrary choice that recognises that the gastrointestinal tract is designed to favour the absorption of ingested substances into the body but that, in most cases, not all of the ingested material will be bioavailable. Thus, in the absence of data, the assumption is being made that effects seen following oral administration have been caused by a fraction of the administered dose and not the entire amount administered. If there is evidence to suggest poor oral bioavailability, for example the substance is a poorly soluble particulate, it may be more appropriate to assume that only 10% of the administered dose is systemically available. Whenever oral absorption data are available, these should be included in the calculations.
For route-to-route extrapolation, experimental data on absorption will be required for both the dermal and oral route. Any route-to-route extrapolation needs to be performed case-by-case, based on expert judgment of scientific information, including the available toxicokinetic information. It can, however, only be performed if there is systemic toxicity, considering the degree of absorption and also possible metabolic transformation.

For nanomaterials, the calculation of the MoS, especially in the case of (very) low absorption via oral, dermal, and/or pulmonary routes of exposure, can be challenging. In case of (very) low absorption, the validity of NOAELs in toxicological studies may be questionable, and for substances that are hardly absorbed, no toxic effects may be noted. However, in such a case, processes such as translocation and accumulation will need to be accurately studied before a decision on the safe use can be taken (SCCS 2012a).

5.3 SCCS opinions on sunscreens (ETH50, ZnO)

Although opinions and general risk assessment documents on nanomaterials in (consumer) products have been published by SCCS (2012a) and SCENIHR (2009), experience with the assessment of specific substances is limited. The ongoing risk assessments being carried out by the SCCS on four specific manufactured nanomaterials for their inclusion in Annex VII (ultraviolet (UV) filters) of the Cosmetics Directive (76/768/EEC), are the first instances in the EU and worldwide with regulatory implications.
In this report, two of these actual risk assessments are analysed with respect to the previously mentioned issues for dermal exposure.

5.3.1 Opinion on 1,3,5-Triazine, 2,4,6-tris[1,1'-biphenyl]-4-yl- (ETH50)

ETH 50 is a new notified substance to be used as an UV-filter in sunscreen products. In November 2005, SCCS received the first submission of this

substance, and an addendum has been received in 2006. During review of the dossier, it became apparent that it would be present in the form of nanosized particles in the formulation to which the consumer is exposed. Therefore, further tests with this form of ETH50 were requested before the evaluation could be completed. The following questions were evaluated in the opinion of the SCCS (2011):
1. Does SCCS consider that the use of ETH50 as an UV-filter in cosmetic products in a concentration up to maximum 10.0% is safe for the consumers taken into account the scientific data provided?
2. Does SCCS have any other scientific concerns for the safe use of the new UV-filter ETH50 in finished cosmetic products?

5.3.1.1 Dermal penetration studies

In the dossier, both human and rat skin were used for dermal penetration studies with nanoparticles of 80 nm (OECD test 428). Based on the results, the test item did not penetrate through the skin membranes to a significant extent. Given the large variability in the absorption values, the mean value ± 2 SD was used for the calculation of the MoS (for details on the penetration results, see SCCS 2011).
Comment: since most of the measured values were below reliably quantifiable concentrations, the calculation of the absorption value can be considered conservative.
Also pre-damaged human skin has been tested with particles of 120 nm. Based on the reported results, the test item with particle mean diameter of 120 nm did not penetrate through the skin membranes to a significant extent and the damaged stratum corneum did not result in a significantly increased penetration rate of the nanosized test item.

5.3.1.2 Safety evaluation

In the safety evaluation of ETH50, the conclusion is that it has low oral bioavailability. From the in vivo ADME study with ETH50, particle size d(0.5) = 86 nm, it was shown that absorption after oral exposure was < 1% of the administered dose. Therefore, for a risk assessment based on route-to-route extrapolation, the NOAEL from the oral 13 week rat study has to be re-calculated to an internal dose, which would lead to a MoS value below 100.
The SCCS is of the opinion that in the case of substances with very low bioavailability, and in addition, the absence of an effect at the highest dose tested, route-to-route extrapolation is not an appropriate approach and prefers to use the dermal 90 day study for the calculation of the Margin of Safety.

Comparative MoS calculations for ETH50 based on human skin in vitro study results for nanosized (80 nm) and micronized particle size (440 nm) on normal skin (SCCS, 2011):

Parameter	**ETH50 d(0.5) = 80nm**	**ETH50 d(0.5) = 440nm**
Adult Body weight	60 kg	60 kg
Body surface area	17.500 cm²	17.500 cm²
Sunscreen applied (if at 1 mg/cm²)	18 g	18 g
ETH50 applied (10%)	1800 mg	1800 mg
Skin absorption (human) RCC B236 24 April '07 RCC A00112 August '05	0.20% of applied dose	0.57% of applied dose
Systemic Exposure Dose (human)	0.06 mg/kg bw/day	0.171 mg/kg bw/day
NOAEL Rat 13-wk dermal study (CIT 32404 TCR, Aug' 08)	500 mg/kg bw/day	Not available; applicant used 1000 mg/kg bw/day for study performed with d(0.5)= 15 µm

Skin absorption (rat) (RCC B23624 April '07)	4.28% of applied dose	12.77% of applied dose
Systemic Exposure Dose (rat)	21.4 mg/kg bw/day	127.7 mg/kg bw/day
SED Rat / SED Human	**357**	**746**

Based on the comparison of the internal dose between rat and man, the MoS for ETH50 is 357 with a d(0.5)= 80 nm.
It should be noted that the above calculations are very conservative, in particular with regard to the skin absorption value used. Most values in the dermal absorption assay were below the limit of quantification, but used for the calculation of the penetration. Moreover, the majority of the dose was recovered from the skin compartment, rather than the receptor fluid.

5.3.2 Opinion on Zinc Oxide (ZnO)

Zinc oxide has widespread use in cosmetic products, with a number of different functions: bulking, skin protection and as a UV absorber. This is besides its authorized use in all cosmetics as a cosmetic colorant. Two former submissions on zinc oxide in pigmentary form as well as in the form of a nanomaterial were submitted to SCCS in order to have zinc oxide approved as a UV-filter in cosmetic sunscreen products at a maximum level of 25%. In the third opinion of SCCS (adopted 18 September 2012), the following main question has been addressed:
Does the SCCS consider zinc oxide in its nano-form (as well as the non-nano form) safe for use as a UV filter with a concentration up to 25% in cosmetic products? Both uncoated and coated forms of zinc oxide have to be considered.

5.3.2.1 Dermal penetration studies

In the opinion (SCCS, 2012b), several *in vitro* as well as *in vivo* penetration studies with different forms of ZnO have been described. From the results of these experiments, the conclusion can be drawn that ZnO is not penetrating healthy human skin. Sometimes penetration into the SC is observed, but not into the lower skin layers. A general comment is that most of the studies mentioned in the opinion provide limited information on dose expressed as surface area and/or number of particles. One *in vivo* study showed low Zn uptake via healthy human skin. The SCCS considered that the Zn that originated from the topically applied ZnO contain in sunscreen was only a fraction of the amount of Zn present in the overall blood zinc pool (they could be distinguished because the Zn in the sunscreen was a stable isotope). Furthermore, there was no information on whether the translocating Zn was present as nanoparticles or soluble Zn ions. Information on dose expressed as surface area and number of particles was missing (Gulson, 2012).

As a conclusion, it is assumed that penetration of the skin, if any, is caused by Zn ions released from ZnO nanoparticles. Therefore, the solubility of ZnO is one of the critical parameters that should be considered in the characterization of ZnO used for sunscreen formulations.

The data provided, and as present in the literature, indicate that ZnO nanoparticles do not penetrate through the skin. However, some minimal absorption of zinc was demonstrated. Although the zinc was determined by methods that do not discriminate between particulate and solubilized forms, and considering the dissolution rate of ZnO, it is likely that this was in the form of solubilized zinc ion.

5.3.2.2 Safety evaluation

As internal exposure is likely to be to ionic Zn, the safety considerations as indicated below in the EU Risk Assessment Report (RAR) on Zinc Oxide (ECB, 2004) are relevant. The SCCS agrees with the NOAEL indicated in the RAR statement. Therefore, this information is used, together with the data from the absorption study provided in the dossier and exposure assumptions from the SCCS Notes of Guidance, for the risk assessment of zinc oxide nanoparticles in sunscreens as follows (SCCS, 2012b):

Calculation of the margin of safety for ZnO (nano)

Amount of sunscreen applied*	18,000 mg
Maximum concentration of ZnO	25%
Absorption through the skin (Reference: 36)	0.03%
Amount absorbed/day	(18,000 × 25/100 × 0.03/100) 1.35 mg
Typical body weight of human	60 kg
Systemic exposure dose	(1.35 mg/60 kg) 0.0225 mg/kg bw/d
No Observed Adverse Effect Level NOAEL	(oral, human, sensitive subpopulation)** 0.166 mg/kg bw/d
Margin of Safety NOAEL/SED =	**7.4**

* Standard amount as indicated in the SCCS Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation SCCS/1416/11

** The internal NOAEL for ZnO is 10 mg Zn2+/day = 10/60= 0.166 mg /kg bw per day (Reference 44, sub III)

The calculation of the exposure via sun protection products to ZnO nanoparticles assuming $Zn2^{+}$ uptake results in a MoS of 7.4. Given that the NOAEL is derived from a study on women (the most sensitive population in zinc supplementation studies), and that in women clinical signs begin to appear only at a dose three times this NOAEL, a minimal MoS of 1 is considered sufficient when comparing the human NOAEL with the exposure levels for workers/consumers/general population (SCCS, 2012b).

5.4 Discussion points based on risk assessment dossiers

For a proper assessment of the (dermal) safety of nanomaterials in consumer products, a dermal penetration study in both human and animal skin is necessary, as well as a dermal repeated dose study. The use of an oral repeated dose is of limited use for the dermal exposure assessment, especially when information on the oral bioavailability is missing.

For a proper use in the calculation of the MoS, the species used in the skin absorption study needs to be the same as in the dermal repeated dose study.

If dermal absorption studies are available, information is needed on:

- the dose applied expressed as number of particles/ cm^2 or particle surface area /cm^2, therefore, the number of particles per mass or surface area per mass should be reported.
- characterisation of the material in the skin and in the receptor fluid: when absorption is observed, it has to be determined whether this uptake is as nanoparticles or ions (form of substance in skin after applying it as nanoparticles needs to be determined)

Dermal penetration studies with pig skin are the best alternative for human skin, however, there are structural and morphological differences between animal and human skin, especially in density of hair follicles, thickness of SC and total skin,

and the amount of skin lipids. Furtermore, for the calculation of the MoS the use of pig skin has its limitations when calculation the MoS, see below.
Dermal penetration studies with rat skin are not that useful since rat skin is more permeable than human skin because of the differences as mentioned above.

In general, a NOAEL is derived from oral exposure, and many nanoparticles have a very low oral absorption and low bioavailability. Route to route extrapolation is not appropriate. If available, a NOAEL of a dermal study has to be used to calculate the margin of safety. However, for substances with a low bioavailability (in combination with a high external exposure), the use of a NOAEL of a dermal study in the calculation of a MOS also has its limitation:

In the guidance document of the SCCS, *in vitro* dermal penetration studies using pig skin is preferred over rat skin, since pig skin is more comparable to human skin.
However, in the risk assessment of UV filters in sun screens, the external exposure is relatively high (default amount applied: 18000 mg, with a % active ingredient of 10%, resulting in an external dose of 30 mg/kg bw/day for an adult of 60 kg.

The equation to calculate the MoS is SED animal/SED man
The SED animal = NOAEL* % dermal absorption animal
The SED in man = external dose * % dermal absorption in man
So the MoS = (NOAEL* % dermal absorption animal) / (external dose * % dermal absorption in man).

In general, in toxicity studies, the highest dose tested is in the range of 1000 mg active ingredient/kg/day. So when no effects are observed in a repeated dose dermal study, resulting in a NOAEL of 1000 mg/kg bw, the corresponding MOS would be:
(1000* % dermal absorption animal) / 30 * % dermal absorption in man.
This can be simplified to: MOS = 33 * (% dermal absorption animal/%dermal absorption in man)

When pig skin is used, and it is assumed that the dermal absorption in the pig is equal to that of the human, the ratio of the SEDs is equal to the ratio of the external exposure, that is 33. This is much lower than 100. This simple calculation example demonstrates, that, in this case a MoS of 100 can only be reached in the case of an NOAEL of > 3000 mg active ingredient /kg/bw or in the case that the dermal absorption in the animal is >30 times higher than in man. A NOAEL of >3000 mg active ingredient/kg/bw is a concentration that is much higher than the range that is normally tested. The fact the MoS of 100 can be reached when the dermal absorption in the animal is >30 times higher than in man, demonstrated the limitations of this safety approach for substances. This would also favour a dermal rat study instead of a pig, since in the rat study the dermal absorption is expected to be higher than the absorption in man, whereas in the pig the dermal absorption is expected to be comparable.

The limitation of this approach is not specific for nanomaterials, but for substances with low bioavailability.

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Appendix 1

Overview of the skin penetration studies of various inorganic particles applied to different skin types under differing experimental conditions

Particle	Diameter (NM)	Skin type (in vitro/ in vivo)	Addtitional enhancement approach	Reported outcome	Reference
QD-COOH	4	Human (in vitro)	– Massage Tape-stripping Tape-stripping + massage	No penetration No penetration No penetration Penetration	Gratieru T, et al, 2010
Fe2O3-TMAOH	4.9 ± 1.3	Human (in vitro)	–	Penetration	Baroli et al, 2007
AuNPs	10	Human (in vitro)	– Dermaportation by pulsed electromagnetic field	No penetration Penetration	Krishnan et al, 2010
AuNPs-alkylated mercapten† AuNPs-lecithin AuNPs-cetrimide† AuNPs-citrate	~6 ~6 ~15 ~15	Human (in vitro)	– – – – CHCl3/methanol Tape-stripping	Penetration Penetration Penetration No penetration Penetration Penetration	Labouta et al, 2011a,b
TiO_2	10–50	Human (in vivo)	–	Penetration‡	Tan et al, 1996
AuNPs	~12.9	Human (in vitro)	– Dermabrasion	Penetration Penetration	Larese et al, 2011
AuNPs	~15	Human (in vitro)	– Urea Tween 80 SLS DMSO	No penetration No penetration Negligible penetration No penetration Penetration	Labouta et al, 2012 (Forthcoming)
ZnO	15–40	Human (in vitro)	–	No penetration	Cross et al, 2007
TiO2	20	Human (in vivo and in vitro)	–	No penetration No penetration	Mavon et al, 2007
TiO2 TiO2 in sunscreens with and without ZnO	20	Human (in vitro)	–	No penetration	Filipe et al, 2009
ZnO	20–30	Human (in vivo and in vitro)	–	No penetration	Zvyagin et al, 2008
TiO2 ZnO	20–70 <200	Human (in vitro)	– –	No penetration No penetration	Durand et al, 2009
AgNPs	25 ± 7.1	Human (in vitro)	– Abrasion	Low penetration (both), but higher for damaged skin	Filon et al, 2007; Larese et al, 2009
ZnO	30	Human (in vitro)	–	No penetration	Roberts et al, 2008
TiO2 (hydrophobic) TiO2 (amphiphilic) TiO2 (hydrophilic)	20- to 100-nm aggregates	Human (in vivo)	– – –	No penetration No penetration No penetration	Pflucker et al, 2001; Schulz et al, 2002
TiO2 platelets ZnO platelets	Size not given (nm range) 116.8 length × 57.5 width	Human (in vitro)	– –	No penetration No penetration	Dussert et al, 1997
AuNPs	Size not stated (nm range)	Human (in vitro)	Electrophoresis	No penetration	Chen et el, 2008
TiO2	Size not given (nm range)	Human (in vivo, healthy) Human (in vivo, psoriatic)	– –	No penetration No penetration	Ghosh et al, 2008; Pissuwan et al, 2011
QD-PEG, QD-PEG-amine, QD-COOH	12.65–29.35 nm (at different pH values)	Human (in vitro)	– Tape-stripping	QD-PEG penetration–pH 8.3 Penetration	Prow et al, 2011
QD-PEG-amine	7 ± 2	Reconstructed human skin	–	No penetration	Jeong et al, 2010
AuNPs	4.6 ± 1.5	Porcine, full-thickness (in vitro) Porcine, dermatomed (in vitro)	Ultrasound and SLS Ultrasound and SLS	Penetration Penetration	Seto et al, 2010
QD-PEG, QD-PEG-	15–45(spherical	Porcine (in vitro)	–	Penetration	Rymann-

QD-PEG, QD-PEG-amine, QD-COOH	15–45(spherical and ellipsoid)	Porcine (in vitro)	–	Penetration	Rymann-Rasmussen et al, 2006
Polymer-coated QDs	20	Porcine (in vitro)	Ultrasound with or without SLS	Penetration (increased with SLS)	Paliwal et al, 2006
AgNPs (uncoated) AgNPs (carbon coated)	20, 50, and 80 25 and 35	Porcine (in vivo)	–	No penetration	Samberg et al, 2010
TiO2 with different coatings	35 uncoated, 35 coated, 10 × 100, 250‖	Porcine (in vitro)	– Tape-stripping Hair removal	No penetration No penetration Penetration for 35-nm coated	Senzui et al, 2010
PEG-coated QDs	Nail-shaped:5.78 width × 8.4 length 39 ± 1 hydrodynamic diameter	Porcine, dermatomed (in vitro)	–	No penetration	Zhang et al, 2008
TiO2 (four formulations)	45–150 length, 17–35 width (lanceolate shape)	Porcine (in vitro)	–	Penetration	Menzel et al, 2004
ZnO TiO2 agglomerates	80 up to 200	Porcine (in vitro)	–	No penetration	Gamer et al, 2006
Au particles	900 ± 600	Porcine (in vitro)	Ballistic delivery	Penetration	Kendall et al, 2004
TiO2 (uncoated submicron sized, uncoated nano- or Al(OH)3, dimethicone/methicone co-polymer-coated	207 ± 53¶, 30 ± 8¶, and fibrils of 57 ± 18 length and 15 ± 5 width¶	Porcine (in vivo)	–	No penetration	Sadrieh et al, 2010

QD-COOH	4.1	Mouse (in vitro and in vivo)	–	Permeation	Chu et al, 2007
Fe2O3	4.6–10	Mouse (in vitro)	Blade incision, 1 μm width	Penetration	Lee et al, 2010
ZnO	10	Mouse (in vitro)	– OA, EtOH, and OA-EtOH	No penetration Penetration	Kuo et al, 2009
AuNPs	11.6	Mouse (in vivo)	–	Penetration	Huang et al, 2010
QD-COOH	~20 and ~33 nm86; ~12–20 nm74	Mouse (in vivo)	– UV exposure	Low penetration for both but higher on UV exposure	Mortensen et al, 2009, 2008
QD-PEG	37	Mouse (in vivo)	– Acetone-pretreated Tape-stripped Dermabraded	No penetration No penetration No penetration Permeation	Gopee et al, 2009
DT-QD-COOH conjugate	Size not stated (nm range)(nail-shaped)	Mouse (in vivo)	Hyperthermia	Penetration§	Upadhyay, 2006
QD-COOH	6 ± 2	Rat (in vitro)	– Flexion Tape-stripping Abrasion	No penetration No penetration No penetration Penetration	Zhang et al, 2008
AuNPs	15, 102, 198	Rat (in vitro)	–	Permeation	Sonavane et al, 2008
TiO2	4, 10, 21, 25, 60, 90 4, 60 10, 21, 25, 60	Porcine (in vitro, 1 day) Porcine (in vivo, 30 days) Mouse (in vivo, 60 days)††	– – –	No penetration Penetration Permeation	Wu et al, 2009
TiO2	width 20 × length 100	Human (in vitro) Porcine (in vitro)	–	No penetration	Lekki et al, 2007
TiO2	20–100	Porcine (in vitro) Human skin grafted on SCID mouse (in vivo)	– – –	No penetration No penetration No penetration	Gontier et al, 2008
TiO2	Size not given (commercial formulation)	Human foreskin grafted on SCID mouse (in vivo)	–	No penetration	Kertesz et al, 2005

Appendix 2

STRUCTURE OF SKIN (adopted from SCCP, 2007)

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 nonpolar 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 m2 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.

Appendix 3

Analytical methods used for monitoring particle penetration in skin barrier (adopted from Labouta and Schneider, 2012)

Analytical method	**Advantages (A)** **Disadvantages (D)**
Microscopic visualisation	
Light microscopy of skin samples	A: easy technique D: artefacts on staining and mechanical sectioning
SEM	A: high resolution D: artefacts on mechanical sectioning
TEM	A: High resolution for electron-dense materials D: artefacts on mechanical sectioning
Fluorescence microscopy	A: higher selectivity, availability D: no depth information
Confocal laser scanning microscopy	A: optical sectioning D: loss of laser power with depth in the skin
Multiphoton laser scanning microscopy	A: intrinsic optical sectioning, less scattering by the tissues, and less phototoxicity than confocal D: loss of laser power with depth in the skin specimen, expensive
MPM-FLIM	A: can study the effect of NPs on the skin metabolism by measuring autofluorescence of the endogenous fluorophores without the need of extrinsic labels D: loss of laser power with depth in the skin specimen, more expensive
Nuclear imaging	A: includes particle-induced x-ray emission, scanning transmission and ion microscopy, and Rutherford backscattering D: determination of the elemental composition of the particles, not the particles themselves, thus an interference possibility
Other analytical techniques	
ICP-optical emission spectrometry	A: quantitative techniques D: determination of the elemental composition of the particles, not the particles themselves, thus an interference possibility
ICP-MS	
Atomic absorption spectroscopy	