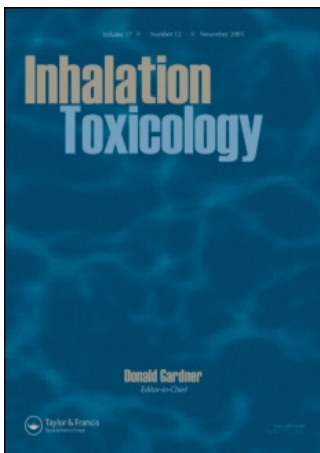


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# Testing Strategies to Establish the Safety of Nanomaterials: Conclusions of an ECETOC Workshop

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The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) convened a workshop in Barcelona, Spain, in November 2005 to develop testing strategies to establish the safety of nanomaterials. It brought together about 70 scientific and clinical experts from industry, academia, government agencies, research institutes, and nongovernmental organizations. The primary questions to be addressed were the following: What can we do today, and what do we need tomorrow? The three major themes of the workshop were: (1) the need for enhanced efforts in nanomaterial characterization; (2) methodologies for assessments of airborne and internal exposures to nanomaterials; and (3) evaluation of the hazard potential—primarily focusing on pulmonary or dermal routes of exposures. Some of the summary conclusions of the workshop included the following: For the development of nanoparticle characterization, the working definition of nanoparticles was defined as <100 nm in one dimension or <1000 nm to include aggregates and agglomerates. Moreover, it was concluded that although many physical factors can influence toxicity, including nanoparticle composition, it is dissolution, surface area and characteristics, size, size distribution, and shape that largely determine the functional, toxicological and environmental impact of nanomaterials. In addition, most of the information on potential systemic effects has thus far been derived from combustion-generated particles. With respect to the assessment of external exposures and metrics appropriate for nanoparticles, the general view of the meeting was that currently it is not possible or desirable to select one form of dose metric (i.e., mass, surface area, or particle number) as the most appropriate measure source. However, it was clear that the surface area metric was likely to be of interest and requires further development. In addition, there is a clear and immediate need to develop instruments which are smaller, more portable, and less expensive than the currently available state of the art instrumentation. With regard to a general testing approach for human health hazard evaluation of nanoparticles, a first step to determine potency may include a prioritization-related *in vitro* screening strategy to assess the possible reactivity, biomarkers of inflammation and cellular uptake of nanoparticles; however this process should

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be validated using *in vivo* techniques. A Tier 1 *in vivo* testing strategy could include a short-term inhalation or intratracheal instillation of nanoparticles as the route of exposure in the lungs of rats or mice. The endpoints that should be assessed include indices of lung inflammation, cytotoxicity, and cell proliferation, as well as histopathology of the respiratory tract and the major extrapulmonary organs. For Tier 2 *in vivo* testing for hazard identification, a longer term inhalation study is recommended, and this would include more substantive mechanistic endpoints such as determination of particle deposition, translocation, and disposition within the body. Additional studies could be designed with specific animal models to mimic sensitive populations. With regard to dermal exposures, currently there is little evidence that nanoparticles at a size exceeding 100 nm penetrate through the skin barrier into the living tissue (i.e., dermal compartment). The penetration of nanoparticles at a size less than 100 nm should be a topic of further investigation. Moreover, considering the impacts of dermal exposures and corresponding hazard potential of nanoparticles, it must be taken into consideration that the dermal uptake of nanoparticles will be an order of magnitude smaller than the uptake via the inhalation or oral routes of exposure. For the evaluation of the health risk of nanoparticles, it has to be determined whether they are harmful to living cells and whether, under real conditions, they penetrate through the skin barrier into the living tissue. For the evaluation of the penetration processes, in principle, three methods are available. Using the method of differential stripping, the penetration kinetics of nanoparticles in the stratum corneum and the hair follicles can be evaluated. This analysis can be carried out *in vivo*. Diffusion cell experiments are an efficient method for *in vitro* penetration studies. Also, laser scanning microscopy is well suited to test penetration kinetics, although requiring fluorescent-labeled nanoparticles. Emerging topics such as (1) environmental safety testing, (2) applications of nanoparticles for medical purposes, and (3) pathways of inhaled nanoparticles to the central nervous system were also briefly addressed during this workshop. However, it has become clear that these topics should be the subjects of separate workshops and they are not further addressed in this report.

Nanotechnology involves creating and using molecules a few billionths of a meter in size. Evaluating the potential hazards of this technology and its products is an emerging area in toxicology and health risk assessment. The development of a safety database and exposure assessments to nanoscale particles are evolving as new particles, materials, and exposure methodologies are being developed. Although similar in size, these engineered nanoscale materials may have different health impacts when compared to combustion-generated ultrafine particles. A related issue is the extent to which nanoparticle toxicity can be extrapolated from existing toxicology databases for macro- and microscale particle types and fibers. One of the aims of this workshop was to provide fundamental information to better understand the rapidly emerging field of testing strategies to establish the safety of nanomaterials. An appreciation of the chemistry and corresponding material science issues related to nanoscale particle composition as well as evolving airborne exposure assessment methodologies are absolute prerequisites to a better understanding of the health

impacts of nanomaterials. The substance of this workshop had some similarities to an earlier report by a Nanomaterials Toxicity Screening Committee sponsored by the International Life Sciences Institute in Washington, DC (see Oberdörster et al., 2005).

The workshop brought together about 70 scientific and clinical experts from industry, academia, government agencies, research institutes, and nongovernmental organizations, and focused on testing strategies to establish the safety of nanomaterials. What can we do today? What do we need tomorrow? It covered three major issues:

- Nanomaterial characterization.
- Exposure, both airborne and internal (particle deposition in lungs and skin).
- Assessment of hazard potential.

This report briefly summarizes the introductory lectures given by key researchers in these scientific areas, as well as the plenary discussions and the outcome of the breakout groups. Before the workshop commenced, the participants were provided with the draft of a background paper on the potential risks of nanomaterials (Borm et al., 2006) and a "Thought Starter" section that was designed to stimulate discussion and is presented next.

## THOUGHT STARTER

### Nanomaterials: What Do We Need to Know?

For hazard characterization, risk assessment, and future regulation of nanomaterials, several crucial issues need to be considered:

1. Effects measured may not be specific or unique for nanoparticles (NP) *per se*, but also present for the same or other materials of larger size or aggregates. In this case, effects of NP are quantitatively different but not qualitatively different, and regulation may be adapted by changing values and/or metrics of respective standards.

*Example:* Exposures to overload concentrations of Nanoparticle TiO<sub>2</sub> or carbon black can induce lung tumors in rats at considerably lower gravimetric lung burdens when compared to their larger sized analogs, and actually the retained particle surface metric has been used to describe the lung tumor rate in chronic animal studies. The overall pattern is one of chronic inflammation that occurs upon saturation of lung clearance by overloading of macrophages, at which point particle accumulation starts and inflammatory cell influx increases sharply. The inflammatory cell influx is held responsible for the lung tumors after chronic particle exposure to low-toxicity particles due to its promutagenic effects and actions on cell proliferation. Still, this surface dose concept is probably an oversimplification, and careful evaluation is needed.

1. Effects may be qualitatively different, based on size, surface chemistry or another specific interaction. In this case normal standard setting could be used, since the critical effect is simply different from the fine-sized analogues. This, however, implies that the same material, based on size differences, may have different standards, also based on different effects.

*Example:* Recently, carbonaceous NP and gold were shown to translocate from the nasal cavity through the olfactory epithelium (2 cm<sup>2</sup>) along the olfactory nerves to the central nervous system (CNS), based on their presence in the olfactory bulb of rats after inhalation. Such a mechanism was first reported for poliovirus (30 nm) and colloidal gold particles (50 nm) moving into the olfactory bulb of various primates. This is a mechanism specific for NP and observed for different materials (carbon, gold, MnO<sub>2</sub>). Similarly, uptake through the gastrointestinal tract (40 m<sup>2</sup>) has also been described for particles of different sizes and is actually now being employed by the food industry to increase bioavailability of compounds that normally have a low bioavailability (vitamins, proteins). To do so, pure chemical substances are synthesized into nanoparticles with crystalline structure and in this way may be taken up through the immune system in the gut.

1. Current regulation of chemicals is driven by area of application. We deal with a growing set of materials of which some properties are largely unknown, and current testing procedures and legislation may produce many false negatives and/or false positives. The second issue already illustrated that the same material dependent on size may exert different (qualitative) effects. The central question here is whether current testing and classification protocols are appropriate and sufficient. Nanotechnology also promotes convergence of technologies, and similar materials may be applied in automotive and life sciences sectors. To stimulate production and marketing of safe nanomaterials, exchange of data between sectors is recommended.

### Nanomaterial Testing: How to Fill the Gaps?

There is a limited amount of data on the toxicity of NP. Moreover, these data are mainly based on a limited number of NP types (combustion derived NP, TiO<sub>2</sub>, carbon black) and the assumption that many effects by PM are driven by the ultrafine particles in it. Due to this background of the data and the specificity of most preparations of engineered nanoparticles, much work needs to be conducted with regard to characterization and biological testing of engineered NP. In this regard, it is recommended to perform testing driven by the anticipated application and classification by risk and not by hazard. From this, it is clear that a range of endpoints should be considered for the testing of NP for potential hazards. Some engineered NP, which become airborne, will pose inhalation hazards, while cosmetics with NP provide dermal exposures.

Each should be tested in the requisite ways focusing on their portal of entry. Other engineered NP are being used as devices to target drugs to specific tissues, to increase their biological half time, or for imaging/sensor purposes. In developing testing procedures and protocols, a number of basic questions need to be addressed:

1. *Which components should be tested?* The following should all be considered: native particle with surface modification, stability of the surface coating, effects of the NP + surface coating, materials used for synthesis of NP.
2. *What type of tests should be used?* A range of in vitro and in vivo tests should provide information that can contribute to hazard assessment, although in vitro hazard studies must be first validated using in vivo methods. Both classical tests and newer models reflecting current insights into the mechanisms of NP should be employed. The key questions for these tests is whether they are suitable to detect the qualitative and quantitative differences that are posed by nanomaterials in comparison to their fine-sized equivalents. Currently the ILSI, ECETOC, Dechema/VDI, and the HSE are starting up explorations and procedures to cope with the emerging industrial need in this matter.
3. *Surface modifications included in testing?* Whatever tests will be used, it needs to be realized that nanoparticles are usually surface modified to prevent aggregation. In fact, about 90% of TiO<sub>2</sub> is coated by organic or mineral (SiO<sub>2</sub>), and it needs to be considered that most suppliers apply post-synthetic strategies to modify engineered and bulk NP to prevent aggregation in order to retain its anticipated properties. Particle coating with polyethylene glycol is a common treatment in drug delivery to prevent recognition by the reticulo-endothelial system and increase the half-life of the particle-conjugated drugs. For fullerenes, such surface modifications have been shown to determine toxicological parameters. Apart from modifying the surface, the compounds used in post-synthetic routes such as 4-dimethylaminopyridine, various thiols, fluoroalkanes, alkoxy silanes, or phosphorus may be released and need to be included in testing protocols.
4. *Particle dissolution: good or bad?* Analogous to the conceptual understanding of fiber-induced adverse effects, during initial discussions particle dissolution has been mentioned as a potential screening property to prevent chronic effects. Current European Union (EU) legislation for new fibers has incorporated in vitro dissolution of fibers based on the body of evidence connecting high in vivo durability (low dissolution) to lung tumors. Although the testing strategies for nanomaterials require further exploration, one should be aware of two major complications. First, some nanoparticles (quantum dots) contain highly reactive or toxic components that may cause effects when dissolved. The second problem is provided by definitions of how to assess nanoparticle dissolution.

## METHODS—ORGANIZATION OF THE REPORT

The remainder of this report is organized in the following manner:

- I. Characterization of nanomaterials
  - A. Characterization from a physicochemical perspective
  - B. Characterization from a toxicological perspective
  - C. Minimum characterization of nanomaterials (Parameters suggested by the workshop participants)
- II. Exposure assessment
  - A. Measuring in the occupational setting
  - B. Experience from carbon black particles
  - C. Dermal exposures
  - D. Measuring exposures (summary of plenary discussion)
- III. Hazard potential
  - A. Tiered testing strategy for pulmonary exposure to nanomaterials
  - B. Complementary testing for mechanistic aspects
  - C. Key safety issues related to inhaling nanomaterials (summary of plenary discussion)
  - D. Testing strategies to establish dermal exposures and hazard potential (summary of breakout group discussion)
  - E. Testing strategies concerning systemic exposures (summary of breakout group discussion)
- IV. Summary and conclusions from the workshop

## CHARACTERIZATION OF NANOMATERIALS

### Characterization from a Physicochemical Perspective

S. Haubold presented the possibilities that nanomaterials offer due to their reduced size. Most materials show a variation in chemical and physical behaviour when reduced to nanometer dimensions. Examples of semiconductors such as CdTe, gold, and TiO<sub>2</sub> were shown. In the case of semiconductors, absorption and emission are strongly dependent on size. Gold nanoparticles change their color from yellow to red and TiO<sub>2</sub> becomes photocatalytically active. Dr. Haubold then presented different methods to produce nanoparticles like spray pyrolysis and wet chemical procedures. Following their production, a careful physicochemical characterization of the particles is needed. Among the methods used, x-ray diffraction (XRD), area under the curve (AUC), ultraviolet/visible (UV/VIS) spectroscopy, and transmission electron microscopy (TEM) are among the most important. It was also demonstrated that the application of the particles strongly depends on their dispersibility in a resin matrix. Therefore, it is crucial to design the particle surface such that it can fulfil its function for each specific application.

### Characterization from a Toxicological Perspective

P. J. A. Borm addressed the sources of evidence for the toxicity of nanoparticles (NP), discriminating three classes of NP: bulk, combustion, and engineered nanoparticles. The current discussion on engineered nanoparticles is mainly driven by data on combustion NP (diesel, ultrafines) and a small set of bulk NP (carbon blacks, TiO<sub>2</sub>). He stressed, however, that the cur-

rent data set on engineered NP is growing and that the qualitative effects (inflammation, atherosclerosis, oxidative stress, Ca transport, etc.) are gradually being investigated with various products such as single-wall nanotubes (SWNT). Some studies allow bridging of data, such as recent work (Radomski et al., 2005) on platelet aggregation with different nanomaterials, but also including ambient PM samples. Since there is an overload of outstanding toxicology questions before gaining a conceptual understanding on nanomaterials, research, and regulation, the following issues need careful consideration.

When compared to ambient-derived ultrafine particles, some effects of nanomaterials are probably similar to the effects of engineered NP; this is no priority for further research, but the validity of current testing effects requires validation. There is a need to identify effects that are novel for (engineered) NP that may occur in populations other than occupational conditions. Almost no data are available on ecotoxicity or ADME of NP, and this area should receive research priority. When choices are to be made in testing and research they should be driven by the application of the nanostructured materials.

### Minimum Characterization of Nanomaterials (Plenary Discussion)

M. Pridöhl introduced this session by stressing that an adequate physical and chemical characterization of nanomaterials was necessary, and should for many materials be carried out more rigorously than has been to date. An appropriate characterization should be based on the current knowledge of potential toxicity, since there was a strong likelihood that physicochemical parameters affect nanomaterial toxicology. He proposed to keep three basic questions in mind when discussing which physicochemical parameters were needed to investigate toxicity to specific target organs:

- Are quantum properties themselves relevant for toxicity?
- What parameters are crucial for translocation?
- Is electrical conductivity (i.e., of CNT) a relevant parameter for toxicity?

Several small breakout groups were formed, generally organized by target organs and for ecotoxicity. Each group was asked to identify the five most important physicochemical parameters with respect to a specific target organ. The following list was suggested for consideration:

Specific surface area; particle size; particle size distribution; porosity; shape (top down, bottom up); state of aggregation (chemical covalent bonding); state of agglomeration (Van der Waals forces); chemical composition and defectivity; crystal phases and/or amorphous content; contaminations (like heavy metals in CNTs); surface modifications (chemistry and kind of attachment); hydrophobicity; surface charge; dissolution (rate) (remark: media of dissolution depend on the target organ considered); catalytic activity (e.g., attachment of proteins, oxidative activity); dustiness; magnetic properties.

The parameters suggested by the target organ groups are:

- Lung
  1. Size and size distribution
  2. Specific surface area and surface modification, adsorption, interference with lung
  3. Dissolution (most bulk materials have data available)
  4. Chemical composition and defectivity (closely related to dissolution / chemistry are only important if the particle is dissolved)
  5. Shape.
- Cardiovascular system (group 1)
  1. Size
  2. Surface area
  3. Solubility
  4. Contaminations
  5. Chemical composition
- Cardiovascular system (group 2)
  1. Surface area
  2. Size and size distribution
  3. Agglomeration / aggregation
  4. Surface modification or chemistry (including contaminations)
  5. Dissolution rate.
- Skin (group 1)
  1. Dissolution
  2. Size
  3. Partition coefficient (but probably not specific for nanoparticles)
- Skin (group 2)
  1. Skin penetration and cytotoxicity / distribution
  2. Size and size distribution
  3. Surface properties (including surface area, chemical composition, hydrophobicity, surface modifications)
  4. Agglomeration
  5. Dissolution
- Brain
  1. Hydrophobicity and surface charge (possibility to cross barriers)
  2. Size and shape
  3. Chemical composition
- Body distribution
  1. Size
  2. Surface properties
  3. Dissolution rate

4. Chemical composition with emphasis on surface
5. Crystalline phase

- Ecotoxicity
  1. Particle size (distribution and uptake in organisms)
  2. Agglomeration state
  3. Contaminations
  4. Hydrophobicity (waterborne/sediment/distribution in soil), dissolution rate; persistence/stability (linked to dissolution, biodegradation). On these parameters no ranking was suggested.

In summary, it was concluded that nanoparticle composition, dissolution, surface area and characteristics, size, size distribution, and shape are parameters needed for any target organ toxicity assessment. Depending on the type of toxicological study undertaken, other physicochemical parameters would also be required.

## EXPOSURE ASSESSMENT

### Measuring in the Occupational Setting

A. Maynard talked about current measuring techniques for workplace exposure of nanomaterials and potential development needs. Since the widespread adoption of mass-based aerosol exposure limits around half a century ago, occupational aerosol exposures have generally been characterized using relatively simple techniques such as filter sampling and gravimetric analysis. However, the size, shape, and structure-related properties of engineered nanomaterials are challenging conventional approaches to exposure measurement. Given the vast range of current and potential engineered nanomaterials, the task of selecting appropriate measures of exposure is daunting. Despite the many possible biologically relevant attributes of nanostructured aerosols, these will most likely be associated with relatively few physical metrics, including number, surface area, and/or mass concentration.

A number of studies have demonstrated an association between aerosol surface area and biological response, suggesting this to be an important exposure metric. Although surface-area measurement methods are currently limited, methods such as diffusion charging are being developed that may lead to viable occupational exposure monitors. However, there is still uncertainty over the general applicability of surface-area concentration measurements, suggesting that viable number and mass concentration measurement methods also need to be considered.

Whichever metric is more relevant, measurement methods will need to be specific to particles within specific size ranges, depending on which regions of the body they are more likely to impact. In some cases, it may be sufficient to rely on current size-selective aerosol sampling standards. However, current research suggests that more sophisticated standards will be required for some materials. This is perhaps one of the greatest

immediate challenges to developing new methods of monitoring nano-aerosol exposure.

### Experience from Carbon Black

T. Kuhlbusch presented data from work area measurements at several carbon black-producing plants. They showed that no ultrafine particles were emitted by the process and bagging during normal conditions. The main sources of ultrafine particles were either related to other combustion sources inside (e.g., forklifts, heaters) or outside (e.g., traffic) of the plants. In the case of maintenance and repair work, emission of ultrafine particles, most likely organic carbon, was observed, as well as in another case with a leak in the production line.

These measurements demonstrated that care has to be taken when measuring ultrafine particles at nanoparticle workplaces with regard to the source. Adequate measurement strategies are needed. These strategies should also include some detailed information on the nanoparticles since the hazard potential may vary on for example particle size, morphology, solubility, and chemical composition.

### Dermal Exposure and Hazard Potential

J. Lademann gave a presentation on a noninvasive method for the investigation of penetration kinetics and penetration pathways of topically applied substances. In the past, it was assumed that the intracellular penetration inside the lipid layers around the corneocytes was the only penetration pathway for topically applied substances. However, recently it has been determined that the follicular penetration also has to be taken into consideration.

Analyzing the penetration of commercial products of titanium dioxide with a size  $\geq 100$  nm, usually used in sunscreens, it was found that these particles are located only on the skin surface or in the uppermost layers of the stratum corneum. No particles could be found in the deeper parts of the stratum corneum, even after long-term application. In regard to skin biopsies, it was found that the nanoparticles penetrate into the opening of the hair follicles; however, not all hair follicles contain nanoparticles. Therefore, concerning penetration, it must be distinguished between open and closed hair follicles. It could be shown that the closed hair follicles were covered with a mixture of corneocyte elements and dry sebum. In contrast, hair follicles are open for penetration, if sebum production or hair growth can be observed. In all cases of investigation, the nanoparticles were located only in the hair follicles, but not in the surrounding living tissue; with time, the hair follicles became depleted of particles by sebum production and hair growth. It can be expected that all nanoparticles that penetrated into the hair follicle openings were subsequently transported back to the skin surface.

Analyzing the penetration of fluorescent dyes in a nanoparticle form and in the nonparticle form, it was determined that the nanoparticles penetrate much better into the hair follicles than the nonparticle form, if massaging action is applied. Addition-

ally, the storage time of the nanoparticles in the hair follicles was up to 10 days, while the nonparticle containing formulation could be detected in the hair follicles only up to 4 days. The reason for the better penetration of the nanoparticles into the hair follicles was found in the action of the moving hair. It seems that the moving hair acts as a geared pump, if the size of the nanoparticles corresponds to the surface structure of the hairs. The moving hair pushes the nanoparticles deep into the follicles, while sebum and hair growth, in time, move the nanoparticles out of the hair follicles.

J. Lademann stated that, up to now, there has been no evidence that nanoparticles with a size  $\geq 100$  nm penetrate into living tissue under real conditions. The results of the diffusion experiments published in the literature, which demonstrated that nanoparticles could pass a skin membrane, should be discussed taking into consideration that the skin samples had a thickness of 500 nm. This means that the thickness of the membranes was less than the length of the hair follicles in the tissue. In this way, the membranes contained open channels, which can act as an efficient pathway for nanoparticles.

Summarizing the results, it was stated that in contrast to the stratum corneum, hair follicles represent an efficient long-term reservoir for topically applied nanoparticles. The optimum size for the penetration into the hair follicles was 300 up to 700 nm; the nanoparticles were removed out of the hair follicles by sebum flow and hair growth. However, no real evidence has been presented, up to now, that nanoparticles at a size larger than 100 nm penetrate through the skin barrier into living tissue.

### Measuring Exposure (Plenary Discussion)

H. Fissan began the session with a short presentation in which he outlined the main metrics (size, number, surface area, mass) that may be used to quantify exposure in the workplace. He also provided information on methods used to measure these metrics and the difficulties associated with measurement of them and in comparing them. In general terms it is necessary, because of lack of sensitivity, to prefer number or surface concentrations instead of mass concentration and to take account of background concentrations, and to subtract these from any measured concentration associated with a task or process.

Mass distributions at high concentration can be measured using low-pressure impactors such as the electrical low-pressure impactor, which can provide information on particle sizes ranging from nanometers to several micrometers. Number distributions can be assessed by various devices, including the scanning mobility particle sizer and the fast mobility particle sizer. These devices are commercially available.

Direct measurement of a surface area distribution or measurement of biological effects as a function of particle size, both of which are of interest in relation to nanomaterials, are not yet widely available. However, a recent development has been the implementation of the surface-area monitor by a leading manufacturer of devices. It uses charge on particles to

develop an estimate of total lung deposited surface area in different compartments of the lung. All of these devices are primarily static devices and are well suited for measurement of concentrations within a room, for example. But they are not particularly well suited to measurements of personal exposure concentrations and may need to be developed toward personal samplers.

R. Aitken then provided a series of questions for the group to consider. These were as follows:

- What is the relative importance of inhalation, dermal, and ingestion exposure routes?
- What is the best choice of metric for each and why? Is this appropriate for all Nanoparticles?
- What strategies (and instruments) are appropriate for demonstration of control for routine surveillance for collection of epidemiological data? What new methods and approaches might be developed?
- How can we better collect and share exposure information?

The discussion broadly followed these questions.

#### *What Is the Relative Importance of Inhalation, Dermal, and Ingestion Exposure Routes?*

It was generally considered that based on current knowledge, the most important route of exposure was inhalation. Although relatively few studies have been reported, there was not much evidence to suggest that dermal exposure is likely to provide a route which results in a systemic dose. (This is not to exclude the possibility that dermal exposure could result in local effects, such as dermatitis). Most of the remaining discussion therefore focused on inhalation as the principal route of exposure. (Again there is almost no information regarding ingestion as a significant route of exposure.)

It was noted in the discussion that exposure did not occur solely in the manufacturing of these products but also in handling, packaging, downstream use, etc. Exposure to nanoparticles also occurs in other processes such as welding where the nanoparticles are incidental by-products of the process, and have been monitored in these and similar industrial scenarios for many years. However, although these are clearly relevant, the focus of the discussion was on engineered nanoparticles rather than on incidental nanoparticles.

#### *What Is the Best Choice of Metric and Why and Is This Appropriate for All NPs?*

The earlier discussion had focused on some of the difficulties associated with the measurement of all of these parameters. The general view of the meeting was that, at this point in time, it was not possible, nor desirable, to select one metric or the other as the best. Currently, the level of the understanding of the toxicological issues does not provide clear

guidance. In different circumstances, alternative measurements may be more appropriate. However, it was clear that the metric surface area was likely to be of interest, but was currently not well covered by existing approaches, and needed further development.

A clear message from the meeting was the need for researchers and others to be very explicit about the way in which they have taken their measurements. Currently many people simply quote a few summary statistics such as mean and and/or standard deviation. This is insufficient, and additional information is required. For example, this should include details of the instruments used, the size range of the instrument, what assumptions had been made, which summary statistic had been used, the duration of exposure, whether background information had or had not been subtracted, etc. In addition, more clarity should be provided when comparing different studies.

#### *What Strategies Are Appropriate for Demonstration of Control, for Routine Surveillance, and for Collection of Epidemiological Data? What New Methods and Approaches Might Be Developed?*

Many of the instruments currently available are large, expensive, and not easily located in industrial scenarios. There is a clear need to develop instruments that are smaller, more portable, and less expensive. In an ideal situation, instruments that were suitable for measurements of personal exposure would be the most useful. Although there are a number of potential technologies that may be used to develop such instruments, none are yet commercially available.

The development of the new surface-area monitor was seen as a welcome step forward. It is likely, though, that much validation work will need to be conducted before a greater understanding of the information obtained from this instrument can be fully integrated and/or utilized, i.e., in relation to its limits of detection, upper and lower size bound, and the range of capability.

General guidance is available, for example, EN 689—Guidance for the assessment of exposure to chemical agents for comparison with limit values and measurement strategy.

In the meantime, it is important to work with the instruments that we currently have utilized and to develop our best understanding of the advantages and limitations of these for the purposes described.

Other issues to be considered included agglomeration. Some instruments such as particle surface-area monitors are likely to be more applicable in situations where agglomerates are present. Relying simply on, for example, particle counting systems where there is significant agglomeration will lead to an erroneous view of the nature of the aerosol. An important question is the following: Do agglomerates consisting out of primary nanoparticles survive mechanical stress and humidity effects during handling and in the surfactant in the lung? If they do, the transport behavior of the big agglomerates is likely to be very different from that of single primary particles.

### *How Can We Better Collect and Share Exposure Information?*

There are real difficulties in sharing exposure information, given some of the preceding discussion. If the details of the methods used are not available, then, for example, the simple count of particle number concentration in two very different scenarios, or comparing an industrial scenario with an environmental scenario, is likely to be of limited utility.

There was a view at the meeting that there might be some data sets available that relate to exposures that are either not yet available or not yet in the public domain. Clearly, if this is the case, then there are some advantages that such data become more widely available. It was recognized, however, that there may be commercial difficulties involved. However, it was a strong view at the meeting that there should be encouragement to share these data perhaps in anonymous form.

### *Other Comments and Issues*

One of the ways to help resolve the issue of the most appropriate metric is to investigate populations that currently are exposed to nanomaterials and to evaluate the various exposure metrics to see if it is possible based on an epidemiological study to associate exposure as assessed by the various metrics with some health effect.

In principle, such a study would be possible: for example, for a population of welders. Although welders have been studied for health effects, it would be important to attempt to utilize some of the emerging techniques for exposure assessment, as well as the new instruments and the new approaches in these studies, and thus to try to ascertain the validity of using these approaches.

## **HAZARD POTENTIAL**

### **Tiered Testing Strategy for Pulmonary Exposure to Nanomaterials**

D. Warheit explained that lung bioassay models can be useful for evaluating the pulmonary hazards related to exposures to nanoparticulate materials. A short-term pulmonary bioassay has been developed to assess the lung toxicity of inhaled particulates. These studies have been designed as hazard screens to determine whether engineered nanoparticle test substances impart significant toxicity in the lungs of rats by assessing numerous biomarkers and comparing the results with other positive and negative reference particle types. The combination of utilizing bronchoalveolar lavage and lung tissue studies concomitant with an experimental design consisting of dose-response, time-course evaluations and the inclusion of reference particle types provides a powerful tool for assessing the acute pulmonary toxicity of the nanomaterial particle test materials.

Key elements of a pulmonary toxicity screening strategy for engineered nanomaterials were outlined in the presentation. The proposed methodology is similar to the lung bioassay models that have been utilized in previous studies, but two important additions or enhancements have been suggested. First, the physicochemical characterization assessment of the nanoparti-

cle test material must become more robust. Thus, in addition to identifying the composition of the nanoparticle, it is important to provide additional characteristics, including the average particle size, shape, surface area, crystal structure, aggregation status, and other defining features—preferably in both the bulk starting material as well as in the dosing preparation utilized in the exposure phase of the study. Second, given that nanomaterials may have a greater tendency (relative to fine-sized particles) to translocate from alveolar regions in the lung to the interstitium or vasculature (and enter the systemic circulation), it will be important to assess the potential adverse effects of nanoparticle exposures on extrapulmonary organs. Thus histopathological evaluation of the major organs is recommended.

A tiered approach in rats is suggested for the assessment of hazards to nanoscale test materials. Tier 1 is viewed as a screening study and would include shorter term exposures (either inhalation or intratracheal instillation) along with several biomarker evaluations for postexposure periods extending through 3 mo. Tier 2 evaluations could include longer term pulmonary exposures, (including regulatory guideline studies), as well as *in vivo* mechanistic studies such as particle deposition, translocation, clearance/biopersistence studies, and animal models of susceptibility.

In summary, the primary features of a lung bioassay study include in the experimental design the following: (1) dose-response characteristics; (2) time-course assessments to evaluate the transient nature or persistence of any measured effects; and (3) the inclusion of appropriate positive and negative control reference materials (particularly for the instillation studies). Accordingly, the major endpoints of the study should include: (1) extensive physicochemical characterization of the test material; (2) pulmonary inflammation and cytotoxicity indices as measured in bronchoalveolar lavage fluids; (3) cell proliferation and histopathological endpoints in lung tissues; and (4) histopathology screening evaluations in major extrapulmonary organs.

### **Complementary Testing for Mechanistic Aspects**

K. Donaldson talked about a number of short-term testing systems that are available to enhance our understanding of the potential toxicity of nanoparticles. Although different particle types may produce different mechanisms that result in adverse cellular effects, there are some common pathways and properties related to the interactions of cells and particles. The dominant hypothesis for the mechanism of the toxic, proinflammatory, and mutagenic effects of nanoparticles (NP) is oxidative stress. Various methods are available to assess the oxidative stress potential of particle samples, such as electron paramagnetic resonance. Particle-derived oxidative stress leads to a number of proinflammatory effects in target cells, such as cellular oxidative stress, calcium flux, and signaling pathway activation. These can all be assessed in cells in culture over a few days. In addition, we need to consider the portals of entry in the selection of

cells—the skin, lungs, and gut—but because of translocation issues, a number of potential target tissues also exist, including endothelium, vessel wall, blood cells, liver, spleen, brain, and fetus. No validated assays exist to measure translocation, but there are potential strategies to develop assays to measure the movement of NP across cell membranes and monolayers and also within cells. The effects of NP on elements of the cardiovascular system can be studied *in vitro* using target endothelial cells, monocytes, platelets, the complement system, etc. Direct effects on the brain and heart could be studied by exposing neurons and heart muscle cells respectively to NP and assessing relevant endpoints. Genotoxicity of NP samples can be assessed using a number of target cells and endpoints such as comet formation and chromosome aberrations.

### Key Safety Issues Related to Inhaling Nanomaterials (Plenary Discussion)

A. Seaton and J. Mauderly cochaired a session on inhalation toxicology of nanomaterials. In their introductory remarks they said that the range of relevant safety issues remains broad because, to date, individual research efforts frame little more than an anecdotal database. An exception is the more systematic study of certain nanoparticles of pharmaceutical interest; however, much of that information is not broadly disseminated. Despite the title of the session, this summary makes no attempt to recite the litany of safety issues. There has been no shortage of meetings convened to explore safety questions and communicate current research approaches and findings. Rather, this summary (and the actual tenor of the discussion that occurred) deals largely with overarching issues that are thought to be key to significant progress. The fundamental safety issues are:

- The extent and nature of adverse health effects that could be caused by plausible exposures to NP.
- The types of NP associated with these hazards.
- The dose and dosing pattern required to induce effects that are sufficiently important to guard against.

Because the known range of physical-chemical species of NP is large and will only grow, the range of potential health hazards and risks can be expected to be similarly broad. There is at present little history of confirmed health problems associated with NP from which to build the knowledge base and research approach, although there is no shortage of speculation. This circumstance could become a fortunate opportunity to do the research necessary to prevent serious harm, were it not for the fact that the development and broad use of NP-based technologies will undoubtedly outdistance the pace of safety research.

A broadly agreed upon taxonomy of NP is a much needed overarching facilitator of progress in the field. Such taxonomy does not presently exist in any systematic or broadly used form. Indeed, clinicians, occupational hygienists, and laboratory researchers are largely left with only particle size as a unifying classifier of NP, despite recognition by all that this is a woefully inadequate (although important) metric. An adequate framework

for communication and development of research strategies requires a mutually intelligible taxonomy that includes not only size, but also shape, composition (notably of the surface), and solubility. Going beyond these minimal descriptors to also address surface and internal structures and chemical moieties will be necessary to fully understand the biological fates and hazards of NPs. No single research group, federal agency, or nation could conduct the full range of NP studies that will be needed in coming years. Establishing a systematic framework for communication and tracking progress among agencies and researchers is absolutely necessary.

Accompanying the need for a systematic NP taxonomy is the need for a coordinated, universally accessible repository of information. For greatest value, the repository should catalogue, according to the taxonomy of both “natural” and “manufactured” NP types, information on where and how exposures may occur, routes of exposure and likely doses (or exposure concentrations), the range of physicochemical composition within categories, biological disposition (see later discussion), and known effects (from molecular to clinical). Great advantage would accrue if the database were to also include indexed listings of ongoing research and publications. This, of course, is a huge undertaking, and would require not only that producers of NP and researchers provide data, but also that a substantial infrastructure be established for database management.

The preceding two needs point to a third general issue, the identification of an entity to take the lead in ensuring that the taxonomy and database efforts are carried through from mere visions to functional realities. It is easy enough to state the needs; it is quite another thing to implement the actions. The truism that “something that is everybody’s business is nobody’s business” is familiar to all. That is, unless some organized entity assumes responsibility for taking the coordinating and financial steps necessary to develop a NP taxonomy and database, they will continue to exist only in effete, fragmented forms. The discussion did not conclusively identify the most appropriate entity; however, all (who spoke) ratified the call for one to be identified.

The fourth overarching need, and one that flows from, and is facilitated by, the first three, is the development and tending of a systematic research strategy that takes into account the types and exposures to NP likely to have the greatest health impact, tracks findings and ongoing research, and marshals research resources (or tracks resources marshalled by others) to ensure that important knowledge gaps are filled. Admittedly, an international research “management” framework is implausible. The point to be taken, therefore, is not that such centralized management could (or should) occur, but that resolving health questions about such a broad range of materials as NP requires systematic research and research management perspectives. If a universal taxonomy and coordinated database of cumulative research and findings were established, individual federal agencies and research groups could pursue better research strategies within their particular realm of interest.

Having dealt with some very broad issues, a more specific need voiced in this discussion was also evident throughout the meeting: There is a general need for better information on the disposition of NP having entered the body via inhalation, ingestion, or dermal penetration. Better information is needed on the fraction of NP that are taken, the pathways by which they are distributed in the body, the transfer rates, the retention time and accumulation rates in different anatomical sites, and the processes, pathways, and rates of dissolution and excretion. There are certainly many other important facets of biological interactions and mechanisms of adverse responses that need investigating. However, a better understanding of the disposition of different types of NP is an "enabling knowledge" that will greatly facilitate the other investigations.

Studies of the disposition of NP could be greatly facilitated by two advances. One is a greater availability of tracer species for quantitative assessment of amounts in tissues and fluids, and (ideally) that can be viewed by some form of imaging. The other is a greater availability of standardized NP species that are representative of different physicochemical types. The purpose of the latter, of course, is so that identical NP can be used in repeated studies and by more researchers. These technical facilitators could greatly improve the systematic pursuit of research issues.

### Testing Strategies to Establish Dermal Exposure and Hazard Potential

(Breakout Group Discussion; Chairman J. Lademann; Rapporteur T. Butz)

The following questions were addressed:

Which tests should be conducted to establish exposure potential through skin barriers?

When is dermal hazard testing necessary?

For the evaluation of a potential health risk of nanoparticles, two aspects have to be considered:

- Potential hazard: Are nanoparticles harmful to living cells?
- Exposure aspects: Do nanoparticles penetrate under real conditions through the skin barrier into living tissue?

The first aspect may be evaluated using cell culture experiments, as broadly applied in toxicological assessments. However, such experiments should always include the appropriate microparticle control groups, in order to assure whether observed adverse effects are substance or nanoparticle related. In addition, the capacity of mammalian cells for phagocytosis/endocytosis of insoluble particles should be considered. It is well established that endocytosis of insoluble particles may cause toxicity in mammalian cells. For example, international guidelines for *in vitro* genotoxicity studies recommend that in

mammalian cells poorly soluble substances should not be tested beyond concentrations producing precipitation.

For the evaluation of the second aspect, in principle, several methods are available for the evaluation of penetration of nanoparticles through the skin barrier. Using the method of differential stripping, the penetration kinetics of nanoparticles in the stratum corneum and the hair follicles can be estimated. The method of differential stripping is based on the well-known method of tape stripping, which is used to completely remove the stratum corneum. After analysis of these tapes, the amount of nanoparticles that remain on the skin surface or the upper layers of the stratum corneum as well as the amount of removed corneocytes can be determined. Based on these measurements, penetration profiles in the stratum corneum may be calculated. Using the method of cyanoacrylate surface biopsies after tape stripping, the amount of nanoparticles penetrating into the hair follicles can be determined, given that cyanoacrylate stripping removes the hair follicles. By analyzing the penetration and storage kinetics of nanoparticles in the stratum corneum and the hair follicles, information on the penetration of these substances through the skin barrier can be obtained. The determination of the amount of nanoparticles removed from the stratum corneum and the hair follicles after penetration permits the establishment of a mass balance. Using this calculation the amount of topically applied substances passing through the skin barrier can be evaluated. Nevertheless, a disadvantage of the method is that only the depletion of the reservoir can be evaluated, whereas the amount of a test substance that has penetrated into or through the skin is not directly measured.

The second method is based on diffusion experiments using skin membranes. Usually the thickness of these membranes is several hundred micrometers, which means that the thickness of the skin samples is less than the lengths of hair follicles in living tissue. Therefore, tissue membranes contain openings whereby topically applied substances as well as nanoparticles may penetrate into the receptor fluid.

Under normal conditions these tissue openings, consisting of the hair follicles, have little effect on the penetration process. One reason could be that a close network of elastin and collagen fibers surrounds the hair follicles. Usually, human tissue samples obtained from surgery are stretched for their application to the diffusion cells. During this procedure, the interfollicular space may be stretched more than the follicles themselves which may produce an opening of the follicular orifice resulting in microscopic holes in the membrane. Therefore, the ratio of intracellular versus follicular penetration could depend on the stretching procedure. Experiments on pig-ear skin are useful for the evaluation of this problem due to the large hair follicles of pig skin. Additionally, mechanical stimulation of hair may affect the penetration of nanoparticles. Usually, in diffusion cell experiments no massage or mechanical force is applied which may move the hair and may push nanoparticles into the tissue. Overall, given these limitations, diffusion experiments appear to be less suited for penetration measurements of nanoparticles.

A highly prospective method for the determination of the penetration of topically applied substances, including that of nanoparticles, into and through the skin barrier is the use of fluorescent dyes in combination with laser scanning microscopy. These noninvasive, online methods allow the determination of the position of topically applied substances in different layers and depths of the stratum corneum, i.e., up to 250  $\mu\text{m}$ . The disadvantage of this method is that laser scanning microscopy cannot be applied to all types of topically applied substances, but only to those with efficient fluorescent properties. The development of two-photon systems, which allow the analysis of the penetration of nanoparticles into deep tissue layers with a high sensitivity, opens new perspectives for the determination of penetration processes in the future.

Another method for the determination of the penetration of topically applied substances, including nanoparticles into and through the skin barrier, is the microscopic analysis of histological sections removed from the skin after topical application of nanoparticles and subsequent biopsy. Thin (approximately 10  $\mu\text{m}$ ) and ultrathin (approximately 50 nm) cross sections can be analyzed by high-resolution transmission electron microscopy (HRTEM) and ion beam techniques such as particle induced x-ray emission mapping ( $\mu\text{PIXE}$ ). HRTEM allows visualizing individual nanoparticles with diameters around 10 nm and larger; in addition, the chemical composition of individual particles is obtained. The disadvantage is that the field of view is limited and substantial sample preparation is required with the risk of introducing preparation artefacts.  $\mu\text{PIXE}$  with a lateral resolution of about 300 nm, on the contrary, does not visualize individual nanoparticles below this size; the advantages are that a large field of view can be scanned with the option to zoom into regions of interest, quantitative elemental maps are obtained, and the risk of introducing preparation artifacts is minimized.

The most sensitive technique uses nanoparticles radiolabeled with positron emitters. This requires skin explants from surgery, to which formulations are applied topically. Thin cross sections are subsequently dipped into nuclear microemulsions, and after exposure individual positron tracks can be detected. Again, there is a risk of contamination during sample preparation.

Summarizing the results of the discussion, it can be established that, up to now, there is no evidence that nanoparticles at a size exceeding 20–100 nm penetrate through the skin barrier into the living tissue. The potential skin penetration of much smaller nanoparticles ( $\ll 10$  nm) will be the subject of further investigations. In this particular case, it must be taken into account that also nanoemulsions, which are applied for cosmetics and medical treatment, form structures with a size of several nanometers, which are comparable to the size of extremely small nanoparticles. On the other hand, solutions contain distinct molecules, which are even smaller than nanoparticles. Therefore, it may be assumed that the skin penetration rate of substances in the form of microemulsions is between that of a particulate form and that of a solution of the same substance.

In conclusion, there is no evidence that nanoparticles exceeding the size of about 20–100 nm pass the barrier in healthy skin. Today's knowledge about penetration of nanoparticles is based on the available methods, which have their limitations, particularly concerning the detection limits of small amounts.

During the discussion of the dermal exposure and the hazard potential it must be kept in mind that the dermal uptake of nanoparticles in any case will be orders of magnitude smaller than the uptake by inhalation or oral uptake.

### Testing Strategies Concerning Systemic Exposure

(Breakout Group Discussion; Chairman G. Oberdörster; Rapporteur E. Haltner).

The following question was addressed: Which tests should be conducted determining whether systemic exposure occurs, and if so, what is the impact on human health?

The responses were subdivided into three categories:

- Exposure measurements at workplaces.
- Physicochemical characterization—for exposure.
- Tiered approach for testing.

#### *Exposure Measurements at Workplaces*

Given that many exposure assessors utilize different procedures, the first step that needs to be taken is a full and detailed description of the measurement methodology that was undertaken. This would include specific descriptions of the conditions for operation, including details of the instruments utilized, the specific sampling techniques, and descriptions of standard operating procedures. Ultimately, it will be important to develop a standardization of methodologies with other organizations such as ISO; however, this process is a lengthy one. In the meantime, it will be important to improve and adjust measurement techniques, as existing systems are not always suitable for the assessment of nanoparticle exposures (e.g., appropriate dose metrics—mass vs. surface area; portability—and appropriate equipment to conduct the required studies). Ultimately, the goal will be to assess personal exposures to nanoparticles using the appropriate dose metrics.

#### *Physicochemical Characterization—For Exposure*

The preferred characteristics should include particle size, particle size distribution, surface properties including surface area, and particle mass. The ultimate goal will be to measure exposure using state-of-the art methods and equipment.

#### *Tiered Approach for Testing*

The important physicochemical parameters for characterizing nanoparticles were determined to be particle size, particle size distribution, chemical composition, surface properties, and stability in physiological systems.

In vitro assays were considered to be important components of a tiered testing system. The following characteristics were

viewed as desirable outcomes for in vitro testing: nanoparticle dose levels, particle uptake within cells, possible absorption through cells, and oxidative stress endpoints.

Ultimately, it will be important to generate in vitro and in vivo databases on a particular nanoparticle type in order to compare the in vitro with in vivo results. In addition, the conditions under which particulate samples were stored prior to testing (e.g., humidity, temperature, containers) will be important to document for comparison with later investigations. Finally, the breakout group suggested testing of typical or generic nanoparticle types—particularly with the inclusion of positive and negative control reference particle types.

## SUMMARY AND CONCLUSIONS

The workshop was concluded by H. Greim, who summarized the discussion on the various parts of the program.

### Background

For nanoparticle characterization the working definition is <100 nm in one dimension or <1000 nm to include aggregates and agglomerates.

Although many physical factors can influence the function and the toxicological and environmental characteristics of nanoparticles, their impact is largely determined by:

- Composition.
- Dissolution.
- Surface area and other surface characteristics.
- Size.
- Size distribution (including aggregation and agglomeration state).
- Shape.

Most of the hazard information on nanoparticle types has been derived from studies with carbon black and ultrafine TiO<sub>2</sub> particles. Currently, available data suggest that nanoparticle-induced toxicity is qualitatively similar to that of traditional, fine-sized particles, with the effects in the lung primarily related to inflammation and possibly secondarily to genotoxicity, while the systemic effects may impact the cardiovascular and central nervous system (CNS) systems. Most of the information on potential systemic effects is derived from combustion-generated particles.

Toxicokinetics are generally determined by size and surface characteristics of particles. The decrease in particle diameter could lead to increased passage through cellular and/or intracellular membranes, along with possible transport through nerve axons. In addition, particle deposition patterns are influenced by particle size.

There currently exist a variety of methods for assessing nanoparticle exposures. However, there is a significant need to develop, improve, and adjust measurement methodologies to obtain accurate information on the most suitable parameters such as particle size, particle number and particle surface area. To this end, the development of standardized procedures is required.

## General Testing Approach

A first step may include an in vitro screening strategy to assess the possible reactivity of nanoparticle types (including biomarkers of inflammation) along with cellular uptake for prioritisation.

This would be followed by repeated dose inhalation studies with completed standard organ evaluation including biochemistry, hematology, and histopathology of the major organs. These tests could be supplemented by specific tests to determine subtle effects including inflammation, transcription factors and corresponding gene expression, oxidative stress related to formation of free radicals, and possible secondary genotoxic effects.

## Specific Recommendations for Tiered Testing Strategy

### *Preliminary Testing and Characterization of Reactivity*

In vitro systems could be developed to determine the reactivity of nanoparticulate types to determine potency when compared to reference materials such as carbon black. However, at present there are no specific test systems that are recommended. As a consequence, it is suggested that 5–10 currently available cellular or acellular systems be selected to develop a database and to compare the results on specific nanoparticle types with results from in vivo studies.

### *Tier I—In Vivo Testing for Hazard Identification*

A short-term inhalation (or intratracheal instillation) study is recommended with predominant evaluation of pulmonary effects in rats or mice. The dosimetric considerations should include particle size, size distribution, surface properties, chemical compositions, shape, and aggregation status. The exposure duration should be a minimum of 2 wk for the inhalation or 1–2 doses for the instillation study. The effects that should be evaluated include lung inflammation and cytotoxicity, cell proliferation, and histopathology of the respiratory tract and the major extrapulmonary organs. Suggested postexposure time periods for the instillation study should include 24 h, 1 wk, 1 mo, and 3 mo. For the inhalation study, the postexposure time periods should include at least one recovery time period.

### *Tier II—In Vivo Testing for Hazard Identification*

A longer term inhalation study is recommended in rats. In addition to the Tier I parameters already described (e.g., substantial physicochemical characterization, histopathology, BAL endpoints, etc.), some suggested mechanistic-based studies could include the following: determination of particle deposition, translocation, and disposition. Additional studies could be designed with specific animal models to mimic sensitive populations. An important caveat for these studies: It is strongly advised to avoid using particle overload concentrations to facilitate a better interpretation of results in the long-term exposure studies.

## Dermal Testing

Up to now, there is little evidence that nanoparticles at a size exceeding 100 nm penetrate through the skin barrier into the

living tissue. The penetration of nanoparticles at a size less than 100 nm should be a topic of further investigation.

Analyzing the dermal exposure and the hazard potential of nanoparticles, it must be taken into consideration that the dermal uptake of nanoparticles will be an order of magnitude smaller than the uptake by inhalation or oral uptake. For the evaluation of the health risk of nanoparticles, it has to be determined whether they are harmful to living cells and whether, under real conditions, they penetrate through the skin barrier into the living tissue.

Cell culture experiments are broadly used for toxicological assessments. For the evaluation of the penetration processes, in principle, three methods are available. Using the method of differential stripping, the penetration kinetics of nanoparticles in the stratum corneum and the hair follicles can be evaluated. This analysis can be carried out *in vivo*. Diffusion cell experiments are an efficient method for *in vitro* penetration studies. Also, laser scanning microscopy is well suited to test penetration kinetics, although requiring fluorescent labeled nanoparticles.

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