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Nanotoxicology

Nanotoxicology: An Interdisciplinary Challenge

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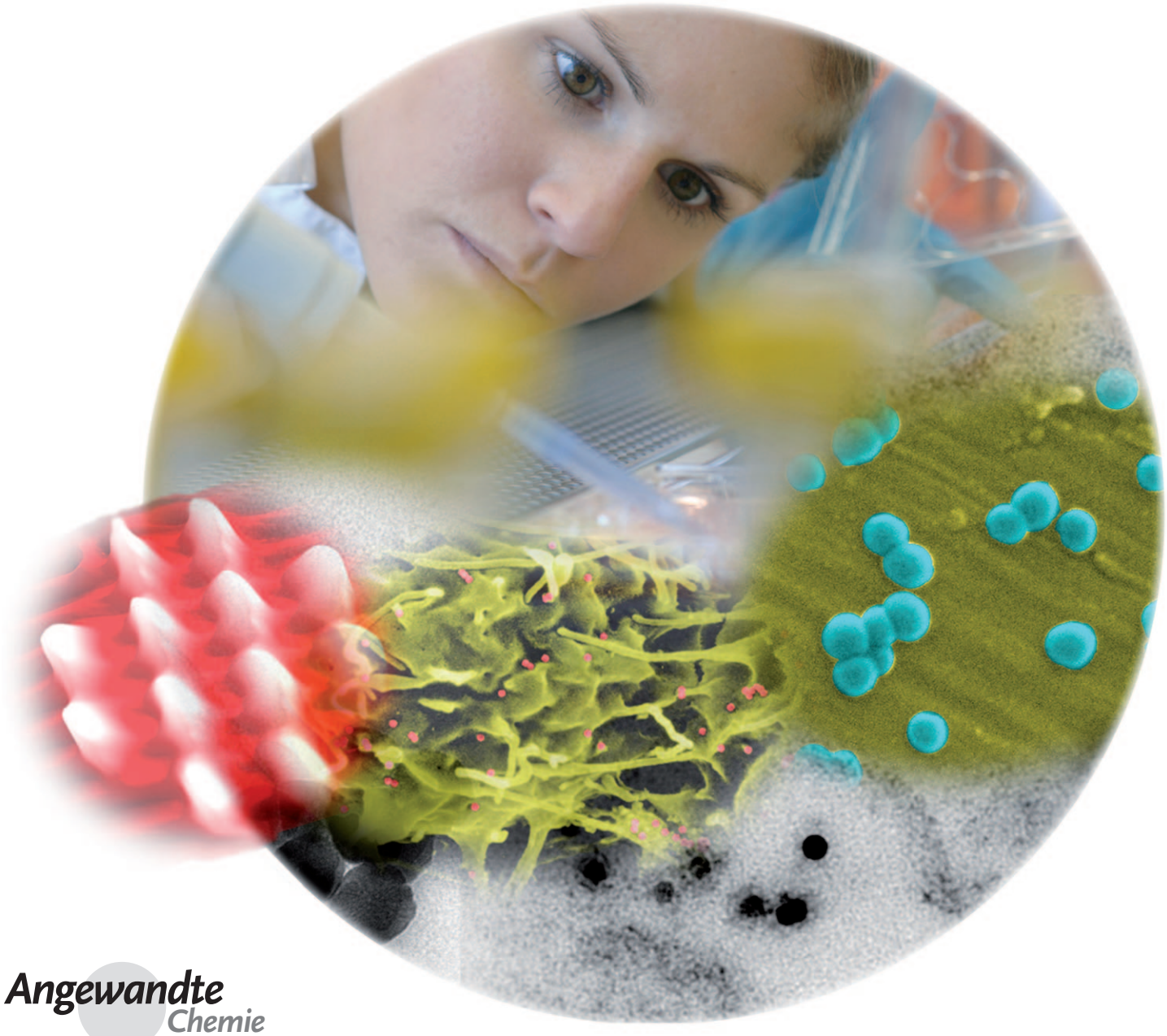

The increasing consumption of products containing nanomaterials that can be currently observed and forecasts of new developments and applications fan the fear of individuals and organizations regarding new risks to health. Considering experiences gained from previous technology developments, such fears are not completely unfounded. But are they really justified? And is it justified, moreover, to speak of "nanotoxicology" as a new discipline? This Review seeks to cast light on the phenomena that may occur as nanoobjects interact with cells, tissues, and organisms. Furthermore, we will demonstrate that the many data made available on the biological effects of nanomaterials do not always come from studies that can be considered reliable. We will point out the aspect of reliability with specific examples from the literature and will not address specific (nano)materials. In particular, inadequate methods will be described together with recommendations how to avoid this in the future, thereby contributing to a sustainable improvement of the available data.

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1. Introduction

Ever since research and scientific efforts have begun to understand the mechanisms of chemistry and to clear up and control the paths of syntheses, concerns have been raised over adverse effects that chemicals and materials may exert on living organisms or on the environment. If the repeatedly experienced fatal damage and severe impairments to health and the environment[1] have caused an increased public attention to impacts of technology in the past are taken into account, it stands to reason and is necessary to take a closer look on this most recent key technology, namely nanotechnology. Before discussing this topic in detail, we have to provide a basic definition of the essence of the issue to be treated. Only a few years ago, the term "nano" was used quite arbitrarily, and it was common practice to speak of nanoparticles when referring to something that is micrometer-sized.[2]

Meanwhile, both national and international institutions and organizations have made it their task to find exact definitions and lay down guidelines (ISO, OECD, BSI, DIN) that fix the range between 1 nm und 100 nm as being relevant (Figure 1). In spite of this clear definition at last, the term "nano" is not uniformly used. The Swiss Action Plan on Nanomaterials, for example, maintains that with regard to the aspects of precaution (see list of links) and biological effects, particles with sizes of up to 300 or even 500 nm may have significance as well, considering the "smallest particles that may reach any part of the body". It is postulated, on the other hand, that the specific "nano-effect" size of particles must be below 30 nm;[3] in other words, below the limit inducing physical or chemical processes that may create unknown, unexpected properties in the materials involved. In fact, as such strict limits, no matter if at 30, 100, or 300 nm, make little sense for the issues of biology and even chemical and physical effects may not appear only within the low nanometer range,[4] the disputing researchers may all be right somehow or other: Depending on the reaction partner interacting with the new materials in the respective cell or biological structure, a larger range than that comprised by the restricted definition given by materials science may be affected (Figure 1).

There is tacit agreement among biologists and toxicologists that particles that can take different, partly not yet defined paths in organisms are referred to as nanoparticles. Being related to sizes of less than about 250 nm, such a definition would also include nanoparticles that are applied in medicine, for example, to act as drug-delivery systems; that is, as materials that are not used for their physicochemical properties but are manufactured for transporting special substances to targeted environments within an organism.[5] As a rule, such delivery systems require particles in the range of 40 to 200 nm or above. However, why should toxicologists treat these in different ways to their larger chemically identical equivalents? The answer to this question is given below in this Review. But we will not initially address specific materials, as generally recognized rules may be more important from our point of view. Thus, we present these concepts using the examples of well-known nanomaterials,[6] whereas materials containing an intrinsic toxicity, such as semiconductor quantum dots, or newly invented materials, such as carbon quantum dots,[7] for which no data exist, will not be addressed.

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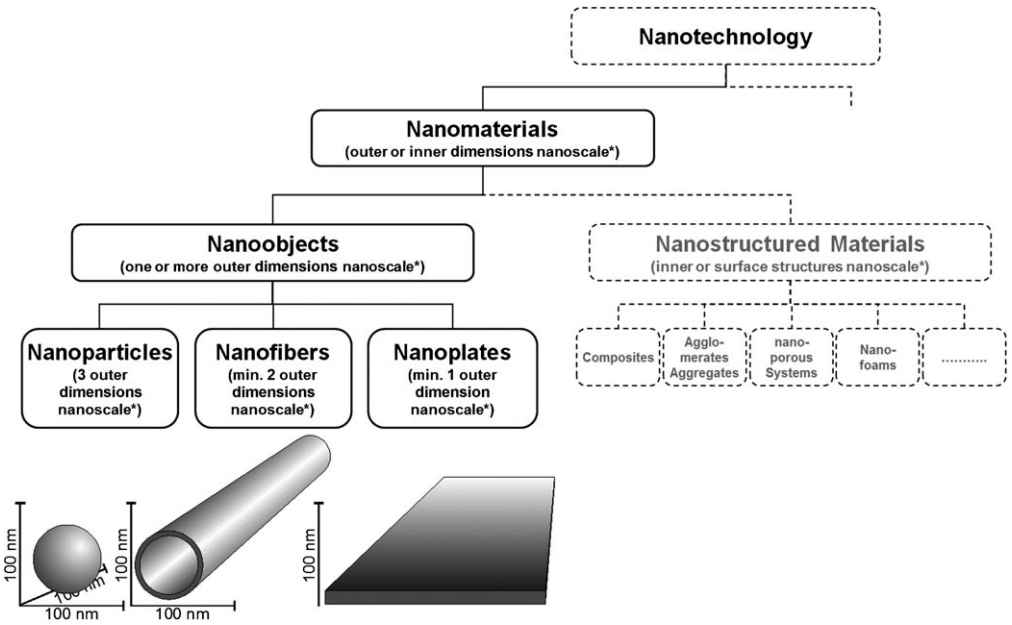

Figure 1. The ISO definition of nanoobjects. Included as nanoobjects are nanoparticles (nanoscale in all three dimensions), nanofibers (nanoscale in two dimensions), and nanoplates or nanolayers (nanoscale only in one dimension). * Nanoscale: a size of between 1 and 100 nm.

Not only experts believe that, once again, we are standing at a technological threshold that promises completely new chances of solving serious problems. In particular, we can expect new applications within the energy sector (energy production and storage), the optical, electronic, mechanical and ceramics industries, the construction industry, and fields of application such as traffic engineering or environmental technology (sewage cleaning, clean-up of soils and air). Moreover, the industry will provide numerous consumer products ranging from cosmetics to medicine, and from mobile phones to flat screens. All of these applications clearly show that when discussing potential health hazards that may come with nanotechnologies, it is necessary to differentiate between two different views: While using new materials with nanoeffects in solid compounds, composite materials, or in ceramics is less significant regarding health, the use of rather freely moving nanoparticles, nanofibers, or nanotubes (for example in cosmetics, pharmaceuticals, on surfaces, or other applications which permit direct contact with the skin) is surely much more critical. Therefore, most current studies[8,9] and projects (see NanoCare und Tracer) are focused increasingly on workplace situations. The public, on the other hand, has meanwhile realized that there are fields and products (cosmetics, for example) wherein using nanomaterials has become common practice. Before discussing the nanotoxicological aspects in specific detail, we need to explain some important facts and issues.

Nanoparticle exposures are not unique to the past decade. Ultrafine particles some hundred nanometers in diameter and smaller are released during all combustion processes and occur in nature during numerous natural processes. Cave dwellers utilized the smallest particles in the form of carbon black or soot to paint the walls of caves they lived in, and stained-glass artists in the Middle Ages used nanoparticulate gold that made the windows of churches appear in brilliant red until the present day. New in our present time is the variety of additional materials and compounds as well as the wide range of possible applications, which are expected to soon increase the loads on man, plants, animals, and environmental compartments and to raise issues of a risk R arising from exposure E to the new materials and of the hazards H that may cause biological effects. The probability P of processes must also be considered, because a risk only occurs when there is a certain probability of the development of biological effects.

$$R = \mathrm{f}_P\{E,H\}$$

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The function of such probability can be explained by a simple example: Primary TiO_2 particles sized 25 nm are added to cosmetic sunscreens to obtain maximum UV protection. TiO_2 exposure takes place each time the sunscreen is applied to the skin. Hence, although E is relatively high, there is no risk unless TiO_2 exerts a biological effect reaching the very site of biological action. Meanwhile, more than 40 studies (for example the European project NanoDerm) have shown that TiO_2 does not penetrate the skin to get into the body and that biological effects are generally rather small. It follows that H is very low and that there is hardly any risk R for humans. As all of the sunscreen constituents eventually are released to the environment, the focus should be on investigating their overall environmental effects. In fact, some recent calculations[10] reveal that most of the products containing and releasing TiO_2 may cause local increases in the respective TiO_2 concentrations within environmental compartments. It is not yet clear whether these may affect living organisms. After all, there is a pronounced natural background (compare Figure 2), as titanium is among the ten most frequent elements in the Earth's crust and as different minerals and metal oxides occur as nanoparticles in the natural environment.[11–14]

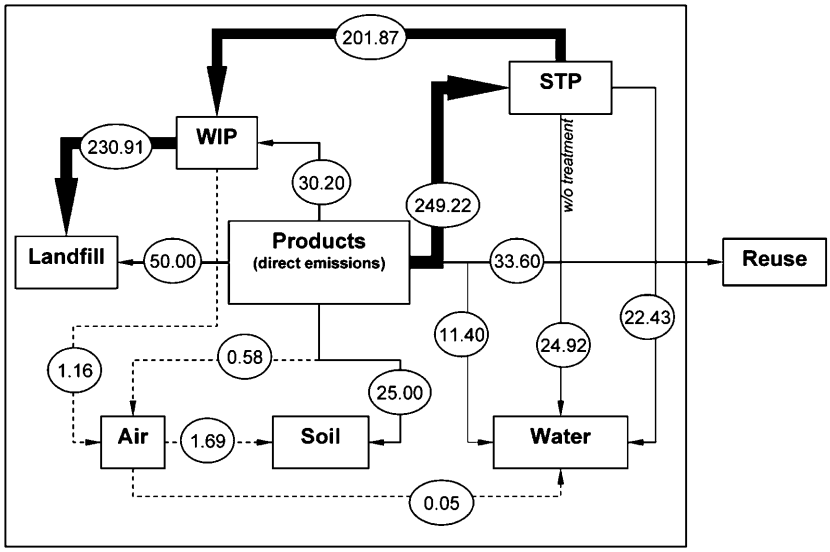


Figure 2. Nano-TiO_2 flows from the products to the different environmental compartments, waste incineration plant (WIP), sewage treatment plant (STP), and landfill (high-exposure scenario). All flows are in tons/year. The thickness of the arrows is proportional to the amount of TiO_2 flowing between the compartments. Dashed arrows represent the lowest volume. (Reproduced from Ref. [10] with permission of the American Chemical Society, copyright 2008.)

It must also be mentioned that there is a lack of suitable standardized detection methods. Hartung has stipulated only recently that three major steps be developed to obtain a viable system for toxicologists to deal with nanotechnology issues in an adequate way (Figure 3). This appears to be easier than it really is: A solution of that kind would require a set of evaluated in vitro systems suited for the new materials and it would have to be capable of assessing adequate biological end points while relying on unaffected parameters for the generation of data sets that allow robust, exact predictions of the toxic responses in the living organisms. As illustrated in Figure 3, this requires assessing the existing systems for their suitability for testing nanoobjects. A new strategy developed on the basis of that evaluation and applying the appropriately adapted OECD guidelines would reduce misinterpretations by using at least two different tests for each biological end point. Besides, it is important to validate each newly developed method through comparisons with existing meth-

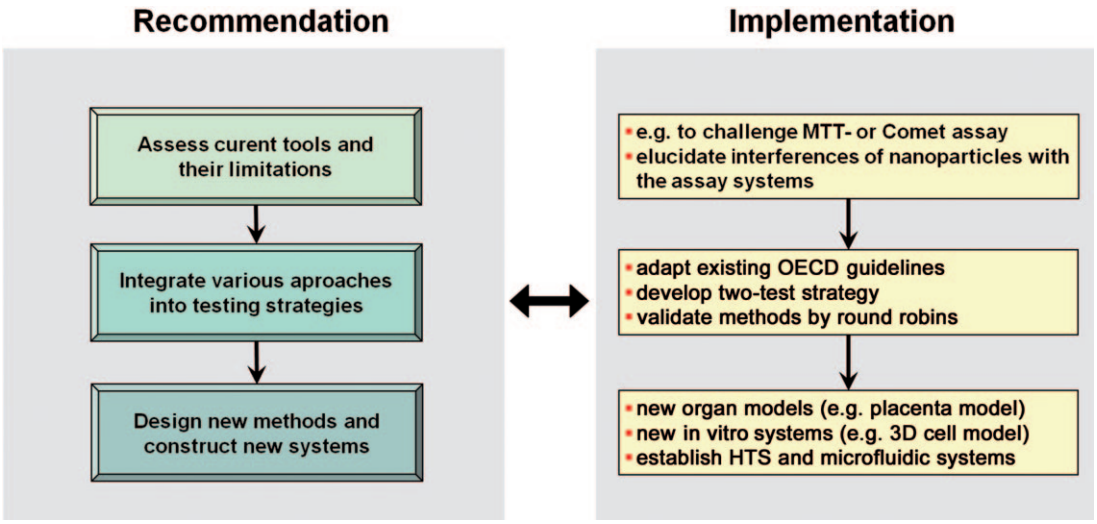


Figure 3. The three main steps that the toxicology community needs to take to arrive at a new system of toxicology (left side; reproduced from Ref. [15] with permission of Macmillan Publishers Ltd, copyright 2009). To achieve this goal, nanotoxicology as a discipline should question the existing testing methods, establish new strategies for testing, and should introduce and evaluate new developments (right side).

ods, for example within international round-robin tests (IANH; see Appendix: Projects). For all that comprehensive effort, this is a tedious, intricate procedure requiring much patience on the part of the participating laboratories and institutes. The complexity of the process will be evident from the sections below.

2. Risk: Does Toxicity Necessarily Imply a Risk?

New technological developments are mostly overestimated, and hardly any attention is paid to their potential crucial aspects. The possible risks of nanotechnology, however, have been discussed at an early stage.[16–20] Each new technology and the developments that it produces bear risks for the society, for the economy, and for health and the environment. Discussing the issues of nanotoxicology, we will be placing emphasis on investigating aspects of health, and thus scrutinizing the possible negative effects on biological systems. Harmful effects do not occur unless there is evidence of the criteria of exposure E and biological effect or hazard H that have been defined above as being the main risk determinants from the point of view of toxicology. Furthermore, the criterion of probability comes into play in gathering the plausibility and likelihood of an adverse occurrence. Once again, we will discuss the example of TiO_2: As mentioned above, nanoscale TiO_2 is used in numerous products as a UV-protecting agent. While its uptake via the skin has been sufficiently understood, TiO_2, as a compound containing both micro- and nanoscale particles, has been used for two decades as an approved food additive (E 171). The consumer protection organizations fear that the nanoscale fractions may be contained in the food and be released into the body via the gastrointestinal tract to cause harmful consequences. The few studies that have examined the relevant gastrointestinal scenarios at least have however not come upon any alarming acute effects. Wang et al.[21] have shown, for example, that extremely high singular doses cause only slight adverse effects. With the relevant dose amounting to 5 g kg^{-1} body weight of the test animals, single doses causing such effects in adults weighing 60 kg would amount to 300 g, which exceeds the acute toxic dose of NaCl (250 g). In spite of this, we add salt to our food using table salt (or sodium chloride) without giving such a "risk" a second thought.

We surely only incur risks when concentrations that we are exposed to are really relevant and occur in our everyday lives. Besides, as many processes in our bodies are either self-healing or part of the "normal" reaction potential of the cells or organs, risks are not necessarily involved by every biological effect. With this in view, risks, and particularly those of the persistent and accumulative substances, are characterized by two main factors: The probability of reaching a biologically effective concentration in the body, and the triggering of a serious biological adverse effect or damage. The related possible time-dependent effects are a particular methodical challenge and an issue to be investigated for the case of stable nanoparticles. An exposure to materials and substances for which effects are not known or have not been determined sufficiently to cause harm (great uncertainty) must be therefore largely avoided, while the risk of substances that are known from sufficient data (high state of knowledge) can largely be avoided or reduced considerably by applying adequate risk management strategies. At the outset, the use of new materials in nanotechnological developments thus requires an increased knowledge about biological effects and to perform measurements and model calculations that can reasonably predict exposure. It is only based on such extensive knowledge that potential risks can be described, and managed if necessary (Figure 4).

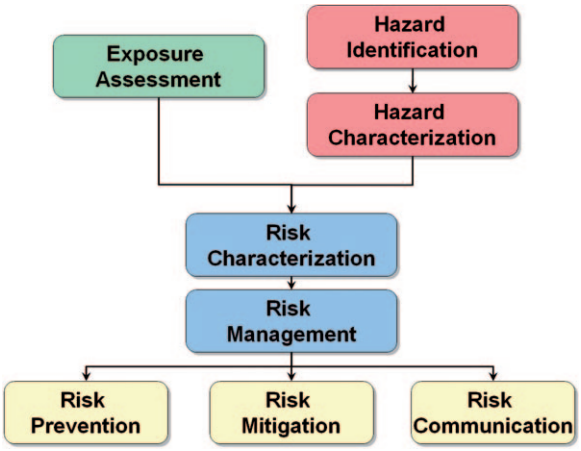


Figure 4. Risk assessment and risk management regarding possible adverse substances or materials.

Different aspects of exposure and of biological effects will be discussed in the following sections, placing emphasis on the "particular" features of nanomaterials and on the approach to finding answers as to whether there can be a special toxicology, namely a nanotoxicology for nanomaterials.

3. Scenarios of Exposure: Possible Uptake Paths

There are numerous applications of nanomaterials with manifold ways for humans to use and be affected by them. This section will be dedicated to determining the routes through which synthetic and free nanoparticles can get into the human body.

Quite plausibly, workplace exposure opens up such routes. To understand the relevant scenarios of exposure, particle measurements can be performed in workplaces themselves[9] or, as in NanoCare, by means of computer models that simulate distributions.[22] Owing to the ever-present workplace background loads, it is a challenge to measure the synthetic nanoparticles without the proper strategies and online characterizations. Instead of outlining such aids in detail, we will describe the toxicologically relevant portals of entry into the human organism.

3.1. The Lung: The Main Portal of Entry for Nanoobjects

The lung is the organ that transports the air via the respiratory tract to the alveoli, where oxygen and carbon dioxide are exchanged with the environment. There are 300 million alveoli to facilitate this gas exchange via diffusion, encompassing a surface area of approximately 140 m^2.[23] The air in the lumen of the alveoli has a close proximity of some hundred nanometers away from the flowing blood. The lumen and alveoli are separated by an epithelial and endothelial layer.[24]

Foreign particles, including nanoobjects that deposit into the lung, are mostly removed by mucociliary transport as they pass the respiratory tract or bronchial tubes. Fine particles (< 2.5 μm) can be transported with the air into the alveoli (Figure 5). Since the alveoli lack a mucociliary clearance

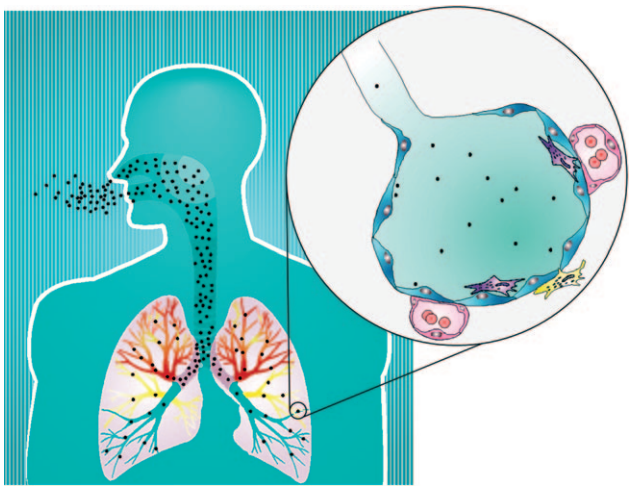


Figure 5. Possible transport pathway for nanoparticles in the lung. Inhaled particles that are smaller than 2.5 μm ($PM_{2.5}$) have access to the alveolar structures of the deep lung and may, in high doses, induce inflammation. A very small portion of the nanoparticles can cross the air–blood barrier and will be distributed via the bloodstream (red). Within the alveoli, most of the particles will be phagocytized by macrophages (purple) or dendritic cells (yellow) or may also be taken up by epithelial cells (blue).

mechanism, such foreign particles are removed by macrophages "eating" all foreign particles that are deposited in the alveoli to transport them to the bronchial regions from where they are removed by means of mucociliary clearance. These clearance mechanisms that have developed through evolution are extremely efficient as long as they are not chronically overstressed, for example by excessive smoking or dust in workplaces. It was shown in animal tests that high doses of nanoscale particles are capable of overcoming the thin air–blood barrier to transmigrate into the blood.[25] The quantity of nanoparticles that reach the bloodstream through inhalation amounts to only a fraction (< 0.05 %) of the quantity administered[26] and, in addition, is dependent on the physicochemical properties of the respective particles.

With the pulmonary uptake of foreign particles, particulate matter, or fumes constituting the most frequent and most probable scenario of exposure, it is not surprising that many of the funded research projects are based upon investigating the effects of synthetic nanoparticles on the respiratory tracts (see the Appendix for internet addresses, databases, and projects).

3.2. Uptake of Nanoparticles via the Olfactory Nerve: Bypassing the Blood–Brain Barrier

Another quite significant uptake pathway is available to nanoobjects owing to their small sizes. The particles can be incorporated via the nerve fibers in the area of the olfactory epithelium. Instillation/inhalation tests on rodents using different particles have demonstrated that nanoscale carbon particles, gold particles, manganese oxide particles, and others are conveyed by transsynaptic transport.[27–30] Nanoparticles can reach the brain directly by passing the olfactory epithelium and the *nervus olfactorius* located in the roof of the nose.[28] It is also conceivable that systemic uptakes take place via the *nervus trigeminus* und the sensoric nerve fibers in the tracheobronchial tract.[31] The quantities reaching the brain via the olfactory nerve are very small; however, they bypass the blood–brain barrier.[32]

3.3. Healthy Skin: An Effective Barrier Against Many Nanomaterials

The healthy skin of humans is a 1.5–2 m^2 organ that protects the organism from environmental stresses and pathogens while avoiding heat and fluid losses. It is composed of three main layers: The epidermis, dermis, and subcutis. The outer layer of the epidermis, the corneal layer (*stratum corneum* and *stratum corneum disjunction*), mostly consists of a 5–20 μm layer of dead squamous epithelial cells (keratinocytes), which is a first mechanical barrier against all nanoparticles and is much thicker than the epithelium of the lung. Below the layer of the squamous epithelial cells, the layers of acanthocyte (*stratum spinosum*) und basal cells (*stratum basale*) that consist of living cells are found. The dead cells of these two layers constitute the corneal layer. With the help of these cells, the outermost layer of the skin regenerates continuously from within. Hair follicles with sebaceous glands (15–20 cm^2 skin) and perspiratory glands (approximately 150 cm^2 skin) are embedded in the dermis. Below, capillary vessels and the so-called lamellar bodies (mechanoreceptors of the skin) are found that are embedded in loose connective and subcutaneous adipose tissue.[33]

The uptake of nanoparticles, especially of the non-lipophilic type that are contained mainly in cosmetics and in sunscreen, is hampered by the very anatomic structure and the continuous regeneration of the human skin from within. Several exposure studies, for example studies as part of the 6th FP EU project NANODERM (NanoDerm, 2008), have shown that TiO_2, modified in different ways, is deposited only in the upper three to five corneocyte layers of the *stratum corneum disjunctum* on the corneal layer or in the hair follicles or folds of the skin but is not detected in the deeper

regions of the skin. Although it was found that the skin does not react acutely to the nanoparticles (NanoDerm, 2008),[34,35] it was shown by another group that very small particles (< 10 nm) are capable of penetrating through to the epidermis or dermis.[36] Particle surface coatings or functionalization, which are often used to prevent agglomeration, may strongly influence the penetration.[36–40] As the corneal layer of stressed or diseased skin is not intact, it is as a rule more permeable to all kinds of particles and must be regarded independently.[41]

3.3.1. Franz Cell Method and Tape Stripping Method: Two Standardized Methods for Determination of the Skin's Permeability to Particles

Intact skin biopsy samples can be tested in Franz cells for their permeability to active molecules or nanoobjects.[42] Since the Franz cell method does not allow conclusions to be drawn about the penetration path, tape stripping, that is, tape-assisted stripping and analysis of the skin in layers after application of the nanoobjects to one single patch, is often used instead. Interpretation of the data obtained in this manner is not always easy; owing to the presence of skin folds and hair follicles, particles may be found after stripping of the corneal layer and may be assigned by mistake to the dermis. Artefacts of that kind were described in detail by the NanoDerm project consortium (NanoDerm, 2008). The newly developed relevant detection methods were summarized in the final report. While nanosized metal oxides were plausibly shown to not penetrate the skin, there are indications that lipophilic or instable (soluble) particles are more likely to penetrate the stressed skin (strain tests)[39,40] or that skin that is affected by solvents is more permeable.[43] The effects of penetrating nanoparticles on cells below the corneal layer will be discussed in Section 4.

3.4. Minor Significance of Uptake via the Gastrointestinal Tract

The gastrointestinal tract is a complex barrier tissue with an area of about 2000 m^2 that fulfils different functions. In the stomach, food is digested at a pH value of approximately 2. The nutrients are taken up by the small and large intestines by the intestinal epithelium and are distributed in the body via the bloodstream. Since the blood vessels are however one or several cell layers below the intestinal epithelium, it is not easy for macromolecules or nanoparticles to migrate into the bloodstream.[33]

Nanoobjects contained in the food (as food additives) or transported to the bronchia through mucociliary return transport after intake of breath can be swallowed unconsciously, thus gaining access into the gastrointestinal tract. There is no consensus about the behavior of nanomaterials in that area. While some animal experiments found that 50 to 100 nm-sized polystyrene particles absorbed through the intestinal wall to get into the lymphoid system,[44] other studies maintain that there is no uptake at all.[45,46] While 98% of the nanoparticles administered orally to the test animals were excreted, approximately 80% of the intravenously administered material was found to have accumulated in the liver after one week.[29] Although the uptake of nanoparticles by the gastrointestinal tract in accordance with these findings could be of minor significance, the current lack of data prevents a final evaluation.

3.5. Injected Nanoparticles Bypassing Vital Barrier Tissue

Although many of the nanotechnological developments are only at an early stage, they are expected to have a great future, especially with regard to diagnostic and therapeutic medical applications.[5] Currently, nanomaterials are tested for use as contrast agents that help to better display body structures and body functions in novel imaging methods (X-ray diagnostics or magnetoresonance tomography).[47] In addition, novel vaccines that are either bound to or incorporated in the nanoobjects are being developed to achieve improvements in immunization compared to conventional products or adjuvants.[48] Specially coated iron oxide particles that may revolutionize cancer therapy are expected to be approved soon.[49] It is common to all applications that the nanoparticles must be injected either in the target tissue or the bloodstream to achieve the desired effect. Natural barriers such as the skin or the intestinal epithelium can be bypassed through injection, while other types of barrier tissue such as the blood–brain barrier[20] or the placenta tissue of pregnant women become relevant.[50] Figure 6 gives an overview of the conceivable paths of uptake and transport.

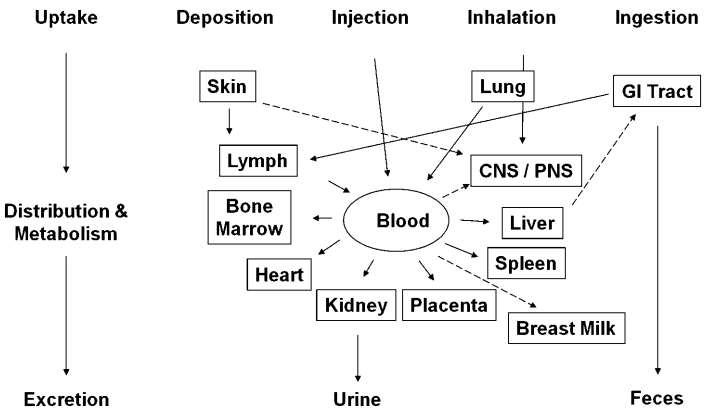


Figure 6. Overview of the demonstrated (solid lines) and hypothetical translocation routes (dashed lines) of nanoobjects within the human body. (Modified and reproduced from Ref. [29] with permission of *Environmental Health Perspectives*.)

4. Evidence of Hazard: Biological Effects of Nanoobjects

To accurately predict the hazards of these new materials for humans, different biological models are used to determine their potential exposure and toxicity. Figure 7 elucidates the in vitro↔in vivo relationship and its extrapolation to humans.

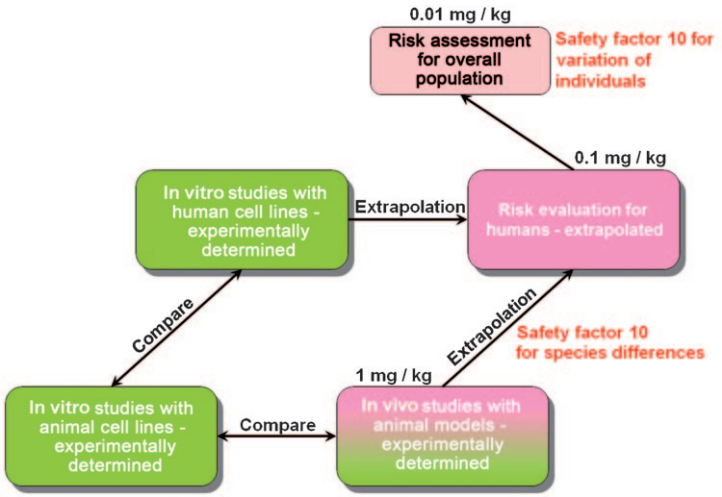


Figure 7. The evaluation process of toxicity of nanoobjects for humans. The interrelationship between experimentally determined thresholds (assumed here to be 1 mg kg^{-1}) and the safety factors for the species differences and the interindividual differences between human beings is shown. This gives a minimum of the factor of hundred for fixing threshold limits for humans.

In vitro studies are understood as being very simplified biological models that enable a rapid, low-cost estimation of the effects of xenobiotic substances or nanomaterials. A comparison of different cell types isolated from different tissues or organisms enables evaluation of more than just the tissue-specific effects. Only animal experiments (in vivo) can provide sufficient answers to the complex issues of absorption, distribution, metabolism, and excretion (ADME). However, the constant improvement of in vitro models to simulate complex multicellular systems[51–54] or entire organs[55] allows an ever more differentiated investigation of possible mechanisms of action and will reduce the need for animal experiments in the long run.

4.1. Nanoparticle Effects in the Lung

Epidemiological studies of (ultrafine) particulate matter have demonstrated that respirable nanomaterials can trigger a variety of diseases of the lung, the cardiovascular system, and the nervous system.[56–60] Although, for lack of the correspondingly exposed collectives, there are no comparable studies of equally sized synthetic nanoparticles, there is no reason to assume that these may cause different effects. However, the same novel properties that make nanoparticles so attractive to nanotechnology may cause hitherto unknown toxic effects and therefore they must be studied carefully prior to large-scale application.

4.1.1. Oxidative Stress, Inflammation, and Genotoxicity

Although the exact mechanisms of action of nanoparticles, nanofibers, or nanoplates are not yet completely understood, it seems plausible that the specific surfaces of the nanomaterials, which for smaller particles are much larger than for larger particles, are key factors in the formation of free oxygen radicals, also referred to as reactive oxygen species (ROS; compare Section 5.2 and Figure 13). Large quantities of free radicals (for example, superoxide anions and hydroxyl radicals) in cells can cause cellular damage by interacting with their components (lipids, proteins, and DNA) in an uncontrolled way. This was shown in vitro and in vivo for different types of nanoparticles and nanofibers (C60 fullerenes, CNTs, TiO_2, diesel exhaust particulates, etc.).[29] ROS formation can have different causes: 1) ROS may form directly on the surfaces of nanoobjects;[61] 2) transition metals may act as catalysts for formation of ROS;[62] 3) nanoobjects cause damage to the mitochondria, thus disturbing the balance in the respiratory chain;[63] and 4) during activation of macrophages and neutrophils by nanoobjects, these cells themselves produce ROS or RNS (reactive nitrogen species).[63]

If not bound by endogenous antioxidants (for example by vitamin C) or degraded by the action of antioxidative enzymes, these radicals trigger inflammatory reactions. Inflammation is a natural reaction to injuries that initiates a healing process and activates the immune system. Cytokines, such as TNFα or interleukins (IL-8, IL-6, IL-2), are released during that process. For an ROS formation that is strong enough to cause a collapse of the defense systems of the cell or tissue, it may happen that the radicals react with the macromolecules of the cells, causing negative consequences.[64] After instillation or inhalation of high doses of CNTs or TiO_2, fibrosis and bronchial granulomas were observed to form in the test animals and to strongly affect their lung function.[26,65–70] While the lung function and long-time inflammatory reactions can be tested only within animal experiments, oxidative stress can only be detected in vitro. It is important to note that the tests and effects that have been described above apply only to high doses (see Section 7.2).

4.1.2. The Fiber Paradigm

In contrast to spherical particles, long, stiff fibers cannot be removed from the lung through mere action of the mucociliary clearance mechanism. Particularly fibers with a length of more than 20 μm and diameters of less than 3 μm and with biopersistent properties (for example, asbestos fibers) cannot be phagocytized and cleared by the macrophages[71] and are likely to cause inflammation, fibrosis, and even cancer in the lung (Figure 8).[72] Single- and multi-walled carbon nanotubes are used increasingly in different materials science applications. They have been attracting major attention owing to their alleged hazards to health; these hazards have been attributed to their morphological similarity to asbestos.[65,69,73–78] Injection of CNTs in the abdominal cavities of mice showed that tissue modifications similar to those caused by asbestos were caused only by very long (> 20 μm) and very thick (> 80 nm) carbon nanotubes,[77] and that shorter tangled CNTs were not capable of triggering such reactions. It was shown in vitro that the CNT toxicity is influenced directly by the way or manner of suspension.[79] In

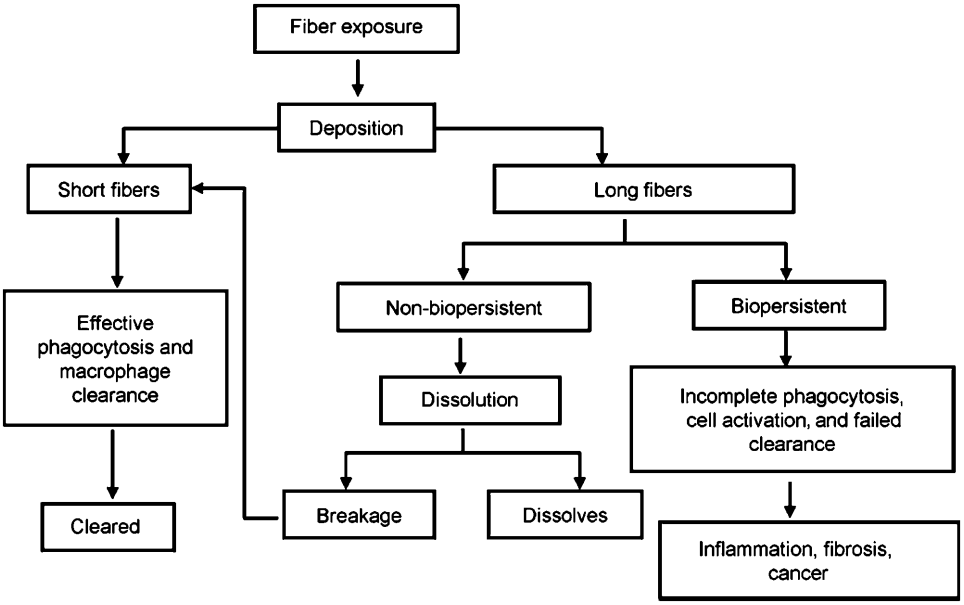


Figure 8. The fiber paradigm. (Reproduced from Ref. [84] with permission of Oxford University Press.)

contrast to these findings, there are indications that the acute toxicity of industrially manufactured CNTs is only small.[80–83]

Tests on other inorganic fibers will be required to determine whether the assumed fiber paradigm plays a greater role than the fibers' chemical composition.

4.2. Effects of Nanoobjects on the Skin

The high protective factors of sunscreens are achieved through addition of coated titanium or zinc oxide nanoparticles that absorb UV radiation. For reasons of precaution, other nanomaterials, such as CNTs,[85] silver nanoparticles,[38,86] quantum dots,[36,87,88] or aluminum,[89] are also being tested for their potential toxic effects, assuming that they are capable of sufficiently penetrating the corneal layer of the *stratum corneum*. Increasingly, fullerenes (C_{60}) are added to cosmetics to be serving as scavengers (Vitamin C60 BioResearch Corporation; see http://www.vc60.com/). These lipophilic particles can penetrate through to the epidermis but are not found in the dermis.[43,90]

4.2.1. Effects in the Skin and on Skin Cells

Several in vivo studies[34,91] show that neither nanoscale TiO_2[92,93] or ZnO[94–96] nor lipophilic C_{60} fullerenes[43,97] can trigger irritation of the skin or signs of allergic reaction, even though it could be demonstrated recently that zinc(II) ions can be found in the body after use of ZnO-containing sunscreens.[98] These results contradict in vitro studies performed on human skin cells (keratinocytes) or stromal cells (fibroblasts). Absorption and significant reductions in the cell function were only determined for high doses of nanoscale TiO_2 (sized 3–10 nm).[68,93] Once taken up in the cell, single- or multi-walled CNTs[99] can trigger cytotoxic reactions, for example oxidative stress,[100] in keratinocytes, induce production of inflammatory factors,[101,102] or even cause cell death (apoptosis or necrosis).[103] Some studies have shown that very small TiO_2 or ZnO nanoparticles in particular can cause photocatalytic effects, inducing the production of DNA-damaging free radicals in the uppermost layers of the skin[104–106] or reducing the functionality of the cells.[68,107] The discrepancy between the results of in vivo and in vitro studies is ascribed to the assumption that most of the nanoparticles hardly penetrate the corneal layer of the skin. This assumption is corroborated by the fact that granulomas are not formed unless CNTs or "hat-stacked" nanofibers are implanted subcutaneously in rats.[108,109]

Separate studies on the supposedly much more severe effects of nanoparticles that penetrate the damaged, injured or diseased skin need to be performed (see the Review in [35]).

4.3. Effects of Nanoobjects on the Intestinal Epithelium

Inhalation and ingestion are considered to be the two major portals of entry for nanoobjects. The majority of the inhaled nanoobjects are transported out of the lung by the mucociliary clearance mechanism and are swallowed afterwards, reaching the gastrointestinal tract.[31] The intestinal epithelium is covered by a mucus layer (glycoproteins) that is secreted by the goblet cells and serves to protect the epithelium from proteases and from gastric acid.[33]

It is expected that the food industry will make increasing use of the possibilities of nanotechnology, for example in the development of new packaging concepts or new kinds of food additives. Microformulations of titanium oxide or silica are approved food additives that have been accepted and used for decades as brighteners or flow-regulating agents.[110] Furthermore, packaging films with multifunctional properties are provided with silicate finishes to prevent oxidation, or with silver nanoparticles to prolong the freshness of food. For reasons of precaution and safety, ever more nanomaterials are tested for their toxicity to the gastrointestinal tract. It remains to be determined how many of the particles are capable of getting into the bloodstream via the gastrointestinal tract. It was reported during early in vivo studies on ^{14}C-labeled fullerenes or ^{192}Ir nanoparticles that only a very small portion of the particles administered were adsorbed, and without causing acute toxic effects.[46,111] In vitro studies are conducted primarily using the human intestinal adenocarcinoma cell line Caco-2. Other studies reveal an acute cytotoxicity and

genotoxicity for ZnO, TiO_2, and SiO_2 at a relatively high concentration of 80 $\mu g\,cm^{-2}$ of monolayer.[112] The very few studies published so far on these issues provide only a preliminary basis for final assessment and evaluation.

4.4. Material Properties and Effects

Particle toxicity has been evaluated so far by applying the mass dose parameter as the dose metric. The DFG Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK) has fixed a maximum workplace exposure of 1.5 $mg\,m^{-13}$ for the respirable fraction *R* (previously referred to as respirable dust *F*) and a value of 4 $mg\,m^{-13}$ for the inhalable fraction *I* (previously referred to as "total dust" *G*). Figure 11 (see Section 5.2) illustrates that important particle parameters change if one uses equivalent masses but scales down the sizes of the particles. For as many as ten years, researchers have been discussing whether the mass or rather the quantities or surface doses are better suited for nanoparticle load criteria. As a linear correlation to the specific overall surfaces of differently sized TiO_2 particles was found regarding the occurrence of inflammation markers after particle administration to rats and mice, it seems that the quantities and surface doses are the more appropriate criteria.[113] A comparable relationship between size and effect was demonstrated in vitro for lung cells for differently sized vanadium oxide particles[114] and in vivo for nickel[115] as well as for differently sized carbon particles from combustion processes.[116] It is evident from these results that the size or the total surface measured in m^2g^{-1} are not only important parameters regarding the physicochemical properties of a nanomaterial but are also suited for prediction of its effects in biological systems. This hypothesis stipulates that surface modifications have a direct influence on the toxicity of nanoobjects. A constant decrease in cytotoxicity was shown for functionalized carbon nanotubes in the presence of an increasing number of functional groups, such as the $C_6H_4SO_3H$ groups, on the surface of each tube.[117] The same trend was observed for functionalized fullerenes.[118] The very toxic quantum dots of CdSe must be coated with layers of a biocompatible material to protect the biological matrix. Using a material of that kind, it was demonstrated that the biological effect is strongly influenced by the coating while the transport to or uptake by the cells remain completely unaffected.[119]

It must be noted nevertheless that this simple relation between size and effect does not apply to all materials: There are examples that show either independence from the size or a more pronounced toxicity of the larger particles. Warheit and co-workers have shown that TiO_2 can act independent of the size but depending on the surface reactivity and crystallinity,[120] and that also quartz acts independent of the surface dose.[121] This is obviously reversed for the case of nickel ferrite particles, which shows that in neuronal cell cultures, large-sized particles are much more effective than the nanosized particles of the same material.[122]

The main results of the studies carried out so far are reflected by the biological interactions of the nanoparticles with the cells, organs, and organisms described herein. Although this overview is not fully exhaustive, the studies cited show that effects could only be detected for the high or highest concentrations. Therefore, results are only relevant regarding mechanistic aspects and are less significant for workplaces or the environment. As at present no significant quantities of the new, synthetic nanoparticles are released into the environment, there are few corresponding epidemiological data available at the moment, and the environmentally relevant information on particulate matter must be resorted to.[60]

5. The Three Principles of Nanotoxicology

This Section is dedicated to elucidating whether there is really something unique about nanotoxicology. The specific uptake paths (see above) and special features of the nanosized materials lead to the assumption that there are special mechanisms that play a role in biological systems. Three principles have been identified that involve unique characteristics of nanoparticles or nanomaterials and justify, therefore, the use of the term "nanotoxicology".

5.1. The Transport Principle

The basic features of this first and perhaps most important principle, which could as well be referred to as the "principle of the Trojan horse", have been described already by former particle toxicity studies that recognized the process of phagocytosis to initiate the toxic effect of nickel and zinc compounds (see the Review in [123]). For nanoparticles, these findings take on other dimensions: Phagocytosis is not the only relevant process. Other mechanisms, too, are responsible for the uptake of metals, metal oxides, or other particulate nanosized systems by the cells,[62] and for the different biological reactions that may follow (Figure 9). Although particles with diameters below 100 nm are capable of getting into the cell by almost any vesicle transport pathway,[124–127] further options are to be considered, for example transport of nanoparticles into cells bound to receptors[128–131] or even "diffusion" through the plasma membranes, which is referred to as an adhesive interaction.[25,126,132] Gehr and co-workers have demonstrated this kind of uptake by showing with erythrocytes that nanoparticles advance into the cell interior, whereas larger particles are unsuccessful.[132] This is surprising, as the erythrocytes lack the conventional uptake mechanisms.

No matter how the nanoparticles gain entry into the cells, the process of infiltration is indeed reminiscent of a Trojan horse invasion because a veritable material package is delivered by introduction of only one particle into the interior of the cell (Figure 10). The effects observed are influenced by the different uptake mechanisms: In the case of uptake by vesicular processes, particles are sheathed by membranes (for example, caveolae). Free transport through the membrane, however, would be assumed to be more critical, as it allows particles to achieve direct contact with the plasma proteins and with other molecules of the cell. The uptake of nano-

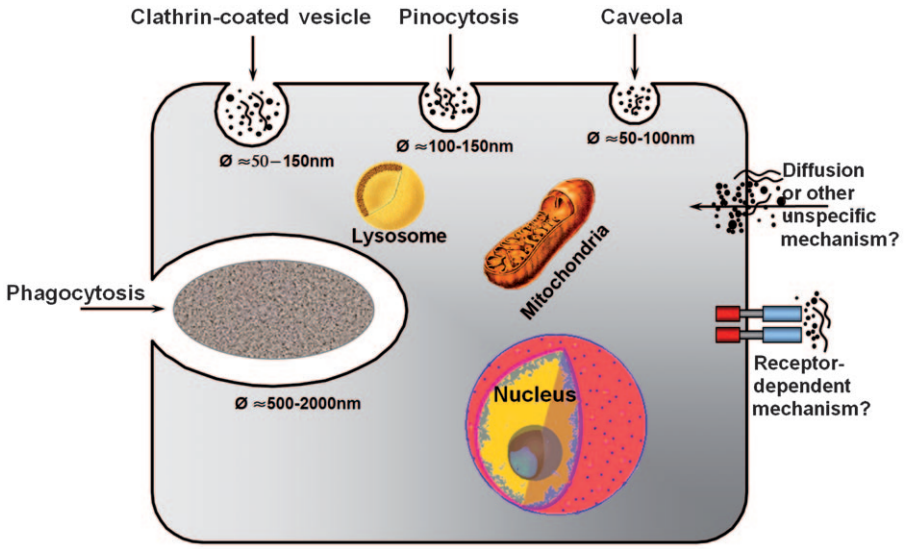


Figure 9. Proposed cellular uptake mechanisms for nanoobjects. In contrast to large particles (>500 nm), which will be exclusively taken up by phagocytosis, nanoobjects may use different translocation routes into the cells. (Modified and reproduced from Ref. [133].)

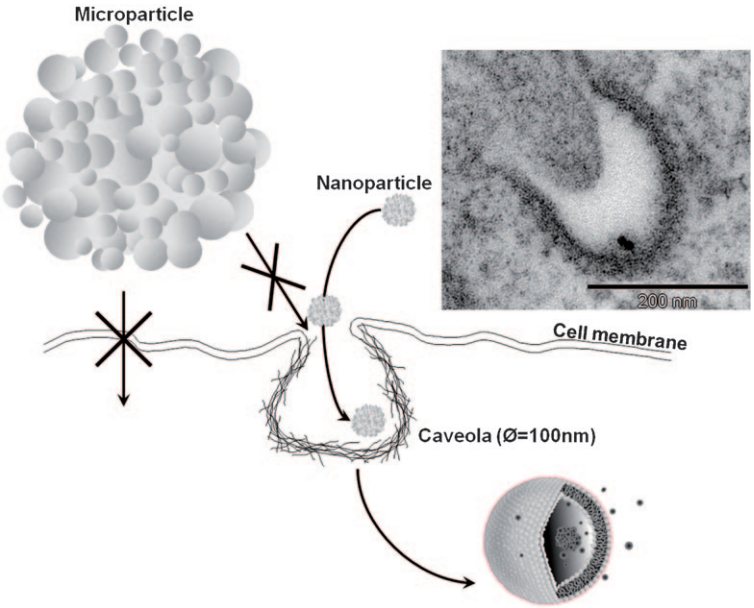


Figure 10. Comparison of nanoparticles and microparticles due to their possible uptake into cells via vesicular pathways (caveoli). Only small particles with a diameter of less than 100 nm fit into vesicular structures such as caveoli with which they will be transported into the plasma. Within the cells, these vesicles may fuse to build up a lysosome with an acidic interior, facilitating the dissolution of materials such as ZnO. The ions can move relatively freely inside the cells (blue dots). The TEM image shows such a situation where two nanoparticles (22 nm) are located within a caveoli of a lung epithelial cell (A549) in culture.

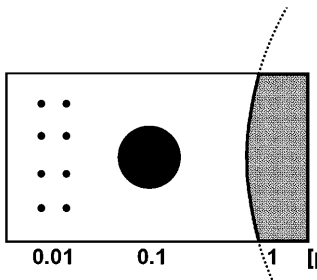
particles may well have fatal consequences for the cell if the material consists of, for example, an incompatible metal and/or is removed owing to physiological conditions: Zinc is an essential element that we need to take in with the food each day to ensure that our body cells and immune system have the power to control important processes such as the regulation of the genes.[134] Overtreatment of a cell with zinc will upset its control functions and cause it to commit a programmed cell death, which is known as apoptosis.[135] A medium-sized nanoparticle consisting of zinc oxide and having a diameter of 10–50 nm contains as many as 50000 to 8 million zinc atoms. With a typical cell volume of approximately 500 femtoliters, this large quantity of atoms, evenly distributed in the cell, would correspond to a concentration of 150 nM to 25 μM. However, as concentrations above 100 μM may already be harmful, toxic amounts of zinc are already deposited in the cell through dissolution of only small quantities of nanoparticles. In fact, this has been shown for zinc oxide nanoparticles.[127,136,137]

The transport principle explains that materials of a certain inherent toxicity may be particularly critical when they are nanosized: Particulate distributions are often controlled less strictly than the transport of individual molecules. Uptake of the latter in the body cells is usually very precisely regulated.

Particles that do not dissolve but remain stable for a long time (biopersistent) or accumulate in cells may become "active" in another way while obeying the second principle discussed below.

5.2. The Surface Principle

Particles that are not soluble but rather stable for extended periods, or biopersistent and able to accumulate in cells, can become active in another way, thus leading to the surface principle. Comparing particles of different sizes, it becomes evident that surfaces and volumes change in parallel with the diameters (Figure 11). Scaling down the diameter by a factor of 10 (for example, from 1 μm to 100 nm), the surface becomes smaller by a factor of 100, and the volume decreases by a factor of 1000. This is not just a purely numerical example but a factor of biological significance because mass has been taken as the measure of effects (dose–effect relationship) when testing a substance for its toxicity. Using particles with three different diameters of 1 μm, 100 nm, and 10 nm of a particular material of unchanged mass, the specific surface of these particles increases each decimal step by a factor of 10, and the number of particles even increases by a factor of 1000 (Figure 11). While a reduction in particle size can improve and accelerate reactions in the case of catalysis or other chemical processes, it increases the reactivity with cells or their components in the biological system.



Ø [µm]	A [µm²]	V [µm³]	Number* [N]	A* [µm²]
1	3.1425000	0.523625000	1	3.143
0.1	0.0314250	0.000523625	1000	31.425
0.01	0.0003143	0.000000524	1000000	314.250

*: same mass

Figure 11. The relationship between size, surface area, and volume (number) of nanoparticles. The two columns on the right demonstrate the ratio between the specific surface area and the number of particles in the case when particle mass is the same but sizes are changed by a factor of ten.

As there are considerably more atoms available on the particle surfaces for smaller particles, they can interact with the environment much more efficiently. Figure 12 shows that particles with sizes of 100 nm or less have a pronounced exponentially increasing number of atoms, or molecules lying on their surface to potentiate both positive (for example antioxidation or transport of therapeutic agents) and negative effects (such as oxidation or protein binding).

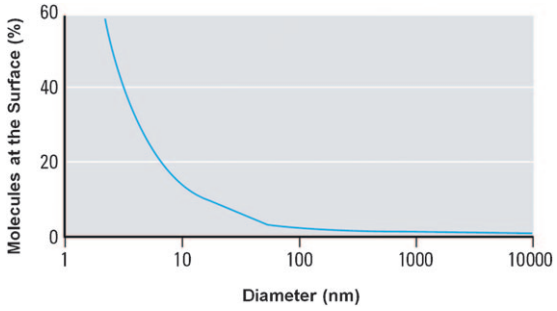


Figure 12. Surface molecules as a function of particle size. (Reprinted from Ref. [29] with permission of *Environmental Health Perspectives*.)

This behavior was described some years ago by Nel et al. in their contribution on the toxicity of nanomaterials,[64] which was updated by the same authors in 2009.[138] It was outlined that small size may in fact cause chemical reactivity not only by the large number of reaction partners on the surface but also by surface effects, such as crystal lattice defects, owing to the enormous curvature of the particles or the adsorption of photons because of to physical effects: The energy absorbed and stored by the particle can be released again by formation of radicals or degradation of hydrocarbons (Figure 13). Furthermore, molecules of the same size as proteins are direct ligands that may adsorb on surfaces[139,140] and may induce deactivation (inhibition) or other protein modifications.

The above dependence on the size of particles has been shown by several studies. Oberdörster and co-workers have

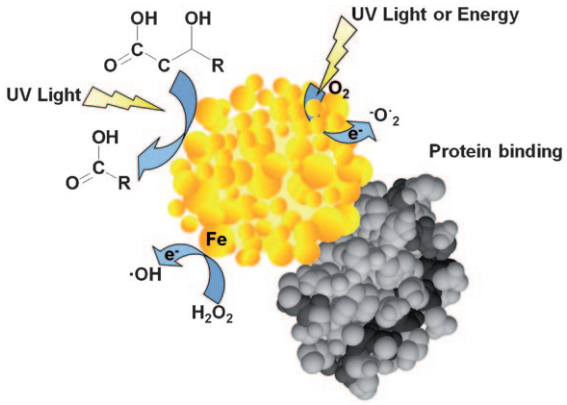


Figure 13. Surface reactivity of nanoparticles. Crystalline structures or quantum effects may provoke energy absorption and transfer, which leads to the formation of oxygen radicals or the degradation of hydrocarbons. Moreover, the nanoparticles may bind to biological macromolecules of comparable size as proteins or DNA. Such reactions may exert adverse effects within the homeostasis of the cellular physiology.

shown in tests on rats and mice that inflammatory reactions in the lung are triggered by TiO_2 particles as a direct function of their specific surfaces.[113] The same phenomenon was observed for combustion particles by Stöger et al.[116] and for inhalation of differently sized nickel particles[115] and other materials[141] by other authors. Moreover, such size-dependent effects were confirmed in cell-culture and animal experiments when using polystyrene particles,[142] carbon particles, and carbon nanotubes,[143,144] SiO_2 particles,[51] or vanadium oxide.[114] In addition to the size of particles, reactivity was found to be a major factor that depends directly on the specific surface. This was shown, for example, for TiO_2,[70,145] copper,[146] and quartz.[147] Alternatively, as some effects may also occur independently of the size of particles, there are contradictions that remain to be considered.[120,121]

Karlsson and co-workers have demonstrated that not only physical but also chemical properties considerably influence the effects of nanoparticles on living systems.[148] They found that the size-dependent toxicity of particles can manifest itself in different ways: Although smaller particles may be more toxic than the larger particles (CuO), larger particles can be more effective than smaller particles (TiO_2); other materials (iron oxides) show no size-dependent effects. With this in view, the third of the three principles has to be explained.

5.3. The Material Principle

Almost all materials (metals, metal oxides, polymers, carbon materials, etc.) can be manufactured as nanosized nanoobjects. Mostly, manufacturing changes the materials' physical or chemical properties. These properties are often determined by the particular characteristics of the surfaces of the particles, fibers, or platelets, but they may also result from the low number of the corresponding molecules or atoms. For biological systems coming into contact with such objects, the materials constituting the nanofractions are rather relevant despite uniform shapes and sizes: For example, nanoobjects

that consist of zinc oxide exert effects that are completely different from those exerted by comparable metal oxides containing iron, silicon, aluminum, cerium, or other elements.[22,149,150] This reveals that, following the transport principle, small size is of relevance to health but is not the only factor that causes a harmful toxic impact. Furthermore, the respective particle must be reactive, meaning that reactions either take place on its surface or are catalyzed (Figure 13) or that molecules or atoms come off the material to trigger the corresponding reactions in the cell (Figure 14).

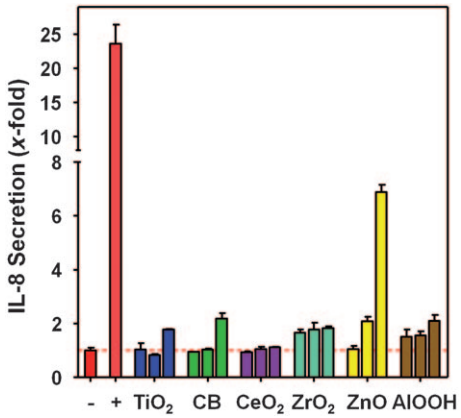


Figure 14. Comparison of the biological effect of nanoparticles of different composition (mean Ø: TiO_2 10–20 nm, carbon black (CB) 15 nm, CeO_2 20 nm, ZrO_2 10-25 nm, ZnO 40 nm, AlOOH 40 nm). Shown is the production of an important mediator of inflammation (Interleukin-8) by human lung cells (A 549; ——: untreated control; +: positive control induced by treatment with 1 ng mL^{-1} TNF-α). The concentrations used for the experiments were (from left to right): 0.5, 5, und 25 µg cm^{-2} cell culture surface (results from NanoCare; see Refs [6, 22]).

The above is also evident when comparing not just different materials of uniform sizes but also different conformations and modifications of one and the same material. Carbon is the best example, as it occurs in very different modifications that cause different reactions in biological systems. Although no adverse effects have been found so far for nanosized diamonds,[151,152] industrial soot (carbon black), mostly when applied in relatively high concentrations, are observed to exert biological effects.[22,141,144] While fullerenes, especially as solvent-free suspensions, seem to remain without effect,[146,153,154] carbon nanotubes can trigger health effects depending on their lengths[77] or states of aggregation.[79] Besides, contaminating substances such as the metals used for catalytic synthesis may cause reactions of the cells.[80,81] The above representative examples point out the importance of material properties, material composition, and impurities.

5.4. Three Principles, and Many Possibilities

The above basic principles of nanotoxicology may be regarded as some kind of a basis for the description of specific reactions and interactions between nanomaterials/nanoobjects and biological systems. Combining these three principles encourages the examination of each nanoobject separately for its specific size, shape, surface, and composition. All of these factors are significantly involved in causing biological effects and must be considered separately for each material to evaluate its potential toxicity. To summarize: Nanomaterials, much like chemical substances, must be tested individually. Since they may be regarded, so to speak, as a special form of chemical, this is not that far-fetched.

6. National and International Safety Research Activities

The opportunities and chances that come with the development of new materials have been recognized at an early stage, and financial means for research into novel applications are being provided by various programs all over the world. The expected enormous increase in the commercial production of nanoparticles and other nanomaterials will make it ever more probable for man and the environment to come into contact with the substances involved. Thus, early on, numerous institutes and research groups have been trying to investigate the measures to be taken if the challenges involved in an increasing number of materials whose potential health hazards are only insufficiently known are to be met. Adequate knowledge about the biological and toxicological aspects of nanotechnology is expected to be obtained from nationally and internationally funded projects. Various research groups believe that the risks associated with a new technology have hardly ever been investigated and assessed so intensely as has been the case for nanotechnological developments. Plans of action dedicated to developing sustainable nanotechnology concepts have been in existence for years in the EU, the USA, and in other countries (see links to Action Plans). Besides, numerous institutions throughout the world, often funded by and acting on behalf of the respective governments, are engaged in establishing databases (DaNa, NanoTrust, Safenano, Woodrow Wilson, ICON, etc.) while evaluating methods (IANH, OECD) and exchanging knowledge during conferences, workshops, and summer schools. A commission of the German government in particular (nanocommission; see www.bmu.de/nanokommission/) was appointed to investigate the opportunities and possibilities of utilization as well as the potential negative effects and urgent needs for research. In Switzerland, a precautionary matrix that was developed by the Federal Office for the Environment and the Federal Office of Public Health enables manufacturers and trade to identify their own specific safety needs.

7. Conclusions and Recommendations

In spite of today's continuous advances in the development of new nanomaterials and an increasing number of publications coping with the potential negative effects, the results available so far are of limited suitability for risk assessment. Among other things, this is due to the fact that

materials have not yet been sufficiently standardized (the ISO TC229 definitions of nanomaterials were only published in 2008[155,156]), that the reference materials that were called for several years ago[83] were not made available until recently (NIST: http://ts.nist.gov/MeasurementServices/ReferenceMaterials; IRMM: http://www.irmm.jrc.be/html/reference_materials_catalogue/catalogue/index.htm), and that the adapted methods have not been established yet. Solutions to overcome this unsatisfying situation have been suggested in recent publications.[6,157] However it has yet to be realized that many of the earlier used methods are often faulty and inaccurate, which will be discussed below for some results of extremely high importance.

7.1. Unreliable Methods (Lacking Reliability)

Tests in the laboratories at our institute and of other research institutions have already shown that different nanomaterials, and carbon nanotubes in particular, can interact with reagents to cause both false positive and false negative results. For example, we determined that it is difficult or even impossible to evaluate MTT assays of cells treated with CNTs.[83,158] This was confirmed by tests performed by other groups and was complemented by the observation that selected dyes bind to the CNTs, thus producing erroneous results.[159,160] There is a very high probability that there further test methods for which the respective nanomaterials are found to interact with the analytes in a similar way. To obtain reliable results, a closer scrutiny of all such interactions is required prior to the tests. Another example concerns the possibility that not the nanomaterial itself, but rather contaminants or solvents may be toxic to the investigated cells or organisms. Fullerenes have been described to be toxic to fish and daphnia, especially via the mechanism of lipid peroxidation.[161,162] After revision of this result by the authors[163] and other research groups,[154,164,165] it was demonstrated that peroxides derived from aging of the solvent tetrahydrofuran exerted this toxic effect, and peroxide-free suspensions had no toxic effect at all.

7.2. Unrealistic Test Conditions: No-effect Studies

Another example of inaccuracy is provided by the unspecific effects caused in the lung or in in vitro cell cultures owing to exaggerated particle doses with little relevance to human exposures. Such doses are administered so as to be able to detect any effects at all that are caused by the nanoparticles. The revised version of a study carried out more than a decade ago by Roller et al.[166] that was published recently shows that the "nanoparticles" applied had induced tumors in the lung,[167] but it does not take into account the fact that extreme doses were administered. Already almost 20 years ago, a single dose of a material of 3 mg or more was found to overload the lungs of rats.[168] This extreme dose had been exceeded considerably in the case of all materials investigated by Roller and co-workers. As most materials will trigger health effects or even induce tumors when applied in cytotoxically high doses, these cannot be said to cause "nanospecific" effects.

An similar study of the effects of carbon nanotubes[67] describes how after inhalation, CNTs are delivered to the deep areas of the lung to penetrate through to the subpleural regions of the tissue. Such results are extremely relevant as regards the current discussion concerning an asbestos-like effect of CNTs. The reported effect on the lungs, however, was only found after treatment of the test animals with $30\,mg\,m^{-13}$ over 6 h, which is more than 20 times the maximum workplace dose identified for respirable particulate matter. No such effect was observed upon administration of a lower concentration of $1\,mg\,m^{-13}$. As high-dosage tests do not allow valid statements of the mechanisms of action of the respective materials, the point of such experiments is arguable. However, this current example of CNT–lung interactions makes clear which fundamental difficulties exist for nanotoxicologists. It can be assumed that the lower dose is ineffective because of the short exposure time. Treatment of cells in vitro is generally very restricted in time, but animal experiments carried out as 5 day or 90 day exposure studies[6] can usually not be compared to real-life scenarios where humans may be exposed over months and years. Apart from the adaptation and improvement of in vitro methods in the future, long-term studies in particular should be carried out.

This point leads us directly to another important point that is currently discussed in the scientific community, namely the "no-effect-studies". Scientific publications are normally requested to present results on mechanisms and effects which increase the present level of knowledge. The international community of nanotoxicologists, however, has recently agreed that many studies on nanomaterials are expected to observe no effects or mechanisms and will thus not be accepted for publication. By depriving the scientific community of important information, such exclusion will also cause different laboratories to repeat experiments more often than is necessary. To save the money and the manpower required for such repetitions, the editors of three scientific journals (Nanotoxicology, Vicki Stone; *J. Nanopart. Res.*, Enrico Traversa; *Part. Fibre Toxicol.*, Paul Borm) have agreed to also publish the results of "no-effect studies" of the kind described above.[82] Nevertheless, the community is aware of the fact that this offer should not open the door for publishing simply all studies, whether or not an effect could be found. The demands on quality to such "no-effects-studies" have to be even higher compared to studies describing a biological mechanism. This can only be achieved if editors and reviewers consider partly the recommendations that are depicted in the next Section.

7.3. Recommendations

For almost two decades, nanomaterials and in partucular nanoparticles have been tested for their potential negative effects on the health of humans. Mmedical applications such as drug targeting systems have also been studied for some time. However, as outlined above, our knowledge of the toxicology of nanomaterials is incomplete. To improve this

situation in the future, we need to enhance the quality and reliability of the studies. Governmental and non-governmental organizations, journalists, stakeholders, or the public can hardly judge whether publications in renowned journals are right or wrong, good or bad, important or irrelevant. The scientific community cannot assure the readers of the quality of studies unless two major aspects are considered:

- As stipulated earlier,[169–171] nanomaterials that are intended to be tested in studies must be characterized sufficiently beforehand.
- Sufficient information must be provided as to the validity and suitability of the selected test methods.

As long as these two preconditions are not fulfilled sufficiently and as long as readers cannot clearly understand which materials are tested and which methods are applied or if the appropriate negative and positive controls have been considered, studies of the same materials and aspects will be repeated over and over again. In addition, data lacking in reliability will raise justified doubts and deprive us of a proper basis for a comprehensive evaluation of the biological effects of nanomaterials. Therefore, we would like to summarize the results of different working groups (DECHEMA, Nanokommission, SCENIHR, IRGC, NanoDialog, and others) in the list below. These groups have argued for a minimum set of information on the properties of nanomaterials for each study, and this set should consist of:

- Chemical composition, purity, impurities
- Particle size and size distribution
- Specific surface
- Morphology (crystalline/amorphous, shape)
- Surface chemistry, coating, functionalization
- Degree of agglomeration/aggregation and particle size distribution under experimental conditions (for example, media with/without proteins)
- Water solubility (differentiation between soluble, metastable, and biopersistent nanomaterials)
- Surface reactivity and/or surface load (zeta potential).

Regarding ecotoxicological issues, octanol–water coefficients may also be important. Along with details on their measurement, these parameters should be included in a section dedicated to "Materials and Methods".

To complement such characterization, some major data are required on the methodology to ensure that the studies are evaluated properly:

- Applied quantities (concentration/dose), to be given in more than one unit and expressed as: $\mu g\,mL^{-1} \Leftrightarrow \mu g\,cm^{-2} \Leftrightarrow N$ (particle)/cell $\Leftrightarrow$ pg/cell.
- Doses administered during animal experiments should be clearly marked as "overload" or "non-overload" doses. Overload doses should be largely avoided as they impede unambiguous statements.
- At least two different tests should be made for each biological end point to exclude cross-reactions.
- As unspecific cell reactions (for example, apoptosis) can cause DNA damage, cytotoxic concentrations should be avoided in genotoxicity studies. Any such study should contain data on the dose–effect relationship of the acute toxic effects (see OECD guidelines for genotox testing, point three under "overload conditions").
- Interference of the nanomaterials with the test system should be taken into account in any case and be excluded if possible.[83,169,172]
- Paths of uptake and an appropriate selection of experimental organisms should also be considered when performing ecotoxicological studies.[173]

If these points are not considered for future publications by authors, reviewers, and editors, the resulting unsuitable manuscripts will certainly impede:

- comparisons of studies on an international level,
- reliable discussions of the biological effects, and
- conclusive arguments for or against a certain nanomaterial for the public, for stakeholders or the non-governmental organizations.

Therefore, we call on reviewers and editors to either reject manuscripts that do not consider the above points or demand that the experiments required are performed or the data needed are provided prior to publication.

Appendix: Internet Homepages on the Safety of Nanomaterials Cited in the Text

Action Plans

- Action Plan of the Federal Government of Germany, BMBF (2007): http://www.bmbf.de/pub/nano_initiative_action_plan_2010.pdf
- Action Plan of the Austrian Ministry on Traffic, Innovation, and Technology (BMVIT; 2009): http://www.bmvit.gv.at/innovation/iktnano/nano_aktionsplan.html
- Action Plan of Switzerland on Nanomaterials (2007): http://www.bag.admin.ch/themen/chemikalien/00228/00510/index.html?lang=de
- European Strategy for Nanotechnology and the Nanotechnology Action Plan (EU, 2004): http://cordis.europa.eu/nanotechnology/actionplan.htm
- National Nanotechnology Initiative (NNI, USA), founded in 2001: http://www.nano.gov/

Databases

- European Nanotechnology Gateway: http://nanoforum.org
- European Union Funded Projects (6th and 7th Framework): http://cordis.europa.eu/nanotechnology/src/safety.htm
- Institute of Technology Assessment of the Austrian Academy of Sciences (2007): http://nanotrust.ac.at/
- International Council on Nanotechnology (ICON): http://cohesion.rice.edu/centersandinst/icon/index.cfm
- International Organization for Standardization (ISO), TC229 on Nanotechnology (2005): http://www.iso.org/iso/standards_development/technical_committees/list_of_iso_technical_committees/iso_technical_committee.htm?commid=381983

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