The novel properties of engineered nanomaterials may alter their interaction with the human body, especially for inhalation of unintentionally released biopersistent material. We discuss the characterization of nanoparticles in interaction with biological media and we review animal inhalation and cell culture studies in comparison to original results. We establish that an intrinsic size-specific toxicity does not exist and identify material-specific indicators of concern that help to select safe uses.
Testing Metal-Oxide Nanomaterials for Human Safety

By Robert Landsiedel, Lan Ma-Hock, Alexandra Kroll, Daniela Hahn, Jürgen Schnekenburger, Karin Wiench, and Wendel Wohlleben*

Nanomaterials can display distinct biological effects compared with bulk materials of the same chemical composition. The physico-chemical characterization of nanomaterials and their interaction with biological media are essential for reliable studies and are reviewed here with a focus on widely used metal oxide and carbon nanomaterials. Available rat inhalation and cell culture studies compared to original results suggest that hazard potential is not determined by a single physico-chemical property but instead depends on a combination of material properties. Reactive oxygen species generation, fiber shape, size, solubility and crystalline phase are known indicators of nanomaterials biological impact. According to these properties the summarized hazard potential decreases in the order multi-walled carbon nanotubes $\gg \text{CeO}_2, \text{ZnO} \gg \text{TiO}_2 > \text{functionalized SiO}_2 > \text{SiO}_2, \text{ZrO}_2, \text{carbon black}}$. Enhanced understanding of biophysical properties and cellular effects results in improved testing strategies and enables the selection and production of safe materials.

1. Introduction

The intentional generation and application of nanomaterials with novel properties is one of the century's key technology developments, offering extraordinary opportunities in various technological fields such as electronics, energy management, structural materials, functional surfaces, construction, and information technology, but also in the pharmaceutical and medical field. Indeed, the appearance of clean-tech, seen as the capture, storage, and conversion of energy and resource-efficient materials, depends critically on nanomaterials, whereof the majority is fabricated by compounding engineered particulate nanomaterials. Since the miniaturization of materials down to the nanometer scale can change physical and chemical properties, nanomaterials

will presumably also influence biological systems—regardless of a human intention behind the material's generation. The natural nanomaterials and the unintentionally man-made nanomaterials by far outweigh the engineered nanomaterials, but the exposure scenarios resemble each other. A systematic risk assessment requires the separate determination of both the hazard potential and the actual exposure levels resulting in a risk characterization (Scheme 1). Typical consumer products combine low exposure to free nanostructures and low hazard potential. Materials with high hazard potential are restricted to professional handling, where safe levels of exposure can be enforced by technical measures.

The scientific community started to evaluate the potential hazard of nanomaterials since 1992, culminating in the current developments of regulatory frameworks in the EU, USA, and Canada. Based on the extreme diversity of hazard potential—from potent to harmless—and diversity of exposure—from occupational to consumer settings—the regulatory framework evolves into a case-by-case risk assessment. Hazard potential and (internal) exposure (Scheme 1) need to be merged into a testing strategy, e.g., in the REACH Implementation Plan. Current knowledge is sufficient to shape the first regulation approaches, but these have to undergo revisions with enhanced risk assessments. The outcome of safety research is also an integral part of the public awareness and confidence in nanotechnology.

In the present contribution, we focus on the potential adverse effects of engineered metal oxide nanomaterials, in comparisons to the frequently discussed toxicity of carbon nanomaterials. Appropriate toxicity testing requires a thorough understanding of nanomaterial specific properties with regard to distribution in the body and possible nano-specific effects on the systemic and cellular level (Scheme 2). The unique nano-specific properties of nanomaterials require a careful adaptation of the test methods, and the OECD recommends that guidelines be newly developed or revised for sample preparation and dosimetry, degradation and fate, for inhalation and for the majority of the physicochemical characterization methods. A base set of applicable toxicity screening systems and characterization tools has been suggested already by Warheit et al. At present, inhalation studies with animals are the most predictive testing of possible adverse effects of nanomaterials on humans. But inhalation studies entail the
sacrifice of animals and are quite expensive and time-consuming.

Traditional methods have to be adapted and in vitro methods must be improved through better understanding of their biophysical mechanisms until the in vitro tests achieve predictive power.

This paper is organized as follows:

Section 2 starts from a wider perspective and discusses exposure levels and possible routes of internalization in humans.

Sections 3–6 track the physiological effects from biophysical to cellular to systemic levels.

In Section 3 we review the physicochemical properties of nanomaterials and their characterization with appropriate biophysical methods. We focus on the biophysical modification of the nanomaterial’s surface and state of agglomeration in cell culture media (Scheme 3).

The in vitro toxicity (cell viability, genotoxicity, inflammation) of metal oxide and carbon nanomaterials is reviewed in Section 4 and is complemented by original results from different titanium dioxide (TiO$_2$) nanomaterials (Scheme 3).

In Section 5 we give an overview on the limited range of existing inhalation studies with engineered nanomaterials. Furthermore, we present original data from our inhalation studies with six metal oxide materials and two carbon materials. These results are excellently comparable due to an identical experiment design (Scheme 4).

Section 6 summarizes the correlations between the in vivo and in vitro chapters, leading to a ranking of hazard potential for the materials tested in Section 7. We identify materials properties and in vitro indicators that should trigger in vivo experiments in a future testing strategy.

2. Emission versus Exposure

2.1. Emission Quantities and Possible Routes of Internalization

Potential human exposure to nanomaterials is as manifold as the potential applications of different nanomaterials. It is beyond the scope of the present contribution to assess all factors in detail.

The following paragraph introduces four questions to guide a prioritization:

(i) Emission of nanoparticles from composites or powders? Touching a composite thermoplastic that was reinforced with silicon dioxide nanoparticles (Scheme 1) is of less concern than being exposed to free nanoparticles. Consumer applications of nanomaterials focus on composite materials from which only the unintended release of fragments containing nanoparticles during use, recycling, or disposal may raise concerns. Given typical product lifetimes on the order of years, the dose of release from composites should be vanishingly low, even for a hypothetical complete degradation. First available evidence supports this assumption: Abrasion of acrylate coatings containing ZnO nanoparticles did not lead to significant release of nanoscale aerosols. Not intending to banalize the issue, one should keep in mind that evolution itself developed most remarkable nanostructured materials: Human bones and human tooth enamel are examples of organic–inorganic hybrid nanomaterials with biopersistence.

(ii) If there is emission of free nanoparticles, are these liquid, soluble or biopersistent? Natural and technological processes can produce ultrane droplets or nanoemulsions (e.g., milk). The ultrafine state may affect the uptake of a substance in the body, but inside the body the substance will dissolve or blend in body fluids and only effects different from those associated with nanometer sizes are expectable. In contrast, biopersistent nanomaterials could exhibit general nanometer-size-specific effects if internalized. Among the natural (biogenic, geogenic, or pyrogenic) sources of biopersistent nanomaterials, black carbon from incomplete biomass combustion dominates with 50 to 270 megatons per year followed by 16 megatons of inorganic dust from desert storms. But also human activity releases nanomaterials as unintended by-products. A typical urban atmosphere contains 10$^{12}$ particles m$^{-3}$ particulate matter (around 10$^7$ particles m$^{-3}$); a candle or a cigarette release 10 g m$^{-3}$ particles (around 2 × 10$^{11}$ m$^{-3}$). Welding fumes consist of...
cases, as demonstrated by the case of nanostructured sun screen pigments\cite{29,30} that prevent exposure, we show here the external exposure. External exposure does not lead to uptake in all thermoplastic nanocomposites (bottom left). In contrast to the toxicologically relevant internal skin damage (bottom right).

Scheme 1. The generally accepted principle assesses risk as: Risk $\propto$ Hazard $\times$ Exposure. Risk is controlled by low hazard or low exposure, ideally both. The examples from present technology show the laboratory synthesis of nanostructured battery materials (top left) and SiO$_2$-reinforced thermoplastic nanocomposites (bottom left). In contrast to the toxicologically relevant internal exposure, we show here the external exposure. External exposure does not lead to uptake in all cases, as demonstrated by the case of nanostructured sun screen pigments\cite{29,30} that prevent skin damage (bottom right).

~$10^9$ m$^{-3}$ metal nanoparticles.\cite{19} These values set a frame of reference, and they justify the use of CB as reference material in safety testing. The world production of CB for tires and printing inks is estimated around 8 megatons per year (in 1996).\cite{20} Emission of CB is relevant in aerosol form, but its quantity is vanishingly small compared to the background of black carbon.\cite{21}

(iii) Is the emission intended or unintended? There are a limited number of applications of biopersistent nanomaterials with intentional (external) exposure of the human body, especially as sunscreens in cosmetics (Scheme 1). The global turnover with engineered nanomaterials\cite{22} can be converted into very rough estimates for the quantities that were actually produced in 2007: metal oxide nanoparticles: 0.02 megatons (=20000 tons); metal nanoparticles: 20 tons; carbon nanotubes (CNTs): 100 tons. Graphene catches up with 15 tons in 2009.\cite{23} These values are worlds apart from CB, but they still outnumber specialties in the OECD sponsorship program like quantum dots, dendraimers or fullerences/C$_{60}$\cite{24,25} The vastly dominant applications are technically bound: CNTs and graphene in polymer nanocomposites, metal nanoparticles in catalysts, electronics, and antimicrobials. Note that nanoscale silver ranks high only when the number of marketed products is counted,\cite{25} but not among the production quantities. Metal oxide nanoparticles find broad application from coatings and plastics over catalysts to sunscreens. Specifically nanoscale TiO$_2$ is used for coatings and sunscreens, with an estimated production of 0.005 megatons per year,\cite{26} expected to grow to 0.06 mega-tons per year until 2025.\cite{27} Sunscreens represent one of the few nanomaterial-containing products to which humans are intentionally exposed. Preliminary scenarios of coating degradation\cite{28} estimate levels of unintended emission around $10^{-2}$ mg m$^{-3}$ in air and around 10 mg L$^{-1}$ in soil compartments.\cite{29} Measure-ments at workplaces in nanoscale TiO$_2$ production did not reveal any significant emission.\cite{30}

(iv) Is the exposure oral, dermal, or by inhalation? Nanotechnology in food processing focuses on nanostructures for encapsulation, whose degradation in the human body is essential to fulfill their purpose. Migration of particles larger than 1 nm from packaging materials into food seems to be no concern.\cite{31} This may explain why relatively few investigations on the absorption and effects of nanoparticles via the oral route are available. TiO$_2$ and ZnO nanoparticles in sun screens were comprehensively tested on skin and several studies demonstrated that the intact human skin is an effective barrier for those nanoparticles.\cite{29,30} The absence of dermal penetration minimizes human internal exposure and hence minimizes the health risks. On the other hand, there is a wealth of information on the effects of ultrafine particles in the air\cite{38,39} indicating the concerns for human health arising from the inhalation of ultrafine particles.

In summary, the highest concern for human health arises from the unintended exposure to biopersistent nanoparticles. These may occur mostly in workplaces, and to a much lesser extent in consumer settings. The existing knowledge of adverse health effects by inhaled ultrafine particles gives priority to the investigation of effects caused by inhaled engineered nanomaterials,\cite{31} and among these, the emission quantities prioritize metal oxides.

2.2. Approaches to Regulation

The US OSHA Permissible Exposure Limit (PEL) for General Industry is 5 mg m$^{-3}$ time-weighted average (TWA) (PEL listed under Inert or Nuisance Dust). The American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value states the same limit value of 5 mg m$^{-3}$ TWA for Particles (insoluble or poorly soluble) Not Otherwise Specified (PNOS). A distinction between inhalable and respirable dust was changed in 2001 to PNOS. Whereas in Germany the legally binding Occupational Exposure Limit (OEL) for inhalable dust is 10 mg m$^{-3}$, there is...
3. Review on the In Situ Biophysics of Nanomaterials

Since the potential nanohazard arises from the nanosize, the full characterization of the pristine or as-produced nanomaterial must be completed with the conditioning effects and the actual state of dispersion in biological media (Scheme 3). In the next two subsections, we discuss the surface and agglomeration state with their in situ dynamic variability (Scheme 2) in more detail. These properties are decisive for biokinetics, biodistribution, and clearance of nanomaterials. At the same time, surface and agglomeration are rather sensitive to the experimental protocols, the nanomaterial, and the surrounding medium. In the third subsection we summarize particle properties influencing measurement procedures of in vitro toxicity tests and suggest suitable technologies for particle characterization.

3.1. Surface Conditioning and Hybridization

The control of fate and biokinetics by surface is standard practice in biological sensing, delivery, and imaging of live cells and tissues. Such issues have been reviewed by De et al.[34] in a previous issue of the same journal, and by Dobrovolskaia and co-workers[35] from a drug delivery perspective. The nanomaterial’s high surface to mass ratio enhances any specific surface characteristics, including the interaction with serum, saliva, mucus, or lung lining fluid components. The adsorbed molecules (certainly proteins, presumably also peptides, carbohydrates, and phospholipids) change the biological identity of the conditioned nanomaterial. Their differential adsorption[36] induces a characteristic “protein corona” around the nanoparticle, a term coined by Dawson and co-workers.[37–42] To date, most work has been devoted to serum interactions. Blood serum contains about 75 mg mL⁻¹ glucose, and 3 mg mL⁻¹ calcium (for the case of Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS)). Using polymer nanoparticles, it is possible to fine-tune the hydrophilicity by the copolymer composition, e.g., by changing the ratio of NIPAM and BAM monomers.[39] It was established by different physicochemical methods that a single

in addition an OEL for respirable dust (<10 µm) of 3 mg m⁻³
(excluding soluble particles, ultrafine particles and coarse-disperse dust). The German MAK commission, a scientific committee for the investigation of health hazards of chemical compounds in the workplace, advises public authorities and established a so-called MAK-value (maximum concentration at the workplace) for respirable nuisance dust of 1.5 and 4 mg m⁻³ for inhalable dust.

The regulatory and political framework evolves into a case-by-case risk assessment that requires knowledge of both hazard and exposure: California’s Division of Toxic Substances Control (DTSC) has requested data regarding CNT hazard, but also regarding uses, life cycle fate, and transport.[32]

Key milestones for a first approach to regulation[38] are a testing strategy in the REACH Implementation Plan and a definition of nanomaterials for regulatory purposes. That definition must complement scientific criteria of size[33] or surface area[31] or others with protocols for cheap and undisputable measurements, e.g., from the revised OECD guideline of ISO standards.[11]
Polymer nanoparticles have the obvious methodical advantage of being nicely homogeneous, spherical, and well dispersible. Corona-related mechanisms are known to much less detail for inorganic nanomaterials. First results indicate that the corona is selective also for different naked inorganic surfaces: For instance, specific pro-inflammatory and anti-inflammatory precursors displayed an up to 30 times higher affinity to Ni and Al particles than albumines, as demonstrated by isotope labeling (Fig. 1). Likewise, diamond nanoparticles showed a high affinity for vitronectin, which can stimulate tumor necrosis factor α (TNF-α) release from alveolar macrophages. However, even with its low relative affinity, the high concentration of albumin in serum still represents a significant portion of the bound protein fraction for all nanoparticles. Quantitatively, albumin adsorption onto carboxylic-acid functionalized inorganic nanoparticles was anti-cooperative and saturated at serum level concentrations and one monolayer. Working with semiconductor (quantum dot) particles, Frangioni and co-workers showed in a series of experiments how surface functionalization controls biodelivery: Particles were filtered by renal clearance and urinary excretion only for diameters below 6 nm and with zwitterionic or neutral organic modification to prevent protein adsorption. The significant corona conservation between different polymer particles is reflected by the uniformity of surface charge of various naked metal oxide nanoparticles when dispersed in serum-containing media (−20 to −10 mV), attributed to a universal coverage of the nanoparticle surface (zeta-potential ranging from −25 to −55 mV) by proteins. Part of the reduction of zeta-potential must be attributed to charge-screening in the physiological buffer with isotonic salt load. As expected, smaller particles adsorb more protein, demonstrated directly by UV and secondary ion mass spectroscopy (SIMS) detection of the colloidal ZnO surface and indirectly by BCA assay (bicinchoninic acid) of the protein fraction that did not adsorb onto TiO₂. On nearly the same series of naked metal oxides as described in Section 5, protein adsorption was shown to even induce buffer depletion, but only at completely unphysiological nanoparticle...
without bilayer disruption. Such phenomena make it difficult to
groups, the other with the same moieties in random distribution,
sub-nanometer striations of alternating anionic and hydrophobic
with identical hydrophobic content, one functionalized with
nanoparticles.\[2\] The direct comparison of conditioning CNTs in
dominant surfactant protein SP-A does adsorb onto metal oxide
particles.\[43\] It has been shown that a higher protein concentration leads to a
smaller average agglomerate size of the nanoparticles
(Fig. 3b).\[51,66\] Working with a 50-fold excess of serum protein
concentration, which is the relevant range for nanoparticles that
translocated into the human blood stream, Richter and cow-
workers\[67\] established that agglomeration of TiO$_2$ and wolfram
carbide is prevented over more than 40 min, compared to 5 min
until complete agglomeration in DMEM. Furthermore, they
showed that BSA alone is sufficient to prevent the agglomeration
process.\[67\] Alternatively, suppression of adsorption and steric
stabilization by PEG functionalization also stabilizes particles in
aqueous suspensions of non-functionalized nanoparticles and
the lung lining fluid, the first conditioning contact
surfaces,\[53\] and we demonstrated by antibody staining that the
adsorbed protein corona is specific and selective for the different pristine nanoparticle surfaces
(Figure redrawn with permission from ref. \[46\]).

3.2. State of Agglomeration

Clearly a correlation between the biological surface conditioning—controlled by the chem-
ical functionalization—and the colloidal interaction between the thus coated particles is to be expected.\[74\] In good qualitative agreement, numerous studies established that
aqueous suspensions of non-functionalized nanoparticles are stabilized against agglom-
eration by the addition of bovine/human serum albumin (BSA/HSA) and some other
proteins. The effect has also been exploited in production for the
debundling and dispersion of graphene and CNT material before
chemical compounding (Fig. 2).\[57,58\] Especially albumins in
water or DMEM have dispersed and stabilized a wide variety of
nanomaterials: CNTs,\[57,59-61\] metal nanoparticles,\[62\] metal
carbide nanoparticles,\[63\] and metal oxide nanoparticles.\[51,63,64-67\]

It has been shown that the focus.

Sometimes, however, minute differences between nanoparti-
cle surfaces strongly change the biodistribution: Surface
functionalization with poly(ethylene glycol) (PEG) of varying
chain length – typically considered an inert molecule – resulted
in major changes in organ/tissue-selective biodistribution and
clearance from the body,\[47\] although 2D gel electrophoresis
showed that immune-competent proteins (IgG, fibrinogen) bind
much more than albumins irrespective of PEG chain length.\[55\]
Verma et al.\[56\] demonstrated that of two nanoparticle “isomers”
with identical hydrophobic content, one functionalized with
sub-nanometer striations of alternating anionic and hydrophobic
groups, the other with the same moieties in random distribution,
only the striated particles penetrated the plasma membrane
without bilayer disruption. Such phenomena make it difficult to
relate materials properties directly to physi-
ological effects without knowing the biophysical
interactions (compare abstract figure).

We conclude that despite the human risk
being dominated by inhalation exposure and
by metal oxide nanoparticles, most work on the
protein corona has been devoted to polymeric
nanoparticles and serum proteins, often
restricted to albumin. In future, metal oxides
and lung lining fluid interaction with their
higher relevance for human safety should be in
the focus.
The use of extensive ultrasonication in these experiments makes it difficult to compare the experimental results to calculations that find that the interaction energy between TiO$_2$-agglomerates cannot be overcome by the interaction between the particles and DPPC. In a comparison of eight metal oxide nanomaterials, we showed that the anticipated interface activity of surfactant proteins is in general not sufficiently strong to overcome the agglomeration or flocculation tendency due to other components in complete BALF. Strikingly, the two particles that were functionalized with synthetic polymers evaded near-complete agglomeration and at the same time differentiated by low overall protein adsorption, but strong SP-A interaction. The physicochemical results are in good agreement with histological studies of lung slices after inhalation exposure of rodents, in which the particulate material that was deposited on the lung surface was found in the form of agglomerates.

While the deagglomeration potential by natural macromolecules certainly changes transport and biokinetics, a deagglomerated nanomaterial is in general not more potent, as demonstrated by the example of polymer-functionalized BaSO$_4$ that stays well-dispersed in a variety of media, but has virtually no in vitro or in vivo effects.

What is the mechanism of dispersion by interface-active proteins? Given the rather low zeta-potential of conditioned nanoparticles, the dispersing effect of the protein corona is not related to electrostatic repulsion. Instead, the stabilization must be a steric mechanism, whereby the entropy decreases if the protein coronas of approaching nanoparticles start to overlap. Electrostatic stabilization collapses in high ionic strength buffers such as DMEM with 0% FBS, then shifts to a steric stabilization by the adsorbed proteins in 100% FBS. The steric stabilization by biopolymers has been exploited industrially for a long time since protection colloids such as gelatine or starch stabilize organic composite particles. Structural models from X-ray diffraction seem to suggest that proteins fold into a single well-defined structure, which would eliminate the entropy stored in the structural degrees of freedom, hence disabling steric repulsion. However, most proteins are minimally stabilized mesoscopic biopolymers whose configuration fluctuates around the time-average structure under physiological conditions—a field that was pioneered by Kai Wüthrich (Nobel Prize 2002).

The quantitative degree of deagglomeration is controversial, due to (i) the use of different dispersion protocols and (ii) the disagreement of measurement techniques, which will be discussed in more detail in the following subsection. The dispersion protocol defining shear rate, energy input and duration of conditioning has a drastic influence on the resulting state of agglomeration as established also in ISO 14887 “Sample preparation—Dispersing procedures for powders in liquids”, One can either mimic the dispersing action that we assume to be active in the human body, and since the blood stream is laminar with rather low shear forces, ultrasonication should be omitted. Alternatively, one assumes that only the most dispersed fraction has a profound effect; then one can try to prepare the total administered dose in the most dispersed state, using wetting agents, vortexing, and ultrasonication. Since both approaches have been pursued, biophysical data published so far are hardly comparable on the quantitative level.

3.3. Characterization of Nanomaterials for Biological Testing

The intrinsic polydispersity and inhomogeneity of nanomaterials represent major obstacles for a biophysical characterization. Traditional methods already fail to characterize nanomaterials in a controlled environment (e.g., distilled water + one surfactant). This situation is drastically aggravated under physiological conditions since at least 40 components add to the colloidal domain and interact with each other and with the nanomaterial. Recent contributions by Hussain, Tiede, Powers, the OECD and others stress the need for a conscious characterization beyond the naïve application of characterization methods that claim to cover the relevant parameters of nanotoxicology. The most important parameters and appropriate measurement techniques are summarized in Table 1.

3.3.1. Intrinsic Properties

Impurities are an issue especially for CNTs, with catalysts (nanoparticulate Co, Fe, Ni, and Mo) and amorphous carbon being present during their synthesis that may impose additional toxic effects. Such trace elements were the subject of previous studies on welding fumes. The distribution of primary particle and aggregate sizes of a pristine nanoparticle powder requires proper statistics of at least ~10$^6$ nanoparticles, corresponding to more scanning electron microscopy (SEM) images than reported usually. Some nanoparticles are not stable in aqueous solutions and can release chemical substrates. If particles are designed to dissolve in aqueous solutions like water-soluble quantum dots or show an intrinsic, size-dependent dissolution in aqueous media like ZnO, particles will release metal ions when...
1 introduced into biological media. Cytotoxicity assays that are
2 sensitive to metal ions may then rather reflect metal ion toxicity
3 than particle effects. A high surface/mass ratio of nanoparticulate
4 materials results in excess surface energy enhancing particle
5 catalytic activity, depending on the crystalline phase. A variety of
6 nanoparticles such as metal oxide nanoparticles, fullerenes and
7 silica (SiO$_2$) particles were reported to produce reactive oxygen
8 species (ROS) in cell free systems.[86–89] ROS production was
9 size dependent, e.g., small 2–4 nm-sized nanoparticles had a
10 100–1000-fold increased kinetics compared to 100 nm-sized
11 nanoparticles.[90] Redox-active nanoparticles may cause false
12 positive signals in assays based on substrate oxidation or in assays
13 detecting cell stress induced ROS production. Few metal oxide
14 nanoparticles like Fe$_2$O$_3$ are magnetic and may generate local
15 magnetic fields leading to the production of free radicals that in
16 turn may interfere with cytotoxicity test methods based on redox
17 reactions.[91,92] ROS measurement by electron spin resonance
18 (ESR) is a valid, but not widely available technique. However, ROS
19 inside cells can be tracked as detailed in the materials and
20 methods.
21 The characterization of chemical composition and purity,
22 crystalline phase, morphology, and specific surface can be
23 regarded as relatively safe and well established—the same does
24 not hold true for the (last two properties of Table 1) state of
25 agglomeration and corona conditioning effects.

### 3.3.2. State of Agglomeration

For inhalation aerosols, the Scanning Mobility Particle Sizer
(SMPS) is widely used to determine size distributions of
submicron aerosols, by balancing the electrostatic force on
particles in an electric field with their aerodynamic drag as they
cross a laminar gas flow.[93] Aerosols of nanoparticles can be
generated using a dry powder aerosol generator and by
nebulization of particle suspensions. The mass concentration
of the particles in the inhalation atmosphere can be determined
gravimetrically, and the particle size using a cascade impactor, an
optical particle counter, or the SMPS. Such dispersion techniques
generate fine aerosols with particle size distributions in the
respiratory range, but with no more than a few mass percent of
ultra-fine material (i.e., agglomerates <100 nm).[93] Intercom-
parison studies indicate a rather high uncertainty between
different SMPS instruments and manufacturers.[94,95] More
appropriate dose metrics need to be developed for a relevant
indication of risk in exposure studies.[96]

Once the nanoparticles are suspended in any physiological
liquid (lining fluid, blood, or serum), size characterization
constitutes a major difficulty due to the enormous colloidial
polydispersity. Ultrafine particles and agglomerates have to be
quantified in an excess of proteins with diameters that are
comparable to the diameter of potentially present dispersed
nanoparticles (Fig. 3a). For characterization of the state of
agglomeration of samples in biological matrices there are a
number of complementary techniques that rely on different
working principles, such as hydrodynamic/sedimentation,
dynamic light scattering (DLS) and fractionating techniques.
The lower working limit of laser Fraunhofer diffraction is
exceeded by the potentially present ultrafine components, thus
introducing a strong bias in results from laser diffraction, where
by principle only coarse agglomerates will be detected. The
nominal working range of DLS does cover all components from
proteins to agglomerates; however, even after filtering some of the
larger agglomerates, DLS still fails to detect the proteins (BSA at
4–6 nm diameter) that constitute 99 wt % of a typical in vitro
sample (Fig. 3a).[51,97] When 99 wt % of the measurable
components (proteins) disappear in DLS measurements, also
the DLS results on the remaining 1 wt % of measurable
components (nanoparticles) are highly questionable.[98] First,
the failure of reverse Mie-scattering calculation with the
overwhelming scattering of the >0.1 wt % agglomerates that are
present in nearly every physiological suspension of
nanoparticles should be considered and second the well-known
fact that retrieving a size distribution from the autocorrelation
function is a mathematically ill-posed problem[99–101] that fails
especially for very broad distributions such as the four orders of
magnitude in physiological suspension of nanoparticles (Fig. 3a).
However, with careful sample preparation and elimination of the
very coarse agglomerates by ultrasonication, Hussain et al.[62]
obtained sub-micrometer average diameters for physiological
suspensions of Cu, Al$_2$O$_3$, Al, Ag, TiO$_2$ nanomaterials, and they
confirm also by DLS that serum-containing media reach the
same, often lower, diameters as in water, thereby drastically
reducing the agglomeration compared to serum-free cell culture
media. The majority of published data from scattering techniques
neglects the very loose structure of nanoparticle agglomerates.
The standard Mie routines such as those that retrieve relative
concentrations and distributions in commercial DLS software

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**Table 1. Most important properties and the appropriate characterization tools.**

<table>
<thead>
<tr>
<th>Property</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical composition and purity (pristine nanoparticles)</td>
<td>XRD, ICP-MS</td>
</tr>
<tr>
<td>Crystalline phase (pristine nanoparticles)</td>
<td>XRD</td>
</tr>
<tr>
<td>Morphology, primary particle size (pristine nanoparticles)</td>
<td>SEM</td>
</tr>
<tr>
<td>Specific surface (pristine nanoparticles)</td>
<td>BET</td>
</tr>
<tr>
<td>Solubility (in water and after conditioning in the test medium)</td>
<td>ICP-MS of supernatant</td>
</tr>
<tr>
<td>Surface chemistry (pristine nanoparticles)</td>
<td>zeta-Pot., SIMS, XPS</td>
</tr>
</tbody>
</table>

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**Advanced Characterization for Mechanistic Understanding of Observed Effects**

<table>
<thead>
<tr>
<th>Property</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalytic activity, esp. ROS generation</td>
<td>ESR</td>
</tr>
<tr>
<td>Protein corona (in vitro: conditioned nanoparticles)</td>
<td>SDS-PAGE, zeta-Pot., SIMS</td>
</tr>
<tr>
<td>State of agglomeration and potential of deagglomeration (in vitro: conditioned nanoparticles/inhalation: aerosol)</td>
<td>AUC, cryo-TEM//SMPS</td>
</tr>
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</table>
To complement the ensemble methods, an in situ imaging technique is desirable, but is not generally available at present. Any optical microscopy does not cover the relevant structural sizes. Standard electron microscopy introduces artifacts of unknown extent by drying and vacuum preparation; cryo-TEM is a compromise, requiring still a number of preparation steps (shock-freezing the liquid, then replicating and etching), but paves the way to a high-resolution image of aqueous structures.

The disagreement between different measurement methods is exemplified for the case of TiO$_2$ B nanoparticles in FBS (Fig. 3b, diamonds). Ensemble techniques (DLS, Fraunhofer diffraction) detect only agglomerates and disagree by many orders of magnitude, while imaging (cryo-TEM) and fractionating techniques (AUC) agree at least within a factor 4.

Hence, it is indispensable to complement and critically compare measurement techniques of different working principles, such as hydrodynamic/sedimentation, imaging, and scattering.[79–82] Some complementary techniques may be field-flow-fractionation (FFF-ICP-MS or FFF-MALLS)[111] or particle tracking. Murdock et al.[97] have mentioned these characterization issues earlier and gave an excellent discussion of the phenomena, but their preference for the simpler, albeit less sensitive method of DLS led them to underestimate the amount of deagglomeration in serum. Hassellöv et al.[80] published an

Figure 3. Colloidal characterization of physiological suspensions of nanomaterials. a) The suspension of nanoparticles in serum spans four orders of magnitude in diameter. A fractionating characterization (interference-AUC, dotted blue line; turbidity-AUC, solid blue line) detects all colloidal components from proteins to agglomerates. The submicrometer fractions are missed by DLS (magenta line). b) The average diameter of the nanoparticle fraction (X-axis) drops significantly with increasing protein concentration in the suspension medium (Y-axis). CeO$_2$ (green triangles), TiO$_2$ B (black squares), and an organically modified ZrO$_2$ (red dots) (redrawn with permission from ref. [51]). Diamonds: inter-method comparison, see text Section 3.3. c) Enlarged sub-10 nm-interval with linear axes in order to facilitate the comparison of the protein signal to the expected value of the BSA monomer at 66 kDa. d) The metal oxide and carbon nanomaterials of the present study in DMEM/10% FBS (interference-AUC, this data enters into Table S1, Phys-bio-chem properties of the test materials).

assume a solid spherical shape for the Stokes–Einstein relation.
1 In reality, diffusion-limited colloidal agglomeration leads to a fractal morphology, and this has been proposed as the dominating transport mechanism.[45] The fractal dimension can be determined explicitly by static (light, X-ray, neutron) scattering, and for many colloids a universal fractal dimension of 2.1 has been found.[102] The fractal shape has been incorporated into DLS evaluation only by specialist particle labs.[103] Concluding the DLS discussion, dynamic, and static light scattering (DLS, multangle laser light scattering(MALLS)) as well as Fraunhofer diffraction provide complementary information only if it is known from other sources that size distributions are narrow.

A mighty tool for the characterization of nanocolloids (0.5–10,000 nm diameter) is the analytical ultracentrifuge (AUC).[104–106] especially the universal interference optics of Beckman XLI with widespread use in the proteome business[107–109] and, only to a lesser extent, also the disc centrifuges (Brookhaven Instruments XDC, CPS Instruments DC24000) with their rather limited detection optics and lower speeds. Schlieren, turbidity, interference, UV–vis absorption, and X-ray absorption detection are published.[105,110] The optical AUC method detects the time- and radius-dependent concentration of the solutes simultaneously with the sedimentation at 600–60,000 rpm. Thereby, we quantify the amount and the diameter of each component independently.[104] At present, AUC is the only method that detects all components from the agglomerates to the dispersed nanoparticles and the sub-10-nm proteins (Fig. 3a): Note that interference-AUC retrieves without prior knowledge the correct molar masses and correct concentrations of 33 mg mL$^{-1}$ of BSA monomer and dimer in serum (Fig. 3c). In contrast to light scattering, AUC is a fractionating technique by which a distribution of sizes is determined with high resolution. Furthermore, in contrast to transmission electron microscopy (TEM)/SEM, the AUC integrates over $10^{12}$–$10^{14}$ particles in approximately 0.5 mL of a test substance, so that statistical relevance even of minor fractions is high. If low concentrations of nanoparticles are present in medium containing high concentrations of proteins, nanoparticles are easily discerned from sedimenting proteins (i) due to their much higher density difference compared to the surrounding medium resulting in faster sedimentation by several orders of magnitude, and (ii) due to their different absorption spectrum and higher refractive index. It is possible, but in general not mandatory to use X-ray detection optics that are inherently only sensitive to the inorganic components with high electron densities. It is straightforward to take into account the fractal morphology of nanoparticle agglomerates[49] and the hydrodynamic sedimentation of fractals has already been derived by Lin et al.[102]
3.3.3. Surface Conditioning

Once the nanoparticles are incubated in some physiological medium, the adsorption of proteins requires a combination of biochemical (for qualitative identification) and physicochemical methods (for quantification of binding mechanisms). By centrifugation, harvesting and washing, conditioned nanoparticles can be isolated from the medium and their adsorbed corona can be assessed by SDS–PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis), ideally by 2D gels for full characterization, performed up to now only with polymer nanoparticles with the exception of an early work on iron oxide. A complementing qualitative method is provided by SIMS, even if the necessity to prepare samples under ultra-high vacuum is prone to introduce preparation artifacts. SIMS records ion fragments from the impact of an energetic primary ion beam; molecular groups on the surface are detected with ppm sensitivity. A purely elemental resolution with ~10 nm depth integration such as from X-ray photoelectron spectroscopy (XPS) is of high relevance for the purity of the pristine nanoparticle surface, but of less value for the identification of organic matter. XPS bombards the sample with X-rays that excite characteristic core electrons, and has the advantage of quantitative information. Quantitative information can be drawn from fluorescence correlation spectroscopy (FCS). Unfortunately, FCS is not generally applicable to industrial metal-oxide nanomaterials due to their lack of fluorescence and to their quenching of the fluorescence of adsorbed dyes.

Surface properties like hydrophobicity and surface charge determine the capacity and kinetics of aqueous solution dispersion and this in turn modulates particle ability to adsorb proteins or assay components. The zeta-potential is closely related to the surface charge density, screened by salts, and is experimentally accessible in many cases. Any changes in zeta-potential should be observed by step-wise addition of buffer components, so that charge-screening cannot be misinterpreted as a surface coating by an organic material. While the zeta-potential records the average surface composition, the dynamic change of the surface can be assessed by gel filtration of conditioned nanoparticles. The longer a protein is desorbed on average, the longer is its elution delay. Finally, the adsorption enthalpy can be determined by isothermal titration (also known as microcalorimetry). Microcalorimetry is a particularly sensitive method to measure the heat flow of a sample normally under isothermal conditions at room temperature or at 37 °C or higher. Detectable heat flows range from a few to 3000 μW. Due to the high baseline stability the dynamics of slow reactions can be studied over minutes up to several days.

3.3.4. Interferences With In Vitro Test Assays

Classic cytotoxicity or genotoxicity assays are often based on the detection of fluorescence or light absorption of indicators and chemical or enzymatic reactions. Undesired particulate interactions interfere with the test mechanism and detection. If undetected by insufficient in situ characterization, such interferences may lead to a misinterpretation of results. Especially CNTs and fullerenes show quite unexpected interactions with the testing systems that induce artifact signals. It has been reported that endotoxin tests are less sensitive, essential nutrients are adsorbed and hence cells starved. Furthermore, carbon nanomaterials have been shown to interact with indicator substances (methylthiazolyl-diphenyl-tetrazolium bromide (MTT)). When protein concentration or protein activity are read outs of cytotoxicity assays these parameters can be influenced by particles as well as by assay components used for the detection of cellular activity (e.g., substrates, dyes) and proteins (lactate dehydrogenase (LDH)) may be adsorbed and hence misleadingly low concentrations detected.

Light absorption or fluorescence emission is used to determine toxicity by most of the in vitro assay systems (Scheme 3). Optical properties of nanoparticles might therefore directly interfere with these detection systems. Metallic nanoparticles with light-adsorptive or light scattering properties like sodium titanate or TiO$_2$ might influence the readout in cell viability assays. Moreover, close proximity of gold nanoparticles and fluorescent dyes, have been shown to result in a quenching of fluorescence signal intensity. We believe that the characterization methods must be adapted to the in situ properties of the nanomaterials—instead of modifying the dispersion procedure until turbidity is low enough to apply widespread methods such as DLS. Since the most common in vitro assays are pH-dependent and may thus be influenced by acidic or basic nanoparticles, acidity/alkalinity should be tested when using nanoparticle concentrations which exceed the buffer capacity of biological media.

3.4. Correlation of Biophysical Properties

To summarize Section 3, the nanometer-sized entity exposed to the organism is not identical to the pristine nanomaterial, but undergoes dynamic changes of both its surface chemistry and its state of agglomeration. The protein corona is partially conserved, and partially selective for specific naked metal or metal oxide or organically functionalized surfaces. Serum tends to decrease the state of agglomeration, whereas lung lining fluid in general does not. One must abandon the attractively simple picture of a naked inorganic nanoparticle in the human body; we have to take proper care that the in vitro buffers are nearly identical to human body fluids, in order to mimic closely the true corona and state of agglomeration that develops in vivo. Due to the complexity and polydispersity of a physiological suspension of nanoparticles, a combination of characterization methods with different physical measurement principles (imaging, hydrodynamic, scattering) is mandatory. Similar statements have been stressed most recently by the “characterization matters” initiative. In the present contribution, we fulfill the criteria (Table 1) of minimal characterization for comparability of studies, and we additionally provide advanced characterization data that may help to elucidate the mechanisms underlying nanoparticle-effect relationships (Supporting Information Table S1).
4. In Vitro Studies With Engineered Nanomaterials

4.1. Critical Aspects of Nanomaterial Test Systems

In comparison to animal models, cytotoxicity testing allows for a simpler, faster and more cost-efficient determination of defined toxicity endpoints. Classic cytotoxicity assays were established for soluble chemicals, not for particles (see Section 3.3.4). Since they are not sufficient at this time to evaluate toxic nanomaterial effects in cells, multiple assays have to be employed.\(^{[125]}\) Nanomaterial specific properties are crucial determinants of biological effects. Recent in vitro screenings have used a variety of well-characterized nanomaterials\(^{[126]}\) or different variants of two kinds of nanoparticles.\(^{[127]}\) In most of the earlier studies, however, nanomaterials were used without prior characterization regarding their composition and physicochemical properties. Physicochemical properties of nanomaterials such as surface charge, size, agglomeration state, and shape have been shown to heavily influence the outcome.\(^{[31,49,128]}\) These difficulties might explain why numerous in vitro studies dealing with nanomaterial toxicology have provided conflicting results with little or no correlation to in vivo data.

Here, we will provide an overview of in vitro toxicity experiments of engineered nanomaterials (especially metal-oxides, and some carbon nanomaterial) and we will focus on studies that have been performed with well-characterized materials and multiple test systems. For the complementary nanomaterial classes of metals (including surface modifications) and quantum dots the reader is referred to the excellent review by Lewinski et al.\(^{[129]}\)

4.2. Review on the In Vitro Toxicity of Engineered Nanomaterials

Cultured cells exposed to toxic agents can respond with various mechanisms that differ in the level of cell damage. Cellular reactions range from reversible short term stress responses to irreversible induction of cell death or the long-term malignant transformation.\(^{[130]}\) Engineered nanomaterials have been studied mainly with established in vitro toxicity assays that analyze major cellular parameters such as cell viability and response to various stress factors.

4.2.1. Oxidative Stress

Evidence is accumulating that oxidative stress induced by nanoparticles is a key route by which these nanomaterials induce cell damage.\(^{[131]}\) Oxidative stress is often detected using a fluorimetric 2’,7’-dichlorofluorescein (DCF)\(^{[132,133]}\) or a colorimetric GSH (reduced l-glutathione) assay\(^{[114]}\) (Scheme 3). An approximate 50% increase in DCF fluorescence has been observed after exposure of cultured human skin fibroblasts to anatase TiO\(_2\) nanoparticles (UV irradiated).\(^{[135]}\) However, cells could be protected against TiO\(_2\)-induced intracellular ROS formation by encapsulation of particles with NaY zeolites (TiO\(_2\)@NaY). Sayes et al. reported that the structure of titania nanoparticles correlates with toxicity (Fig. 4). In their studies with dermal fibroblasts, rutile TiO\(_2\) particles produced two orders of magnitude less reactive oxygen species than similarly sized anatase TiO\(_2\) particles.\(^{[136]}\) Using P25 TiO\(_2\) (anatase/rutile 80:20), Xia et al.\(^{[137]}\) observed TiO\(_2\) to generate ROS in a cell-free system but not in murine macrophages (RAW 264.7). On the contrary, SiO\(_2\) nanoparticles doped with 1.6 wt % iron, cobalt, manganese, and titanium displayed catalytic oxidative effects inside living cells.\(^{[138]}\) Human lung epithelial cells (A549) were exposed to thoroughly characterized particles of the same morphology, comparable size, shape, and degree of agglomeration to determine the influence of chemical composition and catalytic activity on ROS formation. These studies clearly showed that the chemical composition of nanoparticles is a most decisive factor influencing ROS formation in lung epithelial cells.\(^{[138]}\) The role of particle size, shape, and composition to induce oxidative stress in primary mouse embryo fibroblasts was also evaluated for SiO\(_2\), ZnO, CNTs, and CB.\(^{[139]}\) Although all four nanomaterials induced significant ROS generation and GSH depletion in a dose-dependent manner, the effects were different with ZnO inducing significantly more oxidative damage than the other nanomaterials. Since SiO\(_2\) and ZnO had similar crystal shape and particle size this further confirms that toxicity diversity can be attributed to their chemical composition.\(^{[139]}\) Recently, Park and Park\(^{[140]}\) observed both, ROS formation and an increased level of nitric oxide when macrophages (RAW 264.7) were exposed to SiO\(_2\) nanoparticles and ROS formation in these cells may trigger proinflammatory responses observed in vitro and in vivo. On the contrary, Diaz et al.\(^{[79]}\) did not always find a positive correlation between cytotoxicity of SiO\(_2\) nanoparticles and ROS formation in human monocytes and mouse peritoneal macrophages. In vitro toxicity screenings with CeO\(_2\) nanoparticles revealed a dose-dependent induction of ROS and a decreased level of intracellular GSH in BEAS-2B as well as in A549 human lung epithelial cells.\(^{[141,142]}\)

Commercial SWCNTs and MWCNTs (single-walled and multi-walled CNTs) were found to induce a dose- and time-dependent increase of intracellular ROSs in rat macrophages (NR8383) and human lung epithelial cells (A549) that might be related to metal traces present in manufactured nanotubes.\(^{[143]}\)

4.2.2. Cell Viability

Different endpoints for cell viability have been used in nanomaterial toxicity testing. Metabolic activity, for instance, has been widely determined using the colorimetric MTT assay based on the reduction of a yellow tetrazolium dye (MTT) to a purple formazan in cells bearing intact mitochondria. Recently, however, the suitability of MTT for toxicity evaluation of CNTs has been doubted since SWCNTs have been shown to deplete free MTT thereby causing false-negative results.\(^{[117]}\) Moreover, numerous cytotoxicity studies are based on the detection of intact lysosomes via neutral red uptake. Neutral red accumulates in intact lysosomes of viable cells whereas it is excluded from dead cells. The uptake of neutral red may be detected via fluorescence or absorption measurement. Cellular necrosis is another endpoint commonly used in cell viability studies. Upon necrosis, significant amounts of LDH are released from the cytosol. This LDH release can be easily detected using INT (a
yellow tetrazolium salt) as a substrate since LDH catalyzes its oxidation to a red formazan (Scheme 3). Nanomaterial toxicity leading to apoptosis is commonly assessed with caspase-3 assays. Caspase-3 is activated in the terminal apoptotic cascade by cleavage and this step can be detected by measuring the cleavage of chromogenic or fluorimetric Caspase-3 substrates.

In a comprehensive study, Simon-Deckers et al. determined the cytotoxicity of well-characterized metal oxide nanoparticles and CNTs using different cell viability assays. Studying the response of A549 human lung epithelium cells, Simon-Deckers et al. found metal oxide nanoparticles (rutile or anatase TiO₂ and Al₂O₃) to be less toxic than CNTs. Although all nanoparticles were efficiently internalized in A549 cells, their cytotoxicity was generally low with a maximum cell death rate of 25% for TiO₂ (MTT). Since TiO₂ and Al₂O₃ particles were of similar size and shape but different of toxicity (with a maximum cell death rate of 3% for Al₂O₃ compared to 25% for TiO₂) this study revealed again that nanoparticle toxicity can be attributed to their chemical composition. In line with nanotoxicity data previously published by Sayes et al. Simon-Deckers et al. reported that anatase TiO₂ was slightly more toxic than rutile TiO₂.

Redox activity in mouse neuroblastom cells has been shown to decrease significantly when the cells were exposed to ZnO whereas an exposure to other metal oxide nanoparticles such as FeO₄, TiO₂, Al₂O₃, and CrO₃ had no measurable effect on the cells. Similarly, cell viability assays (MTT, LDH) using TiO₂ and metal nanoparticles (Co, Ni), did not reveal any significant toxic effect on A549 cells. Nanometer-sized and fine-sized ZnO particles were also found to be more cytotoxic to L2 lung epithelial cells than SiO₂ particles in LDH assays by Sayes et al. However, a comparison of in vivo and in vitro measurements demonstrated little correlation.

Lin et al. reported that SiO₂ nanoparticles reduce the viability of human bronchoalveolar carcinoma-derived cells in a dose- and time-dependent manner. The cytotoxicity of 15- and 46-nm SiO₂ nanoparticles was investigated by using crystalline SiO₂ as a positive control. Both SiO₂ nanoparticles were more cytotoxic than the bulk material; however, the cytotoxicities of 15- and 46-nm SiO₂ nanoparticles were not significantly different. Moreover, cell viability of A549, endothelial EAHY926 cells, and J774 monocyte-macrophages in response to SiO₂ particles was found to be determined by their total mass, number and surface area as well as by their concentration. A time- and dose-dependent effect of 20 nm-sized CeO₂ particles on cell viability of A549 cells was reported by Lin et al. In their studies, cell viability decreased to 53.9% when a CeO₂ concentration of 23.3 μg mL⁻¹ was used.

Although a variety of cell viability studies using carbon nanomaterials have been published so far, no coherent picture has emerged yet. Davoren et al. found a very low direct cytotoxicity of SWCNTs in cell viability assays using A549 cells but did not observe any acute toxicity on the viability of A549 cells exposed to SWCNTs or MWCNTs but, as mentioned above, observed a dose- and time-dependent increase of ROS formation presumably associated with metal traces found in commercial carbon nanotubes. These confounding findings may be due to interference of the nanomaterials with the employed test systems. Carbon nanomaterials have been reported to distort light absorption and fluorescence measurements due to their optic activity and to interact with dyes and substrates used in classical cell viability test systems. CNTs in particular absorb and interfere of the nanomaterials with the employed test systems. The interaction of MTS with other nanomaterials is still to be tested. Further studies using MTS in addition to multiple other cytotoxicity assays have to be performed for an appropriate assessment of carbon nanomaterial toxicity.
displayed mutagenicity. Recently, Xu et al. demonstrated that different anatase TiO$_2$ particles (5 and 40 nm in size, respectively) and fullerences increased the mutation rate in mouse primary embryo fibroblasts (MEF) in a dose-dependent manner. To elucidate the mechanisms underlying TiO$_2$ genotoxicity, this group also conducted studies using a nitric oxide synthase inhibitor and a chemical inhibitor of cyclooxygenase-2 (COX-2). Both nanomaterials lead to the formation of peroxinitrite anions and induced kilobase pair deletion mutations that could be protected by antioxidants. Furthermore, DNA damage could be reduced via suppression of COX-2. COX-2 plays an important role in cellular inflammation and genomic instability, and the particle induced oxidative stress may activate the COX-2 signaling pathway. In another detailed study of manufactured nanoparticles (ZnO, SiO$_2$, TiO$_2$, CB, and SWCNTs), SWCNTs were found to be more genotoxic than ZnO. Since ROS production induced by ZnO was significantly higher than compared to CNTs, it was assumed that DNA damage induced by carbon nanotubes can be attributed to mechanical injury rather than to an oxidative effect. Furthermore, Yang et al. provided evidence that DNA nanoparticle genotoxicity might primarily be due to particle shape rather than to chemical composition. Comet assays performed with SiO$_2$ nanoparticles in two different laboratories using cultured 3T3-L1 fibroblasts revealed no significant genotoxicity but showed that in vitro toxicity testing can be quantitatively reproducible. Using comet assays, Jacobsen et al. found different carbon nanomaterials (CB and SWCNTs) to induce significant DNA damage. However, MWCNTs did not show any mutagenic effects in chromosome aberration studies using Chinese hamster lung fibroblasts or in bacterial reverse mutation assays. Colloidal SiO$_2$ nanoparticles of different sizes (30, 80, 400 nm) did not exert any genotoxicity in 3T3-L1 fibroblasts.

4.2.4. Inflammatory Response

To assess inflammation by nanomaterial immunotoxicity, the production of inflammatory markers such as the chemokines Interleukin-8 (IL-8), TNF-α, or IL-6 are usually measured in cell culture supernatants using enzyme-linked immunosorbant assay (ELISA). In rat model systems, the production of the inflammatory cytokine MIP-2 (macrophage-inflammatory protein-2) together with that of TNF-α and/or IL-6 are used as cytotoxicity endpoints. Comparing the toxicity of rutile and anatase TiO$_2$ in A549 cells, Sayes et al. demonstrated an overall greater toxicity of TiO$_2$ anatase nanoparticles (Fig. 4). Anatase TiO$_2$ nanoparticles triggered a dose-dependent release of IL-8 in human dermal fibroblasts (HDF) and A549 cells that was significantly lower when the cells were exposed to rutile TiO$_2$ nanoparticles. Ultrafine (P25 rutile/anatase 80:20) but not fine TiO$_2$ particles were found to elicit IL-8 release in A549 cells indicating a size-dependent effect of immunotoxicity. However, TiO$_2$ ultrafine particles remained highly aggregated in cell culture as well as inside the cells. Inflammatory properties of TiO$_2$ particles therefore appear to be driven by their specific surface area. In a comprehensive study aimed to determine the importance of area surface and surface reactivity of particles to induce inflammatory responses, Duffin et al. used a variety of manufactured particles, such as TiO$_2$, CB, and metal nanoparticles (Ni and Co) both for instillation and for treatment of A549 cells. They observed a correlation between particle surface area dose, specific surface activity, and the proinflammatory effects in vivo and in vitro. Their study also demonstrated the utility of in vitro assays for predicting the ability of nanoparticles to cause inflammation in vivo on the basis of their surface area and reactivity.

Recently, Herzog et al. demonstrated that exposure of A549 or normal human bronchial cells to SWCNTs did not induce inflammatory responses but can lead to the suppression of a variety of inflammatory mediators including IL-8, IL-6, and MCP-1 (monocyte chemotactic protein-1) in vitro. In contrast, chemically unmodified MWCNTs caused a dose-dependent IL-8 increase in HEK cells. Since carbon nanomaterials seem to be capable of adsorbing a variety of substances including cytokines in the culture medium, classical toxicity assays may not be appropriate for assessing carbon nanoparticle toxicity. In this context it is important to note that Veranth et al. have observed a significant change of IL-6 response to nanoparticle treatment, either when different cell types were used or when the same cell type was grown in different media. Moreover, inflammatory responses to particles seem to be amplified by contact-dependent interactions between alveolar macrophages and epithelial cells. Therefore, future studies determining inflammatory effects of nanoparticles have to be conducted using co-culture systems with defined cell types and media to generate comparable data.

4.3. Original Results on the Cytotoxicity of TiO$_2$

As reviewed above, the available reports of nanomaterial in vitro testing give a broad overview regarding possible toxicology effects. However, a valid testing strategy is not available. Moreover most of the data are not comparable due to a lack of validated test protocols and a focus on only a few cell lines. Here we report exemplarily in vitro data from the Nanocare project in vitro screening strategy highlighting two critical aspects of reliable nanomaterial in vitro testing: the required number of sensitive cell lines and the selection of essential assays.

Six different stable cell lines (Supporting Information Table S3) were exposed to dispersions of two different types of TiO$_2$ nanoparticles and were tested regarding the formation of ROS, their metabolic activity, and cell death. The cell lines represented six different mammalian organs. A549 and RAW264.7, two of the most commonly used lung derived cell lines in in vitro toxicology, represent the first line of exposure to inhaled nanoparticles. While many studies are restricted to these two cell lines, we also incorporated three cell types representative of other routes of exposure. CaCo2 cells stem from a human colon carcinoma and are characteristic for the colon epithelium, while NRK-52E cells have been widely used as a model for the mammalian kidney epithelium and have been established from a healthy rat kidney. Furthermore, the skin is represented by HaCaT, a cell line isolated from spontaneously transformed human epidermal keratinocytes. A sixth cell line, NIH-3T3, represents the fibroblast phenotype and has been cloned from healthy mouse embryos. NIH 3T3 is a widely used well-known model for sensitive in vitro toxicology testing.
We focused on standardized cell lines instead of using primary cells to allow for a sound reproducibility of results and high throughput suitability of the test systems used.

Cells were exposed to 0.1, 1, and 10 μg cm⁻² anatase/rutile TiO₂ nanoparticles that originate from opposed synthesis routes. TiO₂ A is precipitated in a wet chemical process (sol–gel), while TiO₂ B is formed in flame pyrolysis of titanate salts. Concentrations of nanoparticles above 10 μg cm⁻² interfered strongly with the assay systems which were based on optical detection and were therefore neglected.

Dispersions of TiO₂ A nanoparticles did not induce a significant change in any of the three parameters studied nor in any of the cell lines investigated (Figs. 5a, 6a, and Supporting Information Fig. S3). TiO₂ B, on the other hand, provoked the formation of ROS in all cell lines tested in three or more independent experiments (Fig. 5b). The percent increase in ROS formation was dependent on the cell line and the concentration of TiO₂ applied. The mouse fibroblast cell line NIH 3T3 showed the strongest increase in ROS formation after exposition to 10 μg cm⁻² TiO₂ B. The metabolic activity and the incidence of cell death remained unaffected by TiO₂ B in all cell lines tested (Fig. 6).

It has been suggested that inhaled particles exert their adverse effects primarily by triggering an inflammatory response which is in turn mostly elicited by the formation of ROS by the particles themselves and by the cellular stress response. The observation of ROS formation is therefore a good indication of the inflammatory potential of a given particle. In the present study, TiO₂ B has been shown to cause the formation of ROS in vitro and may therefore trigger an inflammatory response in vivo.

It has been shown that the crystal structure of TiO₂ nanoparticles may influence them in vitro toxicity. Rutile TiO₂ nanoparticles triggered two orders of magnitude less ROS than similarly sized anatase TiO₂ particles in dermal fibroblasts (Fig. 4). However, TiO₂ A, which did not influence any of the parameters tested in vitro, consists mostly of anatase TiO₂ like TiO₂ B. Both particles are in a similar size range which leaves the organic modification detected on TiO₂ A as possible explanation for the different biological activity of the two types of TiO₂ nanoparticles.

In contrast to the extensive inhalation studies presented in the following section, this investigation was designed to provide an exemplary insight into the necessity of using different cell types and test systems when assessing the in vitro toxicity of nanoparticles. The six cell types presented here displayed individual degrees of ROS formation in the presence of TiO₂ B nanoparticles. While some cell lines, such as HaCaT and RAW264.7, behaved more robust, NIH-3T3 seem to be more sensitive to the exposition with TiO₂ B. Unpublished results of our laboratory show that the cell type specific sensitivity is also dependent on the nanoparticle applied. In line with this, Veranth et al. have shown that inflammatory responses to TiO₂ nanoparticles are influenced by the cell type and culture conditions applied. Furthermore, our results show that cell types of the other routes of exposure may also be affected by nanoparticles. As it has been shown that inhaled nanoparticles may be translocated into the body (e.g., ref. [164], the need for the investigation of cell types representing other organs than the lung becomes evident. As described, concentrations of nanoparticles above 10 μg cm⁻² interfered with the quantification of the chosen endpoints. Consequently, in vitro methods and especially those based on optical detection have to be adapted with respect to interference with nanoparticles and are limited regarding the maximum applicable dose. A comparison to adverse effects of high doses used in inhalation studies is therefore impossible. In various studies, higher concentrations of nanoparticles (e.g., P25 and other TiO₂ particles) than those reported here have been applied and found to induce strong effects. However, it remains questionable if the application of for instance 100 μg cm⁻² yields measurement artifacts or reliable results. Based on our findings we argue that the investigation of several parameters at lower particle concentrations is preferable over the application of high doses.

Taken together, our results and recently published data [125] demonstrate that it is...
necessary to use a minimum set of sensitive cell lines and to consider several test systems as nanoparticles may exert particle type specific adverse effects which will arise in different endpoints.

4.4. Correlation of In Vitro Toxicity Data

A number of studies conducted with physicochemical characterization and multiple cytotoxicity assays showed that nanoparticle toxicity can be attributed to size,[33,147] chemical composition,[138,139] surface,[146,165] and structure.[136] Currently, however, sufficient data enabling to predict adverse effects of nanoparticles based on their physicochemical properties are still missing. To allow an appropriate interpretation of test results, it is not sufficient to characterize the intrinsic properties of nanoparticles only since the interaction of nanoparticles with physiological media will also influence the level of toxic effects.[51,115] Furthermore, appropriate control materials and validated protocols for the preparation of particle suspensions should be used in future studies of nanoparticle toxicity.

An increasing number of studies designed to analyze the mechanisms underlying nanoparticle toxicity has been published recently and evidence is accumulating that many toxic effects derive from oxidative stress initiated by the formation of ROS. The ROS generating capacity of nanoparticles seems to correlate with their potential to induce cellular inflammation and DNA damage.[64,136,140,152] Therefore, measurement of oxidative stress potential can be regarded as an important and highly sensitive component of a screening strategy for nanoparticle toxicity assessment. However, intracellular ROS formation induced by nanoparticles may not be predictive of all possible cytotoxic effects. For SiO₂ particles and carbon nanomaterials, for instance, a positive correlation between cytotoxicity and ROS formation could not be found.[70,139] Multiple tests should therefore be used in a comparative manner to enable an appropriate evaluation of nanoparticle cytotoxicity.

Taken together, the presented in vitro testing strategy may be suitable for predicting the in vivo effects of nanomaterials. Currently, however, there is little correlation between qualitative in vitro data generated in different laboratories which might result from a lack of adapted in vitro test systems. Furthermore, in vitro test systems display a lower complexity than living organisms and the transfer of doses applied in vitro to in vivo exposure scenarios is hardly possible. For an appropriate design of in vivo experiments, standardized in vitro testing will be of considerable value.

Scheme 4. Work flow of in vivo inhalation studies for nanomaterials. Aerosols are generated from the nanomaterials (Supporting Information Fig. S2) and monitored (Table S2) in an inhalation chamber, typically with head-only exposure. The study design of the short-term inhalation test developed by the authors[79] is shown on the bottom. X, head–nose exposure to aerosols for 6 h day⁻¹ on five consecutive days; R, post-exposure time; H, histology of selected organs (especially lungs slices, as shown bottom right) including cell proliferation and apoptosis; e, examinations of blood and bronchoalveolar lavage fluid (as shown on bottom left).

5. In Vivo Studies With Engineered Nanomaterials

5.1. Review of Pulmonary Toxicity Studies With Engineered Nanomaterials

Adverse health effects of air pollution have been recognized in epidemiological studies. Part of the pollution is Particulate Matter, mostly black carbon (see Section 2), and has been linked with cardiovascular effects and pulmonary toxicity.[166–168] Here we focus on pulmonary toxicity of engineered nanomaterials, and since study designs are not standardized yet, we report...
Inhalation studies with animals are the best possible approximation to the exposure of the human respiratory tract to nanomaterials. But inhalation studies entail the sacrifice of animals, are quite expensive, consume up to 1 kg of test material and they require care with regard to aerosol preparation. Instillation remedies the last two drawbacks and replaces the aerosol by a suspension; however, nanomaterials in suspension have different surface properties, different states of agglomeration and deposit differently in the lung compared to nanomaterials in aerosols. If these constraints are taken into consideration, the pulmonary exposure by intratracheal instillation, pharyngeal or laryngeal aspiration is only acceptable for hazard identification. Endpoints of concern for pulmonary exposure are organ-specific markers of inflammation, oxidative stress, cell proliferation, and histopathology in the lung as well as measurement of damage to non-pulmonary organs.

5.1.1. Metal-Oxide Nanomaterials

Based on their own intratracheal instillation studies and literature review, Donaldson et al. [165, 169] came to the conclusion that ultrafine particles made of low-solubility, low-toxicity materials are more inflammagenic in the rat lung than fine, respirable particles made from the same material, which is driven by their surface area. In more details, initial findings with nanoparticles after inhalation were published by Heinrich et al. in 1995, [170] describing an increased lung tumor incidence after long-term exposure to ultrafine titanium dioxide (uf-TiO$_2$) P25 and CB. Bermudez et al. [171, 172] performed a multispecies, sub-chronic, inhalation study comparing pulmonary responses to a uf-TiO$_2$ P25 (average primary particle size of 21 nm). Female rats, mice, and hamsters were exposed to aerosol concentrations of 0.5, 2.0, or 10 mg m$^{-3}$ TiO$_2$ nanoparticles for 6 h day$^{-1}$, 5 days week$^{-1}$, for 13 weeks. Following the exposure period, animals were held for recovery periods of 4, 13, 26, or 52 weeks (49 weeks for the uf-TiO2-exposed hamsters) and, at each time point, TiO$_2$ burdens in the lung and lymph nodes and selected lung responses were examined. Inhalation of 10 mg m$^{-3}$ TiO$_2$ nanoparticles for 13 weeks resulted in pulmonary overload in rats and mice but not in hamsters in which the lung burdens were approximately only 23% of lung burdens of other species. While there were various responses in mice and rats, hamsters had very limited responses probably due to the low lung burdens and rapid clearance of particles in these animals. Responses in mice were limited to animals exposed to 10 mg m$^{-3}$, whereas in rats responses were also observed in animals exposed to 2 mg m$^{-3}$. The magnitude and spectrum of responses were, in general, equivalent in rats and mice. The extent and character of the inflammatory responses in rats differed from that observed in mice; in rats the responses had a greater neutrophilic component that diminished over time, whereas in mice significantly increased neutrophil and macrophage numbers remained relatively constant. Histopathological examination of rats and mice uncovered progressive fibroproliferative lesions in rats but not in mice. Taken together, the species-specific differences observed in this study are well in line with results of previously reported chronic exposure studies with rats and mice and poorly soluble particulates. They suggest that susceptibility of the rat to the induction of lung tumors by pulmonary overloads is related both to dosimetry and biological response. The authors concluded that the findings of this multispecies study were consistent with the results of a companion study using inhaled pigmentary (fine mode) TiO$_2$ [171] and demonstrated that the pulmonary responses of rats exposed to nanoparticle concentrations likely to induce pulmonary overload are different from similarly exposed mice and hamsters. Different types of TiO$_2$ nanomaterials were tested by Warheit et al. [12] by intratracheal instillation in rats examining pulmonary effects in the BALF and lung tissue up to three months post-exposure. The TiO$_2$ nanomaterials had different crystal structures and surface coatings, showing differential endpoints of concern for pulmonary exposure are organ-specific markers of inflammation, oxidative stress, cell proliferation, and histopathology in the lung as well as measurement of damage to non-pulmonary organs.

Figure 7. Inhalation of 2–5 nm TiO$_2$ nanoparticles, dark field micrographs of lung tissue (A,B) and alveolar macrophages (C,D). Mice exposed acutely to 0.77 or 7.22 mg m$^{-3}$ nanoparticles demonstrated minimal lung toxicity or inflammation. Mice exposed subacutely (8.88 mg m$^{-3}$) and necropsied immediately and at week 1 or 2 post-exposure had higher counts of total cells and alveolar macrophages in the BAL fluid compared with sentinels, indicating a significant but moderate inflammatory response. However, mice recovered by week 3 post-exposure. Other indicators were negative. Figure reproduced with permission from Grassian et al. [173].
pulmonary effects based on these properties. The rutile and surface treated TiO₂ showed only transient and fast reversible pulmonary inflammatory responses, whereas inflammatory effects, cytotoxicity, and adverse lung effects were described with the anatase/rutile TiO₂. Grassain et al.⁶ performed acute as well as subacute inhalation studies (whole body exposure) with TiO₂ nanoparticles (2–5 nm diameter, pure anatase) in mice (Fig. 7). Mice exposed for 4 h to 0.77 or 7.22 mg m⁻³ titanium dioxide nanoparticles demonstrated only minimal lung toxicity or inflammation. Mice exposed subacutely (4 h day⁻¹ for 10 days) to 8.88 mg m⁻³ (only one concentration) and examined immediately or 1 or 2 weeks after the last exposure had higher counts of total cells and alveolar macrophages in the BAL fluid, no effects were seen after 3 weeks recovery. No effects were found in lung histopathology or any other clinical parameter. A recent study by Sager and Castranova exposed rats via intratracheal instillation to various doses of ultrafine and fine CB and TiO₂, all low toxicity and low solubility materials. Ultrafine TiO₂ was more bioactive than ultrafine CB at equivalent surface area, supporting the hypothesis that surface area, not mass is a more appropriate dose metric to assess pulmonary inflammation.⁴ Previously, Heinrich et al.⁵ reported an increased tumor incidence in inhalation studies using CB and pulmonary inflammation. Moreover, increased chemokine and mutagenic responses after three months inhalation exposure were described by Driscoll et al.⁶ For instance, they observed that mutations in the hprt gene of alveolar epithelial cells encoding the hypoxanthine–guanine phosphoribosyltransferase occurred only after CB exposures and resulted in significant inflammation and epithelial hyperplasia. The no observed adverse effect concentration (NOAEC) in this study, for this specific material (particle size 0.016 μm, 220 m² g⁻¹) was 1 mg m⁻³.⁶

5.1.1. Carbon-Based Nanomaterials

Similar to the study performed by Bermudez et al.,⁷ the inhalation toxicity of low (37 m² g⁻¹) and high surface area (500 m² g⁻¹) CB was examined in rats, mice, and hamster after 3-month inhalation exposure.⁸ Again, rat was the most sensitive species. The NOAEC for high surface area CB was 1 mg m⁻³ for rat. The low surface area CB did not exert any toxicity at the 50 mg m⁻³.⁹ In 2006, Lam et al.¹⁰ compiled the animal studies available with CNTs. By this date, data from inhalation studies were not available, only those from studies on intratracheal instillation. The animal studies of CNT pulmonary toxicity showed that CNTs are capable of inducing inflammation, epithelioid granulomas, fibrosis, and biochemical toxicity changes in the lungs that might impair pulmonary functions. Systematic reduction of the metal content of CNTs did not eliminate their inflammation potential (Fig. 8).¹¹ Muller et al. took a similar approach and systematically modified the structural defects of CNTs. They found that the acute pulmonary toxicity was reduced upon heating but restored upon grinding, indicating that the intrinsic toxicity of CNT is mediated by the presence of defective sites in their carbon framework.¹² The studies reviewed here were conducted using intratracheal instillation or modified techniques to administer CNT suspensions to rodents that had been mechanically ultrasonicated. In a much disputed publication, Poland et al. reported a pilot study,¹³ in which they administered different CNTs by intraperitoneal application to mice. They reported that CNTs may behave in this test system comparable to asbestos. However, the relevance of

Figure 8. Instillation of carbon-based nanomaterials: Lung tissues from mice after a single intratracheal instillation of unprocessed iron containing HiPco SWCNTs (D: 7 days, E: 90 days observation), purified CNTs of the same product (F), laser-produced CNTs (B), nickel and yttrium containing electric-arc CNTs (C), with references CB (A, low toxicity) and quartz (fibrogenic). All CNT samples tested, regardless of the type and amount of metal impurities they contained, induce dose-dependent lesions characterized chiefly by interstitial granulomas in the lungs of mice in the 7-day (C, D) and 90-day group (E, F). Granulomas were not observed in rodents exposed to CB. The authors concluded that if CNTs reach the lungs, they are much more toxic than CB and can be more toxic than quartz. Figure reproduced with permission from Lam et al. [177].
these results for inhalation toxicity is not clear. Even though
intratracheal instillation and modifications of this technique are
common routes of administration used to assess the toxicity of
dust in lungs, the authors concluded that inhalation toxicity
studies are imperative. Only inhalation can demonstrate whether
CNTs can reach the lung to produce those lung lesions that were
observed in the intratracheal instillation studies.

We published in 2009 the first subchronic inhalation study
(OECD TG 413) with MWCNTs. Wistar rats were head–nose
exposed for 6 h day−1, 5 days week−1, 13 weeks, total 65
exposures, to MWCNT concentrations of 0, 0.1, 0.5 or
2.5 mg m−3. Highly respirable dust aerosols were produced with
a proprietary brush generator which neither damaged the tube
structure nor increased ROS on the surface. Inhalation exposure
to MWCNTs produced no systemic toxicity. However, increased
lung weights, pronounced multifocal granulomatous inflamma-
tion, diffuse histiocytic and neutrophilic inflammation, and
intra-alveolar lipoproteinosis were observed in lung and
lung-associated lymph nodes at 0.5 and 2.5 mg m−3. These
effects were accompanied by slight blood neutrophilia at
2.5 mg m−3. Incidence and severity of the effects were concen-
tration-related. At 0.1 mg m−3, there was still minimal granulo-
matous inflammation in the lung and in lung-associated lymph
nodes.

After a single acute inhalation exposure of 30 mg m−3 for 6 h,
MWCNTs reached the subpleura in mice. CNTs were
embedded in the subpleural wall and within subpleural
macrophages. Mononuclear cell aggregates on the pleural surface
increased in number and size after 1 day and nanotube-
containing macrophages were observed within
these foci. Subpleural fibrosis unique to this
form of CNTs increased after 2 and 6 weeks
following inhalation. None of these effects was
seen in mice that inhaled CB nanoparticles or a
lower dose of CNTs (1 mg m−3). The local
and systemic responses in C57BL/6 mice after
exposure via pharyngeal aspiration to CNTs
(single- and multi-wall) can be evaluated with
combination of blood gene expression and
circulating soluble protein analysis to identify
novel biomarkers.

In summary, the available evidence sup-
ports that there is a threshold level for effects
by inhalation of CNTs fibers, but the physiolo-
gical effects are qualitatively different and the
threshold is significantly lower than for
inhalation of metal-oxide particles. Material-
specific differences between different metal
oxides are addressed in Section 5.2.

5.1.3. Other Nanomaterials

Ji et al. exposed rats via inhalation to silver
nanoparticles for 6 h day−1, 5 days week−1, for
a total of 4 weeks. The atmosphere was
generated by a device that generates silver
nanoparticles by evaporation/condensation
using a small ceramic heater. As such, the
generator was able to distribute the desired
concentrations of silver nanoparticles to
chambers containing experimental animals. The male and
female rats did not show any significant changes in body weight
relative to the concentration of silver nanoparticles during the
28-day experiment. Additionally, there were no significant
changes in the hematology and blood biochemical values in
either the male or the female rats. Therefore, the authors
concluded that the current silver dust limit (100 µg m−3) fixed by
the American Conference of Governmental Industrial Hygienists
(ACGIH) did not display any significant adverse health effects. At
the highest dose, increased silver concentrations were measured in
the lung, liver, brain, and olfactory bulbus. The same group
performed a 90-day inhalation study. Although no statistically
significant differences were found in the lavage
parameters, histopathological examinations indicated increases
in lesions related to silver nanoparticle exposure, such as infiltrate
mixed cell and chronic alveolar inflammation, including
thickened alveolar walls and small granulomatous lesions.
Since some of these effects were also observed in the control
animals the outcome of this study is inconclusive.

Different surface functionalizations on the same metal oxide
nanoparticle—a standard procedure in nanocomposite produc-
tion for compatibilization of nanomaterials with an organic
matrix—have received less attention. Initial results by Warheit
et al. indicate that the inhalation hazard from TiO2 particles
does depend on their surface chemistry, but the TiO2 core + SiO2
or Al2O3 shell structures studied there are not representative for
the typical inorganic core with polymer functionalization. Much
more on the effects of polymer functionalization could be learnt
from the biotech community. Most pharmaceutical work is
necessary in this area.

Figure 9. Nanomedicine controls biodistribution by surface functionalization. Quantum dots
were functionalized with a systematically increasing chain length of PEG and injected intrave-
nously in mice, then quantified by gamma labeling. This has no relation to the exposure by
inhalation, but it exemplifies the importance of surface chemistry for biokinetics. Radioscinti-
graphic gamma images of intact animals 4 h post-injection. a) A 4 unit PEG chain directs the
dispersion of quantum dots in the lungs. b) The nanoparticles with 14 PEG units were instead excreted through the liver to the intestines
(Figure redrawn with permission from ref. [47]).
Table 3. Toxicological findings after inhalation of aerosols from nanomaterials.

<table>
<thead>
<tr>
<th>Material</th>
<th>Aerosol concentration (mg m⁻³)</th>
<th>No or low effect concentration (NOAEC/LOAEC)</th>
<th>Clin. Path.</th>
<th>Pathology</th>
<th>Reversibility</th>
<th>Translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂ B</td>
<td>2, 10, 50</td>
<td>LOAEC: 2 mg m⁻³</td>
<td>Inflammation</td>
<td>Histocytosis</td>
<td>Not complete</td>
<td>No indication</td>
</tr>
<tr>
<td>CeO</td>
<td>0.5, 2.5, 10</td>
<td>NOAEC: 10 mg m⁻³</td>
<td>No effects</td>
<td>n.d.</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>SiO₂</td>
<td>0.5, 2.5, 10</td>
<td>NOAEC: 10 mg m⁻³</td>
<td>No effects</td>
<td>No effects</td>
<td>–</td>
<td>Yes (spleen)</td>
</tr>
<tr>
<td>SiO₂ functionalized</td>
<td>0.5, 2.5, 10</td>
<td>NOAEC: 10 mg m⁻³</td>
<td>No effects</td>
<td>No effects</td>
<td>–</td>
<td>Yes (spleen)</td>
</tr>
<tr>
<td>ZnO</td>
<td>0.5, 2.5, 10</td>
<td>LOAEC: 0.5 mg m⁻³</td>
<td>Early inflammation</td>
<td>Lung: inflammation/</td>
<td>Yes</td>
<td>Yes (ions from dissolution)</td>
</tr>
<tr>
<td>CB</td>
<td>0.1, 0.5, 2.5</td>
<td>NOAEC: 0.1 mg m⁻³</td>
<td>Inflammation</td>
<td>Inflammation</td>
<td>No</td>
<td>No indication</td>
</tr>
</tbody>
</table>

5.2. New Results Generated by a Short-Term Inhalation Study for Nanomaterials

Previous studies suggest that short-term animal exposures to synthetic amorphous silicas and crystalline silica can provide toxicity data comparable to those of 90-day studies. In the frame of in-house and collaborative projects, we developed a design for a short-term inhalation test in rats with sufficient power and robustness to allow the prediction of potential adverse effects as accurately as long-term exposure tests do and provide a benchmark for the development of appropriate in vitro test systems.

5.2.1. Aerosol Generation

The results of the atmospheric concentrations and the particle size analysis are presented in Table S5 (for detailed method description see Section 7.4). Overall, the concentrations for all test substances were maintained throughout the study. According to the result of the cascade impactor measurements (Supporting Information Fig. S2), respirable aerosols were produced for all tested materials. For MWCNTs, the measured particle size by optical particle counter (OPC) and SMPS was not reported because the physicochemical properties (e.g., black, wool-robe like, conductive) interfere with the measurement principle of these devices.

5.2.2. Organ Distribution and Biological Effects

The observed biological effects are summarized in Table 3, and the organ distribution is listed in the Supporting Information Table S4.

TiO₂ B 50 mg m⁻³ nano-TiO₂ B resulted in an overload of the lung and caused an increase in lung weight. Light microscopic examination of the respiratory tract revealed effects (e.g., histiocytosis, Fig. 10b), which—at a low grade—were considered a normal and reversible response of macrophages to remove particulate matter from the lung. One of five animals in the 50 mg m⁻³ concentration group showed a minimal multifocal infiltration with neutrophils. The bronchioli and bronchi of the animals exposed to 50 mg m⁻³ nano-TiO₂ B displayed a minimal to mild increase of epithelium thickness which was interpreted as hypertrophy and/or hyperplasia. Nano-TiO₂ B induced concentration-related inflammation reaction in the lung. Lung inflammation was associated with concentration-dependent increases in BALF total cell and neutrophil counts, total protein content, enzyme activities, and levels of a number of cell mediators (Fig. 10c). The effect was minimal, but significant at 2 mg m⁻³ nano-TiO₂ B, thus giving a low observed adverse effect concentration (LOAEC) indicating that the NOAEC is close to this concentration. We found no indications of systemic effects by measurement of appropriate clinical pathology parameters. All effects in the 2 and 10 mg m⁻³ concentration group were reversible and partly reversible in the 50 mg m⁻³ concentration group within the 16 day recovery period (Fig. 11).

The toxicity of TiO₂ after subchronic (90-day) inhalation exposure has previously been studied. The results of our short-term study and the 90-day study are overall comparable. The LOAEC in the 90-day study was an atmospheric concentration of 2 mg m⁻³ determined by cell proliferation rate after the exposure. The same LOAEC was achieved in the current study determined by examination of the lavage fluid.

In comparison to the studies with nano-TiO₂, 5-day inhalation exposure to 274 mg m⁻³ pigmented TiO₂ led to a more than 30% increase of the lung weight. Again, diffuse histiocytosis was noted, but without granulocytic infiltration. In three out of six...
animals, the mediastinal lymph nodes were activated, and pigment-loaded macrophages were found in four out of six animals. Single animals displayed very few particles on the surface or intracellularly in the olfactory epithelium of the nasal cavity. After the recovery period, the numbers of infiltrating histiocytes (only focal infiltrates present) as well as particle numbers decreased, which was reflected in a no longer significantly increased lung weight. The mediastinal lymph nodes of five out of six animals showed activation, and in the lymph nodes of all animals pigment-loaded macrophages were observed.

ZrO$_2$ did not show any effects in the lung or in other organs up to the highest concentration tested (10 mg m$^{-3}$, NOAEC). No indications of systemic effects could be found by measurement of appropriate clinical pathology parameters and there was also no indication of a translocation of the inhaled material from the lung into other organs.

CeO$_2$, like TiO$_2$, induced a concentration-related inflammation reaction in the lung, which was associated with dose-dependent increases in BALF total cell and neutrophil counts, total protein content, enzyme activities, and levels of a number of cell mediators (Supporting Information Fig. S4). The effect was still observed at the lowest tested concentration of 0.5 mg m$^{-3}$ (LOAEC). No indications of systemic effects could be found by measurement of appropriate clinical pathology parameters. The observed effects were only partly reversible within the 16-day recovery period.

SiO$_2$ and functionalized SiO$_2$—both SiO$_2$ materials did not show any effects in the lung up to the highest tested concentration of 10 mg m$^{-3}$ (NOAEC). For the non-functionalized SiO$_2$, no indications of systemic effects could be found and there was also no indication of a translocation of the inhaled material from the lung into other organs. The functionalized SiO$_2$ material was, however, detected in the spleen (Fig. 12), indicating some translocation of the material from the lung to this organ. Additionally, the spleen was significantly enlarged without any other pathological findings. The enlargement was greater than the mass of the SiO$_2$ material deposited in the spleen and probably represented some reaction of this organ to the SiO$_2$ material. There were, however, no other findings in the spleen and no findings in other organs than the spleen. While crystalline quartz particles exhibited a strong toxicity, the toxicity of non-cristalline SiO$_2$ particles seemed to depend on their production process suggested by a 5-day inhalation study on three synthetic amorphous SiO$_2$ nanoparticles. These three substances were produced either by precipitation, sol–gel, or by pyrogenic processes. The target concentrations were 1, 5, and 25 mg m$^{-3}$. In the lavage, adverse effects (increased neutrophiles, increased activities of LDH, NAG, increased protein concentrations) were detected in all test groups at 25 mg m$^{-3}$. The NOAEC for the two substances produced by wet processes was 5 mg m$^{-3}$. The test substance Car-O-Sil M5,
which is produced by pyrogenic process, seems to have a higher inflammatory potential, with a NOAEC of 1 mg m\(^{-3}\). The toxicological findings were reversible for all three substances.\(^{189,190}\)

ZnO induced a concentration-related inflammation reaction in the lung, which was associated with dose-dependent increases in BALF (Supporting Information Fig. S5). In addition to the inflammation reaction, necrosis was detected in the lung and the nose. As ZnO is soluble and zinc ions are cytotoxic at higher concentrations,\(^{189,191}\) the necrosis can be attributed to the zinc ions dissoluted from the ZnO particles. Likewise, elevated zinc levels were detected in various organs, most likely due to zinc ions dissolved from the ZnO particles. There was, however, no indication of systemic effects in other organs than the lung and the airways.

CB did not show any treatment-related adverse effects at any of the concentrations tested. Thus, the NOAEC for these materials was the highest concentration test of 10 mg m\(^{-3}\). One subchronic inhalation study with a similar test material was previously reported.\(^{171}\) In this study, the only tested concentration of 50 mg m\(^{-3}\) was a clear effect level, which was much higher than the concentrations tested in the current study. Thus, no statement concerning the predictability of the short-term study can be made for CB at this time point.

MWCNT exposed animals showed no clinical signs different from normal. The mean body weight and the mean body weight change were not significantly different from the control groups. MWCNTs at the tested high concentration of 2.5 mg m\(^{-3}\) caused significantly increased absolute (+11.5%, p < 0.01) and relative (+11.4%, p < 0.01) lung weights. Increased relative lung weight (+10.5%, p < 0.05) was still determined at the intermediate concentration of 0.5 mg m\(^{-3}\). The increases were observed only directly after the exposure but not after the 3-week recovery period.

Examination of BALF (Fig. 13c) showed moderate increases of the polymorphonuclear neutrophils, total protein, and some enzymes in the 0.5 mg m\(^{-3}\) and in the 2.5 mg m\(^{-3}\) concentration group. Furthermore, there was a slight but not statistically significant increase of polymorphonuclear neutrophiles (PMN) even at the lowest concentration of 0.1 mg m\(^{-3}\). The total cell count in BALF was significantly increased in all concentration groups. These effects were still present, though less pronounced, after a 3-week recovery period.

During necropsy, all treated animals sacrificed directly after the last treatment showed black fibrous particles within the alveolar macrophages (Fig. 13a). In the 0.5 and 2.5 mg m\(^{-3}\) concentration group, the number of alveolar macrophages was slightly increased, and the animals of the 2.5 mg m\(^{-3}\) concentration group showed a diffuse infiltration compared to the other groups indicating a multifocal distribution pattern. One animal of the 2.5 mg m\(^{-3}\) dose group revealed a minimal granulomatous inflammation, containing the fibrous particles within the inflammatory lesion which were interpreted as MWCNTs.

Besides the above mentioned findings, five out of six animals of the 2.5 mg m\(^{-3}\) dose group and one animal of the 0.5 mg m\(^{-3}\) dose group revealed a minimal granulomatous inflammation after the 21-day recovery period. Particles were again observed inside the lesion (Fig. 13b). Based on the changes in the BALF, the NOAEC was 0.1 mg m\(^{-3}\) for MWCNTs. The effect was not reversible within the recovery period.

Our results indicate a high inflammatory potency of MWCNTs. The effects were not reversible but progressive. At 0.1 mg m\(^{-3}\), slight effects were still present. Furthermore, the results were in good correlation with a 90-day inhalation study of MWCNTs performed in our laboratories.\(^{181}\) We have submitted our results to the US EPA under TSCA 8e.\(^{193}\) Apart from our 5- and 90-day inhalation studies and the 1-day inhalation study by Ryman-Rasmussen et al.,\(^{180}\) studies on lung toxicity of MWCNTs were only performed by intra-tracheal instillation. There were strong positive correlations between our findings after 5-day inhalation exposure with 3 weeks post-exposure and those intra-tracheal instillation studies after 90 days post-exposure observation.

5.2.3. Correlations Between the Inhalation Results

The aerosol generation from nanomaterials and the short-term inhalation study for nanomaterials have been established in previous studies\(^{76,93}\) and the resulting test system was used to investigate the inhalation toxicity of eight different nanomaterials in the present study. Selected test concentrations were in the range of internationally established workplace limit values for so-called inert or nuisance dust. For each nanomaterial, a LOAEC or NOAEC was defined along with the characterization of the toxic effects in the lung and translocation and effects in other organs (Tables 3 and S4). These results provide essential information for the safe production and use of these nanomaterials, allowing to define safe exposure levels during production and handling, thus ensuring safe production and use. This may include abandoning of certain applications or trigger the selection of less toxic
nanomaterials—and in fact both has happened based on the results published here.

The results from eight inhalation studies with different nanomaterials presented here represent the largest dataset on nanomaterial inhalation toxicity published so far. Although still limited, this data set allows for some preliminary correlations. 

TiO₂, CeO₂, ZrO₂, and SiO₂ are insoluble nanomaterials of similar particle size and shape, yet the toxic concentrations varies between NOAEC <0.5 to >10 mg m⁻³. Obviously, the chemical composition influenced the toxicity and not-or not only-the size or shape of the material.

CB and MWCNTs are both carbon-based nanomaterials, yet very different in shape and structure. The toxicity in the short-term inhalation study with rats differs by a factor of 100 with a NOAEC of 0.1 mg m⁻³ for MWCNTs and 10 mg m⁻³ for CB.

ZnO and TiO₂ particles were tested as nanometer-sized and as fine-sized particles. Most of the nanomaterial agglomerated to particles similar in size to the micrometer-sized material, resulting in similar particle sizes for both materials. Yet, the agglomerates of TiO₂ and ZnO nanometer-sized particles exhibited stronger effects than the solid fine-sized particles of TiO₂ and ZnO, respectively. Studies with TiO₂ did not reveal any deagglomeration in the body and hence the differences in toxicity between solid particles and agglomerates are most likely due to the fact that the agglomerates are built from nanoscaled primary particles with a different inner structure and higher specific surface area of the agglomerates compared to the solid particles. 

CeO₂ and ZnO generated aerosols of similar characteristics. Yet, ZnO is soluble and releases zinc ions after deposition in the body, whereas CeO₂ is unsoluble. Both nanomaterials had the same LOAEC in the short-term inhalation studies, yet displayed different effects. The additional necrosis found with ZnO may be attributed mainly to the toxicity of released zinc ions.

Non-functionalized and functionalized SiO₂ are very similar in shape and size with the functionalized material having been derived from the non-functionalized material. While both nanomaterials caused no lung effects in the short-term inhalation study, the functionalized material, unlike the non-functionalized one, was translocated to the spleen. The surface modification led to striking differences in biological effects compared to the non-functionalized SiO₂ nanomaterial.

6. Correlation of In Vitro and In Vivo Data

The suitability of in vitro test systems to predict potential adverse health effects of nanomaterials is still a matter of discussion. To date, only a few comparative studies have been performed using the same nanomaterials for toxicity assessment both in vitro and in vivo and some of these studies provided contradictory results. For example, Sayes et al. found little correlation between in vitro and in vivo pulmonary toxicity of different fine- and nanometer-sized particles (SiO₂, ZnO). On the contrary, Donaldson et al. reported a concordance between the in vivo and in vitro dosimetry of different low-toxicity, low-solubility particles (TiO₂, BaSO₄) based on the surface area dose and discussed in vitro studies as a valuable complement to animal studies. More recently, Park and Park observed oxidative stress and proinflammatory responses induced by amorphous SiO₂ nanoparticles both in mice and in mouse macrophage cell lines.

Here, we have collected and analyzed in vivo and in vitro screening data from the literature as well as from our studies on the same well-characterized and categorized nanomaterials. When comparing these data, several correlations of potential adverse effects induced by nanomaterials in vitro and in vivo can be found. For instance, the ROS generating capacity of nanomaterials in vitro seems to correlate with their potential to induce inflammation in vivo (this study). Furthermore, the relationship between crystal structure of TiO₂ nanoparticles and its biological effects have been demonstrated in several in
8. Materials and Methods

8.1. Test Materials for Inhalation

The materials tested were TiO$_2$ A and TiO$_2$ B, ZrO$_2$, CeO$_2$, ZnO, CB, and a type of MWCNT, as well as two amorphous SiO$_2$; the surface of one SiO$_2$ was functionalized with polymeric carbonate while the other SiO$_2$ was not modified. TiO$_2$ A is precipitated in a wet chemical process (sol–gel), and TiO$_2$ B is formed in flame pyrolysis of titane salts. The physicochemical properties of eight test materials are presented in the Supporting Information Table S1, comprising their specific surface area, impurities, surface chemistry, solubility, crystallinity, state of agglomeration in DMEM +10% FBS (Fig. 3), and their monolayer TEM images (Supporting Information Fig. S1).

The surface-functionalized SiO$_2$ was produced from the above-listed SiO$_2$ by covalent surface modification with an acrylate carboxypolymer. The solid fraction of the product suspension was approximately 40%, particle size and BET surface area of the functionalized material were not analyzed, but are expected to be similar to the starting material. Some of the nanoparticles were obtained by partners of the German research project NanoCare$^{[2]}$ (see also www.nanopartikel.info and characterized as previously described.$^{[51]}$ Before entering a cell culture assay, all nanoparticles were analyzed for Endotoxin contamination with the Limulus Amebocyte Lysate (LAL) Kinetic-QCL kit (Lonza, 50-650U).

8.2. Inhalation Study Design

Groups of 14 animals were head–nose exposed to dust aerosols for 6 h a day for five consecutive days. The respiratory tract was evaluated by light microscopy in groups of six animals either
immediately after the last exposure or 3 weeks thereafter (study days 5 and 26), as well as the content of the content of the test material in the lung and in the mediastinal lymph nodes. Bronchoalveolar lavage was performed in satellite animals (five animals per group and time point) 3 days after the exposure and 3 weeks thereafter (study days 8 and 29). Several biochemical, cytological parameters as well as a large panel of cytokines/chemokines were measured in the in BALF. The study design is summarized in Scheme 4. Details of the parameters examined are described in the online Supporting Information.

The inhalation studies with nanomaterials were approved by the competent German authority (Landesuntersuchungsanstalt Rheinland-Pfalz, http://www.lua.rlp.de), the permission was issued under no AZ 177-07/053-1. The testing facility at BASF was certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in 2007 (http://www.aaalac.org/accreditedorgs/) and is in accordance with the German Animal Welfare Act and the European Council Directive 86/609/EEC.

8.3. For the Dispersion of Nanoparticles in Cell Culture Medium

Nanoparticles (19.2 mg) were transferred into 10 mL sterile snap-lid glasses together with a magnetic stir bar (Scheme 3). The glasses were then exposed to 30 Gy in a Biobeam 8000 gamma irradiation device (Gamma-Service Medical GmbH) for sterilization. Following the addition of 6 mL of DMEM/10% FBS gold, dispersions were stirred at 900 rpm for 1 h at room temperature. Dilutions of this stock dispersion were prepared immediately and stirred for 24 h at 900 rpm at room temperature. The mode of dispersion chosen for the presented study was established as Standard operation procedure in the frame of the German BMBF funded project NanoCare and was aimed at modeling a real exposure rather than producing a high fraction of single dispersed nanoparticles.

8.4. Agglomeration Control by Analytical Ultracentrifugation (AUC)

The particle size distribution was determined by AUC of ~500 μL of the above (Section 8.3) described dispersion with 0.1 mg mL⁻¹ nanomaterial in DMEM/10% FBS gold. Simultaneous detection by synchronized interference optics quantified the amount and the diameter of each fraction independently. The evaluation of the AUC raw data incorporated the fractal morphology of nanoparticle aggregates and applied the fractional dimension of 2.1 together with the sedimentation relation as specified in Equation 6 of ref. [102]. This value of the fractional dimension has been shown to be universal for all reaction-limited colloid aggregates. The tabulated materials constant of refractive index allows the interference optics to linearly quantify the fraction that is dispersed to diameters below 100 nm in the actual test preparation, as given in the Supporting Information Table S1, with the full size distributions shown in Figure 3d. The value for the nanodispersed fraction is regarded as an upper limit, judging from the comparison of methods in Section 3.3 and Figure 3b. Additional thorough documentation of the inhalation studies is available as online Supporting Information and includes: animals for inhalation; atmosphere generation and monitoring; biological examinations of inhalation results; histopathology; bronchoalveolar lavage; cytokines and chemokines in BALF and lavaged lung tissue; hematology and acute phase proteins in serum; statistical analysis of inhalation results.

Additional documentation of the cytotoxicity studies comprises: chemicals and cell culture components, cell lines, determination of ROS, metabolic activity, and cell death.

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