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Nanotoxicology

Nanosafety Research—Are We on the Right Track?

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The number of studies that have been published on the topic of nanosafety speaks for itself. We have seen an almost exponential rise over the past 15 years or so in the number of articles on nanotoxicology. Although only a couple of hundred papers had appeared on the topic of "Nanomaterials: environmental and health effects" before 2000, this number has exploded to over 10000 since 2001. Most of these studies, however, do not offer any kind of clear statement on the safety of nanomaterials. On the contrary, most of them are either self-contradictory or arrive at completely erroneous conclusions. Three years ago in this Journal we underscored the deficiencies in the way these studies were designed and pointed out the sources of error in the methods used. Now, on the basis of a comprehensive review of the literature and with the help of selected toxicological end points, we attempt to indicate where the significant weaknesses of these studies lie and what we must improve in the future.

From the Contents

1. Summary

The objective of this study was to screen the literature of the last 10–15 years to establish whether an evaluation is possible for human toxicological end points of engineered nanomaterials (ENMs). More than 10000 publications have been inspected since 2000 for aspects of human health effects or biological end points in animals or cell cultures.

This Review discusses the results obtained for four core topics for which the evaluation has been finished. These are the three uptake pathways in vivo—the lung, the gastrointestinal tract, and the skin—and finally a comparison of inhalation and instillation studies for their significance to evaluate lung exposure.

The main conclusions after the examination of more than a thousand published studies on these topics are:

- It is an undeniable fact that ENMs can pass the lung and the gastrointestinal tract. However, only a very small fraction of the applied dose reaches the bloodstream and is distributed in the body to secondary target organs. The vast majority of the applied ENMs are cleared from the lung by macrophages and/or are excreted through the faeces. Systemic effects have been observed in only a small number of studies, but these results have not been found to be related to a specific "nano effect" of the ENMs because of flaws within the study design or uncertainties in the conclusions.
- Comparison of instillation and inhalation experiments: instillation studies have to be carried out with relatively high local doses and, thus, more often meet overload conditions than inhalation studies. Transient inflammatory effects have been observed frequently in both types of lung exposure, irrespective of the type of ENMs used for the experiment. This finding suggests an unspecific particle effect; moreover, the biological response seems to be comparable to a scenario involving exposure to fine dust. Prominent exceptions are long and rigid carbon nanotube (CNT) bundles, which induce a severe tissue reaction (chronic inflammation) that may ultimately result in tumor formation. Overall, the evaluated studies showed no indication of a "nanospecific" effect in the lung.
- It is frequently disregarded that specific ENMs can dissolve slowly (e.g. Ag) or relatively fast (e.g. CuO, ZnO) in body fluids. This implies a complete new situation with no "nanotoxicity", but a more general element-related toxicity, which is described in the textbooks.
- The presented literature study could give important hints for eminent biological pathways which are affected by ENMs; however, the "Babylonian diversity" in the applied methods allows no comparability between the studies, but explains the often contradictory results of several publications.
- The majority of studies did not consider the necessity to characterize the material properties of the ENMs used for the experiments. This considerably reduces the significance of these studies, in some cases to a total meaninglessness of the presented results. Without an interna-

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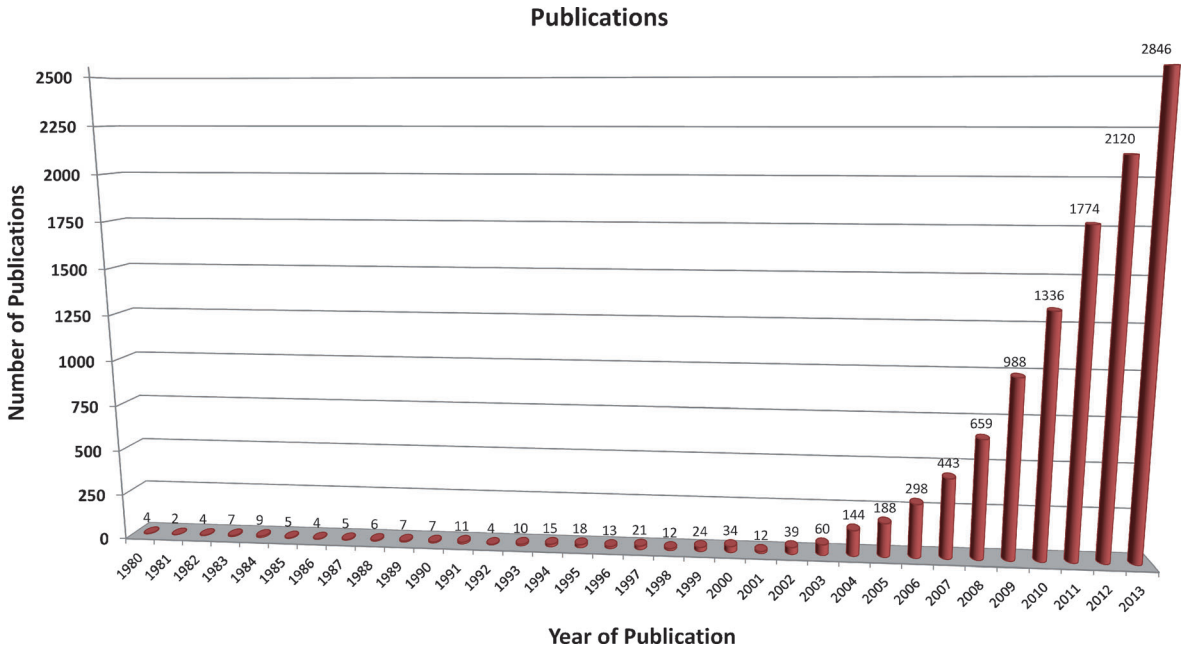
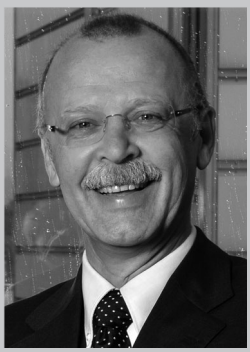


Figure 1. The number of published papers on nanotoxicology from 1980 to 2013.

tional agreement on the need for the characterization of the physicochemical properties to be included within all studies on health hazards, it can be assumed that the significance of these studies will not increase substantially in the future. The use of financial and material means will then be questionable.

One of the most important outcomes of this literature study is the fact that most of the studies are not toxicological, but mechanistic studies, even though the authors discuss "toxicologically relevant" results! This aspect is very puzzling and confusing to the reader of such publications, because this is generally not pointed out. Moreover, the discussions on toxicological end points are very often misleading, as the experiments have been carried out with high concentrations or in a too high a dose range and so only provide mechanistic insights but are not useful for a toxicological assessment of the hazard.

On the basis of the presented results, clear recommendations can be derived for the sponsors of funding programs, for regulators, and also for project leaders in the field of nanotoxicology.



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2. Introduction

When we made our last contribution on the topic of nanotoxicology to *Angewandte Chemie* in January 2011,[1] some 5000 papers[2] had been published on this theme up until the end of 2010. Since then, the total number of studies published on this subject has more than doubled! This means that more contributions have appeared in the past three years than in the previous thirty (Figure 1)! In principle, the availability of this quantity of data should mean that we are in the fairly comfortable situation where we are able to infer possible negative effects of engineered nanomaterials[*] (ENMs) on the environment and human health. After all, does not the increasing number of contributions published in international journals imply a significant enhancement in the general level of knowledge on nanotoxicology, or are we all simply barking up the wrong tree?

The result of a review of the current literature on nanomaterials by Hristozov et al. in 2012, which assessed the studies on the basis of a defined set of criteria,[3] was rather disappointing. They showed that for the given criteria and the six materials selected, only carbon nanotubes (CNTs) and fullerenes demonstrated good results with respect to the characterization of the materials and toxicological information as the basis for risk assessment. For the two nanomaterials on which the most work has been done, the results are also rather disappointing: for titanium dioxide, only 32% of the 302 publications met the conditions for evaluation,

[*] The term "nanomaterial" will be used in this Review to cover the entirety of so-called "nano objects" (for a definition, see Ref. [1]). In all the studies, it is, therefore, the dimension of the primary particle which determines the size and not that of the agglomerate or aggregate. For comparison, see also the EU definition given in the third footnote of this Review.

whilst for zinc oxide, this was a mere 13% of the 279 contributions, when considering the toxicological data. This is, of course, dependent on the criteria applied, which were determined by the Hristozov working group. The project consortium of the DaNa project ("Data and facts on Nanomaterials—processing scientific facts relevant to our society") had a very similar experience. To create their online text they used only original literature which met a given set of criteria (published on the project website www.nanoobjects.info/en/nanoinfo/methods/991-literature-criteria-checklist. This insistence on a publication fulfilling a minimal list of conditions before it could be considered for analysis resulted in up to 90% of the papers reviewed being rejected, depending on the material and subject, because they did not supply sufficient information concerning, for example, the characterization of the materials under investigation. However, this is exactly what has been demanded by large numbers of researchers in the field for a long time,[4–11] and is in principle an essential element of a good publication. It has become clear that through insufficient characterization (applied "*as purchased*" and properties "*as represented by the supplier*") various studies have been subject to significant errors such as when, for example, the particle size distribution as stated by the manufacturer was incorrect. In addition, only a few working groups indicate whether they have tested for contamination of the material under study, since most samples are not manufactured and packed under sterile conditions. This is a particularly important factor when dealing with ENMs. It was demonstrated that authors investigating inflammatory processes who used the release of inflammatory mediators as a measurement end point published false-positive results because their samples were contaminated with endotoxins which produce exactly the same reaction during testing.[5,12–17]

Further weaknesses and sources of error are due to the solvents and dispersion agents employed during testing, their concentration and dosage, the fact that interference effects with the test system are not taken into account, and the complete lack of references for comparison, since no control tests were run simultaneously. My Empa colleagues and I have been able to show, for example, that fullerenes dispersed in tetrahydrofuran do not produce oxidative stress in aquatic organisms and that this effect is, in fact, due to peroxides created by ageing processes in the tetrahydrofuran.[18] Further pitfalls involved in the dispersion of nanopowders have also been uncovered during the NanoCare project.[19]

Much of the erroneous interpretation of data found in the literature can be traced back to the fact that unsuitable concentrations are frequently used during experiments. Wittmaack has been able to show[20] that even a minor overdose of nanomaterial during in vitro experiments in petri dishes leads to a complete coverage of cells by agglomerated nanoparticles, thus making the supply of nutrients and oxygen from the medium difficult and possibly leading to the death of the cells. Another research group in Lausanne used an almost 10 times higher concentration of nanoparticles to investigate the effect on the possible induction of the inflammasome.[21] Knowing that the cells in this experiment were "buried" under a 500 nm thick layer of agglomerated nanoparticles throws the interpretation of the results into a completely different light. An overdose of nanomaterials can also lead to erroneous interpretations of the result in animal experiments, such as when the airways are blocked during the instillation of a bolus into the lungs, thereby leading to death of the test animal by suffocation, as has already been reported.[23,24]

The next point concerns the cross-reactions (interferences) of nanoparticles with one or more analytes in the test system under consideration. As a consequence of their extreme reactivity (because of their enormous surface-to-volume ratio), ENMs can, for example, strongly bind other reactants to their surfaces, thereby removing them permanently from the test environment. Alternatively, when an ENM possesses particular optical properties, these may directly affect optical measurements during testing. We were able to demonstrate this some time ago,[24] and these effects have since been confirmed by other research groups.[5,25–30]

A lack of reference or control samples makes the correct interpretation of an experimentally observed effect almost impossible, a situation which the toxicologist may or may not recognise but the layperson or scientific nonspecialist interested in the toxicological field probably does not. In the medicinal field, it is routinely accepted that an experimental value is compared to reference data, and only after this process has been completed is any consequential effect on existing damage or the state of health of the patient taken as proven. This eminently sensible method seems to have been completely ignored in the field of nanotoxicology. Without citing any specific references in this regard, this factor is certainly one of most frequent sources of error in the interpretation of observed effects. Often the mere fact that an effect has been seen in a treated sample and not in the untreated material is taken as an indication of a toxicologically relevant phenomenon. However, marginal effects are very frequently "sold" as having a significant effect on cells without knowing how strong the effect really is, and whether it has biological consequences for the cells or tissue in question. All the examples cited above should make it absolutely clear why a large number of publications, although frequently carrying the words "toxicological effect" in their title, do not actually prove this in the work they describe. This situation is unfortunately normally only recognizable to experts in the field, thus providing a wide-open window of opportunity to the less well-informed for speculation on the safety of ENMs.

Through the analysis of many thousands of publications, an attempt is now being made to ascertain if, despite the qualitative differences, common ground with respect to experimental data can be found on the uptake processes and possible effects of ENMs[*]. In addition, the knowledge gaps which still exist (even after the publication of reports on more than 10000 studies) should give rise to lively discussions and also help to clarify how, in the future, we can unify this

[*] Information about the database, the search profiles, the criteria, and hypotheses as well as some statistical data including all citations of the chosen and evaluated publications on the topics of this Review can be found in the Supporting Information.

Babylonian plethora of results and generate a common basis for their evaluation and interpretation.

3. Quality Criteria and Their Significance

For some years now, a discussion within the international community of (nano-)toxicologists has been in progress on how to categorize published studies and what significance to attribute to their results. As already mentioned in the introduction to this Review, there are demands that adequate characterization of the nanomaterials should always be given in publications, without which data no well-founded conclusions may be drawn from the results being presented, since not even the relationship to the particle size is defined. This, and other, deficiencies in current publications on the toxicology of ENMs have possibly led to a gradual loss of confidence in the process of ensuring the safety of chemical materials. The appropriate conventions are already in place for dealing with "conventional" chemicals, and they are internationally recognized and accepted.[31] Furthermore, it seems that today we do not respect results which have been known for over 40 years, and only think in terms of two categories: a material is either poisonous or it is safe! Henschler, in a contribution dating from 1973, made it clear that the same problems which were valid then face us now in connection with nanomaterials, and yet we behave as if these challenges are completely new![32] The question "How safe is safe?" can never be answered with finality in relation to toxicology. Paracelsus' theorem—the dose alone determines if a substance is poisonous—still holds, and we must learn to live with this uncertainty. It remains, however, the duty of toxicologists to determine the dosage range and conditions under which ENMs might represent a critical risk to the environment and human health. A satisfactory answer to these questions cannot be obtained with animal trials alone, something that Henschler inferred 40 years ago. In addition, he also reached the conclusion that "*In the interests of better comparability of the results, toxicological laboratories should be subjected to standardization and quality control, like in the areas of analytical and clinical chemistry. Such a policy would greatly reduce or even eliminate duplication of work*" and "*Minimum requirements should be stipulated for toxicity tests, although detailed rules as to procedures and evaluation entail some risk.*"[32] Unfortunately, even in the field of nanotoxicology today, we are a long way from implementing such standardization of test procedures or laboratory quality controls. Duplication is today, above all in the EU, the norm rather than the exception. This has led to the problems considered later in this Review in the discussion on the results of the studies.

It was originally intended in this Review to evaluate to what extent all the studies fulfilled the criteria regarding the quality of published data determined during the DaNa project and published on the Internet (see Section 2). These are very stringent quality criteria which aim to select only reliable studies for publication on the Internet.[33] However, it was not possible to apply the DaNa conditions for the simple reason that the sheer number of studies to be evaluated would have necessitated years of analysis. Consequently, only rather coarse quality-control criteria were applied, which focused on the minimum requirements for the characterization of the materials used in the studies under consideration. Studies which offered no information on the dimensional data of the materials investigated were, for example, not selected for further evaluation. However, it must be clearly stated that in the future, the unification of criteria and standardization of methods are absolutely necessary in order that we arrive at a situation where investigations may be effectively compared and provide reliable data. The call for a "minimum" physicochemical characterization of ENMs and the relationship to their biological effects has again recently been voiced by an international group.[34] Table 1 makes clear the range of shortcomings which can be found in currently published tests. From the large number of variables paired with the various research areas of the groups publishing the data, it is clear that the rules of toxicology, as have been demanded by toxicologists for many years, are not being adhered to in the majority of these studies. This makes comparisons between papers difficult, and consequently in this literature study hypotheses are also presented which are rather more relevant for research purposes than those which focus on topics of a purely toxicological nature.

This Review gives no real indication, therefore, of the quality of the studies analyzed—indeed this is not one of its objectives. Exceptions have, of course, been made, in which case a particular study is expressly described as being of "good quality". This assignment indicates that the individual study under consideration either meets the DaNa list of criteria or that the journal in which it is published has an Impact Factor exceeding 6.0. Despite this limitation, it is perfectly possible to find answers in the large amount of data presented in these studies to questions such as the potential degree of uptake of ENMs in cells or organisms. When the data are contradictory, a paper must be more carefully evaluated and analyzed to discover the cause of the contradiction and to ascertain if this is possibly a methodological effect or due to the use of a completely new material or different experimental conditions.

4. Analyzing the Studies: The Pros and Cons of Standardization

The assessment of several thousand publications in terms of their nanotoxicological relevance has shown that in principle we do indeed find the multiplicity of variables in the design of the studies listed in Table 1. As a result, useful comparisons between papers are possible only very infrequently. This situation could have been avoided if the majority of the studies evaluated had adhered to basic toxicological rules and made use of standard procedures for the various tests described. In the case of epidemiological studies, for example, clear specifications have been laid down governing how these are to be conducted. In addition to a representative group selected from the general population or working place, an internationally comparable study design should be used and the parameters being measured must be

Table 1: Variables in the in vitro toxicity tests during the investigation of ENMs (modified according to Ref. [35]).

Variables associated with the nanomaterial	Variables associated with the tox assay	Variables associated with the biological model
sample purification for the removal of biologically relevant trace elements	selection of the correct test system regarding the biological end points	selection of the biological system
sample characterization of the raw material: composition and purity size shape agglomeration status etc.	different test systems for the same biological end point	cell lines: selection criteria identification age and storage number of passages etc.
sample characterization regarding biological impurities: endotoxins etc.	controls: adapted negative controls adapted positive controls comparison to reference materials	primary cells/organ systems: donor dependency donor variability culture conditions
dispersion in biological media under relevant conditions: temperature humidity gas concentrations (O_2, CO_2) salinity etc.	testing of possible interferences of the ENM with the biological test system binding of indicator molecules light absorption or fluorescence of the materials etc.	culture conditions during the experiments: temperature humidity gas concentrations (O_2, CO_2) salinity etc.
sample characterization in biological media: size and shape agglomeration status protein corona etc.	measurement uncertainty not considered: round robins calibration with standards or reference material	biological parameter: cell density volume of the medium serum content of the medium compatibility of the solvent or dispersion medium

clearly defined (e.g. urine, blood, behavior etc). Furthermore, the analysis of the measured data should generally be performed following the principles of *Good Laboratory Practice* (GLP) and the participation of experienced scientists who are capable of interpreting experimental results in accordance with international standards is a must. None of these points seem to have been taken seriously in the field of nanotoxicology. The representativeness of the biological models used in the studies analyzed is not given serious consideration, and nor are the study designs (experimental set-ups) in any shape or form internationally comparable, since in most cases no SOPs (*Standard Operation Procedures*) are followed. The parameters—that is, the biological end points—are often freely defined and the analyses do not follow standardized procedures. Far more serious, however, is the fact that frequently no toxicological expertize amongst the authors of a study is to be found. Quite the contrary in fact—scientists of all persuasions seem to feel a compulsion to take a stand on a range of toxicological problems. This situation has, over recent years, led to criticism from various working groups, who have pointed out the weaknesses in many published studies and called for this state of affairs to be remedied (Table 2).

These examples, and the points expressed above, will have made clear the fact that whilst this literature study can provide a basic platform for specific statements and conclusions, these must be viewed with a great deal of caution, since the comparability of the evaluated studies on which they are based is far from ideal. Despite this, the assessment of the literature can and should provide the reader with an overview of such important questions as the uptake of nanomaterials in cells and organisms, or the mechanisms through which ENMs influence, or induce effects in, biological systems (i.e. the Mode of Action, MoA). In addition, the assessment of the results from specific individual methods or procedures can provide a valid impression as to whether their use in different laboratories leads to similar results. In the next section we will attempt to explain this in more detail with the help of two examples. On the one hand we will present the results pertaining to important questions such as whether nanomaterials or nanoparticles are always, and to the same degree, taken up by organisms, and on the other hand we compare two methods—inhalation and instillation—which are very frequently cited as "gold standards", although it is unclear whether both techniques are equally suited to the investigation of the characteristics of ENMs and whether they both deliver the same results.

5. Specific Consideration of Two End Points (In Vivo Uptake Pathways and Lung Exposure Methods)

Over the past few years, two critical questions of seemingly particular importance have distilled out of the assessments of ENMs and their biological effects. The first is to what extent are ENMs taken up by cells (in vitro) or organisms (in vivo), and additionally in the latter case how does this permeation of the relevant tissue barrier occur (the air–blood barrier in the lungs, the intestine–blood barrier in the gastrointestinal tract, and the skin). The second question deals with the assumption that the uptake path via the lungs is of critical importance, thus leading to a comparative evaluation of the experimental methods used in instillation- and inhalation-based studies. In this section these two questions

Table 2: Recommendations for the standardization of particular experimental procedures to improve the comparability of the results from nanotoxicological studies.

Ref.	Study parameter	Set of problems addressed
Crist et al., 2012[5]	ENM characterization	sterility and endotoxin physicochemical characterization impurities of the material samples biocompatibility of the components batch-to-batch consistency nanoparticle in vivo stability
Wittmaack, 2011[20]	ENM dosimetry	suspension concentrations too high; sedimentation induces dose distortion
Schulze et al., 2008[19]	ENM dispersion	dispersion protocol agglomerate formation: size distribution surface charge wettability of the ENM adsorption of medium ingredients (protein corona etc.) which induces a shift in the properties of the ENM sterility of the ENM endotoxin: measurement and assay reliability
Wörle-Knirsch et al., 2006[24]	interferences	dispersion and solvents binding of analytes to the ENM multiple methods for the same end point reference material for comparison
Geys et al., 2010[28]	biological matrix	cell density test assay serum (amount, origin, species) solvent (tween, polysorbate etc.)
Hirsch et al., 2011[36]	assay system	calibration with reference materials comparison of multiple methods round robins of different labs cause–effect analysis measurement uncertainty

will be given particular consideration and an attempt will be made to provide answers derived from the evaluated literature.

5.1. The Uptake of ENMs in the Body

ENMs have repeatedly been named as critical factors in connection with both workplace exposure and exposure during the usage of products containing them. Frequently, all materials in powder form are thrown into the same "nanopot", as typified by the example of titanium dioxide. In sun cream, it is certainly most effective when in nanoparticle form,[37] yet in toothpaste or paint it serves best as micro- or millimetre-sized particles! Furthermore, this substance is also permitted as a food additive (E171), where it is also used as a pigment, although generally not in the nanoform. Consequently, one could assume that, according to the current definition by the EU commission[*], in principle all materials in powder form are in fact nanomaterials, since the defined fraction of nano objects permitted out of the total number of particles makes it practically impossible to market a powder which does not fall under this definition. However, at this point the diametrically opposite conclusion would also be correct, namely that all previously manufactured powders would also fall under this definition based on the large number of nanosized particles they contain (even though their mass as a fraction of the total is very low). Consequently, all previous investigations carried out on these powders would also have covered the safety of nanomaterials. I would, therefore, stress at this juncture the fact that the current work only includes studies of materials falling under the ISO definition of nanoobjects,[1] that is synthetically manufactured nanoparticles, nanofibers, or nanoplatelets, which are always abbreviated herein to ENMs.

For a factual discussion of the possible toxicological significance of ENMs it is still important to know the transport pathways into the body and to validate a possible systemic availability. Of the large number of studies considered herein, 153 dealt with the lung as an absorption pathway, 204 considered the gastrointestinal tract, and 201 investigated the skin as a transport mechanism (Figure 2); thereof 151, 116, and 111 have been selected and finally 116, 67, and 74, respectively, have been judged as relevant. As far as the skin barrier is concerned, the situation seems to be fairly clear, with an absolute majority of the studies being of the opinion that no penetration of nanoparticles through the skin into deeper layers of living cells occurs. For the two other barriers mentioned above, however, matters are not so clear. There is strong evidence that ENMs can penetrate these barriers, although here also there are significant differences in the evaluation of these effects. Only a few studies of the lung pathway mechanism have shown that ENMs could enter the circulatory system this way and thereby reach secondary organs, but the proof they demonstrated of this effect was unambiguous.[38–43] In most cases, only a very small fraction of the dosed quantity was able to penetrate the lung barrier. Examples also exist which show that no such transport takes place for particular ENMs.[44] The vast majority of the studies, however, did not take this point into

[*] "Nanomaterial" means a natural, incidental, or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm (Official Journal of the European Union, L 275/38, 2011/696/EU).

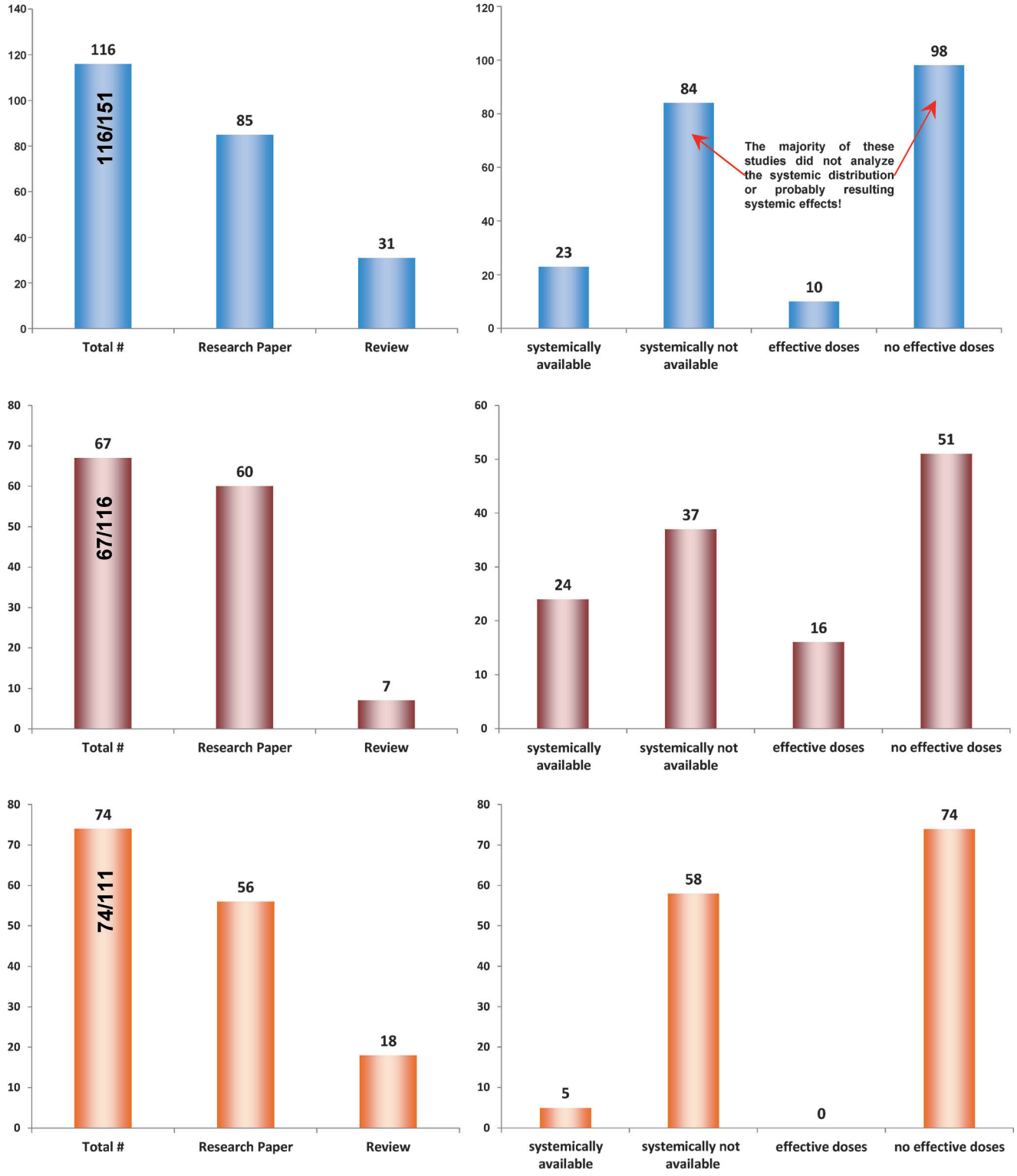

Figure 2. Analysis of in vivo studies on the uptake of ENMs by the lungs (top), the gastrointestinal tract (middle), and the skin (bottom). The columns in the left graphs show the total number of studies selected and analyzed, the number of original studies, and the number of review articles. In each case, the column showing the total number also indicates the number of studies in the category after selection and how many are relevant. The right graphs show the distribution of the relevant studies if a systemic distribution and a possible systemic effect dependent on this has been observed.

account at all, instead only observing and analyzing the direct effects on the lungs (Figure 2, top). As a result, while there is evidence that transport across the lung barrier does occur, this effect has only in exceptional cases been quantitatively evaluated (because of the enormous analytical problems involved) and determined to be relatively small. This by no means implies that the absorbed fraction is insignificant, since a possible lifetime accumulation in the secondary organs has

to date rarely be investigated and cannot be ruled out. Some 8% of the papers reviewed (9 out of 116) even described a systemic effect following the absorption of ENMs by the lungs. One of these was a review which will not be considered further herein. The original publications were, however, carefully evaluated in terms of the following particular points: which nanomaterials were involved, which methods were used, and what critical factors need to be considered in categorizing the results (Table 3)? With the exception of one study covering carbon nanotubes,[45] all the others involved either very high local concentrations achieved through instillation (or even concentrations at overload levels) or they did not consider (or simultaneously analyze) the solubility of the materials used (e.g. in the case of silver). As a result, these studies, which describe a systemic effect after uptake of nanoparticles through the lungs, with a single exception deliver no proven indication of the presence of a systemically effective dose of the ENM in question.

Table 3: Critical appraisal of studies describing an effective dose in the organism after exposure via the lung.

Ref.	ENM/application	Comments
Li et al., 2007[46]	CNT/instillation	repeated instillation, tissue damage not excluded! Transfer into the blood not investigated, systemic effects also discussed as possibly "indirect"!
Song et al., 2011[47]	SiO_2/epidemiological study	the authors use the terms amorphous and crystalline silica synonymously; epidemiological study with low significance; no confounder included.
Stapleton et al., 2012[45]	CNT/inhalation	systemic transfer shown, but only for 0.002% of the applied dose! However, endothelial damage observed.
Sung et al., 2009[48]	Ag/inhalation	no detection of dissolved Ag^+! Systemic effects only for the highest exposure dose.
Umezawa et al., 2011[49]	CB[a]/instillation	local overload![b] Tissue damage not excluded; biological consequences for the fetus possibly through induced inflammation.
Vesterdal et al., 2010[50]	CB/instillation	effects only in high dose range; locally very high concentrations.
Zhu et al., 2009[51]	Fe_2O_3/instillation	4 mg/rat is overload scenario, hence, no conclusion about systemic effects possible.
Zhang et al., 2012[52]	Cu/intranasal instillation	effects observed in mice only at 10 and 40 mg kg^{-1}! Both concentrations are overload scenarios. Effects have been attributed to ENMs *and* released ions.

[a] CB = carbon black. [b] For the explanation of overload, see Figure 5.

Considering the second uptake pathway—the gastrointestinal tract—here also, several particularities stand out (Figure 2, middle). There are 16 papers dealing with this topic, significantly more than with the lung pathway case, and they consider both a translocation within the body as well as systemic effects. The reason for this (among others) lies in the fact that relatively frequently materials were used which have a certain degree of solubility and whose effects may, therefore, be due to the release of ions (ZnO, Cu, Ag; 6 studies). In addition to this, the doses used in more than half the studies were very high (1–5 g kg^{-1} body weight of the test animal). A very recently published review, which only considered a few studies on the gastrointestinal tract (of which only a small fraction described the absorption of an ENM through the intestinal epithelium) has come to a similar conclusion.[53] However, even in this overview, the enormous dosage of 5 g kg^{-1} body weight of TiO_2 which was used in one of the considered studies using mice as test animals,[54] was not called into question even though no systemic effects were found, despite the huge amount of titanium dioxide involved.

The results concerning the skin seemed to be more conclusive. ENMs seem to be practically unable to penetrate the dermal barrier. No publication considered to date has described an effective body dose being transmitted through the skin, and in addition only 5% of the studies have reported a transdermal translocation of ENMs. Among these studies was one which described an experiment with silver nanoparticles in a Franz cell which included neither an adequate description of the study nor an analysis for dissolved silver.[55] Another (good) paper described the work of the research group led by Monteiro-Riviere on peptide-derivatized fullerenes applied to pig skin which was rhythmically stretched for 60 or 90 min.[56] An outstanding summary on the skin and related topics, with particular regard to the two important nanomaterials titanium dioxide and zinc oxide (which are primarily used in sun creams) was published in 2010.[57] Although more reports on this subject have been published in the intervening three years, the core message of this paper is still valid: "*The consistent finding of these different studies is that nano TiO_2 or ZnO does not penetrate beyond the stratum corneum of the skin.*"

This makes it possible to give an answer to our first working hypothesis (Table 4). It is undisputed that ENMs can use the lungs and the gastrointestinal tract as ports of entry into the body. However, in most cases only a very small fraction of the total applied dose actually penetrates into the bloodstream, and is consequently transported to secondary organs. The great majority of the applied dose is taken up in the lungs by macrophages and is removed by the normal clearance processes and/or excreted in the faeces. However, because of the extremely complex analytical procedure involved and the difficulty of finding nanoparticles in the organism, the number of studies which do in fact investigate transport via the air–blood barrier is very small. A similarly poor state of affairs in terms of the quantity of data applies to the gastrointestinal tract. Relatively speaking, only a few

Table 4: Confirmation or rejection of hypotheses on the question of the uptake of ENMs in vivo by the vast majority of the evaluated studies.[a]

Hypotheses on the uptake in vivo	Number of evaluated studies	Yes	No
ENMs[b] can be inhaled with the air and are systemically available	151	X	
ENMs can be ingested with food and are systemically available	116	X	
ENMs can permeate the skin and are systemically available	111		X
ENMs can cross tissue barriers and reach biological effective doses (BEDs) in the body	378	o	o
all ENMs can cross tissue barriers in equal measure, there are no exceptions	378		X
the uptake of ENM is dependent on their	378		
size		o	o
shape (length/thickness ratio)		X	
solubility		X	

[a] An "X" implies here that a hypothesis is either supported or rejected by the overwhelming majority of papers or by a series of very important and qualitatively good studies. (When the evaluated study is described as being of "good quality", this means that either the study fulfils the criteria list laid down by the DaNa project or that the Impact Factor of the publication in which the study appears is greater than 6.) An "o" indicates that there are good reasons (i.e. a reasonable number of good-quality studies) for assuming that the confirmation or rejection of the hypothesis is well-founded. This is mostly dependent on the ENM and its properties. [b] ENMs are nanomaterials in accordance with the ISO definition of "nanoobjects" (see first footnote in the text)

papers cover this topic. The analytical process suffers from the same problem of complexity mentioned above and the variance arising from testing different ENMs with various dosage levels, and the means of administration increases the difficulty of interpretation yet further, so that it is hardly possible to draw any unified consensus from the results.

The situation with respect to the skin is significantly better. Here, only under extremely unfavorable conditions—where the skin is, for example, injured, severely mechanically stressed, or treated with solvents—could the penetration of ENMs into the deeper cell layers (or even as far as living cells) be observed.

Two important results are worth mentioning at this point: barrier penetration seems to be easier for small particles than for large ones, and solubility has a significant influence on their behavior and their toxicological effects. While the dependence on particle size seems to be somehow understandable, despite only very few particles actually migrating through the barrier tissues, the instability of some ENMs in biological fluids raises the question about the "nanorelevance" of such materials. If a material dissolves before or during migration through a tissue barrier then the underlying assumption that we are dealing with a so-called "nano effect" is nullified. If this is in fact the case, then the toxicological process must be measured against different standards than those applicable to nanotoxicology.

An examination of which materials were investigated for specific uptake paths is also interesting (Figure 3). Whilst the fiberlike CNTs were most frequently studied in association with lung absorption, the first place in the case of skin and in gastrointestinal tract studies was taken by titanium dioxide. In this context, both product-dependent and safety-related criteria have been taken into account during selection process by the investigators. The skin studies mostly involve materials used in cosmetics (ZnO, TiO_2) or in bactericidal surfaces (Ag, TiO_2), while those concerning the gastrointestinal tract involve ENMs which are relevant in foodstuffs or significant in the food chain. All in all, those ENMs which were most often investigated are either to be found very frequently in existing products (TiO_2, ZnO, Ag) or are those from which a certain degree of the critical effect might be expected (QD, CNTs).

5.2. Lung Toxicity—Instillation versus Inhalation

The natural roots of nanotoxicology are derived from the critical discussions on fine particulate matter in the air. The very first studies on this topic were, therefore, conducted by those groups worldwide who were studying the health effects of lung exposure to fine dust particles, and who then also considered the consequences of exposure to other ultrafine particles.[58–71] Consequently, the analogies between the effects of ENMs in the lungs and those due to ultrafine dust are a topic which is considered in most studies and discussions. The similarity between environmentally relevant fine particulates and synthetic "model particles" in terms of their lung exposure behavior and biological effects was, appropriately enough, recognized by researchers very early on.[70,72,73] For as long as this topic has been under consideration, there have been discussions on the pros and cons of techniques used in toxicological investigations. In principle, there are two methods which can be used to conduct animal trials on the pulmonary toxicity of particulates: inhalation, which is a complex and elaborate process but near to reality, and instillation, which is simpler and less costly. A comprehensive comparison of the two methods was made more than 15 years ago by a group led by Oberdörster.[74–76] A clear description of instillation and its limitations in association with the investigation of aerosols was published in 2000.[77] One might be justified in assuming that these results have become accepted by those performing experiments on nanomaterials and expect that such studies would now be designed in accordance with these established rules. An evaluation of publications since 2000, however, paints the following picture: generally speaking, significantly more instillation studies have been carried out than inhalation-based ones (Figure 4). One reason for this is certainly that conducting inhalation studies requires elaborate experimental infrastructure and an enormous amount of analytical effort. The creation and maintenance of a controlled aerosol environment over a long period of time (the norm is 4 to 6 h exposure per day for several days or even weeks) is simply beyond the capabilities of many working groups. As a result, researchers very frequently turn to the instillation method as an alternative.

The instillation method is much easier and cheaper to carry out. However, there are several pitfalls in the technique

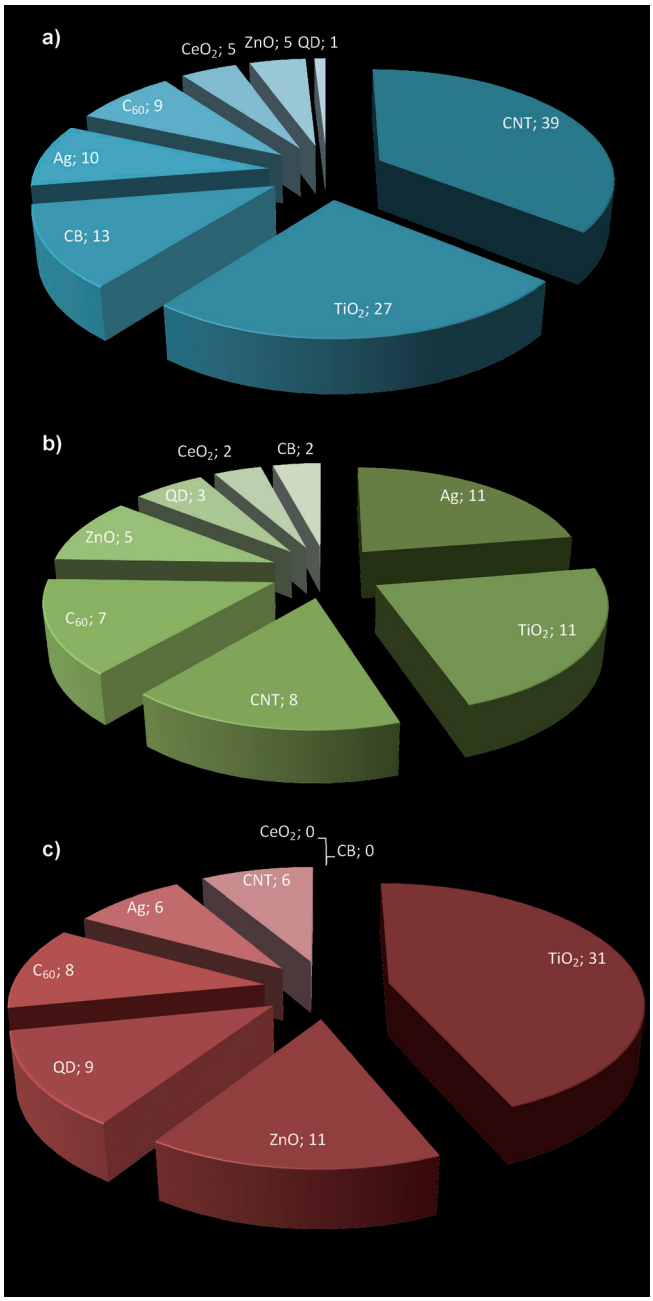

Figure 3. Evaluation of the in vivo studies on the uptake of specific ENMs by the a) lungs, b) the gastrointestinal tract, and c) the skin. The number following the ENM name indicates the number of studies assessed.

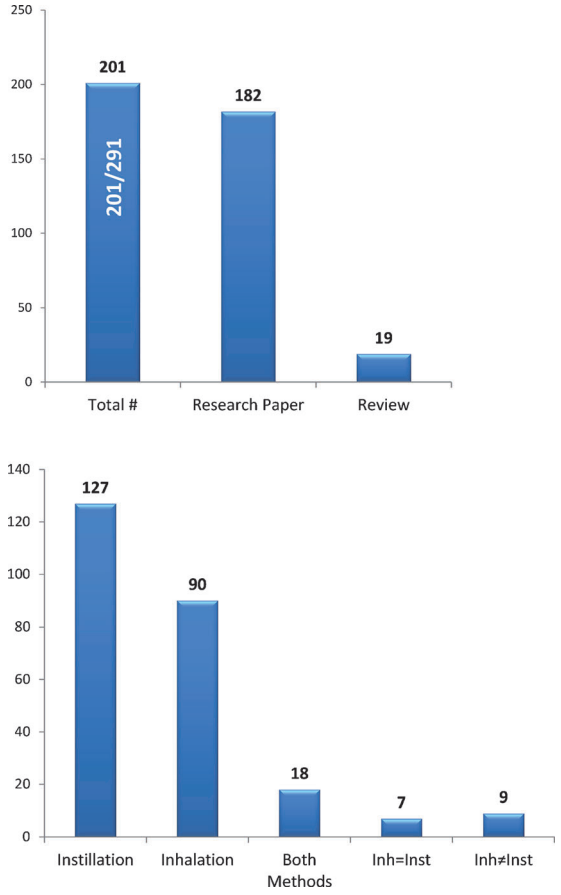

Figure 4. The total number and distribution of studies on the subjects "inhalation studies" and "instillation studies".

which, if not avoided, can lead to a falsification of the results. For example, the insertion of a bolus of nanomaterial into the airways may result in the entire lung volume being no longer ventilated. This is a particular problem with fibrous test material such as CNTs, and as a consequence the test animal may have difficulty in breathing or even suffocate.[22,23] Such stress situations will always generate a reaction from the animal which has nothing whatsoever to do with the administration of the particle suspension. The critical points which must be considered when using the instillation method are:

- the method used for intubation
- the specific medium in which the test particles are suspended
- the total volume that is administered
- the total dosage of test material
- the anaesthetic method used.

In addition, a controlled trial should always be carried out in parallel by using an appropriate reference material for which a good database of results already exists. Furthermore, when using rats as test animals, a maximum quantity of 100 μg per instillation should be used, since the results can be falsified by the effects of agglomeration and excessively high local concentrations. Direct comparisons between the two methods by groups which have applied both techniques in the course of a single study are not conclusive. Opinions are equally divided as to whether or not both methods produce the same or different results (Figure 4, bottom). However, a study just published[78] indicates that instillation, in principle, always leads to significantly greater effects than inhalation, given the condition that the same dose is applied in both cases.

Without at this point wishing to go into detail (for which, see the publication by Driscoll et al.),[77] it is quite apparent after the evaluation of the publications on ENMs that instillation studies are much more frequently carried out in

the overload range than comparable inhalation experiments (Figure 5). The overload region, which was defined over 20 years ago,[64,79] is critical because then the total administered dose of test material exceeds the cleaning capacity of the lungs. The simple fact that overloaded macrophages may start to produce cytokines that, in most, cause cases non-specific inflammatory effects in the lungs (i.e. independent of the type of administered material) makes this an essential point to consider. A study conducted under these conditions cannot, therefore, come to any reliable conclusions relating to the test materials used, although regrettably this does in fact continue to occur. Furthermore, it is a characteristic of the instillation method that very different localized levels of concentration may occur in the lung tissue. This means that while some heavily exposed areas of the lung may be in the overload zone, there may be other areas which are completely unexposed. This frequently makes the interpretation of the results difficult, and yet this factor is not given appropriate consideration in the discussion of results in various publications. Surprisingly, the results agree relatively well (Figure 5, top) when evaluating inhalation and instillation studies in terms of the severity of the induced effect. Although in the case of instillation the tendency is shifted slightly towards more intensive damage (an effect similar to quartz), for both methods the maximum values in the studies for the estimation of ENM effects was found to be comparable to fine particle exposure. The numbers of inhalation and instillation studies for nearly all the six most frequently investigated ENMs is practically the same. Only in the case of silver have more inhalation studies been found (Figure 5, middle). When categorized according to types of effects, however, the six ENMs show very different results. CNTs are, depending on their origin, definitely a group of substances which need to be evaluated very critically. This also makes the relationship between serious effects (asbestos-like and quartz-like) and lighter effects (fine-particle-like, or no effects) significant (Figure 5, bottom). For all other ENMs, the less serious effects predominate and in most cases they are comparable to those caused by fine particulates, with a transient inflammation reaction between the first and seventh day after the start of exposure, and thereafter the subsiding of all symptoms as long as no further exposure occurred.

After detailed consideration of the results of the studies on ENM uptake after lung exposure, one can now attempt to evaluate the hypotheses postulated for these trials (Table 5). Concerning the methods used, it is clear that specific aspects of both the instillation and inhalation techniques are frequently given inadequate consideration. In the instillation case, this often leads to results being given excessive weighting. In addition, the overload factor is often not sufficiently investigated or is simply accepted uncritically in these studies.

In conclusion, it can be stated that whilst instillation studies are eminently suitable for hazard assessment, the results of inhalation studies are also necessary for a comprehensive risk estimation (which includes risk characterization).[84] A further, very important, result is the recognition that the effects caused by ENMs in the lungs are fundamentally not different to those arising from other forms of particulate exposure.

It is also clear that the published results were obtained from studies that were essentially conducted over a very short

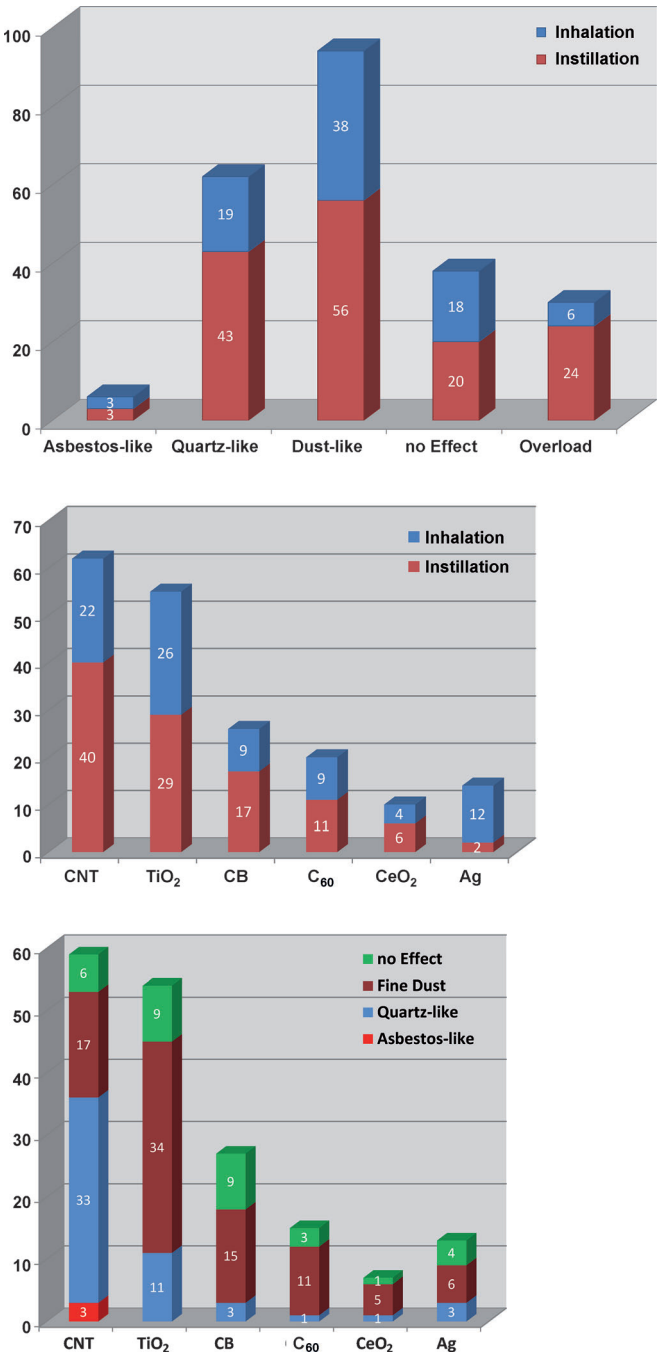

Figure 5. Analysis of all experiments involving lung exposure categorized by the type of effect caused by the nanomaterials and the methods used. Top: number of studies in which a specific effect arising from ENMs was observed. The various effects are categorized as follows: asbestos-like = tumour induction comparable to mesothelioma; quartz-like = inflammation, oxidative stress, fibrosis, granuloma formation; dust-like = effects as for "normal" fine-particle exposure, transient inflammatory processes; no effect = no effects observed under the given conditions (dose, time); overload = for rats and hamsters: $\geq$2.5 mg/animal and lung, mouse: $\geq$0.5 mg/animal and lung. Middle: Breakdown of the methods used for the six most frequently investigated ENMs. Bottom: Breakdown by the type of effect for the six most frequently investigated ENMs.

Table 5: Hypotheses relating to the question of the pulmonary toxicity of nanomaterials in vivo and confirmation or rejection based on the overwhelming majority of the studies evaluated.

Hypotheses on lung toxicity in vivo	Number of evaluated studies	Yes	No
instillation of ENMs into the lung demonstrates reliably the specific effect of this ENM	317		X
instillation studies are not as significant as inhalation studies	317	X	
effects observed after instillation studies have been always confirmed by inhalation studies	317		X
the different doses (dose rates) used for instillation studies compared to inhalation studies induce different effects	317	X	
the biological effects of ENMs differ substantially from those of other or "normal" particle lung burden	317		X

period of time. In addition to this, it seems that it was not possible to conduct any epidemiological studies for ENM exposure. The reason for this was that the workplace effects and those on the general population were too small, and the sample populations were of inadequate size. As a result of these factors, it is not possible to make any reliable claims regarding potential long-term effects. On the other hand, some of the materials investigated have in fact been manufactured in large quantities, with particle sizes on the nanometer scale, for many decades (e.g. carbon black since the 1920s, nanosilver for over a century, titanium dioxide and silicon dioxide for over 50 years). No results giving cause for alarm have been noted in toxicological studies conducted over this long period of time, a point which is supported, in principle, by the studies evaluated for this Review.

6. What Do We Expect from Nanotoxicology as a Discipline?

Even though there appears to be no reason for increased concern, the principles of nanotoxicology (as published some time ago)[1] must still be observed and should be taken into account in the design of all experimental trials. This is because the unusual properties of ENMs (which are due to their small particle sizes, large surface to volume ratios, and material differences) could lead to a modified distribution model and different biokinetic behavior, thereby producing different, unexpected effects. From this point of view, what is demanded of nanotoxicology as a discipline is rather clear: fundamentally, it should deliver an answer to the question as to whether specific—and perhaps special—regulations should be applied when dealing with a specific ENM, based on the particular nano characteristics of the substance in question. However, before this and related questions can be answered, it is necessary to take a closer look at the applicable nanotoxicological rules themselves. It is not possible to derive a set of generally applicable rules because of the uncertainties mentioned above, the inadequate quality of the studies, and frequent significant differences in the ways in which the studies were conducted (Table 1), despite the number of published studies increasing enormously. From a scientific point of view, therefore, the following requirements on future nanotoxicological studies are relatively clear (see Table 2):

- an obligatory and sufficient characterization of the investigated ENM
- the use of correct methods and appropriately modified experimental layouts in accordance with the principles of good scientific practice
- experimental procedures which follow toxicological guidelines, if possible based on SOPs
- consideration of the appropriate dose and/or concentration and the inclusion of a dose–effect relationship in the study design
- testing and monitoring of ENM characteristics under the given experimental conditions
- appropriate choice of biological model
- comparison of experimental results with appropriate positive and negative controls
- the use whenever possible of reference material to enhance comparability.

In the social sphere, the demands on and expectations of nanotoxicology as a scientific discipline are completely different from the points listed above. The disparity in the background of the stakeholders involved is reflected in the wide range of their requirements. Government-related organisations such as senior civil servants, ministries, and political parties demand to know if financial support for the further development of nanotechnologies is justified, without a disastrous safety situation occurring. Industry requires unambiguous proof that ENMs do actually have biological effects; as long as such proof is missing, more and more "nanoproducts" will appear on the market. Non-government organizations (NGOs) raise warning fingers and demand "complete safety" for the user and the environment. Consumers want safe products, but otherwise show little interest in the debate on safety. Science expects answers to questions of biological relevance and would very much like additional financial support for its investigations, generally from government funds.

These disparate demands can currently hardly be fulfilled by nanotoxicology as a scientific discipline. Firstly, as an emerging discipline[80] and subdiscipline of toxicology, nanotoxicology has clearly failed to equip itself with the same fundamental rules as are applied to toxicology (see Section 3). Furthermore, what is true for toxicology in general, naturally also applies to nanotoxicology, namely that it is impossible to prove the absence of an effect, no matter how long one searches for it. Data relating to whether and under what conditions an ENM exhibits no effects and is, therefore, considered to be safe, will never be published. It will,

therefore, never be a matter of general public awareness and consequently not be taken into account during an assessment of the safety of an ENM. Conversely, believers in the paradigm "the dosage makes the poison" maintain that all substances, including of course all ENMs, have some effect, on exposure to a high enough concentration. This reflects the current situation in nanotoxicology, where only "positive" studies are published, that is those in which a biological effect is described. Such effects generally involve cells or tissues and, therefore, give reason for concern. Those familiar with the work of the Maximum Workplace Concentration committee (MAK-Kommission) of the German Research Foundation (DFG) will know, however, that this group does in fact categorize substances as "marketable" which, based on the results of scientific investigations, produce biologically or even toxicologically relevant effects, since one factor in the assessment process is the estimation of how realistic it is that the relevant (toxic) dose will be achieved within an organism or the environment.

Besides mechanistic/biological studies, which simply search for the mechanisms of action, I would also personally wish in the future that true toxicological studies will also be carried out within the discipline of nanotoxicology. Conducted using harmonized or standardized experimental protocols, these would be able to show definitively that a specific ENM either does or does not demonstrate a toxicologically relevant effect and, therefore, must be treated with caution under particular circumstances.

Currently, an overview of the many thousands of publications which have appeared to date leads to the clear conclusion that, despite great efforts by many working groups, no unexpected results that give cause for concern have been shown for technical nanomaterials,[81] with a few not very surprising exceptions such as quantum dots containing cadmium, soluble zinc oxide and copper oxide particles, and fibrous carbon nanotubes (including associated metal catalysts). The uncertainty remains as to whether hazardous effects possibly exist which have not yet been found or whether such effects are in fact absent in the ENMs investigated to date.

7. Recommendations: International Harmonization and the Rules of Toxicology

For over 10 years in most of the technologically advanced countries of the world, and also the European Union, action plans have been implemented which cover developments in nanotechnology. Each of these action plans include a part which deals with questions concerning safety and security, and also contains relevant research and support measures. Since the EU's 6th Framework Programme, research activity relating to nanosafety has also been given encouragement and financial support both collectively and in individual EU states. These programs are, in principle, responsible for the rapid increase in the number of publications now seen on this topic, but they must also be prepared to evaluate the Babylonian plethora of the studies and their results. Too little attention has, however, been paid to the fact that the results of these new studies have made it possible to close a range of gaps in our knowledge of nanotoxicology. This fact notwithstanding, several important aspects have not yet been adequately investigated. The following recommendations for action are derived from the considerations mentioned above:

1. Political bodies must understand that research programs cannot provide an "absolute safety" label for ENMs, because "proof of an effect which is not present cannot be established". We need to return to a risk assessment process in the context of the probability of exposure and the dose–response relationship.
2. Standard protocols and methodical further development processes established during support programs must form an integral part of new incentive projects. Researchers who do not know or apply these rules should no longer be given financial support for toxicologically oriented research programs.
3. An integral part of the harmonization of experimental methods is conclusive and feasible analytics. Thus, the development of appropriate and possibly inexpensive analytical methods should be an integral part of all funding programs, as this is a challenging point for all investigations concerning ENMs.
4. Significant developments in toxicology, such as the international activities on the toxicology of the 21st century[82] and an appropriate nanotoxicology for the 21st century,[83] must be the basis of further research activities. The links between in vitro and in vivo experiments must be greatly improved, as must extrapolations and the accuracy of predictions based on in vitro experiments.
5. Gaps in scientific knowledge (e.g. regarding certain exposure pathways such as the gastrointestinal tract) must be specifically targeted in new research programs.
6. Long-term studies on the possible accumulation of nanomaterials should be integrated into future incentive measures and support programs.
7. The comparability of studies must be achieved by the integration of toxicological expertize into all projects. A quality control system covering the methodological processes would be very desirable. Moreover, a sufficient and competent physicochemical characterization of the investigated ENMs should be obligatory, without which no funding of nanotoxicological projects should be possible.
8. Decisions concerning the regulation of ENMs should be made by appropriately qualified experts who are familiar with the field of toxicology and understand the principles of toxicological effects.

In an international context, it is of course rather difficult to satisfy all these demands to the same extent, but we should at any rate try hard to improve across-the-board harmonization. If we do not insist on employing comparable methods and similar dosing techniques in future experimental work, we will once again be confronted with some results that, whilst generating shocking headlines, are not based on sound fundamentals and which yet again will need to be disproven by new studies.

8. Quo Vadis Nanotoxicology? A Critical Forecast for the Discipline

Forty years ago in a contribution to *Angewandte Chemie*, Henschler wrote "*The above discussion of the limitations of epidemiological methods and animal experiments shows plainly that it is impossible to predict with absolute certainty whether a toxic effect in man must be expected or can be ruled out with particular substances. Despite this, the authorities expect, the press demands, and many manufacturers promise 'safety' from injury to health through environmental poisons.*"[32] If one replaces the words "environmental poisons" with "ENMs", then this sentence is as true today as it was when written. If we do not recognize this fact, and that of the publication of studies which describe exactly this "negative outcome", that is, non-existent effects, then we will not be in a position to make any valid statements on the toxicity of ENMs. A few studies undertaken by groups without specialist toxicological qualifications generate results which are not based on true scientific fundamentals and speak of the "*enormous toxicological potential*" of ENMs. These have a much greater negative effect on public opinion than the many good studies which demonstrate, through careful analysis of the dose–response relationship, that we are operating in a safe area, since neither the effects shown nor the predicted environmental concentrations lead one to expect any impact on human health or the environment.

However, this demands from all of us a clear commitment to adhere to the basic rules of toxicology. If we do not follow and respect these rules covering the design of toxicological investigations, and we continue without the harmonization of experimental processes through, for example, the use of SOPs or other standardized protocols, then future support programs, whether national or international, are doomed to failure too, and their results will only contribute further to the Babylonian plethora of low-value results that exists today.

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