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Food for Thought ... on Alternative Methods for Nanoparticle Safety Testing

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Nano is a big thing in toxicology. Articles, journals, and conferences are mushrooming, paralleling the rise of nanotechnologies but also showing the hunger of toxicology for new objects to study. Perhaps it would be better to focus on new approaches first? Nanomedicine promises new solutions for old problems, but what about the old problems of toxicology? It is a fallacy to assume that one can gain beneficial effects in the human organism without unwanted collateral effects. First, any biologically active agent perturbs physiology, hopefully as a corrective for the patient but at least requiring compensatory reactions of the healthy. Second, few agents are specific enough to have only one effect, but it is rare that we want all the effects and in the given mix of strengths. Third, many agents show excess toxicity – even desired effects often become negative if excessively stimulated. This increase in negative effects is directly linked to more sensitive subpopulations (children, elderly, the diseased, those with genetic polymorphisms, etc.). Thus, the promise of nanoparticles (NP) may be paid for in possible side-effects, i.e., toxicities (Garnett et al., 2006). For most manufactured NP, toxicity data are unavailable, with some exceptions for carbon black, titanium dioxide, iron oxides, and amorphous silica (Di Giacchino et al., 2009).

So far nanoparticles and other nanomaterials (I will use the abbreviation NP for both but primarily thinking of particles or fibers and not e.g. nano-thick films) are, for the most part, treated by regulatory toxicology as chemicals (see last issue of this series, Hartung, 2010b). The most common definitions for NP include materials with dimensions from 1 nm (size of a sugar molecule) to 100 nm (size of a virus). Regulatory frameworks are on the way, opening up possibilities for alternative approaches (Sauer, 2009). Whether the differences between NP and their parent compounds are actually small or big problems, remains to be seen. But to quote Albert Einstein: "*Anyone who doesn't take truth seriously in small matters cannot be trusted in large ones either*." When discussing alternative methods for nanoparticle toxicology, we might first look at some phrase permutations – leaving out one word in each iteration:

– Alternative methods for toxicology
– Methods for nanoparticle toxicology
– Alternative nanoparticle toxicology
– Alternative nano-methods

Consideration 1: Alternative or advanced methods for toxicology?

The term "alternative methods" is most commonly understood as "alternative to animal experiments" or at least using refinement and reduction alternatives to traditional animal experiments. I have been struggling with the term over the last few years.

– "Alternative" has anti-establishment connotations for many, so we might talk instead about "advanced" methods. A lot of support the area receives, however, comes exactly from this "rage against the machine" aspect of the animal welfare movement – an honest, well warranted, ethical disagreement with the way science treats animals – which needs to be accommodated to find societal compromise.
– "Methods" is not very clear, since work is mostly about testing and increasingly about *in silico* approaches or integrated testing strategies, so the phrase "alternative approaches" is used more frequently.
– Most work is not alternative to animal *experiments* but to animal *testing*, as much experimentation describes research and testing the routine application of certain methods, especially in the regulatory context. So the discussion is very much about toxicology, though vaccine testing, efficacy testing for agent discovery, or basic research all utilize far more animals.

Recently, the phrase "toxicology for the 21st century" (Tox-21c) generated tremendous buzz, more on the west side of the Atlantic, emphasizing the technological needs and opportunities for change. CAAT follows a dual strategy, stressing both the "alternative" (3Rs) and "advanced" (Tox-21c) aspects for the different stakeholder groups. Fortunately, the two paths normally converge, and we can see them as two sides of the same coin – the most humane science is also the best science.

There is a broad base of literature, to which this series of articles contributes, highlighting the ethical concerns, costs (Bottini et al., 2008; Bottini and Hartung, 2009), limited predictivity (Hartung, 2008b; Hartung and Daston, 2009; Hoffmann and Hartung, 2005, 2006; Hartung, 2009) and limited throughput (Rovida and Hartung, 2009; Hartung and Rovida, 2009) of current approaches that, for the most part, were developed some decades ago for drug safety testing and subsequently were adapted to pesticides, chemicals, cosmetics, and foodstuffs. These limitations serve as the driving forces for change on both

sides of the Atlantic (Hartung, 2010a). While there is progress, particularly in some areas of topical and acute toxicity (Hartung, 2008c), progress for the more demanding systemic and chronic toxicities has been limited.

Is there any reason to assume that nanotoxicology would not benefit from alternative methods, e.g. that they are less applicable to particles than to dissolved substances? Indeed, some theoretical considerations apply: The *in vitro* kinetics of particles might differ, i.e. their behavior in cell culture. This might include particle clumping (aggregation), binding to plastic, or floating on the cell culture media surface, all of which would alter cellular exposure and, thus, the concentration response curve. Similarly, exposure to air and non-physiological culture conditions might affect the experiments. Also, specific artifacts have interfered with cytotoxicity measures (MTT) as typically applied in alternative methods (Worle-Knirsch, 2006). Later, we will discuss some general problems of alternative methods use for NP. However, altogether nanotoxicology is likely a driving force and not a stumbling block toward the use of modern approaches in toxicology (Hartung and Leist, 2008; Hartung 2008a; Nyland and Silbergeld, 2009).

Consideration 2: Special methods for nanotoxicology?

The first major question for nanotoxicology is: does it even exist? Is it any different from the current risk paradigm, i.e. hazard, kinetics, exposure measurement, and overall risk assessment? First, completely new modes of action for NP have been found – if we think of asbestos as a natural nanofiber, where a key mechanism is macrophage activation after ingestion of needles of asbestos, it also applies to nanofibers in general. However, the hazards are still classical ones, i.e. fibrosis and cancer. We can argue that this is only an additional mode of action, which can either be anticipated by size and shape of particles, or it could simply be added to the assessment, and be found by traditional approaches. From this point of view, it is rather unlikely that a really new hazard that could not be seen in repeated dose studies or cancer bioassays would be attributed to particles.

However, lung toxicology, for example, lags far behind other areas of concern, while NP are especially likely to reach the alveoli of the lung and exert toxicity there (Donaldson et al., 2004; Kagan et al., 2005). Airborne exposure testing is experimentally cumbersome and is avoided when possible, due not only to the effort (costs) involved but also to the poor reflection rodents give of human exposure. Testing for respiratory irritation and sensitization is not standard for industrial chemicals, and guideline tests have yet to be developed. We also should be clear that the particular health effects of industrial chemicals – endocrine disruption, immunotoxicity, and developmental neurotoxicity – are among the more recent additions and are not yet reflected in testing programs. It would just take one scandal, however, to change this.

It may be unlikely that a completely new adverse health effect is induced by NP ("the ears fall off"), but there are many human diseases where we do not suspect any chemical involvement and where, in fact, one might exist. In particular, the chronic effects of chemicals are so poorly understood that we have no idea whether we would get any relevant alert from routine animal tests, which are inadequate even with regard to well-known hazards. Possible examples of continuously increasing health problems include atherosclerosis, male infertility, autism, and diabetes. It is worth noting that air pollution involving natural NP led mainly to excess deaths associated with cardiovascular illness (Seaton and Donaldson, 2005), a hazard not generally addressed in toxicology. Arteriosclerosis, in fact, is very difficult to induce in animals. Determination of the pulmonary and systemic inflammatory hazards typically seen with NP (Kipen et al., 2005) is not among the strengths of the toxicological toolbox.

There might well be hazards not present for a parent compound due to kinetics, as the adsorption, distribution, metabolism, and excretion of NP can differ greatly from that of larger particles or soluble substances. Changes in kinetics (bioavailability) alone (Holl, 2009) are sufficient to create additional hazards not seen with the parent compound, since whenever higher plasma or tissue levels of the substance are obtained, thresholds of toxicity might be exceeded (Fig. 1). We know very well from formulations of drugs that solubility after oral administration depends on particle size influencing peak plasma levels – a crucial determinant for toxicity. Similarly, higher concentrations can be achieved if transports through barriers are accelerated. However, the faster elimination – for example by cellular uptake or chemical reactivity – acts against this (Fig. 2).

For toxic effects, size matters, as a number of studies show size-dependent effects (Gornati et al., 2009). Good examples are gold and silver, which normally are minimally reactive but become much more so at NP sizes. Silver NP, for this reason, are used as bactericidal coatings for clothes, for example ("wash

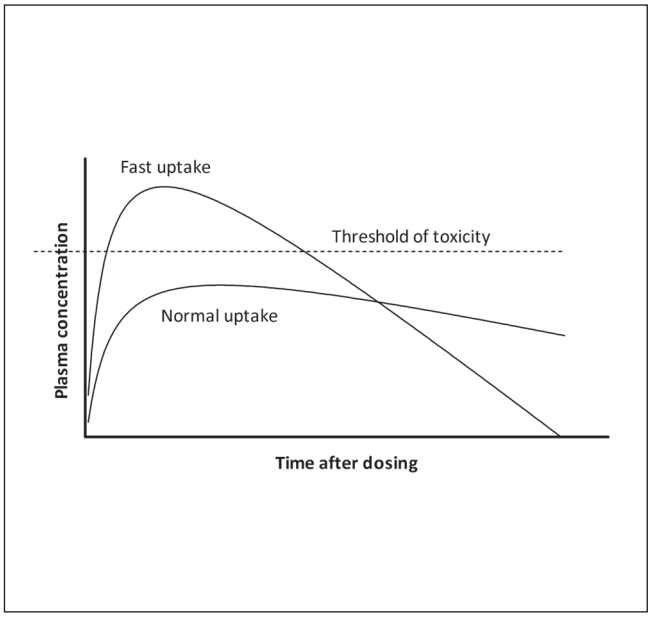

Fig. 1: Relation of biokinetics with the threshold of toxicity

your socks less often, thanks to silver NP coating"). At least for the antimicrobial properties of silver NP, shape dependence has been shown (Pal et al., 2007). How this translates to toxic effects on human cells is not known. Cellular uptake of gold NP has been found to be shape-dependent (Chen et al., 2006), while others reported no differences in a number of cell systems for silica NP (Cha et al., 2007).

Similarly, reactive chemistry, a key feature of many toxicants, is strongly influenced by particle surface. One milliliter of 10 nm-sized NP has the surface area of a soccer field. Thus, we might see hazards with NP at lower concentrations than the maximally tested or testable doses currently applied of the parent compound. As one consequence, the exposure might require different measurements, e.g., instead of dose measures in mg/kg, particle numbers or particle surface might be more meaningful both *in vivo* and *in vitro*.

We might also see some effects only *in vitro*. We should not underestimate the hazards that are masked by current tests because the animals defend themselves successfully. More than 90% of substances that can exert genotoxicity in cells are not mutagenic in animal tests. This is not to say that the cell result was wrong; rather, it usually means we did not achieve the concentrations *in vivo* that we can apply *in vitro* or that some defenses were not reflected *in vitro*. The substance may still present a hazard, which might become relevant for humans or subpopulations. We do not know whether the defense mechanisms against some hazards are as effective when administered as NP. The novel properties of NP also can lead to new biological interactions (Walker et al., 2009), which could result in toxicities not shown by the parent compound. This extends to refinement methods, where, for instance, the exposure to airborne particles for the obligatory nose-breathing rat and mouse require attention to avoid overloading the respiratory tract.

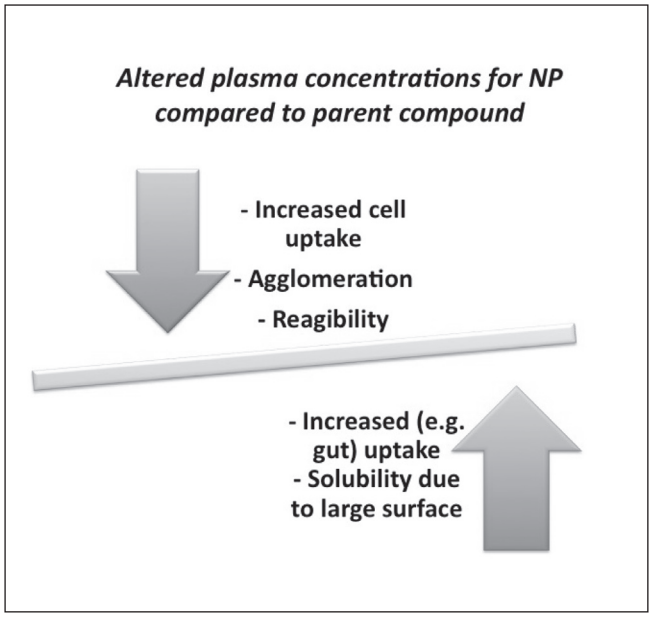

Fig. 2: Relation between plasma levels of parent compound and respective NP

There might be opportunities for reduction alternatives, too. Though it is rather unlikely that lower variability of responses to NP would allow a reduction in animal group numbers, they should still be considered, as should designs with one control group for multiple tests or longitudinal studies following the same group of animals rather than sacrificing a group per time point. Here, the imaging opportunities for NP might make a difference for kinetics experiments. Opportunities to combine studies, e.g. mutagenicity and repeat-dose studies, enhanced chronic studies including carcinogenicity, or the inclusion of developmental neurotoxicity in reproductive toxicity studies should be considered as reduction alternatives. NP do not really differ from other test materials in this regard.

Consideration 3: Do we need a traditional or an alternative toxicology for NP?

The number of different NP we might need to address is potentially extremely high, with various shapes, size distributions, and coatings for each material. This alone suggests the importance of using alternative methods, which often allow higher throughput, replicates, and parallel tests. Thus, the limit of test throughput is more relevant for NP. First, the main health concerns in particle toxicity hint at risks for the most complex endpoints (cancer, lung toxicity), which require most test capacities. Rodent inhalation models are especially prohibitive in terms of time and expense (Hillegass et al., 2010). Second, since any given substance, at least theoretically, can be formulated to particles of very different sizes, size distributions, shapes, and modifications, an almost unlimited testing demand could be considered. Choi et al. (Choi et al., 2009) calculated the costs for traditional testing of NP already on the market to be between $ 250 million and $ 1.2 billion and the time required at 34-53 years.

In addition, we should be aware that current regulatory toxicology was established for drugs under development. A very precautionary approach was taken to avoid putting volunteers and patients at risk (Hartung, 2009). While this might be appropriate for nanomedicine products, we have to ask ourselves what development opportunities we sacrifice if we apply the same precautionary methods to products with lesser exposure to humans or those that are not intended to be biologically active. The problem becomes most pronounced when precautionary methods (many false-positives) are used for substance groups with rare side-effects (Hoffmann and Hartung, 2005; Hartung, 2009).

This reasoning makes evident the need to explore the toxic profiles of a broad variety of NP to learn what we must control for. Furthermore, novel approaches need to be developed, since traditional approaches might have even greater limitations for NP than for other industrial chemicals and may not offer the throughput and velocity to cope with the dynamic developments in nanotechnologies.

What are the specific opportunities to use alternative methods? First, one major concern in cosmetics is dermal penetration

of NP. Rodent and rabbit skin have little to do with human skin, and both artificial human skin and explants offer opportunities with available, accepted methods. Some of these also allow testing for mechanical stress or inflammation, as well as penetration via hair follicles, as specific concerns for NP. Other barrier models for gut uptake, blood-brain barrier, or placental barrier are prevalidated but not yet validated. Still, they might be useful to characterize NP and identify or rule out specific concerns.

Kinetics will be affected, which can shift concentration response curves, limits, and threshold concentrations, as well as no-effect levels, thus altering critical components of the risk assessment paradigm. We will need to explore whether this can be handled with, for example, safety or assessment factors. This means requiring, for example, an additional factor as safety margin for the use of NP in humans. For current estimations of safe-dose uses from animal no-effect levels, we often require one of 10 for interspecies differences and one of 10 for sensitive subpopulations. The numbers appear to correspond more with the decimal system than with sound science – if we had twelve fingers instead of ten, we would likely be 1.44 times better protected by regulation. Completely new ports of entry of substances have already been described, when NP sizes fall below barrier cut-offs (Hillyer et al., 2001). Note that the ease of cellular uptake may also result in bioaccumulation of NP (Oberdörster et al., 2007). Furthermore, the larger surface area per unit weight often makes NP more reactive. Since many forms of toxicity are mediated by chemical reactivity, such as mutagenicity by formation of DNA adducts or sensitization by hapten binding, this raises the possibility of increased toxic potentials.

A number of alternative methods validated for chemicals and drugs might be useful for NP (Tab. 1), but none has been validated for this purpose. The modular approach to validation (Hartung et al., 2004), it should be noted, allows expanding applicability domains for validated methods, a possible fast-track to obtain regulatory acceptance for NP evaluation. The potential carcinogenicity of NP is of concern to toxicologists because of several specific properties – the potential to activate inflammatory mechanisms as promoters of cancer, the ability to reach alveolar compartments when inhaled, altered cellular uptake allowing NP to reach DNA, and the fact that mutagenicity is linked with reactive chemistry, which is often amplified by the large surfaces of NP. A number of *in vitro* models for mutagenicity are available, but they also are known to have many false positive results (Kirkland et al., 2005, 2007). Many of them also can be integrated in enhanced animal studies, allowing a reduction in animal use. A specific opportunity is offered by the cell transformation assays for cancer, which currently are peer-reviewed after validation.

Inflammation can be studied in monocyte activation tests, such as the validated alternative pyrogen tests (Hoffmann et al., 2005; Schindler et al., 2006). Human whole blood assays offer specific opportunities, as a cell suspension is used (Schindler et al., 2009).

Experimental set-ups for airborne exposure of particles to air/liquid interface cultures of cells are available but have not yet been validated.

Consideration 4: Special problems for *in vitro* nanotoxicology

Agglomeration. Not everything called nano is actually nano. Aggregation or agglomeration of NP is very common and difficult to prevent. NP can have complex aggregation behaviors in aqueous solutions (Holl, 2009) with substantial impact on their toxicity. Many of the studies published so far did not exclude aggregation, but even as non-mono-dispersed particles, the smaller particles are more potent in many respects (Oberdörster et al., 2007). Aggregation effects also have been recognized in the ecotoxicity of silica, titanium dioxide, and zinc oxide NP (Adams et al., 2006). Some systematic approaches to dispersed nanoparticles have been proposed (Sager et al., 2007), but the problem still needs to be addressed on a case-by-case basis.

Stability. The stability of NP is not often discussed, but the sheer surface area represents a problem, as it not only attracts substances offering binding sites (for pyrogens, for example) but also lends itself to chemical reactions such as oxidation. Many NP might actually be coated. We know as little about the modification and degradation occurring over time as we do about the metabolic fate of NP.

Dosimetry. In toxicology, we have seen a move from primarily weight-based doses (mg/kg or ppm) to (molar) concentrations, especially when kinetic measures (plasma concentrations, for example) could be assessed or when experiments were done *in vitro*. For NP, weight, particle number, and surface area are typical dose measures, but shape, coating, and electrophysical properties, etc. can have a further impact. The chemical characterization (Powers et al., 2007) and dosimetry clearly require closer attention than for traditional chemicals (Walker and Bucher, 2009). In some cases, toxicity correlated best with NP surface area (Unfried et al., 2007), but it is still to be established whether this is a more general rule. It makes sense for reactive chemistry, which is a leading mechanism of toxicological damage, and for oxidative processes; in fact, generation of reactive oxygen species has been a key mode of action associated with NP toxicity.

In vitro biokinetics. This term has been coined to indicate that test substances *in vitro* also exhibit kinetics: they are adsorbed (e.g., by plastic or the albumin of fetal calf serum), stay soluble or precipitate, are taken up by cells, are oxidized by air or metabolized by the cells, and we interfere with their presence when changing cell culture media. The situation is not as complex as *in vivo* kinetics, but certainly the actual effective concentration reaching the cells is not the one we added. Just as *in vivo* work has been augmented by introducing kinetics, we might likewise give consideration to these factors as we move the field of *in vitro* toxicology forward (Bouvier D'Yvoire et al., 2007). The situation is no less complex for NP, where aggregation and particle coating must be considered. Cell membranes, mitochondria, and nuclei are considered major compartments for NP toxicity (Unfried et al., 2007). Thus, uptake and intracellular trafficking must also be addressed.

Cell contact of NP. Actual exposure of cells to NP needs to be assured, as NP might swim on the culture media. Also, NP are

Tab. 1: Validated alternative methods for chemicals and their likely relevance for nanoparticles

No.	Method	Date of ESAC statement	Suitability for NP
1	3T3 NRU phototoxicity test	03/11/1997	Likely applicable but rare testing demand
2	EpiSkin skin corrosivity test	03/04/1998	Likely applicable
3	Rat TER skin corrosivity test	03/04/1998	
4	Application of the 3T3 NRU phototoxicity test to UV filter chemicals	20/05/1998	See 1
6	Local lymph node assay for skin sensitization	21/03/2000	Reduction / Refinement method likely applicable
7	EpiDerm skin corrosivity test	21/03/2000	See 2-3
8	CORROSITEX skin corrosivity test	06/12/2000	
11	Micromass embryotoxicity assay	01/05/2002	Relevance unclear; might need to be combined with a placenta barrier model
12	Whole rat embryotoxicity assay	01/05/2002	
13	Embryonic stem cell test for embryotoxicity	01/05/2002	
17	Upper Threshold Concentration (UTC) step-down approach for acute aquatic toxicity testing	21/03/2006	Reduction method likely applicable
18	CFU-GM assay for predicting acute neutropenia in humans	21/03/2006	Relevance unclear; possible value for acute toxicity testing strategy
19	Human Whole Blood IL-1 for *in vitro* pyrogenicity testing	21/03/2006	Likely applicable; especially important because of large surface area and thereby questionable applicability of the Limulus assay
20	Human Whole Blood IL-6 for *in vitro* pyrogenicity testing	21/03/2006	
21	PBMC IL-6 for *in vitro* pyrogenicity testing	21/03/2006	
22	MM6 IL-6 for *in vitro* pyrogenicity testing	21/03/2006	
23	Human Cryopreserved Whole Blood IL-1 for *in vitro* pyrogenicity testing	21/03/2006	
24	*In vitro* micronucleus test as an alternative to the *in vitro* chromosome aberration assay for genotoxicity testing	17/11/2006	Likely applicable; high relevance
25	Application of the SkinEthic human skin model for skin corrosivity testing	17/11/2006	See 2-3 and 7-8
27	Bovine Corneal Opacity and Permeability (BCOP) test method	27/04/2007	Likely applicable
28	Isolated Chicken Eye (ICE) test method	27/04/2007	
29	Reduced Local Lymph Node Assay (rLLNA)	27/04/2007	See 6
30	EpiDerm (with MTT reduction) for skin irritation	27/04/2007	Likely applicable, highly relevant
31	EPISKIN (with MTT reduction) for skin irritation	27/04/2007	
32	Fixed dose procedure (FDP)	31/10/2007	Reduction methods likely applicable
33	Acute Toxic Class Method (ATC)	31/10/2007	
34	Up and Down procedure (UDP)	31/10/2007	
35	EpiDerm SIT (with MTT reduction) for skin irritation	5/11/2008	See 30-31
36	SkinEthic (with MTT reduction) for skin irritation	5/11/2008	
37	CellSystems human skin model EST-1000 for skin corrosivity testing	12/06/2009	See 2-3 and 7-8
38	Cellsensor Microphysiometer for eye irritation	10/07/2009	See 27-28
39	Fluorescence Leakage Assay for severe eye irritation	10/07/2009	

Noteworthy, methods 18-23 were not developed for the purpose of chemicals testing, but current validation activities explore their use for acute toxicity testing, which might lead to an extension of the applicability domain. Validity statements not listed are not relevant for chemicals / NP.

known to pass from cell to cell. Cell monolayers resemble pan-fried eggs lying next to each other, giving only minimal cell-to-cell contact areas. Furthermore, cell density in a typical culture is only 1% of normal tissue (Hartung, 2007), which changes dosimetry and the likelihood of NP-to-cell contact.

Special artifacts by NP in vitro. Single-walled carbon nanotubes (SWCNTs) appear to interact with some tetrazolium salts such as MTT but not with others (such as WST1, INT, XTT) (Worle-Knirsch et al., 2006). More such artifacts are likely to be discovered, which may be prompted by large surface area, electrostatic properties, or increased reactivity.

Consideration 5: Alternative nano-methods

The question whether nanotechnologies offer specific opportunities to create new alternative methods deserves some consideration. Nanostructuring the surfaces of cell culture dishes is one example, to induce or maintain the differentiation of cells. Coating techniques also are often required for approaches such as "cells on chips." Imaging technologies using quantum dots might also enhance (non)-invasive imaging technologies for laboratory animals as they are developed in humans. NP already are used to deliver genes or other materials into cells, enhancing and broadening opportunities for *in vitro* approaches. The opportunities offered by nanotechnologies, however, are only starting to be exploited.

Consideration 6: Opportunities for *in silico* alternatives in nanotoxicology

Computational approaches to nanotoxicology so far are rather limited. With increasing datasets, however, modeling some aspects of interest might become feasible. Data mining of large datasets and the interspecies extrapolation of kinetics are most promising. Size and shape variations add dimensions of complexity to the correlative approaches, however, which will require enormous data-sets. The tremendous opportunities and challenges to *in silico* toxicity approaches have been discussed recently (Hartung and Hoffmann, 2009). In the meantime, modeling of kinetics, starting with airway disposition, might hold the most promise. However, nanotoxicology could be very stringent from the beginning, making the best use of biometry and avoiding the many pitfalls repeatedly discussed in this *Food for Thought* series (multiple testing, lack of power analysis, significance vs. relevance, lack of meta-analysis, etc.)

Consideration 7: Are there reasons to make current alternative tests less applicable to NP?

The answer to the above question is yes, unfortunately, since the biokinetics of NP will affect *in vivo* and *in vitro* results very differently. Consequently, many prediction models developed for general chemicals will not work. NP aggregation and the difficulty of application to cells and animals affect the execution of routine tests. Databases allowing computational approaches are so rare that, for the immediate future, no major contribution can be expected.

There might also be reasons to question the extrapolation of the NP parent compound to humans, but it is difficult to say whether animal results for NP are better or worse when extrapolated to humans. NP differ in exposure/kinetics, and, with regard to hazards, even higher potentials for interspecies differences exist. What is required is the systematic evaluation of validated alternatives for their applicability to NP and, if necessary, a modification of the prediction model.

It is disturbing that nanotoxicology is reinventing alternative approaches, often without referring back to the two decades of development and validation already accomplished for chemicals and cosmetic ingredients. Cytotoxicity and mutagenicity assays are broadly used (Kroll et al., 2009; Holl, 2009) without necessarily bridging to the validated methodologies. Others have highlighted the need to optimize validated toxicity and ecotoxicity tests for NP (Oberdörster et al., 2007; Behra and Krug, 2008). A variety of approaches lend themselves to adaptation for NP but none has been formally validated for NP.

Conclusions

The toxicology of NP is a rapidly emerging concern. It is driven by the dramatic increase in industrial uses of NP and by public debate. Increasing funding and studies inevitably will result in reports of toxicological effects of NP – both publication bias for positive findings and the multiple testing fallacy (if 20 experiments or endpoints are analyzed with p= 0.05 for significance, one should be false-positive) will come to bear here. They will spur further research, and it will require decades to sort out what is true and what is relevant. We will need validated tools that offer the throughput, reliability, and relevance to address key features of NP risks. The additional testing demand for NP adds to the urgency of developing new approaches in toxicology. Whether this will only add some tools for NP to the traditional approach or help to create an entire new paradigm for toxicology awaits an answer. So far, it appears that the problem of nanotoxicology is mainly a kinetic one – some safety factors could help to account for differences in ADME, but we need to keep in mind that the enhanced bioavailability of NP on body, organ, and cell level might result in thresholds of toxicity being overstepped, in which case a change in hazards suddenly does become relevant. The fact that higher exposure levels in target cells can be more easily modeled in cell systems than *in vivo*, and thus such hazards identified, argues again for the use of alternative methods.

Taken together, it appears that nanotoxicology, to a large extent, is dependent on the use and further development of alternative approaches. The more we know what we are looking for, the better we can target our testing. If we have no hypothesis, screening in many models and black-box types of animal tests

might be the only way forward. NP are different, but they are not so different that we should expect completely new hazards. Hazards not necessarily shown by the parent compound may be seen, however, due to higher concentrations achieved at target tissues.

In vitro approaches represent a reasonable compromise between effort and information gain, allowing direct comparison of various NP and their parent compounds. A broad, animal-based screening approach is not feasible with regard to laboratory capacities and costs, and it certainly is not desirable from an animal welfare viewpoint.

A number of alternative approaches have undergone the optimization and validation process to make them suitable for regulatory purposes. It appears to be most promising to adapt these to NP in order to have a testing platform for broader characterization. When combined with a somewhat more extensive physicochemical characterization than normally applied to industrial chemicals, this will help us derive some more general rules about the hazards of NP. The field of alternative approaches has paid for its lessons on the importance of good practices and standardization for the success of validation and regulatory acceptance of methods. It is strongly advised that the respective guidance on Good Laboratory Practice for *in vitro* toxicity (OECD, 2004) and Good Cell Culture Practice (Coecke et al., 2005) be followed from the beginning. It is promising that some good practices for how to test NP have emerged from expert workshops (Maynard et al., 2006; Balbus et al., 2007; Warheit et al., 2007; Hoet and Boczkowski, 2008). In the near future, the respective quality assurance for the execution of such tests will be integrated.

Due to the high number and heterogeneity of particle samples and experimental systems, it is still difficult to find common principles of NP toxicity (Hoet and Boczkowski, 2008). We have been rightly warned (Fadeel et al., 2007), however, that we are witnessing only the first generation of NP; more sophisticated NP (active nanostructures, coated NP, integrated nanosystems, etc.) will make this even more complicated. Thus, it might well be that each and every NP formulation of a substance will have to be considered an individual entity requiring at least some risk assessment. K.C. Elliott (Elliott, 2007) characterized nanotoxicology as a pre-normal science, in which researchers have no widely accepted paradigm to guide their investigations.

We must not forget that not only NP themselves, but also contaminations, may have adverse effects. Carbon nanotubes, for instance, were shown to include metals, amorphous carbon, and other compounds (Pulskamp et al., 2007; Fadeel et al., 2007). A special case of high relevance is the contamination with pyrogens due to the large surface area and the high lipophilicity of these compounds (Ashwood et al., 2007). It remains to be seen whether current pyrogenicity tests can retrieve such contamination before applying nanomedicines by injection.

The major problem for NP risk assessment is kinetics. Though we expect differences from the parent compound due to size and shape, we do not really know how to test for them. Species differences are not really well established. A key problem is that we still do not know how NP are metabolically processed (Fisher and Chan, 2007). The field of alternatives mainly has to offer some barrier models, which certainly represent a key priority.

Last but not least, toxicity is not always bad news, since sometimes it can be exploited for therapeutic purposes (Oberdörster et al., 2007). The main difference between toxicology and pharmacology is whether an effect is desired. NP offer fascinating opportunities to interfere with the organism in new ways. We must take care to find the right balance between opportunities and safety concerns. *In vitro* approaches promise to provide an affordable database on the biological activities to help understand the risks and opportunities.

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