Review

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In vitro-ex vivo model systems for nanosafety assessment

Abstract: Engineered nanomaterials have unique and novel properties enabling wide-ranging new applications in nearly all fields of research. As these new properties have raised concerns about potential adverse effects for the environment and human health, extensive efforts are underway to define reliable, cost- and time-effective, as well as mechanistic-based testing strategies to replace the current method of animal testing, which is still the most prevalent model used for the risk assessment of chemicals. Current approaches for nanomaterials follow this line. The aim of this review is to explore and qualify the relevance of new in vitro and ex vivo models in (nano) material safety assessment, a crucial prerequisite for translation into applications.

Keywords: alternative models; nanomaterials; risk assessment.

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Introduction

Pressure on nanomaterial [for a definition see box and (1)] safety comes from society, consumer and regulatory bodies, but also from industry to identify potential adverse nanomaterials as early as possible during development of nanomaterial-based products in order to avoid economic and social drawbacks. Multifunctional, smart or adaptive material concepts envisioned in nanomedicine, with an estimated worldwide market size of US$1 trillion (2), create new requirements for biological risk assessment. This in turn creates a demand for alternative test models which must be necessarily complex, but still as standardized as possible to allow high throughput, content and cost-effectiveness (3), allowing the acceleration of 'faster to fail' processes (4).

> According to the European Commission, nanomaterials are defined as natural, incidental or manufactured (engineered) material-containing particles, in an unbound state or as an aggregate or 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 (1).

In general, risk assessment of chemicals is mainly based on animal testing strategies – an approach that has not changed over the last 40 years (5). Current approaches for the risk assessment of nanomaterials follow very similar lines. However, this strategy is both resource- and time-consuming, leading to a bottleneck and a back-log of materials requiring testing (6). In addition, the number of newly-developed nanomaterial-based material concepts is steadily increasing. A full assessment of the safety of such materials following traditional regulations would be extremely cost-intensive and time-consuming. Moreover the outcome of animal testing regarding its predictive power for human beings poorly correlated, due to physiological and biochemical species dissimilarities (7). Furthermore, the principle of the 3Rs – replacement, reduction and refinement – became an increasing public

and legal demand which for ethical reasons supports the replacement of animal use with more human-relevant alternatives (8). Therefore new concepts for efficient, cheaper and evidence-based testing strategies were proposed, based on the use of human primary cells and cell lines (6).

In Switzerland more than 70 Mio CHF of public funds from the Swiss federation have been invested in research studies with animals, whereas <500,000 CHF have been made available for the development of alternative methods (9). A similar situation is reported for the EU. In the latest report, the use of 11.5 million animals was recorded for the year 2011, with rodents representing more than 80% of the total animal number (10). Consequently, it is clearly time to realize a paradigm-shift towards the development of more complex and realistic in vitro alternatives.

Over the last 3–5 years, intensified efforts have been made towards a systematic development and evaluation of innovative and more reliable in vitro models in the hopes of improving R&D productivity in the pharmaceutical and biomedical industries. Thereby, the focus of this review is to explore and qualify the relevance of new human in vitro and ex vivo models in (nano)material safety assessment. Selected in vitro and ex vivo models are analyzed herein, and current challenges and perspectives associated with these approaches are further discussed, especially with regard to their ability to predict nanomaterial toxicity.

Human exposure to nanomaterials

Due to the enormous diversity of nanomaterials being produced and used in a wide variety of consumer, industrial, and biomedical applications, the exposure routes to which humans may be potentially subjected to nanomaterials are numerous. These specific routes include inhalation, injection, ingestion and permeation through (diseased) skin (11). The availability and toxicity of any nanomaterials to a biological organism is determined by both the toxicokinetics (TK) [administration, distribution, metabolism and transformation and excretion (ADME)] and toxicodynamics (TD) (binding, interaction and induction of toxic effects) (12). As nanomaterials come into contact with the skin, the gastrointestinal or the respiratory tract, these biological compartments are "innately designed" to act as barriers to the passage of foreign materials into the organism (13). The epithelium provides a first interface between biological compartments, and after nanomaterials have passed through the epithelial barrier they may pass through the basement membrane and the subepithelial connective tissue layer and eventually come into contact with endothelial cells lining the capillaries. As endothelial cells play an important role in inflammation processes (14), these nanomaterials might affect endothelial cell function and viability, inducing pro-inflammatory stimuli. Biomedical application of nanomaterials requires most frequently the injection of these materials directly into the blood stream, bypassing the aforementioned classical barrier tissues. Aspects of blood-compatibility, liver- or nephrotoxicity or interactions with internal barrier tissues are thus becoming more relevant in nanomaterial safety assessment.

Towards predictive cell culture models of organs and barrier systems

The human body includes more than 200 different cell types with distinct levels of differentiation, embedded in soft extracellular matrices, organized in different tissues and organs, regulated by complex signalling networks and cross-talk (15). Due to this complexity, predictive models should mimic the key parameters of the in vivo organ. To achieve this, the following approaches are in development [adapted from (3)]:

i) replacement of malignant or cancer-derived cell lines by primary or well-characterized human cell lines
ii) movement from single cell type to multi-cellular cultures
iii) movement from monolayer to organoid-like 3D models
iv) tissue preparation from explants

What is still underestimated in the current cell-based models is the fact that living tissue in its corresponding microenvironment is a dynamic and moving system (e.g., due to the bloodstream, lymph liquid or breathing) or alternatively represent a particular interface (e.g., a barrier between different compartments such as air-liquid). These models would facilitate both the fundamental understanding of nanomaterial-biology interactions, elucidating specific mode-of-action mechanisms, as well as translational research aimed at accelerating the market readiness of nanomaterial-based innovations. These reflections are addressed and consistently emphasized in a number of reports and reviews on the subject (4, 16, 17) and are attracting increasing attention in the scientific community.

Human alternative models

Cell-based in vitro models: the bottom up approach

Since the discovery of the possibility of maintaining animal tissues and cells in artificial media outside of the organism in glass dishes (in vitro) in the late 19th century (18), several key inventions have been made, such as the establishment of the first human carcinoma cell line HeLa (19), the production of monoclonal antibodies by cell fusion of mouse myeloma cells with lymphocytes originating from the spleen (20), or the development of a complete cell culture medium such as Dulbecco's modified Eagle medium developed by Harr Eagle and Renato Dulbecco (21). These achievements in cell culture technology significantly boosted not only the fields of virology and cell-transfection technology in recent decades, but also stem cell research, and have become indispensable tools in modern biomedical research.

Over the last 10 years, well-characterized cells either freshly isolated from tissue (primary cells) or cell lines were used as building blocks for co-culture systems or three-dimensional micro-tissues (22, 23). An important step forward in this field was the development of permeable supports that allow researchers to keep the culture medium on either side of the cultured epithelium separate, leading to increased differentiation of the cultured cells (24) or the growth of different cell types on two sides of the membranes (25, 26). Furthermore, the medium can be removed from the upper side to expose the cells to (for example) air on one side, and to allow them to be fed from the medium in the chamber underneath (27).

These advanced in vitro cultures close the obvious gap between monolayer cultures and animal models, combining the advantages for increased throughput capabilities and increased predictive power. However, most of these systems are still in their infancy and further validation is needed in terms of reliability, relevance and predictive power.

A number of promising examples have been published that carefully address physiological relevance, correlation and validation against established in vivo models. The respiratory tract, being the most sensitive entry port of nanomaterials, has been the focus of several hundred in vitro and in vivo studies [for reviews see (28–30)]. Therefore, it is not surprising that human advanced in vitro models have been developed in order to gain more insight into the mode of action of inhalable aerosols/(nano)particles (Figure 1A, B).

Many monocultures exist that mimic the different compartments of lungs, i.e., conducting airways and lung parenchyma, however, the advantage of co-cultures in this research area was already recognized several years ago. The first two co-culture systems were described using epithelial and endothelial cells to study the impact of nanoparticles (26, 31).

We recently reported on an in vitro triple-cell co-culture model of the human airway wall composed of three main cell types: epithelial cells, human blood monocyte-derived macrophages and dendritic cells cultivated in a transwell system (32). A detailed characterization and validation of the system showed that the cell-cell interactions and communication in the culture system behave similar to that in vivo, indicating not only the proper functioning

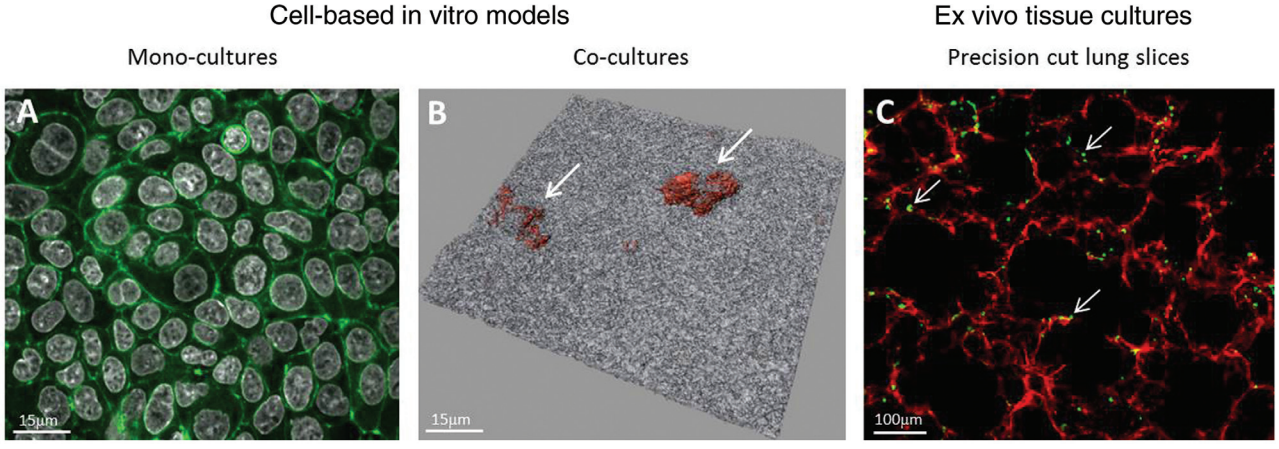

Figure 1: Examples of conventional cell culture models and ex vivo models. Laser scanning micrographs representing epithelial monocultures (A549 lung epithelial type II cell line) labelled for F-actin (green) and cell nuclei (white) (xy projection) (A); co-cultures of epithelial cells forming a tight monolayer (white) with macrophages on top (orange, arrows) (3D shadow projection with transparent renderings) (B), and a precision cut slice from rat lungs stained for F-actin (red) and macrophages (green, arrows) (C).

but also improved biological relevance of the system (29). Further improvements with four cell types were also described recently, showing that epithelial and endothelial cells, macrophages, and mast cells (33, 34) can be cultured together to study the impact of both engineered and environmental particles.

Liver- and nephrotoxicity profiles of nanomaterials used as carriers in nanobiomedical applications must be well defined since the materials have direct access to those organs when injected into the bloodstream. Nanomaterial biodistribution studies elaborated in animal studies show a significantly high accumulation in the mononuclear phagocyte system (MPS) (35). The liver, a multifunctional organ of the digestive system, plays a central role in homeostasis and detoxification of foreign substances (including nanomaterials) reaching the bloodstream (36). A number of in vitro approaches including two- (2D) and three-dimensional (3D) systems used to assess hepatotoxicity were summarized in great detail by Godoy and colleagues in 2013 (37). In short, significant differences were observed in the response of hepatocytes after treatment with different drug substances depending on whether the cells were cultivated in monolayers or as 3D cultures, indicating the importance of the model system on the biological effect assessment. Another recent report shows the potential of 3D liver microtissue models generated by primary hepatocytes for the toxicology assessment of nanomaterial exposures (38). Although promising 3D systems have been reported, the majority of the hazard assessments of nanomaterials to date have still involved hepatocyte monocultures, probably due to lack of clear guidance or validated advanced human in vitro systems.

The kidney, with its central role in metabolism and blood filtration, is continuously exposed to adverse metabolites, drugs or nanoparticles, and therefore must be considered in safety assessment. A recent study developed a 3D organoid kidney proximal tubule epithelial cell system based on isolated proximal tubules from male C57BL/6 mice cultivated in hyaluronic acid hydrogels (39, 40). Well-defined fluorescein isothiocyanate (FITC)-labelled carboxyl-terminated poly(amidoamine) (PAMAM) dendrimers <6 nanometers in diameter were applied to consider an in-depth in vitro - in vivo correlation study. These dendrimers produced a set of toxicity indicators which accurately reflected the damage observed in vivo (41), indicating high predictive power of the system for nephrotoxicity. Further extension to disease models or human proximal tubules would help to strengthen this promising approach.

Despite all the advantages described in these examples, advanced in vitro systems have become more and more time- and cost-intensive, and well-trained experts are also needed to work with complex cell culture systems, all issues that should not be neglected. Therefore, ex vivo tissue preparation might present a viable alternative approach to obtain predictive model systems.

Ex vivo tissue preparation: bringing in vivo tissue into Petri dishes

A promising alternative approach that provides a better in vivo-like environment is the use of precision-cut tissue slices (PCS), which represent an ex vivo model of the organ of study by maintaining the original architecture, i.e., containing all the cells types of the tissue in their natural conformation (42). The advantage of this system is that slices from different species can be prepared and compared, such as from rodents as well as human biopsy material. Particularly impressive progress towards this end has recently been made in the field of lung research [for a review see (43)]. Although most studies have focused on aspects of pharmatoxicology (44, 45), some recent publications have proven that PCS-based approaches are highly relevant for the risk assessment of nanomaterials and xenobiotics in general, in terms of inflammation, organ injury and sensitization (46–48). A recently published study showed, however, that the system might only be useful for ions released from nanoparticles or soluble substances since it was observed that the silver nanoparticles did predominantly attach at the cut surfaces of the PCS from lung tissues (Figure 1C) but could hardly penetrate into deeper regions (49).

Fewer studies have been published using liver PCS to assess the interaction of nanomaterials with the organ slices (50, 51). The disadvantage of this technique is that by cutting the organ into slices the surface is covered with damaged cells, which might themselves induce an inflammatory reaction. However, in general, this system has great potential and warrants further exploration.

Regardless of the chosen approach (bottom up or ex vivo), the balance between relevant output data and throughput capability has to be evaluated carefully. Not only throughput suitability but also the development of high content analysis applicable to advanced in vitro as well as ex vivo models should be considered to be able to gain further mechanistic understanding in (nano)material safety assessment [see also Ref (52) for more detailed insight].

Organs-on-chip approaches

Innovative in vitro platforms based on microfluidic technologies and aimed at assessing nanomaterial safety

and efficacy are currently emerging [for a review see (53)]. These systems, called "organs-on-chip" (54, 55), intend to mimic the physiological conditions and architecture of human tissues. 3D organoids made of human cells are created on bioengineered platforms in order to simulate key organ-level functions (56). These systems, which mimic the in vivo environment of specific organs in an unprecedented way, are widely seen as having the potential to improve in vitro model accuracy and experimental efficiency. Although most of the ongoing efforts target the development of more predictive preclinical in vitro models (57), the potential of these devices for toxicology evaluation of chemicals, such as nanomaterials, is undisputable.

One of the key assets of these novel technologies is their capability to accurately reproduce specific aspects of the cellular microenvironment of various tissues. They not only enable the creation of microstructures with dimensions that are similar to those of mammalian cells, but also allow control of the cellular microenvironment in space and time. The perfusion of cell cultures confined in such systems enables them to mimic the continuous transport of nutrients and oxygen, the dilution of the secreted cytokines and chemokines, in addition to the cellular waste products (58). Insoluble signals can also be reproduced by modifying the cell culture surface topography or stiffness, and/or by the addition of specific extracellular matrices (59). Mechanical stimuli, such as the cyclic mechanical strain of respiration movements, are additional and important factors that affect the cellular and organ homeostasis (60).

Microfluidic technologies offer further possibilities to recreate the cell microenvironment, including the integration of cell culture membranes in microfluidic chambers, allowing for the creation of bioartificial barriers. Microfluidic barriers have recently been reported to recreate a number of in vivo barriers, such as the liver sinusoidal barrier (61), the blood-brain barrier (62, 63), and the gut (64), to name just a few.

Microfluidic air-liquid interfaces mimicking the alveolar barrier using porous membranes have also been described. Nalayanda and colleagues reported a perfused lung alveolar epithelial layer cultured on a PET porous membrane (65), whereas Zheng et al. (66) investigated the mechanical stresses induced by liquid plug propagation in a similar airway model. Recently, even more sophisticated lung alveolar models were reported that were able to mimic the mechanical processes of physiological breathing and the shear stress induced by the blood stream. Douville et al. (67) reported a microfluidic chip equipped with a 100 µm-thick poly(dimethysiloxane) (PDMS) membrane on which epithelial cells were cultured and cyclically stretched. This microfluidic alveolar model allowed the recreation of the fluid and solid mechanical stresses taking place in the alveoli during mechanical ventilation. In contrast to the latter model, Huh et al. (68) reported an innovative "breathing" lung-on-a-chip device in which the air-liquid interface was reproduced, with a 10 µm-thin, porous PDMS membrane on which epithelial and endothelial cells were cultured. In an experiment in which the epithelium was exposed to 12 nm silica nanoparticles, evidence was obtained that "breathing" motions greatly accentuate the pro-inflammatory activities of silica nanoparticles. Furthermore, it was demonstrated that translocation of these nanoparticles across the alveolar-capillary interface was significantly increased in mechanically-stressed alveoli in comparison with static controls. Very recently, a novel lung alveolar barrier model was reported consisting of a 3.5 µm thin, porous and flexible membrane on which epithelial and endothelial cells were cultured and whose actuation mechanism is inspired by the lung diaphragm contraction (Figure 2). A permeability assay carried out with this advanced model revealed that the permeability of small hydrophilic molecules (FITC-Na^+) was significantly increased if the cells were mechanically and cyclically stretched. In contrast, no significant effect was observed for a larger molecule [rhodamine B isothiocyanate-dextran (RITC-dextran)](69). These results are comparable to an in vivo study (70) that showed a higher clearance of hydrophilic solute upon increasing the human lung volume, suggesting that the novel lung alveolar barrier model better mimics the in vivo situation than existing in vitro models.

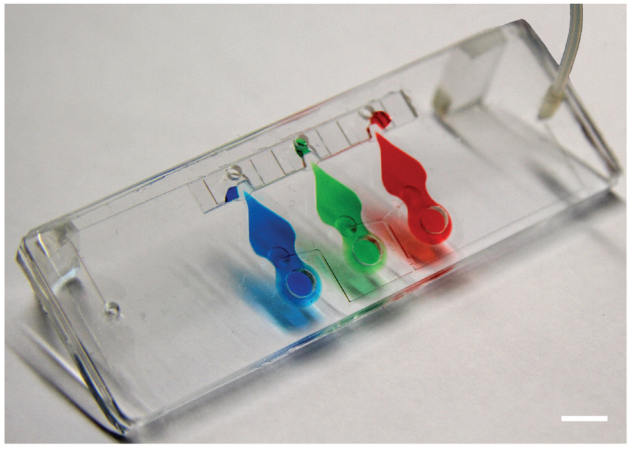

Figure 2: Example of an organ-on-chip: A lung-on-a-chip made of an array of three lung alveoli with a thin, alveolar barrier that can be cyclically stretched to mimic the respiration movements. Scale bar 5 mm.

Although the expectations in the potential of such approaches are very high, their development is still in their infancy. More validation steps will be required to identify their unique possibilities and the benefits of reproducing organ-level functions (e.g., breathing movements). One of the key factors that will need to be improved is the shift towards more relevant cells (e.g., primary cells) from the cell lines that are still broadly used in such models. Once these requirements are fulfilled such models may well provide low-cost alternatives to animal and clinical studies for faster and more efficient drug screening and material toxicological testing.

Qualification of reliable and accurate in vitro models

The early results of 2D model systems assessing the toxicity of nanomaterials were often contradictory, for several reasons that were outlined in a number of publications over the last few years (71–75), and lacked in vitro - in vivo correlation. The reasons can be summarized into five groups:

(i) no correlation between simple in vitro models and in vivo measurements (39–41)
(ii) no informative assay controls documenting the performance characteristics of the model system (76)
(iii) no adequate calculation of the applied dosages of nanomaterials
(iv) absence of appropriate interference controls uncovering potential interference between nanomaterials and the detection system (73)
(v) absent or incomplete characterization of nanomaterials (71)

The first reason (i) illustrates the poor relevance of common in vitro cell assays for the assessment of acute toxic effects of nanomaterials on full biological organisms. Furthermore, recent results of an interlaboratory comparison underline that the last four aspects (ii–v) are important in obtaining an adequate comparability between the results of the different participating laboratories.

A number of recent studies illustrate that basic 2D cell culture models lack a reasonable correlation with in vivo studies (39–41). Therefore their predictability and relevance is also poor. At the same time the correlation between rodent models and humans is around 60% (77), which leads to some augmented risk in early clinical trials. Recently published results demonstrate some correlation between advanced 3D cell culture systems and in vivo models (39–41). This highlights the importance of the adequacy of the selected model systems and their qualification with respect to reliability and accuracy. In a number of instances it proved difficult to determine the reasons for the poor comparability of common cell assays used for nanotoxicity assessments, as the controls were inadequate to document proper functioning of each step of an experimental procedure. A cause-and-effect analysis was an effective tool to categorize the various influences affecting the performance of common MTS cell proliferation assays (78). Based on this outline, a number of controls that monitor the assay performance for the different steps of the procedure were integrated into the design of the standard 96-well plate. Results of a recent interlaboratory comparison showed the combined strength of these controls to uncover potential shortcomings in the standard operating procedure (unpublished observations, Dr. Matthias Roesslein). In addition, the full titration of the chemical positive control and the deduced EC_{50} value that is within tight specifications allows for documentation of the proper functioning of the entire assay itself for each experiment. This enables comparability within and between laboratories over a longer period of time. The six major categories of a cause and effect analysis, such as cell maintenance, pipetting, instrument performance, toxic chemical positive control, assay protocol, and engineered nanomaterial handling and characterization, will also help in determining most influences of advanced 3D cell culture systems. This systematic summarization of effects facilitates the overview of functional principles of even complex cell culture systems. Furthermore, it allows the design of adequate controls that document their performance characteristics, in particular the EC_{50} value chemical positive control.

Until the publication of the in vitro sedimentation, diffusion and dosimetry (ISDD) model in 2010 (76), there was only a limited understanding of the actual dosing levels of nanomaterials during nanomaterial-cell investigations using adhesive cell culture assays. With all relevant information stated in the publications, this allows conversion of the common weight/volume as the dosing unit of the nanomaterials to the actual number of particles interacting with the cells over the duration of the experiment. Even at the early stage of any advanced models, the calculation of the proper dosing of the investigated nanomaterials is examined and potential scenarios have been outlined. The model has been extended by Rodriguez-Lorenzo and colleagues, who included heterogeneity, including polydispersity both in size and mass density to study the influence of heterogeneity on the particokinetics of nanoparticles and on the corresponding delivered dose (79). Therefore it will be essential that at least a full set of

dosing-relevant characteristics is given in a publication so that in the future a direct comparisons of results of the different approaches is feasible.

Interference controls will require further optimizations, as the readout system of any endpoint in a co-culture or advanced 3D model system will be refined due to the more complex structure of these systems compared to a simple adhesive cell culture. In addition, basic materials characterization of any nanomaterial remains essential, regardless of the applied type of in vitro or ex vivo model system (71).

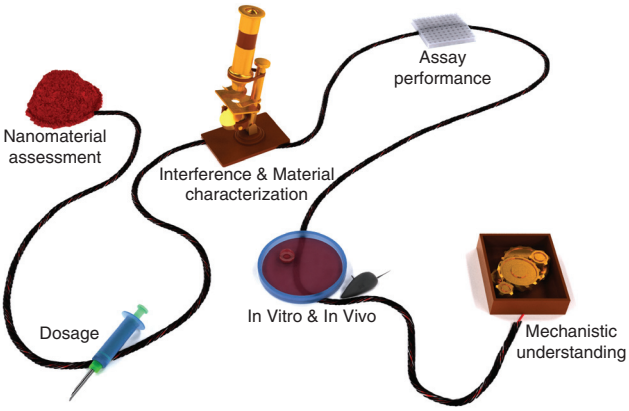

Figure 3: Important components that allow to link nanomaterial assessment with mechanistic understanding in a biological system in a reliable way.

Regulatory environment

This review focuses on the scientific issues, the possibilities and limitations of advanced in vitro – and ex vivo model systems established to date for the risk assessments related to nanomaterial exposure. It summarizes the models that may one day have to qualify for the regulatory review and some unresolved questions about a "fit for purpose" solution within the current regulatory framework. Two different regulatory frameworks address the specific objectives of industrial and medical applications. Industrial nanomaterials are regulated under the REACH and OECD guidelines following the tracks of chemicals with toxicity testing for product fillings. In addition, within the EU there are considerable efforts to replace the expensive and controversial discussed animal testing with in vitro alternatives (5–7). Despite the fact that changing this regulation is a long process, first efforts have been successful within the EU with a ban on animal testing for skin allergies of cosmetics (80). In contrast, the regulatory environment of medical related nanomaterials is highly fragmented with individual national regulations. The testing strategy of the pharmaceutical industry focuses mainly on avoiding failure during clinical trials and their tremendous costs. Therefore they have a considerable interest in novel in vitro models able to better predict the clinical outcome.

Conclusions

Due to the inevitable exposure of nanomaterials to humans it is imperative to gain an understanding of how these materials interact with the human body, since there are increasing concerns as to the potential adverse effects on human health that the production of, and subsequent exposure to, such nanomaterials might pose. In the field of regulatory toxicology, animal testing is still the most prevalent model used for risk assessment. It is, however, time to realize that a paradigm-shift in the understanding of toxicology towards a modern evidence-based research discipline can be supported by advanced in vitro and ex vivo models. It has already been recognized that in vitro and ex vivo models should be able to depict the complexity of an organ or tissue as far as possible, while maintaining the capability for standardization, high throughput and reproducibility. In addition the relevance of these models has to be validated towards animal models and especially towards human related epidemiological studies for nanomaterials exposure. Furthermore clinical trials will be needed to assess the relevance of theses novel models for nanomaterials to be used for medical purpose.

Extensive efforts have been made to simulate different organs in a petri dish, and predictive cell culture or ex vivo models have been generated. It will require, however, a combined effort of many disciplines to focus more on the reliability of such systems by considering the thorough characterization of nanomaterials, but also the cell culture systems. In addition, validation with well-known toxic substances should be emphasized in order to gain a proof-of-concept.

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