Advanced human *in vitro* models to assess metal oxide nanoparticle-cell interactions

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Engineered nanoparticles, in particular metal oxide nanoparticles, with their unique and novel properties, enable a plethora of new applications in various fields of research. These new properties have raised concerns about potential adverse effects for the environment and human health and are nowadays very controversial. A reliable, cost- and time-effective, rapid and mechanistic-based testing strategy is needed to replace current conventional phenomenological assessments. Today's *in vitro* technology, providing human-based advanced cellular models representing different organ barriers such as skin, lung, placenta, or liver, may cover this need. The aim of this article is to present the current changes in (nano) toxicology strategies, the extent to which *in vitro* models have achieved general acceptance, and how the relevance of these models can further be improved using examples of selected metal oxide nanoparticles.

Introduction

Nanotechnology enables the possibility to engineer materials in the nanoscale range with remarkable new physical and chemical properties different from their bulk, which can be used for a broad range of applications. This huge potential has led to increasing growth of research and development activities all over the world and has created an entire new class of materials. Nanoscale metal oxides are a well-developed subclass of nanoparticles known for decades in colloids long before the current explosion of interest in nanoscience.

These new properties and industrial production in high tonnage have raised concerns about potential adverse effects for the environment and for human health. Aside from the broad industrial use of metal oxide particles for nanotechnology, there are substantial research and development activities to apply nanomaterials such as zinc oxide (ZnO)[1] or super paramagnetic iron oxide nanoparticles (SPIONs)[2] in medicine. Therefore a better understanding of the mechanisms show how nanomaterials interact with cells, and the consequence is a prerequisite for their safe and successful use in any application.

The assessment of potentially adverse or off-target effects is currently based on phenomenological analysis of animal testing—a strategy that has not changed in the last 40 years.[3] However, the number of newly developed particles is large and is still increasing, as are consumer expectations about their safety. A full assessment of the potential side effects according to today's regulations would be extremely cost intensive and time consuming, and its relevance for human beings is still doubtful.[4] Therefore new concepts for more efficient, cost-effective, and evidence-based testing strategies toward mechanistical-based understanding of the nanoparticle–biology interaction are proposed.[5]

Despite the broad use of traditional cell-based *in vitro* cell monocultures, it is recognized that these models lack phenotypic details, physiological functions, or depict the complex cross-talks between different cells only partly or not at all. There are multitudes of new co-culture or 3D cell culture systems (i.e., cells cultured on 3D scaffolds or co-cultures of various cell types) for different tissues developed during the last 10–15 years, combining several relevant cell types for the same organ into one culture system. So far, only a few

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have been carefully validated to whole organ or *in vivo* responses.[6] The crux of any *in vitro* technology is the predictive power, reliability, and usefulness of these model systems for regulatory toxicology.[7]

The aim of this article is to present the current developments in complex human cell models describing recent work in relation to the assessment of metal oxide nanoparticles. We demonstrate how far advanced human *in vitro* models have achieved general acceptance and how the relevance and acceptance of these models can be further improved (e.g., for metal oxide nanoparticles hazard assessments).

Metal oxide nanoparticles enable new applications for industrial and medical use

Metal oxide nanoparticles have been identified as the most widely used type of nanoparticles in consumer products.[8] Market estimates for production have been increasing over the last several years, estimated to reach 1,663,168 tons by 2020.[9] A large variety of metal oxides are known, with a vast number of crystallographic structures and with metallic, insulating, or semiconducting electrical behavior.[10] Titanium dioxide (TiO_2) and cerium dioxide (CeO_2) are two important representative metal oxide nanoparticles that are currently used in many products such as sunscreens, paints (TiO_2), energy, and fuel additives (CeO_2). Numerous research papers, reviews, book chapters, and patents focus on synthetic approaches to produce highly defined metal oxide nanoparticles. Since many of their chemical and physical properties depend on their size or shape, much effort has been dedicated to produce "perfect" particles with high yields and develop a synthesis method that is more generally applicable to many different nanoparticle systems[11] (**Figure 1**).

Typical approaches for the synthesis of nano-metal oxides include co-precipitation and sol-gel techniques, microemulsions, solvothermal methods, and chemical vapor deposition (CVD). All methods have their advantages and disadvantages; while the co-precipitation method is usually easy to reproduce, size control is not necessarily easy to achieve. CVD is capable of producing highly uniform and pure nano-metal oxides but requires careful initial set up of the experimental parameters. Nonaqueous solution techniques are an interesting alternative to aqueous sol-gel processes, since they can provide a large degree of crystallinity at low temperatures and allow for controlled crystal growth without the use of surfactants.[12]

With regard to nanomedicine, SPIONs have attracted a lot of interest for numerous therapeutic and diagnostic applications, such as magnetic resonance imaging (MRI), drug delivery, hyperthermia, and cell separation.[2] SPIONs have been synthesized with different compositions and phases.[13] Chemical co-precipitation is an easy and convenient approach to synthesize iron oxides from aqueous solutions, and size, shape, and composition of the resulting particles strongly depend on the reaction conditions, in particular the reactants used, pH, and ionic strength of the media. Monodisperse magnetic nanoparticles can alternatively be synthesized by high-temperature thermal decomposition of organometallic precursors in the presence of stabilizers.[14] The overall process is often compared to seed-mediated growth, which can be explained by the classical LaMer mechanism.[15] Although the particle size distribution can be much better controlled following these organic decomposition pathways, the final particles are dispersed in organic solvents and require a subsequent phase transfer step from the organic solvent into water.

Although significant progress was made in recent years to evaluate the toxicological profile of metal oxide nanoparticles,[16] it is still difficult to compare these studies because of biological variations (e.g., choice of cells, growing conditions, sample preparation, time points, and assays) and material variations (e.g., particle size [distributions], zeta potential [an important indicator of the electronic charge of the nanoparticles' surface], colloidal stability, type of material, batch-to-batch variations, and size and grafting density of surface molecules).

Examples of advanced 3D *in vitro* models

In the field of human health, animal testing is still the most prevalent model used for risk assessment of newly developed

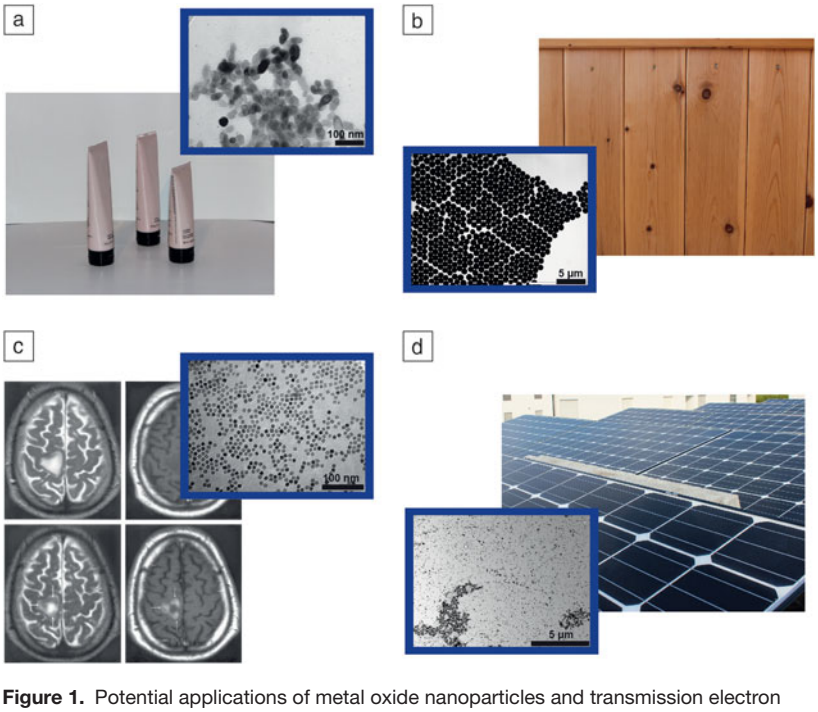

Figure 1. Potential applications of metal oxide nanoparticles and transmission electron microscope micrographs of the respective nanoparticles: (a) TiO_2 in cosmetics, (b) SiO_2 in wood preservatives or paints, (c) iron oxide nanoparticles as contrast agents in magnetic resonance imaging,[70] and (d) ZnO in solar cells.

nanomaterials such as metal oxides. However, three main factors create a strong need for *in vitro* alternatives: regulatory pressure to ban animal testing, pressure to reduce cost in drug development, and concerns with respect to the significance of animal experiments to model human health. In addition, reliable and cost-effective screening systems are required not only for basic research questions, but also for drug discovery and toxicity assessment. It has been recognized during the last few years that not only the 3D scaffold structure seems to be important for the differentiation of certain cells,[17] but also the culturing of different cells together is an important issue for continuous cross-talk through intercellular signaling to maintain homeostasis and to coordinate immune responses.[18] Both aspects (i.e., 3D scaffold and cell–cell interactions) cannot be mimicked by simple monocultures. Therefore, the overview of cell models given within this review will focus only on recent 3D tissue engineering development for (nano) toxicology research (**Figure 2**).

Another aspect is the ongoing discussion about the use of primary cells versus stable cell lines.[19,20] Primary cultures are cells isolated from animal or human tissue, whereas cell lines are immortalized cells that can proliferate indefinitely. Both systems have advantages and disadvantages, and for each model, the reproducibility and reliability have to be considered. If cell cultures are used properly, they represent a sophisticated and reproducible system with which basic questions can be answered and which may help in understanding the physiological processes *in vivo*.

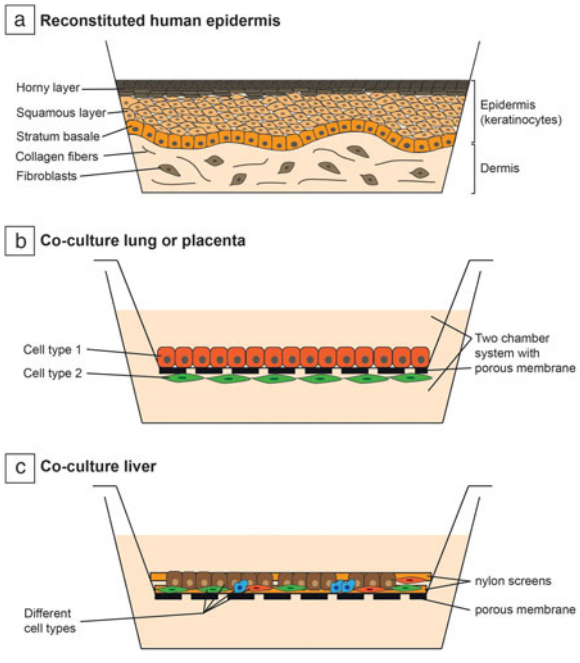

Figure 2. Examples of advanced human *in vitro* models. (a) Skin: All layers of the epidermis are represented in the *in vitro* model for the human skin. (b) Lung or placenta: In this transwell model, the translocation of endogenous and exogenous substances across a biological barrier can be studied. Therefore, the substance of interest is added to the apical chamber, and its appearance in the basal chamber is analyzed. (c) Liver: This 3D model consists of several primary cells isolated from the liver. The porous membrane facilitates an optimal nutrient supply from the medium in the basal chamber, and the nylon screens promote cell growth in 3D. Adapted with permission from Reference 34.

Artificial human skin model accepted for regulatory toxicology

The epidermis, which is the external surface of the skin and the first line of defense, consists of a keratinized squamous epithelium (cornified layer), which is supported and nourished by a thick underlying layer of connective tissue referred to as the dermis, which has many blood vessels and contains many sensory receptors.[21] A major function of the skin, especially the *stratum corneum*, which is the outer most layer of the keratinized squamous epithelium, is to provide a protective barrier against the hazardous external environment. The skin is relatively impenetrable to lipophilic particles larger than 600 Daltons in size (equivalent to 2–3 nm for a structural protein or 0.5 nm for a globular protein), whereas lipophilic particles smaller than this may passively penetrate the skin.[22] Skin can be exposed to metal oxide nanoparticles such as ZnO or TiO_2 present in cosmetic products such as moisturizers and sunscreens as an UV (ultraviolet) adsorber.[23] The skin is also a potential target for drug delivery via nanocarriers.[21] Many studies have been done with simple monocultures using skin epithelial cell lines, such as the epidermal cell line A431[24] or the immortalized human keratinocyte cell line HaCaT,[25] both of which have been used to study the effects of silver,[26] TiO_2,[27] or SiO_2 nanoparticles,[28] to mention some examples. In general, it can be stated that healthy human skin is an efficient barrier against most nanoparticles in cosmetics such as ZnO or TiO_2.[22]

Cytotoxicity testing of chemicals and cosmetics in *in vitro* skin models goes back to the 1980s (OECD guideline 431, Organization for Economic Co-operation and Development), and the first co-cultures of skin tissue composed of human epidermal keratinocytes and fibroblasts (cells that form the connective tissue that makes and secretes collagen proteins) from the dermis, which is between the epidermis and the subcutaneous tissue, were described in the 1990s.[29] In 2013, OECD guideline 431 was adapted to a protocol that does not require the use of animals, and it provides an *in vitro* procedure allowing for the identification of non-corrosive and corrosive substances and mixtures. Within this guideline, the use of reconstructed human epidermis composed of several epithelial cell layers, which closely mimics the histological, morphological, biochemical, and physiological properties of the upper parts of the human skin, is recommended. Commercially available models such as the EpiDerm skin model[30] as well as the EPISKIN[31] are two examples of validated 3D models for this guideline; however, their potential in hazard assessment for metal oxide nanoparticles has not yet been described in the literature and needs to be evaluated more thoroughly.

Lung and liver—Two well-established in vitro *co-culture models for basic research*

As already stated previously, the 3D model of human skin is so far the only accepted *in vitro* alternative in regulatory toxicity. However, many other 3D culture systems have recently been described in the literature for toxicity testing studies. One example is the liver, which plays a central role in the metabolic homeostasis and detoxification of any xenobiotic, which is a foreign chemical substance, reaching the liver,[32] whereas the CYP450 enzyme is responsible for the metabolism of drugs and chemicals in the hepatocytes, which are the main cell types in the liver.[33] A recent paper described an effort to build a 3D human liver chip mimicking the acinus, the smallest functional unit of the liver, including its oxygen gradient;[34] however, the prediction of this liver model for toxicity testing purposes has not yet been described. Another attempt describes a 3D liver cell co-culture composed of hepatocytes, hepatic stellate cells (fat-storing cells), endothelial cells (thin layer of cells lining the interior surface of blood vessels), as well as Kupffer cells (macrophages), which has been validated to assess the toxicity of some well-known liver drugs.[35]

Although promising 3D liver models have been reported, only a few papers describing metal oxide effects in these advanced models have been found so far. The majority of the hazard assessment studies for nanoparticles are still performed in hepatocytes[35] or Kupffer cells[36] in monocultures, and further research is needed until a well-accepted 3D model for the liver has been tested and validated thoroughly.

Another well-described organ for which many co-cultures exist is the respiratory tract. Since the morphology and function of the respiratory tract changes completely from the upper to the lower airways, many cell culture models have been established using primary cells or cell lines to represent the area of interest for a certain study. Since the lung is the main portal of entry for aerosolized nanoparticles, *in vitro* co-cultures mimicking the air-blood barrier with two cell types have been described in the literature to study the impact of nanoparticles in the lung.[37]

Recently, a triple cell culture *in vitro* model of the human airway wall to study the cellular interplay and the cellular response of epithelial cells, human blood monocytes derived macrophages (i.e., professional phagocytotic cells), and dendritic cells (i.e., professional antigen-presenting cells to nanoparticles has been developed (see Reference 38 for a review). In addition, a quadruple-culture containing epithelial and endothelial cells, macrophages, as well as mast cells (i.e., granulocytes containing histamines) has been established.[39] Studies using such co-cultured cell systems have reported different reactions compared to monoculture analysis when the cells were exposed to nanoparticles, including TiO_2;[40,41] however, such reactions observed from a culture containing two, three, or four different types of cells merely cultured in the same dish are not as specific as those that would occur in the human body. Thus, the architecture of the *in vitro* cell co-culture model, in regard to the specific organ they represent, is essential when nanoparticles effects are studied.

However, all these described lung models have thus far failed to provide organ-level functionalities (e.g., molecular transport across tissue-tissue interfaces, contributions of vascular and air flow) that are required for the development of meaningful physiological models.[42,43] A potential solution to this problem is the development of human "organs-on-chips" in which microscale engineering technologies are combined with cultured living human cells to create microfluidic devices that recapitulate the physiological and mechanical microenvironment of whole living organs.[44] These organomimetic microdevices enable the study of complex human physiology in an organ-specific context and, more importantly, they offer the potential opportunity to develop specialized *in vitro* human disease models that could revolutionize drug development.

There is no closing statement about the toxicity of metal-oxide nanoparticles, because the research in regard to cell culture types and nanoparticles exposure is not standardized yet. However, for single types of metal oxides such as ZnO[45–47] or amorphous SiO_2,[48,49] the underlying mechanism is well-elucidated (e.g., dissociation into Zn ions or only transient pro-inflammatory effects, respectively), whereas others such as TiO_2 or AlO_2 are more controversial.

New technologies for complex co-culture systems for human in vitro *placenta model*

The placental barrier is a fascinating and multifunctional organ with the highest species-to-species variability in mammals,[50] which underlines the necessity to have access to human-based models. In addition to the severe thalidomide-induced birth defects in the 1960s[51] and exposure to particulate air pollution,[52] the development of new concepts in nanomedicine[53] requires a valid and predictive human placenta *in vitro* model, which could also be a link toward reproductive toxicology.[54] To fulfill these requirements, the dual perfused *ex vivo* human placenta model was developed in 1967, allowing the placental tissue to stay alive in order to assess the translocation rate across the tissue barrier of substances or particles.[55,56] In the meanwhile, classical *in vitro* approaches, such as transwell systems,[57–60] isolated plasma membrane vesicles, or placental tissue explants,[61] all well-established alternatives to the time-consuming *ex vivo* model, allow higher throughput. Furthermore, the use of microfluidics technology[62] and scaffold-free self-organized organoid cultures[63] are anticipated to stimulate and accelerate the process of *in vitro* model development substantially, and not just for the human placenta. Knowledge of metal oxide particles' off-target effects at the placental barrier is still in its infancy; however, several research activities are ongoing[64–68] to provide more mechanistic insights in the near future.

Today's technology and engineering competences combined with an in-depth knowledge of the physiological responses of a tissue or organ and the appropriate validation strategy will not only create the fundamentals necessary for reliable and predictive models to complement and reduce animal testing, but also help identify faster off-target effects of substances or particles.

Relevance of advanced *in vitro* models: There is space for improvement

Conventional two-dimensional (2D) cell cultures of any desired organ or tissue can be used as the basis for simplified biological models in order to obtain controllable and reproducible data. Such a radical simplification compared to the physiological environment should also imply that the predictive power is limited.[69] The change from carcinoma cell line to human primary cells, from monoculture to co-cultures, from static to perfused systems, or from 2D to 3D culture systems alone or in combination can help significantly improve the physiological function of the modeled tissue or organ toward the *in vivo*-like situation. The example of the *in vitro* skin models that have achieved acceptance as an OECD guideline for skin irritation assessment in 2010 underlines in an impressive way that focused development and validation over years of such models supported by research, authorities, industry, and consumers can be seen as pioneering for additional human *in vitro* models (**Figure 3**).

However, as in the case of all models, the advanced human *in vitro* models have their limitations, too. For the determination of absorption, distribution, metabolization, or excretion, animal models will still have their justification. Close collaboration and strict validation of these newly developed advanced *in vitro* versus mammalian models with well-known benchmarking materials or substances will accelerate the further development and improvement of co-culture and 3D models toward well-accepted alternatives to pure animal testing and contribute significantly in the "fail faster process," the identification of failure as early in development as possible, for nanoparticle-based innovations.

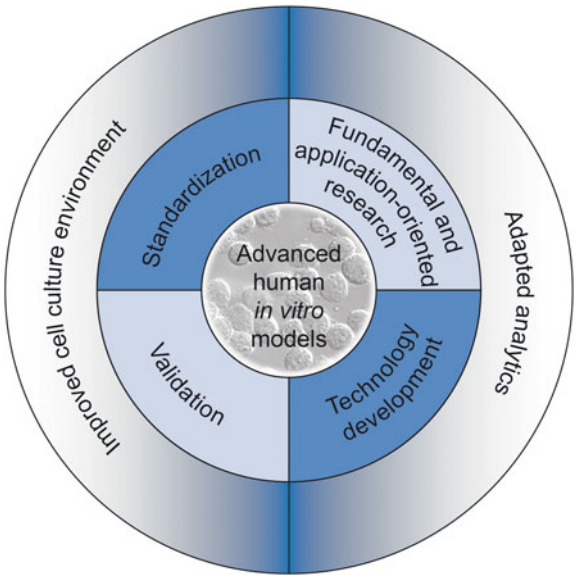

Figure 3. Scheme summarizing the actions to be taken to further improve the relevance of advanced human *in vitro* models.

Conclusions

It is time to realize that a paradigm shift in the understanding of toxicology toward a modern evidence-based research discipline supported by advanced *in vitro* models is inevitable. It is already recognized that *in vitro* 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. However, there are still uncertainties in our knowledge on the toxicity of metal oxides and other nanoparticles that could be reduced by obtaining more comparable and predictive data. Hence, efforts are still required to develop and carefully validate advanced *in vitro* models. Faster and reliable identification of toxic materials by using emerging new technologies will accelerate safe, sustainable, and functional nano-applications.

Acknowledgments

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