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An Alternative Perspective towards Reducing the Risk of Engineered Nanomaterials to Human Health

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To elucidate the impact of human exposure to engineered nanomaterials, advanced in vitro models are a valid non-animal alternative. Despite significant gains over the last decade, implementation of these approaches remains limited. This work discusses the current state-of-the-art and how future developments can lead to advanced in vitro models better supporting nano-hazard assessment.

1. Introduction

Engineered nanomaterials (ENMs) can be defined as "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 1–100 nm."[1] While much is made of which definition of an ENM should be used,[2] the one defined by the European Union (EU) in 2011[1] is most apt even today, as it considers the development of ENMs into products, and how they may interact with other entities, and not simply just the raw materials for focus by scientific research only.[3] This is particularly important, since ENM are advantageous for numerous usages, including industrial, consumer, medical, and environmental applications, all of which will lead to elevated human exposures.[4] Their mass application to such areas drives the total value of the nanotechnology industry to the $1 trillion level, and beyond.[5] To achieve this, significantly large ENM quantities must be produced on a yearly basis. To put it into perspective, it has been calculated that the total amount of titanium dioxide ENMs, often found in many cosmetics[6] (e.g., suncreams, moisturizers) and has received increased attention recently from a regulatory context,[7] are produced at over 45 000 tons per year.[8] Meanwhile, carbon nanotubes, proposed for use as a construction material and found in car tires,[9] which also seemingly receive constant negative attention due to their physical characteristics,[10] are estimated to be produced at only >1200 tons per year.[8] Thus, considering the fact that there are thousands of ENMs already used in industrial applications,[11] and thousands more in production,[12] human exposure is an inevitable outcome of nanotechnology.[13] Yet, to fully deduce the opportunities that nanotechnology-orientated applications and methods can have towards human health, understanding of the potential risks posed must be gained.[14]

The field of nanoparticle toxicology,[15] or as it is known nowadays as "nanosafety,"[16] is a constantly evolving discipline. In the 1990s, the first articles were only just being published in terms of nanoparticle toxicology,[17] leading to the sub-discipline of particle and fiber toxicology, that is, "nanotoxicology," being coined.[18] Further, and only over a decade ago, the field was focused upon determining which specific physico-chemical characteristics should be most prominently defined in order to understand structure–activity relationships.[19] To the present day, research continues to increase almost daily in order to fully elucidate both the potential opportunities and risks posed by ENMs.[20,21] How ENM risk is determined however, is a topic of constant debate and heightened research activity, as well as concomitant to the research actually assessing ENM risk.[22] Notably, enhanced scrutiny is currently focused towards which biological systems are most apt to elucidate the human health impact of inevitable exposure to ENMs.[23] Numerous questions remain as to the relevance, validity, and morality of using invasive animal experimentation. Further, with the EU legislative ban upon in vivo research within the cosmetics industry,[24,25] as well as the recent announcement of the United States Environmental Protection Agency to ban all animal testing by 2035, a heightened emphasis is nowadays given towards alternative approaches, namely, in vitro approaches. Thus, having an (advanced) alternative biological-based model system that can re-create the key components of a complete organism has become the ultimate scientific desire.[23] Despite the great strides taken in the past few years towards this objective there remains a plethora of questions, most importantly which alternative models, if any, have the potential to meet this requirement for not only basic research, but also for industrial and regulatory hazard characterization and risk assessment purposes.[26,27]

2. Motivation to Create Valid Alternatives

Although it is considered, still, the gold standard for toxicology testing,[28] in vivo approaches remain questionable in terms of

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their relevance towards fully understanding the hazard implications towards human health.[29] There have been plentiful examples throughout history in this regard. For example, the drug "Thalidomide" is inert within rodents, yet is significantly detrimental to the human fetus.[30] This highlights the differences within the reproduction systems/cycles of different mammals, and that any form of in vivo approaches to deduce impacts of drugs/xenobiotics upon the reproduction system/cycle must be specific to the species.[31] This is also true of other organs, for example, the lungs. In a canine, the lung tissue has microscopic pores throughout it, which is not the case in the normal human lung.[32] The surface area of a rodents' lungs, while mathematically can be correlated to humans, are different structurally.[33] Further, primates (e.g., rhesus monkey) have significant differences in the structure (i.e., number of lobes) compared to the human lung,[34] and therefore absolute direct comparisons are not possible. Thus, it is questionable as to their relevance as a surrogate of the human lung to determine the impact of inhaling particulates.[35] Further, while the cell types within a rodents' lungs (e.g., mouse), at the macromolecular level can be similar, when understanding the immunological impact of an inhaled foreign body it has been noted that intra-species cells can exhibit different receptors, making it difficult to conclude absolute implications from classical in vivo research towards human health.[36] Therefore, although in vivo (i.e., rodents, monkeys, dogs) provide the "whole body system," numerous questions remain, for example, their metabolism levels,[36] as to the pertinence and relevance of conducting such testing strategies to assess the human health implications of new materials, drugs, and other xenobiotics.[29]

Within the field of nanosafety however, since the EU ban upon animal experimentation in the cosmetic industry took effect,[24] the number of publications using the phrase "in vitro" or "alternative(s)" has increased on a yearly basis.[21] Based upon the decade prior to that, the number of articles, while increasing, was exponentially lower than after the EU ban.[21] This indicates that the ban upon cosmetics testing using in vivo systems was, to an extent, successful; however, it also highlights the fact that when animal experimentation was no longer a valid tool, it was possible to utilize an alternative approach.[37] With the increase in alternative models however, while initiating an intended reduction and refinement within in vivo approaches, it poses the question as to the usefulness of in vitro cell cultures, and furthermore what types of cell cultures should be focused on in terms of any proposed replacement strategy for the previously widely used, invasive animal testing approach.[38]

3. Current Status and Key Points of (Advanced) In Vitro Systems

As previously discussed by Evans and colleagues,[39] and further highlighted in **Table 1**, there are numerous different types of advanced in vitro systems that can be considered, spanning most organs of the human body.[40]

These can focus upon mammalian cell cultures that may be constructed as either single- or multiple-cell cultures, also referred to as mono- or multicellular systems, or as 3D models, otherwise known as "oid" cultures (e.g., organoids, spheroids).[41] Beyond static cultures, in whichever geometry, also include more physiologically relevant systems, such as microfluidic cell cultures,[39] or dynamic breathing approaches.[42] Interestingly, it is reported that for nearly every organ of the human body, these different cell culture approaches can be created and adopted for different experimental questions. However, not many have been created specifically to study the potential for foreign substances to cause toxicity, or even predictability of the human hazard response.[40]

Most commonly, such alternative systems focus on the use of cell-lines, often cancer cell-lines, due to their relatively low

Table 1. Summary of the advantages and disadvantages of the currently available advanced in vitro systems. Adapted under the terms of the Creative Commons Attribution 3.0 Unported (CC BY 3.0) license.[39] Copyright 2016, The Authors, Published by Oxford University Press on behalf of The UK Environmental Mutagen Society.

Type of advanced in vitro system	Structure/cellular construct	Advantages/disadvantages
Multicellular systems	Multiple (i.e., two or more) cell types cultured together, commonly within trans-well membrane inserts. These systems are indicative of a specific cellular construct of a specific tissue type within the human body	Enables cell-to-cell interplay representing important (barrier and immune) cell types relevant to target tissue
		High cost, laborious nature (often needing specific expertise) Considered highly indicative of the specific tissue being studied
Microtissues (e.g., "oid" culture systems)	Cell cultures that undergo a degree of self-assembly that enables the formulation of geometric structure that closely resembles the human tissue of interest (e.g., 3D tissue-like structure (spheroid) of the human liver)	Allows for important cellular interplay and specific geometry of anatomical structure
		High cost, laborious, short culture period and is unable to be used for certain biochemical endpoints depending upon size
Complex 3D structures	Models that are commercially available resembling primary cell cultures/multi-tissue layer systems and are representative of specific tissues	Closely mimics in vivo
		High cost, laborious nature (often needing specific expertise) Considered highly indicative of the specific tissue being studied
Micro-fluidic devices	Models (often mono- and co-cultures) that are incorporated into bio-engineered devices allowing additional in vivo relevance to the in vitro approach (i.e., fluid-flow (mimicking blood-flow) and/or dynamic movement (mimicking breathing pattern))	High cost, requires specific expertise, and additional equipment (i.e., peristaltic pump)
		Laborious to find optimal application of system with in vitro model
		Provides additional and advanced in vivo characteristics beyond static in vitro systems

complexity, although primary cells are also utilized. Importantly though, it is essential that the correct cell type is used to represent the correct organ. In many cases, it has been reported that cells from one organ are used to represent the same particular cell type, but in a different organ.[43] A prime example of this is the human umbilical endothelial cell (HUVEC). These are relatively inexpensive, are easy to culture, and can be used for multiple passages with little to no mutation shift. However, if used as a surrogate of lung endothelial cells, then the relevance of the in vitro system becomes nonsensical. Human lung endothelial cells (HULECs) exist, and while they exhibit similar characteristics to HUVECs, they are pedantically different with respect to organ specificity. Thus, a key factor in the creation of any in vitro model is that the key organ/tissue characteristics are understood at the single cell type level (or 2D) prior to initiating advanced in vitro models (of any geometry). Subsequent confirmation of these characteristics throughout the creation of any advanced in vitro model is also essential. The characteristics monitored however, can be related to the specific cell types being used. For example, immune cells, the phenotype, (pro-)inflammatory mediator baseline, morphology, and maturation status would be important. Whereas a barrier cell, for example, epithelial cell, the growth curve/proliferation index, barrier capacity/membrane integrity, and monolayer morphology would be apt. Irrespective, how the cells culture over passage number, identification of the maximum passage number is essential for any cell-line used. For primary cells, the source must be similar (and ethically approved), but more so a continuous quality control concerning the phenotype and morphology is imperative to maintain consistency across cultures and time.[44] The scaffold, or matrix that the cells are cultured upon is also significant in this regard and needs to be considered in these approaches.[45]

In addition to the baseline approaches and extensive characterization needed to create advanced alternative models, it is a common perspective that any alternative system beyond a monoculture has significant complexity requiring extensive training, increased consumable cost, and is more labor intensive. This is a usual misconception. The difficulty with any advanced, alternative model lies within the technique and characterization of the system (as noted above). Further, the specificity and relevance of the in vitro model to the in vivo (i.e., human) organ is paramount. There are some examples in the literature of complex multicellular systems that simply apply different cell types into the same dish. Human organs consist of complex, cellular structures creating the tissue system organ, and thus this must be represented as such in vitro; for example, the lung requires structural building of cell types, as shown in the model from Rothen-Rutishauser et al.[46] This is also true of spheroid cultures as well, in that the cellular architecture must be carefully constructed in order to allow the cellular self-assembly into the spherical geometry. Thus, knowledge of the anatomy and physiology of the organ-type model being created in vitro is necessary. Most notably, many in vitro systems have been created to study the cell-to-cell interplay, which is the widely cited major advantage of multicellular systems, as well as the role that specific cell types have in disease progression (e.g., fibrosis).[47] Interestingly though, for those systems that have been used for toxicology testing, it has been in the monoculture format, since these relate to many of the regulatory testing approaches (e.g., Organization for Economic Cooperation and Development (OECD) Micronucleus Test that uses TK6 human B-lymphoblastoma cells).[48] Nonetheless, there have been studies comparing the toxicological outcomes of using different cell systems (from monocultures to multicellular systems) to assess their suitability of ENM hazard characterization. For example, in the study of Clift and colleagues,[49] it was observed that when comparing the coculture of Rothen-Rutishauser and its representative cells at the monoculture level, that the toxicological outcome was the same, but the effect level was heightened in the multicellular system when controlled for cellular protein levels. Thus, it is important to deduce the relevance of advanced in vitro systems as an alternative to invasive animal experimentation.

4. Relevance of In Vitro Systems to Humans

One of the key questions related to in vitro systems in general is their relevance to the human system. While the pertinent, scientific research to support any answer to this question remains lacking, and needed. Relevance can of course be attributed to the systems characteristics and architecture (as previously described above). Yet, if we consider this further, many alternative in vitro systems published focus upon static cultures. Very few consider the notion that in the "whole body" in vivo approach all tissues/cells are at least impacted by dynamic flow—of either a liquid (i.e., liver) or air (i.e., lung).[50] In a study by Moore and colleagues,[51] it was reported that when cells were placed within a period of dynamic flow that their phenotype as well as transendothelial transport was significantly altered. It has also previously been noted that dynamic flow can change the maturation and differentiation status of cells in vitro.[52] Thus, this physiological parameter, while being vitally important to mimic a further dimension of the in vivo approach in vitro, seemingly creates a significant change within the cells compared to the static approach. There are a number of commercially available systems on the market, and so it would be essential to start to integrate such important physiological factors into advanced in vitro models in general. It is also important to note the "organ-on-a-chip" movement that is increasing the complexity of model systems and allowing multiple organs models to be connected in vitro.[53] However, as of yet this model system has not showed anything different to what is seen with single cell systems under dynamic flow.

Consideration of such in vivo relevance has been considered with respect to lung and skin in vitro systems. For these systems, it is commonplace now to conduct ENM hazard assessment using cultures at the air–liquid interface (ALI), thus recreating the 1) gas-exchange region in the lower human lung,[54] and 2) the surface of the epidermis.[55] As regards the former however, understanding how dynamic movement,[56] and the role of lung lining fluid (i.e., surfactant)[57] has been explored through preliminary assessments, but knowledge in this area remains elementary at this moment in time. Such advances though, would allow lung in vitro models to take an additional step forward in their physiological relevance when compared to the in vivo situation.

When considering other organs being developed, for example, liver spheroids, the O_2 content is important. Normally

they are kept in the conventional incubator environment (i.e., 37 °C, 5% CO_2, 20% O_2, 75% N_2). Yet, in the human liver, the O_2 content is <2%. Thus, when approaching understanding the ENM impact upon the liver and using spheroids, the O_2 content becomes extremely relevant and important in the development of advanced, alternative models. Such differences in the environment that the cells are cultured within would also have an impact upon cellular metabolism, which would further impact the ENM-cell interaction. In this regard, the temperature that the cell cultures are kept will also impact on how ENMs interact with the cell system (i.e., 4 °C will inhibit any form of endocytosis). On a final note in this topic, the use of cell culture supplements is a component often overlooked by in vitro scientists. Commonly we are focused upon the overall model being an "alternative" system, but remain using animal products, that is, fetal bovine serum and animal-tested antibodies. It is interesting to note that we continue to culture primary human cells with fetal bovine serum (FBS) in our cell culture media, when, in fact, human serum would be more pertinent. Alas, the availability and cost implications of using human serum often result in the continued use of the more prevalent and cost effective FBS. However, the point is that in the attempts to create advantageous, alternative model systems to animal experimentation, it could be more productive to make small, specific alterations to the system, which could have a similar if not heightened improvement towards the in vitro model itself and its output.[58,59]

5. How Predictive Are In Vitro Models for Toxicology Testing

As previously highlighted, there have been limited advanced models used to assess the impact of ENMs. Further, there are no published reports of in vitro models being used to predict the human response to ENM exposure, as yet. However, this is the ultimate goal. In an attempt to close the knowledge gap, Snyder-Talkington and colleagues[60] compared an in vitro biculture of epithelial and endothelial cells to a rodent model and observed significant differences at the gene level for (pro-) inflammatory mediators after carbon nanotube exposure. Alas, this approach, while it should be applauded, highlights an issue that we have in this scenario. As scientists we are creating advanced models using human cells, but then comparing the effects of the human-based model against an animal system (e.g., rats, mice) to then predict a response in a human body. For exposures (e.g., quartz, asbestos), it is possible to compare to human epidemiological and toxicology studies; however, for ENM there is significant lack of such data, and so the approach of in vivo to in vitro extrapolation[61] is essential, especially when considering the exposure concentrations for the in vitro system.

With predictability in mind, it is important to highlight the advantageous role that adverse outcome pathway (AOP) approaches can have towards alternative models. By definition, AOPs are models that identify specific biochemical mechanisms, or "key events" (KE) that are produced following a molecular initiating event in terms of the toxicological response relative to the biological system to an exposed xenobiotic.[62] There are a number of different AOPs currently being produced and tested for, the most notable being that for lung inflammation, namely, AOP173 (lung inflammation leading to fibrosis).[63] In theory, by enabling analysis of each KE (e.g., pro-inflammatory mediator release, cell proliferation, pro-fibrotic mediator release, collagen production), it is possible to use an in vitro model to determine if it predicts the KE(s), and thus the AO. Recently, a study by Barosova and colleagues[64] used AOP173 to assess the pro-inflammatory and pro-fibrotic impacts of different multi-walled CNTs upon an advanced in vitro lung cell system. These approaches are starting to lay the foundation for the use of (advanced) in vitro models to predict the hazardous outcome of human exposure to specific xenobiotics, such as ENMs.

6. Recommendations and Future Perspective

As highlighted throughout this concept article, the approach of alternative model development is to provide a valuable assessment tool that could be easily applied in both a hazard screening approach and also in a regulatory setting, to better predict the human response following exposure to a foreign object, for example, ENMs, without the need to conduct invasive animal testing strategies. From the previous review by Burden and colleagues[23,27] in which the short, medium, and long-term objectives are set-out for alternatives in toxicology testing strategies, it can be considered that the field remains within the short-term goals, that is, acute-phase exposures and response characterization, combination of several hazard endpoints, advanced exposure systems to cell culture models, and in vivo in vitro extrapolation approaches. There is progress towards medium-term objectives, in the sense of chronic exposures and biological response analytics;[63] however, in order to achieve further steps towards medium-term objectives, the following steps should be considered:

- Use specific and relevant cells in the model system being formulated that most closely relate to the anatomy and physiology of the human organ of interest.
- Use human relevant cell culture supplements (e.g., serum), and other analytical approaches (e.g., antibodies) rather than animal based (in order to make it a truly animal alternative model).
- Characterize the system that is being used, from its most primitive form to its most complex. In this context, there are two distinct levels: 1) tissue level characterization (i.e., how representative is the model mimicking that of the in vivo tissue), and 2) hazard response level (i.e., how does the model respond to specific biological endpoints (i.e., oxidative stress, (pro-)inflammatory mediator release) following exposure to specific positive controls. It is important to know precisely what the system is and how it responds to a positive particle, chemical, and assay specific controls.
- Further, albeit that the anatomical set-up might be as specific as possible, when considering the physiological relevance of the system, many systems currently used are static and do not adequately represent the key physiological components of the in vivo (human) system (e.g., fluid flow dynamics, tissue flexing, cellular movement).

- Determine the predictive nature of the in vitro model against currently available in vivo and human exposure data for the same hazard endpoint (use of relevant AOPs are advised here, e.g., AOP173).

Considering these approaches, in terms of future perspective, the field must focus on

- Complete non-animal models/approaches, that is, model systems that are made purely of human cells and supplemented with non-animal supplements. Importantly though, that these "new" systems are then able to detect the same response (and effect level) as previously found with animal-based systems (e.g., those using fetal bovine serum).
- Tailored testing systems that cover the range of (multiple) hazard endpoints required for risk assessment purposes. Then further developing these systems towards AOPs, as well as high-throughput approaches, in order to enhance the predictive nature of the tests coupled to the improved in vitro system(s).

7. Summary and Conclusion

In summary, the development of new alternative model is currently a fast-moving area with novel systems being introduced and further improved upon at a rapid rate in order to understand, at the cellular level how organs develop and function, as well as to provide systems that could be used for more predictive in vitro toxicology testing, for example, for ENMs. Alas, while many of the advanced in vitro systems available have had at least the minimal characterization necessary performed, and their general anatomical and physiological attributes relate to the human organ level, precisely how predictive these model systems are in terms of the human response to, for example, ENMs, is currently limited at best. To be precise, extremely little research has been conducted in order to understand this important point, and thus this aspect is one of the key factors needing focus in order to capitalize on these new developments to promote the 3Rs in toxicology. Questions should start to be asked within the field as to whether or not many more model systems should be created with the intention to be predictive models for toxicology testing regarding human health implications of new products, for example, drugs. With the plethora of models already available, it is arguably more pertinent to test these systems and determine which, if any, characteristics must be improved in order to obtain the closest possible, predictive model of the human response in vitro. While increasing the technology readiness levels of systems may be scientifically interesting, it is abundantly clear that the more complex the model, the more training, cost, and time is needed to conduct system set-up and testing. All of which are key deterrents for industry and legislative bodies when considering which testing approach to employ. Thus, understanding the usefulness, applicability, cost–time efficiency, and predictability of these advanced models are essential moving forward. Furthermore, it is also important to note that since there is a clear commitment towards the development and use of alternative methodologies within the field of nanosafety, that the inception of in vitro models using completely animal free components, as well as biochemical assays based upon animal free approaches is an aspect that should be strongly considered from this point onwards.

Therefore, in conclusion, the field of nanosafety is on a good setting already in terms of utilizing the knowledge and expertise within the area of alternative in vitro models. In order to assess ENM impact upon human health, it is necessary that the predictive nature of any of the plethora of advanced in vitro systems currently available is realized. Through this the overall usefulness of alternative testing strategies will be determined and allow for the further creation of regulation and legislation for their widespread use within risk assessment approaches.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

3Rs, alternatives, in vitro systems, nanosafety, reduction, replacement

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